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MICRONUCLEUS INVESTIGATION IN EXFOLIATED BUCCAL CELLS AMONG TOBACCO CHEWERS/ SMOKERS AND CONTROLS

CAPLASH S*, MEENAKSHI AND KAUR S

Department of Human Biology, Punjabi University, Patiala-147002, Punjab, India

***Corresponding Author: E Mail: shruticaplash@gmail.com**

ABSTRACT

Micronuclei (MN) are cytoplasmic chromatin masses with the appearance of small nuclei that arise from lagging chromosomes at anaphase or from acentric chromosome fragments and are formed by chromosomal damage in the basal epithelial cells. MN assay is a well validated method for genotoxicity testing. In the present study, micronucleated cells (MNC's) were evaluated from exfoliated buccal mucosal cells. A total of 115 subjects including 65 tobacco chewers/ smokers and 50 controls (non-tobacco chewers/ non-smokers) were studied. 500 cells per person were examined. The mean frequency of MN was found to be significantly higher ($p<0.05$) in tobacco exposed individuals (0.76 ± 0.74) compared to the control group (0.03 ± 0.06). Two confounding factors i.e. age and duration of exposure showed significant association while the third confounding factor i.e. alcohol intake did not show any significant association with the MN frequency.

Keywords: **MN assay, Buccal Cell Micronuclei, Tobacco Chewing/Smoking**

INTRODUCTION

Modern man lives in a hazardous environment and is thus continuously exposed to a large variety of natural and synthetic pollutants. These toxic pollutants may either cause mutation of germ cells resulting in accumulation of heritable abnormal genes or may lead to mutation of somatic cells leading to formation of tumors. Tobacco one of such toxic pollutants, is an agricultural product processed from the leaves of plants in the genus nicotiana. In addition to nicotine, it contains carcinogenic agents including nitrites

and alkylating agents. In developing countries like India, it is mainly consumed in two forms: smoked tobacco products and smokeless tobacco **[1, 2]**. Regular consumption of tobacco in any form (chewing or smoking) has been strongly associated with cancers of the mouth, pharyngeal cavity, and upper digestive tract **[3]**. The International Agency for Research on Cancer has declared that there is sufficient evidence for the carcinogenicity of tobacco smoke. Further, epidemiological studies have shown that cancer mortality and morbidity are increased in cigarette smokers. The risk for various cancers has been shown to increase in individuals exposed to tobacco smoke from two-fold to more than 10-fold as compared to controls **[4]**. Chewing of tobacco along with various ingredients like areca nut, catechu, lime, cardamom, permitted spices, unspecified flavoring agents have also been reported to possess cytotoxic, mutagenic and genotoxic properties **[5, 6, 7]**. Positive associations between oral cancer and the habit of chewing areca quid have also been reported **[5, 8-15]**.

Cytogenetic damage is considered as an excellent biomarker for determining the effects of tobacco **[5, 16]**. This marker can be studied by various methods such as chromosomal aberrations (CA's), sister chromatid exchanges (SCE's) and micronuclei test. Micronucleus assay is a well validated method for testing genotoxic effects of various agents. It is the only biomarker that allows the simultaneous evaluation of both clastogenic and aneugenic effects in a wide range of cells. Micronuclei originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division **[17, 18]**. The MN assay in exfoliated buccal cells has great potential to serve as a biomarker and it is an excellent choice to study the genotoxic effects of tobacco because oral epithelial cells are directly exposed to tobacco along with other ingredients. No doubt, the collection of buccal cells is the least invasive method available for measuring DNA damage in humans, especially in comparison to obtaining blood samples for lymphocyte and erythrocyte assays, or tissue biopsies **[19]**.

The purpose of the present study was to evaluate micronuclei (MN) in exfoliated buccal mucosal cells of individuals with tobacco chewing and/or, smoking habits.

MATERIAL AND METHODS

Subjects

The study samples were collected from 115 individuals comprising 65 tobacco chewers/ smokers (beedi or cigarette smokers) and 50 controls (non-tobacco chewers/ non-smokers)

including 24males and 26 females. Informed consents were obtained from each individual. The salient characteristics of the subjects were recorded by interview using a pre-designed questionnaire. It included demographic information, occupational history as well as history of tobacco exposure, and alcohol consumption.

Micronucleus Analysis

Each individual was asked to rinse his mouth thoroughly with water before taking the samples. Exfoliated cells were obtained by scraping of buccal mucosa with a metal scapula. After discarding the first scraping, the second scraping was collected in centrifuge tubes containing PBS and taken to laboratory. After 2-3 washings with PBS, the cells were fixed in 80% methanol in the ratio of 5:1 to make final cell suspension. About 50 microlitre cell suspension was placed onto pre-cleaned slides and air-dried. The slides were stained with Maygrunwald stain and then counterstained with 10% Giemsa. The dried slides were examined under 200X magnification using Zeiss microscope and 500 cells were scored for each individual. Clear intact well stained cells were selected for finding the frequencies of micronuclei.

Data Analysis

Mean values of frequencies of cells with MN obtained from tobacco users were compared with those of non-users (non-tobacco chewers/ non-smokers) by student's t-test and ANOVA using statistical software SPSS (version 16.0).

