Part - I
In Vitro Assays

Short-term tests for potential carcinogens and for risk assessment generally rely on the assumption that the agent in question will operate through a genotoxic mechanism. *In vitro* short-term tests offer more economical, rapid and dependable means for detecting genotoxic chemicals. Moreover, consistency of the results from *in vitro* short-term tests and rodent carcinogenicity tests have proved to be highly encouraging (Purchase et al., 1976). Subsequently, several international organizations have advocated their use in carcinogenicity testing and have formulated guidelines, wherein cytogenetic studies using mammalian cells in culture have been described (Scott et al., 1983; Perry et al., 1984 for UKEMS; WHO, 1985).

Taking into consideration these points, as well as the information cited in tables 1 and 2, the *in vitro* short-term cytogenetic assays were initiated utilizing the aqueous extract of tobacco and its major alkaloid, nicotine, in pure form. Chinese Hamster Ovary (CHO) cells were selected for collecting preliminary data about possible genotoxic effects of tobacco. Since the chewing of tobacco alongwith areca nut is common in this part of Western India and arecoline being the major areca nut alkaloid (Arjungi, 1976), effects of combining nicotine and arecoline were also studied. CHO cell line was preferred for having large and well defined chromosomes, which aided rapid scoring and short cell multiplication time facilitated repeated experiments within short durations.

MATERIALS AND METHODS:

The CHO cell line was kindly provided by National Facility for Animal Tissue and Cell Culture (NFATCC), Pune, India.

CHEMICALS:

Eagle's minimum essential medium (MEM) with Earle's base & non-essential amino acids (NEAA) and the Trypan blue dye (0.5% w/v) were purchased from Centron Research Lab.,Bombay, India. New born calf serum (NCS) was procured from Sera Lab., U.K. Trypsin (1:250) was obtained from Difco Lab., Detroit, Michigan, U.S.A. Nicotine (N.), Arecoline hydrobromide (A.), 5-Bromo-2-deoxyuridine (BrdU), colchicine and Hoechst 33258 (Bis-benzimide) were purchased from Sigma chemical company, St. Louis, U.S.A. Mitomycin C (MMC) was obtained from Biochem pharmaceutical industries, Bombay, India. ^{3}H-Thymidine was procured from Bhabha Atomic Research Centre, Bombay, India and Nuclear track emulsion (L4) of Ilford company, U.K. was a gift from Dr. D. Scott, Manchester, U.K. Unprocessed Sun-dried tobacco (Deshi-N. tabacum) was obtained from local market.

Calcium and magnessium free phosphate buffered saline (CMF-PBS), 1:3 acetic acid-methanol fixative, Sorenson's phosphate buffer (pH 7.0), Giemsa stain and Carbol fuchsin stain were prepared in the laboratory.

PREPARATION OF TOBACCO EXTRACT (T-ext.):

Fine powder of the tobacco was prepared with the help of a blender. 25 grams of this powder was thoroughly mixed with 75 ml of distilled water. The smooth tobacco paste was kept at

4-8^{o}C till the next morning. Then, the mixture was stirred for 3 hrs. at 37^{o}C and the extract was separated by centrifugation. The extraction procedure was repeated two more times by adding 75 ml of distilled water each time to the residue. The pooled extract was sterilized by passing through 0.22 µm porosity filter and stored at -70^{o}C in small aliquotes. The extract represented 25 grams of tobacco in 225 ml distilled water i.e. 1:9 dilution. The extract contained 4.42 gms % water soluble materials.

CULTURE PROCEDURE:

The growth medium (GM), containing 80-90 % MEM with NEAA, 10-20 % NCS, 100 units/ml benzyl penicillin and 100 µg/ml streptomycin sulphate, was used to maintain the CHO cell line. Every fifth day, the cells were subcultured. A single cell suspension was prepared in fresh GM by trypsinization (0.25 % trypsin in CMF-PBS) and cells were then seeded in new culture bottle at a final concentration of 2-2.5 x 10^{4} cells/ml of GM. The cells were allowed to grow at 37^{o}C. On the third day of subcultivation, the medium was replaced with fresh GM. The possible cytotoxic/genotoxic effects were studied in exponentially growing cells.

EXPERIMENTAL PROTOCOLS:

During all the experiments, an untreated culture, as a control and MMC treated culture, as a positive control, were kept simultaneously. Each experiment was repeated once and mean values were derived from the sum of values obtained in both the assays. The alkaloids were dissolved in serum free

GM just prior to use.

Experiment 1:

Possible **cytotoxic effects** of T-ext., nicotine (N.) and nicotine plus arecoline (N.+A.) were studied by exposing the CHO cells for different doses and durations.

Concentrations:

T-ext. : 5 µl, 10 µl, 20 µl per ml of GM.

N. : 250 µg, 500 µg, 1000 µg per ml of GM.

N.+A. : 90N.+50A. µg, 45N.+25A. µg, 150N.+12.5A. µg per ml of GM.

Durations : 24 hrs. and 48 hrs.

Procedure : At the end of the treatment, the cells were detached from the surface with the help of a rubber policeman and pipetted repeatedly to get a single cell suspension. 0.1 ml of trypan blue solution (0.5 % aqueous) was mixed with 0.9 ml of cell suspension and was allowed to stand for 5 minutes. The number of viable and total cells were counted in haemocytometer chamber.

Experiment 2:

Probable effects of the test-substances on **cell division** were examined by **continuously** exposing the cells grown on coverslip in Leighton tubes.

Concentrations:

T-ext. : 5 µl, 10 µl, 15 µl, 20 µl, 50 µl per ml of GM.

N. : 150 µg, 250 µg, 500 µg, 750 µg, 1000 µg per ml of GM.

N.+A. : 150N.+5A. µg, 150N.+12.5A. µg, 150N.+25A. µg per ml of GM.

Treatment Durations : 3, 6, 12, 24, 36 and 48 hrs.

Procedure : After initiation of treatment, the samples were fixed at different time intervals with chilled 1:3 acetic acid-methanol fixative for 30 minutes. The coverslips were dried and mounted on a slide, keeping the cell layer exposed. The cells were stained with 2 % Geimsa stain in Sorenson's phosphate buffer (pH 7.0), differentiated and mounted with DPX.

Experiment 3:

A **recovery** experiment was also conducted to study the ability of cells to regain normal **cell division** after a short treatment with the test-substances.

Concentrations:

T-ext. : 20 µl, 50 µl, 100 µl per ml of GM.

N. : 500 µg, 750 µg, 1000 µg per ml of GM.

N.+A. : 90 N.+50 A. µg, 200 N.+100 A. µg, 300 N.+150 A. µg per ml of GM.

Treatment Duration : 6 hrs.

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

Procedure : After the treatment, the cells were washed twice with prewarmed CMF-PBS ($37^{o}C$) and were allowed to grow in test-substance free GM for different durations. As described above, the cells were fixed and stained with 2 % Giemsa stain.

Experiment 4:

Effect of a short term treatment followed by a **recovery** in test-substance free GM, on **frequency of micronucleated cells**, was also analysed.

Concentrations:

T-ext. : 20 µl, 50 µl per ml of GM.

N. : 500 µg, 750 µg, 1000 µg per ml of GM.

N. +A. : 90 N.+50 A. µg, 200 N.+100 A. µg, 300 N.+150 A. µg per ml of GM.

Treatment duration : 6 hrs.

Recovery durations : 12 hrs. and 24 hrs.

Procedure : As described in experiment 3.

Experiments 5: 6:

With the help of sister chromatid differential staining technique, the experiments were carried out to study the effects of **continuous** treatments on **CA frequency** and **SCE frequency** in CHO cells.

Concentrations:

T-ext. : 5 µl, 10 µl, 15 µl, 20 µl per ml of GM.

N. : 150 µg, 250 µg, 375 µg, 500 µg, 625 µg per ml of GM.

N.+A. : 150N.+2A. µg, 150N.+5A. µg, 150N.+12.5A. µg per ml of GM.

Treatment Duration: A 24 hr. continuous treatment for CA analysis and a 48 hr. continuous treatment for SCE analysis.

Procedure: After 48 hrs. of subcultivation, exponentially growing cells were subjected to the above mentioned concentr-

ations of different test agents. These cultures were incubated at 37°C in GM containing 10 µg/ml BrdU (the concentration by which the adequate differentiation of BrdU substituted chromatid was observed, without increasing the baseline chromosome aberration frequency in CHO cells) for 24 hrs. (for CA analysis)/48 hrs. (for SCE analysis). Colchicine (0.3 µg/ml) was added during the last three hours of incubation. During all the above mentioned steps, the cultures were protected from direct light to prevent the photolysis of BrdU containing DNA (Ikushima and Wolff, 1974). On completion of 24 hrs./48 hrs. of incubation, the cells were scrapped from the surface with a rubber policeman and were mixed thoroughly in the medium. The hypotonic treatment was given by diluting the medium with distilled water (1:1) for 14 minutes at room temperature. This was terminated by an addition of 1.0 ml chilled aceto-methanol fixative. The cells were washed three times with fresh fixative and slides were prepared by air drying.

Sister chromatid differential (SCD) staining was obtained by a method described by Wolff and Perry (1974) and Sugiyama et al. (1976). Coverslips were mounted on the slides with phosphate buffer (pH 7.0) containing 5.0 µg/ml Hoechst 33258 dye and exposed to direct sunlight/U.V. light for 3-4 hrs. The slides were then treated with 2 x SSC at 60°C for 20 minutes, washed with distilled water, dried and mounted in DPX mounting medium after staining with 2.0 % Giemsa in phosphate buffer (pH 7.0).

Experiments 7: 8:

Effects of a short-term treatment followed by a **recovery** in test-substance free GM on **CA frequency** and **SCE frequency** were also studied.

Concentrations:

T-ext. : 20 µl and 50 µl per ml of GM.

N. : 625 µg and 1000 µg per ml of GM.

N.+A. : 90N.+50A. µg, 200N.+100A. µg, 300N.+150A. µg per ml of GM.

Treatment Durations: 2 hrs. and 4 hrs. for T-ext. and N.
4 hrs. for N.+A.

Recovery Duration: 24 hrs. for CA analysis, and 48 hrs. for SCE analysis.

Procedure: Following the treatment, cultures were washed twice with prewarmed CMF-PBS (37°C). The cells were then allowed to grow in fresh, test-substance free GM at 37°C. Cultures were harvested and slides were prepared as described for previous experiments 5: 6.

Experiment 9:

Autoradiographic studies were performed to learn about the effects of the test-substance treatments on **DNA synthesis.**

Concentrations:

T-ext. : 5 µl, 10 µl, 15 µl, 20 µl per ml of GM.

N. : 150 µg, 250 µg, 500 µg, 625 µg per ml of GM.

N.+A. : 150 N.+12.5 A. µg, 150 N.+25 A. µg, 150 N.+50 A. µg per ml of GM.

Treatment Durations: 24 hrs.

Procedure: Coverslip cultures were treated with various concentrations of different test-substances. During the last 20 minutes of the exposure period, a pulse treatment of ^{3}H-thymidine (2 µci/ml of GM) was given. Thereafter, the cells were washed twice with CMF-PBS and fixed with chilled aceto-methanol fixative. Coverslips were mounted on clean slides, keeping the cell layer exposed. These slides were subjected to a 5 minute treatment with 5 % trichloro acetic acid (TCA) at 0-4^{o}C. The TCA treatment was to remove the unincorporated ^{3}H-thymidine and subsequently the slides were washed repeatedly with 70 % ethanol for a period of 4-5 hrs. The cells were stained with carbol fuchsin according to the method of Darlington (1976). The nuclear track emulsion Ilford L4 was mixed with distilled water (1:1 w/v) and melted at 42^{o}C. Taking all the dark room precautions, the slides were coated with diluted emulsion and when dried, the slides were packed in light proof slide boxes alongwith silica gel as the dessicant. These boxes were stored at 4^{o}C. After 4 weeks, the slides were developed with Kodak D-19B developer at 18^{o}C for 5 minutes. Following acid fixer treatment and thorough washing with cold distilled water, slides were dried and mounted with DPX.

SCORING METHODS:

Cell viability: The following formula was used to calculate the percent viability,

$$\% \text{ viable cells} = \frac{\text{Total no.of cells - No. of stained cells}}{\text{Total no. of cells}} \times 100$$

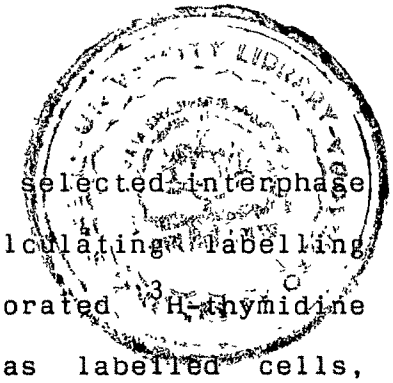
Mitotic index: A minimum of 1000 cells were randomly scored and the number of cells in the process of cell division were counted. The mitotic index (M.I.) was expressed as % dividing cells.

Micronucleus assay: A minimum of 1000 cells were screened from each sample and % micronucleated cells (% MNC) were calculated. The identification of micronucleus was based on the criteria suggested by Schmid (1976) and Sarto et al. (1987) : (1) The chromatin structure and colour intensity were similar to or weaker than that of main nucleus (2) Their edges were distinctly recognizable, suggesting the presence of a nuclear membrane (3) They were almost round in shape (4) They were included in the cytoplasm and (5) They were smaller than or equal to 1/5th of the main nucleus.

