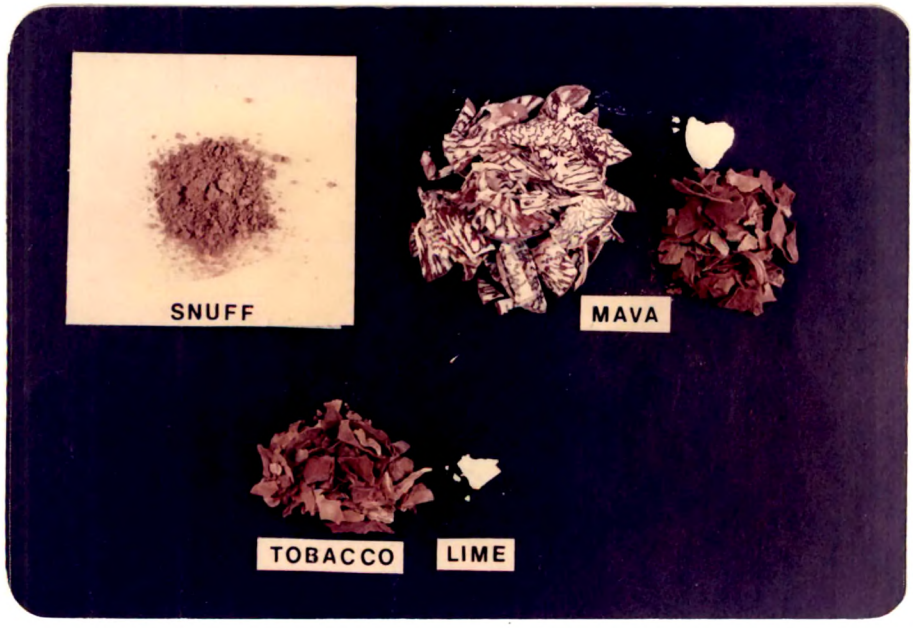
Part - II
Human Studies



17

Extrapolation of results from the *in vitro* short term assays to human beings is still an unresolved issue in carcinogenesis. In real life human beings are exposed to a number of chemicals and complex mixtures simultaneously. The carcinogenic/mutagenic activities of a substance may be modified by the presence of another substance (Boutwell et al., 1982). Hence, in interpreting the potential health hazards of any compound, one should consider the possible synergistic, antagonistic, additive or inhibitory interactions between the agents. Moreover, metabolism of the substance in human body may influence the ultimate carcinogenic effect. Thus it is extremely difficult to simulate combinations of all these conditions in *in vitro* assays. In order to overcome the restrictions inherent to the *in vitro* assays, various biochemical methods and *in vivo* short term tests are in use for detecting and quantitating the *in vivo* exposures to genotoxic/carcinogenic agents.

Numerous epidemiological studies have firmly established an association between oral uses of smokeless tobacco (snuff and chewing tobacco) and cancers of the oral cavity (IARC, 1985). Chemical analysis has revealed the presence of tobacco specific genotoxic/carcinogenic agents in the saliva and urine of the smokeless tobacco users. Nicotine, arecoline, ASNAs and TSNAs have been detected in the saliva of chewers of betel quid containing tobacco. Apart from preformed TSNAs, *in vivo* formation of additional TSNAs in the oral cavity of the chewers has also been reported (Nair et al., 1987). Dura-

tion and frequency of snuff dipping has been correlated with the levels of TSNAs and cotinine in the saliva (Brunnemann et al., 1987). The exposure to tobacco has also been determined by measuring levels of nicotine and cotinine in the urine samples of individuals consuming tobacco (George Palladino et al., 1986) as well as in the samples of those engaged in tobacco related occupations (Govekar, 1991; Ghosh et al., 1985; 1986).

Saliva and urine samples have also been tested for mutagenicity/genotoxicity in order to detect, and to some extent also to quantitate, the exposure to mutagenic/carcinogenic chemicals in tobacco. Curvall et al. (1987) have observed marginal difference between the mutagenic activity of urine from snuff users and tobacco non-users, whereas, Menon and Bhide (1984) have reported weak mutagenic activity for the urine concentrates of tobacco chewers. Increased mutagenicity of urine from bidi rollers (Govekar, 1991) and clastogenicity of saliva of the tobacco chewers have also been documented (Stich and Stich, 1982).

Studies pertaining to the effects of smokeless tobacco consumption on the cells, exposed directly or indirectly to its genotoxins, are of relevance in understanding the mechanism of oral carcinogenesis. Stich and coworkers (1982-1986) have reported high frequency of MNC in the exfoliated buccal mucosa of individuals with different smokeless tobacco habits. However, excepting for a few reports on SCE freqency

in peripheral blood lymphocytes (PBLs) of tobacco plus areca nut chewers (Adhvaryu et al., 1986; 1991; Ghosh and Ghosh, 1984), the *in vivo* effects of other forms of smokeless tobacco consumption remain to be explored.

The present study was carried out to assess the DNA damage caused by chronic low level *in vivo* exposure to **(i)** dry snuff **(ii)** tobacco mixed with lime and **(iii)** tobacco with areca nut and lime (locally known as 'mava'). (Illustration 17). The study was undertaken with the help of three cytogenetic endpoints and two tissues. Exfoliated cells from the buccal mucosa, the putative target tissue, were screened for the detection of micronucleus **(MN)**, whereas, chromosome aberration **(CA)** and sister chromatid exchange **(SCE)** frequencies in PBLs, the non-target circulating cells, were considered as biological indicators of genotoxic effects on somatic cells.

MATERIALS AND METHODS:

Chemicals:

The details of all the chemicals used in the present study have already been provided in Part I. Phytohaemagglutinin (PHA-M) was purchased from Gibco Chemicals Ltd., U.S.A.

STUDY DESIGN:

Selection of subjects:

A total of 136 individuals were included in the study. According to their habit and condition of the oral cavity,

these individuals were classified as under:

Group/Habit	Snuff	Tobacco+Lime	Tobacco+Areca nut+Lime
Controls (20)	-	-	-
Normal consumers	N-SNF **(13)**	N-TC **(15)**	N-TAC **(20)**
OSMF patients	-	-	OSMF-TAC **(20)**
OC patients	OC-SNF **(20)**	OC-TC **(10)**	OC-TAC **(18)**

Values in parenthesis indicate the number of individuals.

where,

Controls were the individuals who had never consumed alcohol, tobacco or areca nut in any form, had no viral disease in near past and were not undergoing any antibiotic therapy.

Normal consumers were the individuals who had clinically normal buccal mucosa, i.e. no appreciable change in their buccal mucosa, despite their tobacco habits of not less than two years duration.

OSMF patients were the individuals who had a positive history of chewing tobacco with areca nut and lime and had an insidious, chronic fibrotic change in their oral mucosa, diagnosed as oral submucous fibrosis by a qualified dental surgeon. Habit of snuff or tobacco plus lime was not found among OSMF patients.

OC patients were the histopathologically confirmed oral cancer (ICD 140-145) cases with a strong history of consuming one of the three forms of tobacco, considered in this study.

Collection of samples:

(1) For micronucleus (MN) assay, exfoliated buccal mucosa was collected after repeated gargling with clean water. With the

help of a blunt spatula, the cells were scrapped from the region where the tobacco was usally placed in the mouth. The cells, smeared on clean slide, were fixed with 1:3 acetic acid-methanol fixative for 15 minutes and air-dried slides were stored until staining.

(2) For lymphocyte culture, venous blood was collected aseptically in heparinized vial.

PBL culture procedure:

One ml of whole blood was added to 7.0 ml growth medium (MEM with Earle's base and nonessential amino acids, containing 20% NCS, 100 µg/ml streptomycin and 100 units/ml penicillin, 3 % PHA-M and 2 µg/ml BrdU). Cultures were incubated at $37^{o}C$ for 72 hours and colchicine, at a final concentration of 0.3 µg/ml, was added during final 3 hours. The cultures were harvested following the hypotonic treatment with 0.56 % KCl for 15 minutes and fixation with chilled aceto-methanol fixative. Cultures were protected from direct light during all these steps. Following three changes of fresh fixative, the slides were prepared by conventional air drying procedure.

Staining and Scoring methods:

(1) Micronucleus staining and scoring: The exfoliated buccal mucosa cells were stained by Feulgen plus Fast Green method. Briefly, the fixed cells were hydrolysed with 5N HCl at room temperature for 20 minutes. After a rinse in distilled water, the cells were stained with Feulgen reagent for 90 minutes in dark. After three changes of 2 minutes each in SO_2 water, the

slides were kept in running water for 10 minutes. Counter staining was done by 0.5 % alcoholic Fast Green for 30 seconds. The slides were rinsed in distilled water, air-dried and mounted with DPX.

At least 1000 cells were scored from each individual for examining the presence of micronuclei. Identification of micronucleus was done according to the criteria proposed by Sarto et al. (1987). Because of presence of abundant necrotic cells in the preparations and since it was not feasible to obtain scorable smears due to poor hygenic condition of the oral cavity, it was not possible to perform the assay for OC patients.

(2) Sister chromatid differential staining and scoring of CA and SCE: Procedures for the diffential staining of the chromosomes and criteria for scoring CA and SCE were essentially the same as described in Part I.

Analysis of the data:

Mean values, standard deviations and standard errors were calculated for each individual as well as for every group, separately. For all the three cytogenetic endpoints, the Student's 't' test was applied to determine the level of significance of difference between the values obtained for control subjects and the group of tobacco users.

RESULTS:

OBSERVATIONS AMONG SNUFF USERS:

The individual frequency of MNC in exfoliated buccal mucosa as well as CA and SCE frequencies in PBLs of controls, N-SNF and OC-SNF are provided in Tables 18-20, respectively, whereas, the comparative data are provided in Fig. 15.

The frequency of MNC in exfoliated buccal mucosa varied from 0.1 % to 0.3 % in controls providing a mean value of 0.19 $\pm$ 0.019 ($\pm$ S.E.). Among the N-SNF, the MNC frequency varied from 0.4 % to 0.8 % giving a mean of 0.56 $\pm$ 0.037 which was almost 3 times higher than that of the controls (Fig. 15). This elevation was statistically significant **($p < 0.001$)** when compared with controls. Illustrations 18-20 portray the micronucleated cells from the exfoliated buccal mucosa.

The mean CA/cell value (including gaps) for controls was 0.052 $\pm$ 0.003 ($\pm$ S.E.), whereas, the values were 0.095 $\pm$ 0.003 and 0.136 $\pm$ 0.006 for N-SNF and OC-SNF, respectively. The increase in CA frequency in both the groups of snuff users, was statistically significant **($p < 0.001$)** compared to that of the control value. The difference between CA frequencies of N-SNF and OC-SNF was also statistically significant **($p < 0.001$)**. Table-21 details the frequency of different types of CAs observed in controls, N-SNF and OC-SNF. Illustrations 21-24 represent some of the aberrant metaphases. Chromatid-type aberrations were more frequent with maximum

Table-18

INDIVIDUAL VALUES OF VARIOUS CYTOGENETIC ENDPOINTS AMONG CONTROLS

No.	Age/Sex	% MNC	CA/cell ± S.E.	SCE/cell ± S.E.
1	17 M	0.2	0.04 ± 0.0196 (0.01)	6.04 ± 0.325
2	19 M	0.3	0.04 ± 0.0196 (0.00)	6.35 ± 0.319
3	23 F	**0.1**	0.05 ± 0.0218 (0.01)	6.46 ± 0.400
4	23 M	**0.3**	0.04 ± 0.0196 (0.03)	5.66 ± 0.411
5	24 M	0.1	**0.03 ± 0.0171** (0.00)	6.31 ± 0.550
6	24 F	0.2	0.03 ± 0.0171 (0.00)	5.86 ± 0.380
7	25 F	0.1	0.05 ± 0.0218 (0.00)	5.81 ± 0.265
8	25 M	0.3	0.05 ± 0.0218 (0.00)	6.63 ± 0.315
9	25 M	0.1	0.05 ± 0.0218 (0.02)	6.28 ± 0.465
10	26 F	0.1	0.05 ± 0.0217 (0.00)	5.74 ± 0.365
11	26 M	0.3	0.06 ± 0.0276 (0.03)	5.70 ± 0.298
12	28 F	0.3	0.06 ± 0.0238 (0.01)	6.19 ± 0.420
13	30 F	0.3	0.05 ± 0.0218 (0.00)	6.35 ± 0.281
14	35 M	0.1	0.05 ± 0.0218 (0.00)	6.36 ± 0.510
15	40 M	0.2	**0.08 ± 0.0271** (0.02)	**5.55 ± 0.330**
16	49 F	0.1	0.04 ± 0.0196 (0.00)	**6.95 ± 0.387**
17	55 F	0.2	0.06 ± 0.0237 (0.01)	6.18 ± 0.263
18	55 F	0.2	0.07 ± 0.0292 (0.01)	6.06 ± 0.346
19	58 F	0.2	0.08 ± 0.0306 (0.02)	5.79 ± 0.228
20	60 M	0.1	0.06 ± 0.0238 (0.01)	6.14 ± 0.207
Group mean		0.190	0.052 (0.009)	6.121
± S.E.		0.019	0.003 (0.002)	0.078

Value in parenthesis indicates CA/cell excluding gaps.

