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# Lymphocyte DNA Damage in Chewing Tobacco Users of Coimbatore, Tamilnadu, by Using Comet Assay

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**ABSTRACT** The Chewing tobacco (CT) has a physically powerful association with the risk of oral leukoplakia (OL), oral submucous fibrosis (OSF), oral squamous cell carcinoma (OSCC) and squamous cell carcinoma of the head and neck (SCCHN). ST components exhibit genotoxicity and may alter the structure of DNA. Present study to investigate the effects of CT with smoking on lymphocyte DNA damage. After signing a consent form, volunteers provided blood samples (76 samples from including experimental and control subjects) to establish Single Cell Gel Electrophoresis (STGE) or comet were evaluated. Present study found significant differences in the genetic damage induction. However, association was found between smokings had significant effect, and it can induce maximum amount of DNA damage. The genotoxic effect of CT should be considered in addition to other known hazards for assessing health risks

## INTRODUCTION

OSCC is a significant public health problem in India. Worldwide, OSCC is the sixth most common cancer; more than 300,000 new cases are diagnosed each year (Parkin et al. 1988). SCCHN and its subset, OSCC arise through an accumulation of genetic alterations, including chromosomal alterations, DNA changes and/or epigenetic alterations. These events are further influenced by exposure to environmental agents, including tobacco consumption, smoking, alcoholic beverages, and viruses (Mork et al. 2001).

CT contains considerable nicotine, much more than contained in cigarette tobacco (Benowitz et al. 2000). ST perceived as a safer alternative to smoking, also contains 28 carcinogenic agents, including nitrites and alkylating agents (Chakradeo et al. 1994; Niphadkar et al. 1994). Numerous different forms of ST have been used worldwide. CT an interesting kind of ST, is commonly used instead of smoking in south India. Tobacco, slaked lime paste and areca nut are the major components in CT, and a small amount of this mixture is applied to the mucosa of the lower or upper lip for 10-15 minutes and then it is spit out. This procedure is repeated many times during the day. The highly addictive nature of the nicotine in oral tobacco products makes it difficult for many young people to quit and the presence of smokeless tobacco lesions among school aged adolescents may be an early indicator of increased risk for future oral cancers (Perry 1999 and US-DHHS).

Previous reports that have demonstrated that karyotypes of SCCHN and OSCC (Gollin 2001; Jin et al. 2002) and also case-control study conducted by Winn et al. (1981) suggests a strong relationship between oral cancer and long-term ST use.

However, several earlier (Balachandar et al. 2008a) reports regarding the use of ST from Tamilnadu region, it being home to a more diverse number of such products. So present study considered whether individuals with CT habit present more DNA damage with the increase of smoking by analysis using the Comet assay. Although CT has been extensively investigated, to the greatest of our knowledge, this work is the first to perform such kind of study.

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## MATERIAL AND METHODS

***Demographic Profile of Selected Area:*** Coimbatore, the Manchester of South India is located in the western region of Tamilnadu, bordered by the panaromic Western Ghats. The total population of the Coimbatore district is 42.25

lakhs (21, 56,280 males and 20, 67,817 females). Due to the existence and predominance of population the silent toll of human lives due to numerous diseases is on the rise.

***Subject Recruitment and Sample Collection:*** The study was conducted on 38 male (84.21%) and female (15.79%) CT users aged 20–40 (29.84±4.29) years in the surroundings of Coimbatore city. Of these workers, 21 (55.26%) were smokers (no more than 20 cigarettes/day) and 17 (44.74 %) were non-smokers and exposure period of CT was 4.33±1.84. The control groups consisted of 38 healthy male (84.21%) and female (15.79%) aged 20-40 (29.79±4.23) years with no history of exposure to clastogenic and/or aneugenic agents and of socio-economic level also similar to that of the experimental subjects.

At the time of blood collection (3 ml/ individual), the workers signed a term of informed consent and replied to a questionnaire elaborated to determine the profile and habits of the study population. The study procedures used in the present study were approved by the local ethical committee.

All cases were exclusively CT users at the time of the study, consumers for a period of 7 years or more. The cutoff of at least 8 cans/ pouches per week was established to ensure subject safety considering that the use of nicotine patch doses up to 78 g/ day.

***Sample Collections:*** Peripheral blood samples (V = 5 ml) were collected under sterile conditions by venipuncture into heparinized tubes for comet assay (Singh 1988).

