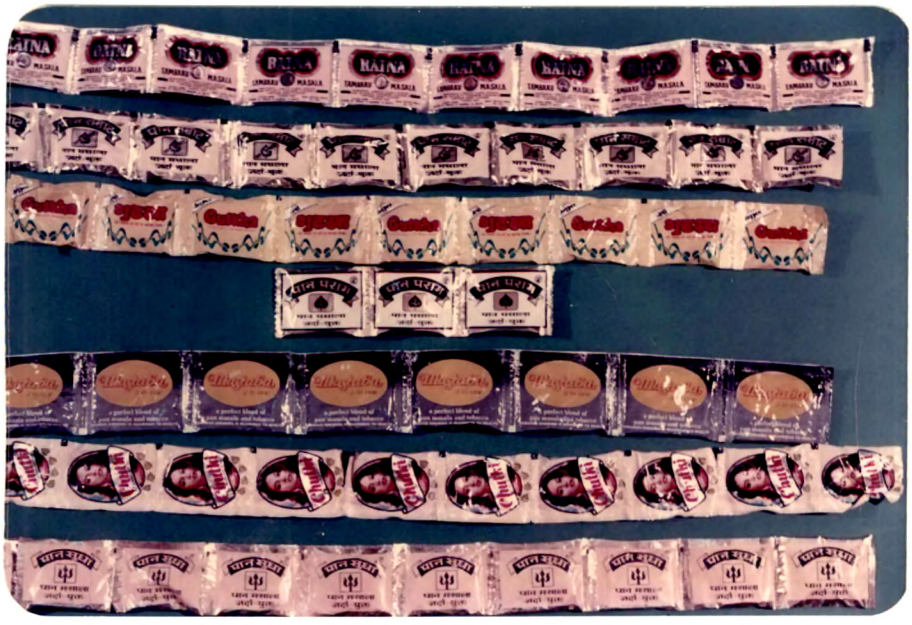
Part - III
Evaluation of
Pan Masala with Zarda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Tobacco has been identified as a major avoidable cause of human morbidity and mortality. Epidemiological studies reveal a close correlation between the manner of tobacco consumption and the development of site specific cancers. In spite of knowing the harmful effects, once initiated, the tobacco consumers find it difficult to give up the habits. Addiction to nicotine, which is the major alkaloid of tobacco, is the prime reason for its habitual use. Secondly, the availibility of various tobacco products in the market provides choice to the consumer. **Pan masala with 'zarda' (PM-T)** is the latest tobacco containing product put forward by the tobacco industry (Illustration 28). It has become highly popular as an alternative to tobacco chewing. As mentioned on its packing, **PM-T** is a dry mixture of tobacco (zarda), areca nut, catechu, lime, spices and flavours.

'**Zarda**' is prepared by cutting tobacco leaves into small pieces, boiling them in water with lime and spices until evaporation, followed by drying and colouring with vegetable dyes (IARC, 1985). It has been documented that compared to unprocessed variety, the processed tobacco contains very high concentrations of **TSNAs** (Tricker and Preussmann, 1988). An increase in the frequency of micronuclei in **CHO** cells, following the treatment with an aqueous extract of zarda as well as with saliva collected from the users of zarda has also been reported (Stich, 1986).

Several epidemiological studies have revealed an

association between the **areca nut** consumption and occurrence of premalignant and malignant oral diseases (Gupta et al., 1980; IARC, 1985; Mehta et al., 1972). Significantly higher frequency of **MNC** in exfoliated buccal mucosa as well as **CA** and **SCE** frequencies in the lymphocytes of chewers of areca nut per se (Dave, 1990; Stich et al., 1982), as well as in chewers of tobacco with areca nut (Adhvaryu et al., 1991) have been reported. The clastogenicity of the saliva of areca nut chewers to **CHO** cells has also been reported (Stich and Stich, 1982). Several animal experiments have provided evidence of the carcinogenicity of areca nut extracts and its tannins (Shirname et al., 1983; Shivapurkar et al., 1980; Stich and Tsang, 1989). Genotoxic effect of areca nut on **CHO** cells (Dave, 1990), mouse bone-marrow cells, V79 Chinese hamster cells (Shirname et al., 1984) and mouse lymphocytes (Panigrahi and Rao, 1986) have been documented. Some of the areca nut specific alkaloids have been found to possess the transforming (Ashby et al., 1979) and clastogenic properties (Dave, 1990; Panigrahi and Rao,1982; Stich et al., 1981; Wary and Sharan, 1988). Wenke and Hoffmann (1983) have reported conversion of these areca nut specific alkaloids to nitrosamines and some of them have even been detected in the saliva and urine of betel quid chewers (Nair et al., 1985; Wenke et al., 1984a).

Catechu, another ingredient of **PM-T**, contains 2-10 % catechin (IARC, 1983). Catechin is a plant phenolic flavonoid and has been shown to be non-toxic (Hennings,1981) and has

hepatoprotective effects (Lapis et al., 1986). Meneith (1990) demonstrated the ability of catechin to alter metabolic pathway of 2-acetyl-aminofluorene (**AAF**) in rat hepatocytes. Nagabhushan et al. (1988) observed that catechin inhibited mutagenicity of **B(a)P** or **DMBA**, and also inhibited the ***in vitro*** binding of ^{3}H-B(a)P metabolites to DNA, in a dose-depe-ndent manner. It has also been reported to inhibit mutagenicity of tobacco (Nagabhushan and Bhide, 1988).

Lime is prepared from sea shells or quarried limestone. Its precise role in the development of oral cancer remains a mystery. However, it causes local irritation to the mucosa and hyperplasia has been observed following the application of lime to the cheek pouch of hamsters (Dunham et al., 1966).

The details of **spices and flavours** used in the preparation of **PM-T** are not revealed by the manufacturers. However, the occasional use of synthetic flavours like musk ambrette and musk xylene, to improve the test and flavour of zarda, is well-known. Nair et al. (1985) have detected both of these agents in the saliva of chewers of betel quid with tobacco. Mutagenicity of these agents has also been reported in Salmonella/mammalian microsome test (Nair et al., 1986).

The quality of the basic ingredients mixed for preparing **PM-T** is also an important factor. Fungal infection, if any, to tobacco and/or areca nut, may affect the ultimate toxicity of the mixture. When the areca nuts are infected with Aspergillus flavus or Aspergillus niger (Borle and Gupta,

1987; Mahdihassan, 1981; Mahdihassan and Rabia, 1988), they are sure to contain aflatoxins, which have been known as potential carcinogens (IARC, 1976; 1982).

In view of the presence of an array of mutagenic and nonmutagenic constituents in **PM-T**, an investigation on its genotoxic potential is necessary for the safety evaluation of its consumption. DNA lesions form the initial steps in carcinogenesis. Short term assays like **MN test, CA analysis and SCE analysis** are the sensitive and well established cytogenetic markers of DNA damage. Hence, effects of **aqueous extract of PM-T (PM-T-ext.)** were tested on ***in vitro*** mammalian test system, employing different cytogenetic endpoints. For the purpose, **CHO** cells were preferred for having large sized small number of chromosomes and having a short propagation time.

In order to make the study more comprehensive, a parallel effort was made to determine the possible genotoxic effects of **PM-T** on individuals regularly consuming it. Screening of exfoliated buccal mucosa for the presence of **MNC** is a simple marker which indicates direct exposure to a DNA-damaging agent (Stich and Rosin, 1984). Moreover, the analysis of **CA** and **SCE** in **PBLs** are one of the most extensively employed indicators in population monitoring for determining the genotoxic effects in individuals exposed to mutagenic carcinogens (WHO, 1985; Carrano and Natarajan, 1988). Hence, analysis of **MNC** frequency in exfoliated buccal mucosa and **CA**

& SCE frequencies in PBLs was carried out to study the effects on **target and nontarget tissues** of individuals having a habit of chewing tobacco containing pan masala.

MATERIALS AND METHODS:

***In vitro* experiments:**

CHO cell line was used to study the effects of an aqueous extract of **PM-T**. The details of chemicals used, culture procedure and experimental steps were essentially the same as described in **part I**. An aqueous extract of **PM-T** (**PM-T-ext.**) was prepared following the procedure detailed in Part I for the preparation of tobacco extract. The extract represented 25 grams of **PM-T** in 225 ml of distilled water.

