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Cytomorphological changes in buccal mucosa cells and to establish correlation with the morphometric analysis of opening of mouth among smokers and non-smokers: A comparative study

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Abstract: Background: Smoking is currently the most preventable cause of diseases and death worldwide and is one of the causative risk factors for developing cancer in different organs. Therefore, smoking patients must be carefully monitored for alterations in buccal mucosa caused by tobacco abuse.
Aim and Objective: The aim of the study is to investigate cytological changes in buccal mucosa cells and establish a correlation with the morphometric analysis of mouth opening among smokers and non-smokers.
Materials and Methods: A comparative study was conducted on 200 individuals aged between 20 and 60 years. The subjects were randomly chosen from inpatients and outpatients of the Medicine Department at Index Medical College and Hospital. Buccal smears of these patients were processed in the Department of Anatomy.
Results: Significant differences were observed between non-smokers and smokers for cells with binucleation, pyknosis, perinuclear halo, cytoplasmic granules, karyolysis, karyorrhexis, and micronuclei in buccal mucosal cells. However, no significant difference was found for cytoplasmic vacuoles between non-smokers and smokers. Similar studies have reported findings consistent with our non-smoking population.
Conclusion: The present study indicates that almost all cytomorphological findings were higher in smokers than in non-smokers. Early detection of oral cancers becomes complex as they are mostly innocuous and asymptomatic during their initial stages. Cytomorphometric analysis can be used regularly to detect these cell alterations.

Keywords: Cytomorphometric; Smoker; Buccal Mucosal; Smoker; Central India.

1. Introduction

India accounts for the second largest tobacco consumption in the world. According to the Global Adult Tobacco Survey India, 2016-17, approximately 267 million adults (15 years and above) in India (29% of all adults) are tobacco users. It is one of India's major causes of death, with nearly 1.35 million deaths yearly [1]. Smoking is currently the most preventable cause of diseases and death worldwide and is one of the causative risk factors for developing cancer in different organs [2]. Therefore, smoking patients must be carefully monitored for alterations in buccal mucosa caused by tobacco abuse [2]. According to the World Health Organization, cancer of the mouth and oropharynx are the common types of head and neck tumors, corresponding to 4% of all cancer cases.

In India, oral cancers affect the lower middle-class strata, ranking as the top 3 cancers in the country [3]. Early detection of oral cancers becomes complex as they are mostly innocuous and asymptomatic during their initial stages [4]. Therefore, despite the ease of accessibility of the oral cavity for visual examination and having well-defined clinical diagnostic findings of oral cancer, poor and delayed prognosis leads to the detection of

cases at advanced stages. Poor healthcare facilities, lack of awareness among people about self-examination of the oral cavity, and expensive treatment make oral cancer control unsuccessful, resulting in increased mortality rates [3].

Hence, it is necessary to detect potentially malignant lesions at their incipient stage. Early diagnosis and initiation of appropriate treatment for early malignant lesions offer the best hope of improving the prognosis [5].

Exfoliative cytology is a non-invasive technique that has been accepted by patients and is a good diagnostic method for the early diagnosis of oral mucosal lesions. Oral exfoliative cytology is particularly valuable for mass screening purposes, with a sensitivity of 94% and specificity of 100%. Recent advances in technology facilitate the use of reliable quantitative techniques such as cytomorphometry, histometry, and computer-assisted image analysis [6].

Papanicolaou is the easiest and most common cytology technique for smear staining and is a routine method for the diagnosis of malignant neoplasms of the cervix. Cytometry is a technique for the characterization and measurement of cells and cellular specifications such as nucleus size, cytoplasm size, nuclear-cytoplasmic ratio, aneuploidy, and diploidy analysis of the nucleus [7].

Oral cavity is the primary site of damage, and the soft tissues of the oral cavity undergo severe insult due to tobacco use. Chronic smokers often experience difficulty in opening their mouth due to changes in the soft tissues, such as the stiffening of the submucosa of the oral cavity, which can lead to oral submucosal fibrosis. The mechanism of opening the mouth involves the temporomandibular joint, the action of mastication muscles, and the adjoining soft tissues. As part of the clinical examination of the oral cavity, it is necessary to understand the range of normal mouth opening. Normal mouth opening is defined as the interincisal distance at maximal mouth opening (MMO). Studies have shown that the measurement of mouth opening varies with age, gender, and race. Our study aims to find a significant correlation between different genotoxic traits in buccal mucosal cells of smokers and non-smokers, as well as their changes across different pack years. Additionally, we will study the effect of smoking on interincisal distance based on the opening of the mouth and its clinical correlation to cytomorphological changes.

