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Assessment of Cytomorphometric Features of Oral Squames from Buccal Mucosa of Tobacco Users using Oral Brush Biopsy: An Exfoliative Cytological Study.

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ABSTRACT

Background: Tobacco comprises of one of the most common addiction in the society. All the forms of the tobacco are harmful, causing alterations in the cellular parameters of the oral mucosal cells. A number of techniques and methods are available along with chair side examination for supplementing the diagnosis of tobacco induced premalignant lesions. Exfoliative cytology is one such technique, which is non-invasive and can be used for mass screening. Therefore, we evaluated the cytological and cytomorphometric changes in the oral squames using brush biopsy from buccal mucosa of tobacco users. **Methods:** A total of 300 patients with age group of 25 to 60 years were included in the study. Detailed history of the patients were taken to known the method (smoking, smokeless or both) and frequency of tobacco intake. Scrapings from the buccal mucosal scrape were obtained, smeared on slides and were subsequently stained with Hematoxylin and Eosin staining. Observation and analyses of the smears was done under microscope followed by analysis in the computer using software Dewinter Biowizard version 3.0. various cellular parameters were assessed and compared in between different groups. Patients were divided into two major groups- Habituers and Non-habituers. The habituers were again divided into: Tobacco smokers, Tobacco chewers and patients having combined habit. Independent-Samples T Test and One-Way ANOVA were used to assess the level of significance. **Results:** Significant results were obtained while comparing nuclear parameter, cellular area, cellular perimeter, cell contour and nucleo-cytoplasmic ratio in between lesional group and tobacco smoking group. While comparing between tobacco smoking group and Non habituers group, significant results were obtained in all the parameters except for cell contour and N/C ratio. Significant results were obtained while comparing between lesions and combined habit groups. While comparing between habituers and non- habituers group, except for cell contour significant results were obtained in all other cellular parameters. **Conclusion:** Early changes detection in clinically normal oral mucosa of tobacco users is possible by using non-invasive, painless procedures like oral brush biopsy and cytomorphometry. Further studies recommended.

Keywords: Cytomorphometry, Exfoliative, Tobacco.

INTRODUCTION

Tobacco remains one of the most important preventable cause of addiction, sickness & mortality in the world. Tobacco can be smoked or chewed in form of smokeless tobacco. Tobacco is most commonly smoked as cigarettes, both manufactured and hand-rolled.

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Pipes, cigars, bidis and other products are used to a lesser extent or predominantly in particular regions. Smokeless tobacco (ST) is tobacco that is not burnt when it is used and is usually placed in the oral or nasal cavities against the mucosal sites that permit the absorption of nicotine into the human body. All forms of tobacco use are addictive and cause harm.[1]

The above habits may cause some changes in the oral mucosa, and the initial changes will eventually give rise to clinically detectable lesions occur in the mucosa. The oral mucosal changes associated with habitual users of smoked and smokeless tobacco are leukoplakia, nicotine palatinus, smoker's melanosis, chewer's mucositis, smokeless tobacco keratosis, oral submucous fibrosis and oral cancer

The different ways in which tobacco is used lead to considerable variation in appearance, site and frequency of the lesions associated with the tobacco habit.[2] A number of techniques have been developed to supplement clinical examination in the diagnosis of these lesions. They include chemiluminescent illumination, toluidine blue supravital staining test, exfoliative cytology and scalpel biopsy. There is sufficient evidence that visual inspection of the lesion alone is not adequate to differentiate potentially malignant and malignant lesions from similar looking benign lesions and instead require evaluation to rule out dysplasia and carcinomas.[3] Histological changes of the buccal mucosa have been reported in human & experimental animals in association with tobacco usage.[4]

Hence, we undertook this study to identify cytological and cytomorphometric changes in the squames obtained using brush biopsy from buccal mucosa of tobacco users.

