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Research Article

CYTOMORPHOMETRIC EVALUATION OF ALTERATIONS IN BUCCAL MUCOSAL CELLS OF SMOKERS AND NON-SMOKERS

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Abstract: The objective of the present study was to assess the effect of smoking on buccal mucosa by using cytomorphometry. A case control study on 50 smoker subjects and 50 non-smoker controls between the age of 28 and 71 years. Buccal epithelial cells of these individuals were collected with a brush and fixed smears were stained with Papanicolaou stain and cytomorphometric analysis performed using image analysis software (Image J v 1.47). Cytomorphometric changes could be the earliest indicators of cellular alterations. There is progressive increase in nuclear parameters i.e. mean nuclear area (MNA), mean nuclear perimeter (MNP), mean of maximum nuclear diameter (Max-ND) and mean of minimum nuclear diameter(Min-ND) and decrease in ratio of cellular to nuclear parameters in smears from smokers, as compared to normal subjects. This indicates that there could be cause–effect relationship between smoking and quantitative alterations. This increase determined in nuclear parameters shows smoking-related cellular adaptation. It is possible to conclude that this adaptive change in the cell nucleus tends to be a progression towards dysplastic change.

Keywords: Cytomorphometry, Smoker, Buccal Mucosa.

INTRODUCTION

Oral cavity cancer is one of the six most common cancers in the world.[1] and is one of ten major causes of death across the globe.[2] 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. Two thirds (2/3) of oral squamous cell carcinoma and 75% of head and neck cancer can be attributed to tobacco use and alcohol consumption.[3,4] 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.[5] Tobacco smoking has been observed to be associated with increasing risk of oropharangeal cancer and oral leukoplakia.[6] The risk increases with the frequency of exposure.[7] The contents of tobacco have been identified as mutagenic in vitro and *in vivo*.[8] Oral exfoliative cytology is a simple, non-invasive, and painless method that involves microscopic analysis of cells collected from the surface of the buccal mucosa. Oral cytology, which is largely based on the presence of nuclear or cytoplasmic alterations, can easily be performed to detect cancer at an early stage and to establish quantitative techniques.[9,10,11] 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.[3] Of these parameters, the nuclear size, cytoplasmic size and their ratio have been shown to be significant in the evaluation of oral lesions.[4,5]

Ogden *et al.*,[11] indicated that quantitative techniques based on cytomorphometric parameters were more accurate, objective, and reproducible. Today, with advanced imaging techniques, computerized systems, and the use of quantitative techniques to verify the reliability of cytomorphometric analysis, this method is gaining in popularity once again. [12]

The purpose of this study was to analyze the cytomorphology of buccal mucosa cells of smokers using computerized image analysis based on quantitative parameters such as cellular and nuclear area, perimeter, minimal diameter and maximal diameter, as well as to evaluate potential dysplastic transformation.

MATERIALS AND METHODS

A total of 50 smokers and 50 non-smokers were selected for the study. The smokers had been using a minimum of 20 cigarettes a day for at least 10 years.

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Patients with systemic disease such as anemia or diabetes, clinically apparent oral mucosal lesions, and previous benign or malignant lesions were excluded from this study. Both control and subjects of smoker groups were non alcoholics.

Informed consent was obtained from all patients before taking the cytological smears. The smears were taken from clinically normal buccal mucosa. The subject was asked to rinse the mouth with drinking water. Taking all the aseptic precautions, a wooden spatula was then used to scrape the sample area (inner side of the cheek) three to four times with firm pressure. The scrapings were smeared on to the center of glass slide. The slides were immediately sprayed with commercially available spray fixative to ensure proper fixation. All cytological smears were stained by Papanicolaou staining technique.

Papanicolaou staining method

Ethyl alcohol fixed smears were hydrated in descending concentrations of 95% alcohol through 70% alcohol to distilled water, for two minutes in each stage. Then the smears were treated with Harris' hematoxylin for five minutes to stain the nuclei, rinsed in distilled water and differentiated in 0.5% aqueous hydrochloric acid for a few seconds, to remove the excess stain. They were then immediately rinsed in distilled water, to stop the action of discoloration. Then the smears were blued in alkaline water for a few seconds and dehydrated in ascending alcoholic concentrations from 70%, through two changes of 95% alcohol for two minutes for each change. The smears were next treated with Eosin Azure 50 for four minutes. For cytoplasmic staining, they were treated with Papanicolaou Orange G6 for two minutes, rinsed in 95% alcohol and then dehydrated in absolute alcohol. The smears were then cleared in Xylene and mounted in DPX (Diastrene Polystyrene Xylene) mount.