***Single Cell Gel Electrophoresis (SCGE) Assay:*** The comet assay was conducted under alkali conditions according to Singh et al. (1988). All chemicals were obtained by Sigma. Two microlitre of whole blood were suspended in 0.5% low melting agarose and sandwiched between a layer, of 0.6% normal melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were kept on ice during the polymerization of each gel-layer. After the solidification of 0.6% agarose layer, the slides were immersed in lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and DMSO 10%) at 4 °C. After 1 hr, the slides were placed in the electrophoresis buffer (0.3 M NaOH, 1 mM Na2EDTA, pH 10) for 20 min at room temperature to allow for DNA to unwind. The buffers were then chilled and the electrophoresis was performed at 300 mA and 19 V in a horizontal electrophoresis platform for 20 min. The slides were neutralized with Tris–HCl buffer (pH 7.5) and stained with 10% ethidium–bromide for 10 min. Each slide was analyzed by using Leitz Orthoplan epifluorescence microscope. For each subject 50 cells were analyzed by automatic digital analysis system Comet assay II (Perceptive Instruments Ltd., Suffolk, Halstead, UK), determining tail length and tail moment (tail length×tail % DNA/100). DNA damage was further quantified by visual classification of cells into categories of 'comets' corresponding to the amount of DNA in the tail according to Anderson et al. (1994).

***Statistical Analysis:*** All calculations were performed using MINITAB RELEASE II Software package for windows. Mean values and standard deviations (S.D.) were computed for the scores and the statistical significance (P/0.05) of effects (smoking) was determined using analysis of variance (ANOVA). Simple linear regression analyses were performed to assess the association between endpoints and independent variables.

## RESULTS

The effect of occupational exposure to CT, on the level of DNA damage in lymphocytes of study group was assessed by the comet assay. A total 76 subjects corresponding to 38 experimental, and 38 controls were recruited for this study.

Tables 1 and 2 represents the age, number of smokers and years of exposure between the two groups involved in this study. The exposed groups displayed significantly higher levels of DNA damage than controls. The ranges of the MTM (mean tail moment) was 0.68±0.29 in experimentals subjects, while the 0.35± 0.42 in controls, respectively. There was significant difference MTM ($P < 0.01$) between experimentals and controls.

Smoking exposure the lymphocytes of the exposed workers expressed higher DNA migration. The smokers had MTM (0.75±0.27) than the non-smokers (0.58±0.31). A significant increase of MTM in smokers was observed in the exposed workers.

A clear and statistically significant increase in DNA migration was found in the study group when compared with the control groups as analyzed by ANOVA. Among the study group, significantly more were observed, the presence

**Table 1: Data showing the general characteristics of control subjects.**

| *Controls* | *Sex* | *Age* | *MTM* |
|---|---|---|---|
| 1 | M | 32 | 0.38 |
| 2 | M | 28 | 0.12 |
| 3 | M | 23 | 2.01 |
| 4 | M | 27 | 0.91 |
| 5 | M | 31 | 0.32 |
| 6 | M | 38 | 0.31 |
| 7 | M | 26 | 0.26 |
| 8 | M | 31 | 0.22 |
| 9 | M | 33 | 0.67 |
| 10 | M | 39 | 0.14 |
| 11 | F | 29 | 0.16 |
| 12 | M | 26 | 0.12 |
| 13 | M | 23 | 0.29 |
| 14 | M | 29 | 0.11 |
| 15 | M | 35 | 0.21 |
| 16 | M | 26 | 0.17 |
| 17 | M | 32 | 0.16 |
| 18 | F | 32 | 0.14 |
| 19 | M | 30 | 0.18 |
| 20 | M | 25 | 0.19 |
| 21 | M | 31 | 0.25 |
| 22 | M | 35 | 0.17 |
| 23 | F | 32 | 0.14 |
| 24 | M | 27 | 0.14 |
| 25 | M | 37 | 0.15 |
| 26 | M | 24 | 0.17 |
| 27 | M | 28 | 0.23 |
| 28 | F | 30 | 0.17 |
| 29 | F | 30 | 0.29 |
| 30 | M | 38 | 0.08 |
| 31 | M | 27 | 0.31 |
| 32 | M | 32 | 0.29 |
| 33 | M | 25 | 0.31 |
| 34 | M | 29 | 0.21 |
| 35 | M | 24 | 0.89 |
| 36 | M | 31 | 0.25 |
| 37 | F | 26 | 0.21 |
| 38 | M | 31 | 1.93 |

