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# Assessment of genotoxicity of nicotine employing in vitro mammalian test system

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**Summary**

*Genotoxicity of nicotine was evaluated employing Chinese hamster ovary (CHO) cells. Two cytogenetic endpoints, viz. frequency of sister chromatid exchange (SCE) and chromosome aberration (CA) were considered. Nicotine was found to induce CA and SCE frequency in a dose and duration dependent manner. Statistically significant elevations in CA frequency were observed only with higher concentrations of nicotine, whereas, SCE frequencies were increased significantly by all the doses utilized. It was genotoxic at the concentration, comparable to the saliva levels of nicotine achieved during tobacco chewing. The results obtained by continuous and pulse treatments with nicotine explain the harmful effects of chronic tobacco consumption.*

**Keywords:** nicotine; genotoxicity; sister chromatid exchange; chromosome aberration; Chinese hamster ovary cells.

## Introduction

Tobacco usage is a major avoidable cause of human morbidity and mortality. Numerous convincing evidences have shown the interrelationship between tobacco consumption and various forms of cancer [17,18]. As a consequence of increasing awareness about the ill effects of smoking and even passive smoking, use of smokeless tobacco has increased in the western countries. Studies carried out at smoking cessation clinic have suggested the use of nicotine chewing gum as a relatively safer alternative to cigarette [13]. The research on tobacco dependence has identified nicotine as the culprit for the addiction [15].

Nicotine was first identified in tobacco by Henri Nicote in 1828. It accounts for about 95% of the total alkaloids in tobacco [21]. It causes addiction and the underlying pharmacological and behavioral processes are similar to heroin and cocaine [23]. It is probably the agent responsible for the acute toxicity produced due to overdose, when tobacco was being used for medicinal purposes [11]. Oral sores or ulcers are some of the side effects reported by the individuals who resorted to nicotine chewing gum [7]. Nicotine had no mutagenic effect in Ames' test, however, it induced a repairable DNA damage in the *E. coli* pol $A^+$/pol $A^-$ system [6,25]. It did not damage the plasma membrane of human lung fibroblasts in vitro in terms of the size of the holes induced [27]. The cellular proliferation

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and DNA synthesis in human leukemic cells in vitro, were reported to be inhibited by nicotine [20]. However, adequate information on genotoxic effects of nicotine, per se, on mammalian test system is not available. It was reported to induce chromosomal aberrations in mice, but had no influence on human leucocytes in vitro [4]. It was very weakly positive in the SCE test [24].

We report here our findings on the genotoxic effects of nicotine per se by analysing; (1) chromosome aberration (CA) frequency and (2) sister chromatid exchange (SCE) frequency in Chinese hamster ovary (CHO) cells, for two different treatment schedules, i.e., low dose — continuous exposure and high dose — short-term exposure followed by recovery in fresh growth medium (GM).

## Materials and methods

### *Chemicals*

Nicotine (L-1-Methyl-2-(3-pyridyl)pyrrolidine) free base was purchased from Sigma Chem. Co. (U.S.A.). Minimum essential medium (MEM, Earle's base with non-essential amino acids) was procured from Centron Research Lab., Bombay, India; New born calf serum (NCS) from Sera Lab, U.K. and Mitomycin-C (MMC) from Biochem pharmaceutical Co., Bombay, India.

### *Cell culture*

Chinese hamster ovary (CHO) cell line, was kindly provided by NFATCC, (National Facility for Animal Tissue and Cell Culture), Pune. The cells were maintained at 37 °C in MEM, supplemented with 20% NCS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cell cultures were treated 42—45 h after the subcultivation with various concentrations of nicotine for different durations. The experiments were repeated once. MMC was used as a positive control in both the experiments.

### *Experiment — I*

Exponentially growing CHO cell cultures were treated with two different concentrations of nicotine i.e., 625 μg/ml and 1000 μg/ml for 2 h and 4 h. After completion of the treatment, the cells were washed with CMF-PBS (calcium magnesium-free phosphate buffer saline) prewarmed to 37 °C and nicotine free growth medium (GM) containing 10 μg/ml BrdU was added to the cultures. For scoring CAs, cultures were harvested after completion of one cell cycle (24 h), whereas, the cells were allowed to grow for two cell cycles for SCE study.

### *Experiment — II*

CHO cells were treated with 5 different concentrations of nicotine i.e., 150 μg/ml, 250 μg/ml, 375 μg/ml, 500 μg/ml and 625 μg/ml of GM. As mentioned earlier, the cells were allowed to grow for 24 h and 48 h for CA and SCE analysis respectively, in GM containing 10 μg/ml BrdU. The test substance was present in the medium till harvesting.

In both these experiments, during the final 3 h of incubation, colchicine was added at a final concentration of 0.3 μg/ml GM. Simultaneously untreated cultures and positive control cultures (MMC — 0.03 μg/ml GM for expt. — I; 0.007 μg/ml GM for expt. — II) were also processed in the same manner. During all the steps, cultures were totally protected from direct light to prevent the photolysis of BrdU containing DNA [19]. The harvesting of cultures and sister chromatid differential staining procedures were essentially the same as reported earlier [2]. Metaphases were identified in M I, M II and M III phases of cell division according to the staining patterns [3]. Following the criteria recommended for CA identification by WHO [12], a minimum of 100 metaphases in the M I phase were analysed, 25 well-spread metaphases in M II phase were considered for the calculation of mean SCE frequency. Student's *t*-test was applied for the statistical evaluation of the results.

**Table 1.** Chromosome aberrations observed after nicotine treatment and recovery in fresh GM.

| Treatment duration (h) | Concentration GM (μg/ml) | Abberant cells (%) | Chromatid | | | | Chromosome | | | | | CA/Cell ± S.E |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | G | B | I | Af | G | B | Dm | R | D | |
| | Control | 7 | 2 | 2 | — | 1 | 3 | — | — | — | — | 0.08 ± 0.0306 |
| 2 | 625 | 11 | 3 | 1 | — | 7 | 2 | 1 | — | 1 | 3 | 0.18 ± 0.0606 |
| | 1000 | 15 | 9 | 3 | — | 3 | 1 | 1 | 2 | 1 | 2 | 0.22 ± 0.0576* |
| 4 | 625 | 13 | 7 | — | — | 1 | 8 | 1 | — | 1 | 2 | 0.20 ± 0.0566 |
| | 1000 | 16 | 7 | 1 | — | 6 | 7 | 1 | — | 1 | — | 0.23 ± 0.0630* |
| | MMC 0.03 | 21 | 10 | 2 | 1 | 5 | 2 | 1 | — | — | 2 | 0.23 ± 0.0470** |

*$P < 0.05$.
**$P < 0.01$.
(*P* excluding gaps — Not significant)
Key: G = gap; B = break; Af = acentric fragment; I = interchange; Dm = double minute; R = ring; D = dicentric.

## Results

*Experiment — I*

The data presented in Table 1 and Table 2 summarizes the effect of nicotine on CA and SCE frequency. The induction in CA frequency was dose-dependent, however, the increase in CA rate was found to be statistically significant ($P < 0.05$), only for the cultures treated with 1000 μg nicotine/ml GM. Cultures treated with 625 μg nicotine/ml GM for 2 h and 4 h had 8.40 ± 0.379 and 9.04 ± 0.465 SCE per cell (mean ± SE.), respectively, and for cultures treated with 1000 μg nicotine/ml GM, the mean values were 9.56 ± 0.460 and 10.68 ± 0.518, respectively. The SCE inductions for all the treated cultures, were statistically significant ($P < 0.001$) compared to 6.12 ± 0.261 (mean ± S.E.) SCE per cell in untreated cultures. Frequency of CA and SCE in MMC-treated cultures were 0.23 ± 0.047 and 20.52 ± 0.495, respectively.