Values in **bold letters** represent 'range'.

Table-19

INDIVIDUAL VALUES OF VARIOUS CYTOGENETIC ENDPOINTS AMONG NORMAL SNUFF USERS (N-SNF)

No.	Age/Sex	% MNC	CA/cell ± S.E.	SCE/cell ± S.E.
1	35 F	0.6	0.09 ± 0.0349 (0.02)	7.61 ± 0.305
2	37 F	0.5	**0.12 ± 0.0354** (0.05)	7.31 ± 0.465
3	41 F	0.7	0.11 ± 0.0313 (0.03)	7.67 ± 0.218
4	45 F	**0.4**	0.10 ± 0.0424 (0.02)	7.79 ± 0.428
5	49 M	0.5	0.10 ± 0.0300 (0.03)	6.84 ± 0.320
6	50 F	0.4	0.09 ± 0.0286 (0.03)	7.12 ± 0.195
7	52 F	**0.8**	0.10 ± 0.0332 (0.03)	7.66 ± 0.375
8	52 F	0.5	**0.08 ± 0.0306** (0.01)	7.61 ± 0.358
9	55 F	0.8	0.09 ± 0.0349 (0.03)	7.13 ± 0.412
10	55 M	0.4	0.08 ± 0.0306 (0.03)	**6.76 ± 0.287**
11	58 F	0.5	0.09 ± 0.0319 (0.01)	7.27 ± 0.446
12	60 F	0.6	0.10 ± 0.0332 (0.03)	7.30 ± 0.478
13	80 F	0.6	0.08 ± 0.0306 (0.05)	**7.92 ± 0.451**
Group mean ± S.E.		0.56 0.037	0.095 0.003	(0.028) (0.003)	7.384 0.096

Value in parenthesis indicates CA/cell excluding gaps.

Values in **bold letters** represent 'range'.

Table-20

INDIVIDUAL VALUES OF VARIOUS CYTOGENETIC ENDPOINTS AMONG SNUFF USERS SUFFERING FROM ORAL CANCER (OC-SNF)

No.	Age/Sex	CA/cell ± S.E.	SCE/cell ± S.E.
1	30 F	0.13 ± 0.0391 (0.03)	8.55 ± 0.494
2	35 F	0.12 ± 0.0354 (0.04)	7.95 ± 0.329
3	40 F	0.12 ± 0.0325 (0.05)	8.52 ± 0.598
4	40 F	0.13 ± 0.0365 (0.03)	8.43 ± 0.400
5	40 F	0.17 ± 0.0428 (0.05)	7.69 ± 0.264
6	45 F	**0.09 ± 0.0287** (0.04)	7.85 ± 0.451
7	45 F	**0.18 ± 0.0552** (0.05)	8.00 ± 0.451
8	45 F	0.13 ± 0.0365 (0.05)	8.22 ± 0.451
9	45 F	0.12 ± 0.0325 (0.04)	8.25 ± 0.483
10	50 F	0.15 ± 0.0357 (0.01)	**10.17 ± 0.485**
11	50 F	0.14 ± 0.0488 (0.04)	**6.30 ± 0.286**
12	52 F	0.12 ± 0.0354 (0.04)	8.41 ± 0.310
13	55 F	0.18 ± 0.0456 (0.05)	8.49 ± 0.412
14	60 F	0.16 ± 0.0418 (0.03)	7.37 ± 0.328
15	60 F	0.10 ± 0.0300 (0.03)	7.28 ± 0.200
16	60 F	0.10 ± 0.0360 (0.03)	8.04 ± 0.402
17	62 F	0.12 ± 0.0325 (0.04)	8.57 ± 0.301
18	62 F	0.15 ± 0.0384 (0.05)	7.28 ± 0.359
19	70 F	0.17 ± 0.0511 (0.05)	8.04 ± 0.402
20	80 F	0.15 ± 0.0473 (0.03)	8.21 ± 0.423
Group mean ± S.E.		0.136 ± 0.006 (0.039) ± (0.002)	8.081 ± 0.163

Value in parenthesis indicates CA/cell excluding gaps.

Values in **bold letters** represent '<u>range</u>'.

FIGURE-15

RELATIVE FREQUENCIES OF MNC, CA AND SCE

AMONG CONTROLS AND SNUFF USERS

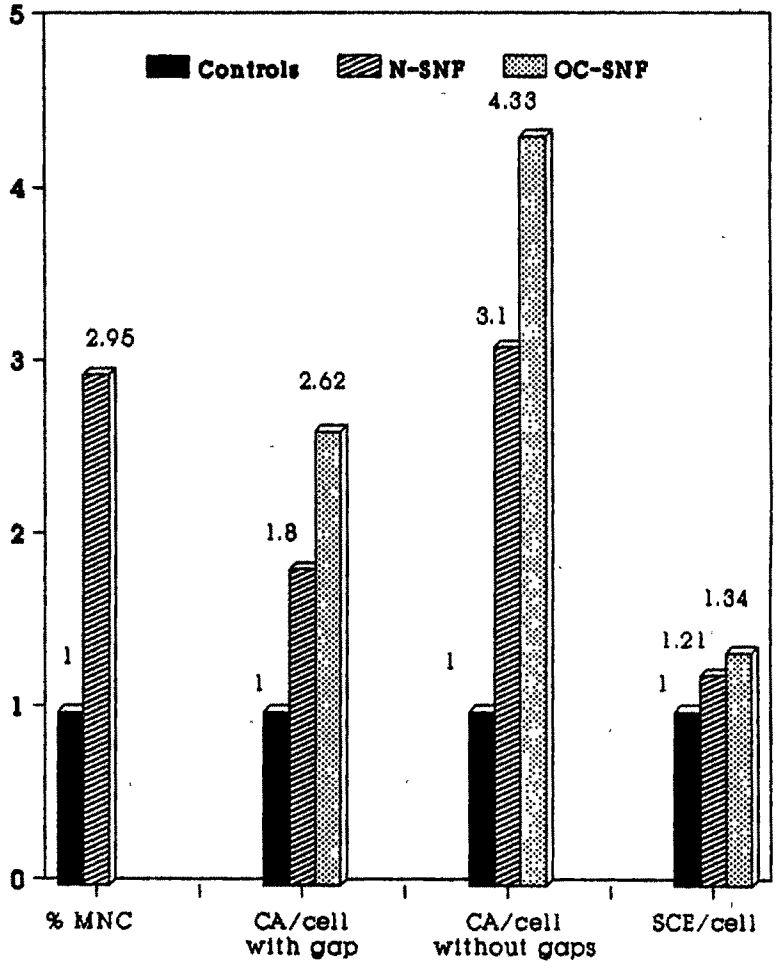


Table-21

DETAILS OF CAs (per 100 cells) OBSERVED AMONG CONTROLS AND SNUFF USERS

Group	Aberrant metaphases	Chromosome Aberrations	Types of aberrations (%) Chromatid				Chromosome					
			G	B/F	I	Ag.	G	B	Dm	R	Dc	Ag.
Control	5.05	5.20	4.1	0.8	-	4.9	0.2	0.1	-	-	-	0.3
N-SNF	8.70	9.50	5.8	2.7	0.1	8.6	0.6	0.3	-	-	-	0.9
OC-SNF	12.28	13.60	8.9	3.2	-	12.1	0.8	0.2	0.4	-	0.1	1.5

where,

G = gaps; B = break; B/F = break and/or acentric fragment; I = interchange

Dm = double minutes; R = ring chromosome; Dc = dicentric chromosome

Ag. = Aggregate chromatid/chromosome-type aberrations.

Table-22

PERCENT DISTRIBUTION OF METAPHASES ACCORDING TO THE NUMBER OF SCEs AMONG CONTROLS AND SNUFF USERS

Group	Range	% metaphases with			
		0 - 5	6 - 9	10 - 15	>15 SCEs
Control	1 - 14	38.6	57.6	3.8	-
N-SNF	3 - 15	14.8	72.9	12.3	-
OC-SNF	2 - 16	10.0	68.0	21.6	0.4

number of gaps in all the three groups. An increase in the frequency of chromatid-gaps from 4.1 per 100 cells in controls to 5.8 and 8.9 per 100 cells in N-SNF and OC-SNF, respectively, was observed. Interchanges, double minutes and dicentric chromosomes were not observed in controls, whereas, interchanges were found in N-SNF and double minutes as well as dicentric chromosomes were found in OC-SNF. Even after excluding gaps from aberrations, the elevations in mean CA/cell values in N-SNF and OC-SNF were statistically significant **($p < 0.001$)** when compared with that of the controls. The difference between the CA frequencies (excluding gaps) of N-SNF and OC-SNF was also statistically significant **($p < 0.001$)**.

For controls, the mean SCE/cell value was 6.121 $\pm$ 0.078 ($\pm$ S.E.), whereas, the values were 7.384 $\pm$ 0.096 and 8.081 $\pm$ 0.163 for N-SNF and OC-SNF, respectively. Both the groups of snuff users had significantly higher mean frequencies of SCE compared to the controls **($p < 0.001$)**. Moreover, OC-SNF had a statistically significant elevation in mean SCE frequency compared to N-SNF **($p < 0.001$)**. Table-22 provides the percent distribution of metaphases according to the number of SCEs for controls, N-SNF and OC-SNF. An increase in number of metaphases with higher SCEs was observed in N-SNF and OC-SNF. Illustrations 25-27 exhibit SCEs observed in the lymphocytes of the controls and tobacco consumers.

OBSERVATIONS AMONG TOBACCO WITH LIME CHEWERS:

Table 23 provides the individual frequency of MNC in exfoliated buccal mucosa as well as lymphocytic CA and SCE frequencies among N-TC. Similar data for OC-TC have been provided in Table-24, whereas, Fig. 16 provides comparison of the data with the controls.

Among the N-TC, the MNC frequency varied from 0.1 % to 0.9 % providing a mean of 0.59 $\pm$ 0.046 ($\pm$ S.E.) which was more than 3 times higher than % MNC in controls. This increase was statistically significant **($p < 0.001$)** when compared with the controls.

