Comparison of cytogenetic abnormality of exfoliative buccal cells among Smokers and Non-smokers

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Abstract

Introduction: Oral cancer is one of the 10 most common cancer among all cancers and oral squamous cell carcinoma is the most common of all oral malignancies. DNA damage can be assessed by considering micronuclei as a biomarker. Oral buccal mucosa is an easily accessible tissue hence DNA damage can be studied conveniently by examining these buccal mucosal cells.

Objective: The aim of the present study was to assess the DNA damage (micronucleus) and cellular death (pyknosis, karyolysis and karyorhexis) of exfoliated buccal mucosal cells.

Materials and Methods: It was a cross sectional study conducted on 50 subjects of young healthy smokers and non-smokers respectively. The buccal mucosal cells were exfoliated using Ayer's spatula and were transferred to in isopropyl alcohol/acetic acid fixative. This specimen was centrifuged and the sediment was stained with PAP stain. Then nuclear phenotypes were checked by counting 500 cells in oil immersion.

Inclusion criteria:

- Smokers who smoked more than 10 cigarettes/day for at least 5 years.
- Only male smokers included.

Exclusion criteria:

- Subjects with history of both alcohol and tobacco intake were excluded.
- Any clinically evident changes in the oral cavity related to the habit.

Results: Among the 50 smokers and 50 non-smokers subjects, Smokers presented with more micronucleated, pyknotic, karyorrhectic and karyolytic cells ($p < 0.0001$) than non-smokers.

Conclusion: Cigarette smoking induces DNA damage and leads to cellular death by increasing the above parameters in buccal mucosa cells and hence these parameters can be considered as indicators in predicting the risk of oral cancer.

Keywords: Exfoliative buccal cells, Micronucleus, Pyknosis, Karyorrhexis, Karyolysis.

Introduction

Cancer is the second most common cause of mortality in developed countries and tenth most common cause of mortality in developing countries like India. The world wide incidence of cancers of larynx, pharynx and oral cavity is approximately 500,000 cases per year with mortality of approximately 270,000 cases per year.(1) The prevalence of oral cancer in the world is mostly associated with the pattern of tobacco products consumption. There exists a dose-response relationship between prevalence of oral cancer and the level of tobacco products consumption.(2)

Major risk factors for oral cavity cancers are chewing of tobacco and cigarette smoking.(3) Lips, palate, tongue and almost all other parts of the oral cavity are susceptible to cancer from tobacco smoking or chewing.(4) Several carcinogens are present in cigarette. Cigarette combustion produces smoke which contains free radicals and other combustion byproducts which are carcinogenic. These free radicals can react with other additives or other combustion byproducts or living cells and cause DNA damage.(5)

Micronucleus assay with exfoliated buccal epithelial cells is a cost effective and a less invasive technique introduced by stich et al and it has been believed that the number of micronucleus is related to increasing effects of carcinogens.(6) Micronucleus (MN), is an oval or round chromatin mass in the cytoplasm which is microscopically visible in the extra nuclear vicinity. It is formed from aberrant mitosis and consists of chromatin fragments, eccentric chromosomes or whole chromosomes, which do not reach the spindle poles during mitosis. For the estimation of DNA damage, MN has been consistently used as a biomarker.(7) The cells are viewed for micronucleus and other cytogenetic anomalies like Pyknosis, karyolysis and karyorrhexis.(8) Hence, the present study was done to assess the DNA damage and cellular death in exfoliated buccal mucosal cells of smokers and non-smokers.

Materials and Methods

Type of study: Cross sectional study

Methodology: This study was conducted in Pathology Department, K.V.G. Medical College and Hospital, Sullia from April 2016 to July 2016. This study was performed on buccal mucosal cells of 100 subjects who were segregated into 2 groups as 50 controls (non-smokers) and 50 cases (smokers) based on the background history of smoking. The volunteers were considered smokers if they had smoked more than 10 cigarettes/day for at least 5 years. The participants were

not consumers of alcohol and tobacco chewing. Ethical Committee clearance was taken and informed consent was obtained from all participants.

Inclusion criteria:

- Smokers who smoked more than 10 cigarettes/day for at least 5 years.
- Both males and females smokers.

Exclusion criteria:

- Subjects with history of alcohol intake and tobacco intake were excluded.
- Any clinically evident changes in the oral cavity related to the habit.

Micronucleus Test in Oral Mucosa Cells: The subjects were asked to rinse the mouth with tap water, followed by which the cells were obtained by scraping the right/left cheek mucosa with wooden Ayre's spatula. Cells were transferred to a tube containing saline solution, centrifuged for 5-10mins then kept in a fixative using 3:1 isopropyl alcohol/acetic acid, and later cells were tranferred onto precleaned slides. The above slides were stained with PAP stain and were examined under 100X oil immersion to determine the frequency of micronucleated cells. 500 cells were scored from each test person. Samples of smokers were obtained approximately 2 hours after the last cigarette.

Data Analysis: Micronuclei were counted as a parameter of DNA damage according to the criteria proposed by Heddle & Countryman. The following nuclear alterations were taken into consideration for measuring the cytotoxicity: pyknosis, karyolysis and karyorrhexis. Results were calculated in percentages.