*Experiment — II*

The details of the effects of continuous nicotine treatment on CA and SCE frequency in

**Table 2.** SCE frequencies observed after nicotine treatment and recovery in fresh GM.

| Treatment duration (h) | Concentration GM (μg/ml) | Range | Percent distribution of cells according to the number of SCEs | | | | SCE/Cell ± S.E. |
|---|---|---|---|---|---|---|---|
| | | | 0—5 | 6—10 | 11—15 | >15 | |
| | Control | 2—10 | 40 | 60 | 00 | 00 | 6.12 ± 0.261 |
| 2 | 625 | 5—12 | 04 | 80 | 16 | 00 | 8.40 ± 0.379* |
| | 1000 | 5—14 | 04 | 68 | 28 | 00 | 9 56 ± 0 460* |
| 4 | 625 | 5—15 | 04 | 76 | 20 | 00 | 9.04 ± 0 465* |
| | 1000 | 7—17 | 00 | 64 | 28 | 08 | 10 68 ± 0 518* |
| | MMC 0.03 | 16—26 | 00 | 00 | 00 | 100 | 20.52 ± 0.495* |

*$P < 0.001$.

**Table 3.** Effect of continuous treatment of nicotine on chromosome aberration.

| Concentration GM (μg/ml) | Abberant cell (%) | Chromatid | | | | Chromosome | | | | | CA/Cell ± S.E. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | G | B | I | Af | G | B | Dm | R | D | |
| Control | 5 | 2 | 2 | — | — | 3 | — | — | — | — | 0.06 ± 0 0276 |
| 150 | 6 | 3 | 1 | — | — | 2 | — | — | — | — | 0.06 ± 0.0237 |
| 250 | 11 | 5 | 1 | 1 | 2 | 3 | — | — | — | — | 0.12 ± 0.0354 |
| 375 | 16 | 5 | 2 | — | 4 | 4 | 1 | — | 1 | — | 0.17 ± 0.0401[a,***] |
| 500 | 21 | 13 | 8 | — | — | 6 | 2 | — | 1 | — | 0.29 ± 0.0653[a,**] |
| 625 | 25 | 15 | 4 | — | 6 | 4 | 1 | — | — | 1 | 0.31 ± 0.0595[b,**] |
| MMC 0.007 | 18 | 8 | 4 | 1 | — | 4 | — | — | 2 | 3 | 0 22 ± 0.0450[b,*] |

*$P < 0.01$; **$P < 0.001$; ***$P < 0.02$.
Excluding gaps — a = $P < 0.05$; b = $P < 0$ 01.
Key: G = gap; B = break; Af = acentric fragment; I = interchange; Dm = double minute; R = ring; D = dicentric.

CHO cells are provided in Tables 3 and 4. Cultures, treated with the last three concentrations of nicotine, showed statistically significant elevations in CA frequencies compared to that of the untreated cultures. The mean SCE frequencies ranged from 7.76 to 11.33 in cultures treated with 150 μg nicotine/ml GM to 625 μg nicotine/ml GM. These values were significantly higher than that of the untreated cultures (6.48 ± 0.392). Frequency of CA and SCE in MMC-treated cultures were 0.22 ± 0.045 and 37.56 ± 0.780 (mean ± SE.), respectively.

## Discussion

The magnitude of tobacco-related cancers has become a universal problem. About 2549 individual chemical constituents have been identified in tobacco [5]. Tobacco specific nitrosamines (TSNA), polycyclic aromatic hydrocarbons (PAH) and polonium (210 Po) have been held responsible for its carcinogenicity [14]. Nicotine serves as a precursor for highly carcinogenic nitrosamines NNN and NNK, which are important in tobacco carcinogenesis [16]. In view of the major role of nico-

**Table 2.** Results of continuous treatment of nicotine on SCE frequencies.

| Concentration GM (μg/ml) | Range | Percent distribution of cells according to the number of SCEs | | | | SCE/Cell ± S.E |
|---|---|---|---|---|---|---|
| | | 0—5 | 6—10 | 11—15 | >15 | |
| Control | 3—11 | 32 | 64 | 04 | 00 | 6 48 ± 0.392 |
| 150 | 4—14 | 08 | 80 | 12 | 00 | 7.76 ± 0.464** |
| 250 | 6—12 | 00 | 88 | 12 | 00 | 8.84 ± 0.320* |
| 375 | 4—14 | 04 | 60 | 36 | 00 | 9.64 ± 0 496* |
| 500 | 7—18 | 00 | 68 | 24 | 08 | 10.52 ± 0.569* |
| 625 | 7—21 | 00 | 44 | 52 | 04 | 11.32 ± 0.551* |
| MMC 0.007 | 24—45 | 00 | 00 | 00 | 100 | 37 56 ± 0.780* |

*$P < 0.001$, **$P < 0.05$.

tine in tobacco habituation, in the formation of carcinogens and as it comes in direct contact with the target tissues of the tobacco consumers, systematic examination of possible genotoxic potentials of nicotine is very important.

Short term in vitro assays have been widely used to detect the mutagenic/carcinogenic potentials of chemicals. Previously Riebe et al. [24] have reported the genotoxic effects of nicotine and other tobacco specific alkaloids, on CHO cells using SCE as the only endpoint. Two cytogenetic markers, namely SCE and CA have been included in the present study to work out the genotoxic potentials of nicotine more conclusively. Even in the absence of metabolic activation, the CA and SCE frequencies were observed to be elevated. This confirms the finding of Riebe et al. [24] that nicotine does not need metabolic activation and is genotoxic by itself.

The results of the present experiments showed that induction of SCE frequency was highly significant for all the concentrations of nicotine, whereas, statistically significant elevation in CA frequency was observed only by highest concentrations for either treatment periods. This confirmed that the mechanisms for the formation of CA and SCE are different [8]. The lowest dose of nicotine employed for continuous treatment i.e., 150 μg/ml correspond to the concentration of nicotine detected in the saliva of tobacco chewers [22]. Even by this concentration, the elevation in SCE frequency was statistically significant with $P < 0.01$, whereas, CA frequency was not affected at all. In other words, SCE emerged to be a more sensitive indicator of nicotine genotoxicity.

In the present study, we have found that nicotine induced SCEs in dose-dependent manner. The induction in SCE frequency was more after the exposure to nicotine (625 μg/ml) for 4 h than for 2 h. The increase in the frequency of SCE by 48 h treatment with the same concentration, was statistically significant compared to 2 h ($P < 0.001$) and 4 h ($P < 0.01$) treatments, i.e., DNA damage, caused by nicotine, increased with the treatment time. Thus, our findings of nicotine genotoxicity provides an explanation regarding the increased risk of oral cancers and duration of tobacco useage [28]. Nicotine is one of the factors responsible for the increased lymphocytic SCEs in individuals chewing tobacco with areca nut [1]. Ghosh and Ghosh have reported the effect of tobacco smoking and chewing betel quid with tobacco on SCE frequency in human lymphocyte chromosomes [9,10]. They have observed significant correlation between the frequency of tobacco consumption and mean SCE value, as well as between the duration of tobacco use and mean SCE frequency. Presently, nicotine chewing gum is being advocated as a substitute for tobacco smoking [26]. Here, daily consumption of several milligrams of nicotine, culminating into a cumulative dose effect should be considered for its adverse effects. Taking all the available reports into consideration and the results of the present study, it can be inferred that nicotine, in addition to being addictive is genotoxic as well.