Chromosome aberration (CA) assay: At least 100 metaphases in M-I phase were scanned for CA analysis. The criteria recommonded by WHO (1985) and in the report of UKEMS subcommittee on guidelines for mutagenicity testing (Scott et al., 1983) were followed for the identification of various types of aberrations. CA/cell values were considered for computing the statistical significance of the data.

Sister chromatid exchange (SCE) assay: A minimum of 25 well spread metaphases in M-II phase of cell division were scored to determine the SCE frequency. A terminal exchange was counted as one SCE and each interstitial exchange was counted as two SCEs. SCE/cell values were used for calculating the stat-

istical significance of the results.



DNA synthesis: A minimum of 1000 randomly selected interphase cells were scored from each sample for calculating labelling index. The number of cells that incorporated ^{3}H-thymidine during a pulse treatment were scored as labelled cells, however, cells with less than 10 grains on nucleus were not considered as labelled ones.

STATISTICAL ANALYSIS: Student's 't' test was applied to find out the statistical significance of the difference in the mean values of the samples.

PHOTOMICROGRAPHY: Photomicrographs were taken on Zeiss Photomicroscope III with the help of bright field optics and Agfa copex film.

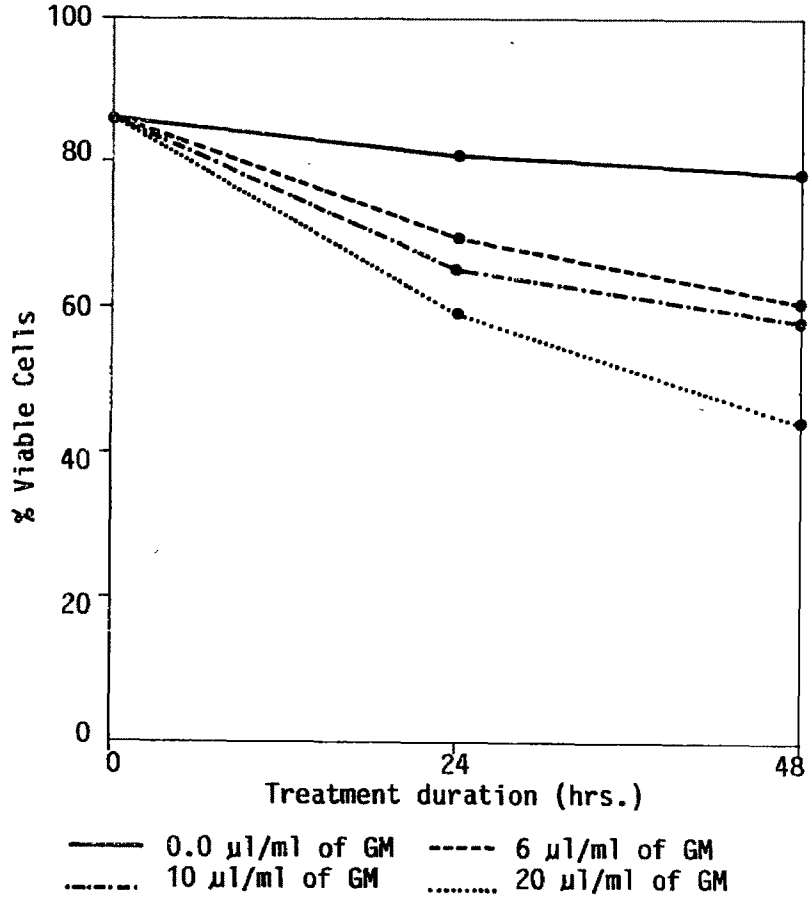
RESULTS:

Experiment 1:

T-ext.:

The results of the cytotoxic effects of T-ext. are depicted in Fig.3. At 0 hr., 85.68 % viable cells were observed in an untreated culture. After 24 hrs., the viable cell count in control culture was 80.65 %, whereas, in cultures treated with 5 µl, 10 µl and 20 µl T-ext./ml of GM, the values were 69.57 %, 65.19 % and 59.11 %, respectively. At 48 hrs., the viable cell count was 77.95 %, 60.63 %, 57.86 % and 44.10 % for the control and the three treated cultures, in increasing

Figure-3
EFFECT OF TREATMENT WITH TOBACCO EXTRACT ON
CELL VIABILITY OF CHO CELLS



order of concentrations, respectively.

Nicotine:

Similarly, the cell viability reduced from 77.95 % in control culture to 71.14 %, 51.02 % and 23.13 % after 24 hrs. treatment with 250 µg, 500 µg and 1000 µg N./ml of GM, respectively. The dead cell population was further increased after 48 hrs. of exposure (Fig.4).

Nicotine + Arecoline:

The cytotoxic effects of combining two alkaloids, nicotine and arecoline, can be observed in Fig.5. A clear dose and duration dependent reduction in the viable cell count was seen after 24 hrs. and 48 hrs. treatment with three different combinations.

Experiment 2:

T-ext.:

The effect of T-ext. treatments on mitotic activity of CHO cells have been detailed in Table-3. Among controls, M.I. ranged from 4.1 to 5.2 during 0 to 36 hrs. of sampling. In cultures treated with 5 µl T-ext./ml of GM, the M.I. reduced from 4.0 at 3 hrs. to 2.7 by 36 hrs. The diminution in M.I. observed was parallel with the increase in concentration of T-ext., i.e. after the exposure to 5 µl through 50 µl T-ext./ml of GM for 3 hrs., the M.I. declined from 4.9 in control cultures to 2.1 with the highest concentration of T-ext. Frequency of mitotic abnormalities, like chromatin bridges, lagging chromosomes at anaphase, micronucleated interphases

Figure-4

EFFECT OF TREATMENTS WITH NICOTINE ON CELL VIABILITY OF CHO CELLS

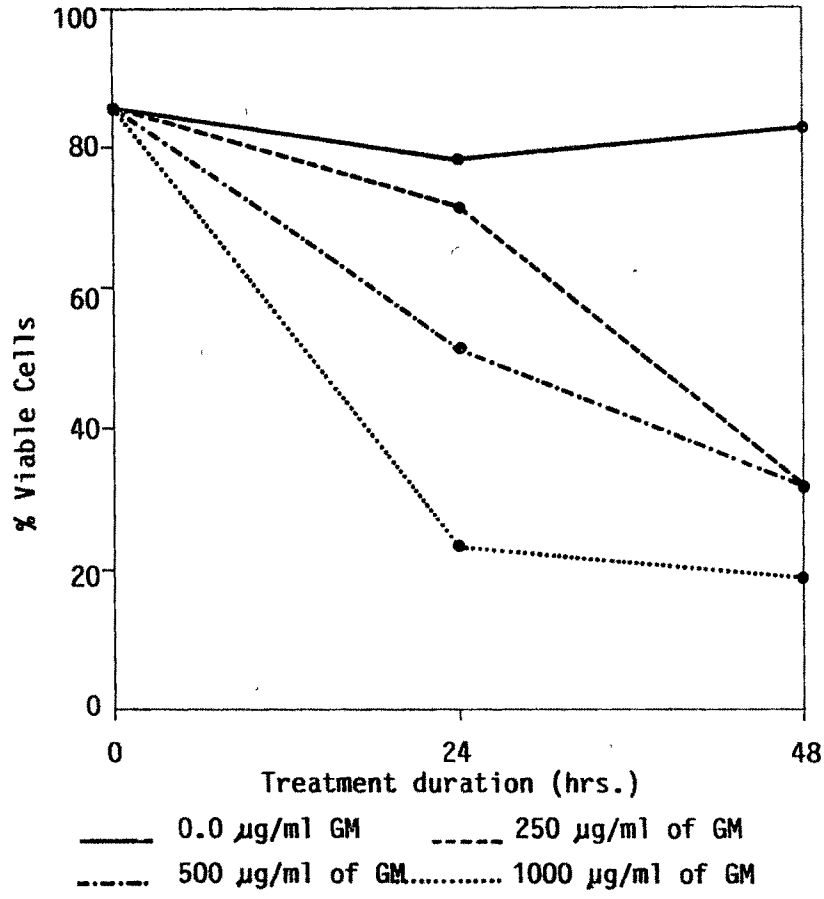


Figure-5

EFFECT OF TREATMENTS WITH NICOTINE PLUS ARECOLINE ON CELL VIABILITY OF CHO CELLS

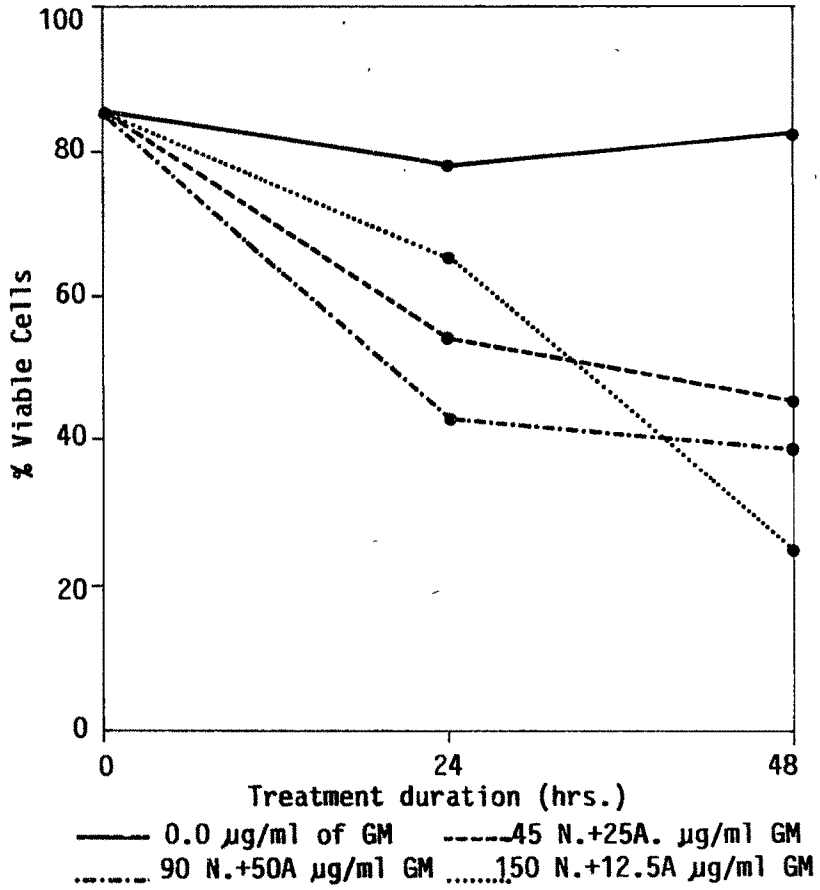


TABLE-3

EFFECT OF CONTINUOUS TREATMENTS WITH TOBACCO EXTRACT ON MITOTIC INDEX OF CHO CELLS

Hours	MMC		concentration µl/ml of GM				
	0.015µg/ml	Control	5	10	15	20	50
0	-	5.2	-	-	-	-	-
3	4.7	4.9	4.0	4.0	3.6	3.4	2.1
6	4.6	5.2	3.8	3.6	-	2.8	1.6
12	2.7	4.7	3.2	3.0	2.9	2.6	1.5
24	1.4	4.8	3.0	2.8	2.6	2.5	1.1
36	0.7	4.1	2.7	2.3	2.5	2.2	0.7

TABLE-4

EFFECT OF CONTINUOUS TREATMENTS WITH NICOTINE ON MITOTIC INDEX OF CHO CELLS

Hours	MMC		concentration µg/ml of GM				
	0.015µg/ml	Control	150	250	500	750	1000
0	-	3.5	-	-	-	-	-
3	3.2	3.3	3.1	3.1	3.0	2.6	2.3
6	2.8	3.1	3.1	3.0	2.6	2.0	1.2
12	2.2	3.8	3.4	3.3	2.6	2.4	1.9
24	1.3	4.3	2.8	2.7	2.0	1.3	0.8
36	0.8	4.4	2.6	2.3	1.9	0.4	0.0

and necrotic cells incresed in cultures treated with higher concentrations and for longer durations (Illustrations 1-7).

Nicotine:

Treatments with N. also affected M.I. in a similar manner (Table-4). The M.I. in untreated cultures varied between 3.1 to 4.4 during 36 hrs. of incubation. Treatment with the lowest concentration, i.e. 150 ug N./ml of GM, lowered the M.I. from 3.1 at 3 hrs. to 2.6 by 36 hrs. A dose dependent mitotic inhibition was clearly evident in the cultures subjected to N. treatments for various durations ranging from 3 to 36 hrs.

Nicotine + Arecoline:

Table-5 describes the effects of combining N. and A. on cell division. There was almost no alteration in M.I. after treating the cells for longer duration with 150N.+5A.ug/ml of GM. However, on keeping nicotine concentration constant, i.e. 150 ug N./ml of GM, and increasing the arecoline concentration, i.e. 12.5 ug and 25 ug A./ml of GM, the mitotic activity of CHO cells was hampered in dose and duration dependant manner.

