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ORIGINAL RESEARCH

Biomonitoring of Buccal Mucosa Cells in Chronic Smokers and Nonsmokers

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ABSTRACT

Background: Despite the well-known presence of carcinogens in tobacco smoke, results in the scientific literature linking smoking habits to micronuclei frequency are rather controversial.

Aim: The study was conducted to compare and evaluate the frequency of micronuclei in chronic smokers and nonsmokers in relation to habit history, personal history, and other related factors like occupation, exposure to radiation, etc.

Materials and methods: A total of 50 randomly selected male subjects were included in the study. Case and control groups (smokers and nonsmokers respectively) comprised 25 subjects each (mean age in controls = 38.24 ± 2.7; mean age in smokers = 39.32 ± 3.8).

Results and conclusion: There was a significant relation between tobacco consumption and frequency of micronucleated cells ($p = 0.05$) and between radiation exposure and presence of micronuclei ($p = 0.05$) in controls and subjects. Within the smokers group, left cheek scrapings showed higher count ($p = 0.05$; significant) for the micronucleated cells as compared with right cheek scrapings.

Clinical significance: In this study, an attempt was made to estimate the cytogenetic damage in oral mucosa in people habituated to smoking beedi. Though tobacco plays an important role in micronuclei generation, other factors like ionizing radiation and personal habits also contribute to micronuclei frequency. Site of smear, sample size, nuclear specific stain usage are some of the contributing factors. In addition, cytogenetic alterations like karyolysis, pyknosis, etc., can be included in future studies to increase the specificity.

Keywords: Buccal mucosa cells, Micronucleus, Tobacco smoking.

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INTRODUCTION

Cigarette smoke is a complex mixture of over 4,000 chemical compounds that readily react to form other reactive substances, which cause deoxyribonucleic acid (DNA) damage and cytogenetic effects.[1,2] Beedi (Bidi) is an indigenous cigarette in which low-grade tobacco is hand-rolled in a tendu (*Diospyros melanoxylon*) leaf and tied with a cotton thread.[3] Despite the well-known presence of carcinogens in tobacco smoke, results in the scientific literature linking smoking habits to micronuclei frequency are rather controversial.[4]

A variety of assays have been proposed as potential biomarkers in biomonitoring studies, but these methods are typically laborious and time consuming or require highly trained technicians to accurately read and interpret slides. For this purpose, a great deal of enthusiasm was raised by the application of the micronucleus test to uncultured exfoliated cells.[2,5,6] Buccal epithelial cells not only provide an alternative source, but also have the advantage of rapid and easy sampling.[5]

Micronucleus arises from acentric fragment or whole chromosomes which are not included in the main nuclei of daughter cells. The formation of micronuclei can be induced by substances that cause chromosome breakage (clastogens) as well as by agents that affect the spindle apparatus (aneugens). The formation of micronuclei is attributed to various exogenous and endogenous factors. Exogenous factors include radiation, chemical agents, and microorganism invasion. Endogenous factors include genetic defects, pathological changes, deficiency of essential nutritional ingredients (e.g., folic acid), and injuries induced by deleterious metabolic products (such as reactive oxygen species).[7-19]

Therefore, a study was conducted to evaluate and compare the frequency of micronuclei in chronic smokers and nonsmokers and the frequency of micronuclei in all the subjects in relation to habit history, personal history, and other related factors like occupation, exposure to radiation, etc.

MATERIALS AND METHODS

Investigated Population

The study consisted of 50 individuals – 25 chronic smokers (aged between 35 and 45 years, with consumption of 10 or over 10 beedi per day for at least 10 years, with no alcohol intake) and 25 nonsmokers (aged between

35 and 45 years with no smoking and alcohol intake history). The subjects were enrolled randomly among apparently healthy subjects attending the outpatient department. A questionnaire recorded patient-specific data for social history and harmful chemical and X-ray exposure (head–neck area). Furthermore, information regarding age, gender, general history, permanent medication, oral hygiene, and nutritional habits was included. Alcohol and tobacco consumption was quantified.