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# Cytogenetic studies reveal increased genomic damage among 'pan masala' consumers

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**Pan masala (PM), a dried powdered mixture containing ingredients like areca nut, catechu, lime, cardamom and flavouring agents, is consumed abundantly by Indians and is also exported to Western countries. Pan masala with tobacco (PM-T) is also available on the market. In view of the role of the ingredients of PM in the causation of oral diseases, the possible harmful effects of consuming this complex mixture were analysed in individuals regularly consuming PM and among healthy non-consuming controls without any habit. Three cytogenetic endpoints and two tissues were employed to assess possible DNA damage. Sister chromatid exchange and chromosome aberrations were estimated in the peripheral blood lymphocytes, tissues indirectly exposed to the substance and the frequency of micronucleated cells was scored in the tissue directly in contact with PM, i.e. the exfoliated buccal mucosa cells. All three cytogenetic endpoints demonstrated a statistically significant increase ($P < 0.001$) among the PM consumers as compared with the non-consuming controls.**

## Introduction

There has been irrefutable evidence that the use of tobacco is a major health hazard and public opinion against its use has heightened. However, with no counter evidence chasing its ever increasing popularity and, under the pretext of being 'safe', in India a new substitute 'pan masala' (PM) is being consumed abundantly, even by those who generally refrain from smoking or other tobacco habits. Unlike betel quid, which is almost always freshly prepared, PM is a dry powdered mixture of various ingredients and is commercially marketed under different brand names. Seeking to determine the genotoxic potentials of this complex mixture, one is immediately confronted by the fact that it is comprised of substances suspected of possessing carcinogenic/mutagenic properties. As printed on their packings, PM consist of areca nut, catechu, lime, cardamom and unspecified flavouring agents (Figure 1). Areca nut, which constitutes 70–80% of the mixture (our estimation for the brand studied), is reported to possess cytotoxic, mutagenic and genotoxic properties (IARC, 1985; Panigrahi and Rao, 1986; Wary and Sharan, 1988; Sundqvist *et al.*, 1989). Giri *et al.* (1987) have reported genotoxic effects of catechu. Lime has also been considered to play an important role in the genesis of oral cancer (Tanaka *et al.*, 1983; Agrawal *et al.*, 1986). The flavouring agents used in PM are not specified; however, the occasional use of synthetic flavours like musk ambrette and musk xylene to improve the flavour of tobacco (zarda) is well known. Both musks have been found to be mutagenic in the Salmonella/mammalian microsome test system (Nair *et al.*, 1986). PM with tobacco (zarda, a kind of processed tobacco, PM-T) is consumed as an alternative form of tobacco chewing.

Our *in vitro* short-term experiments on mammalian test systems, employing cytogenetic endpoints like sister chromatid exchange (SCE) and chromosome aberrations (CA), revealed genotoxic potentials of PM (Adhvaryu *et al.*, 1989a). Recently, Bagwe *et al.* (1990) have reported that the aqueous and chloroform extracts of PM were non-mutagenic while the ethanolic extract was weakly mutagenic in the Ames assay using two tester strains of *Salmonella typhimurium*, i.e. TA98 and TA100. To substantiate the information gained by the *in vitro* studies and with a view of assessing the potential hazards associated with this seemingly harmless product, we have attempted to determine its genotoxic potentials in individuals regularly consuming PM.

The analysis of SCE and CA in peripheral blood lymphocytes (PBL) is one of the most extensively employed indicators in population biomonitoring for determining the genotoxic effects in individuals exposed to mutagenic carcinogens (WHO, 1985; Carrano and Natarajan for ICPEMC, 1988). Exfoliated buccal mucosa cells can be collected non-invasively for screening of micronucleated cells. Micronuclei serve as simple markers indicating direct exposure to a DNA-damaging agent (Stich and Rosin, 1984). Thus in order to make the study more comprehensive, a parallel effort was made to analyse DNA damage in PBLs (the tissue not coming into direct contact with PM) and exfoliated buccal mucosa cells (the tissue directly exposed to the substance), utilizing three cytogenetic endpoints among individuals consuming PM and among normal healthy controls.

## Materials and methods

*Selection of subjects*

A total of 30 healthy vegetarian teetotalers were studied. Care was taken not to include those who had a viral disease or who had had any antibiotic therapy during

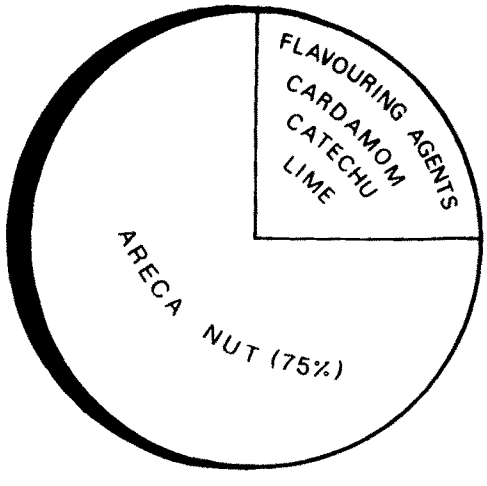


**Fig. 1.** Constituents of PM (plain). The areca nut content remains the same in the tobacco-containing variety of PM.

**Table I.** Individual mean ± SE values of various cytogenetic endpoints among controls

| Subject | Age Sex | SCE/cell | CA/cell | %MNC | AGT |
|---|---|---|---|---|---|
| 1 | 23 M | 5.66 ± 0.411 | 0.04 ± 0.0196 | 0 3 | 35.82 |
| 2 | 23 F | 6.46 ± 0.400 | 0.05 ± 0.0218 | 0 1 | 22 02 |
| 3 | 24 M | 6.31 ± 0.550 | 0.03 ± 0.0171 | 0 1 | 33.57 |
| 4 | 25 M | 6.63 ± 0.315 | 0.05 ± 0.0218 | 0 3 | 31.17 |
| 5 | 25 M | 6.28 ± 0.465 | 0.05 ± 0.0218 | 0.1 | 27.59 |
| 6 | 26 M | 5.70 ± 0.298 | 0 06 ± 0.0276 | 0 3 | 27 91 |
| 7 | 26 F | 6.73 ± 0.360 | 0.05 ± 0.0218 | 0.1 | 35 29 |
| 8 | 27 M | 6.70 ± 0.322 | 0 05 ± 0.0218 | 0.0 | 31 58 |
| 9 | 28 F | 6.19 ± 0.420 | 0 06 ± 0.0238 | 0 3 | 25.81 |
| 10 | 30 M | 5 56 ± 0.271 | 0.03 ± 0.0171 | 0.1 | 29.81 |
| 11 | 32 F | 6.35 ± 0.281 | 0.05 ± 0.0218 | 0 3 | 28.07 |
| 12 | 35 M | 6 36 ± 0.510 | 0.05 ± 0.0218 | 0.1 | 26.97 |
| 13 | 39 F | 5.96 ± 0.236 | 0.04 ± 0.0196 | 0.3 | 29 45 |
| 14 | 40 M | 5.55 ± 0.330 | 0.08 ± 0.0271 | 0.2 | 34.78 |
| 15 | 55 F | 6.18 ± 0.263 | 0.06 ± 0.0238 | 0 2 | 42.86 |
| Mean ± SE | 30.53 ± 2.170 | 6.17 ± 0.0101 | 0 05 ± 0.0031 | 0 1866 ± 0 0264 | 30.560 ± 1 2175 |

the preceding 6 months and to see that they were not engaged in hazardous occupations They were categorized in two groups· (i) PM consumers and (ii) controls.

*PM consumers.* Compared to the deep rooted custom of chewing tobacco–areca nut, PM consumption is a relatively new habit. In the present study we have attempted to select only those individuals who consumed only PM (plain, i.e. without tobacco) or PM-T for at least 1 year, without any other concurrent habit of tobacco or areca nut consumption. Only those individuals who had no clinically detectable change in the oral mucosa and who were consuming the same brand of PM (the one utilized in our *in vitro* experiments, Adhvaryu *et al.* 1989a), were included in the study. Since these criteria for selecting PM consumers were strictly adhered to, we had difficulty in finding appropriate subjects.

*Controls.* Normal, healthy, age-matched individuals not consuming tobacco or areca nut in any form were selected as controls

*Collection of sample*

For lymphocyte culture, venous blood was collected asceptically in heparinized vials.

For the micronucleus assay, exfoliated buccal mucosa cells were collected after rinsing the mouth thoroughly with water. Using a blunt spatula, the cells were scrapped from the region where the PM was usually placed. The cells were smeared on clean slides, fixed with acetic acid: methanol (1:3), air dried and stored until stained.

*Culture and staining protocols*

The culture and staining procedures for PBLs have been described elsewhere (Adhvaryu *et al.*, 1986).