For N-TC, the mean CA/cell values (including gaps) were ranging from 0.07 to 0.12, giving a mean of 0.088 $\pm$ 0.004 ($\pm$ S.E.), whereas, the values were ranging from 0.12 to 0.17 giving a mean of 0.14 $\pm$ 0.005 ($\pm$ S.E.) for OC-TC. The elevations in CA/cell values, in both the groups of tobacco plus lime users, were statistically significant **($p < 0.001$)** compared to mean 0.052 $\pm$ 0.003 ($\pm$ S.E.) CA/cell value in controls. The difference between CA/cell values of N-TC and OC-TC was also statistically significant **($p < 0.001$)**. The mean frequencies of different types of aberrations found in controls, N-TC and OC-TC are presented in Table-25. The frequency of chromatid-gap was almost 1.5 times higher in N-TC and 2.2 times higher in OC-TC compared to that of the controls. Moreover, interchanges and dicentric chromosomes, which were not observed among either controls or N-TC, were

Table-23

INDIVIDUAL VALUES OF VARIOUS CYTOGENETIC ENDPOINTS AMONG NORMAL TOBACCO PLUS LIME CHEWERS (N-TC)

No.	Age/Sex	% MNC	CA/cell ± S.E.	SCE/cell ± S.E.
1	23 M	0.6	0.09 ± 0.0319 (0.02)	7.16 ± 0.336
2	30 M	0.4	0.08 ± 0.0271 (0.02)	8.03 ± 0.375
3	32 M	0.4	**0.12 ± 0.0354** (0.03)	**5.24 ± 0.321**
4	34 M	**0.1**	0.08 ± 0.0271 (0.01)	7.62 ± 0.253
5	35 M	0.6	0.09 ± 0.0349 (0.06)	7.94 ± 0.372
6	35 F	0.5	0.10 ± 0.0300 (0.04)	7.64 ± 0.247
7	38 M	0.7	**0.07 ± 0.0255** (0.02)	7.66 ± 0.283
8	45 M	0.7	0.09 ± 0.0286 (0.01)	**8.05 ± 0.337**
9	45 M	0.7	0.08 ± 0.0271 (0.02)	7.74 ± 0.328
10	48 M	**0.9**	0.07 ± 0.0322 (0.00)	6.74 ± 0.360
11	48 M	0.7	0.08 ± 0.0271 (0.03)	7.97 ± 0.301
12	49 M	0.6	0.10 ± 0.0423 (0.02)	6.63 ± 0.574
13	50 M	0.7	0.08 ± 0.0306 (0.01)	8.00 ± 0.515
14	52 M	0.6	0.08 ± 0.0271 (0.02)	7.12 ± 0.203
15	65 M	0.6	0.11 ± 0.0313 (0.02)	7.16 ± 0.322
Group mean ± S.E.		0.59 0.046	0.088 (0.022) 0.004 (0.004)	7.380 0.188

Value in parenthesis indicates CA/cell excluding gaps.

Values in **bold letters** represent 'range'.

Table-24

INDIVIDUAL VALUES OF VARIOUS CYTOGENETIC ENDPOINTS AMONG TOBACCO PLUS LIME CHEWERS SUFFERING FROM ORAL CANCER (OC-TC)

No.	Age/Sex	CA/cell ± S.E.	SCE/cell ± S.E.
1	45 M	0.13 ± 0.0336 (0.03)	8.62 ± 0.574
2	48 M	**0.17 ± 0.0426** (0.05)	7.45 ± 0.188
3	50 M	**0.12 ± 0.0407** (0.06)	7.37 ± 0.251
4	50 M	0.15 ± 0.0409 (0.02)	8.79 ± 0.290
5	57 M	0.15 ± 0.0409 (0.07)	**9.78 ± 0.670**
6	60 F	0.13 ± 0.0449 (0.00)	7.67 ± 0.314
7	60 F	0.14 ± 0.0347 (0.03)	8.81 ± 0.386
8	60 F	0.15 ± 0.0409 (0.04)	**6.58 ± 0.392**
9	60 M	0.12 ± 0.0354 (0.03)	8.10 ± 0.332
10	70 F	0.14 ± 0.0400 (0.04)	9.24 ± 0.393
Group mean ± S.E.		0.140 ± 0.005 (0.037 ± 0.006)	8.241 ± 0.294

Value in parenthesis indicates CA/cell excluding gaps.

Values in **bold letters** represent 'range'.

FIGURE-16

RELATIVE FREQUENCIES OF MNC, CA AND SCE

AMONG CONTROLS AND TOBACCO PLUS LIME CHEWERS

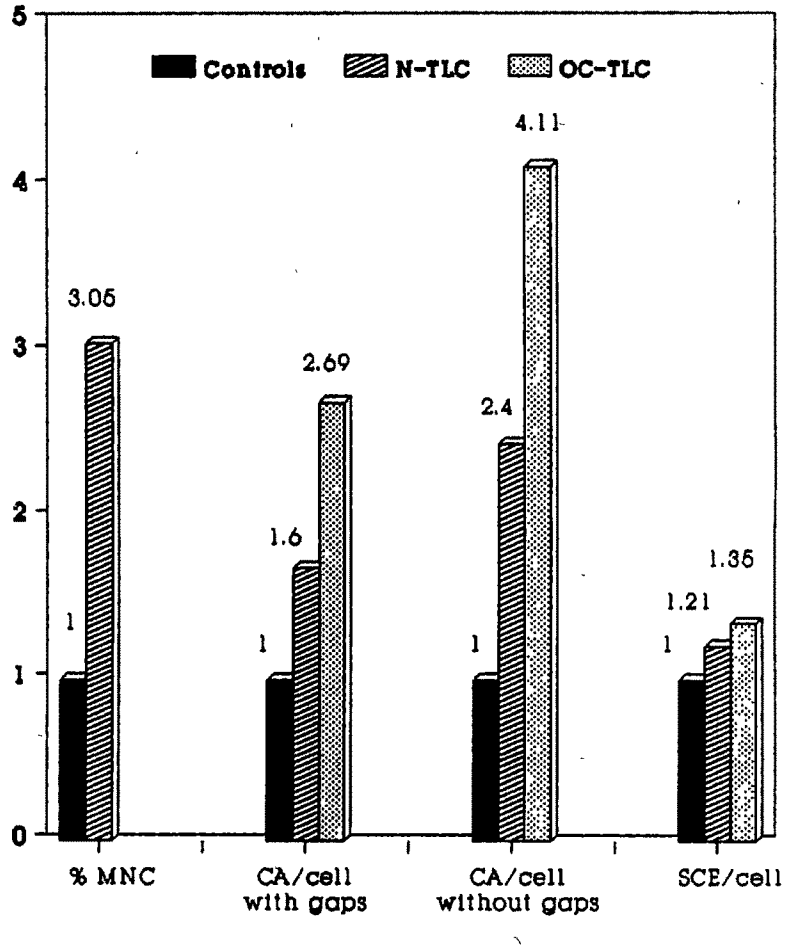


Table-25

DETAILS OF CAs (per 100 cells) OBSERVED AMONG CONTROLS AND TOBACCO PLUS LIME CHEWERS

Group	Aberrant metaphases	Chromosome Aberrations	Types of aberrations (%)									
			Chromatid				Chromosome					
			G	B/F	I	Ag.	G	B	Dm	R	Dc	Ag.
Control	5.05	5.20	4.1	0.8	-	4.9	0.2	0.1	-	-	-	0.3
N-TC	8.40	8.80	6.0	2.1	-	8.1	0.6	0.1	-	-	-	0.7
OC-TC	12.67	14.00	9.2	3.3	0.1	12.6	0.8	0.4	-	-	0.2	1.4

where,

G = gap; B = break; B/F = break and/or acentric fragment; I = interchange

Dm = double minutes; R = ring chromosome; Dc = dicentric chromosome

Ag. = Aggregate chromatid/chromosome-type aberrations.

Table-26

PERCENT DISTRIBUTION OF METAPHASES ACCORDING TO THE NUMBER OF SCEs AMONG CONTROLS AND TOBACCO PLUS LIME CHEWERS

Group	Range	% metaphases with			
		0 - 5	6 - 9	10 - 15	>15 SCEs
Control	1 - 14	38.6	57.6	3.8	-
N-TC	3 - 14	14.1	73.6	12.3	-
OC-TC	2 - 16	5.8	73.4	20.4	0.4

found in OC-TC. Even after excluding gaps, the elevations in mean CA/cell values were statistically significant in N-TC ($p < 0.01$) and OC-TC ($p < 0.001$) compared to 0.009 ± 0.002 (mean ± S.E., excluding gaps,) CA/cell value in controls. The difference between the CA frequencies (excluing gaps) of N-TC and OC-TC was also statistically significant ($p < 0.05$).

For N-TC and OC-TC, the mean SCE/cell values were 7.380 ± 0.188 (± S.E.) and 8.241 ± 0.294 , respectively. These values of SCE/cell were significantly higher when compared with controls ($p < 0.001$). Among the tobacco chewers, OC-TC had statistically higher mean SCE frequency compared to N-TC ($p < 0.05$). The distribution of metaphases according to the number of SCEs for controls, N-TC and OC-TC is provided in Table-26. It was observed that an increase in number of metaphases with greater number of SCEs in N-TC and OC-TC resulted in higher mean SCE/cell values.

OBSERVATIONS AMONG CHEWERS OF TOBACCO WITH ARECA NUT AND LIME:

Tables 27 - 29 present individual frequency of MNC in exfoliated buccal mucosa and CA and SCE frequencies in PBLs of N-TAC, OSMF-TAC and OC-TAC, respectively, whereas, Fig. 17 provides the comparative data of the three groups of tobacco-areca nut chewers alongwith the controls.

The MNC frequencies in exfoliated buccal mucosa were 0.69 % and 0.73 % for N-TAC and OSMF-TAC, respectively. Compared to the control value of 0.19 % MNC, the values among tobacco-

Table-27

INDIVIDUAL VALUES OF VARIOUS CYTOGENETIC ENDPOINTS AMONG NORMAL TOBACCO PLUS ARECA NUT CHEWERS (N-TAC)

No.	Age/Sex	% MNC	CA/cell ± S.E.	SCE/cell ± S.E.
1	21 M	0.8	0.08 ± 0.0351 (0.03)	**9.63 ± 0.453**
2	22 M	0.6	0.12 ± 0.0354 (0.04)	6.99 ± 0.304
3	24 M	**1.2**	0.09 ± 0.0349 (0.03)	7.51 ± 0.516
4	25 M	0.4	0.10 ± 0.0300 (0.02)	6.24 ± 0.432
5	26 M	1.1	0.06 ± 0.0237 (0.02)	**5.86 ± 0.448**
6	27 M	0.5	**0.16 ± 0.0441** (0.02)	6.80 ± 0.228
7	28 M	0.6	0.09 ± 0.0286 (0.03)	7.12 ± 0.299
8	30 M	**0.3**	0.08 ± 0.0306 (0.02)	7.84 ± 0.379
9	33 M	0.9	0.09 ± 0.0286 (0.03)	6.65 ± 0.375
10	34 M	0.7	0.08 ± 0.0306 (0.02)	7.28 ± 0.286
11	35 M	0.9	0.08 ± 0.0271 (0.02)	7.13 ± 0.585
12	37 M	0.5	**0.05 ± 0.0218** (0.00)	7.57 ± 0.503
13	37 M	0.7	0.09 ± 0.0349 (0.01)	7.33 ± 0.584
14	39 M	0.5	0.07 ± 0.0255 (0.00)	7.88 ± 0.385
15	40 M	0.6	0.12 ± 0.0354 (0.01)	7.28 ± 0.405
16	45 M	0.7	0.08 ± 0.0271 (0.02)	7.47 ± 0.344
17	46 F	0.7	0.13 ± 0.0429 (0.05)	6.54 ± 0.408
18	50 M	1.0	0.14 ± 0.0401 (0.02)	6.69 ± 0.288
19	56 M	0.8	0.11 ± 0.0343 (0.04)	7.95 ± 0.430
20	56 M	0.3	0.08 ± 0.0271 (0.04)	7.83 ± 0.549
Group mean		0.690	0.095 (0.024)	7.280
± S.E.		0.054	0.006 (0.003)	0.172

Value in parenthesis indicates CA/cell excluding gaps.

Values in **bold letters** represent 'range'.