2. Materials and Methods

A comparative study was conducted on 200 individuals aged between 20 and 60 years. The subjects were randomly selected from inpatient and outpatient department (IPD and OPD) patients of the Medicine Department at Index Medical College and Hospital. Buccal smears of these patients were processed in the Department of Anatomy. Informed consent was obtained from all participants before the study commenced.

The patients were grouped based on the Pack Year formula [8]:

- Group A: Pack Year < 5
- Group B: Pack Year 5-10
- Group C: Pack Year > 10

A control group (Group D) consisting of 50 samples was also included.

For Cytomorphological Analysis: The area of the buccal mucosa was swabbed using a piece of sterile gauze. Exfoliated buccal cells were obtained by scraping the sides of the cheek 3 to 4 times using a wooden spatula.

The samples were spread on a clean glass slide and immediately fixed with fixation spray to avoid exposure to dry air. The slides were stained with the Rapid Papanicolaou (PAP) staining technique and examined under a light microscope. Various cytomorphological changes, such as binucleated cells, pyknosis, perinuclear halo, cytoplasmic granulation, karyolysis, karyorrhexis, cytoplasmic vacuoles, and cells with micronuclei, were observed.

For Measuring Interincisal Distance, see Figure 1: Subjects were instructed to open their mouth maximally until no further opening was possible. The distance from the incisal edge of the upper incisor teeth to the incisal edge of the lower incisor teeth was measured using a calibrated fiber ruler, and the findings were recorded in ranges of millimeters. Three measurements were taken for each individual, and their average was recorded as the final reading to minimize examiner bias.

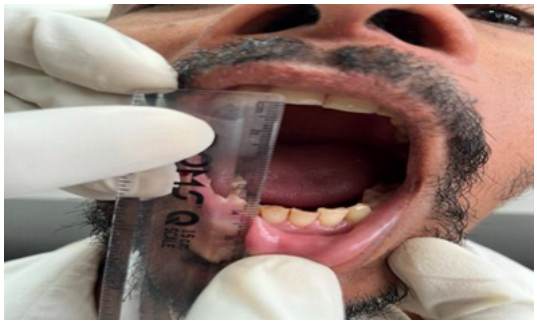

Figure 1. Measuring interincisal distance

2.1. Exclusion Criteria

The following patients were excluded from the study:

- Patients wearing dentures.
- Patients undergoing or having undergone radiation or chemotherapy.
- Alcoholic patients.
- Anaemic patients.
- Diabetic patients.
- Patients with malignant or premalignant lesions of the oral cavity.
- Patients addicted to other forms of tobacco or alcohol.
- Patients with painful oral lesions.
- Patients with a history of maxillofacial trauma.
- Patients with oral malignancies.
- Patients with temporomandibular joint diseases.
- Subjects with reverse overbites.
- Persons with edentulous or no natural front teeth for interincisal distance measurements.

2.2. Inclusion Criteria

The following criteria were considered for inclusion in the study:

- Case group (smokers): Non-anaemic and non-diabetic male patients with clinically healthy mucosa, having only a history of smoking and not having received radiotherapy or chemotherapy in the last 1 month.
- Control group (non-smokers): 50 subjects with no history of smoking, without any systemic illness/anemia, and without diabetes.
- Subjects with submucosal fibrosis were included in the study.

3. Results

The binucleated cells in non-smokers were 0.87 ± 0.83, while in smokers, the mean of binucleated cells was 1.64 ± 1.06. The mean value of pyknosis cells in non-smokers and smokers was 0.93 ± 0.65 and 2.76 ± 1.26, respectively. The mean value of perinuclear halo in non-smokers was 0.51 ± 0.58, and in smokers, it was 1.47 ± 0.86. The mean value of cytoplasmic granules in non-smokers was 0.61 ± 0.49, while in smokers, it was 1.67 ± 0.67. The mean value of karyolitic cells in non-smokers was 0.008 ± 0.34, and in smokers, it was 1.22 ± 0.82. The mean value of karyorrhexis cells in non-smokers and smokers was 0.06 ± 0.24 and 0.46 ± 0.57, respectively. The mean value of cytoplasmic vacuoles in non-smokers and smokers was 0.081 ± 0.34 and 0.29 ± 0.43, respectively. The mean value of cells with micronuclei in non-smokers and smokers was 0.67 ± 0.77 and 3.01 ± 0.94, respectively.