Computerized cytomorphometry

PAP stained smears were examined under a light microscope. 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. A 640 X 400 pixel digital image was taken by a camera on the microscope with 10X eyepiece and 40X objective. Using the image J 1.47 image analysis software, morphometric analysis of around 50cells/case was done. (Figure 1 and Figure 2)

The following cell and nuclear morphometric features were analyzed.

(i) Area was the area within the outlined cell/nuclear perimeter.

(ii) Perimeter was measured as the length around the cell/nuclear border.

(iii) Max diameter was the longest axis of the outlined cell/nuclear perimeter.

(iv) Minimum diameter was measured as the shortest axis of the outlined cell/nuclear perimeter.

(v) Following nuclear shape parameters were calculated based on above measured values:
   a. AR form factor (area divided by $\pi/4$ x Max diameter x Minimum diameter);
   b. Circularity/PE form factor (4π x area divided by the square of the perimeter) with a value of 1.0 indicating a perfect circle.
   c. Nuclear roundness: the inverse of Aspect Ratio (In a circular nucleus, the values of the roundness correspond to 1. If the nucleus is elliptic, the roundness becomes < 1)
   d. Solidity: [Area]/ [Convex area]. [13]

The mean nuclear and cytoplasmic area, perimeter, diameter and cell to nuclear (C/N) ratio of all cases were the parameters of interest in this study and their mean values were obtained in square micrometers for area and in micrometers for perimeter and diameter.

Following statistical parameters (with standard deviation) were calculated for each cell and nuclear feature in each group: mean cell area (MCA), mean cell perimeter (MCP), mean of maximum cell diameter (Max-CD), mean of minimum cell diameter (Min-CD), mean nuclear area (MNA), mean nuclear perimeter (MNP), mean of maximum nuclear diameter (Max-ND) mean of minimum nuclear diameter (Min-ND) and cell to nuclear parameter ratio.

The data obtained was statistically analyzed and compared for the two groups.

RESULTS

This study was conducted on 100 individuals, which included 50 smokers (Group S) who smoked at least 20 cigarettes a day for the last 10 years, and 50 non-smokers (Group N), without any local or systemic diseases.

In the present study most of the smokers were in the age group of 31-40 years (32%), followed by 51-60 years (30%), 41-50 years (24%), 21-30 (8%) 61-70 years (4%) and 71-80 (2%). (Table 1)

Table 1: Age Wise Distribution of Smokers and Non-Smokers Subjects.

Age Range	GROUP S	%	GROUP N	%
21 to 30	4	8	6	12
31 to 40	16	32	14	28
41 to 50	12	24	15	30
51 to60	15	30	14	28
61 to 70	2	4	1	2
71 to 80	1	2	0	0
Total	50	100	50	100

The contents of table 2 show results of nuclear size parameters i.e. MNA, MNP, Max-ND, Min-ND and shape parameters including AR form factor, round, solidity and circularity respectively.

The measured parameters were MNA, MNP, Max-ND and Min-ND. The MNA (μm^2) for Group S and Group N were 80.46±10.07 and 67.92 ±8.39. The MNP (µm) of nucleus was found to be 32.78 ±2.04 and 30.11 ±1.92 for Group S and Group N respectively. Max-ND and Min-ND (µm) for Group S were 11.932 ±0.899 and 8.682± 0.608, whereas for Group N they were 10.786± 0.788 and 8.020 ±0.601 respectively. (p<0.001)

Figure 1: Cellular and Nuclear morphometric analysis of exfoliated squamous epithelial cell in buccal smears of non-smokers using ImageJ v 1.47 image analysis software.

Table 2: Nuclear Morphometric Parameters In Smokers (Group S) And Non Smokers (Group N) Using Image J Software.