EQUIPMENT AND MATERIALS

Probes, mirrors, tweezers, kidney trays, masks, gloves, cotton rolls, glass microscopic slides, beakers, conical flasks, Coplin jars, distilled water, methanol, glacial acetic acid, concentrated HCl, and a binocular microscope were used for the study.

Glacial acetic acid was purchased from Sisco Research Laboratory (India), methanol and ethanol from Ranbaxy (India), and conc. HCl from Qualigens (India).

Equipment and glassware used in the study included beakers (250, 500, and 1,000 mL), conical flasks, and Coplin jars (all from Borosil, India), glass microscopic slides (Blue Ribbon, India), and a binocular microscope (Olympus, New Delhi).

Aceto-orcein (SRL, India) was the nuclear stain used in this study.

Exclusion Criteria

Exclusion criteria included tobacco chewers; alcoholics; gutkha/paan chewers; subjects with oral lesions – ulcers and mucosal lesions (smoker's palate, leukoplakia, carcinoma, etc.).

Inclusion Criteria

Inclusion criteria included age between 35 and 45 years; beedi/cigarette smoker; frequency – ≥10 beedis/cigarettes per day; duration – ≥10 years.

Sample Collection

After thorough rinsing of the mouth, samples were collected from the left and right buccal mucosa using a wooden spatula. The first smears were discarded and the second smears were spread on clean glass slides. For each individual, two slides were prepared (one from each left and right cheek smear) and brought to the laboratory in a wooden box for further processing within 3 to 4 hours.

Fixation

Fixation of the slide preparations was done in chilled, freshly prepared fixative (3 methanol:1 glacial acetic acid) for 20 to 25 minutes for removing debris and fixing the cells. These were then air-dried.

Hydrolysis

The dried slides were hydrolyzed (1N HCl – 1.2 µL concentration HCl and 98.2 µL dH_2O) for 8 minutes at 60°C followed by washing with distilled water and air-drying.

Staining

The slides were first stained in 2% aceto-orcein for 25 min at 40°C, washed in ethanol and water. The slides were rinsed with ethanol followed by rinsing with distilled water, and again air-dried and coded (Fig. 1).

Principle of Staining

Aceto-orcein stains the nucleic acids present in the nucleus as they contain histone proteins.

Scoring

The coded slides were observed under a binocular microscope at 40× magnification. The slides were made permanent by mounting in distyrene plasticizer xylene (DPX) and then covering them with coverslips. A total of 2,000 cells were scored for the presence of Micronuclei (MN) at 40× magnification, and their presence was confirmed under 100× magnification (oil immersion) and also randomly by another scorer.

Statistical Analysis

Data were entered in Microsoft Excel using Windows 7 version and were compiled and prepared for compatibility with the statistical software Statistical Package for the Social Sciences (SPSS) version 19.0. Descriptive statistics and inferential statistics include mean medium mode, Pearson chi square & p value; along with cross tabulations of presence of micronuclei in habit users, frequency and radiation exposure. Mean, standard deviation, variance, and standard error values were calculated for all the

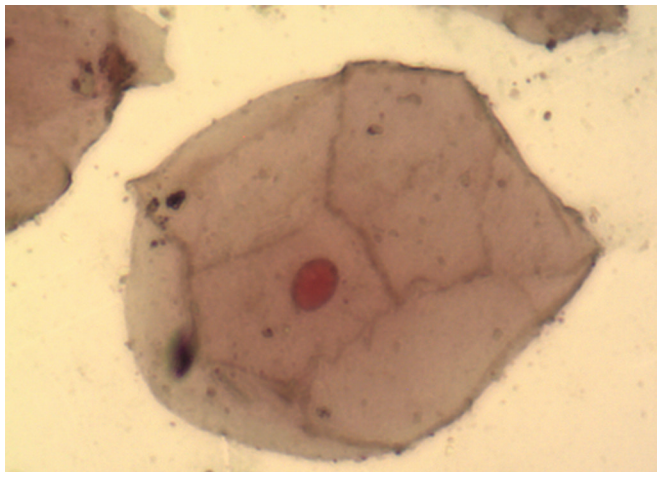


Fig. 1: Micronucleus in buccal mucosa cell

numerical variables wherever needed. For most of the categorical variables, the chi-square test was performed to study the association between various parameters; p values were calculated using $p < 0.05$ as significant association.

