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Genotoxic Studies in Pan Masala Chewers: A High Cancer Risk Group

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KEY WORDS Chromosome aberration; sister chromatid exchanges; micronucleated cells; pan masala; lymphocytes.

ABSTRACT The genotoxic effects of Pan Masala consumption were investigated in the somatic chromosomes of 50 men habituated to chewing Pan Masala (PM) and compared with controls, 50 men who did not consume PM. Chromosomal aberrations (CA) and sister chromatid exchanges (SCE) were investigated in peripheral blood lymphocytes, tissue indirectly exposed to Pan Masala (PM), and the frequency of micronucleated (MN) cells was scored in exfoliated buccal mucosa, tissue directly exposed to PM. When compared with the results obtained in blood samples from the comparable control group, a significant increase ($P<0.001$) was noted in all the above mentioned parameters : CA (0.86-3.36), SCE (3.61-6.64), MN (0.104-0.656), A significant difference occurred between the results for lower and higher age groups (< 30 years and >30 years) in all parameters considered, viz CA (1.49-2.81), SCE (4.93-5.35), MN (0.25-0.49). The increased frequency of these end points was found to be significantly correlated ($P<0.001$) with the duration of consumption (value of b for CA, SCE, and MN being 0.205, 0.044 and 0.333 respectively) and number of pouches consumed per day (b being 0.191, 0.048 and 0.049 for CA, SCE and MN respectively).

INTRODUCTION

Betel chewing is a widespread habit in India. Oral cancer has generally been attributed to the chewing of betel quid, with or without tobacco (Ranadive et al.1979; Jussawala and Deshpande 1981; WHO 1984; IARC 1989). As a result of rapid urbanisation and changing social attitudes, tobacco and betel quid chewing habits have shown a downward trend in recent years (Sanghvi 1989). It has been replaced recently by a new substitute, Pan Masala (PM) which is sold under various trade names. It is a dry mixture of various ingradients like areca nut, catechu, lime, cardamom, permitted spices, unspecified flavouring agents etc. Areca nut which constitutes 70-80% of the mixture, is reported to possess cytotoxic, mutagenic and genotoxic properties (IARC 1985; Panigrahi and Rao 1986; Wary and Sharan 1988). A significant higher frequency of percentage micronucleated cells in exfoliated buccal mucosa; and increased frequency of chromosomal aberrations (CA) and sister chromatid exchanges (SCE) in lymphocytes of chewers of Areca nut has been reported (Dave et al. 1992; Stich et al. 1982a).

Induction of SCE and dominant lethal mutation by 'Catechu' were studied on mice following acute and prolonged oral treatment (Giri et al. 1987).

Lime, another component of PM, causes local irritation to mucosa and hyperplasia has been observed following the application of lime to the cheek pouch of hamesters (Dunham et al. 1966). It has been considered to play an important role in the genesis of oral cancer (Tanaka et al. 1983; Agarwal et al. 1986).

Keeping in view the presence of harmful constituents in Pan Masala an investigation of its genotoxic potential was considered necessary. The cytogenetic end points like chromosomal aberrations (CA) and sister chromatid exchanges (SCE) were investigated in peripheral blood lymphocytes of male individuals only since the habit has not yet substantially caught up with the females. In addition the frequency of micronuclei (MN) was evaluated in exfoliated buccal mucosa cells of Pan Masala consumers.

MATERIALS AND METHODS

A total of 100 healthy male individuals were studied, 50 Pan Parag and Rajnigandha (brands of Pan Masala) consumers and 50 control individuals, who had not been subjected to X-ray treatments or had used any medication three months prior to blood samples were taken. Samples were taken from the subjects living in Kurukshetra, Ambala, Shahabad and nearby villages. Most of the Pan Masala consumers screened in this study worked as gardeners, rickshaw-pullers, labourers and peons employed in Kurukshetra university who belong to lower socio-economic status. Some of the subjects consuming Pan Masala belong to lower middle class viz. Clerks in banks and university. Care was taken to select an individual who consumed Pan Masala (without tobacco) only without any

other concurrent habit of tobacco or areca nut consumption. Control group of individuals contain normal healthy individuals belonging to the same socio- economic group who did not take Pan Masala, areca nut or tobacco in any form, chewing or smoking. They hailed from the same towns/villages to which the exposed individuals belonged. Almost all the sampled individuals exposed as well as controls, took vegetarian diet (wheat, bread, dal/vegetables) only. Both the groups kept away from alcohol consumption.