Experiment 3:

T-ext.:

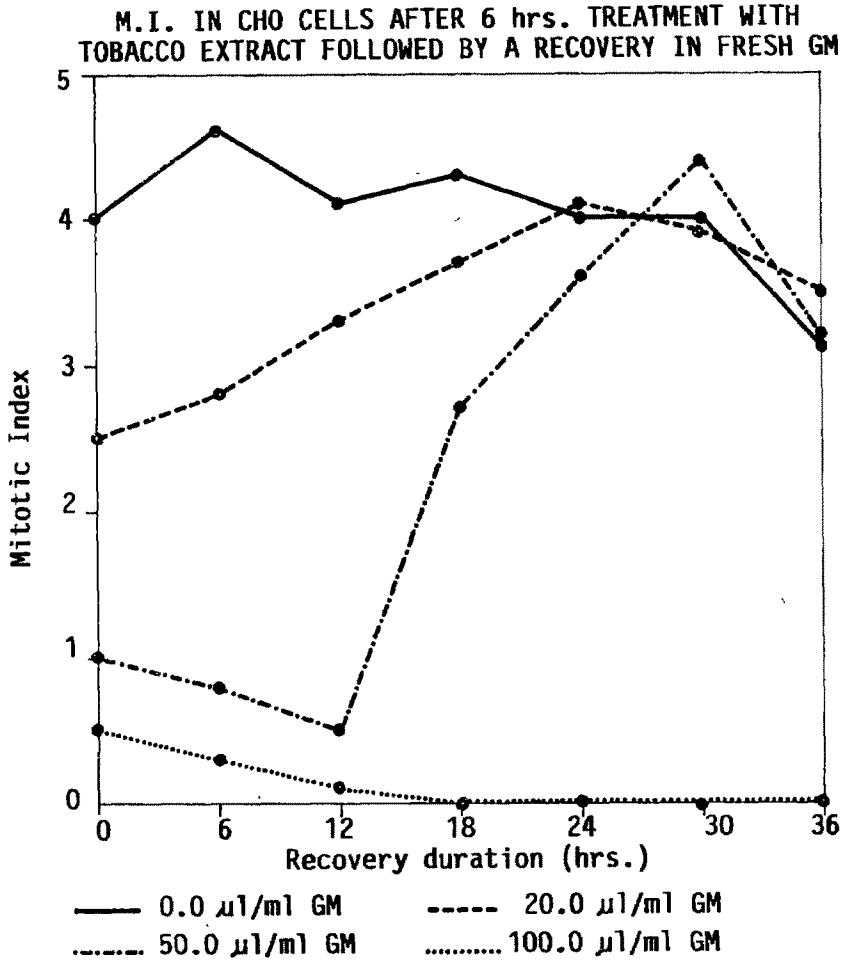
Fig.6 represents the M.I. after a 6 hr. treatment with T-ext. followed by a recovery for various durations in extract free fresh GM. There was a clear diminution in M.I. with three different concentrations tested, viz. from 4.0 in control cultures to 2.5, 1.0 and 0.5 in cultures treated with

TABLE-5

EFFECT OF CONTINUOUS TREATMENTS WITH NICOTINE + ARECOLINE ON MITOTIC INDEX OF CHO CELLS

Hours	MMC		concentration µg/ml of GM		
	0.015µg/ml	Control	150N.+5A.	150N.+12.5A.	150N.+25A.
0	-	4.6	-	-	-
3	4.5	4.6	4.4	4.0	3.9
6	4.1	4.6	4.7	3.8	2.5
12	2.8	4.9	4.4	2.8	2.3
18	2.0	4.5	3.6	3.1	2.2
24	1.3	4.6	3.2	2.9	-

Figure-6


M.I. IN CHO CELLS AFTER 6 hrs. TREATMENT WITH TOBACCO EXTRACT FOLLOWED BY A RECOVERY IN FRESH GM
Mitotic Index
0
1
2
3
4
5
0
6
12
18
24
30
36
Recovery duration (hrs.)
0.0 µl/ml GM
20.0 µl/ml GM
50.0 µl/ml GM
100.0 µl/ml GM

20 µl, 50 µl and 100 µl T-ext./ml of GM, respectively. Mitotic activity was gradually restored when the cells were recovered for various durations, e.g. for 20 µl T-ext./ml of GM treatment, there was an increase from 2.5 to 4.1 by 24 hrs., which was comparable to the M.I. of control cultures. Similarly, the cultures treated with 50 µl T-ext./ml of GM restored the dividing activity after 30 hrs. However, the dose of 100 µl T-ext./ml of GM appeared to be too toxic and the cells failed to recover from the extract induced damage.

Nicotine:

Observations of recovery experiment after nicotine treatment are graphically presented in Fig.7. As can be seen from the line graph, the M.I. of cultures treated with 500 µg N./ml of GM was comparable to the control value by 12 hrs. of recovery, whereas, the cultures treated with higher concentrations required 30 hrs. of recovery in fresh GM for restoring their normal mitotic activity.

Nicotine + Arecoline:

Fig.8 details the M.I. observed after 6 hrs. treatment with different combinations of N. and A., followed by a recovery in fresh GM. For the cultures treated with 90 N.+50 A. µg/ml of GM, the M.I. was comparable to that of untreated cultures by 12 hrs. recovery, whereas, the cultures treated with higher concentrations required 24 hrs. to restore the normal mitotic activity.

Figure-7

M.I. IN CHO CELLS AFTER 6 hrs. TREATMENT WITH NICOTINE FOLLOWED BY A RECOVERY IN FRESH GM

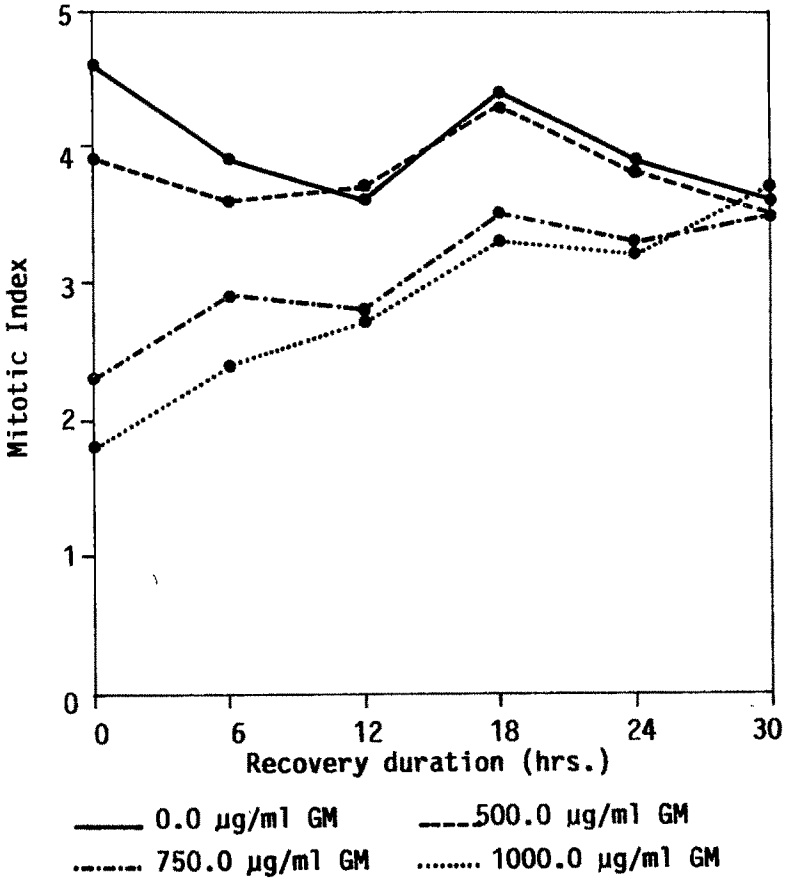
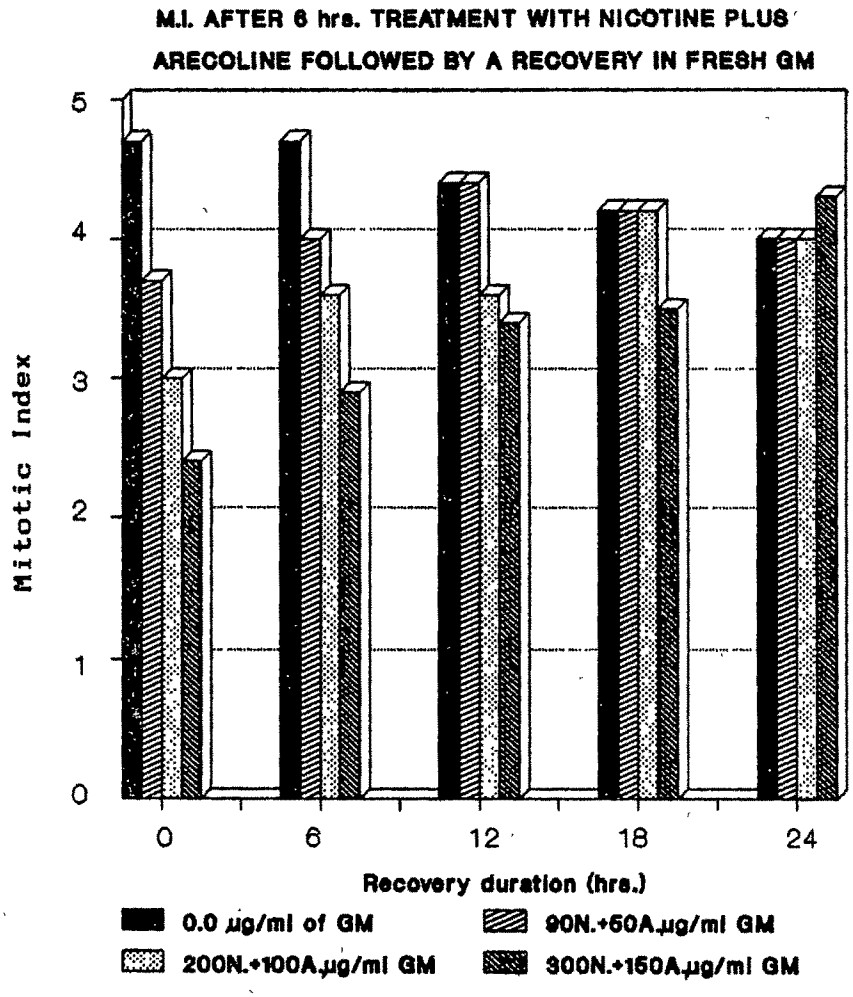


FIGURE-8


M.I. AFTER 6 hrs. TREATMENT WITH NICOTINE PLUS
ARECOLINE FOLLOWED BY A RECOVERY IN FRESH GM
Mitotic Index
0
1
2
3
4
5
0
6
12
18
24
Recovery duration (hrs.)
0.0 µg/ml of GM
90N.+50A.µg/ml GM
200N.+100A.µg/ml GM
300N.+150A.µg/ml GM

Experiment 4:

T-ext.:

Frequency of % MNC observed in the cultures treated with two different concentrations of T-ext. for 6 hrs., followed by a recovery in extract free GM for 12 hrs. and 24 hrs., is shown in Fig.9. The value of % MNC was 0.5 % in control cultures and 1.6 % and 1.9 %, respectively, in 20 µl and 50 µl T-ext./ml of GM treated cultures at 12 hr. and 0.7 %, 2.0 % and 3.4 % at 24 hr. sampling times.

Nicotine:

Fig.10 shows the % MNC scored in cultures treated for 6 hrs. with 500 µg, 750 µg and 1000 µg N./ml of GM and recovered in nicotine free GM. Treatment with 500 µg N./ml of GM, had no effect on MNC frequency, whereas, 2.5 % MNC were observed in cultures treated with 1000 µg N./ml of GM at 24 hrs. which was more than the double the value obtained for control cultures, i.e. 0.9 % .

Nicotine + Arecoline:

Fig.11 shows the % MNC scored in cultures treated for 6 hrs. with combination of N. and A. By 12 hrs. of recovery, the frequency of MNC increased from 0.6 % in untreated cultures to 1.2 %, 1.6 % and 1.8 % in cultures treated with 90 N.+50 A.µg, 200 N.+100 A. µg and 300 N.+150 A. µg/ml of GM, respectively. Following 24 hrs. of recovery, the % MNC, among the cultures treated with 90 N.+50 A. µg/ml of GM, was double than that among untreated cultures. The values of % MNC were

FIGURE-9

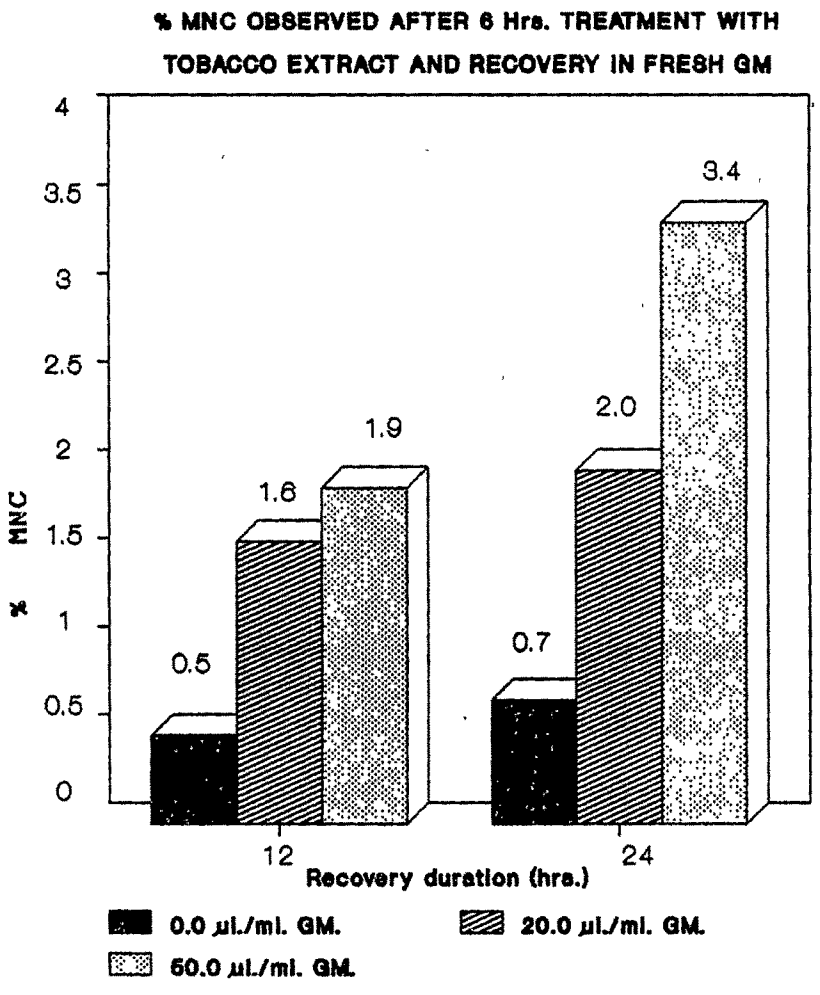

% MNC OBSERVED AFTER 6 Hrs. TREATMENT WITH TOBACCO EXTRACT AND RECOVERY IN FRESH GM
% MNC
4
3.5
3
2.5
2
1.5
1
0.5
0
0.5
1.6
1.9
0.7
2.0
3.4
12
24
Recovery duration (hrs.)
0.0 µl./ml. GM.
20.0 µl./ml. GM.
50.0 µl./ml. GM.

