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Cytogenetic and micronuclei assessment of smokeless tobacco users: A case-control study

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ABSTRACT

Tobacco use is the world's leading cause of death, it is estimated that around 4.9 million deaths occur annually due to tobacco, and it is expected to rise to about 10 million by 2030. The use of smokeless tobacco in most of its forms possesses a substantial oral cancer risk at least in the developing countries where they are closely linked to socio cultural conditions. The primary aim of the study was to evaluate the cytogenetic and genotoxic changes in smokeless tobacco users. About 156 smokeless tobacco users have been enrolled and categorized into two groups on the basis of duration of their habit and equal number controls have been enrolled with age and sex matched non-tobacco users. We observed alteration in the incidence of chromosomal anomaly and micronuclei frequencies among the exposed and controls subjects by using the peripheral blood cells and buccal cells. The smokeless tobacco users exhibited varied levels of damages than non-users and predominantly in group II subjects with the higher duration of habit. In conclusion, use of smokeless tobacco for a longer duration caused more damages in comparison short term use and controls and awareness about the harmful effects of tobacco use is very low in rural populations.

Keywords: Tobacco, Genetic, Chromosome aberration, Micronuclei

INTRODUCTION

Tobacco use is a risk factor for many non-communicable diseases and it is an important cause of avoidable death. It is estimated that one billion people die due to usage of different forms of tobacco in the 21st century (WHO 2011). Today, around the world, tobacco is one of the most widely distributed and commonly used addictive substance (Makwana *et al.,* 2007). Globally, around five million deaths every year are attributable to direct tobacco use, which is the largest preventable cause of death (WHO 2012). There are more than one billion smokers worldwide with nearly 80% of them living in low and middle income countries (WHO 2011). In

India, among people aged 30 years and over, the mortality due to tobacco use is 206 per 100000 in men and 13 per 100000 in women with proportion of deaths attributable to tobacco reaching 12% for men and 1% for women (WHO 2012). In India tobacco is being used in smoking as well as smokeless forms (Rani *et al.,* 2003). The genotoxic effect of smokeless tobacco should be considered in addition to other known hazards for assessing health risks (Sudha *et al.,* 2009). Epidemiological data have shown a correlation between the use of smokeless tobacco (SLT) products, premalignant lesions of the oral cavity, and incidence of oral cancer (Rodu *et al.,* 2004).

SLT use is genotoxic and may affect DNA repair pathways (Ishikawa *et al*., 2005). Genotoxic effects in lymphocytes from smokers are most likely caused by cigarette smoke constituents, providing scientific evidence to encourage national campaigns to prevent tobacco consumption (Monica *et al*., 2004). Tobacco smoke induces an array of genetic aberrations including gene mutations, chromosome aberrations (CAs), sister chromatid exchanges and DNA strand breaks (DeMarini, 2004). One of the best techniques for studying the effects of environmental factors on genetic stability in human cells is the micronucleus (MN) test (Nersesyan *et al.,* 2002). MN may be products of early events in carcinogenesis, especially in the oral cavity, which is directly exposed to carcinogens / mutagens present in tobacco (Ramirez and Saldanha, 2002). The aim of this investigation was to analyze the effect of SLT use on the frequencies of chromosomal aberrations and micronucleus in peripheral blood lymphocytes and buccal epithelial cells respectively.

MATERIALS AND METHODS

A total of 156 smokeless tobacco users and an equivalent number of normal controls (156) were recruited for the present study. The subjects recruited for the present study belonged to hilly areas (mostly Tribals) of Western Ghats of Tamil Nadu such as Bargur, Tamaraikarai and Devarmalai of Erode district. The study was approved by the Medical Review Committee, Dhanvantri College of Nursing, Tiruchengode, Namakkal, Tamil Nadu. The subjects were informed about the study and consent was obtained from them. A standard questionnaire was used to collect information regarding the duration of tobacco use, form of tobacco use (smokeless & smoke), their health status, occupation etc. The recruited SLT users were categorised into two groups (Group I: Low duration of use and Group II: Longer duration of use). Of the 156 SLT users 52 were males and 104 were females. An equal numbers of age and sex matched non tobacco users served as controls. By a skilled medical nurse about 2–3 mL of venous blood was drawn from the SLT users and controls into a heparin-coated vacutainer to analyze chromosome aberrations and micronuclei frequency. The buccal epithelial cells were also collected from SLT users and controls in 0.9% saline solution to analyze micronuclei frequency.