F, Female; M, male; MTL, Mean Tail Length; MTM, Mean Tail Moment;

**Table 2: DNA damage in experimental subjects**

| *Workers* | *Sex* | *Age* | *Smoking* | *Exposure period (yrs)* | *MTM* |
|---|---|---|---|---|---|
| 1 | M | 32 | S | 4 | 0.76 |
| 2 | M | 28 | NS | 6 | 0.20 |
| 3 | M | 24 | NS | 4 | 0.19 |
| 4 | M | 27 | S | 6.2 | 0.72 |
| 5 | M | 31 | S | 5.8 | 0.43 |
| 6 | M | 38 | NS | 5.9 | 0.38 |
| 7 | M | 25 | NS | 3.5 | 0.43 |
| 8 | M | 22 | NS | 4 | 0.53 |
| 9 | M | 33 | S | 4.5 | 1.24 |
| 10 | M | 39 | NS | 11 | 0.96 |
| 11 | F | 28 | NS | 4 | 1.05 |
| 12 | M | 27 | S | 3 | 0.88 |
| 13 | M | 33 | NS | 3 | 0.54 |
| 14 | M | 29 | S | 2.5 | 0.48 |
| 15 | M | 35 | NS | 6 | 0.68 |
| 16 | M | 26 | S | 3 | 0.45 |
| 17 | M | 32 | NS | 3.5 | 0.49 |
| 18 | F | 31 | NS | 4 | 0.64 |
| 19 | M | 29 | S | 3 | 1.15 |
| 20 | M | 25 | S | 2.5 | 0.53 |
| 21 | M | 31 | S | 8 | 0.76 |
| 22 | M | 26 | S | 4 | 0.74 |
| 23 | F | 31 | NS | 5 | 0.51 |
| 24 | M | 27 | S | 2 | 0.36 |
| 25 | M | 37 | NS | 4.5 | 1.03 |
| 26 | M | 35 | S | 6.8 | 0.93 |
| 27 | M | 29 | NS | 3.5 | 0.35 |
| 28 | F | 31 | NS | 4 | 0.89 |
| 29 | F | 30 | NS | 2 | 1.00 |
| 30 | M | 39 | S | 3.5 | 0.63 |
| 31 | M | 24 | S | 4 | 1.41 |
| 32 | F | 27 | S | 3 | 0.55 |
| 33 | M | 34 | S | 3 | 0.67 |
| 34 | M | 31 | S | 2.5 | 0.68 |
| 35 | M | 29 | S | 6 | 0.62 |
| 36 | M | 26 | NS | 3 | 0.09 |
| 37 | M | 25 | S | 3.5 | 0.69 |
| 38 | M | 28 | S | 7 | 1.01 |

F, Female; M, Male; MTL, Mean Tail Length; MTM, Mean Tail Moment;

of cells demonstrating greater DNA damage than among control subjects.

## DISCUSSION

Comet assay can sensitively detect DNA single strand break and alkali-labile site (Hecht et al. 1978; Tice 1995; Oesch et al. 1994). It was used in this study to examine lymphocyte DNA damage of CT users. Our finding is consistent with those were smoking with CT use can increase DNA damage (Betti et al. 1995; Sardas et al. 1995) and our finding also provides further supportive substantiation.

There is sufficient evidence that oral use of CT is carcinogenic to humans. CT addiction is frequent in some places of Tamilnadu. In order to elicit the above issues, the present study has been carried out to determine the DNA damage in this region. Assessment of normal levels of genetic damage in the general population is essential for proper interpretation of data obtained by monitoring of populations that are occupationally exposed to potential genotoxic agents.

CT users an early sign of damage to the oral mucosa and often develop clinically visible whitish lesions and stiffening of the oral mucosa and oral submucous fibrosis (OSF). Almost every tobacco chewing-related oral malignancy is preceded by a clinically distinct premalignant stage at the site of cancer development (WHO 1984; Gupta et al. 1989; Murti et al. 1995).

