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

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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 using Geimsa stain. A total of 80 subjects including 50 tobacco chewers/ smokers/alcoholic and 30 controls (non-tobacco chewers/ non-smokers) were studied. 100 cells per person were examined. The mean frequency of MN was found to be significantly higher ($p<0.05$) in smokeless tobacco (SLT) + smoker + alcohol taking individuals (3.40 ± 1.14) compared to the control group (0.33 ± 0.09). 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.

Key words: *Micronuclei, Smokeless tobacco, Gutkha, Buccal mucosa, Genotoxicity, Oral cancer, carcinogens.*

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 tumours. 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 (Pandey *et al.*, 2009). 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 (IARC monograph, 1986) 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 (IARC monograph, 1985).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 (Panigrahi *et al.*, 1986).

Smokeless tobacco is a method of intra oral application and represents a non-homogenous group of compounds (Greenberg *at al.*, 1984). The term smokeless tobacco also known as dip, plug, chew or spit tobacco, refers to both chewing tobacco (coarse cut) and snuff (fine cut) (Jagriti *at al.*, 2012). Three types of smokeless tobacco are commonly manufactured: loose-leaf chewing tobacco, most snuff and dry snuff.10 Tobacco products which were

usually consumed by a small section of the population are today part of the modern urban and rural lifestyle. Positive associations between oral cancer and the habit of chewing areca quid have also been reported (Patel e*t al.*, 2009).

Cytogenetic damage is considered as an excellent biomarker for determining the effects of tobacco (Fenech *et al.*, 1985). 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 (Holland *et al.*, 2008). 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 (Gupta and Ray, 2003). 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.

Smokeless tobacco products

Smokeless tobacco products can be grouped into those used for chewing, sucking, gargling, sniffing, and as dentifrice. Some products are commercially available or a user can prepare the desired product from ingredients.

Products chewed and sucked

- Orally used products are chewed and placed in the space between the lower lip and gums or in the space between the gums and the cheek.
- *Khaini* prepared from sundried tobacco and slaked lime is commonly used in the states of Gujarat and Maharashtra.
- *Zarda*, a mixture of tobacco, lime, spices, and occasionally, silver flakes is also added to *pan* and chewed.
- *Khiwam*, a mixture of tobacco extract, spices, and additives is a paste-like preparation that may be added to *pan* or chewed as it is.
- *Betel quid or pan* contains four main ingredients, betel leaf (*Piper betel*), areca nut, catechu, slaked lime, and tobacco. Spices and flavouring agents may also be added.
- *Kharra* is a combination of tobacco, areca nut, lime, and catechu that is chewed in some parts of Maharashtra.
- *Mawa*, a mixture of thin shavings of areca nut, tobacco, and slaked lime is widely preferred in Gujarat state.
- *Gutka*or *pan masala* with tobacco is a ready to eat tobacco product has become extremely popular in all parts of India due to its user friendly packaging. It contains arecnanut, slaked lime, catechu, and tobacco as well as flavouring agents and sweeteners that are added to improve taste (IARC monograph,2007).

Common ingredients present in various SLT products/preparations

Sun-cured unprocessed or processed tobacco of *Nicotiana tabacum* species is utilized in many SLT products. *Nicotianarustica* tobacco that contains much higher levels of nicotine and TSNAs is also used. The International Agency for Research in Cancer (IARC) has classified SLT as a Group 1 human carcinogen (Chang *et al.*, 2002)

The International Agency for Research on Cancer (IARC) regards the chewing of betel leaf and areca nut to be a known human carcinogen (Jeng *et al.*, 2001) which have role in

multistage progression of oral cancer (Nair *et al.*, 2004). Smokeless tobacco contains nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; areca nut contains arecoline and 3-(methylnitrosamino) propionitrile, while lime provides reactive oxygen radicals, each of which has a role in oral carcinogenesis(Jacob *et al.*, 2004) Chewing betel quid without tobacco is an independent risk factor for developing oral cancer (Jacob *et al.*, 2004). When betel quid with tobacco is consumed with alcohol and smoking the relative risk increases 11-fold (Subapriya *et al.*, 2007).

