PART II

ARECA NUT CONSUMPTION AND ITS EFFECTS ON HUMAN TARGET AND NON-TARGET TISSUES

The in vitro short term experiments, employing mammalian test system, provided substantial evidence about the genotoxic potentials of areca nut. However, the extrapolation of carcinogenesis data generated from short term assays and laboratory animals to the heterogenous human population is often complicated. The behaviour of a particular substance may be very different in a complex organism such as man. Inside the human body, during the transport and metabolism of the substance, a range of physiological and biochemical factors, different from those present in the in vitro systems or in vivo rodent bioassays, may influence the fate of the substance. These may either inhibit or enhance its toxic potentials. Short term in vitro tests using mammalian cells might not fully mimic all these stages. Long term studies for carcinogenicity in experimental animals also may not necessarily reflect a realistic situation because human beings may be exposed to a vast array of agents that might be participating in the carcinogenic process.

Areca nut, the substance under study, is one of the oldest known masticatories among the Asians. The genotoxic effects of chronic low level exposure to areca nut might well be estimated by cytogenetic analysis in individuals with a habit of chewing areca nut. A positive result would clearly evidence its carcinogenic/ genotoxic potentials in human beings and save the investments and the labour of performing animal bioassays. Thus, to substantiate the information gained by in vitro assays, and also considering the fact there are no reports on cytogenetic studies in human beings in context to ONLY areca nut consumption, the present investigation was extended in this sphere.

In recent evaluation on the association of cancer of oral cavity and the habit of chewing betel quid, it was concluded that there was 'sufficient' evidence that the habit of chewing betel quid containing tobacco is carcinogenic to humans, however, 'inadequate' evidence about the habit of chewing betel quid without

tobacco being carcinogenic to humans was indicated (IARC, 1985). These chewing habits result in exposure inter alia to areca nut alkaloids, N-nitroso compounds derived from these alkaloids, polyphenols and when the habit includes tobacco, to tobacco specific nitrosamines and possibly also to the synthetic musks.

The N-nitrosamines formed by nitrosation of tobacco alkaloids have been known to play a significant role in tobacco carcinogenesis (Hoffmann and Hecht, 1985). Currently, there is a growing concern regarding the carcinogenic potentials of the nitrosation products of the areca nut alkaloid, arecoline. Together with arecoline, areca derived N-nitrosamines namely N-nitrosoguvacoline (NGCO), N-nitrosoguvacine (NGCI), 3-(methyl nitrosoamino)propionitrile (MNPN) and 3-(methyl nitrosoamino)propionic acid have been identified in the saliva of the chewers of betel quid without tobacco (Wenke et al., 1984b; Nair et al., 1985; 1987 Prokopczyk et al., 1987b; Stich et al., 1986; Stich and Anders, 1989). MNPN has been reported to be a potent carcinogen in F344 rats (Wenke et al., 1984a). Its mode of action in the carcinogenic process has been studied in detail (Prokopczyk et al., 1987b; 1988b). The carcinogenicity of NGCO is still an open question with two contradictory reports (Lijinsky and Taylor, 1976; Rivenson et al., 1988).

Preliminary data indicate that higher levels of areca nut alkaloids and areca nut specific nitrosamines are present in the saliva of chewers of betel quid containing tobacco than in the saliva of those who chew betel quid without tobacco. This may be due to the constituents of tobacco which provide additional nitrosation potential (Brunnemann et al., 1986). Excepting a few preliminary reports, including our own reports, on SCEs and adduct formation (Ghosh and Ghosh, 1984; 1988; Prokopczyk et al., 1988a; Dunn and Stich, 1986; Adhvaryu et al., 1986; 1988a), the cellular aspects of the effects of areca nut chewing with or

without tobacco, in humans, remain to be explored in greater depths. Studies in the target tissue i.e. the oral mucosa have been carried out by Stich and co-workers (1982-1986).

The cytological analysis of peripheral blood lymphocytes is a classical and the most extensively employed indicator for the analysis of the genetic effects of the possible exposure to mutagenic carcinogens (Natarajan and Obe, 1980; WHO, 1985a). It measures gross changes occurring in the DNA, including overt breakage and re-arrangement of chromosomes (structural chromosome aberrations) as well as more subtle changes involving the switching of parts of a single chromosome (sister chromatid exchange). These parameters have been most successfully employed as biological dosimeter in individuals exposed to chemical mutagens, occupationally, accidentally or by lifestyle (Lambert et al, 1982; Sandberg, 1982). Even on population basis, increased frequencies of SCEs and CAs are indicators of exposure to carcinogens, a factor that increases the risk of cancer and genetic diseases (Carrano and Natarajan, 1988).

In areca nut-tobacco chewing, the target organ is oral mucosa which is readily assessible for early detection. It was proposed that micronucleus (MN) test in exfoliated cells can be used as an internal index in order to identify and quantitate the damage inflicted by areca nut/tobacco (Stich et al., 1982-1986). As mentioned earlier, a strong association exists between tobacco-areca nut chewing habit and oral cancer. Oral cancers are often preceded by premalignant lesions/conditions (WHO, 1984). Oral submucous fibrosis (OSMF) is now accepted as a premalignant condition and its potency of being converted into oral cancers has been well established by detailed studies (Gupta et al., 1980; Pindborg, 1984b; McGurk and Craig, 1984). Areca nut chewing is considered to be the major etiologic factor responsible for the development of OSMF (Pindborg and Sirsat, 1966; Pindborg, 1980; Gupta et al., 1980; Mehta et al., 1972; Seedat and Wyk, 1988; Sinor P.N.,1990).

In the present study the in vivo DNA damage caused by areca nut consumption was estimated using three short term assays, designed to study the effects on the target as well as the non-target tissues. As areca nut is most frequently consumed in combination with tobacco, the study covered individuals consuming ONLY areca nut and the ones chewing areca nut with tobacco. In both the groups of chewers, i.e. the ONLY areca nut chewers and the areca nut plus tobacco chewers, individuals suffering from OSMF or oral cancer, having similar habits were included to learn in greater depth about habit induced oral disease and the utility of the parameters in detecting the changes at premalignant or malignant stages.

MATERIALS AND METHODS :

CHEMICALS:

The specifications of all the chemicals used in this study have been detailed in Part I. Phytohaemagglutinin-M (PHA-M) was procured from Gibco Chemicals Ltd., U.S.A.

SELECTION OF SUBJECTS:

A total of 88 individuals were selected for the present study and were categorized in four groups viz. (1) Controls-15, (2) Normal chewers-25, (3) OSMF patients-25 and (4) Oral cancer patients-23.

Controls: *Only those individuals were selected as controls, who had never consumed alcohol, tobacco or areca nut in any form and had neither viral disease nor antibiotic therapy in the recent past.*

In all, six groups of chewers were studied. These included three groups of ONLY areca nut chewers i.e. those who consumed areca nut without any concurrent tobacco habit. The other three groups were of chewers who consumed a mixture of areca nut, tobacco and lime (a combination called 'mava' or 'masala' in this part of the country - Illustrations 32-33). The individuals, covered among ONLY areca nut chewers and among those consuming areca nut with tobacco, used ripe, sundried and unprocessed areca nuts (the one used in our in vitro studies). In all the groups the chewing habit was practised at least for two years. The groups of chewers were classified according to the condition of oral mucosa.

Abbreviations used for the six groups of chewers are as follows:

GROUP	ONLY ARECA NUT	ARECA NUT WITH TOBACCO
Normal chewers	NAC	NA-TC
OSMF patients	OSMF-A	OSMF-AT
Oral cancer patients	OC-A	OC-AT

Normal chewers: *These include individuals who, despite their chewing habit, had no change in their oral mucosa.*

(a) NAC -10 (b) NA-TC -15.

OSMF Patients: *OSMF was diagnosed when there was a diffused and progressive fibrotic change in the oral mucosa, characterized by the stiffening of an otherwise yielding mucosa, resulting in difficulty in opening the mouth.*

(a) OSMF-A - 10 (b) OSMF-AT - 15.

Oral cancer patients: *Only histopathologically confirmed oral cancer patients with a positive history of chewing were selected. The samples were collected before any anticancer therapy was initiated.*

(a) OC-A - 08 (b) OC-AT - 15.

The criteria for duration and the specifications of the chewing habit were strictly adhered to, and individuals having smoking or alcohol habit were not included in the study.

COLLECTION OF SAMPLE :

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

(2) For micronucleus assay, exfoliated buccal mucosa cells were collected after rinsing the mouth with water. Using a blunt spatula, the cells were scrapped from the region where the chew was usually placed. The cells were smeared on clean slides, fixed with aceto-methanol, air dried and stored until stained. Since it was not feasible to collect scorable buccal smears from oral cancer patients, this parameter was not carried out in them.

LYMPHOCYTE CULTURE PROCEDURE:

One ml of whole blood was added to 7 ml of MEM containing 20% NCS, 0.3 ml PHA-M, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml BrdU. The cultures were incubated at 37°C for 72 hours. Three hours prior to harvesting, colchicine was added at a final concentration of 0.3 µg/ml. During all the steps, the cultures were protected from direct light.

Harvesting was carried out by subjecting the cells to a hypotonic treatment with 0.56% KCl for 15 minutes and then fixing them with freshly prepared, chilled aceto-methanol (1:3). Slides were prepared following usual air drying protocol.

The differential staining was achieved by employing Flourescence plus Giemsa (FPG) technique detailed earlier, in Part I and the scoring pattern for SCEs, CAs and AGT remained the same.

STAINING PROTOCOL FOR MN:

The exfoliated buccal mucosa cells were stained using Feulgen plus fast green method, with minor modifications. Prefixed cells were hydrolysed with 5 N HCl for a period of 20 minutes at room temperature. After a rinse in distilled water, the slides were stained with Schiff's reagent for 90 minutes. This was followed by three changes in freshly prepared SO_2 water, two minutes each. The slides were kept in running water for 30 minutes. Counter staining was carried out in 0.5% Fast green solution in ethyl alcohol for 30 seconds, rinsed in distilled water, dried and mounted with DPX.

Scoring: *A minimum of one thousand cells from each individual were screened for the presence of micronuclei. Anucleated cells and cells undergoing any kind of degeneration, i.e. karyolysis, karyorhexis, pyknosis and nuclear fragmentation and also MN formed as a result of spindle disturbance (Sarto et al., 1987), were excluded from counting. The identification of micronucleus was based on the criteria detailed in the earlier chapter.*

RESULTS

I. EFFECTS IN ONLY ARECA NUT CHEWERS:

Tables 23-26 present the individual mean values for SCEs, CAs and AGT in peripheral blood lymphocytes and the percentage MNC in exfoliated buccal mucosa cells of the controls, NAC, OSMF-A and OC-A.

Fig. 12 provides the comparative data of SCE per cell frequency among controls and the three groups of only areca nut chewers. As observed in the figure, the controls, with a range of 5.66 to 6.95, gave a mean value of 6.185±0.088 (mean±S.E.). While among the areca nut chewers, in the NAC the SCE frequencies varied

Table-23

Details of various cytogenetic endpoints among controls

No.	Age / Sex	Mean SCE/cell±S.E.	Mean CA/cell±S.E.	Frequency of % MNC	AGT(hr.)
1	17 M	6.04±0.325	0.04±0.0196	0.2	41.03
2	19 M	6.35±0.319	0.04±0.0196	0.3	40.68
3	23 M	5.66±0.411	0.04±0.0196	0.3	35.82
4	23 F	6.46±0.400	0.05±0.0218	0.1	22.02
5	24 M	6.31±0.550	0.03±0.0171	0.1	33.57
6	24 F	5.86±0.380	0.03±0.0171	0.2	22.22
7	25 M	6.63±0.315	0.05±0.0218	0.3	31.17
8	25 M	6.28±0.465	0.05±0.0218	0.1	27.59
9	26 M	5.70±0.298	0.06±0.0276	0.3	27.91
10	28 F	6.19±0.420	0.06±0.0238	0.3	25.81
11	35 M	6.36±0.510	0.05±0.0218	0.1	26.97
12	49 F	6.95±0.387	0.04±0.0196	0.1	44.04
13	55 F	6.06±0.346	0.07±0.0292	0.2	33.57
14	58 F	5.79±0.228	0.08±0.0306	0.2	40.00
15	60 F	6.14±0.207	0.06±0.0238	0.1	37.80
Group Mean:		6.185±0.088	0.050±0.004	0.193±0.022	32.68±1.774

Table-24

Particulars of SCE, CA, MNC and AGT among Normal areca nut chewers (NAC)

No.	Age / Sex	Mean SCE/cell±S.E.	Mean CA/cell±S.E.	Frequency of % MNC	AGT(hr.)
1	27 F	8.79±0.409	0.11±0.0313	0.7	38.71
2	28 F	7.39±0.383	0.13±0.0391	0.3	34.04
3	30 M	5.82±0.343	0.11±0.0343	0.6	31.58
4	35 F	7.51±0.330	0.08±0.0306	1.1	38.40
5	49 M	7.27±0.372	0.13±0.0365	1.2	38.71
6	52 F	5.59±0.380	0.08±0.0306	0.6	33.57
7	56 M	7.58±0.512	0.11±0.0431	0.5	40.00
8	60 M	8.02±0.436	0.08±0.0306	1.0	30.19
9	60 M	6.70±0.482	0.11±0.0371	0.6	38.71
10	78 F	7.51±0.494	0.11±0.0371	0.7	32.88
Group Mean:		7.218±0.288	0.105±0.006	0.730±0.085	35.72±1.055

Table-25

Individual values of the cytogenetic endpoints among areca nut chewers with OSMF (OSMF-A)