During all the experiments, an untreated culture was kept as a control and **MMC** treated culture was kept as a positive control. The concentrations of **PM-T-ext.** and treatment durations applied for the study were as under:

Experiment 1:

To select a suitable dose range for the ***in vitro*** short term genotoxicity assays, the probable cytotoxic effect of **PM-T-ext.** treatment on **CHO** cells was assessed by scoring % viable cells. The viability of the cells, following the continuous treatment with PM-T-ext., was studied by trypan blue dye exclusion test.

Concentrations: 5 µl, 10 µl, 20 µl per ml of GM

Durations : 24 hrs. and 48 hrs.

Experiment 2:

Successful operation of cell division depends on completion of properly sequenced macromolecule synthesizing events. Inhibition or alteration of any one of these will be reflected as abnormal cell division. In order to detect the effect of **PM-T-ext.** on mitotic activity, the **CHO** cells, grown on coverslip in Leighton tubes, were exposed continuously to different concentrations of the extract. The effects on cell division was examined in terms of mitotic index **(M.I.)**.

Concentrations: 2.5 µl, 5 µl, 10 µl, 20 µl and 50 µl per ml of GM

Treatment durations: 3, 6, 12, 24 and 36 hrs.

Experiment 3:

With a view to assess the nature of the damage caused by treatment with **PM-T-ext.** to mitotic apparatus, **CHO** cells were treated for a short duration and then, were allowed to recover in extract free GM, at 37^{o}C. M.I. was calculated at various time intervals to examine the ability of exposed cells to regain the normal mitotic activity.

Concentrations: 20 µl, 50 µl, 100 µl per ml of GM

Treatment duration: 6 hrs.

Recovery durations: 6, 12, 18, 24 and 30 hrs.

Experiment 4:

The detection of micronuclei in cultured mammalian cells offers a simple and rapid screening of chromosomal damage caused by genotoxic agents. As a first step in assessing

possible genotoxic potential of **PM-T**, the frequency of micronucleated cells **(% MNC)** was analysed from the cultures treated with the **PM-T-ext.** for 6 hrs. followed by a 12 hr. and a 24 hr. recovery in fresh GM.

Concentrations: 20 µl, 50 µl, 100 µl per ml of GM

Treatment duration: 6 hrs.

Recovery durations: 12 hrs. and 24 hrs.

Experiments 5: 6:

CA analysis in M-I stage cells is considered to be an extremely sensitive and one of the most unambiguous endpoint for determining chromosomal damage. It also serves to confirm the findings of micronucleus test. Analysis of **SCE** frequency in M-II stage cells, indicates breakage and misrepair of a chromosome following the exposure to genotoxic agents. Hence in order to learn about the possible effects of continuous exposure to **PM-T-ext.** on **CA frequency and SCE frequency** in mammalian cells ***in vitro***, **CHO** cells were treated continuously with different concentrations of the extract.

Concentrations: 2.5 µl, 5 µl, 10 µl, 20 µl per ml of GM

Treatment durations: 24 hrs. treatment for **CA** analysis and 48 hrs. treatment for **SCE** assay.

Experiments 7: 8:

The consequences of a short term treatment with **PM-T-ext.** followed by a recovery in extract free GM on **CA frequency and SCE frequency** were also checked.

Concentrations: 10 µl, 20 µl, 50 µl per ml of GM

Treatment duration: 3 hrs.

Recovery durations: 24 hrs. for CA analysis and 48 hrs. for SCE analysis.

Experiment 9:

The ability of genotoxic agents to interfere with DNA replication has long been recognized (Roberts, 1984). Hence, with the help of autoradiographic analysis of ^{3}H-thymidine incorporated interphase cells, the possible effect of PM-T-ext. on DNA synthesis was also tested. The estimation of DNA synthesizing cells was done by calculating the labelling index.

Concentrations: 2.5 µl, 5 µl, 10 µl, 15 µl and 20 µl per ml of GM.

Treatment duration: 24 hrs.

In vivo **study:**

The pharmacokinetics and metabolic conversion of the substance in human beings often complicate the extrapolation of carcinogenesis data from *in vitro* assays to the heterogeneous human population. Furthermore, for predicting genotoxicity of a carcinogen, it has also been suggested that *in vivo* genotoxicity data would serve well to substantiate the *in vitro* data (Carrano and Natarajan, 1988). Hence, somatic cells were studied from the individuals consuming tobacco containing pan masala. Following a detailed history collection, samples of exfoliated buccal mucosa cells and peripheral blood were

taken from the individuals who were chewing the same brand of PM-T **(PM-T chewers)** that was used for ***in vitro*** studies. Individuals had habit of chewing the **PM-T** atleast for last one year. They were teetotalers and were not consuming tobacco or areca nut in any other form, even in past. **PBLs** were cultured as described earlier in part II (page 101).

The **staining and scoring techniques**, as well as **statistical methods** applied in ***in vitro*** studies, are provided in part I, whereas, that for the evaluation of the effects on cells of **PM-T** chewers were basically the same as explained in part II.

RESULTS:

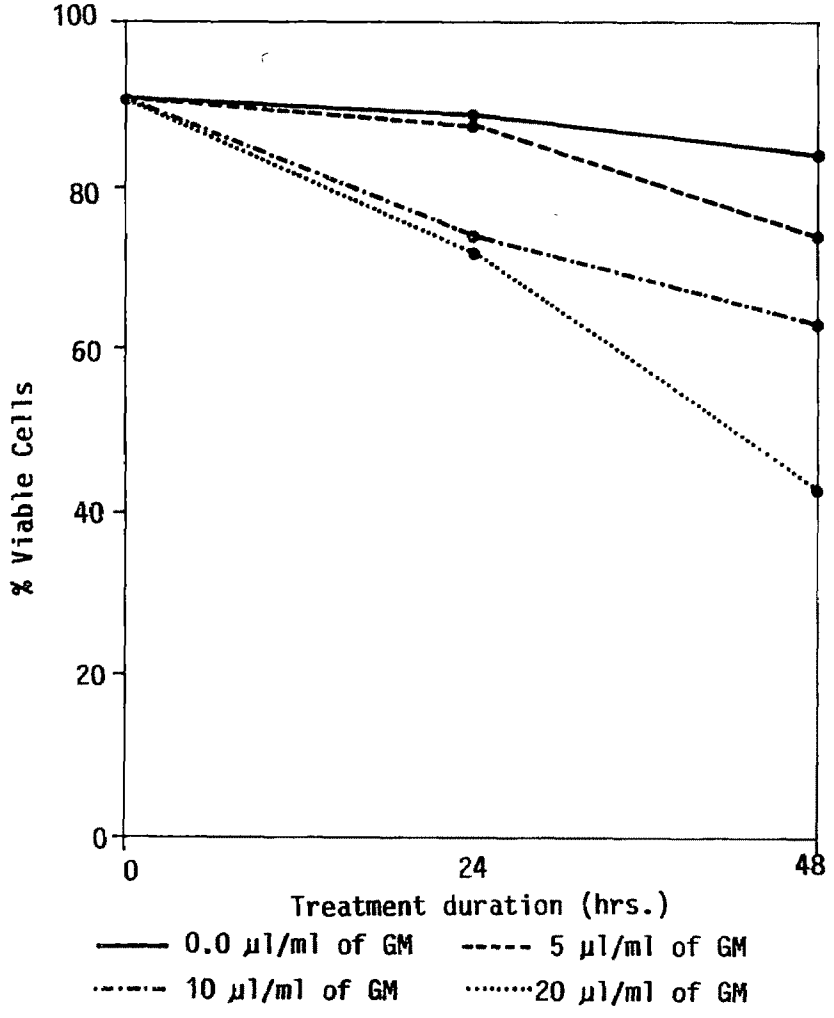
***In vitro* experiments:**

Experiment 1:

Figure 22 depicts the **viable cells (%)** observed after the treatment with **PM-T-ext.** After 24 hrs., the cell viability reduced from 88.32 % in untreated cultures to 87.38 %, 73.77% and 71.70 % in cultures treated with 5 µl, 10 µl and 20 µl **PM-T-ext.**/ml of GM, respectively. At 48 hrs., the viable cell count was 83.47 %, 74.00 %, 62.98 % and 43.04 % for the untreated cultures and those treated with 5 µl, 10 µl and 20 µl **PM-T-ext.**/ml GM, respectively. Thus, a dose and duration dependent cytotoxic effect of the extract was observed in the cell viability experiment.