Chromosome aberration analysis

Lymphocytes cultures were initiated following a standard procedure (Hoyos *et al.,* 1996). A volume of 0.5 ml blood was added to 4.5 mL RPMI 1640 medium supplemented with 15% FBS, 2 mM L-glutamine, 100U/mL penicillin and 100µg/mL streptomycin and 0.2 mL of PHA. The whole culture was incubated at 37ºC for 72 hrs and after 71 hrs, cultures were treated with 0.01 mg/ml colcemid to arrest the cells at mitotic stage. Lymphocytes were harvested after 72 hrs by centrifuging the cells at 800-1000 rpm, thereafter added pre-warmed (37ºC) hypotonic solution (KCl 0.075 M) and left undisturbed for 20 mins. The cells were fixed in 3:1 ratio of methanol: acetic acid. Slides were prepared and cautiously dried on a hot plate (56ºC, 2 min). Later, slides were stained using Giemsa stain. For the chromosome aberration analysis, 50 well spread metaphase spreads were analyzed for each subject under oil immersion lens of Leica light microscope (100X) and well spread metaphases were photographed.

Micronucleus in peripheral lymphocytes

Cytokinesis blocked micronucleus (CBMN) assay was carried out following the procedure described by Fenech and Morley (1986). In brief, Whole blood (0.5 ml) was added to 4.5 mL of RPMI-1640 medium (Hyclone, USA) supplemented with 20% fetal bovine serum (ThermoFischer Scientific, India), 2mM L-Glutamine (ThermoFischer Scientific, India), and 0.2mL of phytohemagglutinin (ThermoFischer Scientific, India). The mixture was incubated at 37°C for 72 hours. Cytokinesis was blocked by the addition of cytochalasin B (Sigma, India) at a final concentration of 6 µg/ml 44 h after stimulation with phytohemagglutinin. After 72 h of incubation, cells were harvested by centrifugation, given 1 min hypotonic treatment (0.075 M KCl) and fixed in fresh fixative solution (methanol : acetic acid, 3 : 1). This

fixation step was repeated twice after 20 min storage at 4°C and eventually, stained in Giemsa stain. About 500 cells from each SLT user were analysed to estimate micronucleus frequency.

Micronucleus analysis in buccal epithelial cells

After rinsing the mouth with tap water, exfoliated buccal mucosa cells were collected by scraping the right/left cheek mucosa with a wooden spatula. Thus collected cells were transferred to a tube containing saline solution (0.9% NaCl). The cells were centrifuged (800 rpm) for 5 min, fixed in 3:1 methanol/acetic acid, and dropped onto a pre-cleaned slide. Later, the air dried slides were stained in Feulgen plus fast green. The identification of micronuclei was based on the criteria proposed by Sarto *et al*. (1987). About 1,000 cells were screened to estimate the frequency of micronuclei in each subject.

Statistical analysis

Statistical analysis was carried out using the statistical software SPSS Version 16. Analysis of variance (one way-ANOVA) was used to evaluate the frequency of chromosome aberration and micronuclei between SLT users and controls. $P<0.05$ was used as the criterion of significance.

RESULTS

Table 1 show the mean age of SLT users and controls and mean duration of SLT usage among group I and group II SLT users. The SLT users were further categorised based on their age into group I (<35 Years) and group II (>35 Years). Of these two groups, group II harboured more SLT users in comparison with group I. The present study also observed that, SLT usage was highly prevalent among females (66.7%) than males (33.4%) in this population.

Chromosome aberrations such as gaps and breaks were observed in SLT users. Elevated levels of chromosome aberrations (2.65±1.29) were found in group II SLT users in comparison with controls (0.65±0.71). Of the two groups of SLT users chromosome aberrations was found to be higher in group II SLT users (Table 2).

Table1. Demographic details of age and year of exposure in smokeless tobacco users.