Figure 2 shows that there was a significant difference observed between non-smokers and smokers for cells with binucleation, pyknosis, perinuclear halo, cytoplasmic granules, karyolysis, karyorrhexis, and micronuclei in buccal mucosal cells. However, no significant difference was found for cytoplasmic vacuoles between non-smokers and smokers. Similar studies were conducted on non-smoking populations.

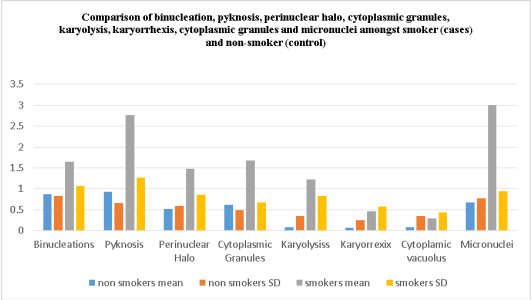

Figure 2. Comparison of binucleation, pyknosis, perinuclear halo, cytoplasmic granules, karyolysis, karyorrhexis, cytoplasmic granules and micronuclei amongst smoker (cases) and non-smoker (control)

We also found a significant difference in interincisal distance among smokers of different pack years. For pack years <5, the mean value was 4.99 ± 1.22; for pack years 5-10, the mean value was 4.79 ± 0.39; and for pack years >10, the mean value was 3.71 ± 0.39. The interincisal distance was found to decrease with an increase in pack years, see Table 1.

Table 2 shows that when comparing the three groups based on pack years, a significant difference was observed between all pack year groups for pyknosis, cytoplasmic granules, perinuclear halo, karyolysis, and micronuclei. However, no significant difference was observed for binucleation, karyorrhexis, and cytoplasmic vacuoles, see Table 3.

Table 1. Comparison of binucleation, pyknosis, perinuclear halo, cytoplasmic granules, karyolysis, karyorrhexis, cytoplasmic granules and micronuclei amongst smoker (cases) and non-smoker (control)

	Non smokers		**Smokers**		**P-Value**
	Mean	**SD**	**Mean**	**SD**	
Binucleations	0.87	0.83	1.64	1.06	0.001 (Significant)
Pyknosis	0.93	0.65	2.76	1.26	0.001 (Significant)
Perinuclear Halo	0.51	0.58	1.47	0.86	0.001 (Significant)
Cytoplasmic Granules	0.61	0.49	1.67	0.67	0.001 (Significant)
Karyolysiss	0.08	0.34	1.22	0.82	0.021 (Significant)
Karyorrexix	0.06	0.24	0.46	0.57	0.035 (Significant)
Cytoplamic vacuolus	0.081	0.34	0.29	0.43	0.65 (Non significant)
Micronuclei	0.67	0.77	3.01	0.94	0.034 (Significant)

Table 2. Comparison of binucleation, pyknosis, perinuclear halo, cytoplasmic granules, karyolysis, karyorrhexis, cytoplasmic vacuoles and micronuclei amungiest smoker groups based on pack <5 years

Pack <5			**Mean value of inter-incisal distance**	**Pack year <5-10**		**Mean value of inter-incisal distance**	**Pack year>10**		**Mean value of inter-incisal distance**	**P value**	
	Mean	SD		Mean	SD		Mean	SD			
Binucleations	1.52	0.84		1.64	1.22		1.87	0.94		0.54	Non-significant
Pyknosis	2.26	1.09		2.38	1.18		4.04	0.85		0.006	Significant
Perinuclear Halo	1.36	0.89		1.67	0.84		1.25	0.73		0.14	Significant
Cytoplasmic Granule	0.73	0.65	5.7+1.29	1.58	1.04	4.79+0.39	2.83	1.27	3.77+0.39	0.002	Significant
Karyolysis	0.42	0.6		0.67	0.63		2.45	0.97		0.008	Significant
Karyorrhexix	0.52	0.69		0.44	0.5		0.54	0.58		0.6	Non-significant.
Cytoplasmic Vacuolus	0.66	0.9		0.58	0.49		0.62	0.57		0.91	Non-significant.
Micronuclei	1.21	0.63		3.11	0.94		4.5	1.1		0.003	Significant

