PART I

IN VITRO ASSAYS EMPLOYING MAMMALIAN TEST SYSTEM

The last decade has witnessed the introduction of a number of relatively rapid and economical tests for detecting mutagenic and carcinogenic substances. Since the animal bioassays are time consuming, complex and highly expensive, scientific studies using a number of animals throughout their life span become a limiting factor many a times, especially during preliminary studies. In view of the advantages that the in vitro short term tests offer, several organizations have advocated their use in carcinogenicity testing and have provided guidelines wherein cytogenetic studies using mammalian cells in culture have been described (Scott et al., 1983 and Perry et al., 1984 for UKEMS; Dean and Danford, 1984 WHO, 1985b). These short term tests can not only measure the ability of the test substance to function as a complete carcinogen, but also detect the events leading to the initiation phase, i.e. the ability to induce a mutagenic or carcinogenic DNA lesion (WHO, 1985b). Hence, in the present studies, as a first step, the in vitro short term assays were employed, to gather vital information about the possible genotoxic effects of areca nut on mammalian cells.

As outlined earlier (Tables 1 and 2), there have been reports that point towards the mutagenicity and carcinogenicity of areca nut. Investigations have also been carried out taking arecoline, the major areca nut alkaloid into consideration. However, the studies on areca nut have been carried out with diverse experimental approaches. There have been wide variations in the extraction procedures of the sample, in the experimental protocol, the choice of animal models, in the exposures and in the routes of administrations. Hence, the results of all these studies led The International Agency for Research on Cancer to derive 'limited' or 'inadequate' evidence regarding the carcinogenicity of areca nut and its alkaloid arecoline (IARC, 1985). Therefore, to elucidate the carcinogenic/genotoxic potentials of areca nut and the active principles responsible for the effect, we have

designed our experiments utilizing the aqueous extract of areca nut as well as arecoline, the major alkaloid of areca nut.

Areca nut, while being chewed, is often coupled with tobacco, which is known to possess carcinogenic properties (IARC, 1987). The most potent carcinogens identified in tobacco are nicotine-derived nitrosamines (Brunnemann et al., 1986). Thus, nicotine, the main addictive factor of tobacco, serves as a precursor for highly carcinogenic nitrosamines (Hoffmann and Hecht, 1985). Nicotine induced chromosomal aberrations in mice, but did not damage human leukocytes in vitro (Bishun et al., 1972). It did not show mutagenic effect in Ame's test, however, it induced repairable DNA damage in E.coli system (Florin et al., 1980; Riebe et al., 1982). Nicotine, at very high concentrations, caused SCE increase in mammalian cells (Riebe and Westphal, 1983). Both arecoline and nicotine, per se, have been reported to leach out in the saliva of chewers consuming betel quid with tobacco (Nair et al., 1985; 1987). It was, therefore, felt worthwhile to study the in vitro effects of combining these alkaloids, which might provide a better insight to the problem of tobacco/areca nut carcinogenesis.

MATERIALS AND METHODS

Chinese Hamster Ovary (CHO) cell line was selected for the present study, which 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 salts and non-essential amino acids (NEAA) was procured from Centron Research Lab., Bombay, India.

New born calf serum (NCS) was obtained from Sera Lab., U.K. Trypsin (1:250) was purchased from Difco Lab., Detroit, Michigan, U.S.A. Arecoline hydrobromide, Nicotine, 5-Bromo-2-deoxyuridine (BrdU), Colchicine and Hoechst 33258 (Bisbenzimide) were acquired from Sigma Chemical Company, St. Louis, U.S.A. Mitomycin C (MMC) was obtained from Biochem Pharmaceutical Industries, Bombay, India. ^{3}H*--Thymidine was purchased from Bhabha Atomic Research Centre, Bombay, India and Nuclear track emulsion used was Ilford* L_4.

CULTURE PROCEDURE:

CHO cells were maintained in growth medium (GM) that comprised of MEM with NEAA supplemented with 10-20% NCS and 100 units/ml benzyl pencillin and 100 µg/ml streptomycin sulphate. The confluent cultures were subcultured every fifth day by trypsinizing the cells with 0.25% trypsin (1:250) in calcium and magnesium free phosphate buffered saline (CMF-PBS). After careful removal of trypsin, the cells detached from the glass surface were repeatedly pipetted to give a single cell suspension in fresh GM, and were then seeded in new culture bottles. On the third day of subcultivation, the medium was replaced with fresh GM containing 10% NCS. For all the experiments the number of cells seeded was always maintained to 2 to 2.5 x 10^4 *cells per ml GM. The assays were initiated in exponentially growing cells after the subcultivation. The culture conditions were maintained at 37°C.*

PREPARATION OF AQUEOUS EXTRACT OF ARECA NUT:

Unprocessed, ripe and sundried areca nuts were purchased from the local market and were cut into pieces. These were then finely powdered with the help of a blender. 25 grams of this was added to 75 ml of distilled water and mixed thoroughly to give a smooth paste. This was allowed to stand overnight at 4-8°C.

Next day it was stirred for three hours at 37°C and the extract was collected by centrifugation. This extraction procedure was repeated two more times adding 75 ml distilled water each time to the residue. All the three extracts were pooled. The pooled extract representing 25 grams areca nut in 225 ml distilled water, was filter sterilized through 0.22 µm porosity filters and stored at -70°C in small aliquotes. The extract contained 1.24 gms% water soluble materials.

EXPERIMENTAL PROTOCOL:

Duplicate cultures were set for all treatments and the mean values were derived from the cells scored from both the cultures. In all experiments MMC was used as positive control.

Experiment 1 :

The experiment was designed to study the possible toxic effects of different doses and durations of areca nut extract (AN ext.) and of arecoline on viability of the cells at the end of 24 hours' and 48 hours' treatments. Arecoline was dissolved in serum free GM just prior to use. Three different concentrations of AN ext. i.e. 5 µl, 10 µl and 20 µl per ml GM were added to different cultures. Similarly, three concentrations of arecoline were added, i.e. 25 µg, 50 µg and 100 µg per ml GM. Since areca nut is most frequently masticated with tobacco, two experiments, using a combination of the major alkaloids of both, i.e. arecoline (A) and nicotine (N), were carried out using three combinations, viz. 5 µg A + 120 µg N, 25 µg A + 45 µg N and 50 µg A + 90 µg N per ml GM. Parallel control cultures were placed for individual time intervals. At the end of treatment, i.e. 24 hours and 48 hours, the cells were scrapped off the surface with a rubber policeman and pipetted repeatedly to give a single cell suspension. 0.1 ml of trypan blue (0.4% aqueous) was added to 0.9 ml of cell suspension, mixed and

allowed to stand for five minutes. Haemocytometer chamber was charged with the suspension and the viable as well as total cells were counted.