MATERIALS AND METHODS

The present study was conducted on patients reporting in the dental OPD of Vananchal dental college for treatment of various oral problems. Patients with age group of 25 to 60 years were included in the study. The study sample size consisted of 200 patients with tobacco habits and 100 non-habitués of tobacco. Patients were examined irrespective of sex, caste and socioeconomic status. Mehta et al criteria was used for general mucosal screening.[5] List of questionnaire/ interview was prepared and was given to each subject of the study to record the subject data and habits .In case of subjects with tobacco habits, detailed history was recorded as to the type, period and frequency of the habit and an informed consent was signed by the patients. Patients having tobacco habit were considered as the study group. Control group included patients with no history of tobacco habits and no lesions. Positive controls included patients with tobacco habits and concurrent presence of lesions formed the positive control group. Scrapings from the buccal mucosal scrape were collected using a cytology brush. Cells were scraped using a gentle scraping motion and were spread on the glass slide and fixed in 95% alcohol. The smears were stained with Hematoxylin and Eosin Smears were observed under microscope and 50 non-overlapping cells with well defined borders were randomly selected. The images were captured with a camera attached to the microscope. All the images of the cells were captured with a 40x achromatic objective. Images thus captured were stored on the computer and analysis was done using the software Dewinter Biowizard version 3.0. The outline of the cell and the nucleus was traced on the screen using a cursor controlled by the mouse, for the area and perimeter. The measurements were carried out using measurement tool and were done in microns. Nuclear contour index, cell contour index and nucleo-cytoplasmic ratio[6] were calculated using the following formulae. Nuclear contour index = N Perimeter/√N Area. Cellular contour index = C Perimeter/ √C Area. Nucleo: cytoplasmic ratio = N Area/(C Area-N Area). Two major groups were included in the study: Habituers and Non-habituers. The habituers were again divided into: Tobacco smokers, Tobacco chewers and patients having combined habit. Lesion group included the patients with lesions in relation to tobacco habit. The nuclear area, perimeter, contour, cell area, perimeter, contour and nuclear/cytoplasmic ratio were compared among the various groups. All the results were analysed by SPSS software. Independent-Samples T Test and One-Way ANOVA were used to assess the level of significance.

RESULTS

All the results are tabulated in Table 1–9. Significant results were obtained while comparing nuclear parameter, cellular area, cellular perimeter, cell contour and nucleo-cytoplasmic ratio in between lesional group and tobacco smoking group as shown in [Table 1]. While comparing between tobacco smoking group and Non habituers group, significant results were obtained in all the parameters except for cell contour and N/C ratio [Table 2]. While comparing between tobacco chewing group and lesions and between tobacco chewing group and Non Habituers, all the parameters showed significant variations [Table 3, 4]. Statistical significant results were obtained while comparing all the parameters except for cell contour and N/C ration between 1tobacco smoking and chewing groups as shown in [Table 5]. While comparing between tobacco smoking group and combined habit group and between tobacco chewing group and combined habit group, significant results were obtained in all the parameters except in nuclear area and N/C ratio as shown in [Table 6 and 7]. Significant results were obtained while comparing between lesions and combined habit groups [Table 8]. While comparing between habituers and non- habituers group, except for cell contour significant results were obtained in all other cellular parameters [Table 9].

DISCUSSION

Tobacco use is widespread throughout the world by men, women and millions of people are involuntarily subjected to environmental tobacco

smoke. Tobacco is consumed mainly in two forms- smoking and smokeless tobacco form. Smoking commonly includes cigarettes and beedis while smokeless mainly includes Betel quid chewing with tobacco or chewing tobacco alone. Mucosal changes are seen due to tobacco in the exfoliated cells.

Table 1: Independent samples test of lesions and tobacco smoking group

Parameters	T-test for equality of means	
	T value	p-value
Nuclear area	-0.568	.452 (n.s)
Nuclear perimeter	2.152	.048 (s)
Nuclear contour	1.428	.215(n.s)
Cell area	3.256	.001(s)
Cell perimeter	5.426	.002(s)
Cell contour	3.865	.008(s)
N/C ratio	-2.856	.002(s)

n.s: Non Significant, S: Significant

Table 2: Independent samples test of tobacco smoking group and Non habituers

Parameters	T-test for equality of means	
	T value	p-value
Nuclear area	6.452	.005(s)
Nuclear perimeter	9.652	.005(s)
Nuclear contour	2.741	.004(s)
Cell area	8.139	.003(s)
Cell perimeter	9.813	.001(s)
Cell contour	1.791	.205(n.s)
N/C ratio	-1.358	.175(n.s)

n.s: Non Significant, S: Significant

Table 3: Independent samples test of tobacco chewing group and lesions.