Table-28

INDIVIDUAL VALUES OF VARIOUS CYTOGENETIC ENDPOINTS AMONG TOBACCO PLUS ARECA NUT CHEWERS SUFFERING FROM O-SMF (OSMF-TAC)

No.	Age/Sex	% MNC	CA/cell ± S.E.	SCE/cell ± S.E.
1	17 M	0.8	0.15 ± 0.0384 (0.02)	8.38 ± 0.568
2	20 M	0.7	0.13 ± 0.0448 (0.05)	**6.21 ± 0.399**
3	20 M	0.7	0.10 ± 0.0300 (0.04)	7.88 ± 0.330
4	21 M	0.6	0.12 ± 0.0325 (0.05)	7.73 ± 0.319
5	21 M	**0.3**	0.08 ± 0.0271 (0.02)	6.55 ± 0.345
6	23 M	**1.8**	0.10 ± 0.0300 (0.03)	7.53 ± 0.335
7	24 M	0.4	0.16 ± 0.0504 (0.06)	8.35 ± 0.319
8	24 M	0.7	0.13 ± 0.0365 (0.03)	8.06 ± 0.416
9	28 M	1.1	0.14 ± 0.0400 (0.03)	7.44 ± 0.400
10	28 M	0.8	0.13 ± 0.0391 (0.04)	7.62 ± 0.315
11	28 M	0.5	**0.07 ± 0.0292** (0.02)	7.21 ± 0.283
12	29 M	0.6	0.09 ± 0.0349 (0.01)	8.11 ± 0.375
13	32 M	1.0	0.14 ± 0.0400 (0.04)	8.07 ± 0.377
14	33 M	0.7	0.19 ± 0.0463 (0.04)	6.66 ± 0.249
15	36 M	0.5	0.11 ± 0.0313 (0.03)	7.36 ± 0.287
16	37 M	0.8	**0.20 ± 0.0469** (0.05)	7.75 ± 0.478
17	39 M	0.7	0.11 ± 0.0343 (0.02)	7.44 ± 0.339
18	40 M	0.3	0.08 ± 0.0271 (0.02)	7.50 ± 0.281
19	43 M	0.6	0.08 ± 0.0271 (0.01)	**8.87 ± 0.504**
20	60 F	1.0	0.10 ± 0.0300 (0.01)	8.02 ± 0.392
Group mean ± S.E.		0.730 0.072	0.121 0.008	(0.031) (0.003)	7.637 0.141

Value in parenthesis indicates CA/cell excluding gaps.

Values in **bold letters** represernt 'range'.

Table-29

INDIVIDUAL VALUES OF VARIOUS CYTOGENETIC ENDPOINTS AMONG TOBACCO PLUS ARECA NUT CHEWERS SUFFERING FROM ORAL CANCER (OC-TAC)

No.	Age/Sex	CA/cell ± S.E.	SCE/cell ± S.E.
1	22 M	0.12 ± 0.0382 (0.01)	7.45 ± 0.384
2	23 M	0.20 ± 0.0490 (0.04)	8.05 ± 0.369
3	34 M	0.15 ± 0.0528 (0.04)	9.61 ± 0.477
4	34 M	0.10 ± 0.0360 (0.03)	8.62 ± 0.487
5	35 M	0.10 ± 0.0332 (0.03)	**6.77 ± 0.233**
6	35 M	0.18 ± 0.0409 (0.03)	7.80 ± 0.255
7	40 F	0.16 ± 0.0484 (0.00)	9.99 ± 0.553
8	40 F	0.12 ± 0.0354 (0.01)	9.18 ± 0.501
9	42 M	0.17 ± 0.0426 (0.04)	7.77 ± 0.248
10	45 M	**0.23 ± 0.0896** (0.13)	8.34 ± 0.276
11	50 F	0.20 ± 0.0658 (0.07)	7.73 ± 0.388
12	50 F	0.19 ± 0.0463 (0.04)	7.96 ± 0.405
13	50 F	0.14 ± 0.0375 (0.03)	8.82 ± 0.431
14	50 M	**0.09 ± 0.0286** (0.01)	7.44 ± 0.247
15	50 M	0.17 ± 0.0448 (0.03)	7.87 ± 0.238
16	55 M	0.13 ± 0.0447 (0.00)	8.62 ± 0.412
17	64 M	0.18 ± 0.0477 (0.07)	**10.36 ± 0.542**
18	70 M	0.14 ± 0.0422 (0.02)	8.56 ± 0.426
Group mean ± S.E.		0.153 (0.035) 0.009 (0.007)	8.386 0.215

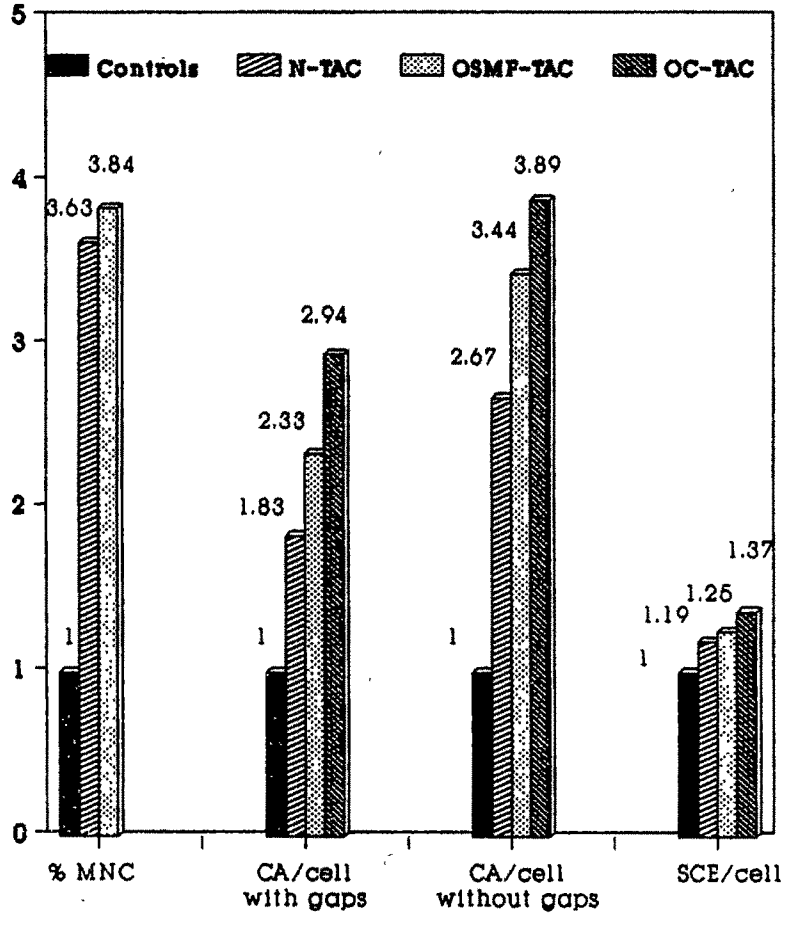
Value in parenthesis indicates CA/cell excluding gaps.

Values in **bold letters** represent '<u>range</u>'.

FIGURE-17

RELATIVE FREQUENCIES OF MNC, CA AND SCE

AMONG CONTROLS AND TOBACCO PLUS ARECA NUT CHEWERS



areca nut chewers were significantly higher ($p < 0.001$). Among the chewers, the frequency of MNC was greater in OSMF-TAC than that of the N-TAC, however, the difference was insignificant statistically.

The mean CA/cell values (including gaps) for N-TAC, OSMF-TAC and OC-TAC were 0.095 $\pm$ 0.006 ($\pm$ S.E.), 0.121 $\pm$ 0.008, and 0.153 $\pm$ 0.009, respectively. The elevations in CA/cell value in all the three groups of chewers were statistically significant ($p < 0.001$) when compared with the controls. The statistically significant differences in CA frequencies was also observed between N-TAC and OSMF-TAC ($p < 0.02$), between N-TAC and OC-TAC ($p < 0.001$) as well as between OSMF-TAC and OC-TAC ($p < 0.02$). Table-30 provides detailed CA analysis for controls, N-TAC, OSMF-TAC and OC-TAC. Compared to the controls, frequency of chromatid-gaps was 1.5 times greater in N-TAC, 1.9 times more frequent in OSMF-TAC and 2.7 times more frequent in OC-TAC. The elevations in mean CA/cell values, excluding gaps, in N-TAC, OSMF-TAC and OC-TAC were statistically significant ($p < 0.001$) when compared with the controls. However, the CA frequencies (excluding gaps) among the different groups of tobacco-areca nut chewers were not significantly different.

The mean SCE/cell values were 7.280 $\pm$ 0.172 ($\pm$ S.E.), 7.637 $\pm$ 0.141 and 8.386 $\pm$ 0.215 for N-TAC, OSMF-TAC and OC-TAC, respectively. The elevations in values of all the three groups of chewers were statistically significant ($p < 0.001$)

Table-30

DETAILS OF CAs (per 100 cells) OBSERVED AMONG CONTROLS AND TOBACCO-ARECA NUT CHEWERS

Group	Aberrant metaphases	Chromosome Aberrations	Types of aberrations (%)									
			Chromatid				Chromosome					
			G	B/F	I	Ag.	G	B	Dm	R	Dc	Ag.
Control	5.05	5.20	4.1	0.8	-	4.9	0.2	0.1	-	-	-	0.3
N-TAC	8.67	9.50	6.3	2.1	0.1	8.5	0.9	0.1	-	-	-	1.0
OSMF-TAC	10.80	12.10	7.8	2.9	0.1	10.8	1.2	0.1	-	-	-	1.3
OC-TAC	12.84	15.30	11.2	2.6	0.1	13.9	1.0	0.1	0.3	-	-	1.4

where,

G = gap; B = break; B/F = break and/or acentric fragment; I = interchange

Dm = double minutes; R = ring chromosome; Dc = dicentric chromosome

Ag. = Aggregate chromatid/chromosome-type aberrations.

compared to the control value of 6.121 ± 0.078 SCE/cell (mean ± S.E.). Among the chewers, the SCE frequency in OSMF-TAC was marginally higher than N-TAC, whereas, OC-TAC had a statistically significant increase in SCE/cell value compared to N-TAC ($p < 0.001$) as well as OSMF-TAC ($p < 0.01$). Table-31 describes the distribution of metaphases according to the number of SCEs in controls, N-TAC, OSMF-TAC and OC-TAC. With the chewing habit and the disease status of the individuals, a gradual decrease in the number of cells with low SCEs, and a concurrent increase in the number of cells with high SCEs was observed.

Figures 18-20 provide group mean frequencies of % MNC, CA/cell and SCE/cell for controls as well as for individuals consuming tobacco in different forms. Table-32 highlights the statistical significance of the results obtained for different groups.

Comparison of different cytogenetic endpoints between the groups of normal individuals consuming tobacco in different forms is provided in Table-33. Although the frequency of MNC in exfoliated buccal mucosa and CA & SCE frequencies in PBLs of normal tobacco consumers (snuff or tobacco with lime or tobacco with areca nut and lime) were significantly higher compared to those of control individuals. However, the mean values of all the three cytogenetic endpoints, when compared between the three groups of normal consumers, i.e. N-SNF, N-TC and N-TAC, the differences were negligible. Similar resul-

Table-31

PERCENT DISTRIBUTION OF METAPHASES ACCORDING TO THE NUMBER OF SCEs AMONG CONTROLS AND TOBACCO-ARECA NUT CHEWERS

Group	Range	% metaphases with			
		0 - 5	6 - 9	11 - 15	>15 SCEs
Control	1 - 14	38.6	57.6	3.8	-
N-TAC	2 - 16	20.9	63.9	15.0	0.2
OSMF-TAC	3 - 14	12.3	73.4	14.3	-
OC-TAC	4 - 17	6.4	66.8	26.1	0.7