The smears of exfoliated buccal mucosa cells were stained using the Feulgen plus fast green method with minor modifications. Briefly, the cells, hydrolysed at room temperature with 5 M HCl for 20 min, were stained with Schiff's Reagent for 2 h, followed by three changes in $SO_2$ water and were then counter-stained with 0.5% alcoholic fast green.

*Scoring*

The slides were coded by a person not involved in scoring Metaphases were identified in the first (M1), second (M2) or third (M3) cycle according to their differential staining patterns, and average generation time (AGT) was calculated by computing M1, M2 and M3 values by the following formula (Tice and Ivett, 1985).

$$\text{AGT (h)} = \frac{\text{Hours since onset of BrdU exposure}}{\text{Proliferation index (PRI)}}$$

where

$$\text{PRI} = \frac{1 \times \text{M1} + 2 \times \text{M2} + 3 \times \text{M3}}{100}$$

For SCE analysis a minimum of 25 well spread metaphases in M2 were counted, while for CA analysis 100 metaphases in M1 were considered. At least 1000 cells from each individual were screened for MNC frequency The identification of micronuclei was based on the criteria proposed by Sarto *et al.* (1987). Student's *t*-test was applied for statistical analysis of the results.

## Results

The details of age/sex and individual mean expressions of SCE, CA, %MNC and AGT among the controls are given in Table I. Similar particulars for PM consumers, together with the frequency and duration of the chewing habit are given in Table II. As seen from the tables, a statistically significant increase in the mean values of all the three endpoints of PM consumers (jointly, or as separate groups of PM and PM-T consumers) was observed when compared to those of the controls ($P < 0.001$). The mean AGT values of PM consumers and controls remained comparable.

Since the number of PM consumers is small and also a wide variation in the frequency of PM consumption was observed, it was not desirable to search for a correlation between the frequency/duration of the habit with the mean values of the cytogenetic endpoints. It was interesting to note that the mean SCE per cell value among all the PM consumers was higher than the highest mean SCE per cell observed among the control subjects, i.e. 6.70. The same was true for %MNC frequency which was 0.3% (highest) among controls, whereas all the PM consumers had values above 0.3%. For CA frequency, only one PM consumer expressed a CA per cell value (0.07) lower than the highest observed in the controls, i.e. 0.08 CA per cell (Tables I and II). The increase in the CA per cell frequency remained statistically significant ($P < 0.001$) even when gaps were excluded from the calculations (Table III).

As shown in Table II, the mean values of SCE, CA and %MNC did not vary among PM and PM-T consumers. The respective values for SCE being 7.592 and 7.576; for CA 0.122 and 0.120 and for %MNC 0.70 and 0.82 among PM and PM-T consumers.

Table III gives a comparative view of the mean values of all the three cytogenetic endpoints among controls and PM consumers. The elevated levels of SCE, CA and %MNC among PM consumers clearly implicate increased genomic damage in the tissue directly exposed, as well as in the cells exposed to biologically effective doses (i.e. absorbed in the body) of various constituents of PM.

Table II. Details of different cytogenetic markers among PM consumers (mean ± SE)

| Subject | Age Sex | SCE/cell | CA/cell | %MNC | AGT |
|---|---|---|---|---|---|
| *Pan masala (plain)* | | | | | |
| 1 | 22 M | 7.40 ± 0.388 | 0.10 ± 0.0332 | 0.9 | 25.81 |
| 2 | 26 M | 7 36 ± 0.466 | 0.08 ± 0.0271 | 0.5 | 33.33 |
| 3 | 28 M | 7 82 ± 0.477 | 0.15 ± 0.0357 | 0 8 | 40.00 |
| 4 | 31 F | 7 16 ± 0.405 | 0.17 ± 0.0506 | 0.8 | 41 38 |
| 5 | 32 F | 8.00 ± 0.449 | 0.10 ± 0.0300 | 0.8 | 29.27 |
| 6 | 34 M | 7 80 ± 0.431 | 0 13 ± 0.0391 | 0.6 | 25.40 |
| 7 | 36 F | 7.93 ± 0.480 | 0.16 ± 0.0367 | 0.4 | 39.67 |
| 8 | 38 F | 7.89 ± 0.501 | 0.09 ± 0.0320 | 0.6 | 34.78 |
| 9 | 39 M | 7.68 ± 0.407 | 0.14 ± 0.0375 | 0.7 | 28.57 |
| 10 | 55 F | 6.88 ± 0.617 | 0.10 ± 0.0360 | 0.9 | 41.03 |
| Mean ± SE | 34.10 ± 2.726 | 7.592 ± 0.1121 | 0.122 ± 0.0096 | 0.70 ± 0.051 | 33.924 ± 1.923 |
| *Pan masala (with tobacco)* | | | | | |
| 11 | 20 M | 7.95 ± 0.315 | 0.07 ± 0 0255 | 0.6 | 31.79 |
| 12 | 25 M | 7.22 ± 0.224 | 0.11 ± 0.0313 | 0.8 | 25.40 |
| 13 | 27 M | 7.45 ± 0.278 | 0.16 ± 0.0393 | 1.1 | 26.52 |
| 14 | 33 M | 7.79 ± 0.355 | 0.12 ± 0.0382 | 0.8 | 36.64 |
| 15 | 35 M | 7.47 ± 0.253 | 0.14 ± 0.0425 | 0.8 | 28.92 |
| Mean ± SE | 28.00 ± 2.433 | 7.576 ± 0.1165 | 0.12 ± 0.0135 | 0.82 ± 0.0715 | 29 854 ± 1.806 |
| Aggregate Mean ± SE | 32.07 ± 2 123 | 7.586 ± 0.0842 | 0.121 ± 0.0078 | 0 740 ± 0.0440 | 32.567 ± 1.4948 |

## Discussion

Human populations are exposed to various carcinogenic substances accidentally, occupationally or by life style. When contact with such substances is habit associated, it is much easier to curtail the exposure by creating an awareness and changing the human perspective towards the habit. Since a long latent period may exist between the exposure and the manifestation of the disease, the harmful effect of a habit will be observed only after a prolonged period of time. The indiscriminate use of PM, even by women and children, the escalating sales in India and neighbouring countries, and also its export to Western countries, needs to be carefully watched.

Our *in vitro* experiments, employing short-term assays on mammalian cell cultures, revealed the genotoxic potential of an aqueous extract of PM (Adhvaryu *et al.*, 1989a). The genotoxic effects were marginally more pronounced on treating the cells with an aqueous extract of PM-T (unpublished data). When interpreting the genotoxic potentials of a complex mixture, the implications of synergistic or antagonistic interactions between individual substances should be carefully considered. However, as outlined earlier, the areca nut forms the major fraction of PM (70–80%) and the results of experiments from our laboratory with aqueous extract of areca nut correlates well with that of PM extract. The findings of the present study are also in accordance with the cytogenetic studies among individuals chewing areca nut without tobacco (data not presented here). Thus, it can be speculated that areca nut plays an important role in the genotoxic effects of PM. It is to be noted that due to the addition of tobacco to the mixture, one would theoretically expect increased damage among PM-T chewers compared to PM chewers; however, the values of the cytogenetic endpoints remain almost analogous in both these groups. An explanation for this may be provided on the basis of differences in chewing pattern. Due to the bitter taste of tobacco, the consumers of PM-T frequently expectorate the saliva while chewing the mixture. In contrast, the PM chewers swallow the mixture as well as the saliva. This increases the possibility of more severe genotoxic effects.

Table III. Comprehensive data of the cytogenetic endpoints among controls and PM consumers (mean ± SE)

| Group | SCE/cell | CA/cell | %MNC |
|---|---|---|---|
| Controls | 6.170 ± 0.010 | 0.050 ± 0 003 (0.007 ± 0.002) | 0.186 ± 0.0264 |
| PM chewers | 7.586 ± 0.084[a] | 0.121 ± 0.008[a] (0.043 ± 0.006)[a] | 0.740 ± 0.0440[a] |

[a] $P < 0\ 001$

Values in parenthesis indicate CA/cell excluding gaps.