FIGURE-10

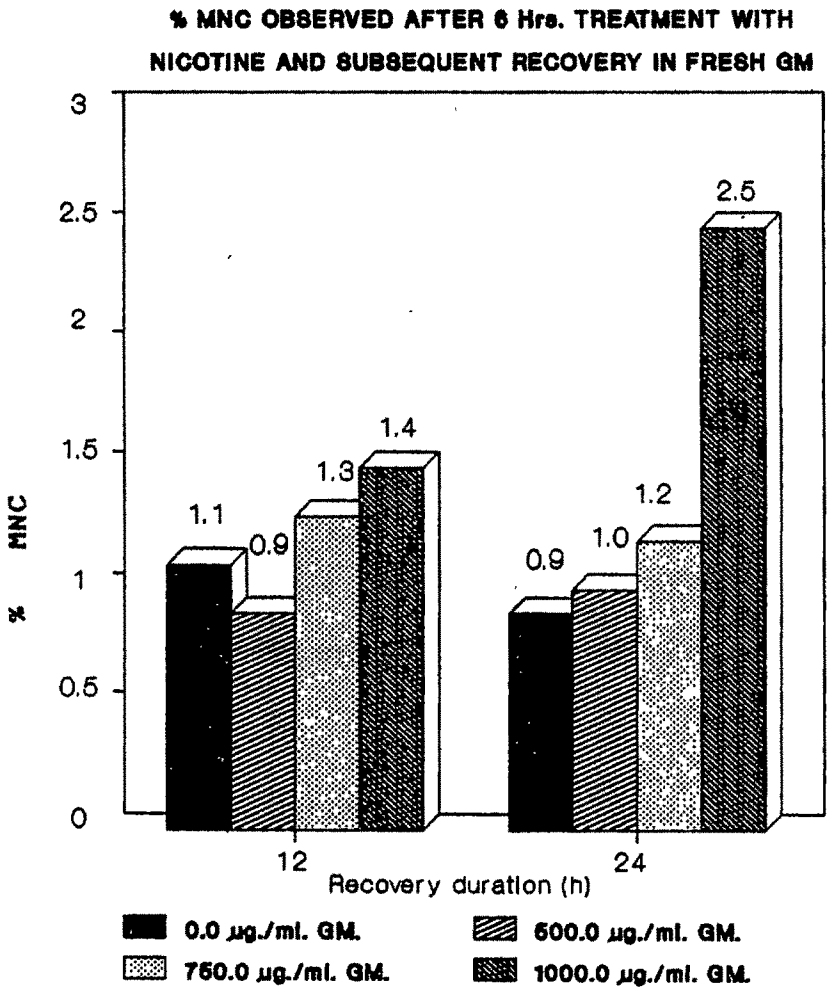
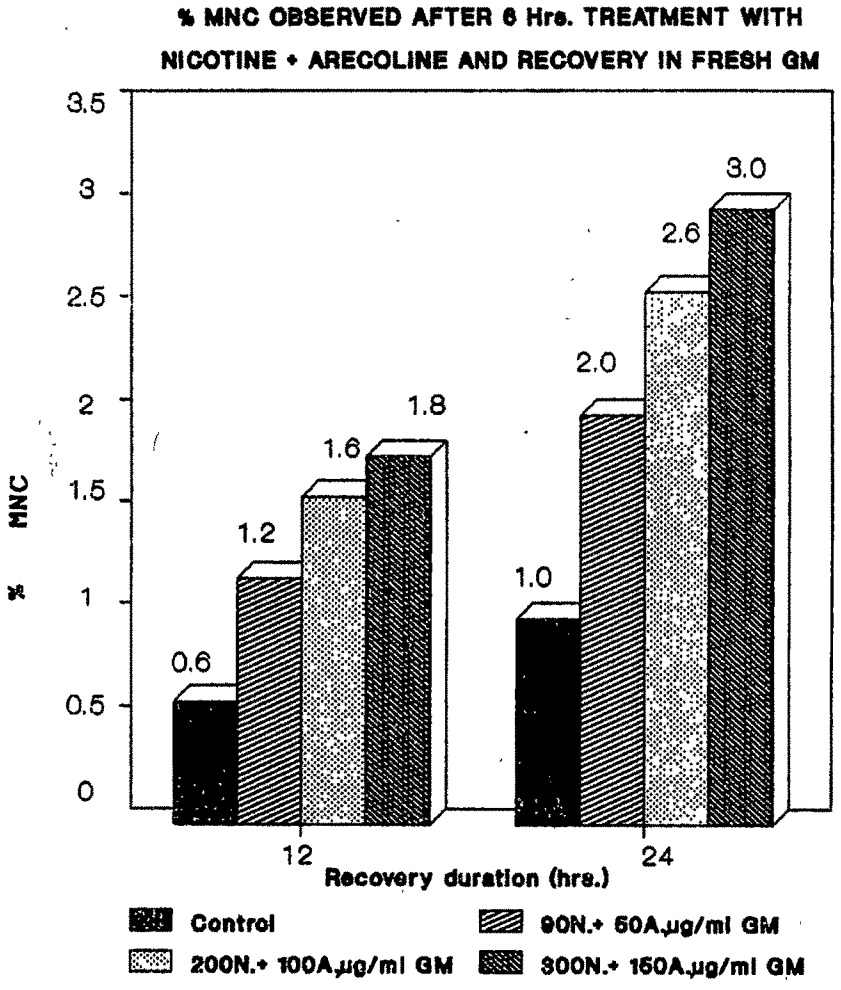

% MNC OBSERVED AFTER 6 Hrs. TREATMENT WITH NICOTINE AND SUBSEQUENT RECOVERY IN FRESH GM
% MNC
3
2.5
2
1.5
1
0.5
0
1.1
0.9
1.3
1.4
0.9
1.0
1.2
2.5
12
24
Recovery duration (h)
0.0 µg./ml. GM.
500.0 µg./ml. GM.
750.0 µg./ml. GM.
1000.0 µg./ml. GM.

FIGURE-11


% MNC OBSERVED AFTER 6 Hrs. TREATMENT WITH
NICOTINE + ARECOLINE AND RECOVERY IN FRESH GM
% MNC
3.5
3
2.5
2
1.5
1
0.5
0
0.6
1.2
1.6
1.8
1.0
2.0
2.6
3.0
12
24
Recovery duration (hrs.)
Control
90N.+ 50A.µg/ml GM
200N.+ 100A.µg/ml GM
300N.+ 150A.µg/ml GM

higher among the cultures treated with higher concentrations.

Experiment 5:

T-ext.:

Table-6 details the types and number of CAs induced following the continuous treatment with four different concentrations of T-ext. for 24 hrs. Illustrations 8-11 exhibit some of the CAs observed after the treatment with test-substance. The number of aberrant metaphases increased from 6 % in control cultures to 12 %, 22 %, 26 % and 27 % in cultures treated with 5 µl, 10 µl, 15 µl and 20 µl T-ext./ml of GM. Consequently, an increase in mean CA/cell value was also observed with an increase in the concentration of T-ext., however, a statistical significance, **($p < 0.01$ - with gaps and $p < 0.02$ - without gaps)**, was observed only for the cultures treated with 15 µl and 20 µl T-ext./ml of GM.

Nicotine:

The results of the continuous N. treatment on CA frequency are provided in Table-7. Cultures, treated with 375 µg, 500 µg or 625 µg N./ml of GM, showed statistically significant elevation in CA frequencies compared to that of the control cultures.

Nicotine + Arecoline:

As can be seen from Table-8, the cultures treated with different combination of N. and A. showed a dose dependent increase in the number of aberrant metaphases as well as CA/cell values. The increase in CA frequency by 150 µg N. +

TABLE-6

EFFECT OF CONTINUOUS TREATMENT WITH TOBACCO EXTRACT

ON CA FREQUENCY IN CHO CELLS

Concentration µl/ml of GM	Aberrant cells (%)	Chromatid-type			Chromosome-type					CA/cell ± S.E.
		G	B/F	I	G	B	Dm	R	Dc	
Control	06	4	5	-	-	1	-	-	-	0.10 ± 0.0436
5	12	5	8	-	1	-	1	-	2	0.17 ± 0.0530
10	22	11	8	-	1	1	3	-	3	0.27 ± 0.0563 B
15	26	10	6	1	-	2	6	1	6	0.32 ± 0.0614 C,b
20	27	15	16	-	1	3	1	1	2	0.39 ± 0.0835 C,b
MMC 0.007 µg	22	16	11	-	3	1	-	2	3	0.36 ± 0.0660 C,b

where,

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

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

B = $p < 0.02$ C = $p < 0.01$ (including gaps)

b = $p < 0.02$ (excluding gaps)

TABLE-7

EFFECT OF CONTINUOUS TREATMENT WITH NICOTINE

ON CA FREQUENCY IN CHO CELLS

Concentration µg/ml of GM	Aberrant cells (%)	Chromatid-type			Chromosome-type					CA/cell ± S.E.	
		G	B/F	I	G	B	Dm	R	Dc		
Control	05	2	1	-	3	-	-	-	-	0.06 ± 0.0276	
150	06	3	1	-	2	-	-	-	-	0.06 ± 0.0237	
250	11	5	3	1	3	-	-	-	-	0.12 ± 0.0354	
375	16	5	6	-	4	1	-	1	-	0.17 ± 0.0401	B
500	21	13	8	-	6	2	-	-	-	0.29 ± 0.0653	D
625	25	15	10	-	4	1	-	-	1	0.31 ± 0.0595	D
MMC 0.007	18	8	4	1	4	-	-	2	3	0.22 ± 0.0450	C

where,

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

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

B = $p < 0.02$; C = $p < 0.01$; D = $p < 0.001$ (including gaps)

TABLE-8

EFFECT OF CONTINUOUS TREATMENT WITH NICOTINE + ARECOLINE

ON CA FREQUENCY IN CHO CELLS

Concentration µg/ml of GM	Aberrant cells (%)	Chromatid-type			Chromosome-type					CA/cell ± S.E.
		G	B/F	I	G	B	Dm	R	Dc	
Control	07	4	2	-	-	-	2	-	-	0.08 ± 0.0306
150 N.	08	6	-	-	2	-	-	-	-	0.08 ± 0.0271
150 N.+2 A.	09	6	-	-	3	2	-	-	-	0.11 ± 0.0371
150 N.+5 A.	12	6	3	-	3	-	-	-	-	0.12 ± 0.0325
150 N.+12.5 A.	18	11	3	-	3	-	-	-	1	0.18 ± 0.0384 A
MMC 0.007	20	8	5	2	4	2	-	2	3	0.26 ± 0.0530 A

where,

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

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

A = $p < 0.05$ (including gaps)

12.5 µg A./ml of GM was statistically significant compared to that of the 150 µg N. treated cultures **($p < 0.05$)**.

Experiment 6:

T-ext.:

The details of SCE frequencies observed following the continuous treatment with T-ext. are provided in Table-9. Illustrations 12-14 show examples of metaphases with low and high SCEs observed in untreated cultures or in cultures treated with test-substance, respectively. The cultures, treated with 5 µl, 10 µl, 15 µl and 20 µl T-ext./ml of GM, yielded the mean values of 6.88, 7.60, 8.32 and 9.40 SCE/cell, respectively. The SCE elevations in T-ext. exposed cultures were statistically significant compared to 5.72 SCE/cell value for control cultures **($p < 0.02$ for 5ul T-ext./ml of GM and $p < 0.001$ for 10 µl, 15 µl and 20 µl T-ext./ml of GM)**. The distribution of metaphases according to the number of SCEs clearly indicated an increase in number of metaphases with higher SCEs, with a concurrent increase in the concentrations of T-ext.