Criteria for describing micronuclei are:[9]

- A diameter of less than 1/3rd of main nucleus.
- Non-refractile
- Colour identical to or lighter than the main nucleus
- Location 3 or 4 nuclear diameters in the vicinity of main nucleus; and not touching the nucleus

Statistical Methods: To compare the frequencies of cytotoxicity among the samples of smoking and non-smoking individuals, Mann-Whitney non-parametric test was used. Micronucleus frequencies of controls (non smokers) and cases (smokers) were estimated.

Results

The mean age of the smokers was 32 years and that of the non smokers was 30 years. Chi-square test showed that the two groups have no significant difference regarding the age. T-test was used for statistical analysis and $p<0.0001$ was considered as statistically significant. Mean and standard deviation of data were calculated. In our study all 100 subjects constituting smoking and non smoking individuals were of male gender.

Table 1: Distribution of micronuclei in exfoliated buccal mucosal cells of smokers and non-smokers

Groups	Micronucleus	
	Mean	SD
Controls	2.32	1.77
Cases	9.4	1.96

Table 1 shows the mean and standard deviation of micronucleated cells in buccal mucosa cells of smokers and non-smokers. Mean of micronucleated cells among smokers was 9.4 with a standard deviation of 1.96 where as that of non- smokers was 2.32 with a standard deviation of 1.77. There was a significant difference observed that smokers have presented with a higher number of micronucleus compared to non-smokers.

Table 2: Cytotoxicity parameters (pyknosis, karyorrhexis, karyolysis) in buccal mucosa cells of smokers and non-smokers

Groups	Pyknosis		Karyorrhexis		Karyolysis	
	Mean	SD	Mean	SD	Mean	SD
Controls	27.04	6.28	1.14	0.88	0.56	0.57
Cases	55.58	17.39	3.72	2.20	1.94	1.30

Table 2 shows the cytotoxicity parameters in buccal mucosa cells of smokers and non-smokers. There was an increase in pyknosis, karyorrhexis and karyolysis among smokers with a mean pyknosis of 55.58, mean karyorrhexis of 3.72 and mean karyolysis of 1.94 compared to non-smokers. There was significant difference among the cytotoxic parameters of smokers and non-smokers.

Table 3: Distribution of all the parameters with their t-value and p-value

Parameters	Cases	Controls	t- value	p- value
Micronucleus	9.4±1.96	2.32±1.77	18.8754	< 0.0001
Pyknosis	55.58±17.39	27.04±6.28	10.912	< 0.0001
Karyorrhexis	3.72±2.20	1.14±0.88	7.687	< 0.0001

Karyolysis	1.94±1.30	0.56 ± 0.57	6.86	< 0.0001

Table 3 shows the frequency of all the parameters studied in our study with their t- value and p- value. Frequency of micronuclei was found to be significantly higher in smokers (9.4±1.96) as compared to non-smokers (2.32±1.77). Frequencies of pyknosis (55.58±17.39), karyorrhexis (3.72±2.20) and karyolysis (1.94±1.30) were also seen to be significantly higher in smokers as compared to the non-smokers. T-value and p-value for each parameter was calculated and showed that there was significant statistical difference among all the parameters of smokers and non-smokers with a p value<0.0001.