Figure-22

EFFECT OF TREATMENTS WITH PM-T EXTRACT ON CELL VIABILITY OF CHO CELLS



Experiment 2:

Mitotic indices in **CHO** cells, detected after **continuous treatment with PM-T-ext.**, are presented in Table-37. During 0 to 36 hrs. of sampling, the **M.I.** varied between 4.1 and 5.2 in untreated cultures. The **M.I.** declined from 3.6 by 3 hrs. of treatment to 2.5 by 36 hrs. of treatment with 2.5 µl **PM-T-ext.**/ml of GM. The mitotic inhibition was more clearly evident in the cultures treated with higher concentrations of the extract and with longer durations. Thus, mitotic activity was inversely proportional to the dose of **PM-T-ext.** and to the duration of the treatment. Illustrations 29-32 feature some of the abnormally dividing cells and micronucleated interphase cells noticed following the treatment with **PM-T-ext.**

Experiment 3:

The **M.I.** observed after a 6 hr. treatment with **PM-T-ext.**, followed by a **recovery for various durations in test substance free GM**, are graphically presented in Fig.23. For the cultures treated with 20 µl **PM-T-ext.**/ml of GM, an increase in **M.I.** from 3.4 to 4.2 was observed by 18 hrs. of incubation in extract free GM, which was comparable to that of the untreated cultures. Similarly, the cultures treated with 50 µl **PM-T-ext.**/ml of GM restored the normal mitotic activity by 24 hrs. in fresh GM. However, following a 6 hr. treatment with 100 µl **PM-T-ext.**/ml of GM, an increase in **M.I.** from 1.1 to 3.7 was noticed only after 30 hrs. of incubation in extract free GM.

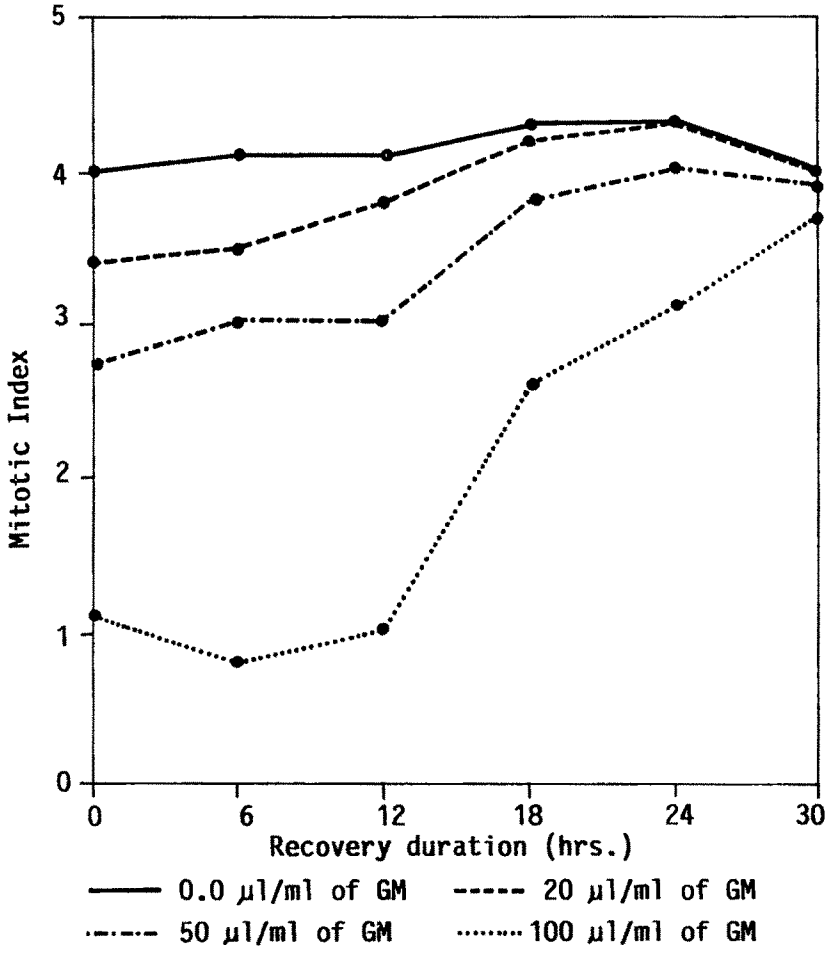
Table-37

MITOTIC INDEX OF CHO CELLS IN CONTINUOUS PRESENCE OF PM-T-Ext.

Treatment duration (h)	µl PM-T-Ext./ml of GM					
	0.0	2.5	5.0	10.0	20.0	50.0
0	5.2	-	-	-	-	-
3	4.2	3.6	3.7	3.1	2.8	2.4
6	5.2	3.3	3.7	3.1	3.3	2.2
12	4.7	3.0	2.5	2.3	2.2	1.6
24	4.8	2.8	2.4	2.2	1.9	0.8
36	4.1	2.5	2.3	1.9	1.5	0.4

Figure-23

M.I. IN CHO CELLS AFTER 6 hrs. TREATMENT WITH PM-T-EXTRACT FOLLOWED BY RECOVERY IN FRESH GM.



Experiment 4:

Fig.24 shows the **% MNC** recorded in cultures treated with 20 µl, 50 µl and 100 µl **PM-T-ext.**/ml of GM for 6 hrs. followed by a 12 and a 24 hrs. of recovery in fresh GM. For control cultures, the **% MNC** after a 12 and a 24 hrs. of recovery were 0.5 % and 0.7 %, respectively. The values were 1.6 % and 2.0% for the cultures treated with 20 µl **PM-T-ext.**/ml of GM; 2.0% and 2.4 % for the cultures treated with 50 µl **PM-T-ext.**/ml of GM, and 2.3 % and 3.7 % for the cultures treated with 100 µl **PM-T-ext.**/ml of GM, by 12 and 24 hrs. of recoveries, respectively.

Experiment 5:

The details of **CA** frequencies observed after the **continuous treatment** with **PM-T-ext.** are provided in Table-38. Illustrations 33-34 show some of the CAs observed following the treatment with PM-T-ext. Following the treatment with **PM-T-ext.**, an increase in almost all types of aberrations, i.e. chromatid-type as well as chromosome-type, was observed. The number of aberrant metaphases increased from 6 % in untreated cultures to 13 %, 17 %, 22 % and 26 % in cultures treated with 2.5 µl, 5.0 µl, 10.0 ul and 20.0 ul **PM-T-ext.**/ml GM. However, the elevations in mean **CA/cell** values were statistically significant only for the cultures treated with 10 µl and 20 µl **PM-T-ext.**/ml of GM **($p < 0.001$-including gaps; $p < 0.05$-excluding gaps)**, compared to that of untreated cultures.

FIGURE-24

% MNC OBSERVED AFTER 6 hrs. TREATMENT WITH PM-T-ext. FOLLOWED BY A RECOVERY IN FRESH GM

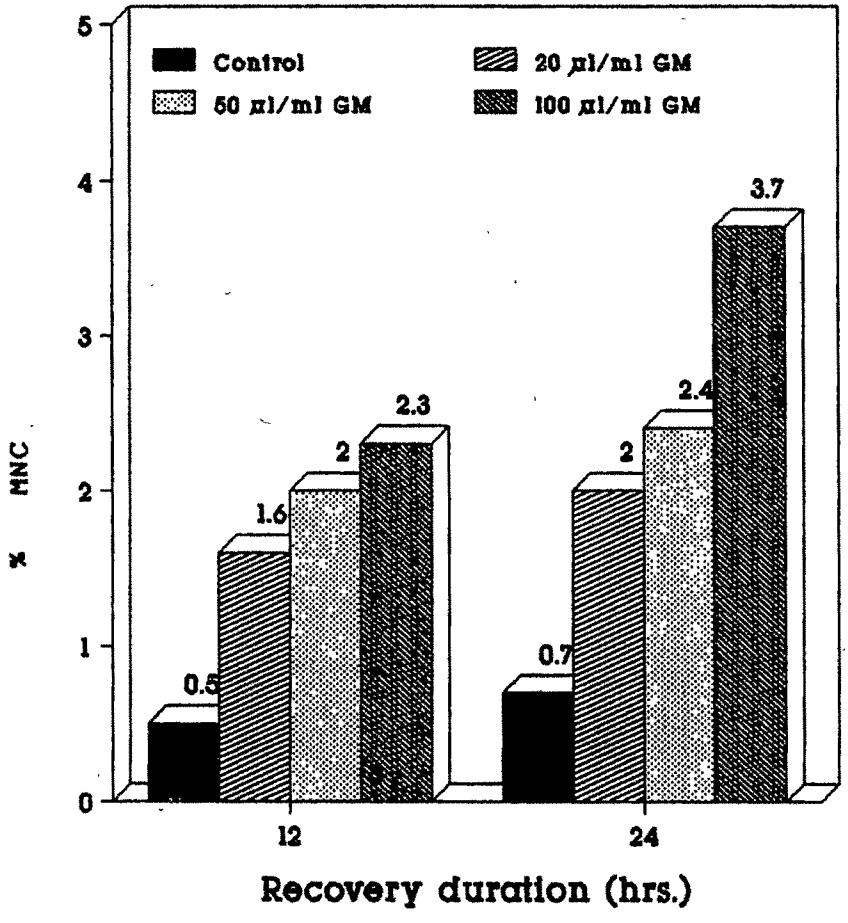


Table-38

DETAILS OF CA FREQUENCY IN CHO CELLS AFTER CONTINUOUS TREATMENT WITH PM-T-Ext.