No.	Age / Sex	Mean SCE/cell±S.E.	Mean CA/cell±S.E.	Frequency of % MNC	AGT(hr.)
1	12 M	8.14±0.058	0.19±0.0504	0.7	37.80
2	20 M	5.98±0.215	0.19±0.0504	0.4	44.04
3	20 M	6.95±0.479	0.11±0.0313	0.7	31.58
4	23 F	7.88±0.494	0.13±0.0365	0.7	27.12
5	38 F	6.60±0.427	0.12±0.0354	0.6	44.44
6	47 M	8.05±0.274	0.16±0.0367	0.4	44.04
7	50 F	6.64±0.240	0.11±0.0371	1.1	30.00
8	50 M	8.09±0.431	0.15±0.0409	0.7	45.28
9	55 M	7.42±0.488	0.12±0.0354	0.8	32.88
10	65 M	9.45±0.637	0.09±0.0286	1.2	32.88
Group Mean:		7.520±0.303	0.137±0.010	0.730±0.078	37.01±2.082

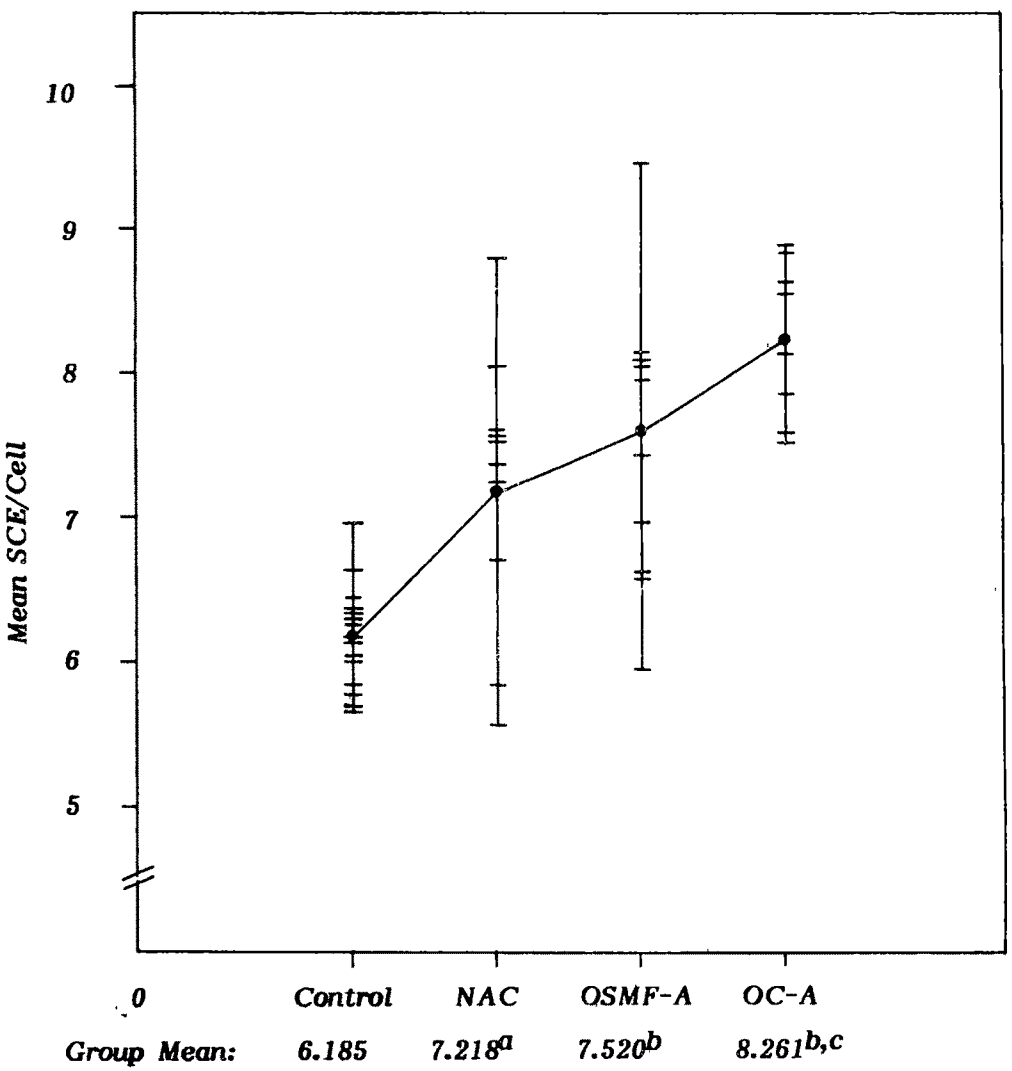
Table-26

Frequency of lymphocytic SCE, CA and AGT among areca nut chewers suffering from oral cancer (OC-A)

No.	Age / Sex		Mean SCE/cell±S.E.	Mean CA/cell±S.E.	AGT (hr.)
1	27	M	8.16±0.499	0.14±0.0400	45.28
2	32	M	8.58±0.388	0.13±0.0365	38.71
3	35	F	7.88±0.292	0.19±0.0440	43.64
4	45	F	8.85±0.515	0.21±0.0454	35.04
5	55	F	8.90±0.559	0.17±0.0426	41.38
6	60	F	7.51±0.420	0.14±0.0465	44.86
7	67	F	8.63±0.470	0.13±0.0391	36.92
8	80	M	7.58±0.498	0.15±0.0433	45.28
Group Mean:			8.261±0.184	0.158±0.010	41.39±1.340

Fig. 12

Mean SCE/Cell values among controls and various groups of areca nut chewers



a = $p<0.01$ *when compared to controls*
b = $p<0.001$ *when compared to controls*
c = $p<0.01$ *when compared to NAC*

from 5.59 to 8.79 providing a mean value of 7.218±0.288. The OSMF-A exhibited a variation from 5.98 to 9.45 giving a mean of 7.520±0.303, whereas, in the OC-A, a mean of 8.261±0.184 SCE per cell was computed from the values spanning from 7.51 to 8.90.

On comparing the mean values of different groups, it was observed that all the three groups of chewers showed a statistically significant elevation in SCE frequencies when compared to the controls. Among the chewers a marginal increase in SCEs was observed between NAC and OSMF-A, but when the values of NAC were compared to OC-A the difference was significantly higher with $p < 0.01$. Although a clear increase was demonstrated between the mean values of OSMF-A and OC-A, the difference was not statistically significant.

When the total metaphases scored, were clubbed on the basis of the number of SCEs, as shown in Fig. 13, it was observed that SCEs in controls followed binomial distribution, however, the other groups (chewers) deviated from normality, with more number of cells with higher SCEs, a shift towards the right in the figure. Illustrations 37-39 exemplify SCEs observed in the lymphocytes of the controls and the chewers.

Fig. 14 details the mean values of CAs observed in the controls and in different groups of areca nut chewers. The mean CA values in controls was found to be 0.05 CA per cell which increased to 0.105 in NAC, 0.137 in OSMF-A and 0.158 among OC-A. All the three groups of areca nut chewers showed a highly significant elevation in CA frequency when compared to that of the controls ($p < 0.001$). Within the chewers when the values for NAC were compared to that of both the diseased groups, the increase was found to be statistically significant. Among the diseased areca nut chewers, inspite of an apparent increase in CA frequencies in OC-A over OSMF-A, the increase was not statistically significant.

Fig. 13

Distribution of SCE frequency among controls and areca nut chewers

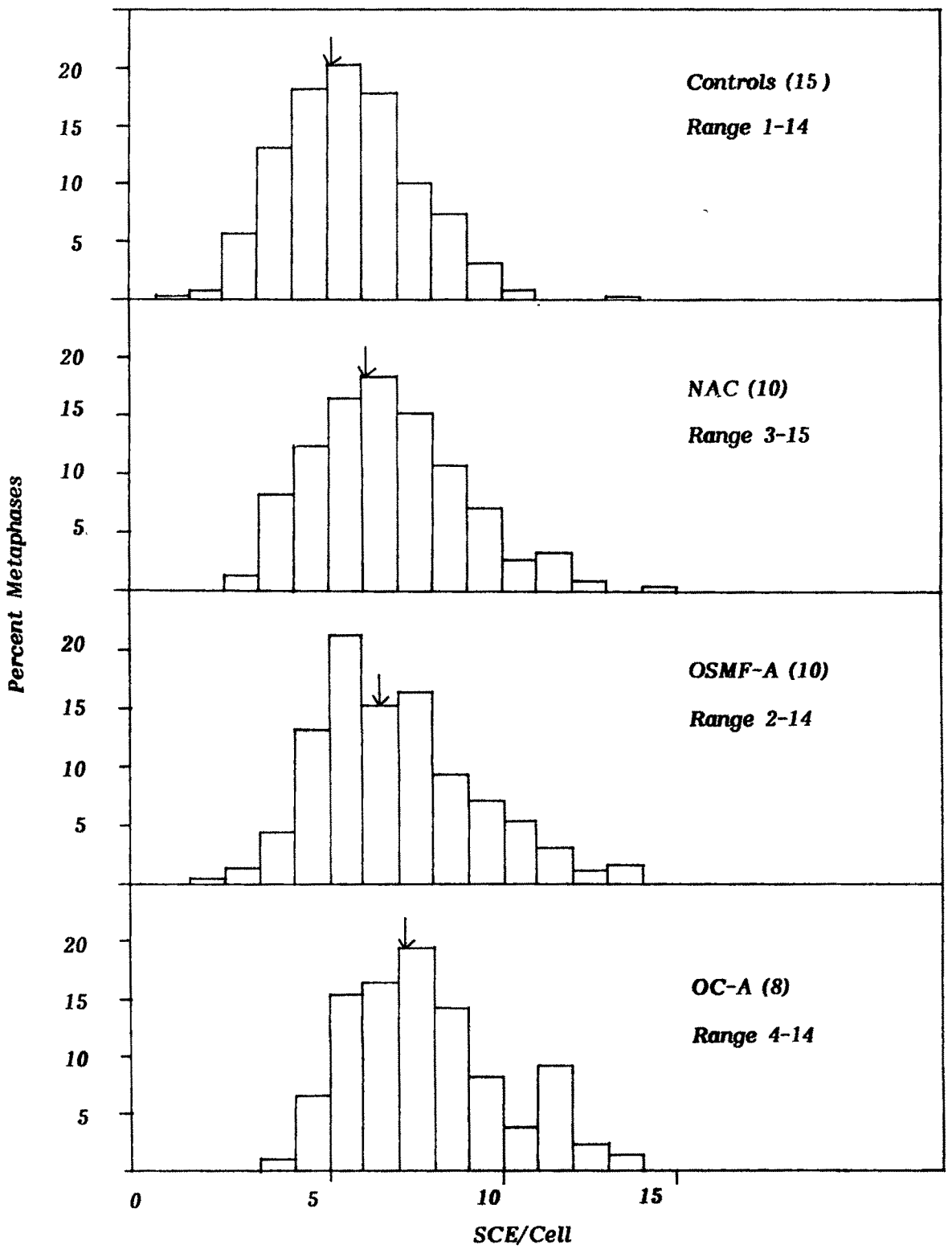
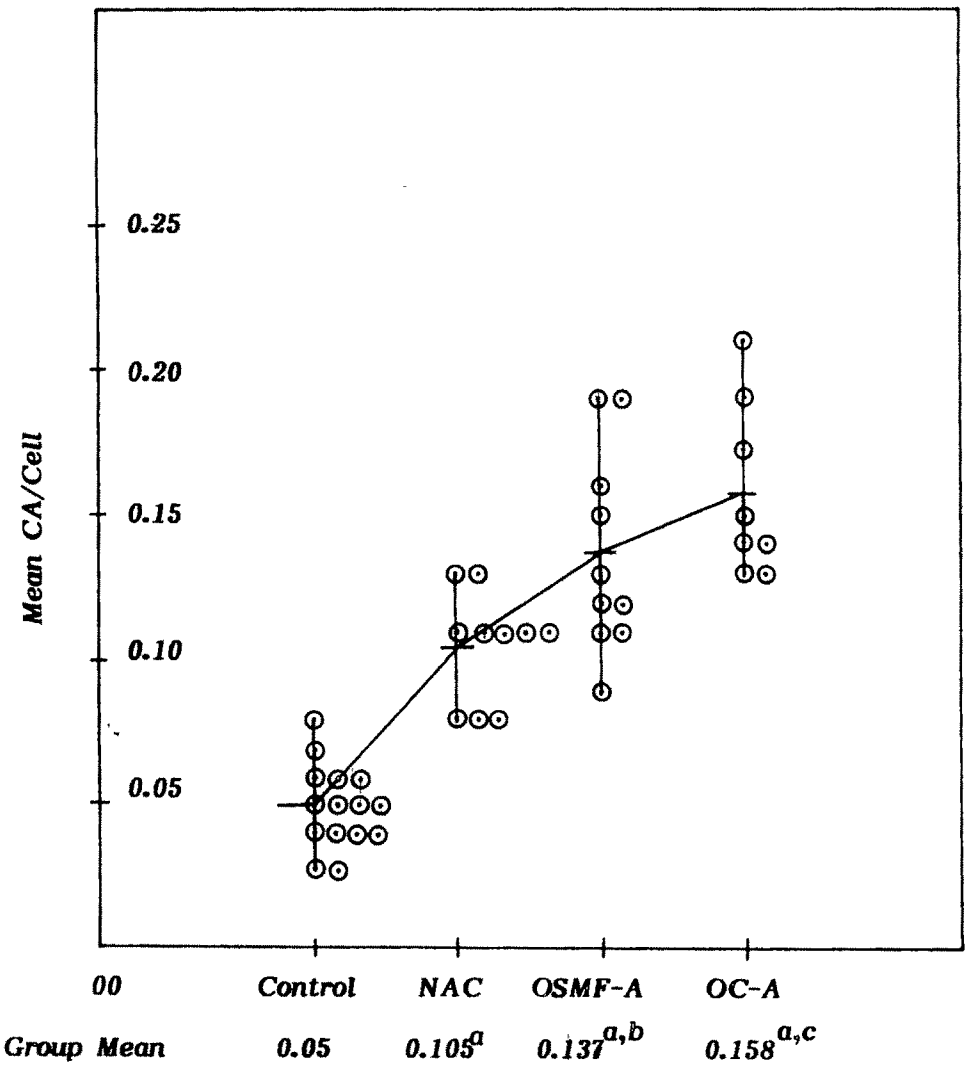


Fig.14

Mean CA/cell values among controls and different groups of areca nut chewers



a = p<0.001 when compared to controls
b = p<0.02 when compared to NAC
c = p<0.001 when compared to NAC

The 4.8% aberrant metaphases observed in the controls, almost doubled i.e. 9.28% in NAC and escalated to 12.20% in OSMF-A and 13.51% in OC-A. Table-27 describes the various types of CAs observed in all the groups. Illustrations 40-51 portray some of the representative aberrant metaphases. There was an increase in all types of aberrations among the areca nut chewers. Chromatid gaps raised from 3.7% in controls to 9.5% in OC-A, whereas, acentric fragments were five times more common in NAC and ten times more frequent in OC-A than in the controls.