Table 3. The distance from the incision edge of the upper incisor teeth to the incision edge of the lower incisor teeth (mm)

Non Smokers		**Pack <5**		**Pack<5-10**		**Pack >10**	
Mean	SD	Mean	SD	Mean	SD	Mean	SD
5.15	0.40	4.99	1.22	4.79	0.39	3.77	0.39

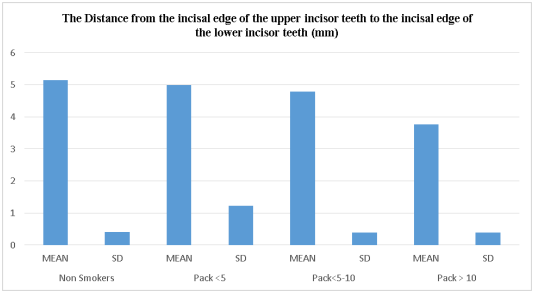

Figure 3

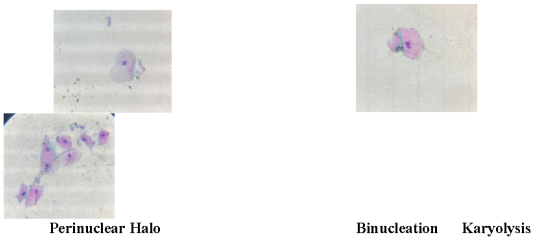

Figure 4

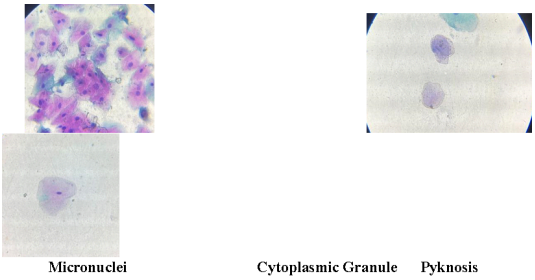

Figure 5

Figure 6

4. Discussion

Among the various cancers prevalent across the world, oral cancer ranks 6th globally. India, being second to China in tobacco consumption, has the highest rates of oral cancer. Researchers have shown that people tend to neglect their oral hygiene status as they are not aware of the relationship between systemic disorders and oral hygiene. Due to the innocuous and asymptomatic nature of this disease, early detection of oral cancer becomes difficult. Detecting potentially malignant lesions at its incipient stage has become the need of the hour. Despite the easy accessibility of oral cancer self-examination, it is usually diagnosed at advanced stages, resulting in poor prognosis and survival rates among patients [9].

The only way to cure the problem of rising trends in oral cancer is through early detection, histopathology investigation, creating awareness for tobacco cessation, and treating tobacco-related oral cancer patients, especially in their pre-malignant state, which may be the only hope in reducing the burden of this disease. In our study, cytomorphological changes were studied in buccal mucosal cells. The study was performed on

150 male smokers from the central Indian population. Pyknosis, binucleation, karyolysis, karyorrhexis, and micronuclei were the nuclear changes observed in the buccal mucosal cells.

Studies suggest that the frequency of binucleation increases in smokers, making binucleation an indicator of cytotoxicity. In the present study, a significant increase in binucleation was seen compared to the control group. Binucleation showed a statistically significant difference between smokers and non-smokers. The mean value for binucleated cells was found to be 1.64 ± 1.06 in smokers and 0.87 ± 0.83 in non-smokers. Twinky M Thomas in 2017, Parmar et al. in 2019, and Sharma et al. in 2021 conducted similar studies on binucleation of buccal mucosal cells in smokers [3,5].

Pyknosis is defined as cells with a small shrunken nucleus having high density of nuclear material, which is intensely stained all over. Our study found that the occurrence of pyknosis was higher in smokers compared to non-smokers. The occurrence of pyknosis was statistically significant, and a positive correlation was obtained with different pack years. In our study, the mean value for pyknosis was found to be 1.64 ± 1.06, which was similar to Sharma et al. in 2021 and Parmar et al. in 2019. In their studies, the pyknosis values were found to be 3.041 ± 0.916 and 2.71 ± 1.74, respectively [3]. However, Hugo V et al. in their study in 2015 found no statistical significance for pyknosis between smokers and non-smokers [10].

Perinuclear Halo is a morphologic finding referring to the presence of a vacuolated area that surrounds the nucleus. It results from nuclear shrinking. In our study, the mean value of perinuclear halo was found to be 1.47 ± 0.86, which is similar to Parmar et al. in 2019 with a mean value of 1.45 ± 1.63 [3].