Blood samples were collected in disposables pre-sterlized heparinized syringes and transferred to laboratory without delay for lymphocyte culture. Short term lymphocyte cultures were set up using the technique of Moorhead et al. (1960) with minor modifications.

Lymphocytes were grown by adding 0.5 ml. of blood in 5 ml. of RPMI 1640 medium (Hi media) supplemented with 20% foetal calf serum and 0.1 ml phytohaemagglutinin (Sigma). Colchicine (10 ug/ml; Sigma) was added to the culture 1h prior to harvesting.

For studying chromosomal aberrations, lymphocytes were harvested after 48 h. Slides were prepared by air drying method and stained with 4% Giemsa (E. Merck). As many as 100 good metaphases were scored.

For sister chromatid exchanges, 5-bromo-deoxyuridine (10 ug/ml, Sigma) was added 24 h after setting up the cultures. Cells were harvested after 72 h. Slides were prepared by air drying method and stained with Hoechst 33258 and 4% Giemsa, following the method of Perry and Wolf (1974). For calculating frequency of SCE per cell, 30 metaphases were analysed as per international practice.

For micronucleus assay, the buccal smears on glass slides were transported to laboratory on ice and processed within 3-4 h of sample fixation. The air dried samples were hydrolyzed for 8 min. in HCl at 60°C. After a rinse in tap water and staining in Aceto-orecine (2% in 60% acetic acid for 20 min. at 40^0C), the samples were given a brief washing in ethanol and distilled water. Counter staining was done with fast green solution with final rinse in ethanol and distilled water. Slides were air dried and coded. At least 1000 cells were scored. The criteria of Tolbert et al. (1992) were followed for scanning cells for micronuclei. Slides were screened in a double blind manner to obviate the risk of bias.

The results were analyzed statistically using two-way analysis of variance (Edwards 1971) to examine the effect of exposure and age of individuals on frequency of CA, SCE and MN. Also the contribution of two variables (duration of consumption and number of pouches consumed per day) to the incidence of CA, SCE and MN was determined separately by linear regression analysis.

RESULTS

The data obtained during the present investigation are presented in Tables 1-3. A perusal of Table 1 reveals that frequencies of CA, SCE and MN increase linearly with duration of consumption. The mean value of highest incidence of CA, SCE and MN in individuals who have been consuming Pan Masala for over 20 years is 6.00, 7.19 and 1.15, respectively.

Table 2 reveals that frequencies of CA, SCE and MN show increase with increase in the frequency of pouches consumed. Highest values of CA, SCE and MN observed in individuals consuming more than 20 pouches per day, were

Table 1: Frequency of Chromosomal Aberrations (CA), Sister Chromatid Exchanges (SCE) and Micronuclei in buccal mucosal cells (MN) in pan masala consumers and controls with duration of consumption

Particulars	*Numbers of samples*	*CA (Mean ± SD)*	*SCE (Mean ± SD)*	*MN (Mean ± SD)*
Control individuals	50	0.86 ± 0.72	3.61 ± 0.27	0.147 ± 0.094
Consumers	50	3.36 ± 2.18*	6.64 ± 4.59*	0.656 ± 0.331*
0-5	21	1.86 ± 1.59	6.23 ± 0.32	0.385 ± 0.253
6-10	10	3.30 ± 1.76	6.80 ± 0.23	0.72 ± 0.187
11-15	11	4.45 ± 1.69	6.92 ± 0.31	0.80 ± 0.184
16-20	4	5.75 ± 1.50	7.11 ± 0.14	1.0 ± 0.182
21-25	4	9.00 ± 1.63	7.19 ± 0.09	1.15 ± 0.191

Values marked with asterisks are significantly higher than the corresponding values for controls (ANOVA test)
*$p<0.001$

Table 2: Frequency of Chromosomal Aberrations (CA), Sister Chromatid Exchanges (SCE) and Micronuclei in buccal mucosal cells (MN) in pan masala consumers and controls with the number of pouches consumed per day

Number of pouches[1] consumed (per day)	*Numbers of samples*	*CA*	*SCE*	*MN*
		(Mean ± SD)	*(Mean ± SD)*	*(Mean ± SD)*
Control individuals	50	0.86 ± 0.72	3.61 ± 0.27	0.104 ± 0.094
Consumers	50	3.36 ± 2.18*	6.64 ± 4.59*	0.656 ± 0.331*
6-10	18	1.83 ± 1.61	6.32 ± 0.44	0.450 ± 0.386
11-15	14	3.57 ± 1.91	6.64 ± 0.43	0.671 ± 0.243
16-20	12	4.16 ± 1.94	6.94 ± 0.18	0.808 ± 0.192
21-25	6	5.83 ± 1.60	7.04 ± 0.27	0.933 ± 0.175

Values marked with asterisks are significantly higher than the corresponding values for controls (ANOVA test)
*$p<0.001$
[1]one pouch contains 4 g of PM

found to be 5.83, 7.04 and 0.933, respectively.