Nicotine:

The details of the effects of continuous N. treatment on SCE frequency are provided in Table-10. The mean SCE frequencies ranged from 7.76 to 11.32 in cultures treated with 150 µg to 625 µg N./ml of GM. These values were significantly higher **($p < 0.05$ for 150 µg/ml of GM and $p < 0.001$ for the rest of the concentrations)** compared to that of the control

TABLE-9

EFFECT OF CONTINUOUS TREATMENT WITH TOBACCO EXTRACT

ON SCE FREQUENCY IN CHO CELLS

Concentration µl/ml of GM	Range	% metaphases with				SCE/cell ± S.E.
		0-5	6-10	11-15	>15 SCEs	
Control	3 - 8	48	52	00	00	5.72 ± 0.318
5	5 - 10	32	68	00	00	6.88 ± 0.322 B
10	5 - 11	08	88	04	00	7.60 ± 0.320 D
15	5 - 14	04	80	16	00	8.32 ± 0.437 D
20	7 - 17	00	80	08	12	9.40 ± 0.570 D
MMC 0.007 ug	20 - 40	00	00	00	100	36.08 ± 0.880 D

where,

B = $p < 0.02$; D = $p < 0.001$

TABLE-10

EFFECT OF CONTINUOUS TREATMENT WITH NICOTINE

ON SCE FREQUENCY IN CHO CELLS

Concentration µg/ml of GM	Range	% metaphases with				SCE/cell ± S.E.
		0-5	6-10	11-15	>15 SCEs	
Control	3 - 11	32	64	04	00	6.48 ± 0.392
150	4 - 14	08	80	12	00	7.76 ± 0.464 A
250	6 - 12	00	88	12	00	8.84 ± 0.320 D
375	4 - 14	04	60	36	00	9.64 ± 0.496 D
500	7 - 18	00	68	24	08	10.52 ± 0.569 D
625	7 - 21	00	44	52	04	11.32 ± 0.551 D
MMC 0.007	24 - 45	00	00	00	100	37.56 ± 0.780 D

where,

A = $p < 0.05$; D = $p < 0.001$

cultures (6.48 $\pm$ 0.392).

Nicotine + Arecoline:

An increase in mean SCE frequency after the treatment with 150 ug N.+ 2 µg A./ml of GM was not statistically significant compared to that of cultures treated with 150 µg N./ml of GM (Table-11). A statistically significant dose dependent increase in mean SCE/cell value was observed on combining 150 µg N. with two higher concentrations of A. **($p < 0.05$ and $p < 0.001$, for 150 µg N.+ 5 µg A./ml of GM and 150 µg N.+ 12.5 µg A./ml of GM, respectively).**

Experiment 7:

T-ext.:

The data presented in Table-12 describe the effects of a short duration treatment of T-ext. and a recovery in extract free GM for 24 hrs. on CA frequencies. The induction in CA/cell value was dose dependent, however, the elevations were statistically significant only for the cultures treated with 50 µl T-ext./ml of GM **($p < 0.01$).**

Nicotine and Nicotine + Arecoline:

Similarly, the increase in CA/cell value was found to be statistically significant **($p < 0.05$)** only for the cultures treated with 1000 µg N./ml of GM (Table-13). As evident from Table-14, treatment with all the three combinations of N. and A. increased the CA rates, which were significantly higher compared to the ones obtained for the control cultures.

TABLE-11

EFFECT OF CONTINUOUS TREATMENT WITH NICOTINE + ARECOLINE

ON SCE FREQUENCY IN CHO CELLS

Concentration µg/ml of GM	Range	% metaphases with				SCE/cell ± S.E.
		0-5	6-10	11-15	>15 SCEs	
Control	3 - 8	60	40	00	00	5.20 ± 0.259
150 N.	4 - 14	08	80	12	00	7.76 ± 0.464
150 N.+2 A.	6 - 13	00	84	16	00	8.76 ± 0.355
150 N.+5 A.	7 - 13	00	92	08	00	8.96 ± 0.250 A
150 N.+12.5 A.	7 - 13	00	68	32	00	9.76 ± 0.295 D
MMC 0.007	20 - 40	00	00	00	100	36.56 ± 0.895 D

where,

A = $p < 0.05$; D = $p < 0.001$

TABLE-12

EFFECT OF SHORT TERM TREATMENTS WITH TOBACCO EXTRACT FOLLOWED BY 24 hrs. RECOVERY IN FRESH GM ON CA FREQUENCY IN CHO CELLS

Concentration µl/ml of GM	Aberrant cells (%)	Chromatid-type			Chromosome-type					CA/cell ± S.E.
		G	B/F	I	G	B	Dm	R	Dc	
Control	06	4	1	-	-	1	-	-	-	0.06 ± 0.0276
2 hr. treatment										
20	08	3	1	-	2	1	-	1	-	0.08 ± 0.0271
50	20	9	6	1	6	-	-	-	-	0.22 ± 0.0460 C
4 hr. treatment										
20	09	3	3	-	5	1	-	-	2	0.14 ± 0.0490
50	21	13	6	-	6	1	-	-	1	0.27 ± 0.0563 D
MMC 0.03 µg	24	8	8	1	2	4	-	2	1	0.26 ± 0.0480 D

where,

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

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

C = $p < 0.01$; D = $p < 0.001$ (including gaps)

TABLE-13

EFFECT OF SHORT TERM TREATMENTS WITH NICOTINE FOLLOWED BY 24 hrs. RECOVERY IN FRESH GM ON CA FREQUENCY IN CHO CELLS

Concentration µg/ml of GM	Aberrant cells (%)	Chromatid-type			Chromosome-type					CA/cell ± S.E.
		G	B/F	I	G	B	Dm	R	Dc	
Control	07	2	3	-	3	-	-	-	-	0.08 ± 0.0306
2 hr. treatment										
625	11	3	8	-	2	1	-	1	3	0.18 ± 0.0606
1000	15	9	6	-	1	1	2	1	2	0.22 ± 0.0576 A
4 hr. treatment										
625	13	7	1	-	8	1	-	1	2	0.20 ± 0.0566
1000	16	7	7	-	7	1	-	1	-	0.23 ± 0.0630 A
MMC 0.03	21	10	7	1	2	1	-	-	2	0.23 ± 0.0470 C

where,

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

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

A = $p < 0.05$; C = $p < 0.01$ (including gaps)

TABLE-14

EFFECT OF A 3 hr. TREATMENT WITH NICOTINE + ARECOLINE FOLLOWED BY 24 hrs. RECOVERY IN FRESH GM ON CA FREQUENCY IN CHO CELLS

Concentration µg/ml of GM	Aberrant cells (%)	Chromatid-type			Chromosome-type					CA/cell ± S.E.
		G	B/F	I	G	B	Dm	R	Dc	
Control	08	4	4	-	1	1	-	-	-	0.10 ± 0.0306
90 N.+50 A.	18	5	6	-	6	5	-	1	2	0.25 ± 0.0589 A
200 N.+100 A.	28	12	11	3	6	5	-	2	4	0.43 ± 0.0828 D,c
300 N.+150 A.	35	9	20	1	2	5	1	-	7	0.45 ± 0.0669 D,d
MMC 0.03	21	10	8	1	2	2	-	-	2	0.25 ± 0.0530 D

where,

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

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

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

c = $p < 0.01$; d = $p < 0.001$ (excluding gaps)

Experiment 8:

T-ext.:

The data presented in Table-15 summarize the SCE frequencies after a short term treatment with T-ext. and a recovery in T-ext. free GM. Cultures treated with 20 µl T-ext./ml GM for 2 hrs. and 4 hrs. had 6.68 ± 0.238 and 7.80 ± 0.240 SCE/cell (mean ± SE), respectively, and for cultures treated with 50 µl T-ext./ml of GM, the mean values were 8.40 ± 0.265 and 9.52 ± 0.316, respectively. The elevations in SCE frequencies by all the concentrations of T-ext. were statistically significant ($p < 0.001$) compared to 5.52 ± 0.227 (mean ± SE) SCE/cell in control cultures.

Nicotine and Nicotine + Arecoline:

Similar results were obtained when cultures were treated either with only nicotine for 2 hrs. and 4 hrs. or different combinations of nicotine and arecoline for 4 hrs. (Table-16 or Table-17, respectively).

Experiment 9:

The labelling index observed after the treatment with T-ext. N. and N.+A. for 24 hrs. are presented in Fig.12, Fig.13 and Fig.14, respectively. Illustrations 15-16 represent interphase cells observed after autoradiography in untreated and treated cultures, respectively. A dose dependent reduction in the number of DNA synthesizing cells was observed following the treatment with T-ext., N. or N.+A. The labelling indices were reduced from 65.15 % in control cultures to 49.30 %,

TABLE-15

EFFECT OF SHORT TERM TREATMENTS WITH TOBACCO EXTRACT FOLLOWED BY 48 hrs. RECOVERY IN FRESH GM ON SCE FREQUENCY IN CHO CELLS

Concentration µl/ml of GM	Range	% metaphases with				SCE/cell ± S.E.
		0-5	6-10	11-15	>15 SCEs	
Control	3 - 7	40	60	00	00	5.52 ± 0.227
2 hr. treatment						
20	5 - 9	12	88	00	00	6.68 ± 0.238 D
50	6 - 10	00	100	00	00	8.40 ± 0.265 D
4 hr. treatment						
20	6 - 11	00	96	04	00	7.80 ± 0.240 D
50	7 - 14	00	72	28	00	9.52 ± 0.316 D
MMC 0.03	17 - 28	00	00	00	100	20.81 ± 0.512 D

where,

D = $p < 0.001$

TABLE-16

EFFECT OF SHORT TERM TREATMENTS WITH NICOTINE FOLLOWED BY 48 hrs. RECOVERY IN FRESH GM ON SCE FREQUENCY IN CHO CELLS

Concentration µg/ml of GM	Range	% metaphases with				SCE/cell ± S.E.
		0-5	6-10	11-15	>15 SCEs	
Control	4 - 9	40	60	00	00	6.12 ± 0.261
2 hr. treatment						
625	5 - 12	04	80	16	00	8.40 ± 0.379 D
1000	5 - 14	04	68	28	00	9.56 ± 0.460 D
4 hr. treatment						
625	5 - 15	04	76	20	00	9.04 ± 0.465 D
1000	7 - 17	00	64	28	08	10.68 ± 0.518 D
MMC 0.03	16 - 26	00	00	00	100	20.52 ± 0.495 D

where,

D = $p < 0.001$

TABLE-17

EFFECT OF 3 hr. TREATMENT WITH NICOTINE + ARECOLINE FOLLOWED BY 48 hrs. RECOVERY IN FRESH GM ON SCE FREQUENCY IN CHO CELLS

Concentration µg/ml of GM	Range	% metaphases with				SCE/cell ± S.E.
		0-5	6-10	11-15	>15 SCEs	
Control	3 - 9	40	60	00	00	5.72 ± 0.351
90 N.+50 A.	6 - 15	00	76	24	00	9.16 ± 0.511 D
200 N.+100 A.	8 - 15	00	48	52	00	10.68 ± 0.395 D
300 N.+150 A.	10 - 19	00	04	60	36	14.92 ± 0.542 D
MMC 0.03	17 - 28	00	00	00	100	21.92 ± 0.585 D