Betel leaf contains large amounts of carcinogens called safrole, which is readily metabolized and excreted in urine. Chewing of betel quid and areca nut leads to oral sub-mucous fibrosis: a painful disabling and potentially precancerous condition of the oral mucosa. Betel quid chewing is a major risk factor for cancer in mouth, pharyngeal cavity and upper digestive tract (Dava *et al.*, 1992)

Carcinogens in pan masala and gutkha ingredients

The main carcinogens in pan masala and gutkha are derived from their ingredients areca nut, lime, catechu and tobacco. Several carcinogens are derived from tobacco but also from areca nut (Hoffman *et al.*,1994).Carcinogenic nitrosamines that could be derived from major ingredients of pan masala (areca nut) and gutkha (areca nut and tobacco). The carcinogenic TSNAs(Tobacco-specific nitrosamines) , N-nitrosonornicotine (NNN), 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosoanabasine (NAB), as well as the volatile nitrosamines N- nitrosodimethylamine and N-nitrosodiethylamine, have been detected in the saliva of chewers of BQ with tobacco(Wenke *et al*,1984). Chemical products of smokeless tobacco are nicotine, nitrosamines, nitrosamine acid, polycyclic aromatic hydrocarbons (PAHS), aldehydes and metals (Stepanov *et al.*, 2008). Tobacco specific nitrosamines are formed from alkaloids during the processing of tobacco leaves. The average consumption in regular users of snuff is about 10-15 g per day. In general the snuff is kept in the oral cavity for several hours per day (Pershagen, 1996).

Chewing a mixture of betel leaf, areca nut and tobacco is a complex behavior and is poorly studied. Betel and areca nut chewing has been extensively studied in populations in many part of the world. However genotoxic effect of combinational use of betel quid with snuff has received less attention. The purpose of this study was to evaluate the MN and CA of individuals regularly using a mixture of betel leaf, areca nut and tobacco with snuff tobacco.
The present study aimed to detect micronuclei in exfoliated buccal mucosal calls in healthy tobacco users (smokeless tobacco users, smokers and alcoholic) and healthy non-tobacco users (control).

MATERIALS AND METHODS

Subjects

The study samples were collected from 80 individuals comprising 50 tobacco chewers and 30 controls (non-tobacco chewers) including 55males and 25 females. Informed consents were obtained from each individual. Chewers used indigenous tobacco forms called *gutkha, zarda or khaini* in India. The control group consisted of age-matched healthy individuals without history of tobacco use in any forms.

Micronucleus Analysis

In this study, micronucleus assay was used to assess buccal cell nuclear changes (Kashyap and Reddy, 2012). Prior to collection of buccal smear samples, mouth of the individuals was washed carefully. Buccal smear was collected with a pre-moistened spatula and a smooth

smear was prepared on a grease free slide. The slides were air dried and fixed with absolute methanol within 24 hours of collection. Each slide was coded according to the study groups. Finally the slides were stained with Giemsa stain. Giemsa staining technique has been shown to give the best result for micronuclei (Neresesyan *et al.*, 2006). The MN analysis was done with a light microscope, at x 100 magnification, using coded slides. 100 cells from each individual were examined. Only unfragmented cells that were not smeared, clumped or overlapped and that contain intact nuclei were included in the analysis. Criteria used for identification of micronuclei were according to the method of Countryman and Heddle (Countryman and Heddle, 1976).