Experiment 2 and 3 :

The probable effects of arecoline on cell division were examined by subjecting the cells, grown on coverslips in Leighton tubes, to five different concentrations of arecoline. Control cultures contained arecoline free GM.

A continuous treatment with following concentrations, viz. 25 µg, 50 µg, 100 µg, 150 µg and 200 µg arecoline per ml GM, was given to the cells for various time intervals ranging from 0 to 48 hours. Cultures were fixed at 3, 6, 12, 24, 36 and 48 hours using chilled 1:3 aceto-methanol for 30 minutes. The coverslips were dried and mounted keeping the cell layer exposed.

To observe the ability of the cells to recover after arecoline treatment and to study its effect on micronucleus formation, a recovery experiment was conducted. Three concentrations of arecoline, viz. 50 µg, 100 µg and 200 µg per ml GM, were employed. The cultures were treated separately for 6 hours and 24 hours. After the treatment, the cells were rinsed with prewarmed CMF-PBS and were then supplemented with arecoline free GM. The cultures recovered for 0, 6, 12, 18, 24, 30 and 48 hours were fixed and processed further as described in the previous experiment.

The monolayers were stained with 2.0% Giemsa in Sorenson's phosphate buffer (pH 7.0), differentiated and mounted with DPX.

Experiment 4, 5, 6:

The experimental protocol was designed in such a way that the effect of the test substances on chromosome aberration (CA) frequency, sister chromatid exchange (SCE) frequency and cellular kinetics could be studied simultaneously.

After subcultivation, five concentrations of AN ext. i.e. 2.5 µl, 5 µl, 10 µl and 20 µl per ml GM were added to exponentially growing CHO cultures. The extract remained present in GM containing 10 µg BrdU per ml GM till harvesting.

In another set of experiment, exponentially growing cells were subjected to three concentrations of AN ext. i.e. 10 µl, 20 µl and 50 µl per ml GM for a period of three hours. After the treatment the cells were washed twice with prewarmed CMF-PBS and were supplemented with a fresh, extract free GM containing 10 µg BrdU per ml GM. The cells were allowed to undergo only one cell cycle for the CA assay, while for SCE frequency and cell kinetics studies they were grown for two cell cycles in BrdU containing medium. Colchicine (0.3 µg/ml) was added to the cultures during the last 3 hours of incubation. During all the above mentioned steps the cultures were protected from direct light to prevent photolysis of BrdU containig DNA (Ikushima and Wolff, 1974). At the end of the treatment, the cells were detached from the glass surface with a rubber policeman and suspended in the medium. The hypotonic treatment was given by diluting the medium (1:1) with distilled water and allowing it to stay at room temperature for 14 minutes. This was then terminated by the addition of 1.0 ml of chilled aceto–methanol (1:3) fixative. After three changes of cold fixative, air dried chromosome preparations were made on clean slides, according to the conventional methods.

Similar experiments were carried out to learn about the effect of arecoline. Four different concentrations, i.e. 100 µg, 150 µg, 200 µg and 250 µg of arecoline per ml GM were utilized for three hours' treatment. The final concentration i.e. 250 µg per ml GM was also used to observe the effect of various durations. The cells were recovered in arecoline free GM after one hour, two hours' and three hours of treatment with this concentration of arecoline. For continuous

treatment, five concentrations of arecoline were utilized, viz. 12.5 µg, 25 µg, 50 µg, 75 µg and 100 µg per ml GM. The experimental protocol remained the same as described earlier.

Assuming that experiments utilizing arecoline in combination with nicotine would provide more details regarding the ultimate effects of combining tobacco and areca nut on the cells, a different set of experiments was formulated. The experimental design remained the same as described previously, however, since both alkaloids were used in combination and in three different concentrations, viz. 50 µg A + 90 µg N, 100 µg A + 200 µg N and 150 µg A + 300 µg N per ml GM, the duration of treatment was reduced to two hours. For continuous treatment of 48 hours, keeping arecoline concentration at 5 µg per ml GM, three different concentrations of nicotine were added in increasing order, i.e. 5 µg A + 60 µg N, 5 µg A + 90 µg N and 5 µg A + 120 µg N per ml GM. Two other combinations, viz. 25 µg A + 45 µg N and 50 µg A + 90 µg N per ml GM were also studied for their effects on CAs, SCEs and cell kinetics. Each set of experiment contained an untreated sample as control and an MMC treated sample, as positive control.

STAINING PROCEDURE: *The modified method of Perry and Wolff (1974) was applied for SCD staining. The slides were flooded with Hoechst 33258 (0.5 µg/ml in Sorenson's buffer) and exposed to U.V. light for 4-5 hours. They were then rinsed in distilled water and treated with 2X SSC at 60°C for 20 minutes. After a rinse in distilled water they were stained with 2.0% Giemsa in Sorenson's phosphate buffer and distilled water (1:1) at pH 7.0. At optimum chromatid differentiation, the slides were air dried and mounted with DPX.*

Experiment 7 :

To gain information regarding the effects of AN ext. and arecoline on DNA synthesis,

autoradiographic studies were performed. Coverslip cultures were treated for 12 hours and 24 hours with four concentrations of AN ext. viz. 2.5 µl, 5 µl, 10 µl and 20 µl per ml GM and with 25 µg, 50 µg, 75 µg and 100 µg of arecoline per ml GM. Four different combinations of arecoline plus nicotine were also studied. Control cultures received GM free of test substances. After the treatment with the test substance, a pulse treatment of ^{3}H-thymidine (2 µci/ml GM) was given for a brief period of 20 minutes. The cells were washed twice with CMF-PBS before fixation of cells with chilled acetomethanol. Coverslips were mounted on clean slides with DPX, keeping the cell layer exposed. They were then treated with 5% TCA for 5 minutes at 0-4°C to remove unincorporated radioactive precursor and washed repeatedly with 70% ethanol over a period of 4-5 hours. Coverslips were stained with carbol fuchsin according to the method of Darlington (1976). These slides were processed for autoradiography. The nuclear track emulsion Ilford L_4 was diluted 1:1 with distilled water and was melted at 42°C. Taking all dark room precautions, the slides were coated with emulsion and dried slides were packed in light proof slide boxes containing silica gel as dessicant. After keeping the slide preparations at 4°C for four weeks they were developed at 18°C for 5 minutes with Kodak D19B developer, followed by acid fixer treatment. The slides were then thoroughly washed with cold distilled water, dried and mounted with DPX.

PHOTOMICROGRAPHY

Using bright field optics and Agfa copex films, photographs were taken on Zeiss photomicroscope III.