where,

D = $p < 0.001$

FIGURE-12

% LABELLED CELLS OBSERVED AFTER 24 hrs.
TREATMENT WITH TOBACCO EXTRACT ON CHO CELLS

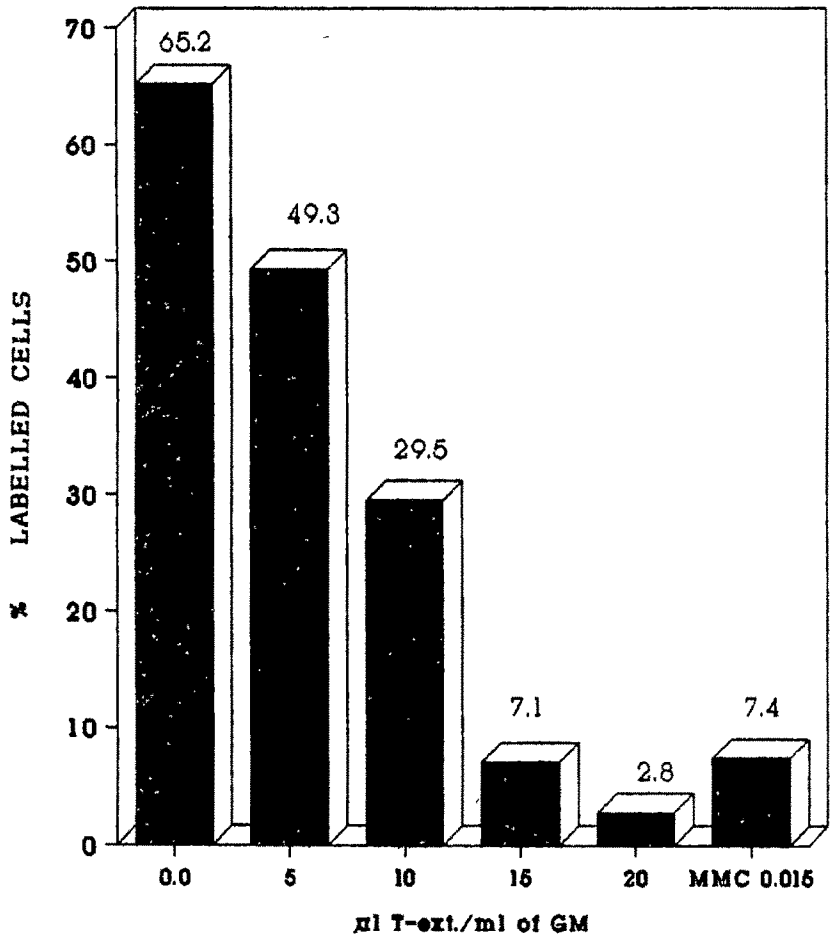


FIGURE-13

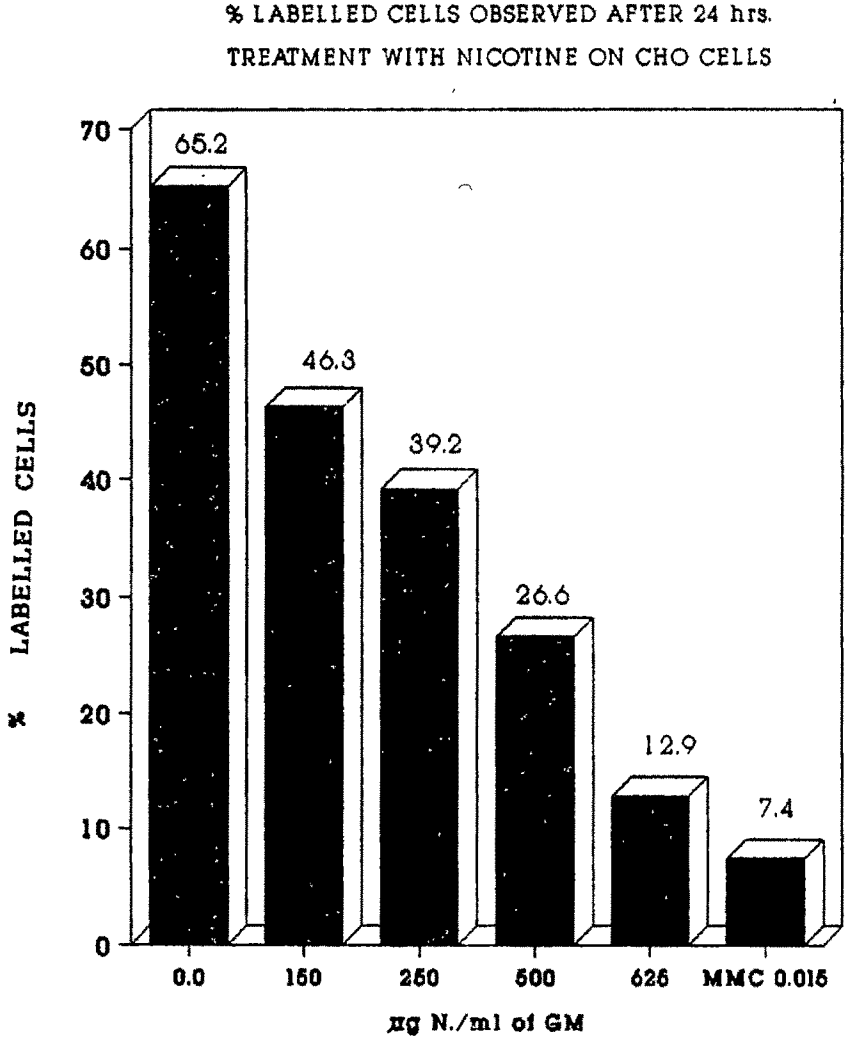
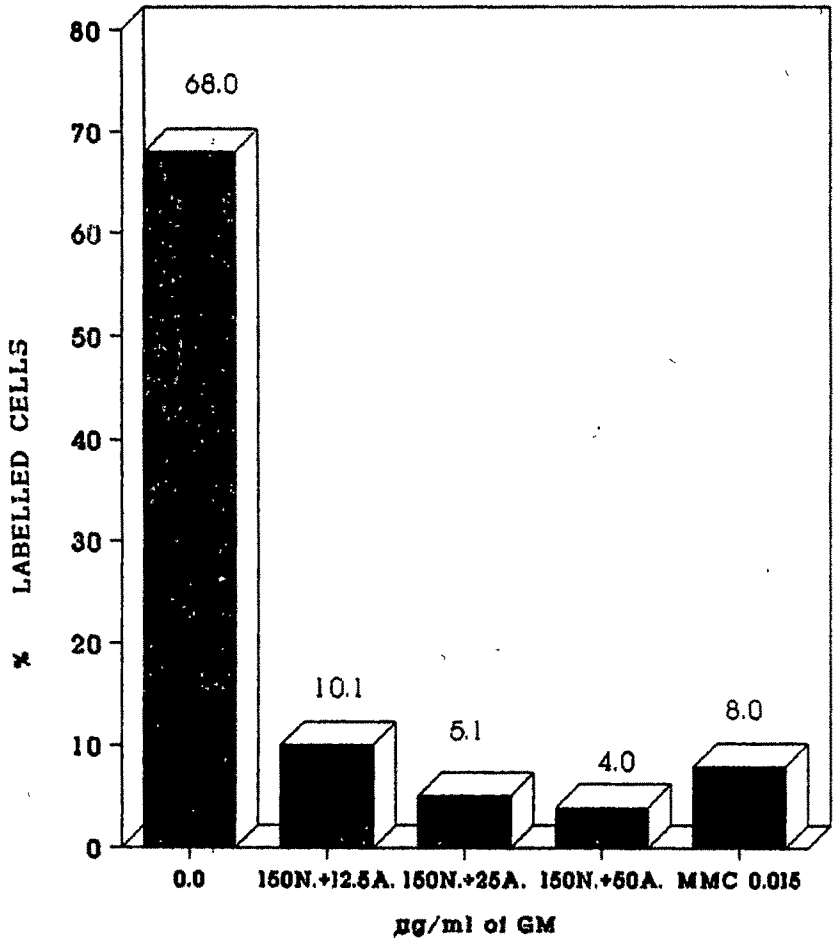

% LABELLED CELLS OBSERVED AFTER 24 hrs.
TREATMENT WITH NICOTINE ON CHO CELLS
% LABELLED CELLS
70
60
50
40
30
20
10
0
65.2
46.3
39.2
26.6
12.9
7.4
0.0
150
250
500
625
MMC 0.015
µg N./ml of GM

FIGURE-14

% LABELLED CELLS OBSERVED AFTER 24 hrs.
TREATMENT WITH NICOTINE + ARECOLINE ON CHO CELLS



Illustrations 1-7 Photomicrographs exhibiting some of the abnormalities observed in the treated cultures.

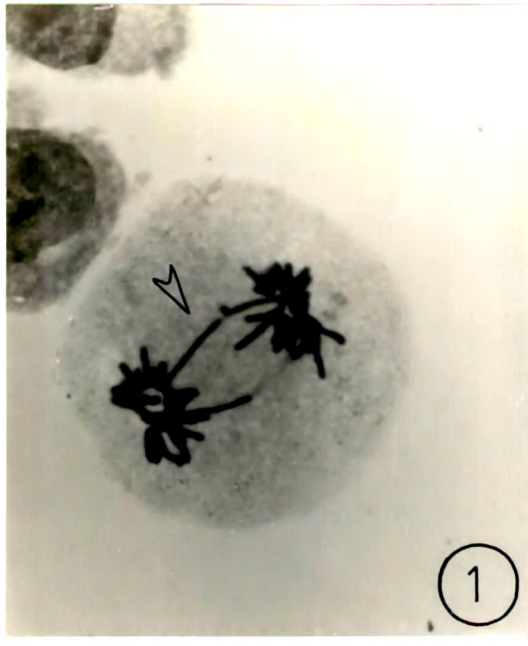
1 Anaphase showing chromatin bridge.

2 Anaphase with lagging chromosome.

3 Tripolar anaphase with lagging chromosome.

4 Degenerating nuclei seen after the treatment with high concentrations of test-substance.

Magnification: 1400x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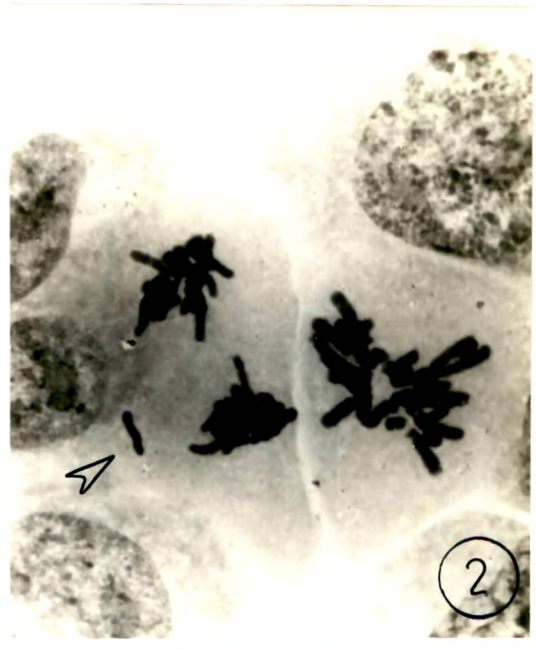
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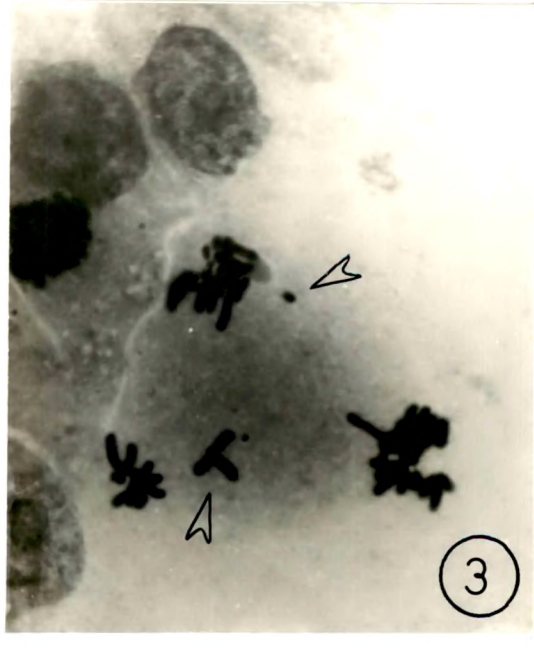
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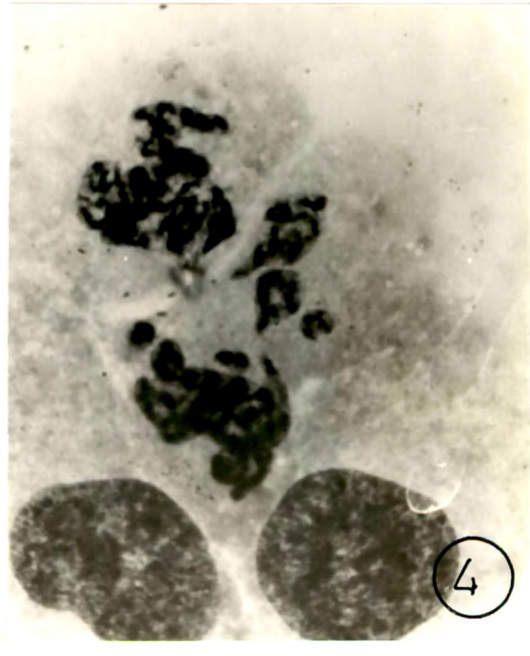
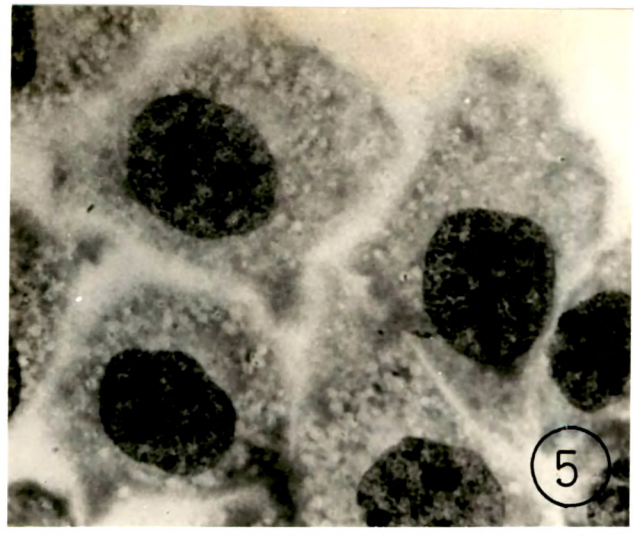

4

Illustration	5	Normal CHO cells.
	6	A cell with one macronucleus and two micronuclei.
	7	A cell with one micronucleus.
Magnification:	5	1250x
	6-7	1400x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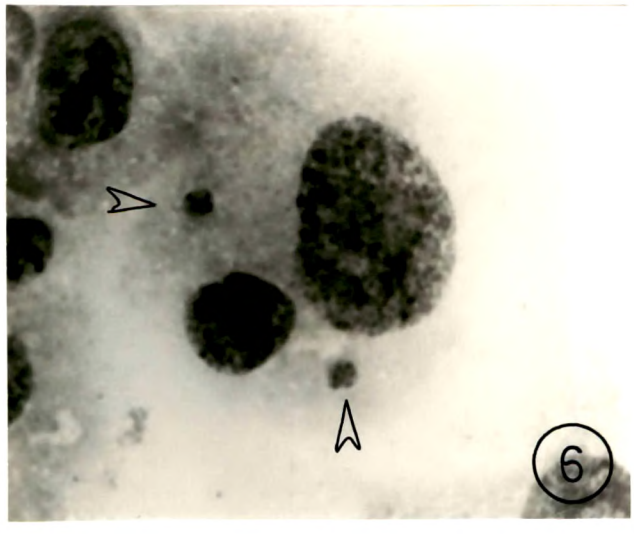
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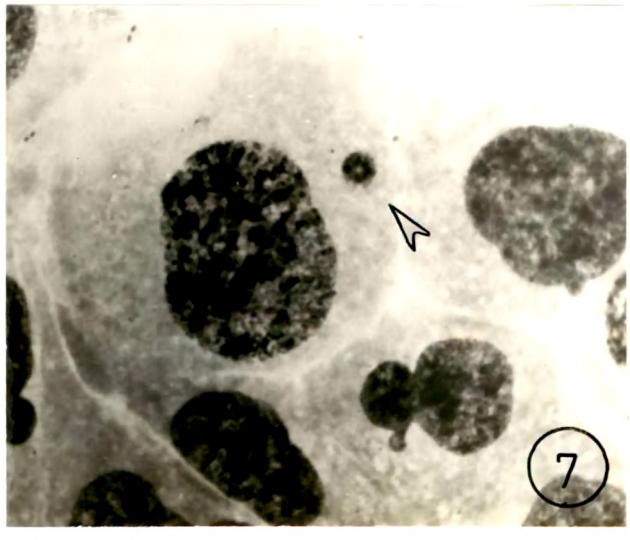

7

Illustrations 8-11 Photomicrographs showing some of the aberrant metaphases.