Stich and Stich (1982) observed that saliva of Pan Bahar chewers was clastogenic to CHO cells. (Pan Bahar is a PM containing areca nuts, catechu, cardamom, lime, copra, menthol and perfumes.) The presence of clastogenic substances or PM in the saliva of chewers indicates the ability of the substance to damage the cells of the oral mucosa. It has been observed that areca nut/tobacco-specific alkaloids, *N*-nitroso compounds derived from these alkaloids, polyphenols and tannins can be released in the saliva while chewing these substances (Wenke *et al.*, 1984; Nair *et al.*, 1985, 1987; Prokopczyk *et al.*, 1987; Stich and Anders, 1989; Sundqvist *et al.*, 1989). These might be responsible for the increased damage in the oral mucosa of areca nut/tobacco chewers. A very high frequency of micronucleated cells has been observed among Indians chewing betel quid, areca nut and/or tobacco (Stich *et al.*, 1982a,b). Similar information has been gathered from our ongoing studies among tobacco and/or areca nut chewers. Given these, little remains to be explained about the increased micronuclei frequency in the buccal mucosa cells of PM consumers. It implies that even in this complex mixture, the DNA damaging effects of areca nut and tobacco persist. The *in vitro* experiments and

endogenous formation of nitrosamines imply that the nitrosation of areca nut and/or tobacco alkaloids occurs in the mouth leading to increased exposure of buccal mucosa to specific nitrosamines. However, chewers who swallow the betel quid, a situation comparable to PM consumption, may form even higher amounts of these nitrosamines in their stomach at pH 2.0 (Nair *et al.*, 1985). To explore the possibilities of the effects of PM at sites other than the oral cavity and the oesophageal lining, cytogenetic analysis in peripheral blood lymphocytes was carried out. Since most lymphocytes belong to the redistributional pool, lymphocytes exposed to a mutagen anywhere in the body can eventually occur in peripheral blood. The system thus offers an advantage to test the *in vivo* mutagenic/genotoxic effects of chemicals on human somatic cells. Previously, we have reported increased frequencies of lymphocytic SCE among tobacco and areca nut chewers (Adhvaryu *et al.*, 1986). Ghosh and Ghosh (1984, 1988) have also presented similar observations among betel quid chewers. CA are considered to be one of the most unambiguous cytogenetic manifestations of genotoxicity among individuals exposed to suspected genotoxins. Recently, Brogger *et al.* (1990) have reported that subjects with a high frequency of CA develop cancer twice as often as others. The rate of CA has been found to be higher among tobacco/areca nut chewers in our studies (Adhvaryu *et al.*, 1989b). To the best of our knowledge, except this report from our laboratory, CA have not been reported among tobacco/areca nut chewers. However, elevated levels of CA have been witnessed among smokers and alcoholics (Obe *et al.*, 1980; Vijayalaxmi and Evans, 1982). Thus it appears that areca nut, which is present in PM, and areca nut plus tobacco, which is present in PM-T, both influence the SCE and CA frequencies among PM chewers.

It has been emphasized that while assessing the exposure to possible genotoxins, the use of a battery of cytogenetic markers is always more beneficial (Carrano and Natarajan for ICPEMC, 1988). The present report, considering the target as well as the non-target tissues, once again justifies this approach. Further, proper regard was paid to undertaking a blind study with carefully matched controls and eliminating most of the confounding factors that might interfere in the cytogenetic analysis.

The number of PM chewers reported here might appear to be small. This is because of strictly adhering to the specific selection criteria (detailed in Materials and methods). Nevertheless, considering the fact that the proposal of implementing a statutory warning on PM (without tobacco) has met with a lot of controversy, it was felt that the present report, being the first of its kind among humans consuming PM, will provide a scientific basis and help the authorities in making more rational decisions. Although the results confirm that consuming PM, *per se*, can cause DNA damage in the target as well as the non-target tissues, close surveillance and follow-up epidemiological and cytogenetic studies will prove to be more meaningful.

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# Cytogenetic surveillance of tobacco-areca nut (mava) chewers, including patients with oral cancers and premalignant conditions

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## Summary

Three cytogenetic endpoints were studied in non-chewing healthy controls and 3 groups of tobacco-areca nut chewers, viz. normal chewers, chewers with oral submucous fibrosis and chewers with oral cancer. Frequencies of sister-chromatid exchanges and chromosome aberrations in peripheral blood lymphocytes and of micronucleated cells in exfoliated buccal mucosa were evaluated. All the parameters showed statistically significant elevations in all 3 groups of chewers compared to the controls. The data indicate possible application of the parameters as sensitive endpoints for monitoring tobacco-areca nut chewers, the group of individuals at higher risk of developing oral cancer, the commonest cancer among Indian males.

Consuming tobacco is the major avoidable cause of human morbidity and mortality. A number of epidemiological studies have established a close correlation between the consumption of tobacco in various forms and a higher incidence of site-specific cancers (Jussawala and Deshpande, 1971; Sanghvi, 1981). The ill effects of smoking have been studied in great depth, however, tobacco is carcinogenic irrespective of the mode of its consumption. As a result of these findings, coupled with reported harmful effects of passive smoking, a strong public opinion against tobacco smoking has been generated. This has partly culminated in the increasing acceptance of smokeless tobacco, under the pretext of being a safer alternative (Chassin et al., 1985; Schaefer et al., 1985).

Chewing of tobacco as an ingredient of betel quid (areca nut, tobacco, catechu, lime and some flavouring ingredients wrapped in betel leaf (*Piper betel* Linn.)), is a widespread habit in India and South-East Asia. This habit has been held responsible for oral cavity cancers, which form almost one third of total cancer cases in India (WHO, 1984). In Gujarat, a part of Western India, a mixture of sun-cured unflavoured tobacco, dried pieces of areca nut and lime (locally known as *mava*), which does not contain betel

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TABLE 1

INDIVIDUAL FREQUENCIES OF SISTER-CHROMATID EXCHANGES (SCE) PER CELL (MEAN ± SE) IN BLOOD LYMPHOCYTES OF TOBACCO-ARECA NUT (MAVA)-CHEWERS AND CONTROL PERSONS

| Subject | Age/Sex | Controls | Subject | Age/Sex | NC | Subject | Age/Sex | OSMF | Subject | Age/Sex | OC |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 17 M | 6.04 ±0 325 | 16 | 21 M | 9.63 ±0.453 | 31 | 17 M | 8.38 ±0.568 | 46 | 22 M | 7.45 ±0.384 |
| 2 | 19 M | 6 35 ±0.319 | 17 | 24 M | 7.51 ±0.516 | 32 | 20 M | 6 21 ±0.399 | 47 | 23 M | 8.05 ±0.369 |
| 3 | 23 M | 5.66 ±0.411 | 18 | 25 M | 6.24 ±0 432 | 33 | 21 M | 6.55 ±0.345 | 48 | 34 M | 9.61 ±0.477 |
| 4 | 23 F | 6 46 ±0 400 | 19 | 26 M | 5.86 ±0.448 | 34 | 23 M | 7.53 ±0.335 | 49 | 34 M | 8.62 ±0.487 |
| 5 | 24 M | 6.31 ±0.550 | 20 | 27 M | 6.80 ±0.228 | 35 | 24 M | 8.35 ±0 319 | 50 | 35 M | 6.77 ±0.233 |
| 6 | 24 F | 5 86 ±0.380 | 21 | 33 M | 6.65 ±0.375 | 36 | 24 M | 7 33 ±0.264 | 51 | 40 F | 9.99 ±0.553 |
| 7 | 25 M | 6.63 ±0.315 | 22 | 34 M | 7.28 ±0.286 | 37 | 28 M | 7.62 ±0.315 | 52 | 40 F | 9.18 ±0.501 |
| 8 | 25 M | 6.28 ±0 465 | 23 | 35 M | 7.13 ±0.585 | 38 | 28 M | 7 44 ±0.400 | 53 | 45 M | 8.34 ±0.276 |
| 9 | 26 M | 5.70 ±0.298 | 24 | 37 M | 7.57 ±0.503 | 39 | 29 M | 8.11 ±0.375 | 54 | 50 M | 7 44 ±0.247 |
| 10 | 28 F | 6.19 ±0 420 | 25 | 37 M | 7.33 ±0 584 | 40 | 32 M | 8.07 ±0.377 | 55 | 50 F | 8.82 ±0.431 |
| 11 | 35 M | 6.36 ±0.510 | 26 | 40 M | 7.28 ±0.405 | 41 | 33 M | 6.66 ±0 249 | 56 | 50 F | 7 96 ±0.405 |
| 12 | 49 F | 6.95 ±0.387 | 27 | 46 F | 6.54 ±0 408 | 42 | 36 M | 7 36 ±0.287 | 57 | 50 F | 7.73 ±0 388 |
| 13 | 55 F | 6.06 ±0.346 | 28 | 50 M | 6 69 ±0.288 | 43 | 37 M | 7 75 ±0 478 | 58 | 55 M | 8 62 ±0.412 |
| 14 | 58 F | 5.79 ±0 228 | 29 | 56 M | 7.95 ±0 430 | 44 | 43 M | 8.87 ±0.504 | 59 | 64 M | 10.36 ±0 542 |
| 15 | 60 F | 6 14 ±0.207 | 30 | 56 M | 7.83 ±0.549 | 45 | 60 F | 8 02 ±0.392 | 60 | 70 M | 8 56 ±0.426 |
| Mean | | 6.185 ± 0.088 | | | 7.219 ± 0.221 [a] | | | 7 617 ± 0.183 [a] | | | 8.500 ± 0.248 [a,b,c] |