SCORING METHODS

Cell Viability: *For calculation of percentage viability, the following formula was applied,*

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

Mitotic Index: *The mitotic index (M.I.) was determined as percent dividing cells in a randomly counted cell population. A minimum of one thousand cells were counted for each sample.*

Micronucleus: *The scoring of micronucleus was based on the criteria detailed by Schmid (1976) and Sarto et al. (1987): (1) The chromatin structure and colour intensity were similar to or weaker than those of the main nucleus, (2) Their borders were distinctly recognizable, suggesting the presence of a nuclear membrane. (3) They were almost round in shape. (4) They were included within the same cell cytoplasm. A minimum of one thousand cells were screened from each sample and percent micronucleated cells (MNC) were calculated.*

Chromosome aberrations: *The identification of cells in I, II and III division was based on the differential staining property of the BrdU substituted chromosome. Cells in I division, designated as MI, had both chromatids darkly stained. Due to bifilar substitution with BrdU, the cells in II cycle (MII) had one chromatid darkly stained and the other lightly stained. The cells in third division (MIII) showed two third of the chromosome with light stain as a result of complete substitution with BrdU.*

At least one hundred well spread metaphase cells in first division, as evidenced by SCD staining, were analysed for scoring CA frequency (Evans, 1976). Following types of aberrations were considered: chromatid and isochromatid gaps and breaks,

chromosomal fragments, dicentric chromosomes, ring chromosomes, exchanges, double minutes. The identification was carried out as recommended in guide to short term tests for detecting mutagenic and carcinogenic chemicals (WHO, 1985b) and report of the UKEMS sub-committee on guidelines for mutagenicity testing (Scott et al., 1983).

Sister chromatid exchanges: *For every sample the frequency of SCEs was determined by analysing a minimum of twenty five well spread metaphases in second division. A terminal exchange was counted as one SCE and each interstitial exchange was counted as two SCEs.*

Cellular Kinetics: *A minimum of one hundred consecutive well differentiated metaphases in MI, MII and MIII were considered to compute cellular kinetics. The cellular kinetics were expressed as Average Generation Time (AGT) which was calculated utilizing proliferation index (PRI) as described by Tice and Ivett (1985).*

$$AGT = \frac{\text{Hours since onset of BrdU exposure}}{PRI}$$

$$\text{where } PRI = \frac{MI \times 1 + MII \times 2 + MIII \times 3}{100}$$

DNA Synthesis: *The estimation of cells synthesizing DNA at a given time was done by calculating the labelling index. The number of cells that incorporated ^{3}H-thymidine, during a brief pulse, were scored as labelled cells, however, a cell with less than ten silver grains over nucleus was considered as unlabelled. The labelling index was determined as percent labelled interphase nuclei in a randomly counted cell population. A minimum of one thousand cells were scored for each sample.*

STATISTICAL ANALYSIS:

Standard deviation and standard error were calculated for each sample and Student's 't' test was applied for assessing the statistical significance of difference between the values for control and treated cultures.

RESULTS

1. IN VITRO EFFECTS OF AN EXT.

Cell Viability:

The results of the toxic effects of AN ext. are depicted in Fig. 3. At 0 hour there were 92.43% viable cells in the control samples. After 24 hours, they were 90.12% in the control samples, while in cultures treated with 5 µl, 10 µl and 20 µl of the extract the viable cell count was reduced to 59.80%, 42.00% and 35.93%, respectively. At 48 hours, the control cultures showed 89.46% viability but in cultures treated with the extract, the viable cell count further reduced to 46.93%, 33.20% and 24.98%, respectively. Taking together the values for 24 and 48 hours and all the three doses, it was evident that the cytotoxicity was dose and duration dependent.

Chromosome aberrations:

The data presented in Table-3 describes the number and types of CAs induced by AN ext. in the cultures treated for three hours and recovered in extract free GM. Illustrations 15-26 show some of the CAs observed after treatment of AN ext. and arecoline to CHO cells. The 7% aberrant metaphases found in the control samples, increased to 11%, 15% and 16% in the cultures treated with 10 µl, 20 µl and 50 µl of the extract, respectively. The positive controls showed 21% aberrant metaphases. The mean aberrations per cell values were also elevated with the increase in the concentration of the extract, however, statistically signifi-

Fig. 3

Cytotoxic effects of AN ext. on CHO cells

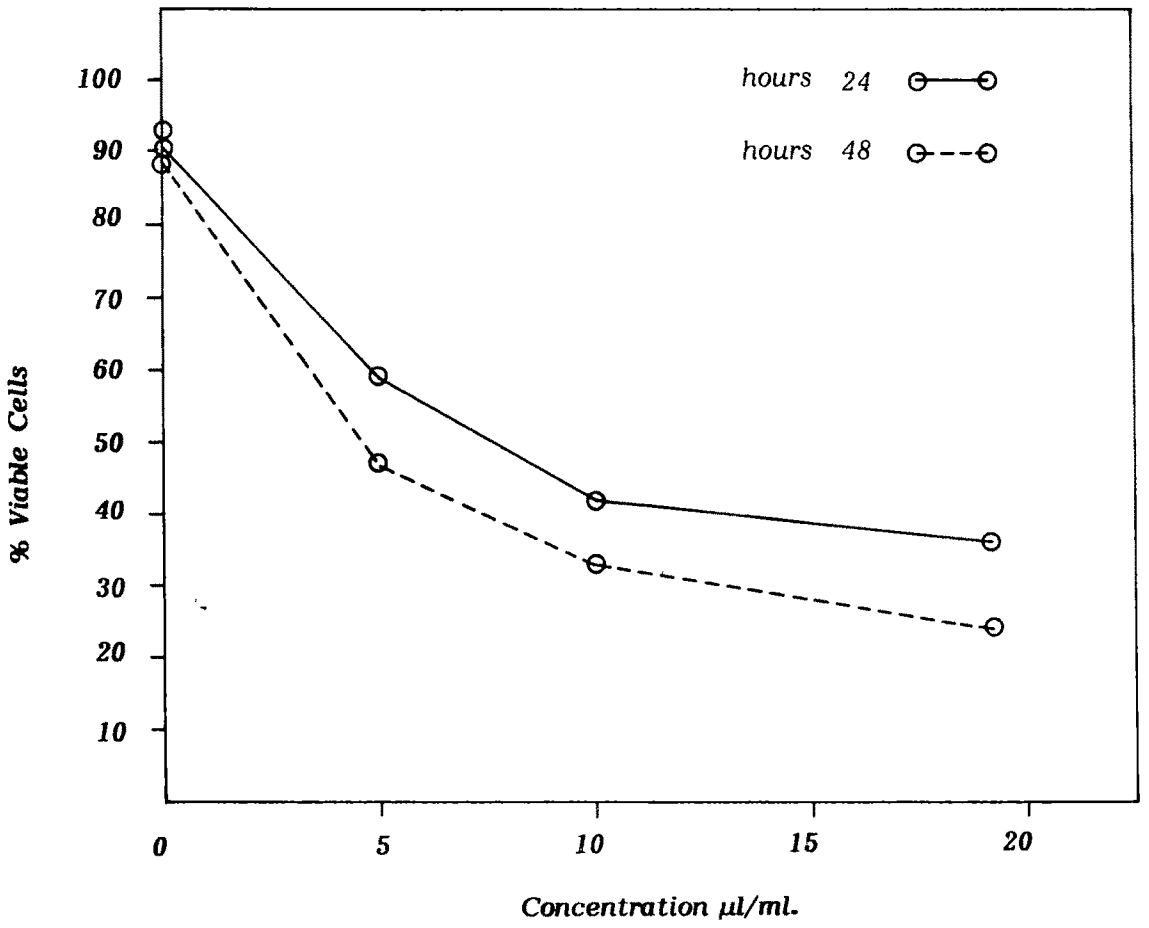


Table-3

Details of CAs observed in CHO cells recovered for 24 hours after 3 hours' treatment with AN ext.