FIGURE-18

MEAN FREQUENCY OF MNC (%) AMONG CONTROLS AND DIFFERENT GROUPS OF TOBACCO CONSUMERS

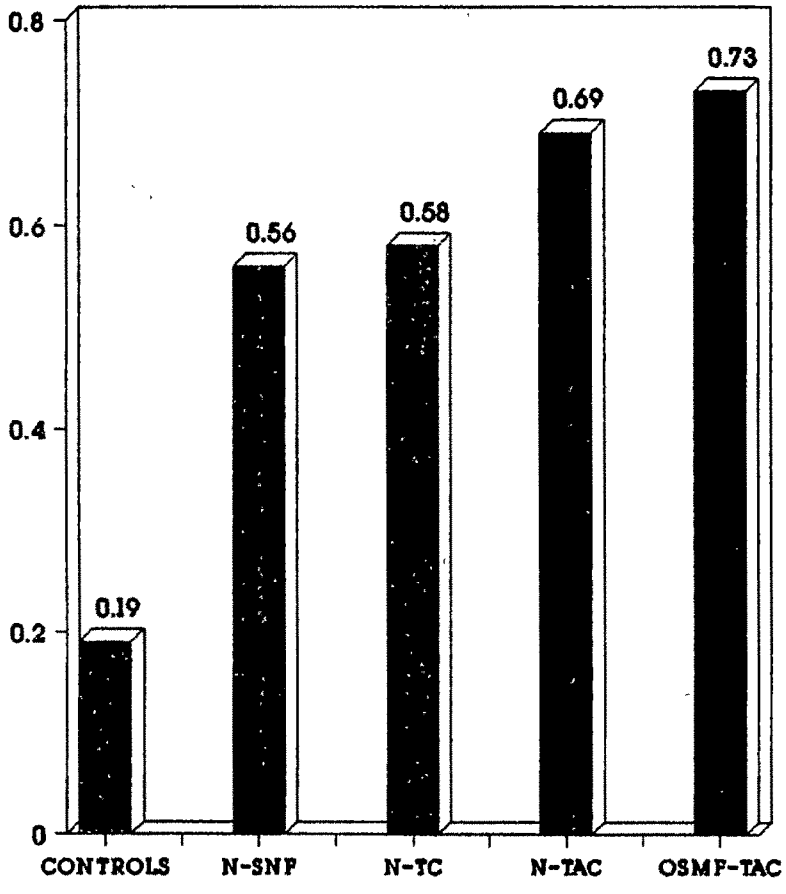


FIGURE-19

MEAN FREQUENCY OF CA AMONG CONTROLS

AND DIFFERENT GROUPS OF TOBACCO CONSUMERS

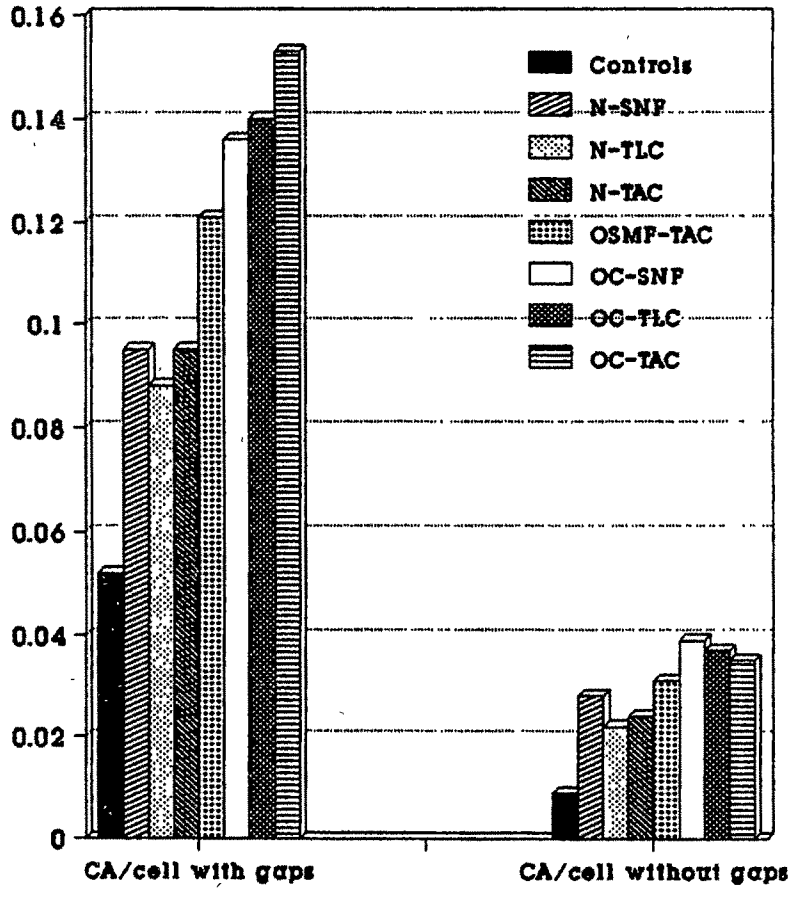


FIGURE-20

MEAN FREQUENCY OF SCE AMONG CONTROLS

AND DIFFERENT GROUPS OF TOBACCO CONSUMERS

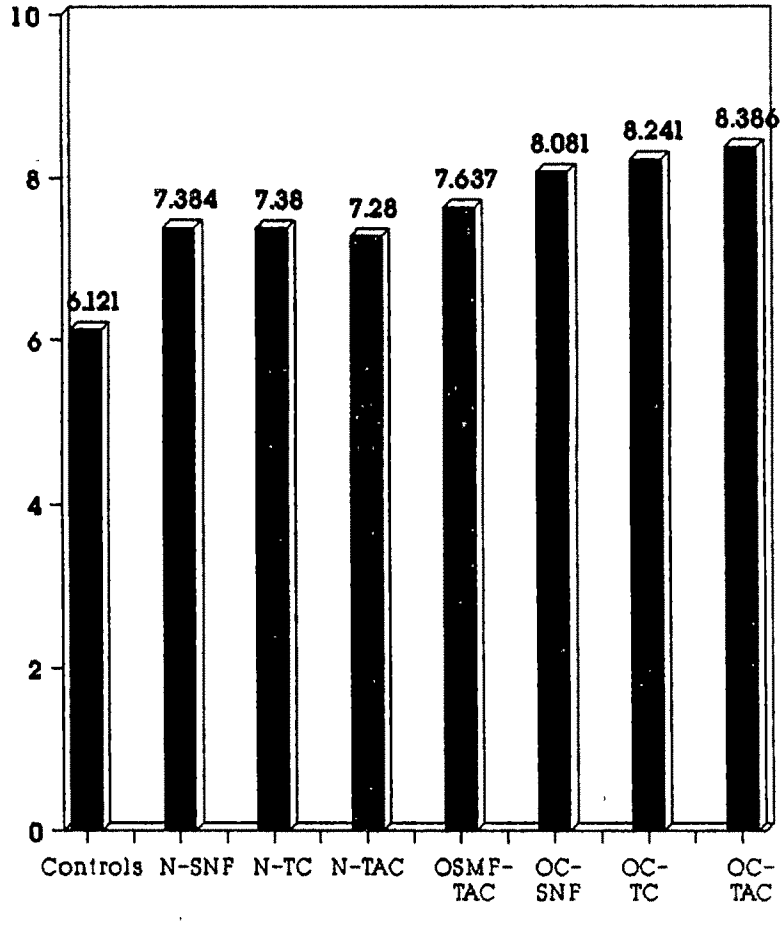


Table-32

STATISTICAL SIGNIFICANCE OF THE DIFFERENCE OBSERVED IN VARIOUS CYTOGENETIC ENDPOINT AMONG DIFFERENT GROUPS ($p <$)

Group	% MNC	CA/cell including gaps	CA/cell excluding gaps	SCE/cell
N-SNF vs. Control	0.001	0.001	0.001	0.001
OC-SNF vs. Control	NA	0.001	0.001	0.001
N-TC vs. Control	0.001	0.001	0.01	0.001
OC-TC vs. Control	NA	0.001	0.001	0.001
N-TAC vs. Control	0.001	0.001	0.001	0.001
OSMF-TAC vs. Control	0.001	0.001	0.001	0.001
OC-TAC vs. Control	NA	0.001	0.001	0.001
N-SNF vs. OC-SNF	NA	0.001	0.01	0.001
N-TC vs. OC-TC	NA	0.001	0.05	0.05
N-TAC vs. OSMF-TAC	NS	0.02	NS	NS
N-TAC vs. OC-TAC	NA	0.001	NS	0.001
OSMF-TAC vs. OC-TAC	NA	0.02	NS	0.01

where,

NA = not applicable since MN scoring was not done in OC patients.

NS = not significant

Table-33

COMPARISON OF VARIOUS CYTOGENETIC ENDPOINTS AMONG CONTROLS, N-SNF, N-TC AND N-TAC

Group	% MNC ± S.E.	CA/cell ± S.E. including gaps	CA/cell ± S.E. excluding gaps	SCE/cell ± S.E.
Control (20)	0.19 ± 0.019 *	0.052 ± 0.003 *	0.009 ± 0.002 *	6.121 ± 0.078 *
N-SNF (13)	0.56 ± 0.037 *	0.095 ± 0.003 *	0.028 ± 0.003 **	7.384 ± 0.096 *
N-TC (14)	0.58 ± 0.049 *	0.088 ± 0.004 *	0.022 ± 0.004 *	7.380 ± 0.188 *
N-TAC (20)	0.69 ± 0.054	0.095 ± 0.006	0.024 ± 0.003	7.280 ± 0.172

* $p < 0.001$ and ** $p < 0.01$ compared to controls.

ts were observed when the values of these markers were compared between the oral cancer patients with different tobacco habits (Table-34).

Individuals with different tobacco habits were also grouped together and were then classified on the basis of their disease status. The data, thus calculated, are provided in Fig. 21 and Table-35. The frequency of % MNC among the N-STC (normal smokeless tobacco consumers, i.e. all the individuals consuming either snuff or tobacco with lime or tobacco with areca nut) was 0.620 ± 0.030 (mean ± S.E.), which was significantly higher compared to 0.190 ± 0.019 (mean ± S.E.) frequency of % MNC among control individuals.

The mean CA/cell values (including gaps) for N-STC, OSMF-STC and OC-STC were, 0.093 ± 0.003 (± S.E.), 0.121 ± 0.008 and 0.144 ± 0.004, respectively. The elevations in CA/cell values were statistically significant compared to 0.052 ± 0.003 (mean ± S.E.) among controls. The differences between the mean CA/cell values (excluding gaps) among N-STC, OSMF-TAC and and OC-STC and among the control individuals were also statistically significant.

The mean SCE/cell values were 7.339 ± 0.097 (± S.E.), 7.637 ± 0.141 and 8.228 ± 0.124 among N-STC, OSMF-STC and OC-STC, respectively. The elevations in values of all the three groups of smokeless tobacco consumers were statistically significant ($p < 0.001$), compared to the control value of 6.121 ± 0.078 SCE/cell (mean ± S.E.).

Table-34

COMPARISON OF VARIOUS CYTOGENETIC ENDPOINTS AMONG CONTROLS, OC-SNF, OC-TC AND OC-TAC

Group	CA/cell ± S.E. including gaps	CA/cell ± S.E. excluding gaps	SCE/cell ± S.E.
Control (20)	0.052 ± 0.003 *	0.009 ± 0.002 *	6.121 ± 0.078 *
OC-SNF (20)	0.136 ± 0.006 *	0.039 ± 0.002 *	8.081 ± 0.163 *
OC-TC (10)	0.140 ± 0.005 *	0.037 ± 0.006 *	8.241 ± 0.294 *
OC-TAC (18)	0.153 ± 0.009	0.035 ± 0.007	8.386 ± 0.215

* $p < 0.001$ compared to controls.