Parameters	T-test for equality of means	
	T value	p-value
Nuclear area	3.125	.004(s)
Nuclear perimeter	5.545	.001(s)
Nuclear contour	4.002	.003(s)
Cell area	7.102	.001(s)
Cell perimeter	11.230	.008(s)
Cell contour	4.185	.008(s)
N/C ratio	-3.112	.005(s)

n.s: Non Significant, S: Significant

Table 4: Independent samples test of tobacco chewing group and Non Habituers.

Parameters	T-test for equality of means	
	T value	p-value
Nuclear area	12.226	.000(s)
Nuclear perimeter	15.425	.001(s)
Nuclear contour	7.263	.002(s)
Cell area	18.253	.004(s)
Cell perimeter	19.123	.035(s)
Cell contour	1.526	.025(s)
N/C ratio	3.154	.012(s)

n.s: Non Significant, S: Significant

Exfoliative techniques are of great significance in those patients in which malignancy is suspected and repeated smears have to be taken. In irradiated areas of the body where biopsy is contraindicated, it can also be used as a prognostic tool.[3] Hence we conducted this study to assess the cytological and cytomorphometric changes in the squames obtained using brush biopsy from buccal mucosa of tobacco users.

Table 5: Independent samples test of tobacco smoking and chewing group.

Parameters	T-test for equality of means	
	T value	p-value
Nuclear area	5.958	.002(s)
Nuclear perimeter	6.774	.002(s)
Nuclear contour	3.685	.003(s)
Cell area	8.425	.004(s)
Cell perimeter	11.458	.005(s)
Cell contour	0.455	.227(n.s)
N/C ratio	1.458	.315(n.s)

n.s: Non Significant, S: Significant

Table 6: Independent samples test of tobacco smoking group and combined habit group.

Parameters	T-test for equality of means	
	T value	p-value
Nuclear area	0.337	.859(n.s)
Nuclear perimeter	1.785	.042(s)
Nuclear contour	2.774	.015(s)
Cell area	2.138	.003(s)
Cell perimeter	3.958	.001(s)
Cell contour	2.452	.020(s)
N/C ratio	0.258	.758(n.s)

n.s: Non Significant, S: Significant

Table 7: Independent samples test of tobacco chewing group and combined habit group.

Parameters	T-test for equality of means	
	T value	p-value
Nuclear area	1.236	.005(s)
Nuclear perimeter	2.385	.142(n.s)
Nuclear contour	3.442	.002(s)
Cell area	3.568	.003(s)
Cell perimeter	2.115	.012(s)
Cell contour	2.748	.005(s)
N/C ratio	0.425	.425(n.s)

n.s: Non Significant, S: Significant

Table 8: Independent samples test of lesions and combined habit group.

Parameters	T-test for equality of means	
	T value	p-value
Nuclear area	0.508	.758(n.s)
Nuclear perimeter	2.209	.002(s)
Nuclear contour	2.439	.014(s)
Cell area	4.448	.002(s)
Cell perimeter	7.185	.001(s)
Cell contour	5.002	.018(s)
N/C ratio	2.748	.008(s)

n.s: Non Significant, S: Significant

While comparing the nuclear area between different groups, statistically significant difference was observed while comparing between tobacco smoking group and non habituers, tobacco chewing group and lesions, tobacco chewing group and non habituers, tobacco smoking and chewing group, tobacco chewing and combined habit group, habituers and non-habituers. While comparing between tobacco smoking group, tobacco smoking and combined habit group, combined habit group