Conc/ml.	%Aberrant Metaphase	Chromatid				Chromosome					CA/Cell±S.E.
		G	B	I	Af	GI	BI	DM	R	Dic	
Control	7	4	-	-	2	1	-	-	-	-	0.07±0.026
10 µl	11	6	-	-	4	2	-	-	-	-	0.12±0.035
20 µl	15	7	-	-	2	4	-	-	-	2	0.15±0.036
50 µl	16	7	2	-	6	2	1	-	1	-	0.19±0.048*
MMC 0.03µg	21	10	2	-	6	2	1	-	-	2	0.23±0.047**

* $p<0.05$ ** $p<0.01$ including and excluding gaps

Table-4

Particulars of CAs observed in CHO cells treated with AN ext.

Conc/ml.	%Aberrant Metaphase	Chromatid				Chromosome					CA/Cell±S.E.
		G	B	I	Af	GI	BI	DM	R	Dic	
Control	08	8	-	-	1	-	-	-	-	-	0.09±0.032
2.5 µl	11	4	1	1	3	2	-	-	1	2	^{c}0.14±0.045
5 µl	17	14	1	-	4	1	-	-	-	2	^{c}0.22±0.056*
10 µl	19	14	2	-	5	1	-	-	-	4	^{b}0.26±0.059**
15 µl	21	12	5	1	5	1	2	2	1	1	^{b}0.31±0.070***
20 µl	21	13	2	-	6	2	3	1	2	3	^{a}0.32±0.066***
MMC 0.007µg	18	8	4	1	-	4	-	-	2	3	^{b}0.22±0.045**

* $p<0.05$ ** $p<0.02$ *** $p<0.01$

a - $p<0.001$ b - $p<0.01$ c - $p<0.05$ - excluding gaps

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

cant increase was found only in the cultures treated with 50 µl AN ext. per ml GM.

In cultures continuously treated with five different concentrations of the extract, the aberrant metaphases increased to 11%, 17%, 19%, 21% and 21%, respectively, while the control value was 8% and the positive control value was 18%. Compared to the controls all the concentrations yielded statistically significant elevations in the CA per cell values (Table-4).

Sister chromatid exchanges:

The cells were subjected to three hours' treatment with 10 µl, 20 µl and 50 µl of AN ext. and recovered in extract free GM. It was observed that the mean SCE per cell frequency was 6.28 for untreated cultures and the treated cultures provided 7.92, 9.84 and 10.56 SCE per cell values for the three doses employed respectively. In the positive control the mean SCE per cell was 21.92. The increase was statistically significant (Table-5).

A continuous treatment with five different doses of AN ext. conceded a dose dependent increase in SCE frequency. Table-6 provides the details of the observations on SCE frequency. Compared to the controls, a statistical significance was observed in the values of all the five concentrations of the extract utilized. Illustrations 27-29 exhibit some examples of metaphases with low and high frequency of SCEs induced by treatments with AN ext. or arecoline.

Cellular kinetics:

AN ext. added for a brief period of three hours in the GM could affect the generation time of the CHO cells. The control cultures had 2% metaphases in MI which increased to 8%, 12% and 55% in the samples treated with 10 µl, 20 µl and 50 µl of AN ext., respectively. The AGT increased from 21.82 hours in controls

Table-5

SCE frequency in CHO cells recovered for 48 hours after 3 hours' treatment with AN ext.

Conc./ml.	Mean SCE/Cell±S.E.	Range	% Metaphases with SCE			
			0-5	6-10	11-15	15
Control	6.28±0.286	4-9	32	68	-	-
10 µl	7.92±0.265*	6-10	-	100	-	-
20 µl	9.84±0.570*	6-15	-	60	40	-
50 µl	10.56±0.400*	6-15	-	60	40	-
MMC 0.03 µg	21.92±0.495*	16-26	-	-	-	100

* $p<0.001$

Table-6

SCE frequency in CHO cells treated with AN ext.

Conc./ml.	Mean SCE/Cell±S.E.	Range	% Metaphases with SCE			
			0-5	6-10	11-15	15
Control	7.28±0.443	4-12	20	68	12	-
2.5 µl	9.96±0.538*	6-15	-	60	40	-
5 µl	10.96±0.478*	7-18	-	48	48	4
10 µl	11.92±0.518*	8-19	-	32	60	8
15 µl	12.36±0.542	7-17	-	24	64	12
20 µl	12.60±0.580*	9-21	-	24	60	16
MMC 0.007µg	37.56±0.780*	24-45	-	-	-	100

* $p<0.001$

to 33.10 hours in cultures treated with 50 µl of AN ext. In the positive controls the AGT was 28.24 hours (Table-7).

The cellular kinetics were also found to be affected when the cultures were continuously treated with AN ext. for 48 hours. As detailed in Table-8, although at lower concentrations there were minor changes in the AGT, at higher doses the AGT increased by 4 to 6 hours. Illustrations 12-14 portray representative cells in MI, MII and MIII of the cell division.

DNA Synthesis:

Fig. 4 reveals the effect of AN ext. on DNA synthesis after 12 hours' treatment and 24 hours' treatment. It was observed that with the increase in concentration of the extract, there was a fall in the labelling index, which expresses the number of cells synthesizing DNA at a given time. A dose and duration related reduction in DNA synthesis was visualized. The number of cells synthesizing DNA, observed at 12 hours, were further reduced at 24 hours. When the control value was 65.15 and the positive control value was 8.0 at 24 hours, the values for the treated samples were 33.50, 25.00, 10.10 and 3.1 for 2.5 µl, 5 µl, 10 µl and 20 µl AN ext. per ml GM, respectively. There was a reduction in the intensity of ^{3}H-thymidine labelling in all the treated cultures when compared to controls. Photomicrographs in Illustrations 30-31 detail the same very clearly.