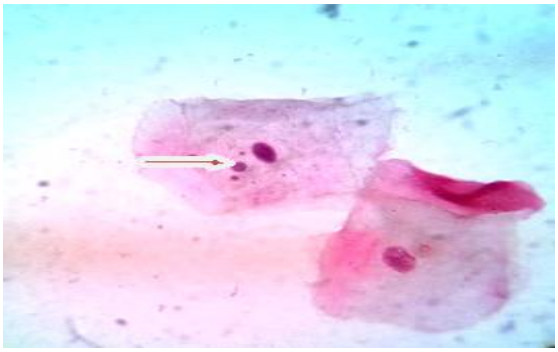

Fig. 1: The photomicrograph at high power shows Micronuclei

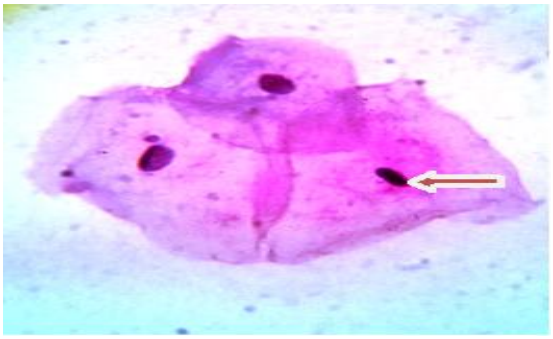

Fig. 2: Pyknotic nuclei

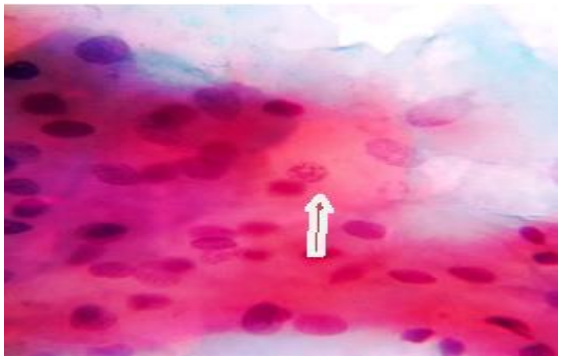

Fig. 3: Karyorrhexis

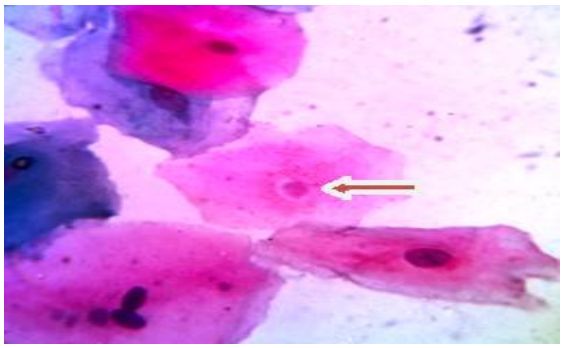

Fig. 4: Karyolysis

Discussion

Oral cancer is one of the most devitalizing disease which afflict humans. The world wide incidence of cancer is on a rise today despite the best efforts of clinicians and researchers.[1] The purpose of recent studies now is prevention and early diagnosis of malignant lesions because of expensive therapeutic and rehabilitating procedures of these lesions.[2]

Buccal cells form the primary barrier for the ingestion and inhalation route. They are capable of metabolizing proximate carcinogens to reactive products. Epithelial cells give rise to approximately 90% of human cancers. Hence, oral epithelial cells serve as a main target for early genotoxic events caused by carcinogens which enter the body through ingestion and inhalation.[10]

The use of the micronucleus test on exfoliated cells as a means to detect genotoxic damage in human tissues which are the main targets for organ specific carcinogens is well-established. Micronuclei are produced in the daughter cells as a result of chromosomal damage due to carcinogens in dividing basal cells.[7] The formation of micronuclei and other cellular anomalies from either acentric chromosome fragment or a whole lagging chromosome occurs as a result of chromosome breakage due to unrepaired or misrepaired DNA strand breaks or malsegregation of the chromosomes due to mitotic malfunction.[6]

For estimating genomic damage, micronuclei can be easily assessed in lymphocytes, erythrocytes and exfoliated epithelial cells (e.g., oral, nasal, urothelial). Therefore for studying the genotoxic effect, micronuclei assay can be carried out in buccal cells. In general population, the mean prevalence of cells having micronucleus is only 0.0 to 0.9%. Any difference in this range of micronucleus can be due to chromosomal alterations.[11]

The results of mean number of micronuclei among men and women were controversial. Some researchers have reported higher micronuclei values in women and the others have shown higher values in men. Also, controversies are prevailing regarding the age of the subjects. In the present study all the cases were men

and were age matched, to exclude the effect of age and sex on the results.

In previous studies, tobacco and its different forms with cigarette smoking were compared and it was observed that the subjects were consumers of several different agents. In the present study, the effect of cigarette smoking alone over nuclear anomalies has been examined. The role of confounding factors especially alcohol consumption has been removed as all the subjects studied were not consumers of alcohol.

In our present study, micronuclei count in smokers group was assessed and compared with micronuclei count among non-smokers group to identify which population group was at a higher risk of cancer. A definite correlation between the duration of smoking and occurrence of micronuclei was seen in the study done by Kamath VV et al.[7] In our study a significant increase in the micronuclei was noted among smokers compared with non-smokers denoting the genotoxic effect of cigarette smoking.

In our study we have also studied the effect of smoking on other nuclear components such as pyknosis, karyorrhexis and karyolysis. Pereira da Silva et al performed a similar study on smokers and non smokers and studied the effect of smoking on all the parameters such as micronucleus, pyknosis, karyorrhexis and karyolysis in a cell and showed that there was significant increase in frequency of micronucleus in buccal smears of smokers but the study did not show increase in pyknosis, karyorrhexis, karyolysis in them.[8] In our study we have evaluated that the frequencies of pyknosis, karyorrhexis and karyolysis was found to be significantly higher in the smokers compared to the non-smokers which was in conformity to a study conducted by Yadav A S et al.[6]

Several reports have concluded that cigarette smoking and other forms of tobacco consumption increases the DNA damage in buccal mucosal cells which was in discordance with Stich and Rosin who obtained different results. These differences can be due of different staining techniques, varying number of samples and difference in smoking and smokeless agents consumed.[6] Hence, standardization of the applied techniques is required to draw more decisive results.

Conclusion

This study demonstrated that the frequency of micronucleus, pyknosis, karyorrhexis and karyolysis in the buccal mucosal cells of smoking individuals were significantly higher when compared to that of nonsmoking subjects ($p<0.001$). Therefore, study of PAP stained oral buccal smears is a non-invasive procedure and an easy method for oral cancer screening and can also be advocated as an educational tool in smoking cessation counseling. However, to obtain justifiable and reliable values, larger groups with history of number of packs smoked per day and the smoking period should be considered.

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