Particulars	SLT users Mean ± SD	Control subjects Mean ±SD
Total samples	156	156
Male	52(33.33%)	52(33.33%)
Female	104(66.66%)	104(66.66%)
Age		
Group I (age below 35)	64(34.28±5.56)	64(34.76 ±5.18)
Group II (age above 35)	92(57.56±8.65)	92(53.54±6.68)
Year of exposure		
Group I	64(9.14±3.13)	-
Group II	92(18.57±8.56)	-

SD= standard deviation

Table 2. Frequencies of chromosome aberrations in smokeless tobacco users and controls

Subjects	Number of samples	Chromosome aberrations
SLT users		
Group I	64	1.90±1.17
Group II	92	2.65±1.29*
Controls		
Group I		
Group II	64	0.65±0.71
	92	0.92±0.77

*significantly elevated compared to controls subjects

Table 3. Frequencies of Micronuclei Frequency in smokeless tobacco users and non users

Subjects	Micronuclei Frequency (blood cells/500)	Micronuclei Frequency (buccal cells/1000)
SLT users		
Group I (64)	1.56±1.12*	2.21±1.56#
Group II (92)	2.95±1.61**	3.10±1.46##
Controls		
Group I (64)	0.64±0.60	0.95±0.80
Group II (92)	1.01±0.88	1.15±1.07

*, # significantly elevated compared to controls subjects

**, ## Significantly elevated compared to controls and group I experimental subject

A significantly elevated level of micronuclei frequency was observed in the blood lymphocytes of group II SLT users (2.95±1.61) when compared to the group I SLT users (1.56±1.12) and controls (p<0.05). Similarly, buccal epithelial cells of group II SLT users had significantly increased number of micronuclei (3.10±1.46) than group I SLT users and controls (p<0.05, Table3).

DISCUSSION

Epidemiological studies related to tobacco use are very important to predict cancer risk and mortality and morbidity associated with tobacco related cancer (ICRC, 2003). In South Asia over 250 million people are using SLT products, about 17% of total population in South East Asia use oral tobacco; of which 95% belong to India (82%) and Bangladesh (13%; WHO, 2004). Smoking is responsible for a substantial number of human health problems worldwide (Weir *et al*., 2003). Tobacco smoke induces an array of genetic aberrations, including gene mutations, chromosome aberrations (CAs), micronuclei (MN), sister chromatid exchanges (SCEs), DNA strand breaks, and oxidative DNA adduct in various models (DeMarini, 2004).

CAs and MNs are excellent biomarkers for predicting cancer risk in individuals who are habitual tobacco users. In the present study, CAs like gaps and breaks were observed in elevated levels in group II SLT users than group I and controls. Chromosomal damage has been shown to increase progressively with age and few studies had identified smoking habit as a significant factor that induces alterations in the genetic material (Moacir *et al*., 2010). Jagetia *et al*. (2001) reported that, the chemical carcinogens present in tobacco cause structural alterations in the DNA of target cells, leading to genomic instability in the form of chromosomal abnormalities. Furthermore, Sierra-Torres *et al*. (2004) observed a significantly higher frequency of CAs among smokers compared to non-smokers. The CAs observed in the present study are consistent with a previous study in which CA frequency was significantly increased in smokers in comparison with non-smokers (Tawn and Whitehouse, 2001).

In the present study, the frequency of MN in blood lymphocytes and buccal epithelial cells were found to be higher in group II SLT users than group I SLT users (p<0.05) and controls. Our results are in agreement with a previous study which reported increased MN frequency in smokers (Haveric *et al.,* 2010). In addition to this, Kamboj and Mahajan reported that, even abnormal oral habits significantly increases the frequency of micronuclei (Kamboj and Mahajan, 2007). An another possible reason for the higher micronuclei frequency in group II SLT users may be the age of subjects. Orta and Gunebakan (2012) reported that, micronuclei frequency increases with age of the subjects.

The study also observed that, literacy level of people including the SLT users of the present study inhabiting in these places is very low and also they are very less aware or unaware of the potential health risks associated with tobacco use. Immediate measures need to be taken to educate or create awareness about the ill effects of SLT usage that is extensive among all age groups in this tribal population.

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