DATA ANALYSIS

Mean values of frequencies of cells with MN obtained from tobacco users were compared with those of non-users (non-tobacco chewers) 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 38 years and 28 years respectively.The mean number of micronuclei observed in five groups is presented in **table-1**. The mean frequency of micronucleated cells in smokeless tobacco chewers, SLT+ smokers, SLT+ alcohol, SLT +ST +alcohol and Controls were 2.91±1.40, 3.00±1.51, 2.50±0.57, 3.40±1.14 and 0.33±0.55 respectively. The mean number of micronuclei found were more in SLT +ST +alcohol as compared with smokeless tobacco chewers , SLT+ smokers, SLT+ alcohol and controls(Table 1). 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. The mean value and the mean differences, which are obtained from the five groups were compared and are shown in Table-2

Table -1: Mean number of micronuclei in five study groups.

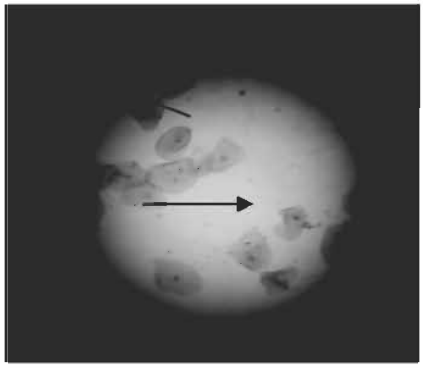
Groups	Numbers	Mean	SD
smokeless tobacco chewers	32	2.91	1.40
SLT+ smokers	8	3.00	1.51
SLT+ alcohol	4	2.50	0.57
SLT +ST +alcohol	6	3.40	1.14
Controls	30	0.33	0.54

Table -2: Multiple comparison of MN Cells in five study group.

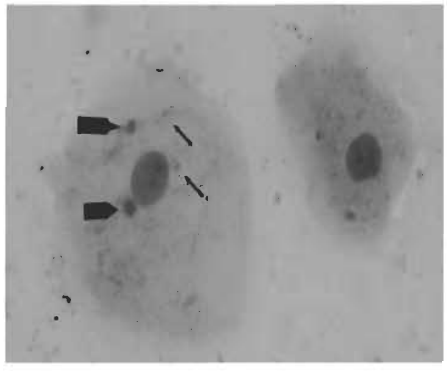
Groups	N	Mean±SD	Mean difference	P-Value
smokeless tobacco (SLT)	32	2.91±1.40	2.57	0.000
Controls	30	0.33±0.55		
SLT+ smokers	8	3.00±1.51	2.66	0.002
Controls	30	0.33±0.55		
SLT+ alcohol	4	2.50±0.57	2.16	0.006
Controls	30	0.33±0.55		
SLT +ST +alcohol	6	3.40±1.14	3.06	0.004
Controls	30	0.33±0.55		

In comparison, the mean difference between the number of micronuclei in smokeless tobacco chewers and control was 2.75 and was statistically significant ($P < 0.05$) .the mean difference between the number of micronuclei in SLT+ smokers and control was 2.66 and was statistically significant ($P < 0.05$) . the mean difference between the number of micronuclei in SLT+ alcohol and control was 2.16 and was statistically significant ($P < 0.05$) . the mean difference between the number of micronuclei in SLT +ST +alcohol and control was 3.06 and was highly statistically significant ($P < 0.05$) [Table -2].
The MN cells observed are shown in figure -1

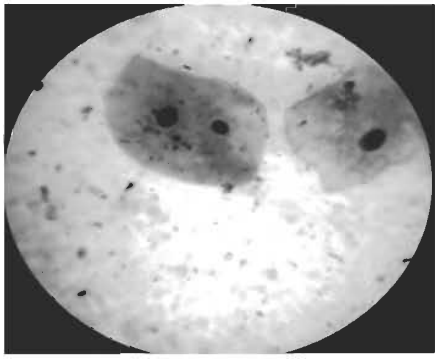
a.



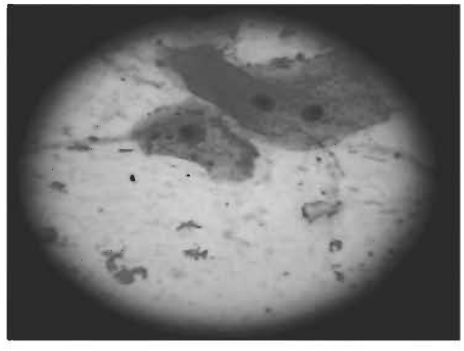
b.