A key initiating step in the carcinogenic process is the formation of DNA adducts. Some miscoding DNA adducts that could be formed by use of CT. Persistence of these adducts during DNA replication can cause miscoding, leading to mutations and derangement of cellular growth control processes. The tobacco-specific nitrosamines can induce miscoding DNA adducts, including O6-pyridyloxobutyl and O6-MeG adducts (Hecht 2003), that could initiate the tumourigenic process in the oral cavity. Leading to focal areas that progress at different rates towards invasive cancer (Califano et al. 1996; Oh and Mao 1997). Microsatellite analysis in SCCHN for allelic loss at 10 major chromosome loci demonstrated that the spectrum of chromosomal deletions progressively increases at each histopathological step from benign hyperplasia to dysplasia to carcinoma in situ to invasive cancer (Califano et al. 1996). The most common gains in tobacco chewing associated oral cancers are on chromosomes 8p, 9p, 9q, 11q, 17q and 20q and the most frequent losses are in chromosome arms 3p, 4q, 5q, 9q and 18q (Mahale and Saranath 2000; Lin et al. 2002b; Pai et al. 2002).

In addition to the separate effect of occupational exposure and CT users, a significant positive interaction observed indicated some synergistic effect of external factors. So present study analysed combined effect of CT and smoking. Oesch et al. (1994) reported a negative interaction that cigarette smoking can protect mononuclear blood cells from DNA single strand breaks in taxi drivers, painters and ethylene oxide-exposed workers.

In many studies the levels of carcinogen-DNA adducts have been shown to be higher in tissues of smokers than in tissues of non-smokers (Nakayama et al. 1984). In terms of biological activity, cigarette smoke and its conductors have been shown to form adducts with DNA protein and to induce chromosome damage. Simple calculation of the mean response would limit the sensitivity of the method. Present study (Table 2) confirmed that smoking factor was one of the risk factor of DNA damage.

Although former cigarette smokers were more likely than those who had never smoked to have a smokeless tobacco lesion, we found little evidence for an independent effect of cigarette smoking on the present lesions (Balachandar et al. 2008b; Sasikala et al. 2003). Jayakumar and Sasikala (2008) reported cigarette smoking routine has a synergistic effect on inducing DNA damage among the jewellery workers are occupationally exposed to nitric oxide.

Hence, the assessment of genetic damage performed in the present study shall aid understanding the mode of action of these obnoxious agents, which are a cheap alternative to the available smoking. The data presented in table 2 show a larger number of damage in DNA was identified in the experimental subjects compared to controls.

Carcinogenic and mutagenic compounds, including tobacco-specific nitrosamines present in ST forms are believed to be responsible for the induction of Genes (Hecht et al. 1978). Moreover, the carcinogenic and mutagenic effects of tobacco forms have been attributed to tobacco-specific Nitrosamines (Erenmemisoglu et al. 1995; Ozkul et al. 1995).

Furthermore, *in vivo* evaluations allow for considering the influence of the individual susceptibility in screened humans. Therefore, the chromosome breakpoints could produce deletions or disruptions of functional genes, producing developmental defects or genetic disorders, including cancer. The aqueous CT with/without lime was shown to be mutagenic in Ames test (Niphadkar et al. 1994).

On the other hand, carcinogenic and mutagenic compounds, including tobacco-specific nitrosamines present in smokeless tobacco forms (Hecht and Hoffmann 1981), are believed to be responsible for the induction of micronuclei. These compounds are produced from nicotine by bacterial or enzymatic activity. The same formation occurs in the mouth under the influence of saliva (Winn et al. 1981).

Our findings may indicate an emerging public health problem, since our subjects were young and adult have lesions that may be markers for increased risk of developing oral malignancies.

Consistent with this findings, a study of Navajo students found that the prevalence of oral lesions tends to increase with duration and frequency (days per week) of use of oral tobacco (Wolfe and Carlos 1987). Also, they found minimum significant increase in the presence of oral lesions among oral tobacco users that were associated with the concomitant use of smoking.

In conclusion, current scientific evidence in India has hitherto well established that tobacco is a demerit good and has adverse effects on health, economics and environment. These re-

search findings have been efficiently utilized in India to form sound scientific foundation for public health policies. Issues related to Smokeless tobacco control have featured prominently in the Indian courts of law and the judicial verdicts have had a major impact on Government policies as well as tobacco trade practices. In view of these findings, the present study indicates that tobacco users should be considered a high risk group and need to be monitored for health hazards including cancer.

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