RESULTS AND OBSERVATIONS

Different parameters were studied after obtaining baseline data of all the respondents like age, education, occupation, dietary habit, brushing habit, frequency of brushing, habit of smoking, duration of smoking, frequency of smoking, and radiation exposure. The Micronucleated (MNd) cells were calculated from the scrapings of left and right buccal mucosa. Total MNd cells were also calculated for each subject.

Cross-tabulations of tobacco consumption habit by the subjects and total MNd cells showed significant association and was done using Pearson chi-square (5.89) having degrees of freedom (df = 2) and p = 0.05 (Table 1).

Cross-tabulation for age of respondents (smokers/controls) showed no significant association (p = 0.18) (Graph 1).

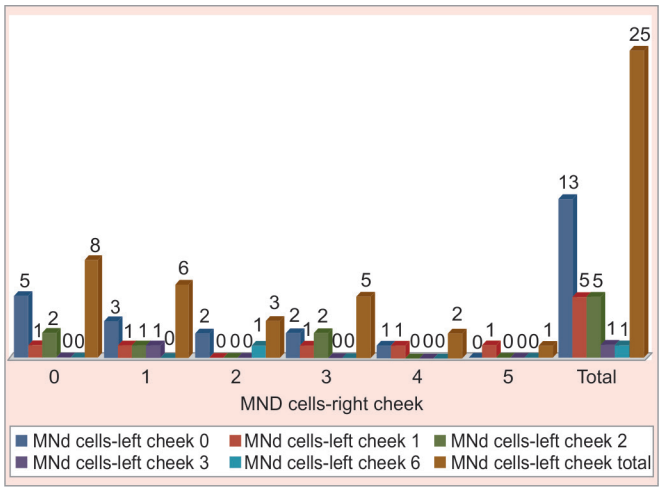
Cross-tabulation of micronucleated cell count from right and left buccal mucosa scrapings in the smokers group showed higher micronucleated cell count in left buccal mucosa scrapings compared with right buccal mucosa scrapings. The association was found to be significant with p = 0.05 (Graph 2).

Table 1: Cross-tabulations of tobacco consumption habit by the subjects and total MNd cells

	Total MNd groups			
Smokers/Controls	*<3*	*>6*	*3–6*	*Total*
Controls	18	0	7	25
Smokers	20	3	2	25
Total	38	3	9	50

Pearson chi-square = 5.89, df = 2, p = 0.05, significant association

The frequency of habit also had a significant bearing among smokers and controls (Table 2). Various frequency groups – 0, 10 to 15, 15 to 20, 20 to 25, >25 – were compared in smokers and controls. The Pearson chi-square test was used and four degrees of freedom were assessed (Graph 3). A significant association between radiation exposure and presence of micronuclei was seen. Five subjects exposed to radiation either daily or for less than 15 days showed three to six MNd cells, whereas a similar number