The mean AGT values calculated for controls and different groups of areca nut chewers, from the percentage of cells in MI, MII and MIII, have been expressed in Table-28. A gradual increase in the MI cells was witnessed in NAC, OSMF-A and OC-A, with a concurrent decrease in the MIII cells. A statistically significant increase was found ($p < 0.001$) when mean AGT of the controls was compared to that of OC-A. Also among the chewers, the OC-A mean AGT was higher than NAC ($p < 0.01$). Illustrations 34-36 exhibit representative cells in MI, MII and MIII cycle of the cell division.

The direct damage caused by the areca nut chewing habit on oral mucosa was analysed by screening the exfoliated buccal mucosa cells for the presence of micronucleus. Illustrations 52-55 feature some micronucleated cells from buccal mucosa. Fig. 15 expresses the details of percentage MNC observed in controls, NAC and OSMF-A. As witnessed in the scatterogram, the controls had a mean percentage MNC as low as 0.193%, which increased to 0.73% in NAC and 0.73% in OSMF-A. In controls, the percent MNC frequency ranged from 0.1 to 0.3%. In most of the chewers the percent MNC values were observed to be higher than the highest value among the controls. A more than three times higher frequency of MNC was observed among the chewers than the controls with $p<0.001$. It was noteworthy that in OSMF-A, although the % MNC remained analogous

Table-27

Variations in the types of CA (%) observed among controls and three groups of areca nut chewers

Group	%Aberrant Metaphases	Chromatid				Chromosome				
		G	B	I	Af	GI	BI	DM	Dic	R
Controls	4.80	3.7	0.6	-	0.3	0.3	0.1	-	-	-
NAC	9.28	7.3	0.6	0.3	1.5	0.6	0.1	-	-	-
OSMF-A	12.20	8.8	0.8	0.1	2.7	0.9	0.6	-	-	-
OC-A	13.51	9.5	1.3	0.1	3.0	1.3	0.3	0.1	0.3	-

G - Gap, B - Break, I - Interchange, Af - Acentric fragment,
GI - Gap Isochromatid, B - Break Isochromatid, DM - Double Minutes,
Dic - Dicentric, R - Ring.

Table-28

Comparison of cell cycle distribution and mean AGT among controls and different groups of areca nut chewers

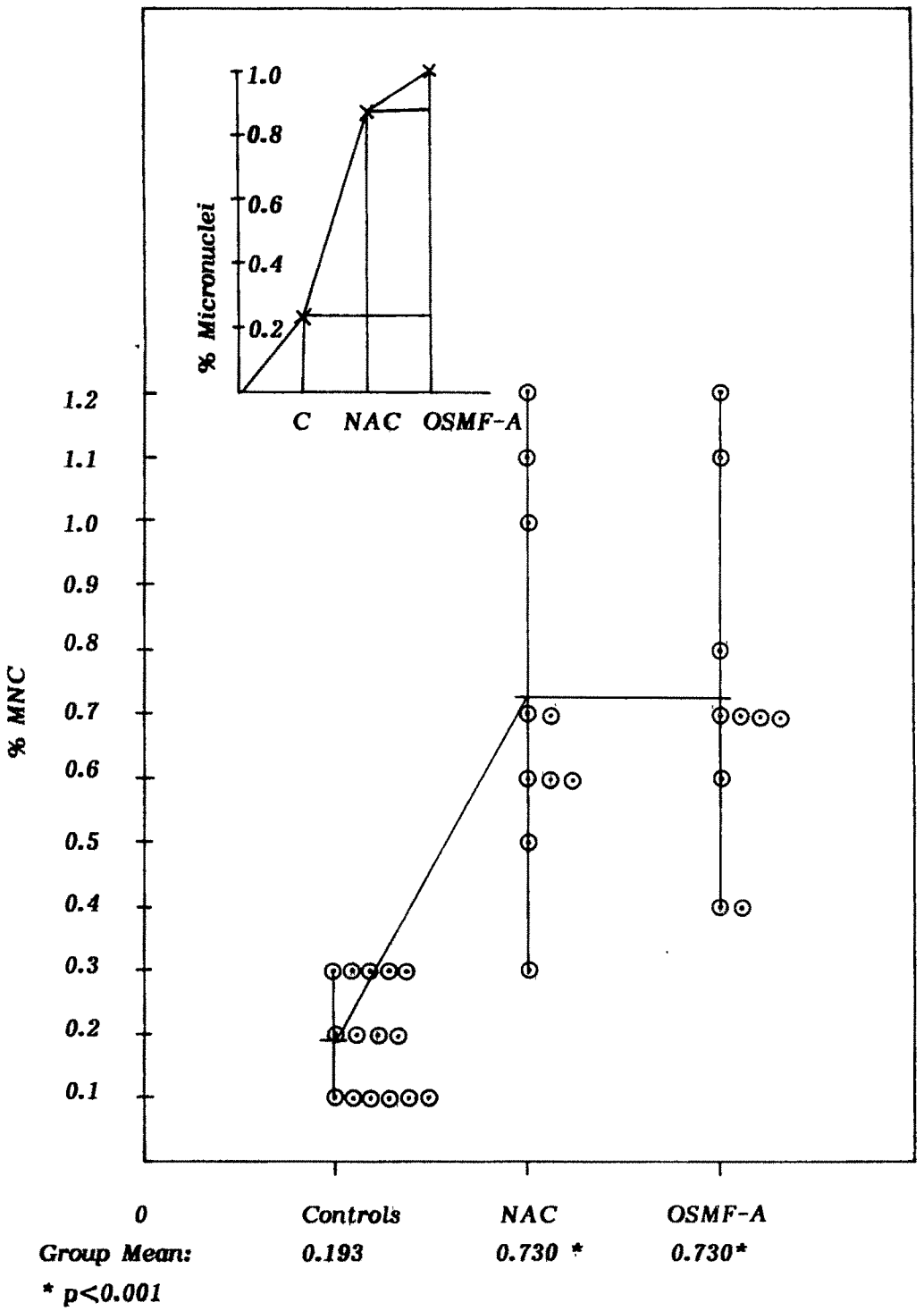
Group	MI (%)	MII (%)	MIII (%)	Mean AGT (h)±S.E.
Controls	58.9	32.2	8.9	32.68±1.77
NAC	67.3	29.6	3.1	35.72±1.06
OSMF-A	71.3	23.4	5.3	37.01±2.08
OC-A	84.3	14.1	1.6	$41.39\pm1.34^{a,b}$

a - $p<0.001$ when compared to controls.

b - $p<0.01$ when compared to NAC.

Fig. 15

Individual frequency of micronucleated cells (MNC) and group mean frequency of micronuclei among controls and areca nut chewers



to NAC values, the percentage of the number of micronuclei per cell was higher than that observed in NAC i.e. 0.87% in NAC and 1.06% in OSMF-A.

II. OBSERVATIONS AMONG CHEWERS OF ARECA NUT WITH TOBACCO (AN + T):

The particulars of age, sex, frequency of SCEs, CAs, and MNC among 45 chewers consuming areca nut with tobacco are contained in Tables 29 to 31.

The individual mean SCE per cell values observed among the three groups of AN + T chewers and the controls are detailed in Table-32. Controls exhibited a mean of 6.185 SCEs per cell. In comparison, the NA-TC provided a mean value of 7.219. For OSMF-AT it was 7.617 and the highest mean value of 8.50 SCEs per cell was observed in the OC-AT. When the group mean values of SCE per cell were compared, all the three groups of chewers presented highly significant elevations when compared to the controls ($p<0.001$). Within the groups of AN+T chewers the difference between the NA-TC and the OC-AT was statistically significant.

The total metaphases scored for SCEs were further grouped according to the number of SCEs (Fig. 16). As clearly visualized in the figure, because of the chewing habit and with the progression of change from normal mucosa to oral cancer, a shift is observed towards right, i.e. more number of cells with higher SCEs resulting in higher mean SCE frequencies for the group.

The mean values of CAs scored in AN + T chewers, together with that of the controls are included in Table-33. In normal chewers the CA values ranged from 0.05 to 0.16 CA per cell, while, in the OC-AT the range was 0.09 to 0.20 CA per cell. The mean values among the chewers were 0.097 in NA-TC, 0.127 in OSMF-AT and 0.144 in OC-AT. When the mean values were compared with those of the controls the elevations were found to be statistically significant ($p<0.001$). The differences were statistically significant when mean value of NA-TC was compared with that of OSMF-AT($p<0.02$) and with OC-AT ($p<0.001$).

Table-29

Individual expressions of cytogenetic endpoints among Normal areca nut plus tobacco chewers (NA-TC)

No.	Age / Sex	Mean SCE/cell±S.E.	Mean CA/cell±S.E.	Frequency of % MNC	AGT(hr.)
1	21 M	9.63±0.453	0.08±0.0351	0.8	35.04
2	24 M	7.51±0.516	0.09±0.0349	1.2	30.00
3	25 M	6.24±0.432	0.10±0.0300	0.4	24.87
4	26 M	5.86±0.448	0.06±0.0237	1.1	25.13
5	27 M	6.80±0.228	0.16±0.0441	0.5	28.07
6	33 M	6.65±0.375	0.09±0.0286	0.9	39.02
7	34 M	7.28±0.286	0.08±0.0306	0.7	30.19
8	35 M	7.13±0.585	0.08±0.0271	0.9	24.24
9	37 M	7.57±0.503	0.05±0.0218	0.5	28.24
10	37 M	7.33±0.584	0.09±0.0349	0.7	30.19
11	40 M	7.28±0.405	0.12±0.0354	0.6	21.82
12	46 F	6.54±0.408	0.13±0.0429	0.7	38.40
13	50 M	6.69±0.288	0.14±0.0401	1.0	36.92
14	56 M	7.95±0.430	0.11±0.0343	0.8	29.63
15	56 M	7.83±0.549	0.08±0.0271	0.3	35.36
Group Mean:		7.219±0.221	0.097±0.007	0.740±0.064	30.48±1.346

Table-30

Individual values of cytogenetic markers among areca nut plus tobacco chewers with OSMF (OSMF-AT)

No.	Age / Sex	Mean SCE/cell±S.E.	Mean CA/cell±S.E.	Frequency of % MNC	AGT(hr.)
1	17 M	8.38±0.568	0.15±0.0384	0.8	32.00
2	20 M	6.21±0.399	0.13±0.0448	0.7	24.24
3	21 M	6.55±0.345	0.08±0.0271	0.3	33.57
4	23 M	7.53±0.335	0.10±0.0300	1.8	25.95
5	24 M	8.35±0.319	0.16±0.0504	0.4	38.10
6	24 M	7.33±0.264	0.11±0.0467	0.2	28.24
7	28 M	7.62±0.315	0.13±0.0391	0.8	39.67
8	28 M	7.44±0.400	0.14±0.0400	1.1	31.79
9	29 M	8.11±0.375	0.09±0.0349	0.6	36.92
10	32 M	8.07±0.377	0.14±0.0400	1.0	40.00
11	33 M	6.66±0.249	0.19±0.0463	0.7	45.71
12	36 M	7.36±0.287	0.11±0.0313	0.5	44.44
13	37 M	7.75±0.478	0.20±0.0469	0.8	42.48
14	43 M	8.87±0.504	0.08±0.0271	0.6	38.71
15	60 F	8.02±0.392	0.10±0.0300	1.0	43.64
Group Mean:		7.617±0.183	0.127±0.009	0.753±0.097	36.36±1.693

Table-31

Values of lymphocytic SCE, CA and AGT among areca nut plus tobacco chewers suffering from oral cancer (OC-AT)

No.	Age / Sex		Mean SCE/cell±S.E.	Mean CA/cell±S.E.	AGT (hr.)
1	22	M	7.45±0.384	0.12±0.0382	42.86
2	23	M	8.05±0.369	0.20±0.0490	39.34
3	34	M	9.61±0.477	0.15±0.0528	42.11
4	34	M	8.62±0.487	0.10±0.0360	43.24
5	35	M	6.77±0.233	0.10±0.0332	42.11
6	40	F	9.99±0.553	0.16±0.0484	25.95
7	40	F	9.18±0.501	0.12±0.0354	27.12
8	45	M	8.34±0.276	0.14±0.0566	32.88
9	50	M	7.44±0.247	0.09±0.0286	44.44
10	50	F	8.82±0.431	0.14±0.0375	36.92
11	50	F	7.96±0.405	0.19±0.0463	44.86
12	50	F	7.73±0.388	0.20±0.0658	38.10
13	55	M	8.62±0.412	0.13±0.0447	38.71
14	64	M	10.36±0.542	0.18±0.0477	28.74
15	70	M	8.56±0.426	0.14±0.0422	36.09
Group Mean:			8.500±0.248	0.144±0.009	37.56±1.568

Table-32

Comparison of SCE/cell values among controls and different groups of areca nut plus tobacco chewers (Mean±S.E.)