For investigating the effect of age on these cytogenetic markers in Pan Masala consumers the data were divided into two groups : (1) those aged above 30 years and (ii) those below 30 years (Table-3). Mean CA in the lower age group was found to be 1.49, whereas it was 2.81 in the higher age group. Mean SCE count also differed, it was 4.93 in the lower age group and 5.35 in the higher age group. In the <30 age group mean MN was 0.25 while in the >30 age group it was 0.49.

The F-ratio for the effect of age on CA, SCE and MN is 21.01, 21.12 and 17.43 respectively ($p<.001$). This showed significant effect of age on these cytogenetic markers.

The F-ratio for studying the effect of exposure on CA, SCE and MN was also computed. . The value of F for CA, SCE and MN is 69.027, 1117.53 and 145.85 respectively showing significant effect ($p<.001$) of exposure on these parameters.

The interactive effect of Age X Exposure has also been found to be significant. The F-ratio for CA and MN being 10.72 and 15.27 ($p<.001$) and for SCE 10.352 ($p<.002$).

Linear regression analysis was applied to study the influence of duration of consumption and number of pouches consumed per day on the parameters used. Value of multiple correlation (multiple R) for dependent variables CA, SCE and MN came out to be 0.855, 0.872, 0.807, respectively showing highly significant values ($p<.001$). The values of R^2 (which determines the proportion of variance in the parameters studied) jointly determined by variation in duration of consumption and number of pouches consumed per day came out to be 0.732, 0.762 and 0.651 respectively.

Regression coefficients (b) are found to have highly significant influence of duration of consumption and number of pouches consumed per day for all the parameters investigated.

The higher significant values of b for duration of consumption are .044,.205, and .033 respectively ($p <.001$) showing linear influence of duration on SCE, CA and MN (Fig.1). The values of b for number of pouches consumed per day for SCE, CA and MN being 0.048, 0.191, 0.049 respectively showed significant positive influence ($p<.001$) of number of pouches consumed per day on the parameters considered (Fig 2).

DISCUSSION

In the modern life an individual can be exposed to mutagens/ carcinogens either at the work place, accidentally or as a part of life style. Of late the consumption of Pan Masala with or without tobacco has escalated owing to an advertising bombardment, especially on T.V, and

Table 3: Frequency of Chromosomal Aberration (CA), Sister Chromatid Exchanges (SCE) and micronuclei in buccal mucosal cell (MN) in 100 individuals (control and exposed) of two different age groups

Age group	*No. of subject*	*Mean value of CA*	*Mean value of SCE*	*Mean value of MN*
<30	57	1.49	4.93	25
>30	43	2.81*	5.35	.49*

*Significant at $p<0.001$

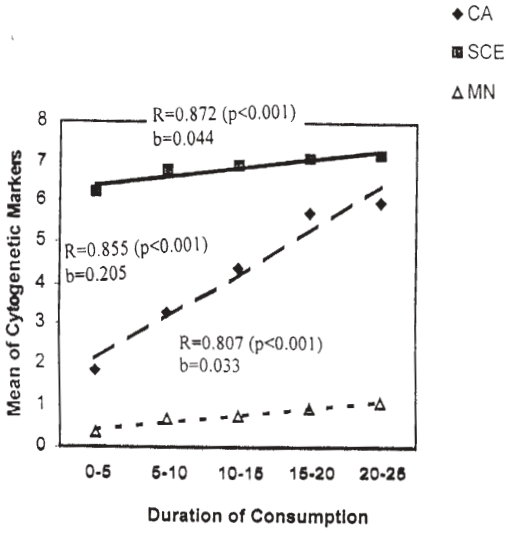

Fig. 1. Linear regression curves showing relationship between duration of consumption of pan masala with frequency of CA, SCE and MN