FIGURE-21

MEAN FREQUENCIES OF % MNC, CA/cell AND SCE/cell

AMONG CONTROLS AND SMOKELESS TOBACCO CONSUMERS

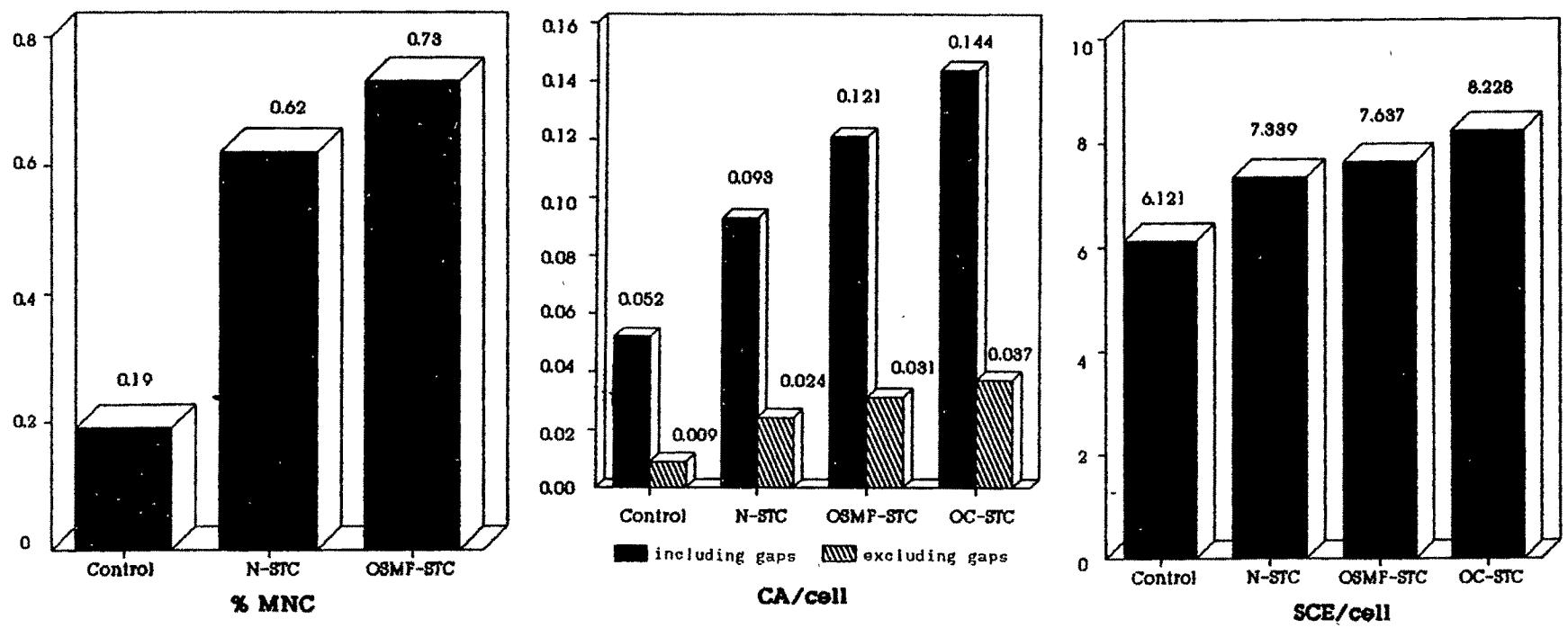


Table-35

STATISTICAL SIGNIFICANCE OF THE DIFFERENCE OBSERVED IN VARIOUS CYTOGENETIC ENDPOINTS AMONG DIFFERENT GROUPS ($p <$)

Group	% MNC	CA/cell including gaps	CA/cell excluding gaps	SCE/cell
N-STC vs. Control	0.001	0.001	0.001	0.001
OSMF-STC vs. Control	0.001	0.001	0.001	0.001
OC-STC vs. Control	NA	0.001	0.001	0.001
N-STC vs. OSMF-STC	NS	0.01	NS	NS
N-STC vs. OC-STC	NA	0.001	0.001	0.001
OSMF-STC vs. OC-STC	NA	0.02	NS	0.01

where, STC includes all the individuals consuming smokeless tobacco in any form.

NA = not applicable; NS = not significant

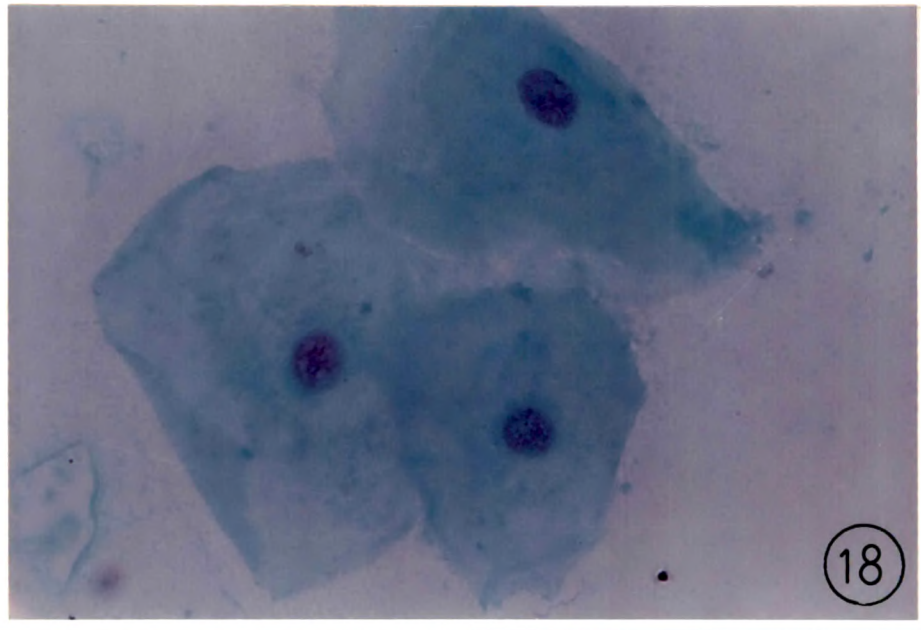
Illustrations 18-20 Exfoliated cells from human buccal mucosa stained with feulgen plus fast green.

18 Normal cells without micronucleus.

19 A micronucleated cell.

20 A cell with several micronuclei attributable to spindle disturbance.

Magnification: 875x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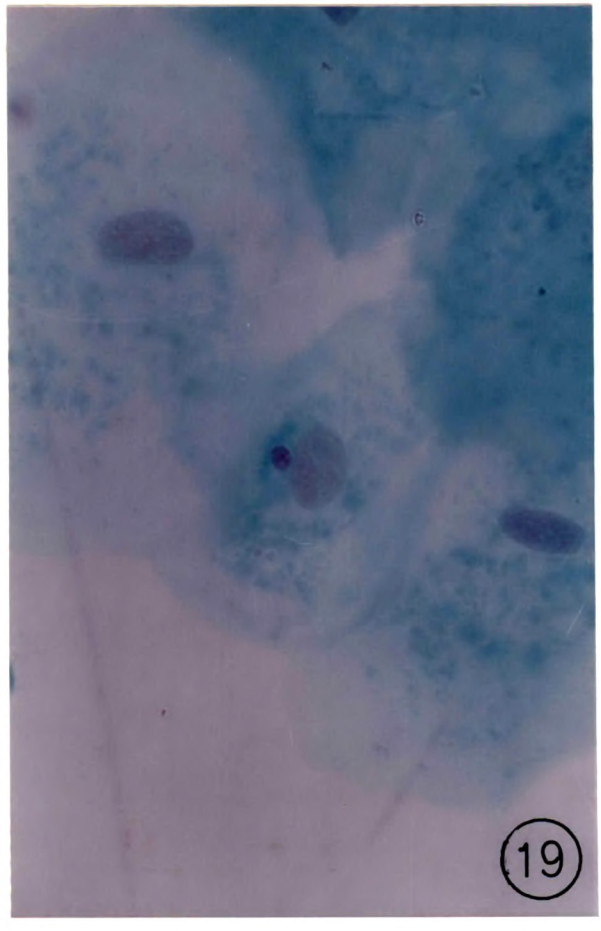
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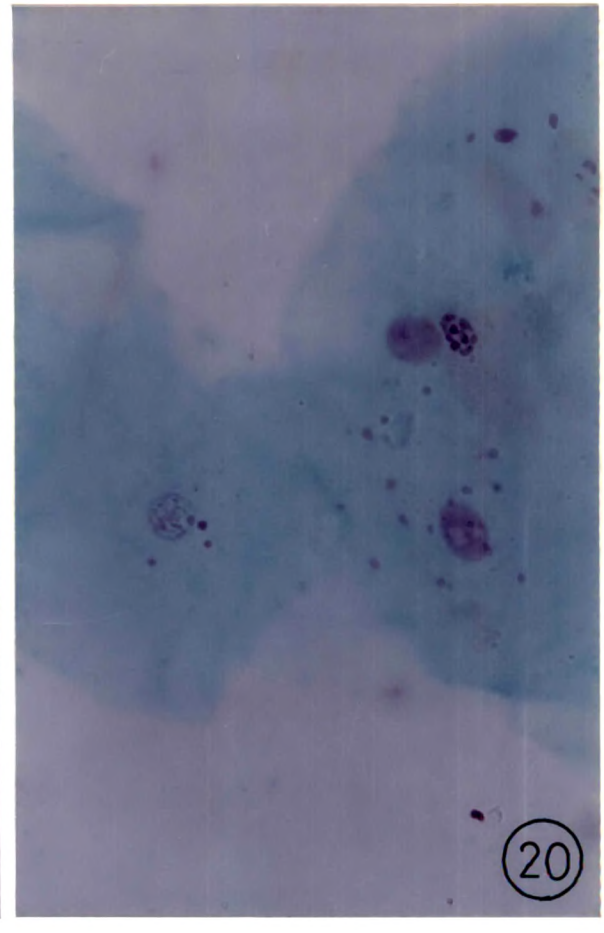

20

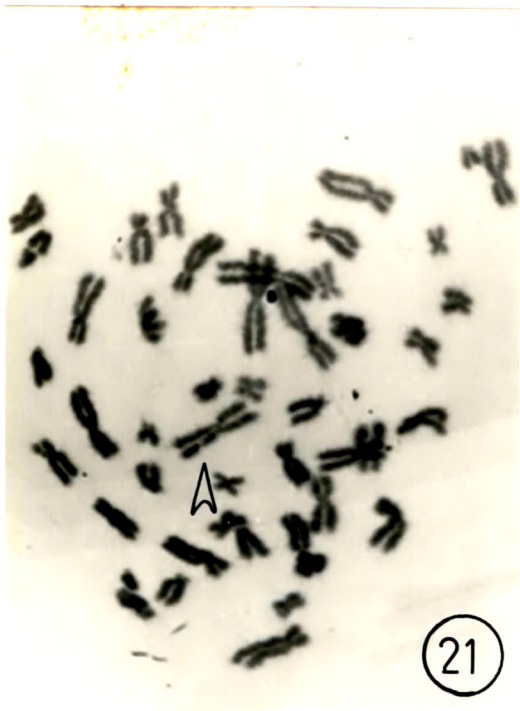
Illustrations 21-24 Photomicrographs showing aberrant metaphases observed in lymphocytes of smokeless tobacco consumers.

21-22 Arrows indicate chromatid-type gaps.

23 Metaphase with chromatid-type break.

24 Arrow pointing towards interchange.

Magnification: 1600x



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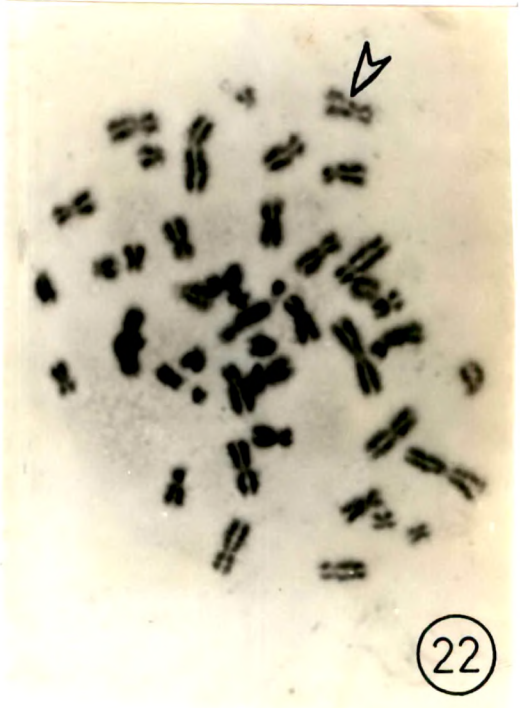
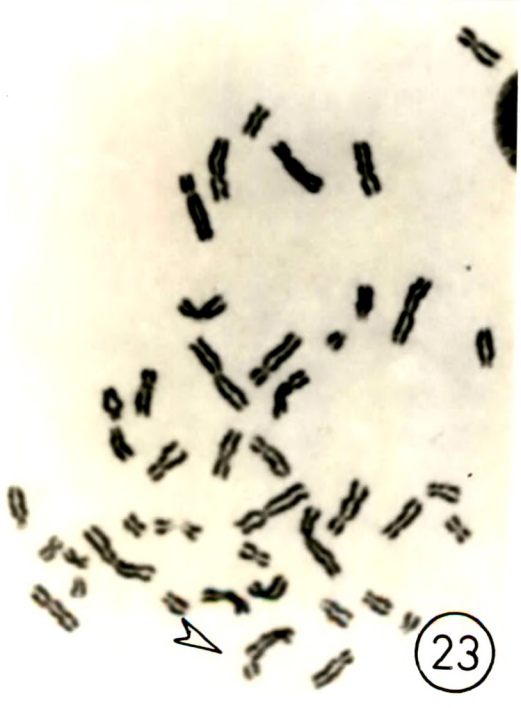