2. IN VITRO EFFECTS OF ARECOLINE, THE ARECA NUT ALKALOID

Cell Viability:

The cell kill brought about by 25 µg, 50 µg and 100 µg arecoline per ml GM at 24 and 48 hours can be observed from the slopes in the graphs (Fig. 5). As seen in the Figure, 95.00% viable cells were found in the controls at 0 hour. At 24 and 48 hours the values were 89.11% and 89.00%, respectively. In the cultures

Table-7

CHO cell kinetics examined after 3 hours' treatment with AN ext. followed by recovery for 48 hours

Conc./ml.	MI	MII	MIII	PRI	AGT (hr.)
Control	02	76	22	2.20	21.82
10 µl	08	89	03	1.95	24.62
20 µl	12	88	00	1.88	25.53
50 µl	55	45	00	1.45	33.10
MMC 0.03 µg	30	70	00	1.70	28.24

Table-8

Cellular kinetics in CHO cells treated with AN ext.

Conc./ml.	MI	MII	MIII	PRI	AGT (hr.)
Control	01	40	59	2.58	18.60
2.5 µl	01	49	50	2.49	19.28
5 µl	03	53	44	2.41	19.92
10 µl	04	76	20	2.16	22.22
15 µl	02	80	18	2.16	22.22
20 µl	10	84	06	1.96	24.49
MMC 0.007 µg	11	89	00	1.89	25.40

Fig. 4

CHO cells synthesizing DNA after treatment with AN ext.

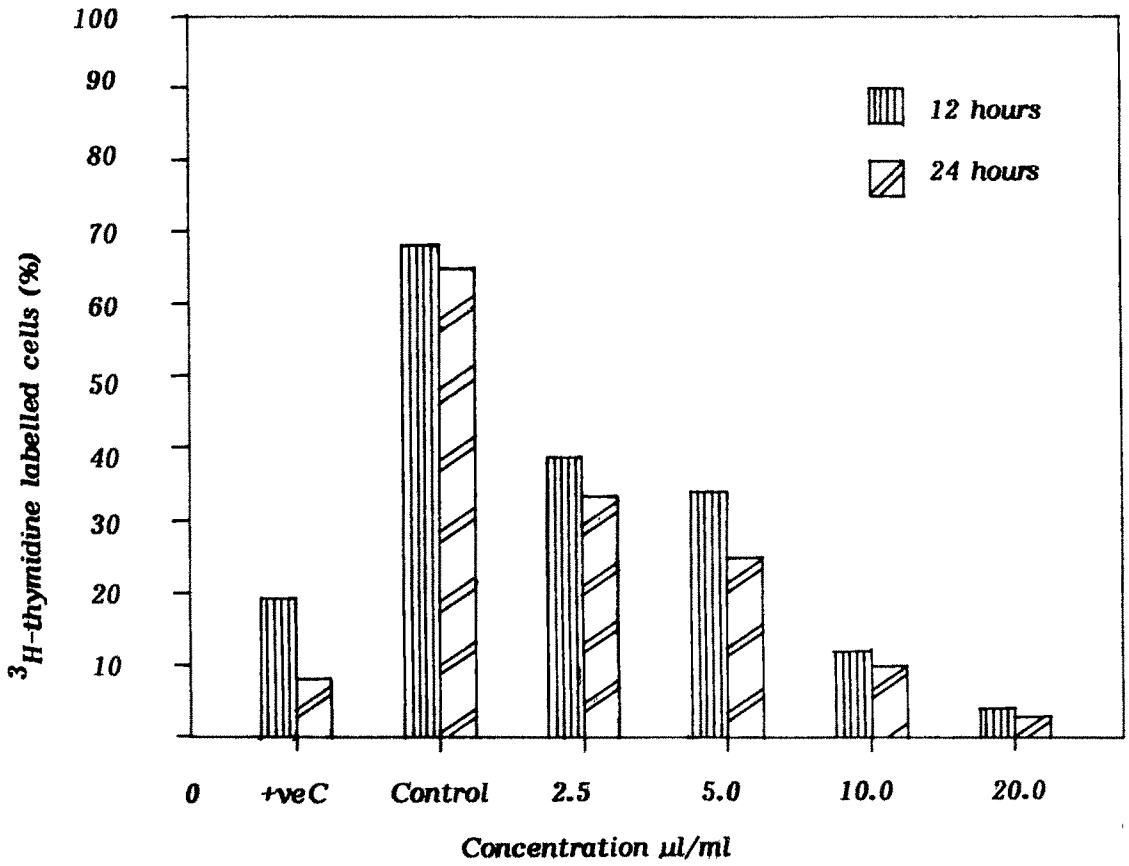
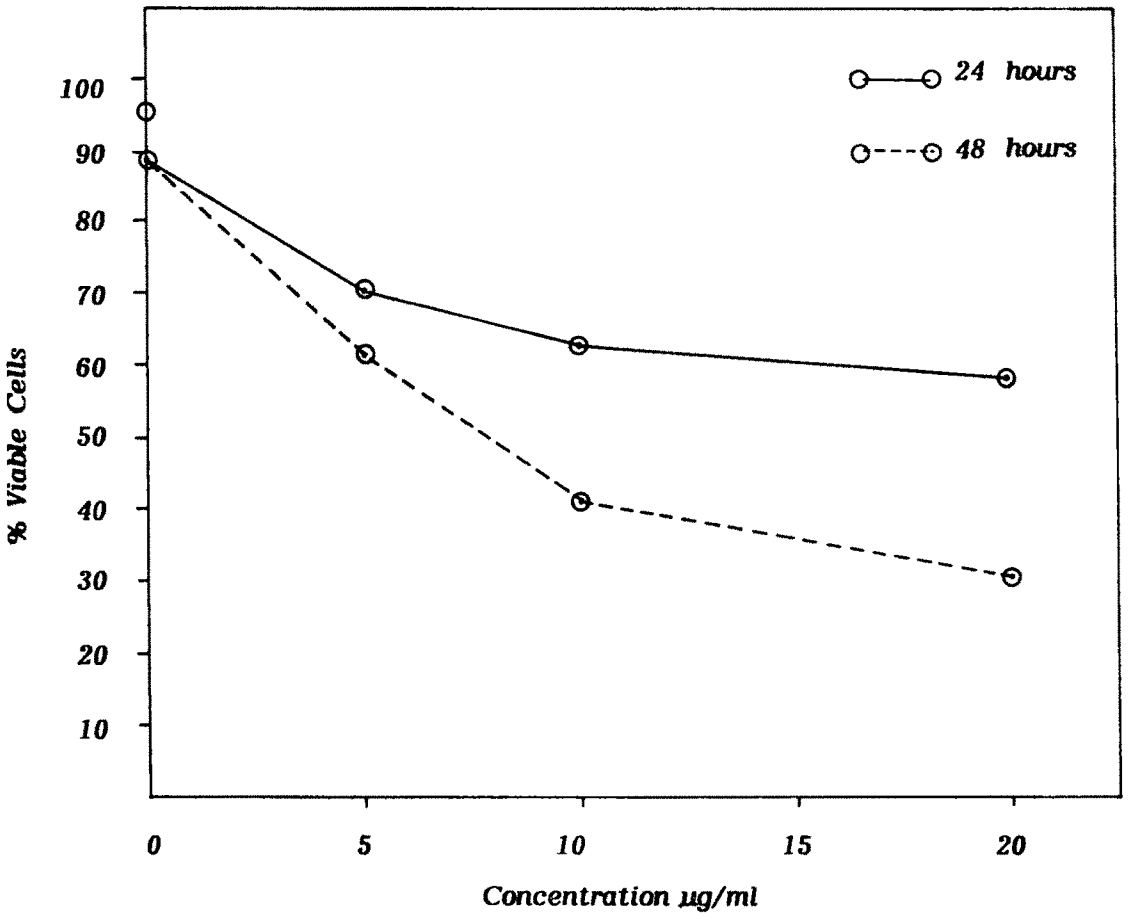