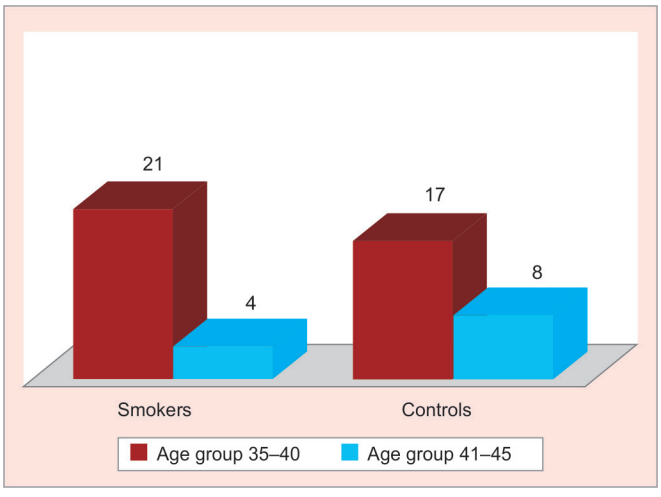


Graph 2: Micronucleated cells from right and left buccal mucosa

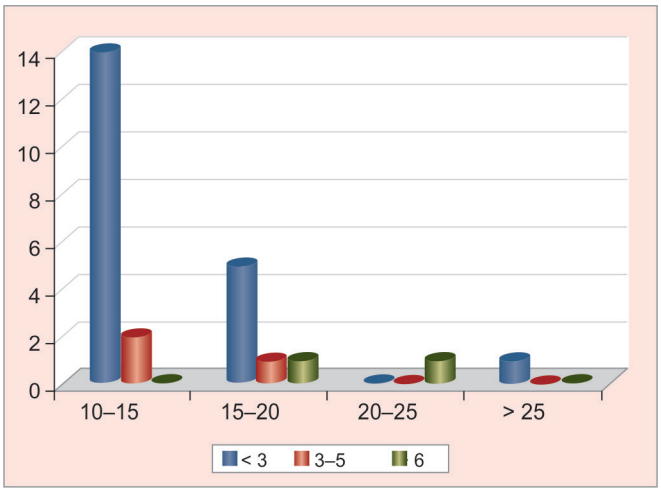
Table 2: The frequency of habit among smokers and controls

Habit Freq Groups Smokers/Controls Cross-tabulation			
	Smokers/Controls		
Habit freq groups	*Controls*	*Smoker*	*Total*
0	25	0	25
10–15	0	16	16
15–20	0	7	7
20–25	0	1	1
>25	0	1	1
Total	25	25	50

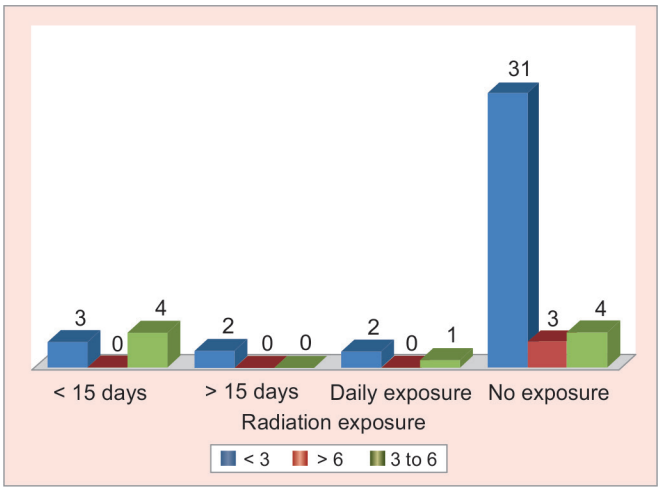
Pearson chi-square = 50, df = 4, p = 0.00014, strongly significant



Graph 1: Age of subjects among smokers and controls



Graph 3: Micronucleated cells in relation to habit frequency of subjects in the smokers group



Graph 4: Micronucleated cells in relation to radiation exposure

of subjects not exposed to radiation still exhibited an equal number of MNd cells. However, radiation exposure was not significant among those having a higher number of total MNd cells (Graph 4).