No.	Controls	NA-TC	OSMF-AT	OC-AT
1	5.66±0.411	5.86±0.448	6.21±0.399	6.77±0.233
2	5.70±0.298	6.24±0.432	6.55±0.345	7.44±0.247
3	5.79±0.228	6.54±0.408	6.66±0.249	7.45±0.384
4	5.86±0.380	6.65±0.375	7.33±0.264	7.73±0.388
5	6.04±0.325	6.69±0.288	7.36±0.287	7.96±0.405
6	6.06±0.346	6.80±0.228	7.44±0.400	8.05±0.369
7	6.14±0.207	7.13±0.585	7.53±0.335	8.34±0.276
8	6.19±0.420	7.28±0.405	7.62±0.315	8.56±0.426
9	6.28±0.465	7.28±0.286	7.75±0.478	8.62±0.412
10	6.31±0.550	7.33±0.584	8.02±0.392	8.62±0.487
11	6.35±0.319	7.51±0.516	8.07±0.377	8.82±0.431
12	6.36±0.510	7.57±0.503	8.11±0.375	9.18±0.501
13	6.46±0.400	7.83±0.549	8.35±0.319	9.61±0.477
14	6.63±0.315	7.95±0.430	8.38±0.568	9.99±0.553
15	6.95±0.387	9.63±0.453	8.87±0.504	10.36±0.542
Mean	6.185±0.088	7.219±0.221[a]	7.617±0.183[a]	8.500±0.248[a,b,c]

a - $p<0.001$ when compared to controls.

b - $p<0.001$ when compared to NA-TC

c - $p<0.01$ when compared to OSMF-AT.

Fig. 16

Distribution of SCE frequency in controls and areca nut + tobacco chewers

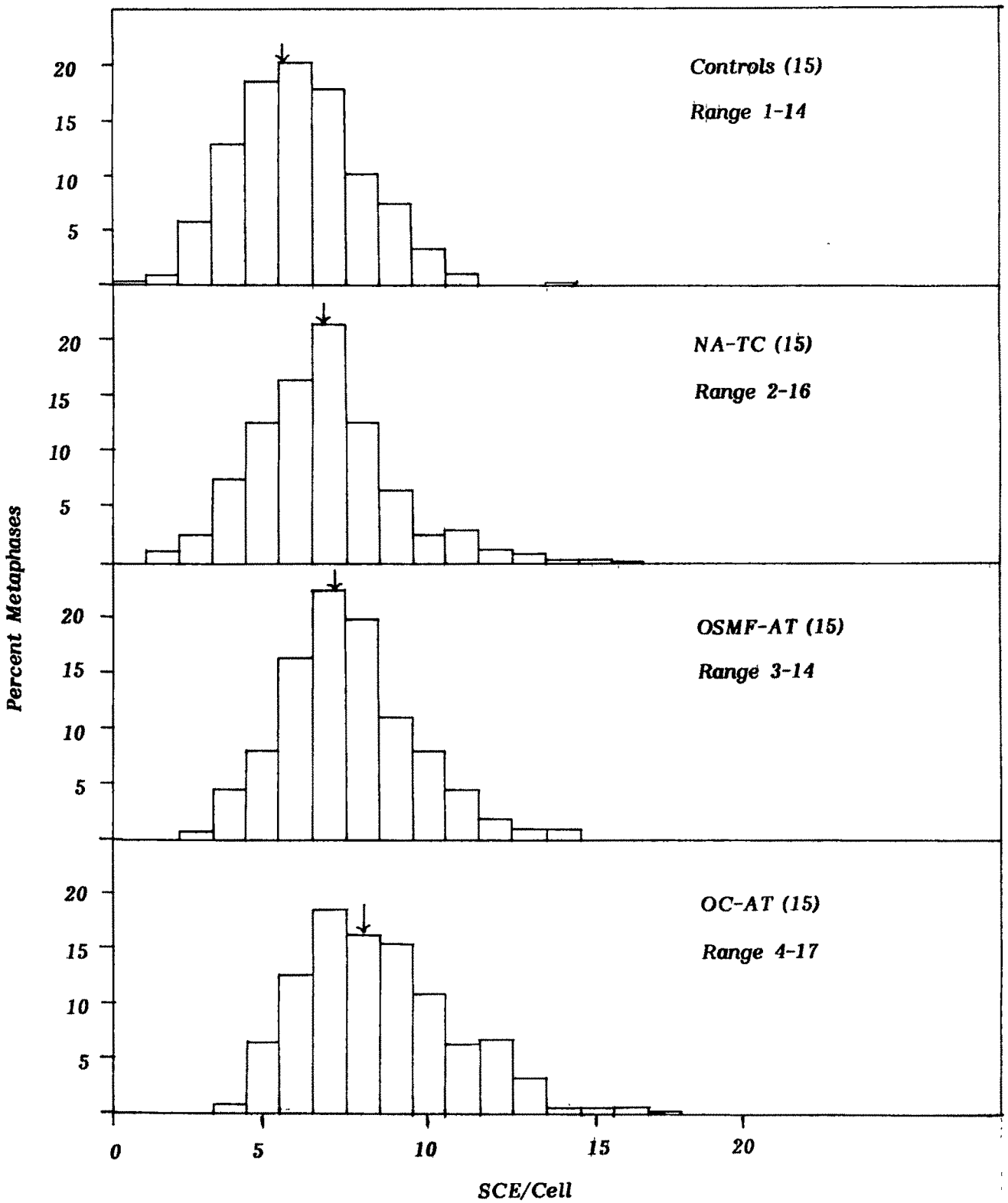


Table-33

Comparison of CA/cell values among Controls and different groups of areca nut plus tobacco chewers (Mean±S.E.)

No.	Controls	NA-TC	OSMF-AT	OC-AT
1	0.03±0.0171	0.05±0.0218	0.08±0.0271	0.09±0.0286
2	0.03±0.0171	0.06±0.0237	0.08±0.0271	0.10±0.0332
3	0.04±0.0196	0.08±0.0271	0.09±0.0349	0.10±0.0360
4	0.04±0.0196	0.08±0.0351	0.10±0.0300	0.12±0.0382
5	0.04±0.0196	0.08±0.0271	0.10±0.0300	0.12±0.0354
6	0.04±0.0196	0.08±0.0306	0.11±0.0467	0.13±0.0447
7	0.05±0.0218	0.09±0.0349	0.11±0.0313	0.14±0.0566
8	0.05±0.0218	0.09±0.0286	0.13±0.0391	0.14±0.0375
9	0.05±0.0218	0.09±0.0349	0.13±0.0448	0.14±0.0422
10	0.05±0.0218	0.10±0.0300	0.14±0.0400	0.15±0.0528
11	0.06±0.0238	0.11±0.0343	0.14±0.0400	0.16±0.0484
12	0.06±0.0276	0.12±0.0354	0.15±0.0384	0.18±0.0477
13	0.06±0.0238	0.13±0.0429	0.16±0.0504	0.19±0.0463
14	0.07±0.0292	0.14±0.0401	0.19±0.0463	0.20±0.0658
15	0.08±0.0306	0.16±0.0441	0.20±0.0469	0.20±0.0490
Mean:	0.050±0.004	0.097 ± 0.007^{a}	$0.127\pm0.009^{a,b}$	$0.144\pm0.009^{a,c}$

a - $p<0.001$ when compared to controls.

b - $p<0.02$ when compared to NA-TC

c - $p<0.001$ when compared to NA-TC

A comprehensive data of the types of aberrations scored in controls, NA-TC, OSMF-AT and OC-AT are given in Table-34. Chromatid aberrations were more frequent with maximum number of gaps in all the four groups including the controls. The number of gaps nearly doubled in NA-TC and were three times more frequent in OC-AT when compared to the controls. Interchanges (exchanges) were never observed in the controls but were found in all the three groups of chewers. Double minutes were observed only in the AN + T chewers with oral disease.

The group mean AGT value of NA-TC was found to be lower than the controls. The mean values being 32.68 in the controls, 30.48 in NA-TC, 36.36 in OSMF-AT and 37.56 in OC-AT. The reason for higher AGT values in OSMF-AT and OC-AT was the increased number of cells in MI and a concurrent decrease in the cells in MIII of the cell division. Table-35 gives the values of mean AGT in all the four groups and provides the details of cell cycle variations among them. A statistically significant difference was observed when AGT value for OC-AT was compared to that of controls ($p<0.05$) and to NA-TC ($p<0.01$).

The MNC frequency in the exfoliated buccal mucosa cells represent the DNA damage in the target tissue. The values observed in the controls, NA-TC and OSMF-AT have been plotted in Fig. 17. The percent MNC ranged from 0.3 to 1.2, providing a mean of 0.74 in NA-TC and with a range of 0.2 to 1.8, a mean of 0.75 percent MNC was observed among OSMF-AT. Both the mean values were significantly ($p<0.001$) high compared to the control mean of 0.193 percent MNC.

Fig. 18 gives a collective data of controls and areca nut chewers for all the parameters analysed and Fig. 19 provides a data for areca nut+tobacco chewers. In both the groups all the cytogenetic markers exhibited distinct increase. Table-36 depicts the statistical significance observed among the groups of chewers and

Table-34

Types of CAs (%) observed among Controls and areca nut plus tobacco chewers

Group	%Aberrant Metaphases	Chromatid				Chromosome				
		G	B	I	Af	GI	BI	DM	Dic	R
Controls	4.80	3.7	0.6	-	0.3	0.3	0.1	-	-	-
NA-TC	8.76	6.4	0.9	0.1	1.3	1.1	-	-	-	-
OSMF-AT	11.07	8.0	1.3	0.1	1.7	1.5	0.1	0.1	-	-
OC-AT	12.28	10.6	1.3	0.1	1.2	0.8	0.1	0.2	-	-

G - Gap, B - Break, I - Interchange, Af - Acentric fragment, GI - Gap Isochromatid, BI - Break Isochromatid, DM - Double Minutes, Dic - Dicentric, R - Ring.

Table-35

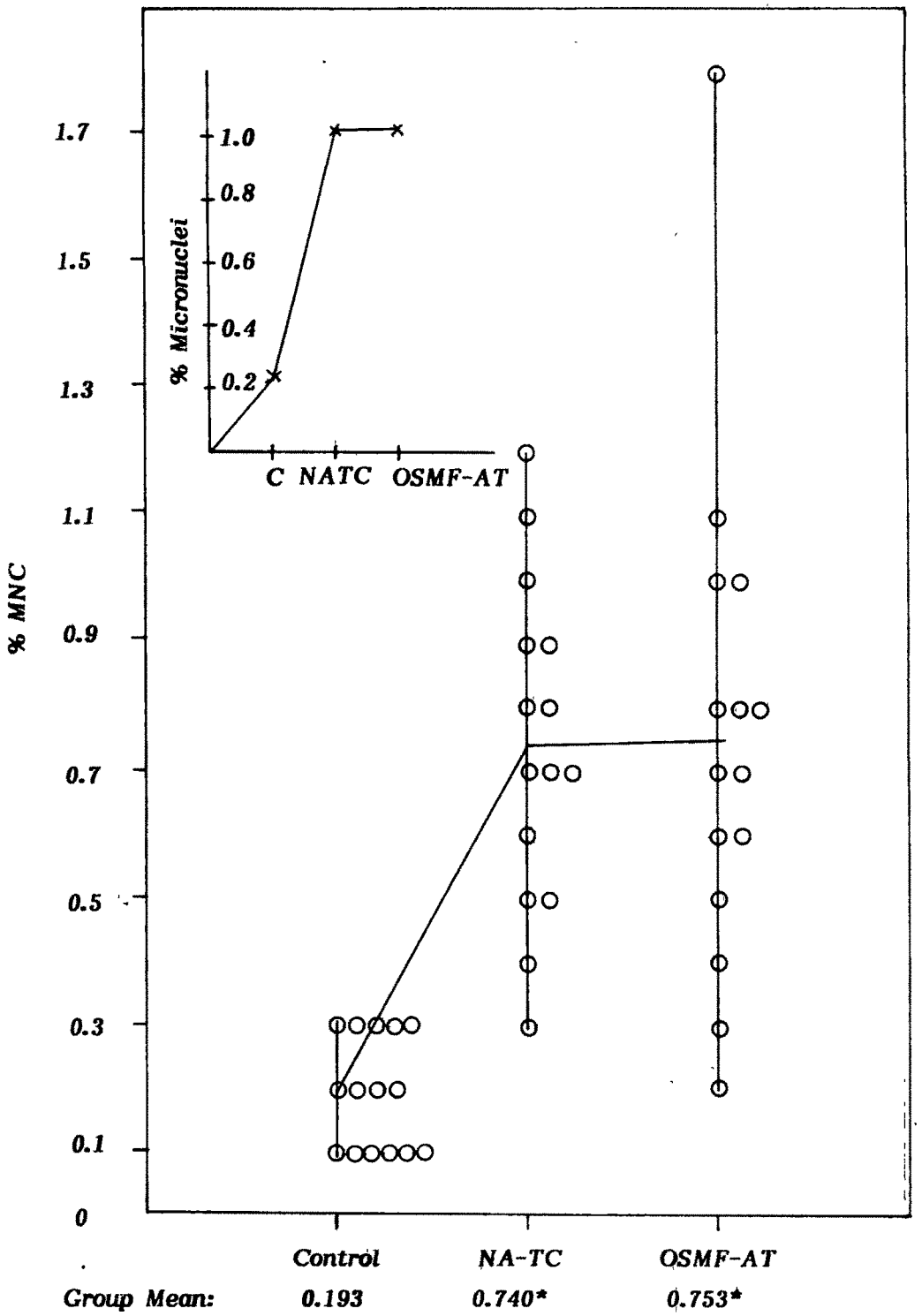
Comparison of cellular kinetics in different groups of areca nut plus tobacco chewers and controls

Group	MI (%)	MII (%)	MIII (%)	Mean AGT (h)±S.E.
Controls	58.9	32.2	8.9	32.68±1.774
NA-TC	51.4	35.9	12.7	30.48±1.346
OSMF-AT	68.8	25.5	5.7	36.36 ± 1.693^{c}
OC-AT	71.8	23.7	4.5	$37.56\pm1.568^{a,b}$

a - $p<0.05$ when compared to controls
b - $p<0.01$ when compared to NA-TC.
c - $p<0.02$ when compared to NA-TC.