NC, normal chewers; OSMF, oral submucous fibrosis patients, OC, oral cancer patients.

[a] $p < 0\ 001$ compared to controls.

[b] $p < 0.001$ compared to NC.

[c] $p < 0.01$ compared to OSMF

leaf or any other ingredient, is chewed very commonly. It is a mixture of ingredients well known for their carcinogenic/cocarcinogenic properties. The problem of oral cancers associated with tobacco-areca nut chewing is likely to assume very serious dimensions, as it is gaining acceptance among adolescents and women. Equally alarming is a recent report showing that genotoxic effects of betel quid chewing were more pronounced in pregnant women (Ghosh and Ghosh, 1988). These facts present an urgent need of evolving parameters for monitoring the genotoxic hazards of the tobacco-areca nut chewing habit.

Cytogenetic endpoints, such as sister-chromatid exchange (SCE), chromosome aberration (CA) and micronucleated cell (MNC) frequencies, have been advocated as sensitive parameters for assessing genotoxic effects of chemical or physical mutagens. When employed in combination, the data collected would provide more comprehensive information (Carrano and Natarajan, 1988; Gebhart, 1981; Hsu, 1982; Stich and Rosin, 1984). We have therefore selected scoring of CA and SCE frequencies in peripheral blood lymphocytes (PBLs) and scoring of MNC frequencies in exfoliated buccal mucosa cells of 4 different groups of individuals. Such an experimental design was expected to provide useful information about possible genotoxic effects of the tobacco-areca nut (mava) chewing habit on target as well as non-target tissues.

## Materials and methods

*Selection of subjects*

Altogether we studied 60 non-smoking teetotalers, including 45 chewing a mixture of tobacco, areca nut and lime only (hereafter referred as chewers) and 15 healthy non-chewing individuals as controls. The 45 chewers were further classifiable as (1) normal chewers (NC) without any clinically detectable change in oral mucosa, (2) chewers suffering from oral submucous fibrosis (OSMF), an oral premalignant condition and (3) chewers with oral cancers (OC), squamous cell carcinoma of the oral cavity. All 3 groups of chewers include individuals who chewed a mixture of tobacco, areca nut and lime (locally known as mava, which is not a betel quid) with comparable frequency and duration (average 6 chews/day). An equal number of individuals, who had never consumed tobacco or areca nut in any form, had had no viral disease or antibiotic therapy during the last 6 months and who had normal oral mucosa, were included as controls. The OSMF patients and OC patients were histologically confirmed cases, however, blood samples were collected before any anticancer therapy was started. Informed consent was obtained from all the individuals studied before sample collection.

Blood collection and culture were done in 9 batches. The controls and the normal chewers were pre-identified from the staff members of our hospital and adjoining institutions. Whenever appropriate OC and/or OSMF patients were available, the sampling was done on the same day and cultures were set with at least one control and one normal chewer sample.

*Culture procedure*

PBL culture procedures were essentially the same as detailed earlier (Adhvaryu et al., 1985). Briefly, aseptically collected heparinised whole blood (1.0 ml) was added to 7.0 ml growth medium comprised of 80% MEM (Earle's base and non-essential amino acids) and 20% newborn calf serum. PHA-M at 3% and 5-bromodeoxyuridine (BrdU) at 2.0 $\mu$g/ml final concentration were added. Following colchicine treatment (0.3 $\mu$g/ml) for the final 3 h, cultures were harvested at 72 h after the usual hypotonic (0.56% KCl) and fixation (3:1 methanol:acetic acid) protocol. Air-dried slides were stained by the fluorescence plus Giemsa (FPG) method for sister-chromatid differential staining which helped to identify cells in the M1, M2 and M3 phases of cell division.

A minimum of 100 well-spread metaphase cells in M1 phase were scored per sample for CA analysis. The identification of CA was carried out as recommended by WHO (1985). 25 metaphase cells in M2 phase were counted per sample for SCE scoring.

*Cytological preparations and micronucleus staining procedure*

After cleaning the mouth thoroughly by repeated gargling with drinking water, the cheek mucosa was scraped with a blunt spatula and

smears of exfoliated buccal mucosa cells were prepared on clean glass slides. Cells were fixed with 1:3 acetic acid:methanol fixative for 15 min. The cells were stained using the Feulgen plus fast green method with minor modifications. Briefly, after hydrolysis with 5 N HCl for 20 min at room temperature, cells were stained with Schiff's reagent for 2 h. Following 3 changes of $SO_2$ water, the cells were counterstained with 0.5% alcoholic fast green, dried and mounted with DPX.

A minimum of 1000 cells was screened from each individual for examining the presence of micronuclei. Micronucleus identification was done according to the criteria detailed by Sarto et al. (1987). The assay was not carried out for oral cancer patients as it was not feasible to obtain scorable smears due to poor hygienic condition of the oral cavity and because of abundant necrotic cells in the preparations.

For all 3 parameters studied, slides were coded and read blindly by 2 microscopists. The person responsible for coding distributed the slides in such a way that both microscopists had almost comparable numbers of controls and patients in each group. Results for the 4 groups examined, were compared with each other. Student's *t*-test was applied to check the statistical significance of the results.

## Results

Table 1 provides mean lymphocytic SCE/cell values for all 4 groups studied. For controls the mean SCE/cell value was $6.185 \pm 0.088$ (mean $\pm$ SE), whereas the values were $7.219 \pm 0.221$, $7.617 \pm 0.183$ and $8.500 \pm 0.248$ for NC, OSMF patients and OC patients, respectively. All 3 groups of tobacco-areca nut (mava) chewers had a statistically significant elevation in mean SCE/cell values compared to the controls ($p < 0.001$), who did not consume tobacco or areca nut in any form. Among the chewers, OSMF patients had a marginally elevated mean frequency compared to NC, whereas OC patients had a statistically significant elevation in mean SCE value compared to both NC ($p < 0.001$) and OSMF patients ($p < 0.01$). The metaphases scored for SCEs were further separated according to the number of SCEs