	Nuclear parameters			
	Parameters	Group S	Group N	p value
Size parameters	MNA (μm^2)	80.46±10.07	67.92 ±8.39	<0.001
	MNP (µm)	32.78 ±2.04	30.11 ±1.92	<0.001
	Max-ND (µm)	11.932 ±0.899	10.786± 0.788	<0.001
	Min-ND(µm)	8.682± 0.608	8.020 ±0.601	<0.001
Shape parameters	AR	1.364 ±0.096	1.340 ±0.123	0.279
	Round	0.754 ±0.052	0.761 ±0.068	0.564
	Solidity	0.998 ±0.005	0.991 ±0.015	0.002
	Circularity	0.931 ±0.021	0.917 ±0.034	0.015

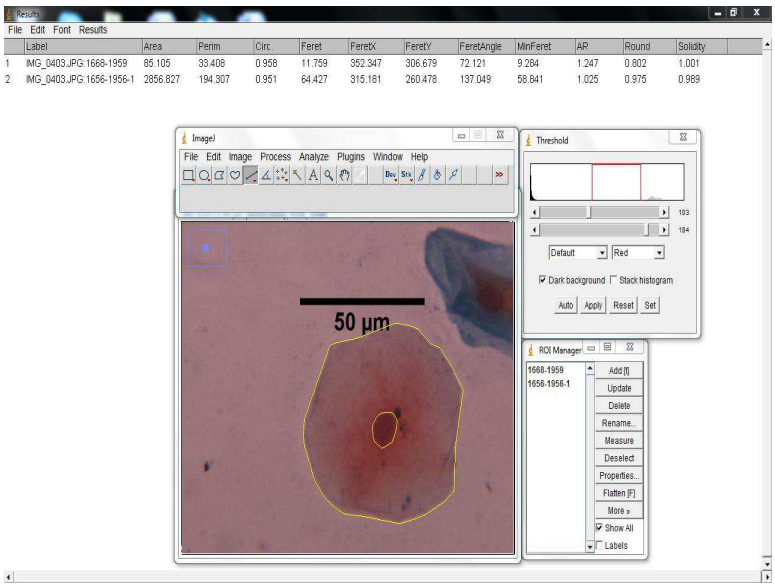

Figure 2: Cellular and Nuclear morphometric analysis of exfoliated squamous epithelial cell in buccal smears of smokers using ImageJ v 1.47 image analysis software.

The calculated nuclear parameters were AR form factor, round, solidity and circularity. These parameters show shape related features of the nucleus. The AR form factor, round, solidity and circularity in Group S were found to be 1.364 ±0.096, 0.754 ±0.052, 0.998 ±0.005 and 0.931 ±0.021 respectively. Group N showed this value to be 1.340 ±0.123, 0.761 ±0.068, 0.991 ±0.015 and 0.917 ±0.034 respectively. P value in shape parameters was not found to be significant.

The cellular morphometric parameters shown in table 3 include MCA, MCP, Max-CD and Min-CD respectively. The MCA (μm^2) for Group S and Group N were 2519.72 ±603.97 and 2646.57 ±365.71. The MCP (µm) of cell was found to be 194.61 ±18.08 and 213.58 ±27.01 for Group S and Group N respectively. Max-CD and Min-CD (µm) for Group S were 68.39 ±8.22 and 50.13 ±5.9, whereas for Group N they were 74.12 ±6.81 and 48.23 ±4.4 respectively.

Table 3: Cellular Morphometric Parameters in Smokers (Group S) And Non Smokers (Group N) Using Image J Software.

CELLULAR PARAMETERS		
Parameters (C/N)	Group S	Group N
MCA	2519.72 ±603.97	2646.57 ±365.71
MCP (µm)	194.61 ±18.08	213.58 ±27.01
Max-CD (µm)	68.39 ±8.22	74.12 ±6.81
Min-CD (µm)	50.13 ±5.9	48.23 ±4.4

Table 4: The Cell to Nuclear (C/N) Parameter Ratio of Area, Perimeter, Max-D And Min-D With P Value.