Fig. 17

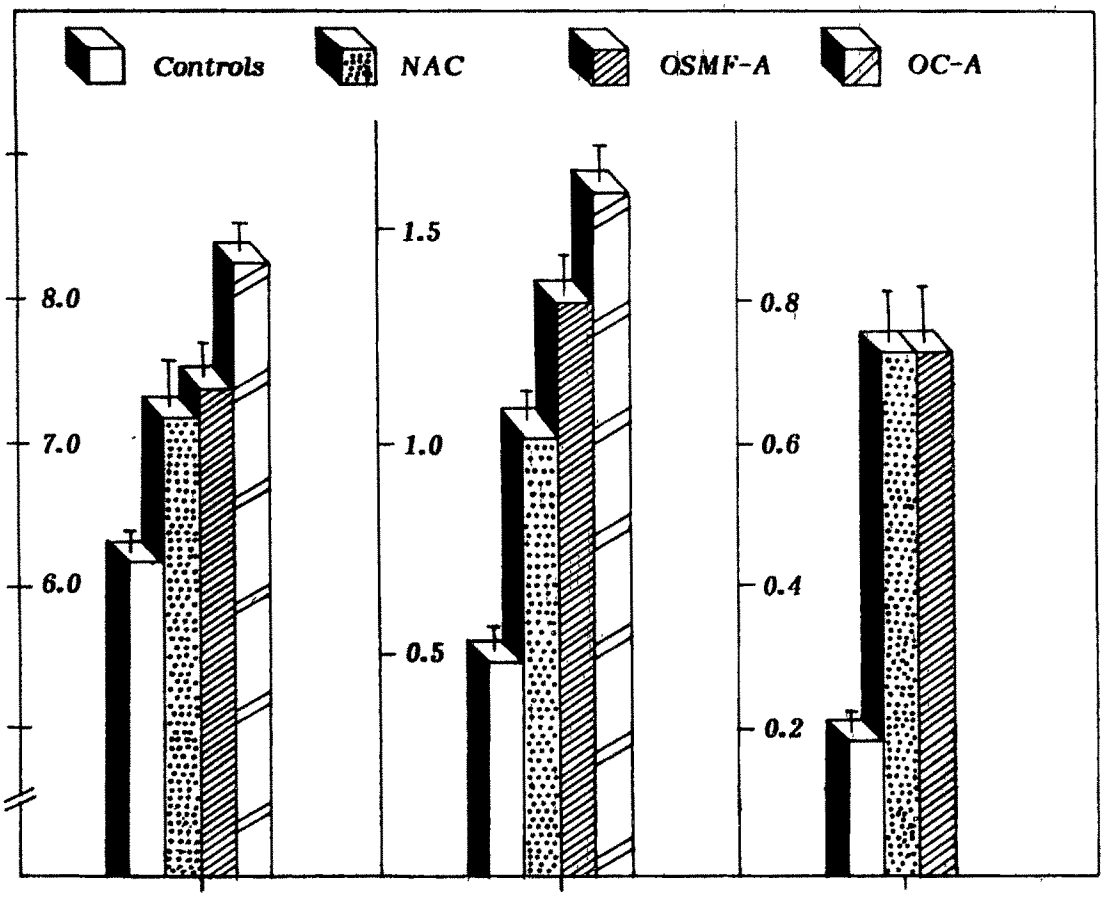
Frequency of % MNC and % Micronuclei among Controls and Arecanut+ Tobacco chewers



* $p<0.001$

Fig. 18

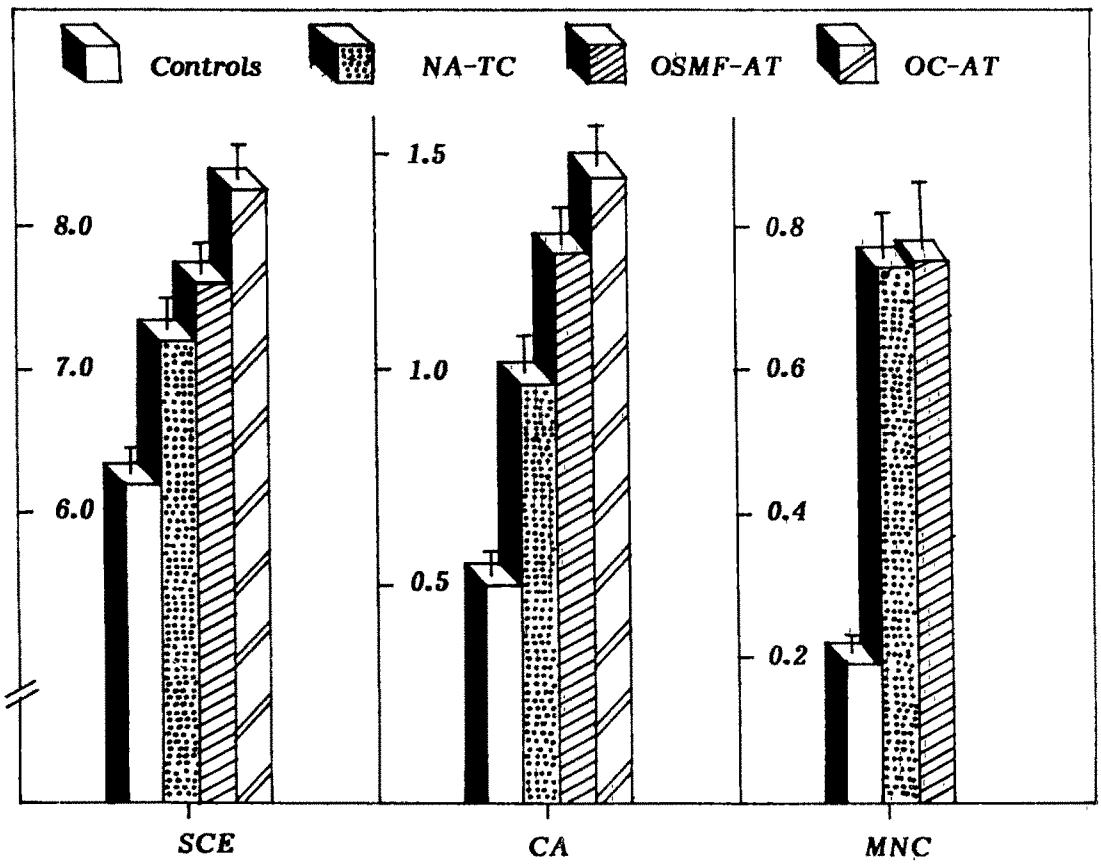
Comprehensive data of group mean values (± S.E.) of the cytogenetic endpoints in controls and areca nut chewers



	SCE	CA	MNC
Control	6.185	0.050	0.193
NAC	7.218	0.105	0.730
OSMF-A	7.404	0.136	0.730
OCA	8.261	0.158	-

Fig. 19

Comprehensive data of group mean values (± S.E.) of the cytogenetic endpoints in controls and areca nut + tobacco chewers



	SCE	CA	MNC
Control	6.185	0.050	0.193
NA-TC	7.219	0.097	0.740
OSMF-AT	7.617	0.127	0.753
OC-AT	8.500	0.144	-

Table-36

Statistical appraisal of mean values for cytogenetic endpoints within different groups (p'value)

Groups	SCE	CA	MN
Controls Vs NAC	<0.01	<0.001 (0.01)	<0.001
Controls Vs OSMF-A	<0.001	<0.001 (0.001)	<0.001
Controls Vs OC-A	<0.001	<0.001 (0.001)	na
NAC Vs OSMF-A	NS	<0.02 NS	NS
NAC Vs OC-A	<0.01	<0.001 (0.02)	na
OSMF-A Vs OC-A	NS	NS (NS)	na
Controls Vs NA-TC	<0.001	<0.001 (0.01)	<0.001
Controls Vs OSMF-AT	<0.001	<0.001 (0.001)	<0.001
Controls Vs OC-AT	<0.001	<0.001 (0.01)	na
NA-TC Vs OSMF-AT	NS	< 0.02 (NS)	NS
NA-TC Vs OC-AT	<0.001	<0.001 (NS)	na
OSMF-AT Vs OC-AT	<0.01	NS (NS)	na

NS = Not Significant na = not applicable

Parentheses indicate 'p' value excluding gaps.

Illustrations 34-36 *Human lymphocytes differentiated in MI, MII and MIII with the help of FPG staining.*

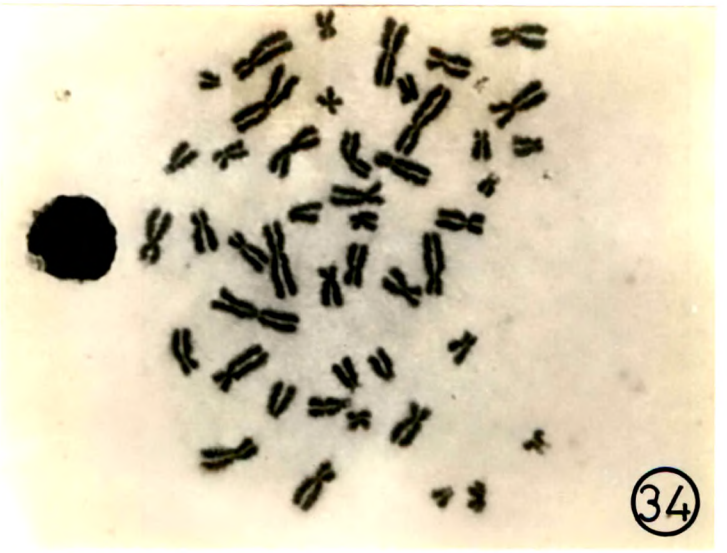
34 *An MI metaphase with both the chromatids darkly stained.*

35 *A metaphase in MII with differentially stained chromatids.*

36 *A metaphase in MIII.*

Magnifications: 34-35 *1400x*

36 *1800x*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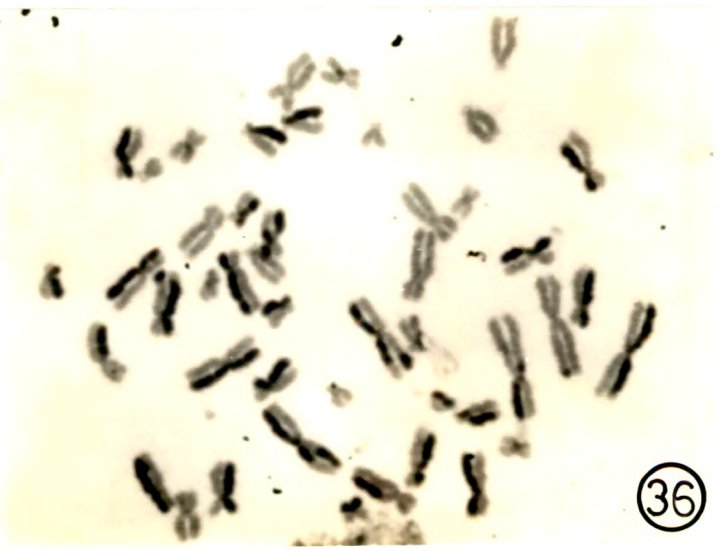
35


36

Illustrations 37-39 *Metaphase chromosomes from human lymphocytes stained by FPG technique for detection of SCEs.*

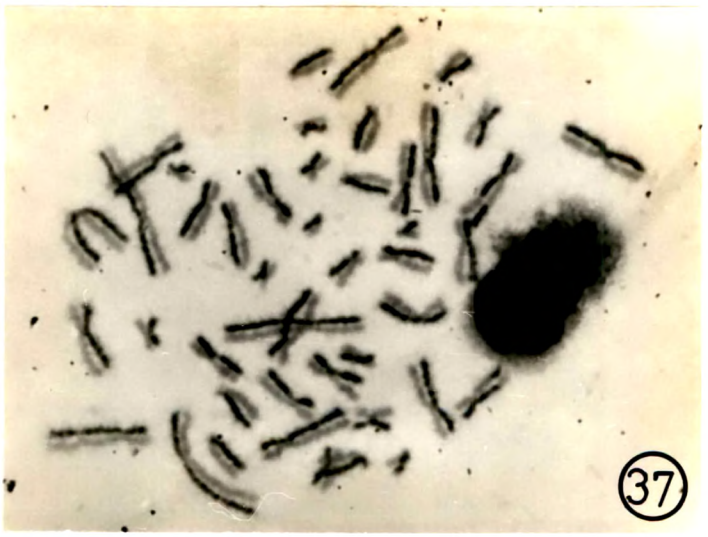
37 *A metaphase from a control subject showing only 2 instances of SCEs.*

38-39 *Chromosomes of areca nut chewers exhibiting more than 9 instances of SCEs.*