Parmar et al. in 2019 concluded that the mean value of cytoplasmic granules was 1.61 ± 1.50. In our study, the mean value was 1.67 ± 0.0067, and there was a significant difference [3]. Karyolysis refers to the disintegration and dissolution of the nucleus of a necrotic cell, resulting in a ghost-like appearance on staining. In the current study, the mean karyolytic value in smokers was 1.22 ± 0.82, significantly higher than in non-smokers with a value of 0.08 ± 0.34. Navya BN et al. in 2017, Parmar et al. in 2019, and Prihastuti et al. in 2022 also observed karyolytic cells in buccal mucosal cells of smokers [3,11,12].

Karyorrhexis is characterized by the presence of nuclear fragmentation. It is a step in apoptosis of cells, resulting in the loss of integrity of the nucleus. In our study, karyorrhexis in smokers was more common than in non-smokers, with mean values of 0.46 ± 0.57 and 0.06 ± 0.24, respectively. Parmar et al. in 2019 and Yarmohammadi and Jalayer Naderi in 2023 are a few of the studies with similar findings on karyorrhexis [3,13].

Cells with cytoplasmic vacuoles show multiple clear spherical vacuolization of variable size, which are due to partial or temporary disturbances in the cell membrane permeability [14]. Parmar et al. in 2019 also noted a mean value of cytoplasmic vacuoles of 0.18 ± 0.55, and the mean value in our study was similar, i.e., 0.29 ± 0.43 [3]. Sharma VL et al. in 2013 showed cytoplasmic vacuoles to be 27%, and Seifi S et al. in 2014 found the occurrence of cytoplasmic vacuoles to be 30.8% [7,14].

The Micronucleus assay is a potential biomarker for malignancy. A micronucleus (MN) is a small extra nucleus separated from the main one, generated during cellular division by late chromosomes or by chromosome fragments [15]. It is a microscopically visible round to oval cytoplasmic chromatin mass in the extra-nuclear vicinity [16]. The mean value of micronuclei for smokers was significantly higher than non-smokers, with values of 3.01 ± 0.94 and 0.67 ± 0.77, respectively, see [16].

On comparison of the 8 parameters across different pack years, Binucleation, Karyorrhexis, and Cytoplasmic Vacuoles were not statistically significant. This shows that these parameters are independent of the duration of exposure to smoking. According to Noushin Jalayer Naderi et al., the cytotoxicity effect of cigarette smoking was not significantly correlated to time exposure. Parmar et al. also found no significant difference in karyorrhexis, binucleation, and cytoplasmic vacuoles across different pack years [3,17]. Pyknosis, Perinuclear halo, Cytoplasmic granule, Karyolysis, and Micronuclei show a significant correlation across different pack years.

In our study, a total of 50 non-smoking and 150 smokers' participants were enrolled and equally divided according to three different pack years. The groups were as follows: non-smokers with a mean of 5.15 ± 0.40 and pack year < 5 with a mean value of 4.9 ± 1.22, pack years 5-10 with a mean value of 4.79 ± 0.39, and pack year >10 with a mean of 3.79 ± 0.39. The mean mouth opening in a study comprising of Pakistani, Indian, and Arab population was found to be 53.12 ± 7.95, and in Nishant et al., it was 5.2 ± 6.5. In our study, the mean value was 5.15 ± 0.4, similar to the study on non-smoking populations.

We also found a significant difference in interstitial distance among smokers of different pack years (<5, 5-10, and >10). The interstitial distance was found to decrease with an increase in pack year.

5. Conclusion

The present study indicates that almost all cytomorphological findings were higher in smokers than non-smokers. Early detection of oral cancers becomes complex as they are mostly innocuous and asymptomatic during their initial stages. Cytomorphometric analysis can be used regularly to detect these cell alterations. Currently, the use of exfoliative cytology has increased as an adjunct to screening precancerous lesions and malignancies of the oral cavity. There is little data available on the interincisal distance and its clinical significance. Most of the studies conducted include gutkha and pan masala chewers. Our study is the first to measure the interincisal distance in smokers and discover a significant correlation between interincisal distance and pack years. Through our study, we could conclude that smoking is a causative factor for the reduced interincisal distance of the mouth.

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Conflicts of Interest: Authors declare no conflict of interests.

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