Concentration µl/ml GM	Aberrant cells	Chromosome aberrations	Chromatid-type				Chromosome-type						CA/cell ± S.E.
			G	B/F	I	AG.	G	B	Dm	R	Dc	Ag.	
0.0	6	6	4	1	-	5	-	1	-	-	-	1	0.06 ± 0.0276
2.5	13	14	7	3	-	10	3	1	-	-	-	4	0.14 ± 0.0375
5.0	17	19	10	5	1	16	2	1	-	-	-	3	0.19 ± 0.0440 B
10.0	22	24	13	7	1	21	3	-	-	-	-	3	0.24 ± 0.0472 D,a
20.0	26	30	16	6	1	23	6	-	-	-	1	7	0.30 ± 0.0539 D,a
MMC 0.007 µg	21	23	9	5	1	15	3	1	2	-	2	8	0.23 ± 0.0470 D,a

where,

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

B = breaks; Dm = double minutes; Dc = dicentric chromosome;

R = ring chromosome

Ag. = Aggregate chromatid/chromosome-type aberrations.

B = $p < 0.02$; D = $p < 0.001$ (including gaps)

a = $p < 0.05$ (excluding gaps)

Experiment 6:

Table-39 contain the details of **SCE** frequencies witnessed following a **continuous treatment** with **PM-T-ext.** Illustrations 35-36 show SCEs observed in the untreated cultures and in cultures treated with PM-T-ext. The mean **SCE/cell** value increased from 7.08 in untreated cultures to 11.92, 14.16, 15.08 and 17.16 in cultures treated with 2.5 µl, 5 µl, 10 µl and 20 µl **PM-T-ext.**/ml of GM, respectively. It is evident from the Table-39 that higher **SCE/cell** values resulted from an elevation in number of cells with higher **SCEs**, with a concurrent drop in cells having lower **SCE** frequencies.

Experiment 7:

Table-40 describe the effects of a 3 hour treatment with **PM-T-ext.** followed by a **recovery in extract free GM. on CA frequency.** An increase in chromatid-type as well as chromosome-type aberrations was found in the treated cultures. Frequencies of **CA** were 14 %, 17 % and 26 % in cultures treated with 10 µl, 20 µl and 50 µl **PM-T-ext.**/ml of GM, respectively. Elevations in **CA** frequency following the treatment with 20 µl and 50 µl **PM-T-ext.**/ml of GM were statistically significant compared to 6 % **CAs** in untreated cultures.

Experiment 8:

Data presented in Table-41 summarize the effects of short term treatment with **PM-T-ext.** followed by a **recovery in fresh GM**, on **SCE** frequency. For cultures treated with 10 µl, 20 µl and 50 µl **PM-T-ext.**/ml of GM, the mean **SCE/cell** values were

Table-39

MEAN SCE FREQUENCY AND PERCENT DISTRIBUTION OF CELLS ACCORDING TO THE NUMBER OF SCEs OBSERVED AFTER CONTINUOUS TREATMENT WITH PM-T-Ext.

Concentration µl/ml GM.	Range	Number of metaphase cells with				SCE/cell ± S.E.
		0-5	6-10	11-15	>15 SCEs	
0.0	3 - 10	12	88	00	00	7.08 ± 0.304
2.5	9 - 15	00	40	60	00	11.92 ± 0.415 D
5.0	11 - 18	00	00	76	24	14.16 ± 0.418 D
10.0	11 - 20	00	00	64	36	15.08 ± 0.515 D
20.0	13 - 23	00	00	40	60	17.16 ± 0.598 D
MMC 0.007 µg	21 - 48	00	00	00	100	36.28 ± 0.876 D

where,

D = $p < 0.001$

Table-40

DETAILS OF CA FREQUENCY (%) IN CHO CELLS AFTER 3 hour TREATMENT WITH PM-T-Ext. FOLLOWED BY A RECOVERY IN EXTRACT FREE GM

Concentration µl/ml GM	Aberrant cells	Chromosome aberrations	Chromatid-type				Chromosome-type						CA/cell ± S.E.
			G	B/F	I	AG.	G	B	Dm	R	Dc	Ag.	
0.0	6	6	4	1	-	5	-	1	-	-	-	1	0.06 ± 0.0276
10.0	12	14	7	5	-	12	2	-	-	-	-	2	0.14 ± 0.0400
20.0	16	17	11	2	1	14	3	-	-	-	-	3	0.17 ± 0.0401 A
50.0	22	26	12	5	-	17	3	4	-	1	1	9	0.26 ± 0.0522 D,c
MMC 0.03 µg	19	22	9	4	1	14	3	2	-	2	1	8	0.22 ± 0.0430 D,c

where,

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

B = breaks; Dm = double minutes; Dc = dicentric chromosome;

R = ring chromosome

AG. = Aggregate chromatid/chromosome-type aberrations.

A = $p < 0.05$; D = $p < 0.001$ (including gaps)

c = $p < 0.01$ (excluding gaps)

Table-41

MEAN SCE FREQUENCY AND PERCENT DISTRIBUTION OF CELLS ACCORDING TO THE NUMBER OF SCEs OBSERVED AFTER 3 hour TREATMENT WITH PM-T-Ext. FOLLOWED BY A RECOVERY IN EXTRACT FREE GM

Concentration µl/ml GM.	Range	Number of metaphase cells with				SCE/cell ± S.E.
		0-5	6-10	11-15	>15 SCEs	
0.0	4 - 8	**44**	**56**	00	00	6.00 ± 0.305
10.0	7 - 16	00	**80**	**16**	04	9.48 ± 0.497 D
20.0	9 - 14	00	**44**	**56**	00	11.00 ± 0.315 D
50.0	11 - 25	00	00	**52**	**48**	15.56 ± 0.645 D
MMC 0.03 µg	18 - 29	00	00	00	**100**	21.81 ± 0.602 D

where,

D = $p < 0.001$

9.48 ± 0.497 (± S.E.), 11.00 ± 0.315 and 15.56 ± 0.645, respectively. These values were significantly higher than 6.00 ± 0.305 (mean ± S.E.) **SCE/cell** value for the untreated cultures. This can be explained on the basis of more number of cells with higher **SCE** frequency in treated cultures.

Experiment 9:

The **labelling index** reduced from 68.00 % in control cultures to 42.80 %, 26.90 %, 11.70 %, 2.30 % and 0.50 % after a 24 hr. exposure to 2.5 µl, 5 µl, 10 µl, 15 µl and 20 µl **PM-T-ext./ml.** of GM., respectively (Fig.25). Illustrations 37-38 show the labelled cells observed in control and **PM-T-ext.** treated cultures.

In vivo:

The individual frequency of **MNC in exfoliated buccal mucosa** as well as **CA and SCE frequencies in PBLs** of controls and normal persons who had habit of chewing **PM-T (PM-T chewers)**, are provided in Table-42 and Table-43, respectively.

The mean frequency of **% MNC** in **PM-T** chewers (0.72 %) was significantly higher compared to control value of 0.190 **% MNC ($p < 0.001$)**. Illustrations 39-40 feature the cells with or without micronucleus from the exfoliated buccal mucosa.

The mean **CA/cell** value (including gaps) for **PM-T** chewers was 0.112 ± 0.008 (± S.E.). The increase in **CA** frequency was statistically significant **($p < 0.001$)** compared to that of the controls (0.052 ± 0.003). Table-44 details the frequency of

FIGURE-25

INHIBITION OF DNA SYNTHESIS FOLLOWING

THE TREATMENT WITH PM-T-ext.