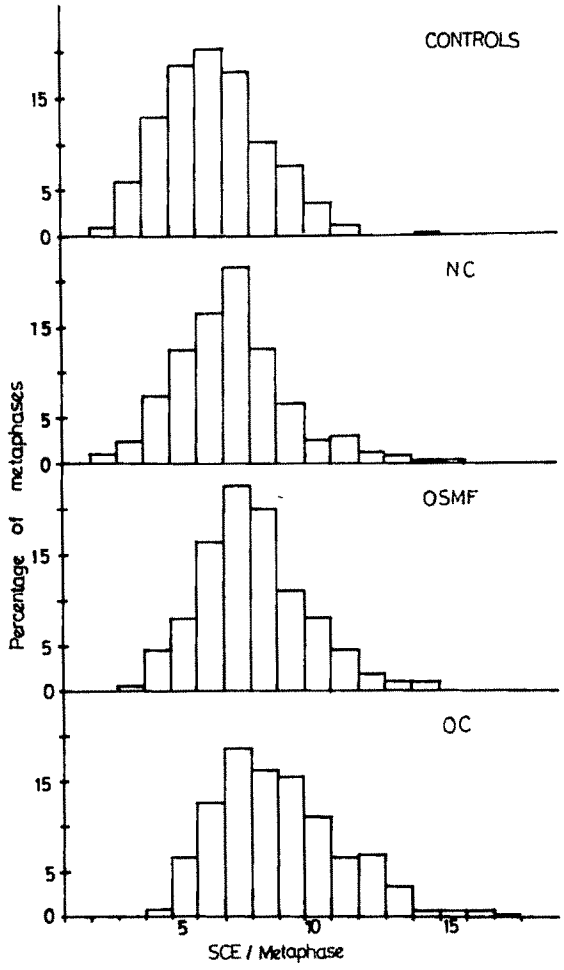


Fig. 1. Percentage distribution of metaphases according to the number of SCEs NC, normal chewers; OSMF, oral submucous fibrosis patients; OC, oral cancer patients.

(Fig. 1). As visualised in the figure, with the chewing habit and progression from normal to oral cancer, a shift is observed towards a greater number of cells with higher SCEs, thus resulting in higher mean SCE frequencies.

Details of lymphocytic CA analysis are presented in Tables 2 and 3. The CA/cell values varied from 0.03 to 0.08 in controls, providing a mean of $0.050 \pm 0.004$ (mean $\pm$ SE). Among the chewers, the CA/cell frequencies varied from 0.06 to 0.16 in NC giving a mean value of $0.097 \pm 0.007$. For OSMF patients, the values ranged from 0.08 to 0.20 yielding a mean value of 0.127

$\pm$ 0.009 and in OC patients CA values from 0.09 to 0.20 resulted in a mean of 0.144 $\pm$ 0.009. The increase in CA frequency (including gaps) in all 3 groups of chewers was statistically significant ($p < 0.001$) when compared with controls. The difference between CA frequencies of NC and OSMF patients ($p < 0.02$) as well as NC and OC patients was statistically significant ($p < 0.001$). Chromatid aberrations, the majority of which were gaps, were more frequent than chromosome-type aberrations. Interchanges were not observed in controls, but were found in all 3 groups of chewers. Even after excluding gaps from aberrations, elevations in CA/cell value in all 3 groups of chewers were significant when compared to the controls (Table 3).

Table 4 details the frequency of percent MNC observed in the smeared exfoliated buccal mucosa cells of different individuals. NC and OSMF patients had 0.74% and 0.75% MNC, respectively. These elevations in the frequency of MNC compared to the control value of 0.19% MNC were statistically significant ($p < 0.001$).

## Discussion

In India tobacco-areca nut chewing is very popular and has been strongly implicated in the

TABLE 2

INDIVIDUAL FREQUENCIES OF CHROMOSOME ABERRATIONS PER CELL (MEAN $\pm$ SE) IN BLOOD LYMPHOCYTES OF TOBACCO-ARECA NUT (MAVA) CHEWERS AND CONTROL PERSONS

| Subject | Controls | Subject | NC | Subject | OSMF | Subject | OC |
|---|---|---|---|---|---|---|---|
| 1 | 0.04 $\pm$0.0196 | 16 | 0.08 $\pm$0 0351 | 31 | 0 15 $\pm$0.0384 | 46 | 0 12 $\pm$0.0382 |
| 2 | 0.04 $\pm$0.0196 | 17 | 0 09 $\pm$0.0349 | 32 | 0.13 $\pm$0.0448 | 47 | 0.20 $\pm$0 0490 |
| 3 | 0.04 $\pm$0.0196 | 18 | 0.10 $\pm$0 0300 | 33 | 0.08 $\pm$0.0271 | 48 | 0.15 $\pm$0.0528 |
| 4 | 0.05 $\pm$0.0218 | 19 | 0 06 $\pm$0 0237 | 34 | 0.10 $\pm$0.0300 | 49 | 0.10 $\pm$0.0360 |
| 5 | 0.03 $\pm$0.0171 | 20 | 0.16 $\pm$0.0441 | 35 | 0.16 $\pm$0 0504 | 50 | 0.10 $\pm$0.0332 |
| 6 | 0 03 $\pm$0.0171 | 21 | 0 09 $\pm$0 0286 | 36 | 0.11 $\pm$0.0467 | 51 | 0.16 $\pm$0.0484 |
| 7 | 0.05 $\pm$0 0218 | 22 | 0.08 $\pm$0.0306 | 37 | 0.13 $\pm$0.0391 | 52 | 0 12 $\pm$0.0354 |
| 8 | 0 05 $\pm$0.0218 | 23 | 0.08 $\pm$0 0271 | 38 | 0.14 $\pm$0 0400 | 53 | 0.14 $\pm$0.0566 |
| 9 | 0.06 $\pm$0 0276 | 24 | 0 05 $\pm$0.0218 | 39 | 0 09 $\pm$0.0349 | 54 | 0.09 $\pm$0.0286 |
| 10 | 0.06 $\pm$0.0238 | 25 | 0.09 $\pm$0 0349 | 40 | 0 14 $\pm$0.0400 | 55 | 0 14 $\pm$0 0375 |
| 11 | 0.05 $\pm$0 0218 | 26 | 0 12 $\pm$0.0354 | 41 | 0.19 $\pm$0.0463 | 56 | 0.19 $\pm$0.0468 |
| 12 | 0.04 $\pm$0.0196 | 27 | 0.13 $\pm$0.0429 | 42 | 0 11 $\pm$0.0313 | 57 | 0.20 $\pm$0 0658 |
| 13 | 0.07 $\pm$0.0292 | 28 | 0.14 $\pm$0.0401 | 43 | 0.20 $\pm$0 0469 | 58 | 0 13 $\pm$0.0447 |
| 14 | 0.08 $\pm$0.0306 | 29 | 0.11 $\pm$0 0343 | 44 | 0 08 $\pm$0.0271 | 59 | 0.18 $\pm$0.0477 |
| 15 | 0.06 $\pm$0 0238 | 30 | 0 08 $\pm$0.0271 | 45 | 0.10 $\pm$0.0300 | 60 | 0.14 $\pm$0.0422 |
| Mean | 0.050 $\pm$ 0.004 | | * 0 097 $\pm$ 0 007 [a] | | ** 0.127 $\pm$ 0 009 [a,b] | | * 0 144 $\pm$ 0 009 [a,c] |

NC, normal chewers; OSMF, oral submucous fibrosis patients, OC, oral cancer patients. $P$ values including gaps: [a] $p < 0.001$ compared to controls; [b] $p < 0$ 02 compared to NC, [c] $p < 0$ 001 compared to NC. $P$ values excluding gaps: * $p < 0.01$ compared to controls; ** $p < 0$ 001 compared to controls

TABLE 3

NUMBER OF DIFFERENT TYPES OF CHROMOSOME ABERRATIONS PER 100 CELLS IN BLOOD LYMPHOCYTES OF TOBACCO-ARECA NUT (MAVA) CHEWERS AND CONTROL PERSONS

| Group | Total aberrations | Chromatid-type | | | Chromosome-type | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | G | B | I | G | B | Dm | D | R |
| Controls | 5.0 | 3.7 | 0 9 | – | 0.3 | 0.1 | – | – | – |
| NC | 9 7 | 6 4 | 2.2 | 0.1 | 1 1 | – | – | – | – |
| OSMF | 12 7 | 8.0 | 3.0 | 0 1 | 1.5 | 0.1 | 0 1 | – | – |
| OC | 14.4 | 10 6 | 2.5 | 0.1 | 0 8 | 0.1 | 0 2 | – | – |

NC, normal chewers, OSMF, oral submucous fibrosis patients, OC, oral cancer patients. G, gap, B, break, I, interchange; Dm, double minute, D, dicentric, R, ring.