RESULTS

Mean ages of tobacco exposed individuals and controls were approximately 39 years and 28 years respectively. **Table 1** represents the average number of micronucleated cells in the subjects exposed to tobacco and controls. The mean frequency of micronucleated cells in tobacco exposed group (0.76±0.74) was found to be significantly higher ($p<0.05$) as compared to the control group (0.02±0.06).

Intra-group comparison among tobacco exposed group in **Table 2** shows that 40% and 43% of the study subjects were habitual smokers and chewers, respectively; 17% were both. The results revealed that mean MN frequency was highest for smokers who were not chewers (SNC), followed by non-smokers but chewers (NSC) and, least for both smokers and chewers (SC). The differences among these three groups were statistically significant ($p=0.019$).

The frequencies of micronuclei in tobacco exposed group stratified by each variable are listed in **Table 3**. The group under study was analyzed based on age (intervals: 20-34, 35-49, 50 and above), duration of tobacco exposure (intervals: 1-20 and 21-40 years)

and alcohol drinking. Age and duration of exposure were significantly associated with MN frequency. The older the individual and more exposed to tobacco (years), the higher was the MN frequency. However, there were no significant differences between the mean percentages of MN cells in alcohol drinkers and non-drinkers ($p>0.05$).

Table 1: Comparison of Frequency of MN in Tobacco Exposed and Control Subjects

Category	N	Mean Age (Age Range)	Cells With MN Mean ± S.D.	SEM	P Value[a]
Tobacco Exposed	65	27.7 (21-60)	0.76±0.74	0.09	0.000*
Controls	50	39 (20-65)	0.02±0.064	0.008	

NOTE: [a] By student's t-Test; *p<0.05

Table 2: Comparison of Frequency of MN Among Tobacco Exposed Subjects

	Category	N	Mean age (Age Range)	Cells with MN Mean ± S.D.	P Value[b]
Tobacco exposure	Smokers, Non-Chewers (SNC)	26 (40%)	0.95 ± 0.60	0.11	0.019*
	Non-Smoking Chewers (NSC)	28 (43%)	0.80 ± 0.89	0.16	
	Smoking Chewers (SC)	11 (17%)	0.22 ± 0.20	0.07	

NOTE: [b] By ANOVA; *p<0.05

Table 3: Comparison of Frequency of MN Cells Stratified by Potential Confounding Variables

Variables	Stratification	N	Cells with MN Mean ± S.D.	SEM	P Value
Age	20-34	28 (43%)	0.51 ± 0.83	0.15	0.03*[b]
	35-49	23 (35%)	0.76 ± 0.60	0.13	
	50 & above	14 (22%)	1.25 ± 0.52	0.14	
Duration of Tobacco Exposure (Years)	0-20	45 (69%)	0.62 ± 0.78	0.11	0.01*[a]
	21-40	20 (31%)	1.07 ± 0.54	0.12	
Alcohol Drinking	Yes	48 (74%)	0.85 ± 0.75	0.11	0.10[a]
	No	17 (26%)	0.52 ± 0.67	0.16	
• Smokers and Alcohol Drinkers • Non-Smokers and Non-Drinkers		21 45	1.01 ± 0.60 0.015±0.04	0.13 0.007	0.0000*[a]

NOTE: [a] By Student's t-Test; [b] By ANOVA; *p<0.05

DISCUSSION

The micronucleus (MN) test has been receiving increasing attention as a simple and sensitive short-term assay for the detection of environmental genotoxicants **[20]**. MN are small fragments of extranuclear DNA formed during cell division which provide a non-specific but quantifiable marker of DNA

damage, so it is used to identify cellular damage caused by carcinogenic agents like tobacco **[21]**. The present study was conducted to analyze tobacco-related genotoxic effects in tobacco exposed individuals by evaluating MN from buccal mucosal cells. We observed an elevated incidence of MN in buccal cells of smokers and smokeless tobacco users than in non-smokers/ non-users. This result was statistically significant ($p<0.05$) and was compatible with findings of some other investigators **[22-28]**.

The potential confounding factors, including age and duration of exposure were found to be associated with significant increase in MN frequency. Although many studies have reported the age of subjects but only a fraction of these studies were able to establish a statistically significant effect of advanced age on MN frequency **[23, 26, 29-30]**. The effects of increase in age on increasing MN frequency might reflect accumulated genetic damage occurring during the life span. Alcohol too, is considered as a genotoxic agent, being cited as able to potentiate the development of carcinogenic lesions. In our study, alcohol consumption did not appear to influence the frequency of micronuclei ($p=0.10$) although drinkers had higher mean frequency. Stich and Rosin **[20]** studied alcoholic individuals and reported the absence of significant differences concerning MN frequencies in buccal cells. However, the same study **[20]** concluded that neither alcohol nor smoking, alone, increased MN frequency in buccal cells, but a combination of both resulted in a significant elevation in micronucleated cells in the buccal mucosa. Similar results were seen in our study, combination of both smoking or chewing tobacco and alcohol consumption resulted in a significant elevation in micronucleated cells in the buccal mucosa. This result is at variance with some previously reported studies, in which a highly significant increase in MN frequency was observed in alcoholics **[29, 30]**. Nevertheless, the low number of subjects in each group in our study might not allow a definitive conclusion to be drawn on the effects of these habitual factors on MN frequency in our study population.

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