Magnification: 37 *1600x*

38 *1400x*

39 *1800x*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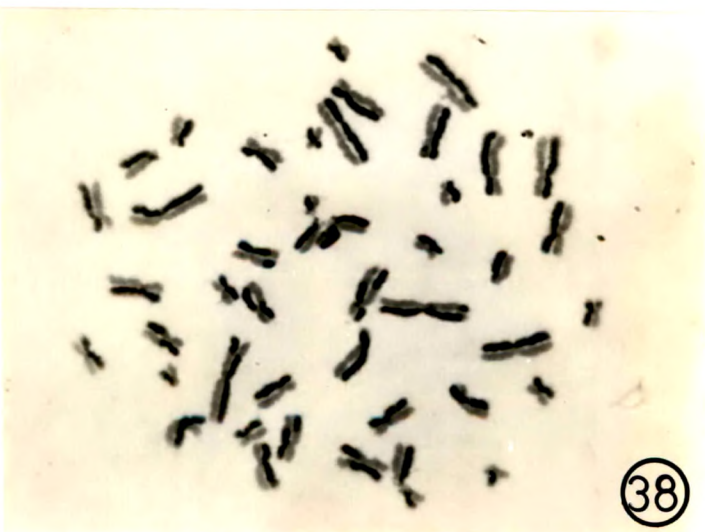
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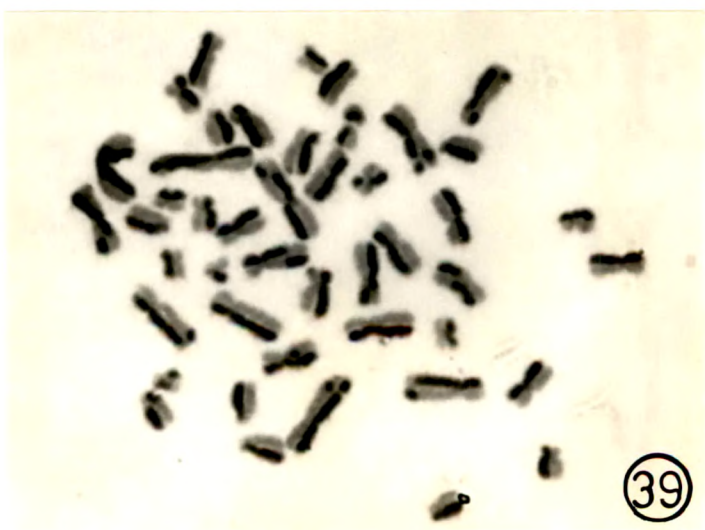
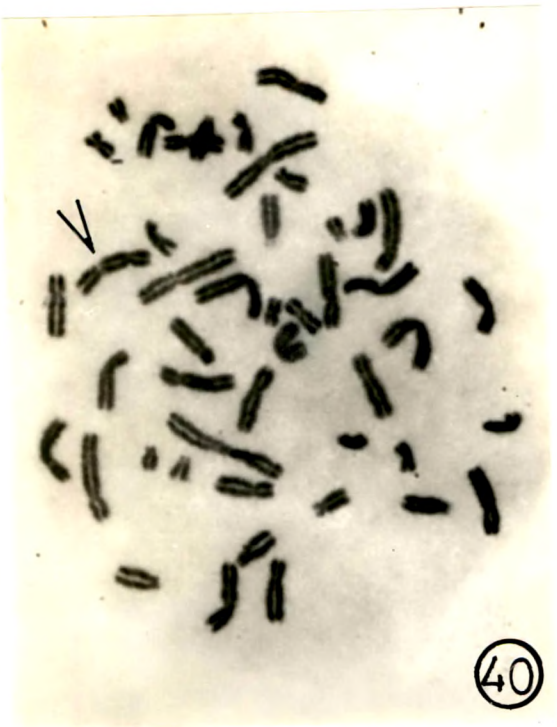

39

Illustrations *40-51* *Photomicrographs depicting chromosome aberrations observed in lymphocytes of areca nut chewers as well as areca nut + tobacco chewers.*

40-43 *Metaphases showing chromatid and isochromatid gaps (arrows)*

Magnifications: *40* *1800x*

41-43 *1600x*



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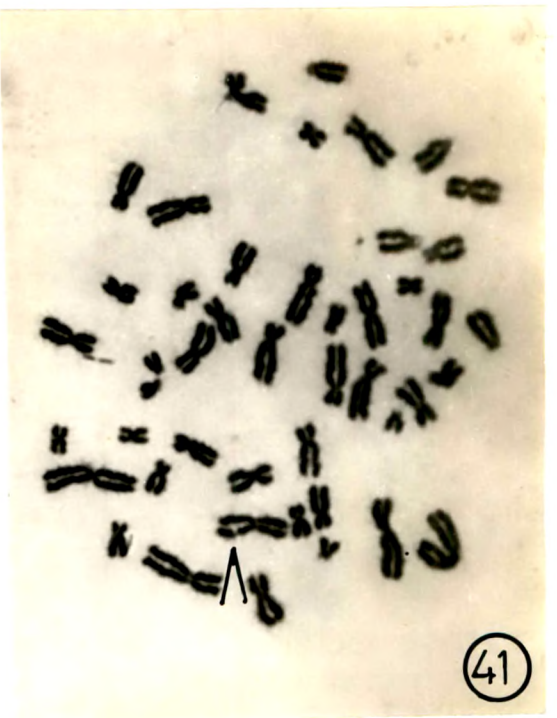

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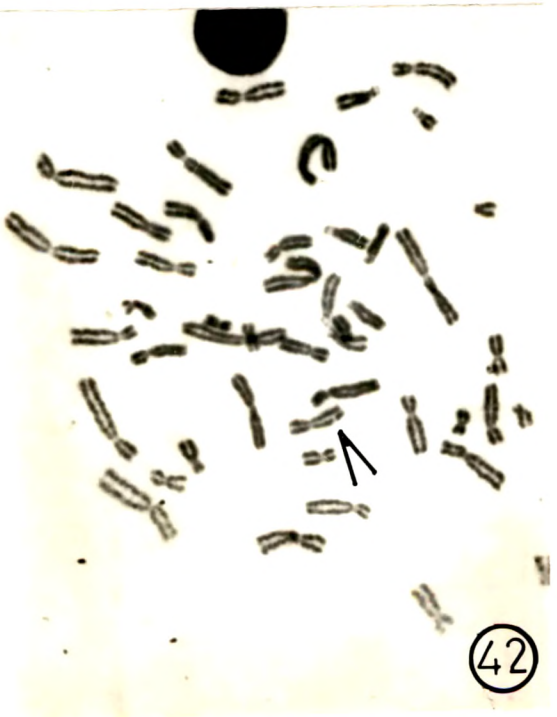

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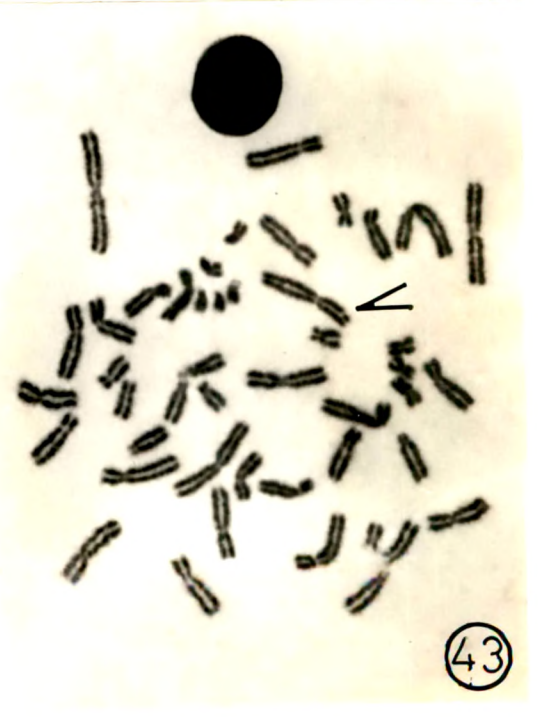
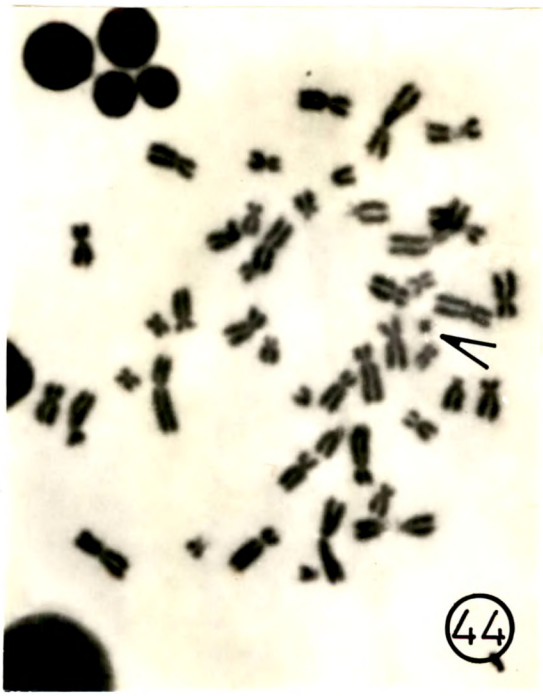


43

Illustrations 44-47 *Arrows pointing towards acentric fragments and interchanges.*

Magnification: *1800x*



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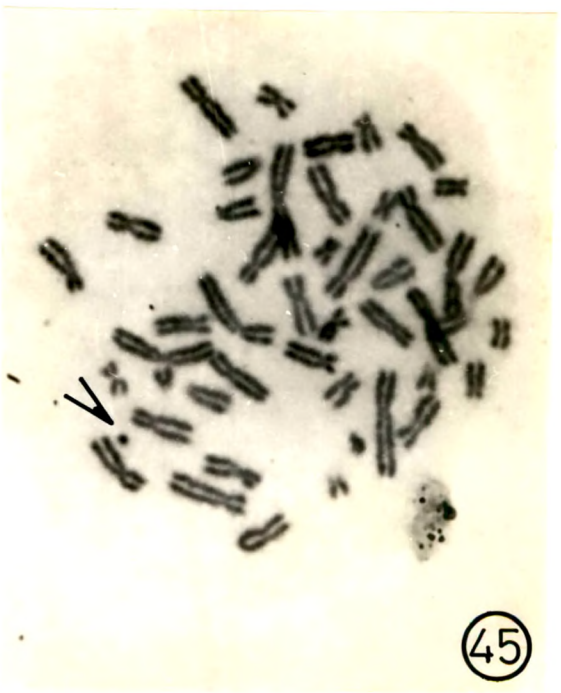

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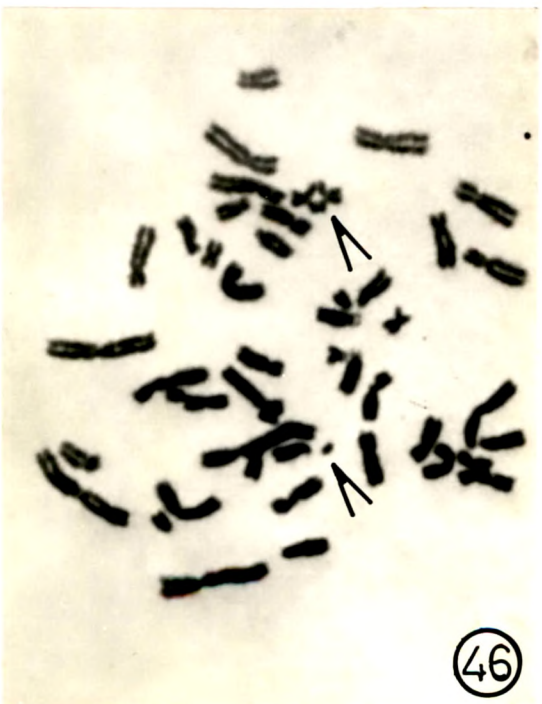

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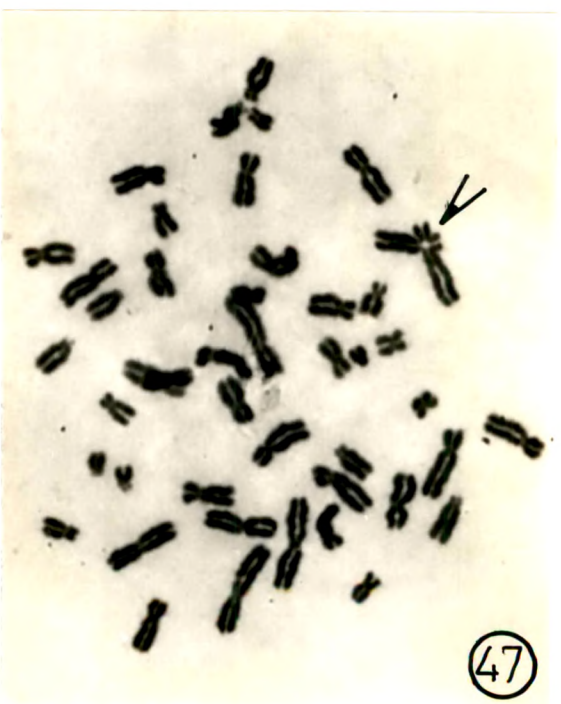


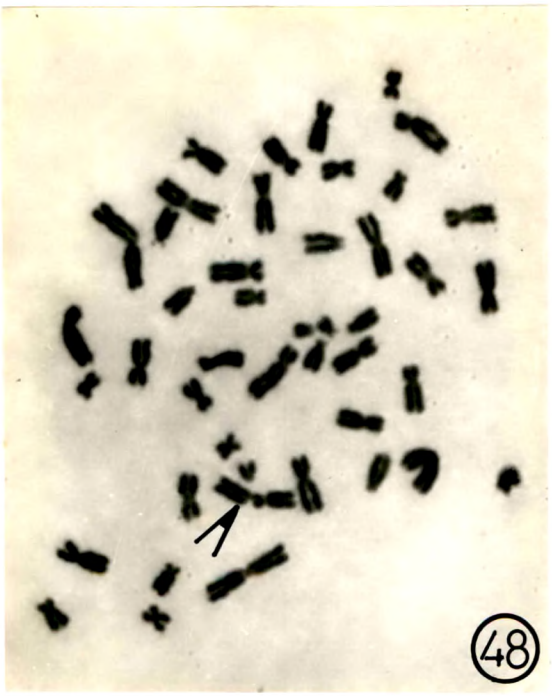
47

Illustrations *48-50* *Lymphocyte chromosomes with chromatid breaks indicated by arrows.*