22



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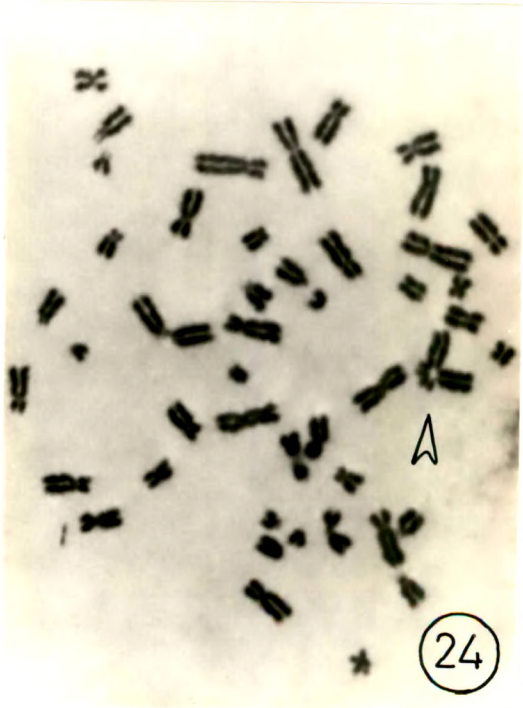
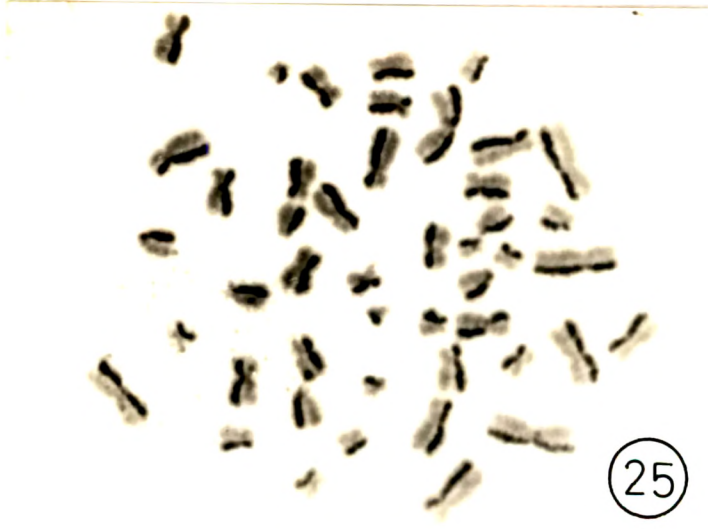


24

Illustrations	25-27	Metaphase in M-II phase of cell division stained by FPG technique.
	25	A metaphase from a control subject showing 2 instances of SCEs.
	26-27	Metaphases from smokeless tobacco consumers exhibiting more number of SCEs.
Magnification:		1800x



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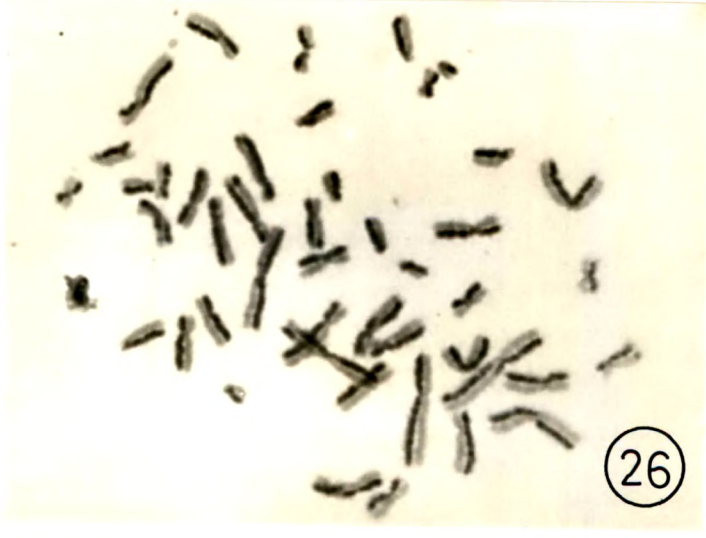
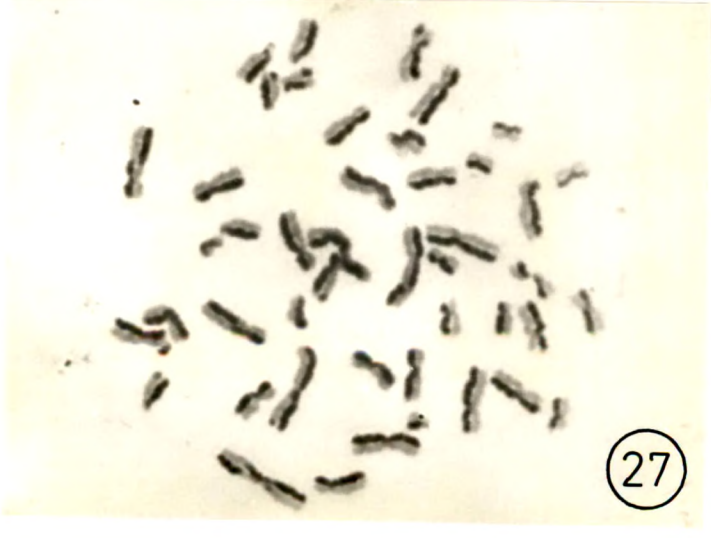


26



27

DISCUSSION:

The International Agency for Research on Cancer (1987) has listed the betel quid containing tobacco and smokeless tobacco products (oral use) in the group of substances for which there is sufficient evidence of carcinogenicity to humans. The causation of oral cavity cancers has been strongly associated with different smokeless tobacco habits. Nair et al. (1985) demonstrated presence of TSNAs viz. NNN and NAT, and two ASNAs viz. N-nitrosoguvacoline (NGCO) and N-nitrosoguvacine (NGCI), in addition to nicotine and arecoline, in the saliva of individuals chewing betel quid with tobacco. NNN has also been detected in the saliva of habitual chewers of tobacco (Sipahimalani et al., 1984). Clastogenic effect of saliva samples from tobacco chewers has also been reported by Stich and Stich (1982). However, for several reasons, it is difficult to assess the genotoxicity of a complex mixture solely by *in vitro* assays. Various interactions, enhancing or suppressing the mutagenicity of individual compound, are expected between the chemicals present in the saliva of tobacco chewers. Human saliva by itself has been reported to reduce the mutagenic effects of several carcinogenic compounds (Nishioka et al., 1981). A reduction in the incidence of tumours following a combined application of 4NQO and DMBA with saliva (Wallenius, 1966), and an increase in tumour frequency following an inhibition of salivary secretion (Wallenius and Lekholm, 1973), have been documented in rats. Thus, simple chemical estimation of mutagens/carcinogens in

various body fluids is not enough to understand the overall effect on human systems. To this end, several *in vivo* short term assays have been proposed to detect and to quantitate the exposure to the genotoxic compounds. Though cytogenetic monitoring is time consuming and expensive, it is the most reliable and reproducible technique available at present, for routine biological monitoring of human exposures to genotoxic agents.

The present study reports the genomic damage inflicted by chronic low level exposure to snuff, to tobacco plus lime and to tobacco plus areca nut plus lime, in human beings. Three cytogenetic endpoints, namely frequency of **MNC** in exfoliated buccal mucosa and frequencies of **CAs & SCEs** in PBLs, were used to monitor the individuals habituated with any one of the above mentioned smokeless tobacco products.

Stich and co-workers (1982-1984) have proposed the use of MN test on human exfoliated cells as 'an internal dosimeter' for identifying the clastogenic effects of carcinogenic agents. High frequency of MNC in exfoliated buccal mucosa reflects the genotoxic effect on the oral tissue. Micronucleus originate from chromosomal regions lagging behind or irregularly migrating at anaphase. Micronucleus, which results from acentric chromosome fragment or aberrant chromosome, survive during the passage of cells from the basal layer to the surface of the mucosa of the oral cavity. Sarto et al. (1987) have differntiated MNC originating as a result of

chromosome breaks and those derived from spindle disturbances. As described in Part I, only the micronuclei originating as a consequence of chromosome breaks were considered for the present study.

The MNC frequency observed among controls, N-SNF, N-TC and N-TAC were 0.19 %, 0.56 %, 0.58 % and 0.69 %, respectively. It was also observed that 100 % (13/13) N-SNF individuals, 92.96 % (13/14) N-TC individuals and 90 % (18/20) N-TAC individuals had higher frequency of MNC than the highest (0.30 % MNC) value observed among the control individuals. The mean frequencies of MNC among all the groups were lower than that reported by Stich and co-workers (1982-1984). This may be due to differnces in the criteria used for the identification of a micronucleus. Alongwith the micronuclei formed due to chromosome breaks, Stich and co-workers have also included the micronuclei formed as a result of spindle disturbances as well as karyorrhexis. However, irrespective of differnces in absolute values, the statistically significant elevations in MNC frequencies among N-SNF, N-TC and N-TAC, compared to the controls, were in accordance with their observations. A gradual increase in the frequency of MNC among N-SNF, N-TC and N-TAC was also observed. However, the MNC frequency in the three groups of tobacco users were comparable. Contrary to this, Stich and Rosin (1983b) have reported significant difference between the MNC frequencies among the individuals chewing khaini tobacco only and those chewing tobacco with areca nut ($p < 0.001$). Significantly higher frequency of MNC

has been reported for chewers of betel quid containing tobacco than the one in chewers of the raw areca nut (Stich et al., 1982). Induction of micronuclei by smoking, a factor which is known to increase the risk of buccal cavity cancers (Wynder and Stellman, 1977), has also been observed (Sarto et al., 1987). However, Stich and Rosin (1983a) did not find any significant increase in MNC frequency in exfoliated buccal mucosa of smokers.

Since the number of individuals in each group was small and since there were wide variations in the frequency and duration of tobacco consumption among the individuals studied, a comparison of the frequency of MNC with the duration of the habit was thought meaningless. As evident from the Table-36, the difference in grams of tobacco consumed per day, as well as number of pack years, was not statistically significant between the three groups of normal tobacco consumers. It has also been observed by Stich et al. (1982) that the frequency of micronuclei does not increase with the number of years that a person chews either raw areca nut or the betel quid containing tobacco.

Frequency of MNC among the OSMF-TAC was comparable to that of N-TAC. Since presence of micronucleus is not known to indicate the preneoplastic or fully neoplastic changes (Stich and Rosin, 1984), the MNC frequency among the OSMF-TAC might be a reflection of the genotoxic effect of chewing tobacco with areca nut and not of the diseased condition of the oral

Table-36

COMPARISON OF TOBACCO HABITS BETWEEN NORMAL INDIVIDUALS AND PATIENTS WITH SIMILAR TOBACCO HABIT

Group	Gram tobacco/day [a]		Pack years [b]	
	Range	Mean ± S.E.	Range	Mean ± S.E.
N-SNF	1.0-11.0	4.04 ± 0.804	3.5-270.0	68.48 ± 19.44
OC-SNF	1.0-4.5	2.65 ± 0.187	6.3-150.0	64.90 ± 10.04
N-TC	0.8-6.0	2.25 ± 0.375	7.5-320.0	91.13 ± 24.56
OC-TC	0.8-2.25	1.43 ± 0.169	12.3-280.0	142.73 ± 29.12
N-TAC	0.8-7.5	3.29 ± 0.415	12.0-300.0	90.00 ± 18.56
OSMF-TAC	1.0-6.3	2.69 ± 0.344	5.0-162.0	45.84 ± 10.41
OC-TAC	1.0-12.5	3.03 ± 0.677	9.0-437.5	113.40 ± 28.03

where,

a = Weight of tobacco consumed per day was calculated by considering

1.0 gm tobacco/chew for SNF-individuals;
0.3 gm tobacco/chew for TC-individuals;
0.5 gm tobacco/chew for TAC-individuals.

b = Pack years = Number of chews per day X Number of years since onset of the habit.

OSMF-TAC vs. N-TAC (Pack years) $p < 0.05$

cavity.