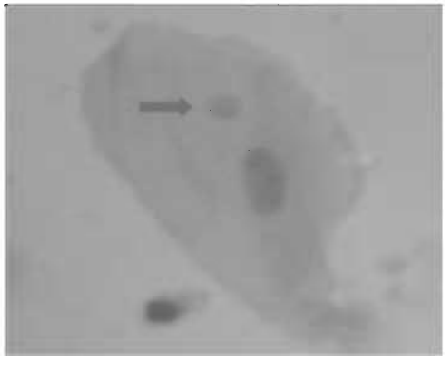
c.



d.



e.



f.

g. h.

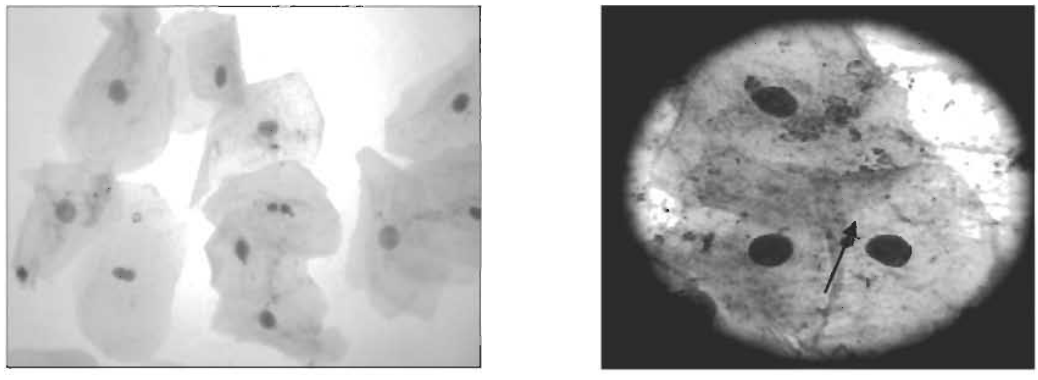


Fig 6.1: Cells with micronucleus, a, b ,c , d , e , f ,g , h.(arrows show micronucleus cells) [a,g,f -10x][b,c,d,e,h – 100x]

DISCUSSION

The micronucleus (MN) test has been receiving increasing attention as a simple and sensitive short-term assay for the detection of environmental genotoxicants (Stich and Rosin, 1983) MN are small fragments of extranuclear DNA formed during cell division which provide a nonspecific but quantifiable marker of DNA damage, so it is used to identify cellular damage caused by carcinogenic agents like tobacco (Fenech and Morley, 1985). The present study was conducted to analyze tobacco related genotoxic effects in tobacco exposed individuals by evaluating MN from buccal mucosal cells. I 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 (Suhas *et al.*, 2004)

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 (Ozkul *et al.*,1997). 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 my study, alcohol consumption did not appear to influence the frequency of micronuclei. Stich and Rosin studied alcoholic individuals and reported the absence of significant differences concerning MN frequencies in buccalcells (Stich and Rosin, 1983). However, the same study 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 my study, combination of both smoking, 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 (Nair *et al.*, 1991). 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 my study population.

CONCLUSION

From the present study, increase in the number of micronuclei provides the evidence that smokeless tobacco chewers and smokers may be at a high risk for developing oral cancer. In

comparison, the cellular changes associated with smokeless tobacco use were more than that in smokers and it is higher when taking both, thus indicating more carcinogenic potential of smokeless tobacco. The genotoxic effects of tobacco on buccal cells are partly age-related and increases with duration of expouser. Micronucleus assay can be used as a biomarker of genotoxicity and epithelial carcinogenic progression. However more research is required to establish it as a potential biomarker for oral carcinogenesis.

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