Fig. 5

Effect of arecoline treatments on percentage viability of CHO cells



treated with arecoline, a clear dose and duration dependent decrease in viability was seen. The percent viable cells being 70.61%, 63.00% and 58.76% at 24 hours and 62.34%, 41.66% and 30.98% at 48 hours for the three concentrations of arecoline tested.

Cell Division:

With five concentrations of arecoline, viz. 25 µg, 50 µg, 100 µg, 150 µg and 200 µg per ml GM the effects were studied on cell division at six different time intervals. As detailed in Table-9, in the control cultures the M.I. ranged from 4.9 to 3.5 between 0 to 48 hours. In the positive control cultures the M.I. spanned from 4.5 to 0.7 between 0 to 36 hours. Cell division was found to be affected even by the lowest concentration of arecoline utilized, i.e. 25 µg per ml GM. The number of percent dividing cells gradually diminished from 4.0 at 3 hours to 1.6 at 48 hours. With the increase in the concentrations of arecoline, the diminution in the M.I. was observed at shorter durations. The M.I. of 1.6 was observed at 48 hours with 25 µg per ml and at 36 hours with the concentration 50 µg per ml GM. At 100 µg per ml the reduction in M.I. was obvious from the initial duration of 3 hours till 48 hours. The M.I. ranged from 3.2 to 0.8. However, the diminution was more clearly evident in cultures subjected to 150 µg and 200 µg per ml GM. At 24 and 48 hours hardly a few mitotic figures were observed. Necrotic cells were visualized in the monolayers following continuous treatment with 150 µg per ml and 200 µg per ml GM after 24 hours.

Observations of recovery experiments are graphically presented in Fig. 6 and 7. Six hours' treatment was given with three concentrations viz. 50 µg, 100 µg and 200 µg arecoline per ml GM. The cells were able to restore the dividing capacity within 18 hours of recovery in arecoline free GM. At 0 hours, with a control M.I. of 4.0, the treated samples provided a M.I. of 3.6, 3.6 and 2.5, respectively for the three concentrations employed. After 18 hours the cell divi-

Table-9

Effect of different doses and durations of arecoline treatments on mitotic index of CHO cells

Hours	MMC 0.05µg/ml.	Control	25µg/ml	50µg/ml	100µg/ml	150µg/ml	200µg/ml
0 hrs	-	4.9	-	-	-	-	-
3 hrs	4.5	4.6	4.0	3.6	3.2	2.9	2.4
6 hrs	4.0	4.5	3.8	3.5	3.0	3.0	2.3
12 hrs	2.7	4.7	3.2	3.0	2.9	2.6	1.8
24 hrs	1.4	4.8	2.6	2.8	2.3	1.4	1.1
36 hrs	0.7	4.1	2.0	1.6	1.5	1.2	0.8
48 hrs	0.0	3.5	1.6	1.2	0.8	0.4	0.2

Fig. 6

Mitotic recovery of CHO cells in fresh growth medium after a 6 hour treatment with arecoline