DISCUSSION

The micronucleus test has been used extensively in a variety of exploratory and mechanistic investigations to understand the basic mechanisms underlying genotoxicity.[8] Factors that can influence genetic damage include chemical and physical genotoxins, diet and climatic changes, etc. The presence of Micronuclei (MNi) can also be seen after irradiation in actively proliferating tissues. MNi provides a convenient and reliable index for both chromosome breakage and chromosome loss.[20-22]

Tobacco-specific nitrosamines (TSNA) have been found to be soluble in saliva. They are potent carcinogenic agents responsible for the induction of chromatid and chromosome aberrations, resulting in increased frequencies of MNi.[1] Stich and Rosin[23] found an eightfold increase in MNd mucosa cells among alcohol drinkers who smoked three or more packs of cigarettes per day, and an approximately 4.2-fold elevation when one to two packs were consumed. Alcohol and tobacco both act synergistically to cause chromosomal defects resulting in the formation of MNi. Another study by Stich et al[6] also showed a significant association between reverse cigar smokers and controls as TSNA present in the cigar act as potent clastogenic and mutagenic agents. In a study by Suhas et al,[3] a significant association was seen between smoking and frequency of MNi in buccal mucosa cells as beedi smoke containing tobacco has TSNA, which are potent carcinogens causing MNi formation. On the contrary, Angilieri et al[2] demonstrated no increase in MNi frequency between smokers and nonsmokers in exfoliated buccal mucosa cells and cells from lateral borders of the tongue. In our study, a significant relation between tobacco consumption and number of MNi cells in controls and subjects was found as tobacco increases clastogenic and aneugenic effects on the buccal mucosa cells, although no significant association ($p = 0.14$) was seen between total MNd cells obtained from smokers and controls. The results can be correlated with the results of the study done by Nersesyan et al, which concluded that micronuclei assays in exfoliated oral mucosa cells of smokers and nonsmokers depend strongly on the staining methods. Higher micronuclei frequencies were found in smokers with non-DNA-specific stains like Giemsa, and no significant differences were seen with acridine orange, Feulgen, etc.[24] However, in our study, aceto-orcein stain was used as the nuclear stain which specifically reacts with histone proteins in the nucleus. According to Bonassi et al, smokers do not experience an overall increase in MNi frequency in lymphocytes, although when the interaction with occupational exposure is taken into account, heavy smokers were the only group showing a significant increase in genotoxic damage as measured by the micronucleus assay in lymphocytes. A small decrease in MNi frequency in current smokers (frequency ratio (FR) = 0.97, 95% confidence interval (CI) = 0.93–1.01) and in former smokers (FR = 0.96, 95% CI = 0.91–1.01) was seen when compared with nonsmokers. This is because lymphocytes are not directly exposed to the carcinogens in tobacco to induce cytogenetic alterations.[4]

The frequency of habit showed significant ($p = 0.00014$) bearing among smokers and controls. As the frequency of habit increases, i.e., as the number of beedis smoked per day increases, the number of micronucleated cells in the buccal mucosa also increases due to the proportional increase in the potential carcinogenic environment. Bloching et al showed a highly significant correlation between increasing tobacco consumption (daily cigarettes and pack per years) and a higher MNi count because of increased resultant cytogenetic damage. The amount and duration of tobacco abuse is directly correlated with increased exposure to carcinogenic contents in tobacco.[25] Gabriel et al could not detect dose effect, because there was no significant difference in the micronucleus frequency between those who smoked <20 cigarettes per day and those who smoked >20 cigarettes per day (9.3 ± 0.4 and 10.1 ± 0.8 respectively) as only a small number of subject ($n = 8$) showed increased habit frequency. However, a higher sample size was required to confirm the test results.[26]

The increase in MNi with age is due to a combination of factors which include (i) the cumulative effect of acquired mutations in genes involved in DNA repair, chromosome segregation, and cell cycle checkpoint and (ii) numerical and structural aberrations in chromosomes

caused by exposure to endogenous genotoxins, inadequate nutrition, exposure to environmental or occupational genotoxins, as well as a wide range of unhealthy lifestyle factors. Popova et al[27] showed a significant association with the frequency of MNi in buccal mucosa of the investigated population ($p < 0.01$). Suhas et al observed a weak correlation between age and micronucleus cell count for both cases ($r = 0.27$) and controls (0.36).[3] Piyathilake et al[28] also showed no significant difference in the distribution of age in chronic smokers and nonsmokers. However, in the present study, there was no significant association ($p = 0.18$) between smokers and controls in relation to age. This could be due to the narrow age range (between 35 and 45 years) in the study.