51 *Metaphase from lymphocytes of a patient with the habit of chewing ONLY areca nut and suffering from oral cancer. Note the extensive chromosomal damage in the cell.*

Magnifications : 48-50 *1800x*

51 *1600x*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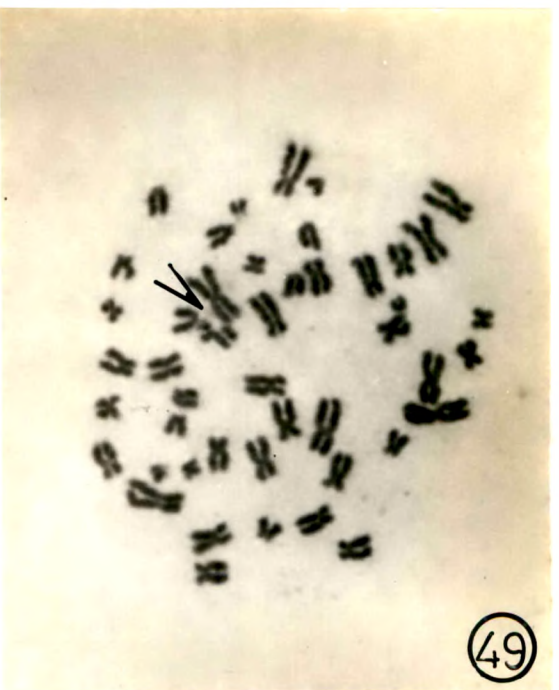
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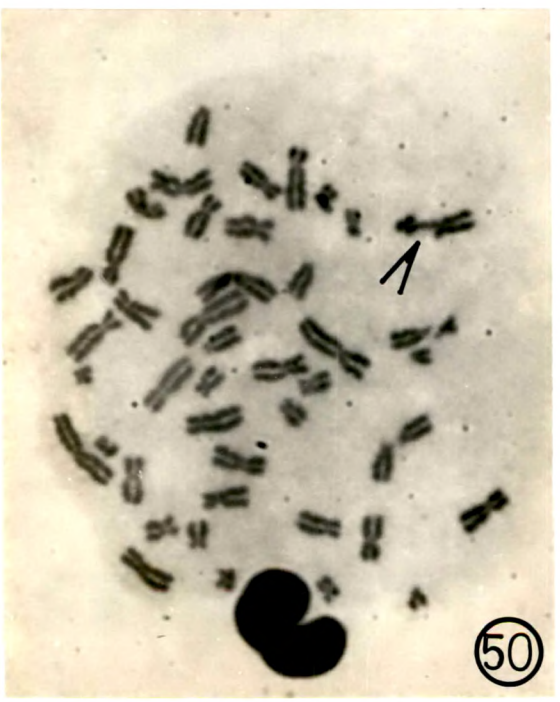
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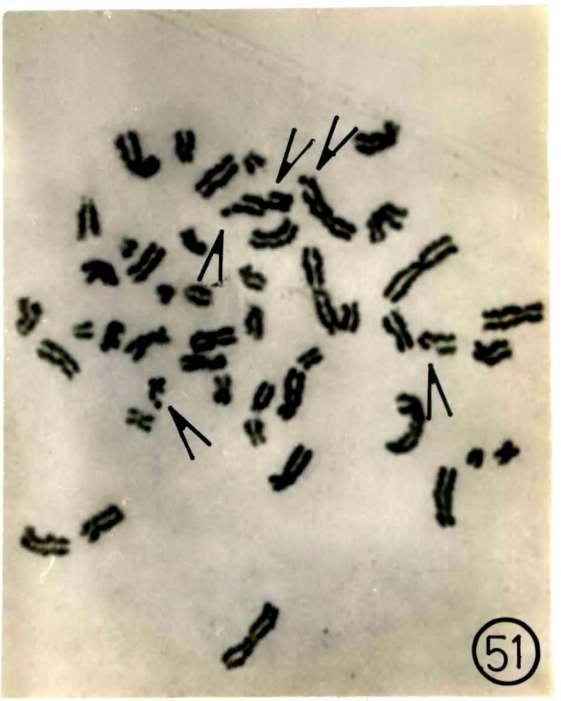
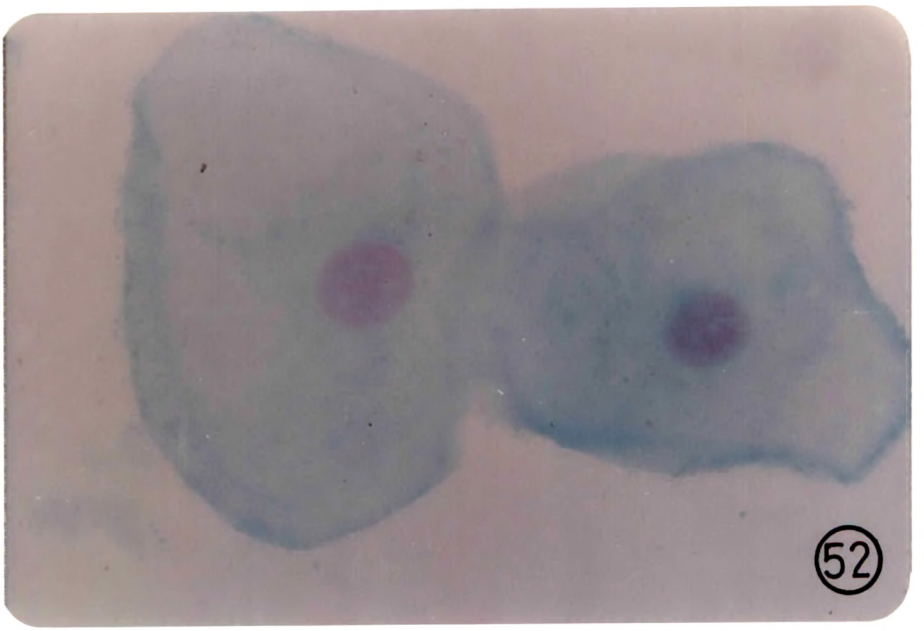

51

Illustrations 52-53 *Exfoliated cells from buccal mucosa stained with Feulgen plus Fast green.*

52 *Normal cells without micronucleus.*

53 *A cell with two micronuclei together with some normal cells.*

Magnification: *875x*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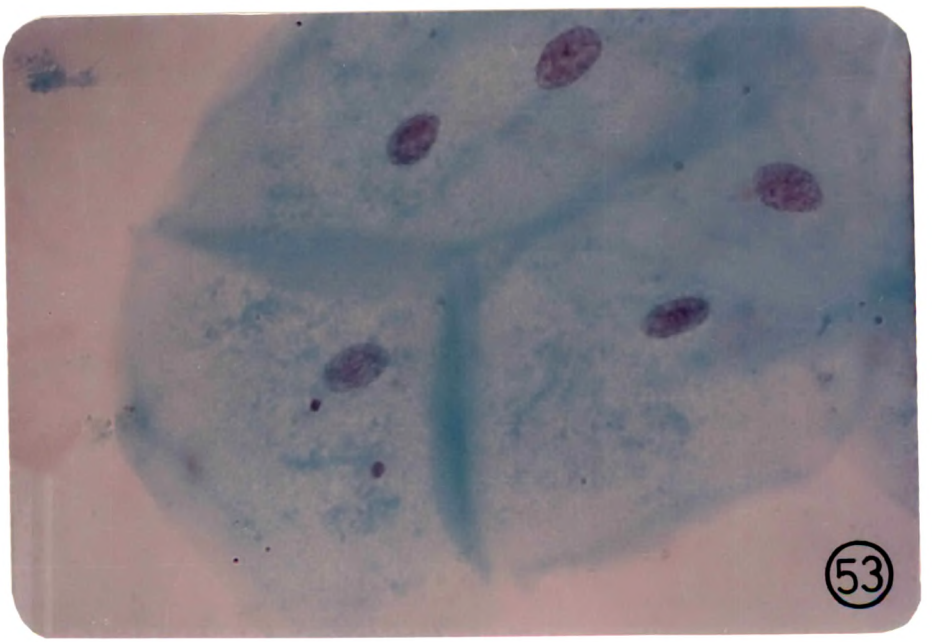
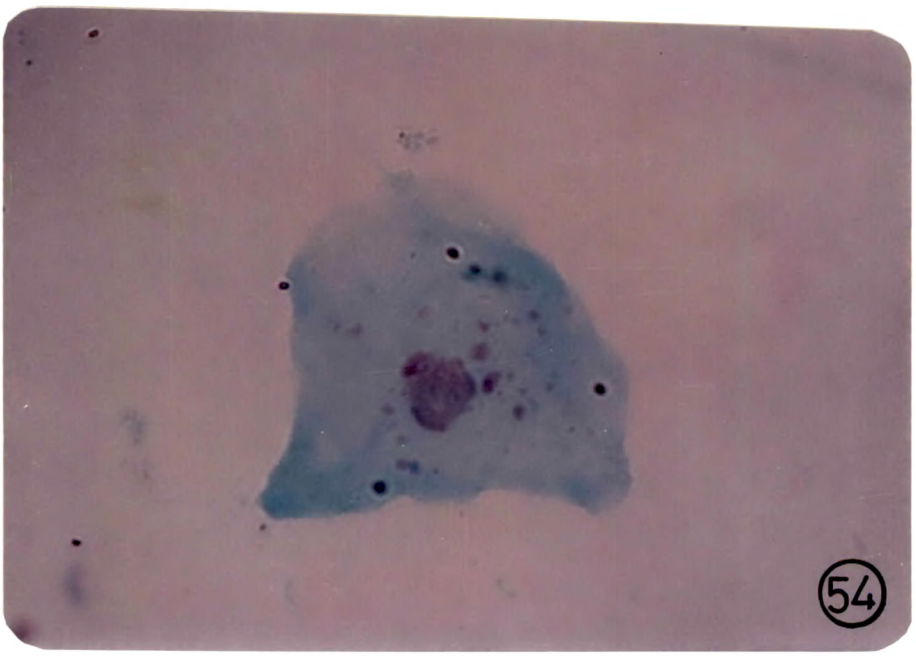

53

Illustrations 54-55 *Exfoliated cells not considered while scoring % MNC.*

54 *A cell with several micronuclei attributed to spindle disturbance.*

55 *Buccal mucosa cells collected from a patient suffering from oral cancer.*

Magnification: *875x*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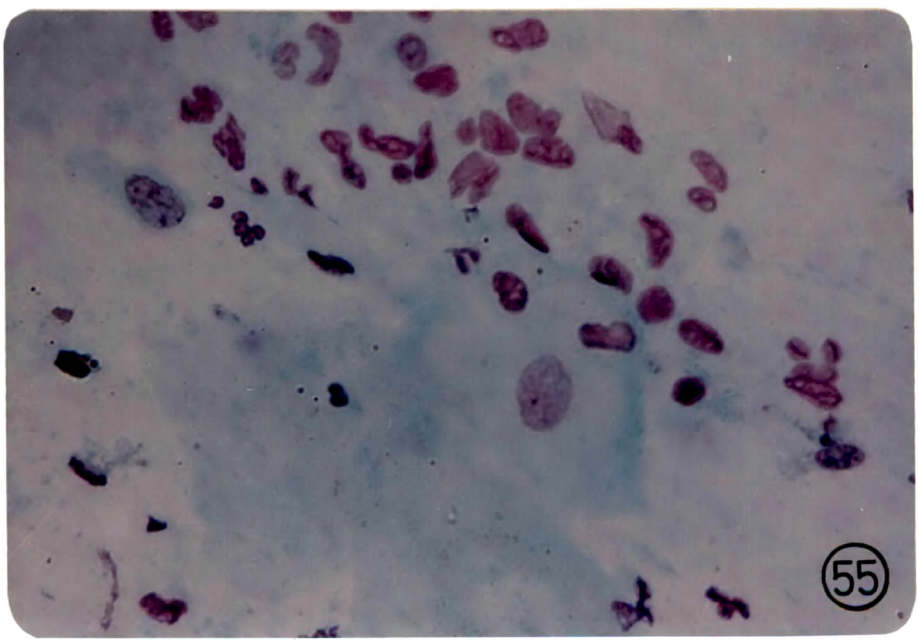
55

controls for all the cytogenetic endpoints.

DISCUSSION

The causal relationship of chewing betel quid with tobacco and oral cancer has been well documented (IARC, 1985). Tobacco and areca nut alkaloids have been detected in the saliva and urine samples of tobacco-areca nut chewers (Nair et al., 1985; Wenke et al., 1984b). It has been suggested that tobacco specific nitrosamines play a major role in the etiology of oral cancers (Hoffmann and Hecht, 1985; Nair et al., 1985; Bhide et al., 1986). But recently there have been reports indicating that apart from the preformed N-nitroso compounds in betel quid with or without tobacco, a substantial fraction of N-nitroso compounds are synthesized in vivo in the oral cavity (Nair et al., 1987). Numerous recent reports prove that saliva of betel quid chewers contain nitrosamines derived from areca nut alkaloids (Wenke et al., 1984b; Nair et al., 1985; 1987; Prokopczyk et al., 1987b). Traces of MNPN, which is a powerful carcinogen in F344 rats (Wenke et al., 1984a), have been reported in saliva of betel quid chewers without tobacco and it showed organ specific carcinogenic activity (Prokopczyk et al., 1987a). Investigations regarding its mechanism of action have been reported to be in progress (Prokopczyk et al., 1987b; 1988b). Stich and Anders (1989) have recently reported the appearance of N-nitrosoguvacine as well as relatively large amounts of tannins in the saliva of betel quid chewers. They observed that these areca nut specific compounds appear in the saliva within a few minutes of chewing. The saliva was found to be genotoxic. The effects of several areca nut specific N-nitroso compounds have been investigated on cultured human epithelial cells (Sundquist et al., 1987) and it has been suggested that induction of cyto and genotoxic effects of areca nut specific compounds may be of importance

for understanding the relationship between areca nut chewing and carcinogenesis in human oral epithelium.