Cell to Nuclear (C/N) parameter ratio			
Parameters (C/N)	Group S	Group N	p value
Area	31.46 ±7.46	39.49 ±6.83	<0.001
Perimeter	5.947 ±0.547	7.105 ±0.896	<0.001
Max. dia	5.739 ±0.621	6.899 ±0.711	<0.001
Min. dia	5.789 ±0.717	6.043 ±0.681	<0.001

The cellular versus nuclear parameter ratios of both the groups were calculated and are shown in Table 4. In Group S the ratio of cell to nuclear area was 31.46 ±7.46 compared to 39.49 ±6.83 in Group N. Similarly in Group S the C/N ratio of perimeter, maximum diameter, and minimum diameter were 5.947 ±0.547, 5.739 ±0.621, and 5.789 ±0.717 respectively. The corresponding values in Group N were 7.105 ±0.896, 6.899 ±0.711 and 6.043 ±0.681 respectively. ($p<0.001$)

DISCUSSION

All of the major forms of tobacco use like cigarettes, cigars, pipes and smokeless tobacco are known to cause oral cancer.

There is a rapid increase in consumption of smoking tobacco products. Tobacco contains carcinogens that influence the DNA repair, cell cycle control and may produce chromosomal aberrations.[14] The strong association between cancers of the oral cavity and pharynx with the use of tobacco is well established. The risk tends to increase with the duration of smoking. In a study Mohammed S. Abdelaziz, Tagwa E. Osman reported that alcohol consumption and cigarette smoking are risk factors for oral atypical cellular changes and possibly oral infection.[15]

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.[16]

In various studies quantitative cytomorphometric evaluation of exfoliated buccal mucosa cells obtained from premalignant and malignant lesions has revealed significant differences at the cellular level.[17,18]

Ogden *et al.*, studied the effect of smoking on the oral mucosa in individuals over 40 years of age using cytomorphological methods. They reported a 5% average increase in the NA values of smokers when compared to non-smokers. Our findings being are consistent with those of Ogden *et al.*,[19.]

Einstein and Sivapathasundraham[20] also analyzed the effect of smoking and betel quid chewing on the oral mucosa, using cytomorphological methods, and determined an increase in the average value of nuclear diameter, and a decrease in cytoplasmic diameter values of smokers.

Ramaesh *et al.*,[21] reported that the cytoplasmic diameter of individuals who smoked cigarettes and chewed betel quid and practiced both these habits was significantly smaller than that of the control group individuals. They also reported that the nuclear diameter of the buccal mucosa cells in individuals who smoked cigarettes, chewed betel quid was significantly greater than that of the control group individuals. In our study the differences in results of nuclear parameters including area, perimeter, maximum, minimum diameter and cellular to nuclear parameter ratio were found to be significant between smokers and non-smokers.

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.[17]

Cowpe JG *et al.*, reported that increase in the nuclear diameter could be due to increased DNA content of the nucleus and increase in ratio of nuclear diameter to cellular diameter is due to the changes in nuclear size and cytoplasm.[22] 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.[23]

Our results revealed that the MNA, MNP, Max-ND and Min-ND values of the buccal mucosa cell nuclei of smokers were higher than those of non-smokers, and the difference was statistically significant in the case of MNA, MNP, Max-ND and Min-ND values. The cell to nucleus ratio was lower in smokers as compared to non–smokers and can be attributed to increase in nuclear size in smokers.

CONCLUSIONS

Cytomorphometric changes could be the earliest indicators of cellular alterations. There is increase in nuclear size parameters and decrease in ratio of cellular to nuclear size parameters in smears from smokers, as compared to normal subjects. This indicates that there could be cause–effect relationship between smoking and quantitative alterations. This increase determined in NA shows smoking-related cellular adaptation. It is possible to conclude that this adaptive change in the cell nucleus tends to be a progression towards dysplastic change.

BIBLIOGRAPHY

1. Gupta PC, Sinor PN, Bhonsle RB, Pawar VS, Mehta HC. Oral submucous fibrosis in India: a new epidemic? Natl Med J India 1998 May-Jun;11 (3):113-116.

2. Razavi SM, Sajadi S. Epidemiological Study of Oral and Perioral Cancers in Isfahan. Dent Res J 2007;4(1):18-25.

3. Kademani D. Oral cancer. Mayo Clin Proc 2007 Jul;82(7):878-887.

4. Hashibe M, Brennan P, Benhamou S, *et al.,* Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. J Natl Cancer Inst. 2007 May 16; 99(10):777-89.