Micronuclei frequency tends to be greater in females than in males. The increase in MNi frequency in females can be accounted for by the greater tendency of the X chromosome to be lost as MNi relative to other chromosomes, and to the fact that females have two copies of the chromosome compared with only one in males.[29] However, in our study, only males were taken into consideration.

Various studies related to MNi frequency and site specificity have been done. Suhas et al observed a significant difference in mean MNd cell count between cases and controls ($p < 0.01$) for the buccal mucosa and palate, but not for the tongue. The dilution effect of pooled saliva from the submandibular and sublingual salivary glands and the relatively short time, due to swallowing of saliva, during which TSNA dissolved in saliva are actually in contact with tissues from the oral sites in question, may be the reason for the low MNd cell count in scrapings from the tongue.[3] In the smokers group, left buccal mucosa scrapings showed higher count for the MNd cells as compared with right buccal mucosa scrapings, i.e., with a significant association ($p = 0.05$). The probable reason behind this can be due to differential force in scraping the mucosa. However, no significant association was found between the locations of scrapping of MNd cells in the smokers group and the controls.

Cerqueira et al showed that the frequency of MNi was significantly higher after exposure ($p < 0.05$), as were the frequencies of nuclear alteration ($p < 0.001$). The ionizing radiation interacts with the DNA and other nuclear components resulting in mutations of the genetic material. These cytogenetic alterations cause the formation of MNi.[30] Angelieri et al[2] found no statistically significant difference ($p > 0.05$) in MNd cells before *vs* after X-ray exposure for buccal mucosa and lateral border of the tongue in either smokers or nonsmokers, although increased cytogenetic alterations were seen in the cells. Popova et al found no significant increase in the frequency of cells with MNi and total number of MNi after panoramic tomography. Most of the damage that leads to MNi formation takes place in the basal layer, where cells undergo mitosis. Rapid turnover of epithelial tissue brings the cells to the surface when they exfoliate. As a result, the maximum rate of micronuclei formation in exfoliated cells is seen 1 to 3 weeks after exposure to the genotoxic agent.[2,29,30] In the present study, a significant association ($p = 0.05$) between radiation exposure and presence of MNi was seen. Five subjects exposed to radiation either daily or for less than 15 days showed three to six MNd cells, whereas a similar number of subjects not exposed to radiation still exhibited an equal number of MNd cells. However, radiation exposure was not significant among those having a higher number of total MNd cells.

CONCLUSION

It can be concluded from this study that tobacco plays a very important role in the generation of micronuclei. However, other factors like site of smear and ionizing radiation also seem to contribute to the micronuclei frequency. A significant relation ($p = 0.00014$) between tobacco consumption and frequency of MNd cells and between radiation exposure and presence of MNi ($p = 0.05$) in controls and subjects was seen. Within the smokers group, left buccal mucosa smears showed higher count for the MNd cells as compared with right buccal mucosa smears ($p = 0.05$). There was no significant association between age ($p = 0.18$) and total MNd cells obtained from the smokers and controls group ($p = 0.14$). The extent to which environmental, chemical, and physical carcinogens play a role in the formation of micronuclei remains to be investigated to arrive at a definite conclusion. Cytogenetic alterations like karyolysis, pyknosis, broken egg, etc., have not been included for biomonitoring in our study. To eliminate the bias resulting from these parameters, they also can be included to increase the specificity of the study.

CLINICAL SIGNIFICANCE

In this study, an attempt was made to estimate the cytogenetic damage in oral mucosa in people habituated to smoking beedi. Various biomarkers are used in biomonitoring studies, but these are typically laborious and time consuming or require highly trained technicians to accurately read and interpret slides. For this purpose, use of the micronucleus test to uncultured exfoliated cells is gaining momentum these days. Buccal epithelial cells facilitate economical, rapid, and easy sampling.

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