8 Metaphase with chromatid-gap.

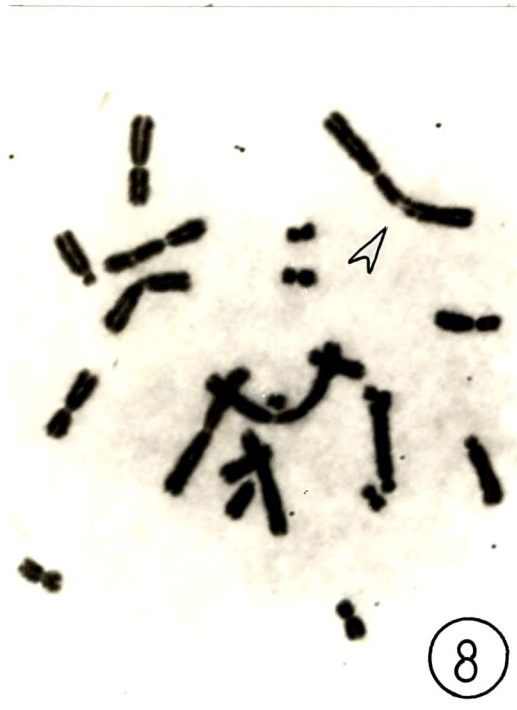
9 Metaphase with chromosome-gap.

10 Metaphase with chromatid-break.

11 Metaphase with ring chromosome.

Magnification: 8-9 1600x

10-11 1400x



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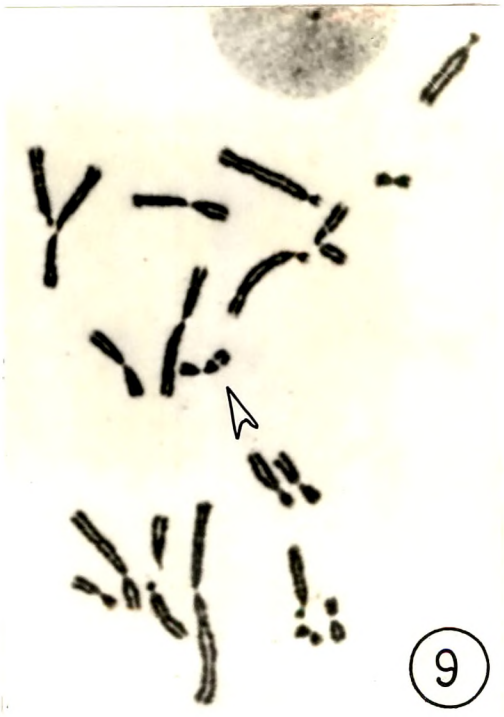

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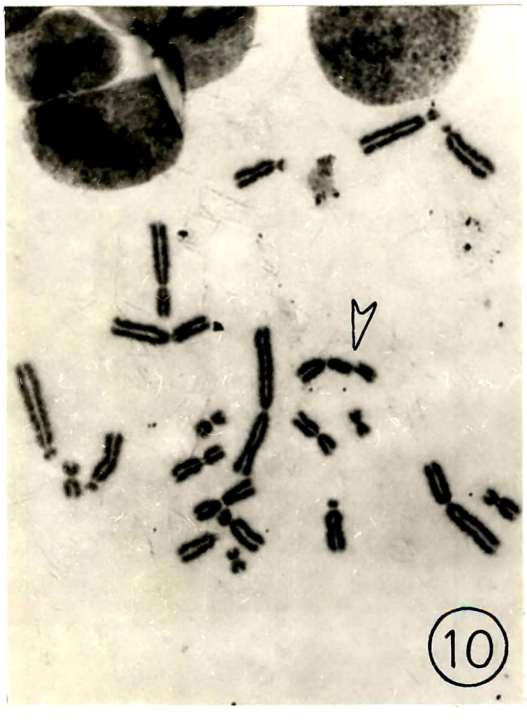

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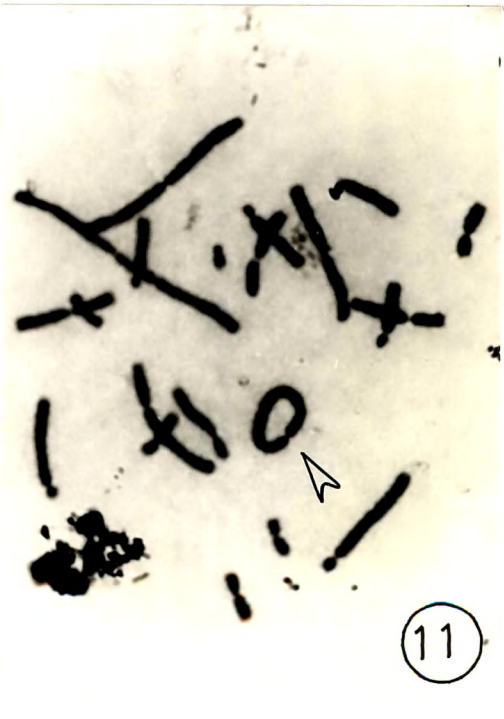
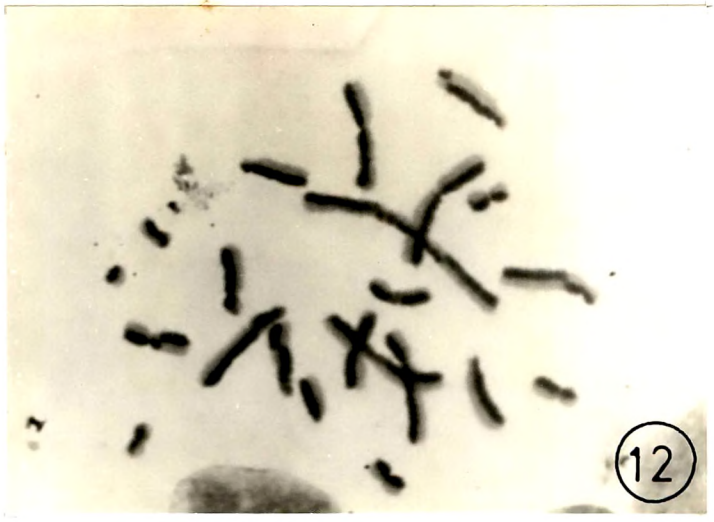


11

Illustrations	12-14	Sister chromatid exchanges in CHO cells.
	12	A metaphase from an untreated culture showing 6 SCEs.
	13-14	Metaphases from treated cultures showing 15 SCEs and 9 SCEs.
Magnification:	12-13	1600x
	14	1400x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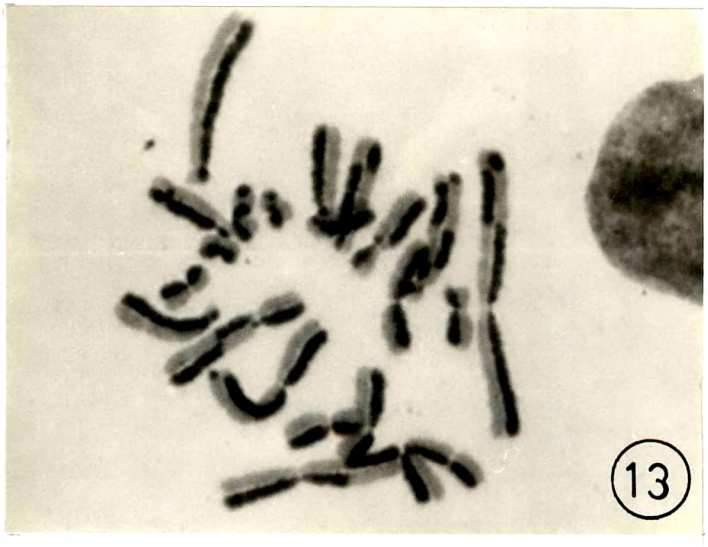
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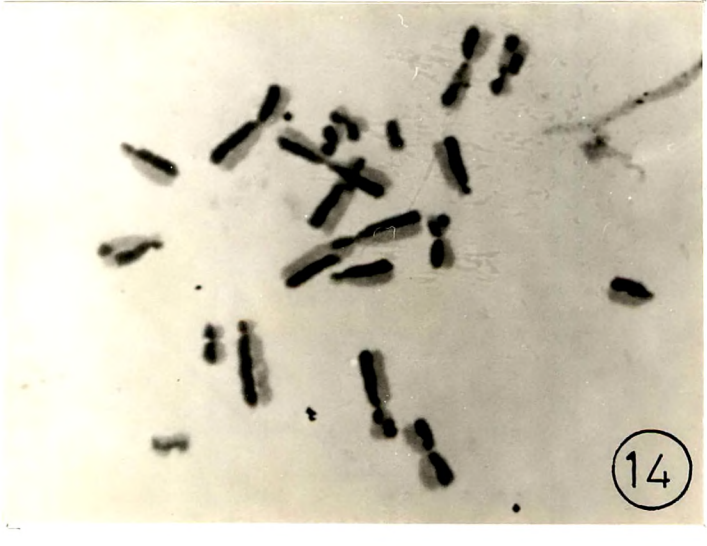
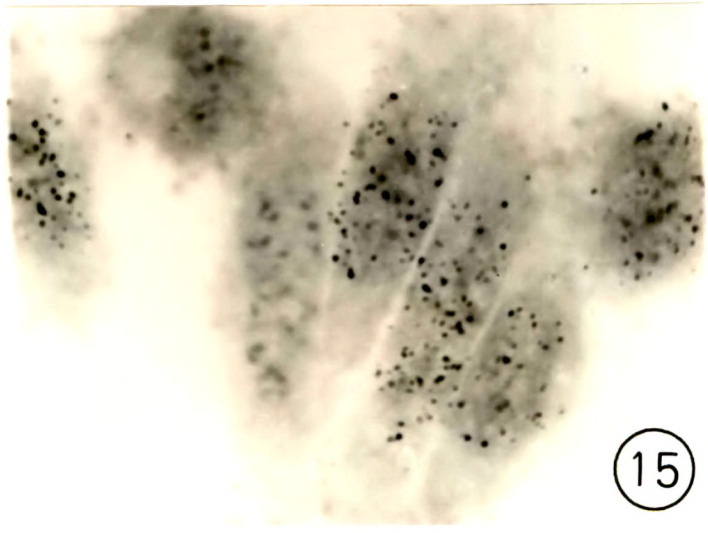

14

Illustrations 15-16 Autoradiograms showing incorporation of ^{3}H-thymidine in CHO cells.

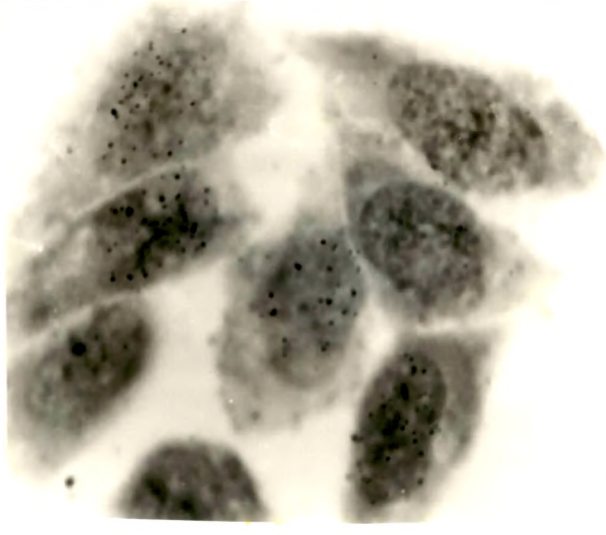
15 Cells from untreated CHO cultures.

16 Labelling intensity observed after the treatment with the test-substance.

Magnification: 1400x



15



16

29.50 %, 7.10 % and 2.80 % with 5 µl, 10 µl, 15 µl and 20 µl T-ext./ml of GM, respectively.

DISCUSSION:

The carcinogenicity of smokeless tobacco consumption has been documented primarily by several epidemiological studies. The findings have been supported by the chemical analysis revealing the presence of carcinogenic chemicals in it. About 2549 individual chemical constituents, with or without carcinogenic potentials, have been identified in tobacco (Dube and Green, 1982). Number of factors have been identified to affect the chemical composition of tobacco, e.g. harvesting period, position of the leaf on plant, aging and curing processes etc. (Andersen et al., 1987). The formation of a major class of powerful carcinogens, the TSNAs, from the tobacco alkaloids has been observed during curing and fermentation processes (Hoffmann et al., 1984). Compared to green tobacco, TSNAs have been detected at a higher level in sun-dried tobacco and highest concentrations have been reported in the processed tobacco (Bhide et al., 1987b). PAHs, another class of chemical carcinogens, have been identified in trace amounts only in the processed tobacco. Passay et al. (1971) have reported lower level of PAHs in air-cured tobacco than flue-cured tobacco. Moreover, distinct differences in genotoxic effects of saliva samples from the chewers of Indian tobacco and Western tobacco, on CHO cells, have explained the differences in the carcinogenic potential of these two types of

tobacco (Stich and Stich, 1982).