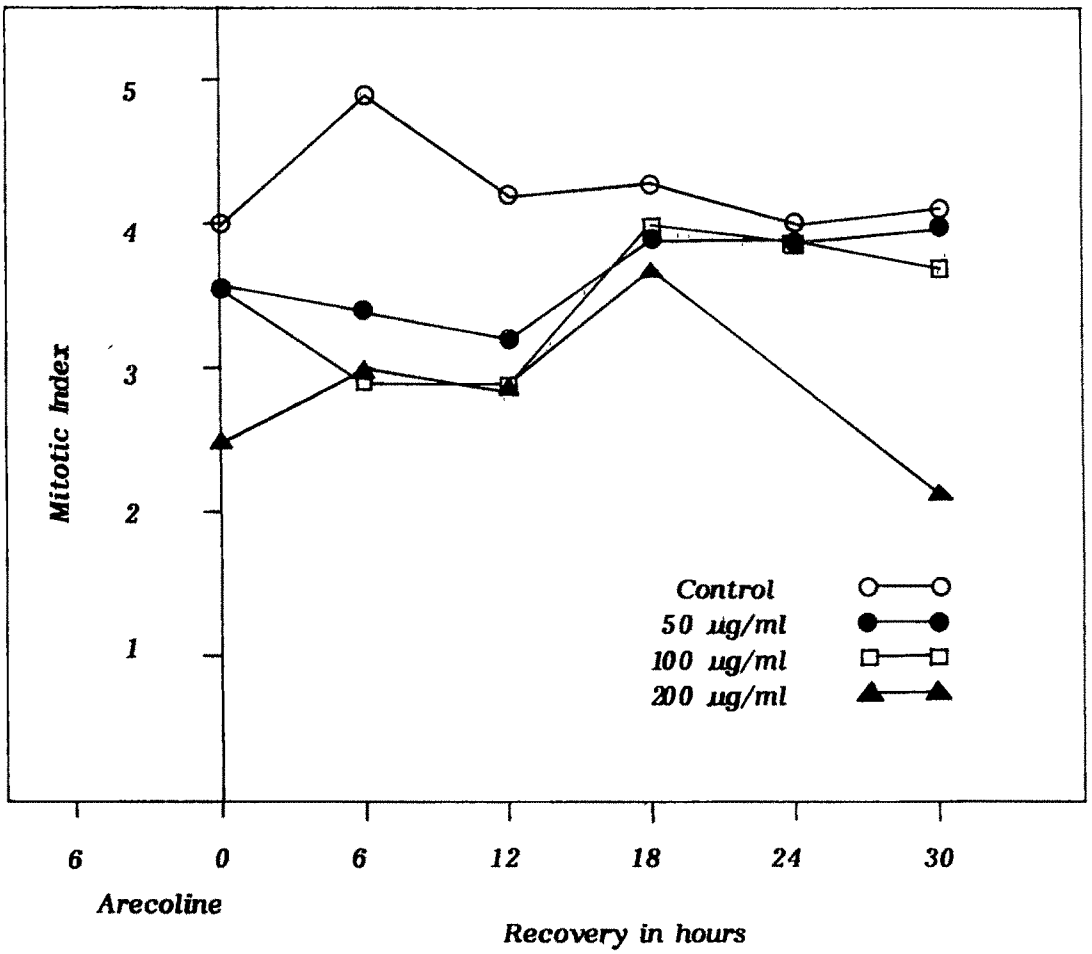
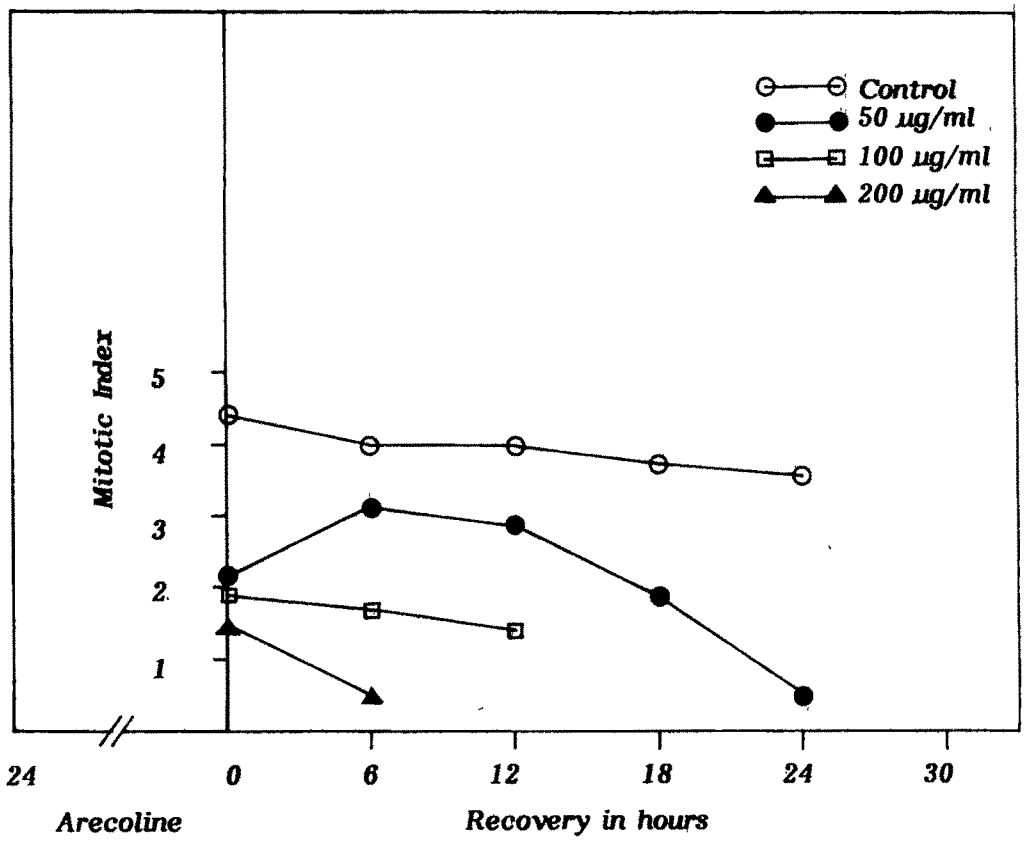


Fig. 7

Effect of 24 hours' treatment with different concentrations of arecoline on CHO cells



sion rates were almost comparable to the controls (M.I.=4.3) giving the M.I. of 3.9, 4.0 and 3.9, respectively. It remained consistent till 24 hours. The presence of abnormally dividing cells with lagging chromosomes, chromatin bridges, micronucleated interphases were very frequently noticed (Illustrations 1-11).

In another set of experiment, the cells were treated for 24 hours and then allowed to recover in fresh, arecoline free GM. At 0 hour the control samples exhibited a M.I. of 4.4, whereas the treated cultures had M.I. of 2.1, 1.9 and 1.5, respectively. It was observed that the cells could not fully restore their dividing capacity and the M.I. of the treated samples could not reach the control levels. The dividing capacity of the cells went on decreasing in the consecutive hours of the treatment.

Micronucleus:

Fig. 8 shows the percent MNC observed in the cultures after a treatment of 6 hours with 50 µg, 100 µg and 200 µg arecoline per ml GM followed by a recovery in arecoline free GM. Two sampling times were considered, 12 hours and 24 hours. As observed in the graph, a linear relationship was observed between the dose and the number of MNC. The MNC seen at 12 hours were 0.4% in the control samples and 1.2%, 1.4% and 2.6% in the treated samples, respectively. At 24 hours the control cultures had 0.4% MNC while the treated samples had 1.3%, 1.5% and 3.3% MNC, respectively for the three concentrations of arecoline.

Chromosome aberrations:

A three hours' treatment with 100, 150, 200 and 250 µg arecoline per ml GM was found to be potent enough to increase the number of CAs. Table-10 gives the details of the types and the number of CAs observed. A gradual dose dependent increase in the number of aberrant metaphases was demonstrable with the

Fig. 8

Elevations in micronucleated cell (MNC) frequency in CHO cells treated with arecoline for 6 hours and recovered in fresh growth medium