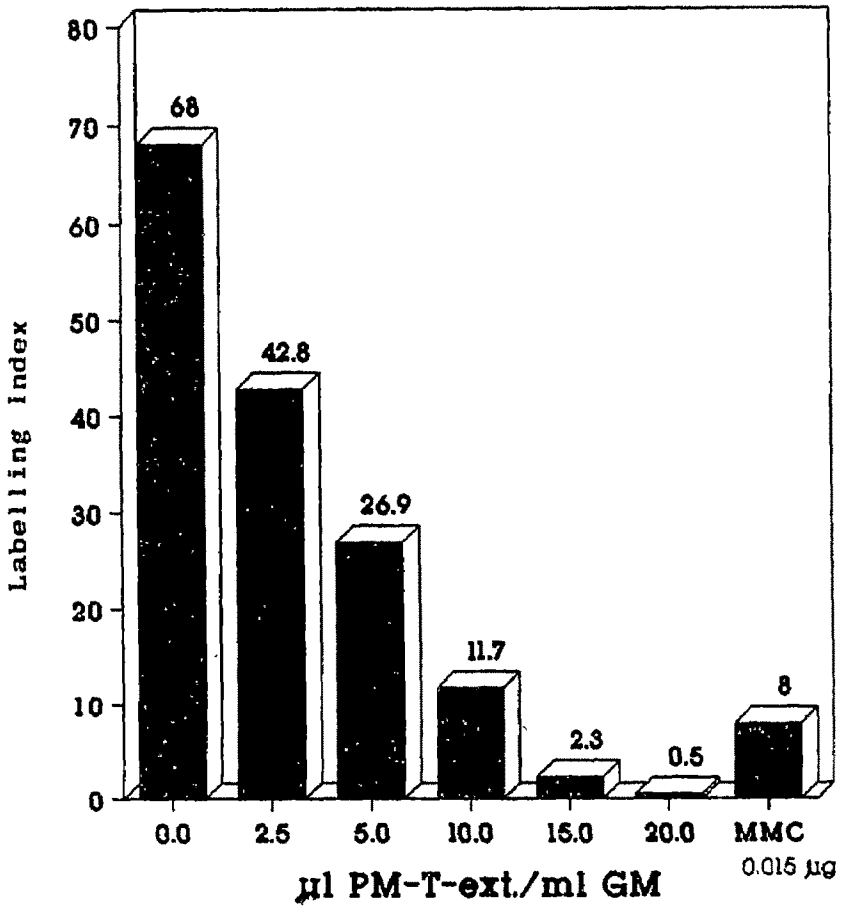


Table-42

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-43

INDIVIDUAL VALUES OF VARIOUS CYTOGENETIC ENDPOINTS AMONG NORMAL CHEWERS OF PAN MASALA WITH TOBACCO (N-PM-T)

No.	Age/Sex	% MNC	CA/cell ± S.E.	SCE/cell ± S.E.
1	20 M	0.6	**0.07 ± 0.0255** (0.02)	7.95 ± 0.315
2	22 M	**0.4**	0.11 ± 0.0343 (0.03)	**8.46 ± 0.612**
3	25 M	0.8	0.11 ± 0.0313 (0.02)	7.22 ± 0.224
4	27 M	0.8	0.10 ± 0.0300 (0.03)	7.42 ± 0.339
5	28 M	0.7	0.13 ± 0.0391 (0.04)	**7.19 ± 0.193**
6	30 M	0.6	0.09 ± 0.0317 (0.02)	7.24 ± 0.358
7	33 M	0.8	0.12 ± 0.0382 (0.05)	7.79 ± 0.355
8	35 M	0.8	0.14 ± 0.0425 (0.07)	7.47 ± 0.253
9	35 M	0.6	0.09 ± 0.0286 (0.03)	7.19 ± 0.333
10	37 M	**1.1**	**0.16 ± 0.0393** (0.05)	7.45 ± 0.278
Group mean ± S.E.		0.72 0.056	0.112 (0.036) 0.008 (0.005)	7.538 0.124

Values in paranthesis indicate CA/cell excluding gaps.

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

Table-44

DETAILS OF CAs (per 100 cells) OBSERVED AMONG CONTROLS AND NORMAL CHEWERS OF PAN MASALA WITH TOBACCO (N-PM-T)

Group	Aberrant etaphases	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-PM-T	10.50	11.30	7.0	3.4	-	10.4	0.6	0.3	-	-	-	0.9

where,

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

B = breaks; Dm = double minutes; Dc = dicentric chromosome;

R = ring chromosome; AG. = Aggregate chromatid/chromosome-type aberrations.

Table-45

PERCENT DISTRIBUTION OF METAPHASES ACCORDING TO THE NUMBER OF SCEs AMONG CONTROLS AND NORMAL CHEWERS OF PAN MASALA WITH TOBACCO (N-PM-T)

Group	Range	% metaphases with			
		0 - 5	6 - 9	10 - 15	>15 SCEs
Control	1 - 14	38.6	57.6	3.8	-
N-PM-T	4 - 15	10.0	77.2	12.8	-

different types of CAs observed **in controls and PM-T** chewers. Illustrations 41-42 portray representative aberrant metaphases. Chromatid-type aberrations were more frequent with maximum number of gaps in both the groups. An increase in the frequency of chromatid-gaps from 4.1 per 100 cells in controls to 7.0 per 100 cells in **PM-T** chewers, was observed. Even after excluding gaps from aberrations, the elevation in mean **CA/cell** value in **PM-T** chewers was **statistically significant ($p < 0.001$)** when compared with that of the controls (0.009 $\pm$ 0.002).

The mean **SCE/cell** values were 6.121 $\pm$ 0.078 ($\pm$ S.E.), and 7.538 $\pm$ 0.124 for controls and **PM-T** chewers, respectively. The difference was statistically significant ($p < 0.001$). Table-45 provides **the percent distribution of metaphases according to the number of SCEs** for controls and **PM-T** chewers. An increase in number of metaphases with higher **SCEs** was observed in **PM-T** chewers. Illustrations 43-44 exhibit represntative metaphases with low and high SCE frequency observed among controls and PM-T chewers, respectively.

Illustrations 29-32 Photomicrographs depicting some abnormalities observed in CHO cells after treatment with PM-T-ext.

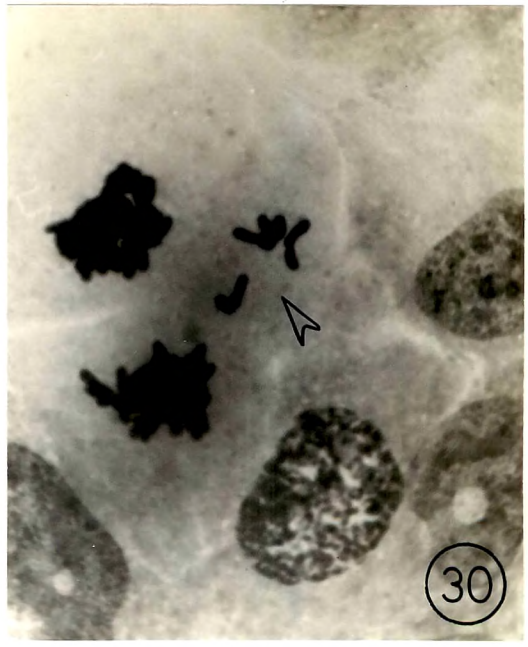
29 Anaphase showing chromatin bridge.

30 Late anaphase with lagging chromosome.

31-32 Cells with micronuclei.