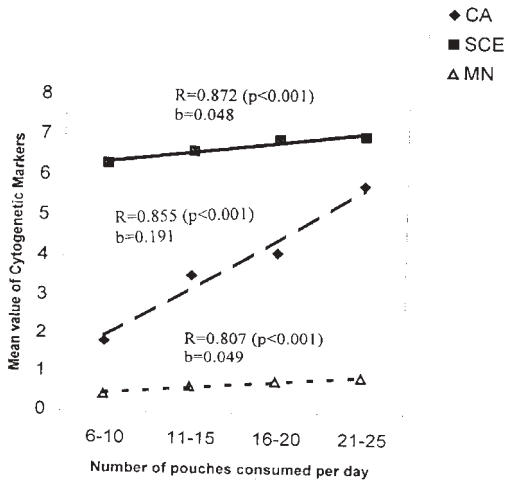

Fig. 2. Linear regression curves showing relationship between number of pouches consumed perday with frequency of CA, SCE and MN.

under peer influence even among women and children.

There is an ever increasing use of pan masala in urban population. As the trend of betel quid chewing is now replaced rapidly by this new chewing substitute in 'Pan Masala', there is possibility of oral cancer epidemic in near future due to absence of betal leaf and the much higher dry weight of PM ingradients (Babu et al. 1996). *In vitro* short term experiments on mammalian test systems, employing cytogenetic end points like SCE and CA revealed genotoxic potential of an aqueous extract of PM (Adhvaryu et al. 1989). Subsequentely the cytogenetic end points – SCE, CA and the frequency of micronucleated cells in the exfoliated buccal mucosa cells demonstrated a statistically significant increase among the PM consumers as compared with the non-consuming controls (Dave et al. 1991). A preliminary review has been published by Trivedi et al. (1996).

CA are considered to be one of the most important cytogenetic parameters for the menifestation of genotoxicity. Recently Brogger et al. (1990) have reported that persons with high frequency of CA develop cancer twice as often as others. During the present investigation PM consumers showed significantly increased CA compared with their matched controls. The background frequency of CA (0.86) matched very well with those reported for control individuals in various populations investigated by our group. (Yadav and Kaushik 1996, 1997; Yadav and Seth 1998a, b; Yadav and Thakur 1999, 2000) .

Significantly higher values of SCE compared with respective controls have been recorded. The increase in the frequency of SCE has been reported in betel and tobacco chewers (Ghosh and Ghosh 1984). Using *in vitro* short term assays, the dimethyl sulphoxide (DMSO) extract of PM has been found to increase the frequency of CA, SCE and MN in cultures without metabolic activation (Patel et al. 1994).

Increase in the frequency of micronucleated buccal mucosal cells has been found during the present investigation. The results are in accordance with those reported by Dave et al.(1991). Stich and Stich (1982) observed that saliva of Pan Bahar chewers was clastogenic to CHO cells. A very high frequency of MN has been observed among Indians chewing betel quid, arecanut and /or tobacco (Stich et al. 1982a, b). Similarly increase in frequency of MN in PM consumers has also been reported by Gandhi and Kaur (2000).

Age plays significant role in affecting the frequency of spontaneous micronucleus formation or DNA damage in lymphocyte populations of male and female individuals (Tice and Setlow 1985; Ghosh et al. 1990). Fenech and Morley (1985) found four fold increase of micronuclei in 80 year old persons as compared with younger ones. Similarly Galloway et al. (1986), and Ghosh et al. (1991) have reported the influence of age on enhancement of spontaneous chromosomal aberrations. Our investigations have also demonstrated the influence of age on these cytoge-

netic endponts viz. CA, SCE and MN. All these parameters showed significant differences between lower (<30) and higher (>30, age groups by applying two way analysis of variance. Beside this, interactive effect of Age X Exposure has also been found to be significant for all these variables.

The present investigation has also revealed significant positive correlations of duration of consumption and frequency of daily use with all dependent variables i.e. CA, SCE and MN by applying linear regression analysis. Significant positive correlation between incidence of MN in buccal mucosa and consumption of tobacco (smoking gm. /day) was earlier recorded by Sarto et al. (1987).

Pan Masala is thus highly genotoxic and is likely to be associated with cancer of the oral cavity as manifested by the frequency of Oral Submucosa Fibrosis, a precursor of oral cancer (Babu et al. 1996). The chewers of PM must be warned and persuaded to give up the habit. Steps must be taken by state and central governments to protect the children from the use of Pan Masala in any form by controlling its sale.

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