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Assessment of DNA Damage in Peripheral Blood of Tobacco Users

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Abstract

Smokeless Tobacco is associated with Oral Leukoplakia, Oral Submucous Fibrosis, Oral Squamous Cell Carcinoma, and Squamous Cell Carcinoma. It exhibits genotoxicity and may alter the structure of DNA, proteins and lipids, resulting in the production of antigenicity. The paper investigated the effects of chewing tobacco (CT) with smoking on lymphocyte DNA damage to establish SCGE (Comet) and concluded that chewing tobacco and smoking lead to significant DNA damage.

Keywords: Oral Cancer, Comet Assay, DNA Damage

1. Introduction

Oral Squamous Cell Carcinoma (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. OSCC arise through an accumulation of genetic alterations,

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including chromosomal alterations, DNA changes and/or epigenetic alterations. These events are further influenced by exposure to environmental agents, including tobacco consumption, smoking, consumption of alcoholic beverages, and virus attacks [12].

Smokeless Tobacco (ST) contains significantly more nicotine than Cigarette Tobacco. ST, perceived as a safer alternative to smoking, also contains 28 carcinogenic agents, including nitrites and alkylating agents [14]. Numerous different forms of ST have been used worldwide. Chewing tobacco (CT), an interesting kind of ST is commonly used instead of smoking in Tamil Nadu, especially in Chennai, Nilgiris, and the Coimbatore district. 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 is spit out. This procedure is repeated many times during the day. The highly addictive nature of nicotine in oral tobacco products makes it difficult for many young people to quit and the presence of Smokeless Tobacco among school-aged adolescents may be an early indicator of increased risk for future oral cancers [21].

Previous studies have demonstrated that karyotypes of SCCHN and OSCC [10] and also a case-control study conducted by Winn et al. (1981) suggested a strong relationship between oral cancer and long-term Smokeless Tobacco use. Several reports regarding the use of smokeless tobacco from Tamil Nadu region and the effect of a diverse number of such products were reported as well [23]. The present study examined whether individuals who use Chewing Tobacco have more DNA damage with the increase of smoking habit using the Comet assay. Although the effect of CT has been extensively investigated, this work is perhaps the first to study the combinatorial effect of chewing tobacco and smoking on the health status of the individual.

2. Material and Methods

2.1 Subject Recruitment and Sample Collection

The study was conducted on 25 male (84.21%) and female (15.79%) CT users aged 20–40 (29.84±4.29) years in the surrounding areas of

Coimbatore city. Of these individual 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. The socioeconomic levels of the control group were similar to that of the experimental subjects.

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

All cases were exclusively CT users at the time of the study. Consumers were addicted to CT for a period of 7 years or more. A 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.

2.2 Sample Collections

Peripheral blood samples (V = 5 ml) were collected under sterile conditions by venipuncture into heparinized tubes for the Comet assay [24].

2.3 Single Cell Gel Electrophoresis (SCGE) Assay

The Comet assay was conducted under alkali conditions, following the protocol laid out by [19]. All chemicals were obtained from Sigma. Two microlitres of whole blood were suspended in 0.5% low melting Agarose and placed 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 polymerisation 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 the 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 analysed using a Leitz Orthoplan Epifluorescence microscope. For each subject, 50 cells were analysed by automatic digital analysis system Comet assay II (Perceptive Instruments Ltd., Suffolk, Halstead, UK), determining the tail length and tail moment (tail length×tail % DNA/100). The DNA damage was further quantified by visual classification of cells into categories of 'comets' corresponding to the amount of DNA in the tail following [1]. □

3. Statistical Analysis

All calculations were performed using MINITAB RELEASE II Software package for windows. The 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.

4. Results

The effect of occupational exposure to CT, on the level of DNA damage in the leucocytes of the study group was assessed by the Comet assay. A total of 76 subjects corresponding to 25 experimental and 25 controls were recruited for this study.

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
38	M	31	1.93

F, female; M, male; MTL, Mean tail length; MTM, Mean tail moment

Table 2: Showing the 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

F, female; M, male; MTL, Mean tail length; MTM, Mean tail moment

Table 1 and 2 represent the age, number of cigarettes 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 range of the MTM (Mean Tail Moment) was 0.68±0.29 in experimental subjects, while the MTM was 0.35± 0.42 in controls respectively. There was significant difference MTM ($P < 0.01$) between experimental and controls.

Smoking exposure for the lymphocytes of the exposed workers expressed higher DNA migration. The smokers had higher MTM (0.75±0.27) than the non-smokers (0.58±0.31). A significant increase of MTM 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 analysed by ANOVA. Among the study group, significantly greater DNA damage was observed than the control subjects.

5. Discussion

Comet assay can sensitively detect DNA single-strand break and Alkali-Labile site [18], [20], [25]. It was used in this study to examine lymphocyte DNA damage of CT users. Our finding is consistent with those subjects who were smoking with CT [3], [18] 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 Tamil Nadu. In order to elicit the above issues, the present study was carried out to determine the DNA damage in this region.

CT users have an early sign of damage to the oral mucosa and often develop clinically visible whitish lesions and stiffening of the oral mucosa and result in Oral Submucous Fibrosis (OSF) [26]. A key

initiating step in the carcinogenic process is the formation of DNA adducts. Some miscoding DNA adducts 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 [8], that could initiate the tumourigenic process in the oral cavity leading to focal areas that progress at different rates towards invasive cancer [15]. Microsatellite analysis in SCCHN for allelic loss at 10 major chromosome loci demonstrated that the spectrum of chromosomal deletions progressively increases at each histo-pathological step from benign hyperplasia to dysplasia to carcinoma in situ to invasive cancer [4]. 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 [27].

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 the present study analysed the combined effect of CT and smoking. Oesch et al. (1994) reported a negative interaction, that is, 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 the tissues of smokers than in the tissues of nonsmokers [13]. In terms of biological activity, cigarette smoke and its conductors have been shown to form adducts with DNA protein and to induce chromosome damage. A simple calculation of the mean response would limit the sensitivity of the method. The present study (Table 2) confirmed that smoking was one of the risk factors for DNA damage. Although former cigarette smokers were more likely to have a smokeless tobacco lesion than those who had never smoked, we found little evidence for an independent effect of cigarette smoking on the present lesions [23], [28].

Hence, the assessment of genetic damage performed in the present study shall aid the understanding of the mode of action of these agents which are a cheap alternative to smoking. The data

presented in Table 2 show a larger number of DNA damage identified in the experimental subjects compared to the controls.

Carcinogenic and mutagenic compounds, including tobacco-specific nitrosamines present in ST forms are believed to be responsible for the induction of genes [8]. Moreover, the carcinogenic and mutagenic effects of tobacco forms have been attributed to the effect of tobacco-specific nitrosamines [16]. The aqueous CT with/without lime was shown to be mutagenic in the Ames test [14].

On the other hand, carcinogenic and mutagenic compounds, including tobacco-specific nitrosamines present in smokeless tobacco forms [8], are believed to be responsible for the induction of micronuclei. Similar compounds were produced from nicotine by bacterial or enzymatic activity. Similar formation occurs in the mouth under the influence of saliva [29].

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

In conclusion, current scientific evidence in India has established that tobacco has adverse effects on health, economics, and the environment. These research findings will help agencies to form scientific foundation for public health policies. Issues related to smokeless tobacco have featured prominently in the Indian courts of law and the judicial verdicts. 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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