Magnification: 1400x


29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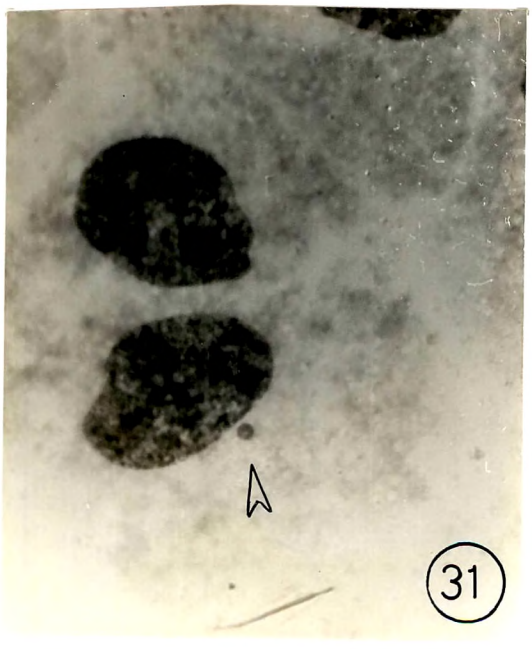
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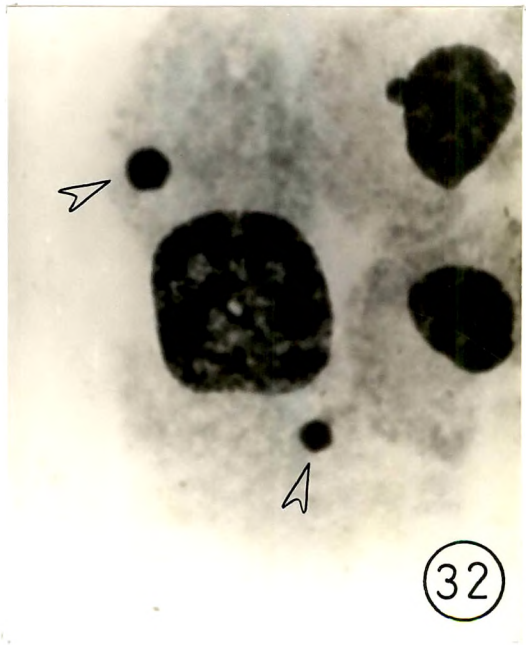
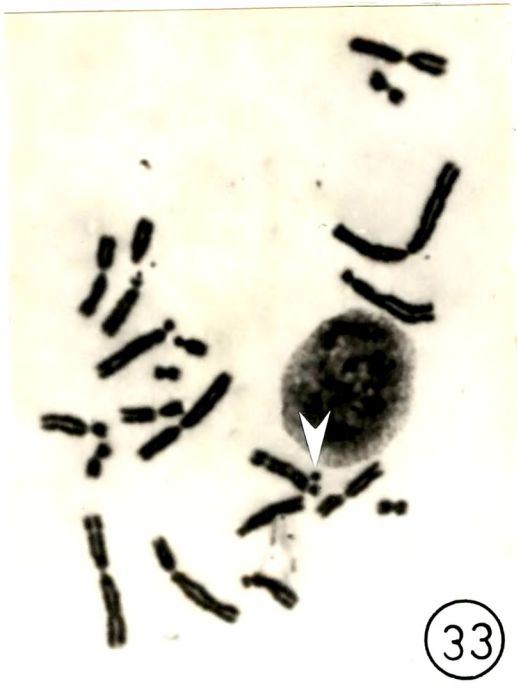

32

Illustration	33	A metaphase with acentric fragment.
	34	A metaphase with chromatid-type break.
	35	A metaphase from an untreated culture showing few number of SCEs.
	36	A metaphase from treated culture showing more number of SCEs.
Magnification:	33-34	1600x
	35-36	1800x



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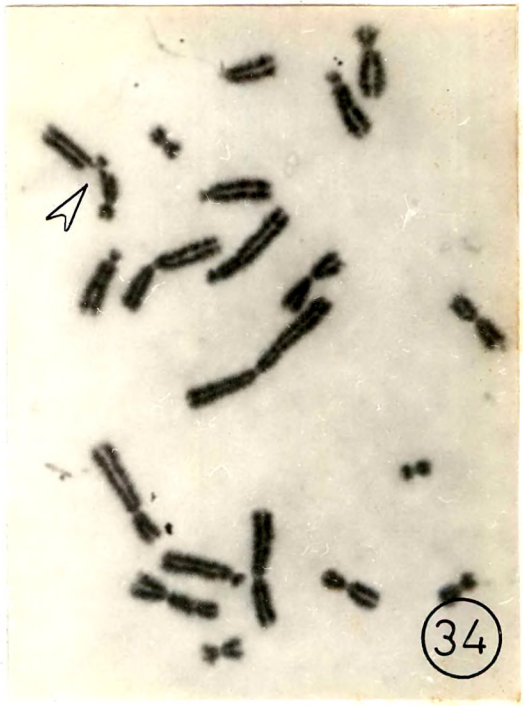
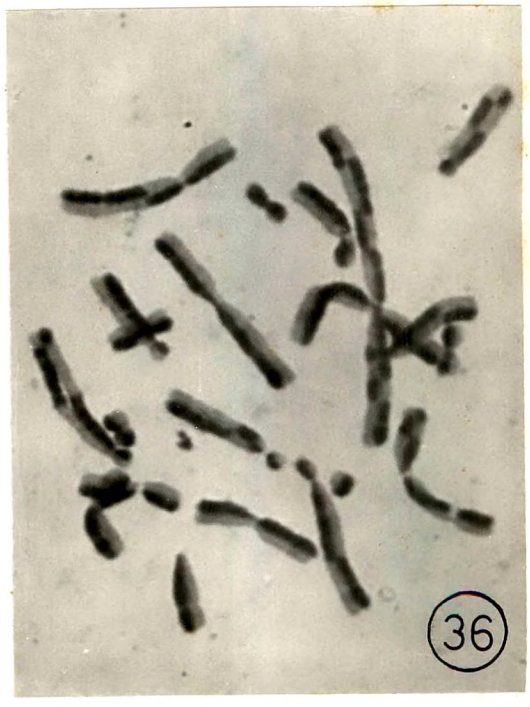


34

35



36

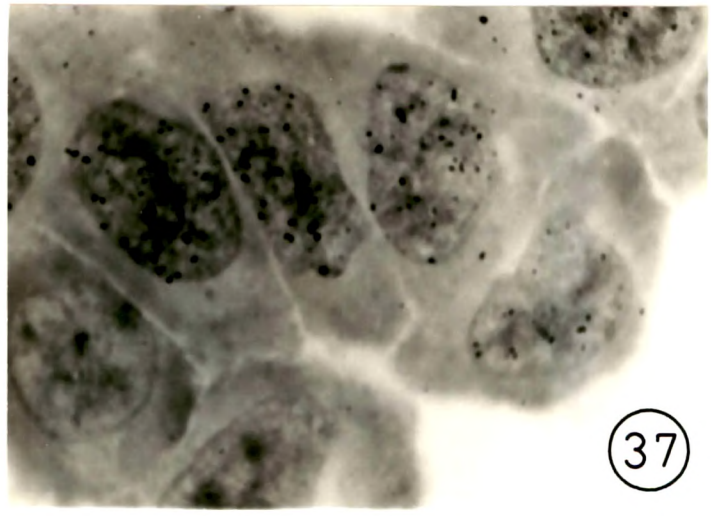
Illustrations 37-38 Autoradiograms exhibiting ^{3}H-thymidine incorporation in CHO cells.

37 Untreated CHO cells showing ^{3}H-thymidine incorporation in the nucleus.

38 CHO cells treated with PM-T-ext.

Magnification: 37 1400x

38 1600x



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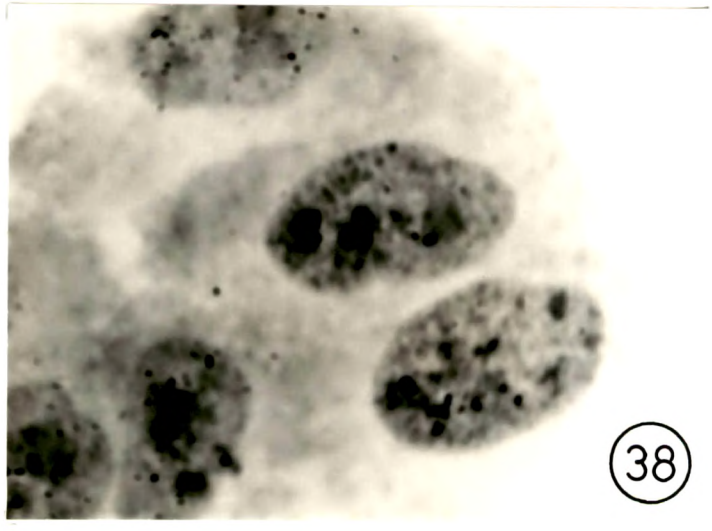