5. WINN DM, Tobacco use and oral disease, J Dent Educ, 2001, 65(4):306–312.

6. Mayne ST, Morse DE, Winn DM. Cancers of the oral cavity and pharynx. In: Schottenfeld D, Fraumeni JF Jr (eds) Cancer epidemiology and prevention. Oxford University Press, New York, 2006; 674–696.

7. Morse DE, Psoter WJ, De La Torre Feliciano T, Cruz G, Figueroa N. Detection of very early oral cancers in Puerto Rico. Am J Public Health 2008 Jul;98(7):1200-1202.

8. Wu PA, Loh CH, Hsieh LL, Liu TY, Chen CJ, Liou SH. Clastogenic effect for cigarette smoking but not areca quid chewing as measured by micronuclei in exfoliated buccal mucosal cells. Mutat Res 2004; 562:27-38.

9. Paiva RL, Sant'Ana Filho M, Bohrer PL, Lauxen IdaS, Rados PV. AgNOR quantification in cells of normal oral mucosa exposed to smoking and alcohol. A cytopathologic study. Anal Quant Cytol Histol 2004 Jun;26(3):175-180.

10. Koss LG. Koss's Diagnostic Cytology and its Histopathologic Bases. 5th ed. New York Lippincott company 2006:11.

11. Ogden GR, Cowpe JG, Wight AJ. Oral exfoliative cytology: review of methods of assessment. J Oral Pathol Med 1997; 26: 201-5.

12. Pektaş ZO, Keskin GO, Karslioğlu Y. Evaluation of nuclear morphometry and DNA ploidy status for detection of malignant and premalignant oral lesions: quantitative cytologic assessment and review of methods for cytomorphometric measurements. J Oral Maxillofac Surg 2006; 64: 628-35.

13. EMBL CMCI ImageJ Basic Course 1.6 Appendices 1.6.4 App.2 Measurement Options or (http://imagej.nih.gov/ij/docs/guide/146-30.html#toc-Subsection-30.7 SET MEASUREMENTS.

14. Souto GR, Caliari MV, Lins CE, de Aguiar MC, de Abreu MH, Mesquita RA. Tobacco use increases the number of aneuploid nuclei in the clinically healthy oral epithelium. J Oral Pathol Med 2010; 39: 605-10.

15. Mohammed S. Abdelaziz, Tagwa E. Osman.Detection of Cytomorphological Changes in Oral Mucosa among Alcoholics and Cigarette Smokers Oman Medical Journal (2011) Vol. 26, No. 5:349-352

16. KAUGARS GE, BRANDT RB, CHAN W, CARCAISE–EDINBORO P, Evaluation of risk factors in smokeless tobacco-associated oral lesions, Oral Surg Oral Med Oral Pathol, 1991, 72(3):326–331.

17. Ramaesh T, Mendis BRRN, Ratnatunga N, Th attil RO. Cytomorphometric analysis of squames obtained from normal oral mucosa and lesions of oral leukoplakia and squamous cell carcinoma.J Oral Pathol Med 1998; 27: 83-6.

18. Zink D, Ficher HA, Nickerson AJ. Nuclear structure in cancer cells. Nat Rev Cancer 2004; 4: 677-87

19. Ogden GR, Cowpe JG, Gren MW. Quantitative exfoliative cytology of normal buccal mucosa: eff ect of smoking. J Oral Pathol Med 1990; 19: 53-5.

20. Einstein TBA, Sivapathasundharam B. Cytomorphometric analysis of the buccal mucosa of tobacco users. Ind J Dent Res 2005; 16: 42-6.

21. Ramaesh T, Mendis BR, Ratnatunga N, Th attil RO. The effect of tobacco smoking and of betel chewing with tobacco on the buccal mucosa: a cytomorphometric analysis. J Oral Pathol Med 1999; 28: 385-8.

22. Cowpe JG, Longmore RB, Green MW, Quantitative exfoliative cytology of abnormal oral mucosal smears, J R Soc Med, 1988, 81(9):509–513.

23. Franklin CD, Smith CJ, Stereological analysis of histological parameters in experimental premalignant hamster cheek pouch epithelium, J Pathol, 1980, 130(3):201–215.

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