Though the validity of MN test as a marker of genotoxic effect on target tissue, being non-invasive and less demanding, is very high, several drawbacks have been observed during the present study. Most important is difficulty in obtaining scorable smears from most of the oral cancer patients due to ulcerated areas in the oral cavity resulting in poorer oral hygene. Secondly, the differences in the criteria employed for MN scoring by different laboratories made the interlaboratory comparisons difficult.

Nevertheless, the utility of frequency of MNC as a rapid marker in chemoprevention trials is of importance. The administration of chemopreventive agents should prevent the formation of micronucleated cells by blocking the entry of carcinogens into cell, either by interrupting the interaction of the reactive carcinogenic species with DNA, or by inactivating carcinogens, or by scavenging free radicals. Success of a chemopreventive agent should be reflected as reduced incidence of MNC in the target tissues. Following the supplementation of retinol and beta-carotene for 3 months, Stich et al. (1984b) have observed almost a threefold decrease in MNC frequency in exfoliated buccal mucosa of areca nut and tobacco chewers. Later on, individual protective effects of both these compounds have also been reported (Stich et al., 1984a). An intervention trial revealed a reduction in the frequency of MNC in the oral mucosa of snuff users following

administration of beta-carotene over a 10-week period (Stich et al., 1985). Recently, Stich et al. (1990) have suggested automated scoring of MNC, which would make this test applicable to large-scale studies of human population groups.

Analysis of chromosomal alterations in PBLs is another most extensively employed method for assessing the genotoxic effects of exposure to mutagenic/carcinogenic substances. The advantages of PBLs, as an ideal test system for analyzing the effects of chronic exposures to low doses of mutagens, have been detailed by Natarajan and Obe (1982). A large number of cells are easily available from a few ml of peripheral blood. T lymphocytes, which were studied after PHA-M-stimulation *in vitro*, belong to the redistributional pool, i.e. following the exposure to a mutagen anywhere in the body - e.g. in oral cavity, a site of relevance for tobacco chewers - they can eventually occur in the peripheral blood. The lymphocytes may spend long duration in the body which provide time for the repair of lesions before they are stimulated *in vitro*. Thus, as present study deals with low dose, chronic exposure to tobacco, the observed cytogenetic abnormalities were the products of at least three competing processes: **(i)** chronic induction of lesions, **(ii)** continual repair of the lesions, and **(iii)** removal and redistribution of lymphocytes from the circulating pool.

CA and SCE frequencies in PBLs are the well validated cytogenetic endpoints for the biological monitoring of the

population exposed to suspected/proven carcinogens (Carrano and Natarajan, 1988). Even after having sufficient evidence of carcinogenic effect of different smokeless tobacco products in human beings, excepting reports from our laboratory (Adhvaryu et al., 1986; 1991), there is a paucity of data on CA and SCE frequencies in PBLs of individuals consuming: snuff (oral use), mixture of tobacco and lime or tobacco, areca nut and lime (mava). Elevated frequency of lymphocytic SCEs have been reported in betel quid (with and without tobacco) chewers (Ghosh and Ghosh, 1984) as well as in pregnant women and women using oral contraceptives with a concurrent habit of betel chewing (Ghosh and Ghosh, 1988).

CAs are still considered to be the most unambiguous cytogenetic manifestation of clastogenicity (Evans, 1982). A correlation between DNA adducts and CAs has also been reported (Talaska et al.,1987). Tabin et al. (1982) observed that under appropriate circumstances, mutation, in turn, can allow a cell to escape the growth controls and transform to a neoplastic cell. Since there is no efficient and easy technique to study the frequencies of induced point mutations in human beings one has to rely on induced CAs for assessing possible hazards (Natarajan and obe, 1980).

Elevations in CA frequencies (including gaps) among all the groups of tobacco consumers, i.e. normal consumers as well as individuals suffering from the precancer or cancer of the oral cavity, were statistically significant compared to

that of controls. Increased CAs have been witnessed in lymphocytes of alcoholics and smokers (Obe et al., 1980; Vijayalaxmi and Evans, 1982). Compared to controls, all types of CAs were found at a higher frequency among the tobacco consumers. Though most of the chemicals induce chromatid-type aberrations in the ***in vitro*** studies, both, chromosome-type and chromatid-type aberrations may be elevated in exposed populations (Obe and Beek, 1982). As seen in Tables-21, 25 and 30, alongwith chromatid-type aberrations, the frequencies of chromosome-type aberrations (out of the total CAs) were elevated from 0.3 % among the controls to 0.9 % among the N-SNF, 0.7 % among N-TC and 1.0 % among N-TAC. OSMF-TAC patients and oral cancer patients had still higher frequencies of chromosome-type aberrations. Thus present observations are in accordance with those of others. Moreover, even after excluding gaps from the total aberrations, the mean CA frequencies observed among all the groups of tobacco consumers, were significantly higher than that of the controls. These suggest that CA assay can provide useful index in estimating ***in vivo*** DNA damage caused by the oral use of smokeless tobacco.

SCE is the cytological manifestation of DNA breakage and misrepair involving homologous sites on the two chromatids of a chromosome. The most efficient inducers of SCE appear to be substances, which form covalent adducts to the DNA or interfere with DNA precursor metabolism or repair (Latt, 1981; Perry and Evans, 1975; Wolff, 1977). The positive

linear correlation between induced SCE and mutation (Carrano et al., 1978) or cell transformation (Popescu et al., 1981), demonstrated the validity of SCE assay as an indicator for both mutagenesis and carcinogenesis. Several studies have demonstrated that frequency of SCE increases when human beings are exposed to mutagens and carcinogens (Lambert et al., 1982). Increased SCEs have been observed in PBLs of cigarette smokers (Carrano, 1982; Hopkin and Evans, 1980; Lambert et al., 1978) as well as in PBLs of bidi smokers (Ghosh and Ghosh, 1987). Present findings of the SCE assay among the normal tobacco consumers, are in agreement with that on chewers of betel quid containing tobacco (Ghosh and Ghosh, 1984).

Percent distribution of metaphases according to the number of SCE per cell showed an increase in number of metaphases with higher SCEs among the tobacco consumer, compared to the controls. Carrano (1982) has suggested use of estimation of the proportion of lymphocytes with a high SCE frequency, i.e. **high frequency cells (HFCs)** for the quantification of the genomic damage caused by chronic exposures to mutagens/carcinogens. These HFCs might represent either lymphocytes that are deficient in repair of lesions, or lymphocytes that are long-lived and have accumulated damage, or a combination of both. Following the method suggested by him, HFCs were determined as number of cells having SCE frequency beyond the 95 % tolerance level (The tolerance level was defined as the SCE frequency in the control population below which 95 % of the cells fall). The 95 % tolerance level for concurrent controls

was 9 SCE/cell. In the present study, compared to 3.8 % HFCs among controls, values of HFCs for normal tobacco consumers as well as OSMF-TAC patients were more than 3 times higher and for oral cancer patients, the values were 5 to 7 times higher (Tables-22, 26 and 31). Such an analysis explains the elevated mean frequencies of SCE among the tobacco consumers.

The increase in CA and SCE frequencies can be explained on the basis of clastogenic/genotoxic potential of tobacco and areca nut. Tobacco and areca nut contains TSNAs and ASNAs, respectively. Various tobacco-areca nut specific nitrosamines have been detected in the saliva samples from chewers of betel quid containing tobacco (Brunnemann et al., 1986). Some of these nitrosamines are potent mutagens and carcinogens (IARC, 1985). Zimonjic et al. (1989) reported induction of SCE by NNK (TSNA) in V79 cells and in cultured human PBLs. They have suggested that the formation of O^6-methylguanine may be responsible for SCE induction. Induction of CAs in V79 cells by NNK has also been reported (Alaoui-Jamali et al., 1988).

The saliva samples from tobacco and areca nut chewers have been reported to cause chromosomal damage in CHO cells (Stich and Stich, 1982). The clastogenic effects of an aqueous extract of tobacco and of nicotine have been discussed in detailed in previous chapter on ***in vitro*** studies. The clastogenic/genotoxic effect of areca nut consumption (without tobacco) in humans has also been documented (Dave, 1990). Therefore,

alongwith tobacco, areca nut should also be considered for the observed elevated frequencies of CA and SCE among tobacco plus areca nut (mava) chewers. Increase in CA and SCE frequencies have been reported in different premalignant and malignant conditions (Adhvaryu et al., 1986; 1988a; 1988b; 1988c; 1988d; Murty et al., 1985; 1986). At the same time, lowered (Kakati et al., 1978) as well as unaltered (Adhvaryu et al., 1985; Bazopoulou-Kyrkanidou et al., 1986; Brown et al., 1986) SCE frequencies have also been reported from patients with different cancers. Hence, the elevation in the values of these two parameters among OSMF-TAC patients and oral cancer patients may be due to either the effect of the habit, or of the occurrence of the disease or both.

Since CAs and SCEs are produced by different mechanisms, they should be used as complementary tests rather than as alternative of each other. Moreover, a joint application of both these parameters for assessing genotoxic potentials of chemical carcinogens, has also been suggested (Abe and Sasaki, 1977; Carrano and Natarajan, 1988; Das, 1988; Gebhart, 1981). Following table shows the number of individuals (%) among the different groups of normal tobacco consumers, who had higher than the highest frequency observed among the controls.

Group	CA only	SCE only	CA + SCE
N-SNF	76.92	84.62	92.31
N-TC	46.67	80.00	93.33
N-TAC	90.00	70.00	95.00

Thus, in comparison to a single marker, simultaneous application of these two parameters allow a more precise determination of genotoxic effects of tobacco.

Biological factors, affecting the expression of the endpoints in question, include age, sex, diet, medication, occupation, lifestyle, genotype etc. Reports regarding the effect of age and sex on frequencies of CAs and SCEs are controversial (Das, 1988; Husum et al., 1986; Livingstone et al., 1983). For more than 5 years, we have been studying these parameters in different groups of cancer patients and controls. So far we have not observed age or sex to have any bearing on SCE or CA frequencies. Moreover, most of the individuals included in the study were vegeterians and in case of precancer or cancer patients, the blood samples were taken before the start of any anticancer therapy. Controls were normal, healthy and habit free individuals. Furthermore, subjects engaged in the occupation which are known to affect the frequencies of CA and SCE, were not included. The most important confounding factor was the lifestyle of individuals under investigation. As described under the title 'selection of subjects', all the individuals were teetotalers. Tobacco users were consuming tobacco (With or without areca nut) in one specific form only. The difference between number of pack years for normal tobacco consumers and oral cancer patients with similar habits was not significant (Table-36). However, mean frequency of pack years for OSMF-TAC patients was significantly low compared to that of N-TAC ($p < 0.05$). This

indicates involvement of factors other than tobacco in the aetiology of the disease. Role of genetic factors in the causation of oral cavity cancers at younger age has been postulated (Sankarnarayanan et al., 1989).

The tobacco habits included in the present study are causally associated with oral cancers, however, data currently available are insufficient to come to any conclusion regarding the relationship of smokeless tobacco use to cancers at sites other than oral cavity. In the case of tobacco smoking, lung cancers are the major cancer, however, risk of cancer at other sites like urinary bladder, pancreas etc. is also elevated. The elevated frequencies of CA and SCE in PBLs of tobacco consumers pinpoint to the possibility of genomic damage caused to other systems/tissues as well. Hence, for individuals consuming smokeless tobacco in various ways, possibility of an elevated risk of cancers at sites other than oral cavity indeed deserves a closer scrutiny.

In short, **(i)** Frequency of MNC in exfoliated buccal mucosa and CA and SCE frequencies in PBLs are the efficient indicators of genomic damage caused by the tobacco habits considered, on the target as well as nontarget somatic tissues.

(ii) A comparable mean frequencies of all the three cytogenetic endpoints among N-SNF, N-TC and N-TAC (Table-42) suggest that oral use of smokeless tobacco in different forms is equally harmful.

(iii) Compared to N-TAC, the significantly low level exposure observed among the OSMF-TAC indicates that factors other than tobacco and areca nut may be involved in causation of the disease.

(iv) During epidemiologic studies related to the cancers at sites other than the oral cavity, analysis of oral smokeless tobacco habits as a risk factor may furnish information about the role of smokeless tobacco (oral use) in the causation of cancer at sites which are not coming in direct contact.