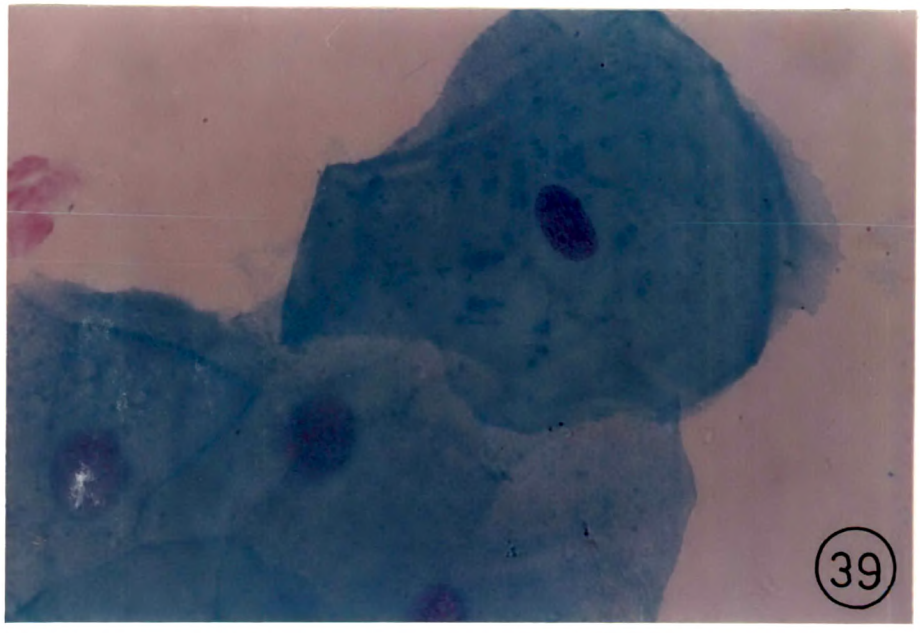
38

Illustrations 39-40 Cells from the exfoliated buccal mucosa stained with Feulgen plus Fast green.

39 Normal buccal mucosa cells.

40 A cell with micronucleus.

Magnification: 875x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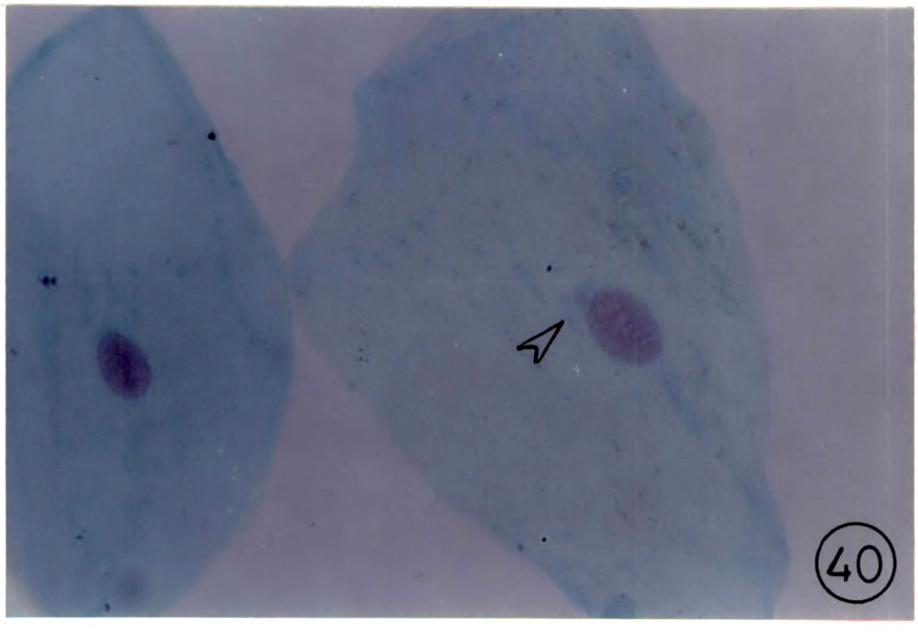
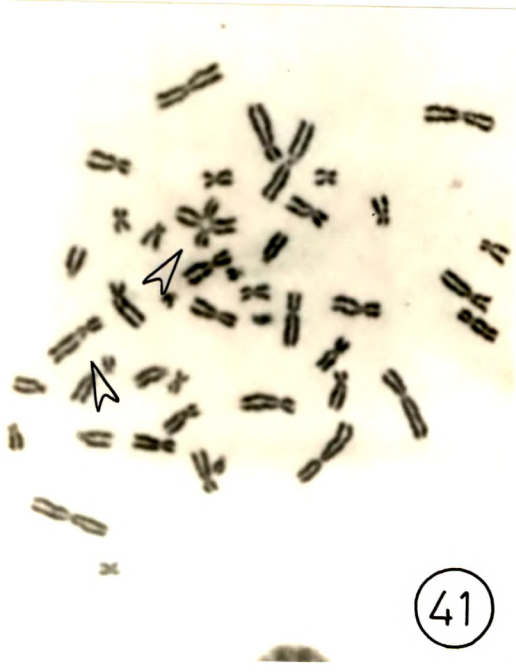
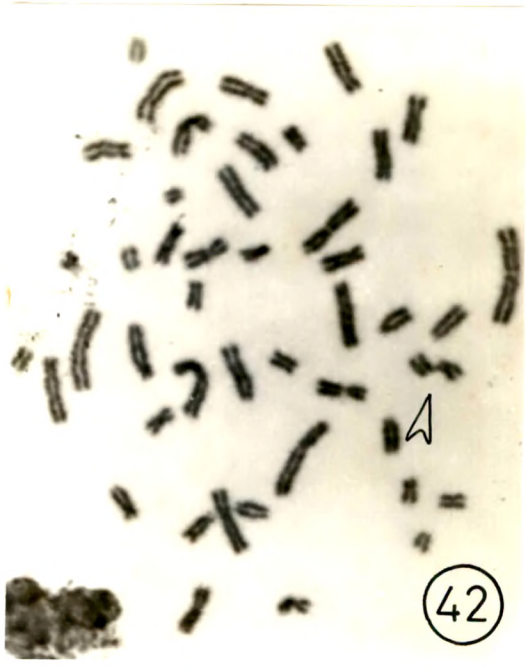

40

Illustrations	41-42	Some of the metaphases observed in lymphocytes of PM-T chewers.
	41	A metaphase with interchange.
	42	A metaphase with chromatid-break.
Illustration	43	A metaphase from a control subject showing 2 SCEs.
	44	A metaphase from the lymphocyte culture of a PM-T chewer showing 11 SCEs.
Magnification:	41	1400x
	42-44	1600x



41



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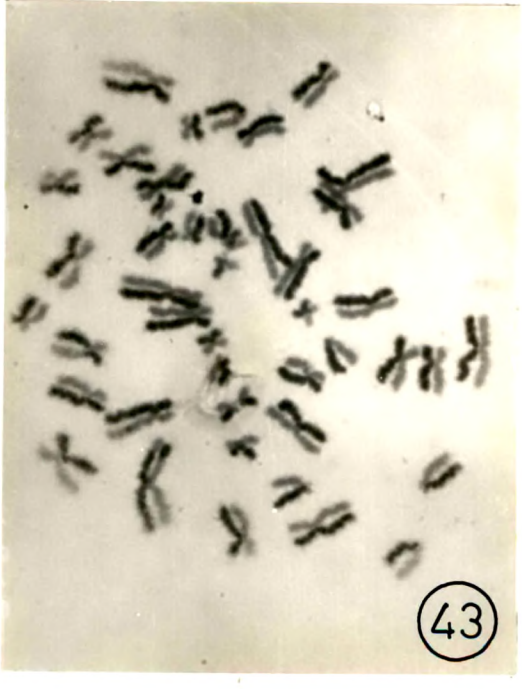

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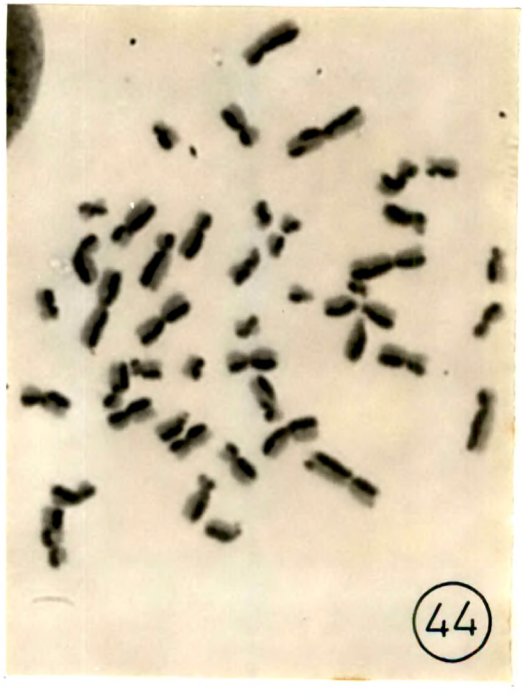

44

DISCUSSION:

Smokeless tobacco is comsumed worldwide with numerous variations in the form of tobacco used, which mainly depend on personal preferences. In India about 85 % of the tobacco consumed is in raw form, i.e. sun-dried, without further processing (IARC, 1985). The figures are likely to change with introduction of a number of processed tobacco varieties (even with liquor flavours) in small pouches. However, they are often processed with additives and flavouring agents. Zarda, a kind of processed tobacco, is abundantly produced and used in India (Sinha, 1984). It is usally chewed in combination with finely-cut areca nut and spices. **Pan masala with 'zarda'** is one of such products. It consists of zarda, areca nut, catechu, lime, spices and flavouring agents. Unlike betel quid, which is almost always freshly prepared, **PM-T** is a dry powdered mixture of various ingredients and is commercially marketed under different brand names. At almost every 'pan shop', it is available in pouches. Due to the feasibility of carrying and flavour of the mixture, it is gaining rapid popularity even among the youth.