We have attempted to quantify the extent of DNA damage caused by chronic, low level in vivo exposure of areca nut and areca nut plus tobacco, with the help of cytogenetic endpoints, utilizing PBLs, the non-target tissue and exfoliated buccal mucosa cells, the target tissue. The findings revealed a positive genotoxic action of areca nut in both the tissues.

The PBLs comprise of two major cell types, depending on their origin and function, namely T and B cells. Our analysis is based on T lymphocytes. Now, most T lymphocytes belong to the redistributional pool i.e. they leave blood, pass through the different organs and re-enter the peripheral blood. Thus, lymphocytes exposed to a mutagen anywhere in the body can eventually occur in the peripheral blood. Majority of lymphocytes are in G_0 stage of the cell cycle i.e. with unreplicated chromosomes. Thus, lymphocytes of exposed persons may spend even years, circulating in the body before they are stimulated in vitro, which allows a considerable time for cellular repair of the lesions. Only the few unrepaired lesions that remain, can be expressed as CAs or SCEs when they pass through S-phase in vitro. It is to be emphasized that in the present study since we are dealing with the low dose chronic exposure of a potential genotoxic agent, the DNA damage observed, is a product of two competing processes, prolonged induction of lesions and their continual repair.

SCEs are efficiently induced by those substances that form covalent adducts to the DNA or interfere with DNA precursor metabolism or repair (Perry and Evans, 1975; Wolff, 1977; Perry, 1980; Latt et al., 1981; Natarajan et al., 1981; Carrano and Thompson, 1982; Abe and Sasaki, 1982). Recently, haemoglobin adducts of arecoline, the major areca nut alkaloid have been reported (Prokopczyk

et al., 1988a). Increased lymphocytic SCE rates among individuals chewing tobacco and areca nut have been reported by us previously (Adhvaryu et al., 1986; 1988a) and among chewers of betel quid with and without tobacco by Ghosh and Ghosh (1984; 1988). In the present study we have also carried out studies among ONLY areca nut chewers. The elevation of SCE frequency, observed in them, can be attributed to the habit of chewing areca nut. In the AN+T chewers it may be a combined effect of areca nut as well as tobacco. The information gathered from the in vitro studies employing CHO cells (Part I), as well as the available epidemiological data, suggest that the combination of areca nut and tobacco provides potentiation to their carcinogenic properties. In the present study, SCE values in those chewing only areca nut and in individuals chewing areca nut with tobacco, remained comparable. The lack of difference in the lymphocytic SCEs among NAC and NA-TC may be due to the possibility that tobacco-areca nut chewers spit out the juice periodically, whereas, areca nut, when chewed without tobacco, is consumed in toto. This might increase the body burden of tannins as well as alkaloids and the in vivo formation of nitrosamines from these alkaloids. The genotoxic effects of the saliva of the chewers have been reported (Stich and Stich, 1982; Stich and Anders, 1989). Tannins were also found to induce SCEs in human lymphocytes (Morimoto and Wolff, 1980).

The elevation of SCE frequency in the OSMF patients as well as in oral cancer patients may be due to the effect of the habit or the occurrence of the disease, or a combined effect of both. SCE elevations have been reported in patients with precancerous conditions (Adhvaryu et al., 1986; Murthy et al., 1986; 1988). Increase in SCE frequency in many types of cancers including oral cancer has been reported from our laboratory as well as by other investigators (Skolnick et al., 1980; Mitra et al., 1982; Raposa, 1978; Kurvinik et al., 1978; Slavutsky

et al., 1984; Adhvaryu et al., 1988a,b,c,d). Findings contrary to these have also been reported for many cancers including oral cancer (Bazopoulou-kyrkanidou et al., 1986; Husum et al., 1981; Adhvaryu et al., 1985; Crossen et al., 1981; Hollaender et al., 1978; Cheng et al., 1979). Hence, the exact reason for additional SCE elevations in oral cancer patients remains to be determined.

CAs are still considered to be the most unambiguous cytogenetic manifestation of genotoxicity (Evans, 1982) as the mechanisms involved in their formation have been clearly delineated. Exposures to physical or chemical agents can lead to somatic or gametic mutations in human populations. Since there is no efficient and easy technique to study the frequencies of induced point mutations in humans, one has to rely on induced CAs to assess a possible hazard (Natarajan and Obe, 1980). Though in the in vitro studies, most of the chemicals induce chromatid type aberrations, in exposed populations, both, chromosome and chromatid type aberrations may be found to be elevated (Obe and Beek, 1982). Increased CAs have been witnessed in lymphocytes of alcoholics and smokers (Obe et al., 1980; Vijayalaxmi and Evans, 1982). However, to the best of our knowledge CAs in areca nut and tobacco chewers have not been reported so far. A statistically significant elevation of CAs was found among areca nut chewers. As already discussed in the previous chapter, clastogenic action of the aqueous extract of areca nut as well as the areca nut alkaloids have been observed by us and some other investigators. Thus the chromosome breaking ability of areca nut derived alkaloids, polyphenols/tannins and nitrosamines may be jointly responsible for chromosome breakage observed among the areca nut chewers. Nicotine as well as aqueous tobacco extract possess the chromosome damaging capacity (our observation). Hence, in AN+T chewers the clastogenic action may be assigned to both, areca nut as well as tobacco.

Stich and Stich (1982) have observed the chromosome damaging activity of the saliva of areca nut and tobacco chewers in CHO cells. As explained earlier, only the areca nut chewers swallow the saliva completely, whereas, the AN+T chewers expectorate it periodically. This, probably, might be the reason for the analogous CAs observed in both the groups. The potentiating effect was not evidenced in lymphocytic CAs. The swallowing of the saliva has been accused for the increased rate of oesophageal cancers among these individuals (Shanta and Krishnamurthi, 1963; Jayant et al., 1977; Jussawalla, 1981).

As observed in our in vitro studies on CHO cells, chromatid type of damage is more frequently observed in the lymphocytes, however, almost all types of chromatid and chromosome aberrations were doubled among the NAC and NA-TC compared to the controls. This signifies that CA can provide a useful index in estimating in vivo DNA damage caused by areca nut chewing. Talaska et al. (1987) have found a correlation between DNA adducts and CAs which may lead to neoplasia. Mutation and aberration, under appropriate circumstances, can allow a cell to escape the growth controls and become a neoplastic cell (Tabin et al., 1982). Lymphocytic CAs were found to increase in precancerous conditions (Murthy et al., 1985; 1988). It is clear from the normal chewers that the habit of chewing areca nut alone or with tobacco, exhibits a chromosome damaging capacity in the lymphocytes which further increases in OSMF and OC patients.

The chewing of areca nut/tobacco is causally associated with cancer of oral cavity, however, the increase in CA and SCE frequencies in the PBLs, signifies that areca nut might have some tumour enhancing activity in other organs also. Bhide et al. (1979) have shown that areca nut extract can induce liver neoplasms in mice. The enhancing effects of dietary administration of areca nut on hepato-carcinogenesis and the upper digestive tract, was observed by Tanaka et al. (1986).

Areca nut with calcium hydroxide causes epithelial thickening of the upper digestive tract in rats (Mori et al., 1979). This is accelerated in vitamin A deficiency states (Tanaka et al., 1983). Rao (1984) described the modifying influence of areca nut ingredients on benzo(a)pyrene induced carcinogenesis. The promoting activity of areca nut has also been observed (Stich and Tsang, 1989; Stich and Anders, 1989). This suggests that areca nut can potentiate the action of other carcinogens. Thus, with the DNA damage observed in the lymphocytes, it can be presumed that areca nut may also act as an enhancer where damage due to other carcinogen has already occurred.

It has been suggested that the alkaloids of areca nut may influence the process of carcinogenesis by disturbing the immune surveillance (Rao and Das, 1989). It has also been accounted that administration of arecoline, resulted in suppression of immune responses in mice (Shahabuddin et al., 1980; Selvan and Rao, 1988). We could not observe a significant difference between the AGT of the controls and that of the normal chewers (NAC and NA-TC). A chronic low level exposure of areca nut, only marginally affected the AGT values in areca nut chewers which was not statistically significant. There was a wide individual variation, hence, the change in AGT can hardly be assigned to the habit of chewing areca nut alone. The lower mean AGT value among the NA-TC than the controls, further justifies the assumption that the chewing habit does not have any bearing on the AGT in lymphocytes of normal individuals with areca nut chewing habit (NAC and NA-TC).

In patients with oral precancerous condition, impaired cellular immunity has been reported (Abrol, 1977; Sharma et al., 1983; Pillai et al., 1987). Oral cancer patients show impaired cell mediated immune competence, exhibited as suppressed lymphocytic response to PHA stimulated cell proliferation (Bazopoulou-kyrkanidou

et al., 1983). The lymphocytes of the oral cancer patients show a slower proliferation capacity (Rao et al., 1985; Nair et al., 1978; Sarnath et al., 1985; Scully, 1982). The higher AGT observed in OSMF and Oral cancer patients (with either habit i.e. areca nut alone or with tobacco) in the present series can therefore be assigned to a poorer lymphocytic response to PHA among these patients.

The target organ to the genotoxic action of areca nut is the oral mucosa. The problem of obtaining a dividing cell population from human epithelial tissue to observe genotoxicity in the target organ, has been resolved by adapting the MN test to the human exfoliated cells which can easily be collected in a noninvasive manner. Micronuclei are simple markers which can be routinely examined in cytological preparations. The origin of MN is well understood. A correlation exists between carcinogenicity and induction of MN and they are relatively easy to score. These reasons have stimulated the application of MN test in exfoliated cells of the putative target tissues.

The mean frequency of percent MNC in the controls was 0.19 which was comparable to that observed by Sarto et al. (1987). This frequency differed from that observed by Stich and Rosin (1984). The reason may be that we have excluded pyknotic and karyorhexic nuclei. Also the MN resulting due to spindle disturbances were excluded. The percent micronucleated cells as well as percent micronuclei were found to increase significantly among NAC and NA-TC. The release of tobacco and areca nut alkaloids and tannins during chewing and also the formation of N-nitroso compounds in the saliva, together with increased nitrite and thiocynate levels (Wenke et al., 1984b; Nair et al., 1985; 1987; Sipahimalani et al., 1984; Shivapurkar et al., 1980a; Stich and Anders, 1989) may contribute to the increased MNC frequency. The capacity of areca nut alkaloids to induce MN in CHO cells has been discussed in the previous chapter. The saliva of betel

quid chewers with tobacco and also areca nut chewers was found to be clastogenic in CHO cells (Stich and Stich, 1982). Increased MNC frequency has been observed among areca nut chewers as well as tobacco chewers and a more severe effect has been found among the chewers of betel quid containing tobacco (Stich et al., 1982; 1986). The MNC frequency in the group of individuals either chewing areca nut alone or areca nut plus tobacco, showed three times higher values than the controls ($p<0.001$). We failed to observe a synergistic effect of tobacco and areca nut by analysing the MN frequency. Sarto et al. (1987) and Stich and Rosin (1983a) observed that alcohol consumption did not significantly affect MN frequency in smokers.

In precancerous conditions the MNC frequency remained analogous to the chewers. In using the MN test, one should bear in mind that an increase in the frequencies of MN does not necessarily indicate the formation of preneoplastic lesions or carcinomas (Stich and Rosin, 1984). The MN is an internal index of exposure to carcinogen (Stich and Rosin, 1983b). Thus, elevated frequencies of MNC reveal the genotoxic action of areca nut (and tobacco when consumed together) to which the mucosa is directly exposed.

Almost all the reviews regarding SCE and CA have stressed the need of employing both the parameters jointly (Abe and Sasaki, 1977; Gebhart, 1981; Carrano and Natarajan, 1988 Das, 1988). SCEs and CAs are produced by different mechanisms and hence, both are complementary and should be considered together in studying individuals exposed to potential genotoxins. Our results detailing the degree of significance observed after a statistical appraisal of the data in different groups (Table-36) strongly justify the suggestion of combining the two assays. Coupled together, both the endpoints used to estimate the genetic damage inflicted by

the habit in the non-target tissue, could clearly determine the deleterious effect among normal chewers, chewers with premalignant conditions and chewers with oral cancer. Further, MN assay, being less demanding and providing vital information about the genotoxic effect on the target tissue, proved to be beneficial and could efficiently distinguish chewers from nonchewers. Thus, a combined application of these endpoints could prove to be highly sensitive indicators in quantitating the genetic injury in human beings.

While accepting the carcinogenic potentials of areca nut, the fact remains that considerable heterogeneity exists among individuals regarding the response to a common insult and, hence, interpretations should be made cautiously. However, interindividual differences observed in different markers may also reflect the variation in the risk of developing malignancy, and long term follow up studies of these individuals only may resolve the enigma.

SUMMARY

1. *The increase in the DNA damage, as estimated by SCE and CA frequency in PBLs of the individuals consuming only areca nut, signifies the in vivo genotoxic effects of areca nut on the nontarget tissue. This indicates the possible role of areca nut in elevating the risk of causation of cancers at sites other than oral cavity.*

2. *Increased MN frequency in the exfoliated buccal mucosa cells of areca nut chewers indicates the in vivo clastogenicity of areca nut on the target tissue.*

3. *Analogous results in individuals consuming areca nut alone (always consumed in toto) and areca nut with tobacco (saliva partly expectorated due to the presence of tobacco) points to the fact that areca nut in combination with tobacco also remains equally genotoxic.*

4. *A battery of cytogenetic endpoints may prove beneficial in surveillance of habit induced diseases.*

5. *Contemplating the in vivo DNA damage, henceforth, in cytogenetic monitoring at population level, the habit of chewing areca nut should also be taken into account, and be considered equally harmful as tobacco.*


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