and lesions, non significant results were obtained (p-value>0.05) [Table 1-4]. Cowpe[7] and Robert[8] et al observed increased nuclear area in dysplastic lesions in contrast to our study. Difference in sampling methods and sample size might contribute to this difference. The significant difference observed between the habituers and non habituers reflects the ability cytomorphology to detect these changes and also on the alterations occurring in oral mucosa due to tobacco.[7] While comparing the nuclear parameter in between different groups, statistically significant results were obtained. Similar results were obtained by Singh[9] et al and Hande[10] et al who observed significant alterations in the nuclear parameters in between tobacco habituers and non-habituers groups. While comparing the mean nuclear contour index ((in µm) statistically significant differences were found between all the groups except for tobacco smoking group and lesions in which non-significant results were seen (p-valus>0.05) [Table 5-7]. Hande et al observed similar changes in their study and stressed on the fact that the initial changes seen in tissue exposed to smoking is cytomorphologically similar to changes occurring lesional tissue.[10] We also compared the cellular area in between different groups and observed statistically non significant difference was found among tobacco smoking group and lesions (p-value>0.05). Statistically significant results were seen while comparing all the remaining groups (p-value<0.005) [Table 8 & 9]. Different results were reported in the studies of Reichart[11] et al and Hillman[12] et al who observed non-significant reduction in the cellular area between lesional groups. Difference in sample size and parameters chosen for the study might be responsible for this conflict in the results seen.[12] Statistical significant results were obtained while comparing cellular perimeter in between all the groups. Similar results were reported by Ramesh[12] et al who also reported a significant alteration in cellular perimeter in between different groups. From the above results, it can be interpreted that cytometry can play a significant role in analyzing changes in cell perimeter in all habit groups. Contour index is a measure of regularity of particle outline. Cells of premalignant and malignant lesions showed more pleomorphism and hence will show higher values. We observed statistically significant results while comparing mean cell contour index (in µm) between tobacco smokers and lesions, tobacco chewing group and lesions, tobacco chewing group and non habituers, tobacco smoking group and combined habit group, lesions and combined habit group. Statistically non significant differences were seen among tobacco smoking group and non habituers, tobacco smoking group and tobacco chewing group, tobacco chewing group and combined habit group, habituers and non habituers. To the best of our knowledge, no studies for contour index in exfoliated cells have been done. In our study, though the cell contour values were different for most groups, a considerable overlap between scores was also observed. On basis of these findings, this parameter does not appear to be reliable in exfoliative cytology.

Table 9: Independent samples test of habituers and non-habituers group.

Parameters	T-test for equality of means	
	T value	p-value
Nuclear area	12.256	.001(s)
Nuclear perimeter	14.485	.002(s)
Nuclear contour	5.758	.001(s)
Cell area	15.145	.003(s)
Cell perimeter	16.286	.001(s)
Cell contour	1.196	.110(n.s)
N/C ratio	2.358	.028(s)

n.s: Non Significant, S: Significant

Statistically significant differences were found while comparing Mean nuclear-cytoplasmic ratio between tobacco smoking group and lesions, tobacco chewing group and lesions, tobacco chewers and non habituers, combined habit and lesions, habituers and non habituers. A statistically non-significant difference was found among tobacco smoking and chewing group, tobacco smoking and combined habit group, tobacco chewing and combined habit group. Nucleo-cytoplasmic ratio of habituers was found to be statistically higher than non habituers. This increase in nucleo-cytoplasmic ratio in habituers is a common finding reported in other studies also.[13-15] Singh et al assessed the morphometric parameters of oral mucosal cells in tobacco smokers and chewers and to evaluate the variations and concluded 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.[9] Saranya et al also assessed the assess the cytoplasmic diameter and nuclear diameters of normal buccal mucous membrane in different age groups of khaini chewers and concluded that Oral exfoliative cytological techniques could be utilized as a non-invasive alternative prognostic marker for detecting early oral malignancy.[16] This study highlights the cause-effect relationship between tobacco usage in varied forms and quantitative cellular and nuclear alterations. It also suggests that detection of early changes in clinically normal oral mucosa of tobacco users is possible by using non-invasive, painless outpatient-based procedure like toothbrush oral brush biopsy and cytomorphometry.

CONCLUSION

This study highlights the cause-effect relationship between tobacco usage in varied forms and quantitative cellular and nuclear alterations. From the results, we conclude that detection of early changes in clinically normal oral mucosa of tobacco users is possible by using non-invasive, painless outpatient-based procedure like toothbrush oral brush biopsy and cytomorphometry. Further studies are recommended with larger study group and more parameters in search of most reliable non-invasive technique for cancer diagnosis and post-radiotherapy prognosis.

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