Areca nut and Zarda, the processed scented tobacco, are the major constituents of **PM-T**. The oral uses of smokeless tobacco have been associated with an increased risk of oral cancer. Tobacco chewing appears to be the greatest exogenous source of exposure to N-nitrosamines (Hoffmann and Hecht, 1985). Saliva of the tobacco chewers contain nicotine, the major alkaloid of tobacco, and TSNAs (Nair et al., 1985).

Since very high concentrations of TSNAs have been reported in zarda (Tricker and Preussmann, 1988), its use exposes its user to relatively higher concentrations of TSNAs. Secondly, several epidemiological and experimental studies have indicated the carcinogenic potential of **areca nut** (IARC, 1985), the major constituent (by weight) of **PM-T**. Enhancement of carcinogenesis in the hamster cheek pouch has been documented following the addition of areca nut extract to that of tobacco (Ranadive and Ghothoskar, 1978). Stich et al. (1981b) have observed potentiation of the clastogenic effect of chlorogenic acid (a phenolic compound present in Nicotiana tabacum) by arecoline, the major alkaloid of areca nut. Moreover, preliminary data indicated higher levels of areca nut specific alkaloids and areca nut specific nitrosamines in the saliva of chewers of betel quid with 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). This gives an explanation for assuming more severe harmful effects of consuming tobacco and areca nut, in combination. The same combination is present in **PM-T** as well. In view of all the above mentioned facts, it is reasonable enough to consider the **PM-T chewers** at an elevated risk of developing oral cancer. Hence, an attempt was made to assess its possible genotoxic effects.

As a first step, an **aqueous extract** was prepared from the most popular and widely consumed brand of **Pan masala with 'zarda'** and tested on **CHO** cells. The results of *in vitro* expe-

riments revealed the **cytotoxic/clastogenic/genotoxic effects of an aqueous extract of PM-T on mammalian cell culture. A significantly higher frequency of MNC in exfoliated buccal mucosa as well as CA and SCE frequencies in PBLs of individuals consuming PM-T,** compared to controls, **confirmed the findings of *in vitro* analysis.** The effects can be attributed to the constituents of **PM-T.** Similar effects of aqueous extract of tobacco **(T-ext.)** have been discussed in **Part I.** However, the genotoxic effects elicited by **PM-T-ext.** were more pronounced compared to that observed after the treatment with **T-ext.** at similar concentrations. This can be explained on the basis of effects of PM-T constituents other than tobacco, which might have added to the gemomic damage caused by tobacco alone.

Following the treatment with aqueous extract of 'pan masala (without tobacco)', an increase in **CA** and **SCE** frequencies in **CHO** cells has also been reported (Adhvaryu et al., 1989). Recently, Bagwe et al. (1990) reported a weak mutagenic response in Salmonella typhimurium strain TA98 following the treatment with ethanolic extract of 'pan masala' (without tobacco), only in absence of metabolic activation system, and thereby, demonstrated the presence of direct-acting frameshift mutagens in 'pan masala'.

These effects can be attributed mainly to the presence of **areca nut** which constituted about 70-80 % (by weight) of **PM-T** examined. Sundquist et al. (1989) and Wary and Sharan (1988)

have also observed toxic effects of areca nut. Following exposure to an aqueous extract of areca nut, an increase in number of **MNC** in **CHO** cells (Dave, 1990) and in mouse bone-marrow cells (Shirname et al., 1984) have been reported. A very high frequency of **MNC** in the buccal mucosa has been observed among Indians chewing areca nut, with or without tobacco (Adhvaryu et al., 1991; Dave, 1990; Stich et al., 1982). The clastogenic effect of **PM-T** was further validated by CA assay. A dose related increase in the frequency of aberrant cells as well as in CA/cell value was observed following a short term or long term exposure to **PM-T-ext.** Arecoline, the major alkaloid of areca nut, and areca nut extracts have been reported to increase **CA** and **SCE** frequencies in animal studies (Panigrahi and Rao, 1982; 1986) as well as in the ***in vitro*** test system (Dave, 1990; Stich et al., 1981; 1983; Stich and Tsang, 1989). One of the nitrosamines of arecoline, 3-(methylnitrosamino) propionitrile (**MNPN**), is a powerful carcinogen in F344 rats (Wenke et al., 1984). It has also been observed that areca nut specific alkaloids, areca nut specific nitrosamines, polyphenols and tannins can be released in the saliva while chewing areca nut (Nair et al., 1985; 1987; Prokopczyk et al., 1987; Stich and Anders, 1989; Sundqvist et al., 1989; Wenke et al., 1984). Saliva of the areca nut chewers has been reported to induce **CAs** in **CHO** cells (Stich and Stich, 1982). Higher frequencies of **CA** and **SCE** among individuals chewing areca nut without tobacco has also been documented (Dave, 1990).

Nevertheless, possible role of other ingredients present in **PM-T** should also be considered. Lime provides alkaline pH to the mixture which influences the liberation of arecoline from areca nut (Boyland and Nery, 1969; Nieschultz and Schmersahl, 1968). It has also been reported that at pH 11.0 or more, nicotine is completely unprotonated and its rate of absorption is thus accelerated. 'Nass' contains lime and its pH is remarkably high (pH 11.0-11.8). When Nass samples are placed into the oral cavity, nicotine reaches the central nervous system more quickly, the speed being positively correlated with the lime content of Nass (Brunnemann et al., 1985). With Giri et al. (1987) reporting clastogenic effects of catechu extracts and Nagabhushan et al. (1988) reporting antimutagenic effects of catechin, contribution of catechu as PM-T constituent remain an open issue. Musk ambrette and musk xylene, which might have been used to improve the flavour of tobacco, have been found to be mutagenic in Salmonella/mammalian microsome test system (Nair et al., 1986). Aflatoxin B_1, which might occur in **PM-T** due to the fungal contamination of areca nut, is an extremely potent mutagen in the Salmonella/mammalian microsome assay (IARC, 1976). It has been reported to induce **CAs** (Iskandar and Vijayalaxmi, 1981) and **SCE** (Batt et al., 1980; Thomson and Evans, 1979) in mammalian cells ***in vitro*** and **CAs** in mouse bone marrow ***in vivo*** (Fabry and Roberfroid, 1981).

Since the synergistic, antagonistic, additive or

inhibitory interactions between these constituents may take place in a mixture, it is difficult to hold any single compound responsible for the observed genotoxic effects of **PM-T**. However, it indeed infers that consumption of **PM-T** is not a safer alternative of tobacco smoking or tobacco chewing.

In short, the findings of ***in vitro*** assays revealed that:

(i) an aqueous extract of **PM-T** is cytotoxic/clastogenic/genotoxic to **CHO** cells, and

(ii) **PM-T-ext.** contains water soluble constituents, other than tobacco, which add to the genotoxicity of tobacco. Areca nut, being the major constituent of **PM-T**, might be the main factor responsible for the additional damage.

In genotoxicity studies on actual PM-T consumers, the number of chewers reported might appear to be small, however, it is to be emphasised that care was taken to see that PM-T consumption is the ONLY confounding factor between controls and the PM-T chewers. The present study provided enough indications that use of **PM-T**, as a new chewing substitute, may prove to be a more harmful habit than other modes of tobacco consumption. Since a long latent period may exist between the exposure and manifestation of the disease and since epidemiological proof will come very late, the present findings should serve as a caution against the use of **PM-T**.