TABLE 4

INDIVIDUAL FREQUENCIES (%) OF MICRONUCLEATED BUCCAL MUCOSA CELLS AMONG TOBACCO-ARECA NUT (MAVA) CHEWERS AND CONTROL PERSONS

| Subject | Controls | Subject | NC | Subject | OSMF |
|---|---|---|---|---|---|
| 1 | 0.2 | 16 | 0.8 | 31 | 0.8 |
| 2 | 0.3 | 17 | 1.2 | 32 | 0 7 |
| 3 | 0.3 | 18 | 0.4 | 33 | 0.3 |
| 4 | 0.1 | 19 | 1.1 | 34 | 1.8 |
| 5 | 0.1 | 20 | 0 5 | 35 | 0.4 |
| 6 | 0.2 | 21 | 0.9 | 36 | 0 2 |
| 7 | 0.3 | 22 | 0.7 | 37 | 0 8 |
| 8 | 0.1 | 23 | 0.9 | 38 | 1 1 |
| 9 | 0.3 | 24 | 0.5 | 39 | 0.6 |
| 10 | 0.3 | 25 | 0.7 | 40 | 1 0 |
| 11 | 0.1 | 26 | 0.6 | 41 | 0.7 |
| 12 | 0.1 | 27 | 0 7 | 42 | 0.5 |
| 13 | 0.2 | 28 | 1.0 | 43 | 0 8 |
| 14 | 0.2 | 29 | 0.8 | 44 | 0 6 |
| 15 | 0 1 | 30 | 0.3 | 45 | 1.0 |
| Mean ±SE | 0 193 ±0.022 | | 0.740 ±0.064 * | | 0.753 ±0.097 * |

NC, normal chewers; OSMF, oral submucous fibrosis patients.

* $p < 0.001$ compared to controls.

causation of oral premalignant and malignant diseases. Considerable interindividual variations have been observed regarding dose, duration of chewing mava and occurrence of the disease. Hence, a great deal of human morbidity and mortality can be prevented by watchful monitoring. In the present study, it was attempted to explore the possible utility of a combination of 3 cytogenetic endpoints in assessing the extent of genomic damage caused by the habit of mava chewing on target as well as non-target tissues.

Previously we reported elevated lymphocytic SCEs in normal chewers and chewers suffering from OSMF (Adhvaryu et al., 1986). Ghosh and Ghosh (1984, 1988) reported similar results among betel-quid chewers. Most striking in their studies was the SCE elevation in pregnant women. Stich et al. (1982a, b) also studied chewers from India and reported a significant elevation of MNC frequencies in exfoliated buccal mucosa cells. They attributed this to the genotoxic effects of tobacco and/or areca nut alkaloids. Arecoline, the major areca nut alkaloid, and areca nut extracts have been reported to increase SCE and CA frequencies in animal studies (Panigrahi and Rao, 1982, 1986) as well as in the in vitro system (Stich et al., 1981, 1983). Though nicotine, which is a major tobacco alkaloid, is by itself a weak clastogen, it induces SCEs in CHO cells (Riebe and Westphal, 1983). Many of the tobacco-areca nut-specific nitrosamines are detected in the saliva of chewers (Nair et al., 1985; Wenke et al., 1984; Sipahimalani et al., 1984; Prokopczyk et al., 1987) and some of these are potent mutagens and carcinogens (IARC, 1985).

SCE elevations have also been documented in PBLs of smokers (Sarto et al., 1985; Husum et al., 1982; Ardito et al., 1980). The SCE elevations observed in the present study are thus in accordance with the reports of others. However, in an ongoing work, individuals consuming only areca nut also exhibited higher lymphocytic SCEs than controls. The subjects included in the present study consumed a mixture of tobacco and areca nut. Since alkaloids of both these substances also exert genotoxic effects individually, both of them should be considered responsible for the elevated SCE frequencies in mava chewers. To the best of our knowledge, CAs have not been reported previously for tobacco and/or areca nut chewers. The lymphocytic CA rates were significantly higher in all 3 groups of chewers than in the controls. This increase can also be explained on the basis of the clastogenic potential of tobacco and areca nut alkaloids. However, since increases in SCE and CA frequencies have been recorded in different premalignant and malignant conditions (Adhvaryu et al., 1986, 1988a, b; Murty et al., 1985, 1986), the elevations in SCEs and CAs among OSMF patients and OC patients may be due to the habit, the occurrence of the disease, or a combination of both.

In the present study, the OC patients were older (mean age 44.1 years) than the other groups (mean ages 32.7, 36.5 and 30.3 years for controls, NC and OSMF patients, respectively). Reports regarding the effect of age and sex on the frequencies of SCEs and CAs are contradictory (Carrano and Moore, 1982; Livingstone et al., 1983; Husum et al., 1986; Margolin and Shelby, 1985; Steenland et al., 1986). Here, due to the small sample size in each group, the evaluation of

the effect of these confounding factors will not be meaningful. However, it was observed during previous studies in our laboratory that age and sex have no influence on the frequency of the cytogenetic endpoints in question.

Scoring of MNC in exfoliated oral mucosa cells is technically less demanding than scoring of SCE and CA frequencies, but this simple assay is flawed with several shortcomings. Most important is the difficulty in obtaining scorable smears from most OC patients. This is because of ulcerated areas in the oral cavity resulting in poorer oral hygiene. Secondly, different laboratories utilising this parameter have employed different criteria for MN scoring, making interlaboratory comparisons difficult. Stich et al. (1982b) have reported elevated frequencies of MNC in the buccal mucosa cells of tobacco-areca nut chewers, but they did not observe any such elevation in smears obtained from smokers and those consuming alcohol or both (Stich and Rosin, 1983). Similarly, Mandard et al. (1987) also reported a negative MN test in patients with upper digestive tract cancer and controls, both consuming alcohol and tobacco. Sarto et al. (1987) proposed more stringent criteria for MN scoring and reported a highly positive MN test in smokers. We have followed the criteria suggested by them. The frequencies of MNC were almost 4 times higher in NC and OSMF patients. As already stated, the MN assay was not conducted in OC patients; however, considering the reports of Stich et al. on tobacco-areca nut chewers and that of Sarto et al. on smokers, the validity of the MN test, being non-invasive and less demanding, is very high.

All 3 parameters studied, viz. frequency of lymphocytic SCE and CA and MNC in exfoliated oral mucosa cells, showed statistically significant elevations in all 3 groups of tobacco-areca nut (mava) chewers compared to controls. Considering the commonness of the chewing habit, the number of 45 chewers (15 each, in 3 groups) may sound insufficient; however, it should be emphasised here that the individuals in all 3 groups (1) chewed the mixture with comparable frequency/duration, (2) had no concomitant habit of smoking or alcohol consumption and (3) were not engaged in any occupation which might affect the parameters studied. Chewing tobacco has been causally associated with oral cancers; however, looking to the elevated frequencies of CA and SCE in PBLs, the possibility of damage caused to other systems/tissues should also be considered, as is the case with smoking which causes lung cancer but also elevates the risk of cancer at other sites like the bladder, pancreas etc. SCE frequencies were higher in OC patients compared to NC and OSMF patients, whereas CA frequencies were higher in OSMF patients compared to NC. It is recommended that, when studying individuals exposed to potential genotoxins, both parameters be employed in combination. Further, the MN assay, being less demanding and providing vital information about the genotoxic effect on the target tissue, would prove to be most useful for monitoring tobacco consumers, not only in estimating the extent of genomic damage caused by the habit, but also in appraisal of the advantages of either quitting the habit or, as already documented by Stich et al. (1984, 1985), for assessing the benefits of chemopreventive measures.

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