Taking all these facts into consideration, an attempt was made to evaluate the genotoxic potential of sun-dried tobacco leaves of N. tabacum which is the variety consumed widely in this part of India. Since it would better simulate the habit of chewing tobacco and its effects on the oral mucosa than an organic extract would, an aqueous extract of tobacco (10 µl extract ≡ 1.1 mg tobacco) was selected for the study. In view of the major role of nicotine in: (i) tobacco habituation (Hoffmann and Hecht, 1985), (ii) in the formation of carcinogens (Hoffmann et al., 1985) and (iii) as it comes in direct contact with the target tissues of the tobacco consumers, systematic examination of possible genotoxic potential of nicotine, per se, was also carried out. The lowest concentration used for the present study, i.e. 150 µg N./ml of GM, was comparable with the concentration of N. detected in the saliva of tobacco chewers (Nair et al., 1985).

Since, tobacco is mostly chewed with areca nut (betel quid with tobacco, mava/masala or pan masala with tobacco), the analysis of effects elicited on combining nicotine and arecoline, the major alkaloids of tobacco and areca nut, respectively, was thought worth carrying out. An average of about 90 µg nicotine (range 1.67 - 310.55 µg) and 50 µg arecoline (range 2.41 - 142.93 µg) have been measured per ml of saliva, collected from the chewers of betel quid with tobacco. Hence, experiments were planned to study the possible effects of

treating the CHO cells with 90 ug N.+50 ug A./ml of GM, or higher (maximum 300 ug N.+150 ug A./ml of GM), for a short duration followed by a recovery in test-substance free GM. Moreover, the possible effects of continuous long term exposure to various concentrations of arecoline, at low concentration in combination with 150 ug N./ml of GM (the concentration equals to that observed in one ml of saliva from the chewers of tobacco alone), were also examined.

Establishing the test material's cytotoxicity was necessary for selecting a suitable dose range for the *in vitro* short term genotoxicity assays. Hence, as a first step, the effects of T ext., N. and N.+A. on the viability of CHO cells, was studied by trypan blue dye exclusion test.

A dose and duration dependent cell death was observed with T-ext. A reduction in the colony forming capacity of C3H/10T1/2 cells, from 100 % in controls to 19 % in cultures treated with 2.5 mg of an aqueous extract of chewing tobacco (Zafrani) per ml of growth medium for 7 days, has been reported by Stich and Tsang (1989). 25-30 % cell kill has been observed by 973.2 ug nicotine per ml of GM in HL-60 cells (Konno et al., 1986). Even in the present study, the cytotoxic effects of nicotine were found to be dose and duration dependent. However, considering 2.05-3.87 % nicotine content of Indian chewing tobacco, (Brunnemann et al., 1985), one ml of the aqueous T-ext. would contain approximately

444 µg nicotine. 5 µl T-ext./ml of GM was the lowest dose used, which would equal to 2.2 µg N./ml of GM. The cytotoxicity after 24 hrs. treatment, with this concentration of T-ext., was comparable to that of the 250 µg of pure N./ml of GM. This clearly suggest that, in addition to nicotine, the extract contains other water soluble component(s) of tobacco, which add to the cytocidal action of nicotine.

Compared to N. alone, a more severe toxic effect was also observed after treating the cells with a combination of N. and A. Following a 24 hrs. of treatment with 90 µg N.+50 µg A./ml of GM, the concentration which has been measured in the saliva of individuals chewing betel quid with tobacco (Nair et al.,1985), the cell viability was lower than the one observed with 250 µg N./ml of GM for 24 hrs.

A dose and duration dependent reduction in M.I. was revealed with the continuous exposure to T-ext., N. and N.+A. Increased frequency of chromatin bridges and lagging chromosome at anaphase was observed after the treatments. The treatment with N.+A. accumulated the cells in metaphase, whereas, no such effect was observed with either T-ext. or N. treatment. Leuchtenberger et al. (1973) showed an increase in DNA content as well as in frquency of lagging chromosomes by cigarette smoke condensate treatment to cultured human lung cells. Increased frequency of cells in metaphase following the treatment with N.+A. leads to assumption that arecoline might as well be affecting the spindle functions. Comparable

metaphase accumulation, after arecoline treatment, has also been reported (Dave, 1990). The restoration of normal mitotic activity in fresh GM after a short term treatment with T-ext., N. and N.+A. suggested that damage to mitotic apparatus is repairable. However, the treatment with 100 µl T-ext./ml of GM was too toxic to the cells, and as a result the cells failed to recover.

Looking to the presence of chromatin bridges and lagging chromosomes in the cell division experiment, it was thought worthwhile to analyse the frequency of micronucleated cells from the cultures recovered in fresh GM after being treated with test compounds for a short duration. Presence of MN indicate chromosomal breakage and/or malfunction of the spindle apparatus. Micronuclei formed by chromosomal aberrations are smaller than those originating as a consequence of exposure to spindle damaging agents (Hogstedt and Karlsson, 1985; Yamamoto and Kikuchi, 1980). In the present study, a dose and duration related increase in MNC frequency indicated the clastogenic effect of T-ext., N. and N.+A. on CHO cells. The treatment with 500 µg N./ml of GM had no effect on MNC frequency. However, when N. and A. were tested in combination, the values of of % MNC were higher than that observed following the exposure to N. and A. separately (our obsevation). The induction of micronuclei in mouse bone-marrow cells by tobacco and its extract has also been reported (Shirname et al., 1984; Bhide et al., 1989). Stich and Stich (1982) have observed elevated frequency of MNC in CHO

cells follwing treatment with the saliva of tobacco chewers. Aqueous extracts of snuff, chewing tobacco, zarda, khaini tobacco and nass have also been reported to increase the MNC frequency in CHO cells (Stich, 1986).

Analysis of MNC frequency has revealed the clatogenic property of T-ext. and N. These findings were further substantiated by CA analysis. The quantitative analysis, following the treatment with T-ext., N. and N.+A., showed a dose dependent elevation in the frequency of aberrant metaphases as well as number of CAs per cell. However, the elevations were statistically significant only with higher concentrations. It was also observed that, long term treatments with N. (150 µg/ml of GM) or A. (12.5 µg/ml of GM) individually, did not increased the CA frequency significantly, however, the treatment with 150 µg N.+12.5 µg A./ml of GM increased the CA/cell value significantly ($p < 0.05$), compared to untreated cultures. The qualitative analysis showed that chromatid-type aberrations were more frequent than chromosome-type aberrations in the treated cultures. Moreover, the gaps and breaks were the most common type of aberrations observed. It is known that the aberrations induced in G-1 and early S phase are chromosome-type, whereas, those induced in late S and G-2 phase are chromatid-type. The inhibitors of DNA synthesis also induce a very high frequency of gaps when the cells are treated in late S or G2 phase (Natarajan and Obe, 1982).

Stich and Stich (1982) have reported induction of CA

frequency in CHO cells by the saliva of Indian tobacco chewers. Addition of either S9 mix., catalase or superoxide dismutase could not alter the capacity of nass extract to induce CA in CHO cells *in vitro* (Zaridze et al., 1985). Aqueous extracts of snuff, zarada, khaini etc. elevated the frequency of CAs (Stich, 1986). After a treatment with aqueous extract of chewing tobacco, similar elevation in CA frequency has been observed in C3H/10T1/2 cells *in vitro* (Stich and Tsang, 1989). Nicotine has been found to induce CAs in mice, however, it failed to influence human leucocytes *in vitro* (Bishun et al., 1972). In the present study significant elevations in CA frequency were observed only at very high concentrations, indicating that nicotine, per se, is a very weak clastogen. Looking to the nicotine content of T-ext. and elevations in CA/cell value following the treatment with T-ext., it appears that T-ext. contains other clastogens which add to the chromosome breakage caused by the treatment, compared to the parallel experiments with nicotine alone. The clastogenicity of several phenolic compounds present in tobacco leaves has also been reported (Stich et al., 1981a). Among the TSNAs so far studied, only NNK induced CAs in peripheral blood lymphocytes *in vitro*, at a 100 µg/ml GM concentration (Padma et al., 1989).

A significantly higher frequency of CA was detected when cells were treated with a combination of nicotine and arecoline, rather than nicotine alone. Following combined application of arecoline with either eugenol, or quercetin or

chlorogenic acid (the compounds present in betel quid), Stich et al. (1981b) have observed more severe genotoxic effect, compared to treatments with individual compounds. The enhancing effect of areca nut in tobacco carcinogenesis has also been documented (Ranadive and Gothoskar, 1978).

Analysis of SCE frequency has been suggested as one of highly sensitive indicators for the screening of mutagens and carcinogens. The presence of SCE may reflect occurrence of DNA breakage and misrepair which are closely associated with mutational changes (Popescu et al., 1981). Statistically significant elevation in SCE frequency was observed following the treatment with T-ext., N. and N.+A. for all the concentrations studied. Umezawa et al. (1981) have reported significant induction of SCEs in virally transformed human lymphocytes and in PHA-stimulated human lymphocytes treated with ethyl acetate extract of Jaffna tobacco. Riebe and Westphal (1983) have reported SCE induction in CHO cells treated with nicotine at very high concentrations in absence of metabolic activation system. SCE frequency observed after exposure to nicotine plus arecoline indicated that arecoline adds to the genomic damage inflicted by nicotine.

The results of the CA and SCE assays showed that induction of SCE frequency was highly significant for all the concentrations of the test-substances, whereas, CA induction was significant with higher concentrations only. This difference can be explained on the basis of different mechanisms

involved in the formation of SCE and CA (Gebhart, 1981). When the cells were treated with 150 µg N./ml of GM, which equals the concentration of nicotine detected in the saliva of chewers of tobacco alone (Nair et al., 1985), the elevation in SCE frequency was statistically significant ($p < 0.001$), whereas, CA frequency remained unaffected. Thus, SCE emerged to be more sensitive indicator of nicotine genotoxicity. Same was true for the assessment of the genotoxicity of tobacco extract as well as of nicotine plus arecoline.

In view of inhibition of cell growth and induction of CA and SCE frequencies following the treatment with T-ext., N. and N. + A., the possible effects of these substances on DNA synthesis was also studied. A dose-dependent inhibition of DNA synthesizing cells was observed following a continuous treatment of 24 hrs. Similar effects have been reported with aqueous extract of tobacco in PHA-stimulated lymphocytes (Yang et al., 1979) and with nicotine in HL-60 cells (Konno et al., 1986).

Thus, the present study indicated that nicotine, the habituating factor in tobacco, also contribute significantly to the carcinogenic potential of tobacco. The results of experiments with aqueous extract of smokeless tobacco indicated presence of constituent(s) which were capable of potentiating the genotoxic effect of nicotine. Because of the presence of a variety of complex chemical constituents like alkaloids, polyphenols and amines in tobacco, it is difficult to

attribute its genotoxicity to certain specific chemicals. Moreover, mixture of a number of chemicals with diverse mutagenic/carcinogenic activities can alter the ultimate genotoxic effects (Boutwell et al., 1982). Chlorogenic acid, present in tobacco leaves has been found to potentiate the clastogenicity of arecoline in a dose-dependent manner (Stich et al., 1981b). Hecht et al. (1986) have suggested nicotine as a compititive inhibitor of metabolic activation of NNN and NNK (TSNAs) in F344 rat oral mucosa. Murphy and Heiblum (1990) have shown the inhibitory effects of nicotine on the metabolism of NNN and NNK by cultured rat oral tissue. Since nitrosamines require metabolic activation for binding to DNA and other cellular macromolecules (Preussmann and Stewart, 1984), TSNAs are not likely to be responsible for the genotoxic effects of the aqueous extract.

The experiments with S9 mix. were not included in the present protocol, as it is known that S9 mix. can be used only during the pulse and chase treatments, whereas, the present study dealt with two types of treatment schedules, i.e. continuous exposure and short-term exposure followed by recovery. Studying continuous treatments was vital, because, in case of chewing associated oral cancers, the oral mucosa is exposed daily to the tobacco alkaloids for hours together and in extreme cases the chewers even sleep with the chew in their mouth (Bhonsle et al., 1979). Moreover, since liver enzymes may not be involved in oral cavity cancers, experiments without S9 mix. would mimic the local action of tobacco

in a better way. Riebe and Westphal (1983) have also reported increased SCE rates in CHO cells after nicotine treatment without metabolic activation, whereas, there was no increase in SCE frequency by nicotine in presence of S9 mix. Thus, the results of the present work expressed the cytotoxicity/genotoxicity of the T-ext., N. and N.+A. on CHO cells, in absence of metabolic activation system.

Thus, taking all the available reports as well as results of the present study into consideration, it can be inferred that:

(I) nicotine, in addition to being addictive, is genotoxic as well,

(II) tobacco also contain other water soluble chemical constituent(s) which are capable of potentiating the genotoxic effect of nicotine, and

(III) even a short term treatment with a combination of nicotine and arecoline, at concentrations comparable to one detected in the saliva of chewers of betel quid with tobacco, followed by a recovery in fresh GM, resulted in significant genomic damage.