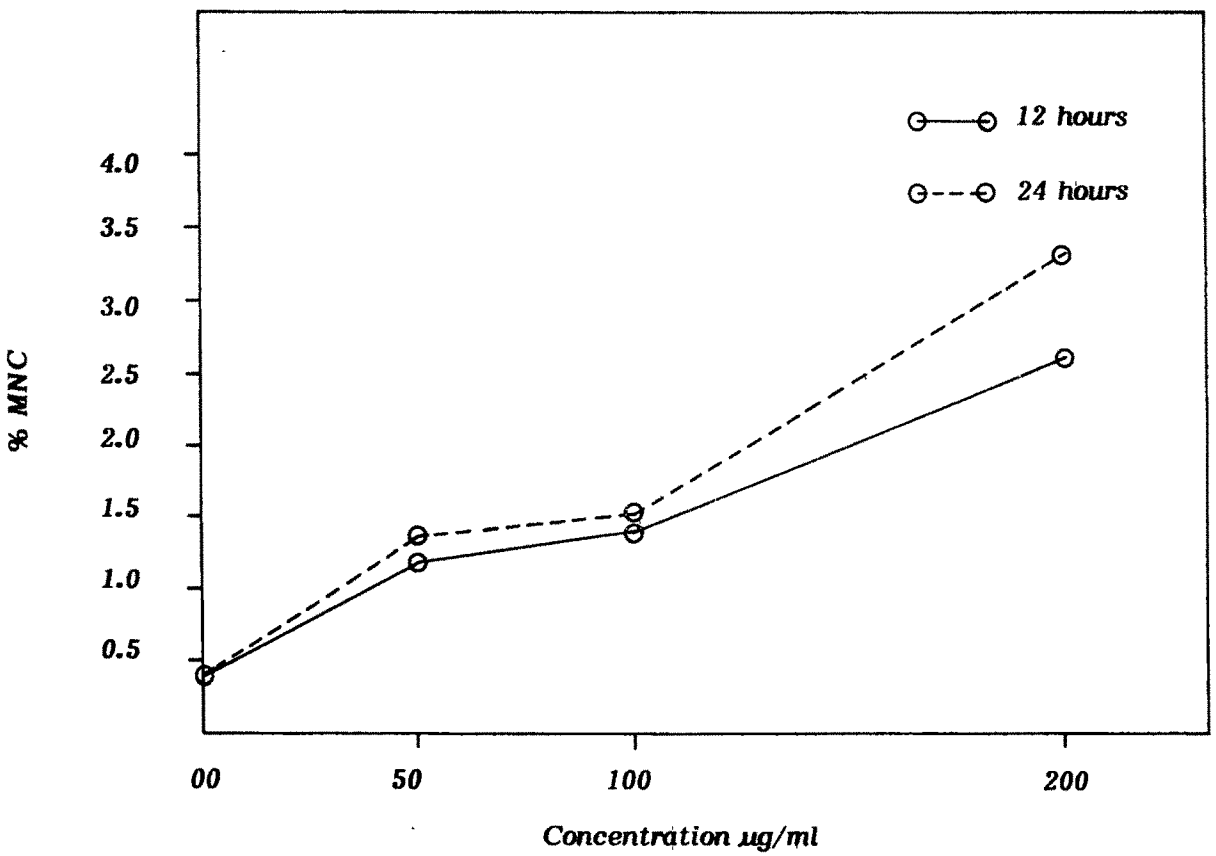


Table-10

Types of CAs visualized in CHO cells after 3 hours' treatment with arecoline and subsequent recovery for 24 hours

Conc./ml.	%Aberrant Metaphase	Chromatid				Chromosome					CA/Cell±S.E.
		G	B	I	Af	GI	BI	DM	R	Dic	
Control	7	3	1	-	1	2	-	-	-	-	0.07±0.026
100 µg	14	5	-	-	3	2	-	2	2	1	0.15±0.038
150 µg	11	5	2	2	3	3	-	-	1	2	0.18±0.065
200 µg	14	5	3	1	5	-	-	1	1	3	[a]0.19±0.054*
250µg	15	7	2	-	1	2	-	2	3	5	[a]0.22±0.059**
MMC 0.03µg	21	10	2	1	6	2	2	-	-	2	[b]0.25±0.053***

* $p<0.05$ ** $p<0.02$ *** $p<0.01$

a - $p<0.05$ b - $p<0.02$ - excluding gaps

Table-11

Effect of arecoline on frequency of CAs in CHO cells

Conc./ml.	%Aberrant Metaphase	Chromatid				Chromosome					CA/Cell±S.E.
		G	B	I	Af	GI	BI	DM	R	Dic	
Control	8	4	3	-	-	1	-	-	-	-	0.08±0.027
12.5 µg	10	3	-	-	-	7	2	-	-	-	0.12±0.038
25 µg	13	4	1	-	4	4	-	1	-	-	0.14±0.037
50 µg	16	5	2	-	3	5	2	1	-	-	0.18±0.043***
75 µg	19	6	2	2	5	2	2	2	2	1	[b]0.24±0.053**
100 µg	33	23	8	8	14	2	4	2	1	-	[a]0.62±0.105*
MMC 0.007µg	20	8	4	2	1	4	2	-	2	3	[b]0.26±0.053**

* $p<0.001$ ** $p<0.01$ *** $p<0.05$

a - $p<0.001$ b - $p<0.01$

G - Gap, B - Break, I - Interchange, Af - Acentric fragment,
GI - Gap isochromatid, BI - Break isochromatid, DM - Double minutes,
R - Ring, Dic - Dicentric

increase in the concentration, however, a statistically significant increase was observed only in samples treated with 200 µg and 250 µg arecoline per ml GM.

Different durations of the treatment, viz. 1 hour, 2 hours and 3 hours with 250 µg arecoline followed by a recovery in fresh GM, resulted in comparable number of CAs per cell, however, it increased the number of aberrant metaphases. The values were 13%, 18% and 17%, respectively. A statistically significant elevation was observed in CA per cell values for all the samples when compared with the controls.

A continuous treatment with five different concentrations of arecoline, exhibited a dose dependent increase in the rate of CAs. The number of aberrant metaphases rose from 10% at the lowest and 33% at the highest concentration utilized, compared to 8% in controls. The increase in CA per cell values was found to be statistically significant in 50 µg, 75 µg and 100 µg arecoline per ml GM, when compared with the control value. 20% aberrant metaphases were observed in the positive control cultures. The details of these data and the types of aberrations are provided in Table-11.

Sister chromatid exchanges:

Table-12 summarizes the mean SCE frequency observed in untreated as well as treated cultures. After a 3 hour treatment with arecoline and recovery in arecoline free GM, the SCE per cell values were 8.68, 10.04, 11.04 and 13.48 for the four different concentrations of arecoline utilized. The control value being 6.88 and the positive control value being 21.92 SCEs per cell. A statistically significant increase was observed for all the concentrations. A twofold induction of SCEs was observed at the highest concentration.

As visualized in Table-13, the induction of SCE was also found to be duration dependent. The SCE per cell values increased from 10.44 after 1 hour treatment

Table-12

SCE frequency in CHO cells recovered after a 3 hour treatment with different doses of arecoline

Conc./ml.	Mean SCE/Cell±S.E.	Range	% Metaphases with SCE			
			0-5	6-10	11-15	15
Control	6.88±0.428	4-11	28	56	16	-
100 µg	8.68±0.475**	5-14	8	68	24	-
150 µg	10.04±0.567*	6-16	-	60	36	4
200 µg	11.04±0.583*	7-16	-	52	36	12
250 µg	13.48±0.606*	9-19	-	28	44	28
MMC 0.03 µg	21.92±0.585 *	17-28	-	-	-	100

* $p<0.001$ ** $p<0.01$

Table-13

Effect of various durations of arecoline treatment on SCE induction in CHO cells

Conc./ml.	Mean SCE/Cell±S.E.	Range	% Metaphases with SCE			
			0-5	6-10	11-15	15
Control	6.88±0.428	4-11	28	56	16	-
250 µg 1 hr	10.44±0.557*	6-15	-	52	48	-
2 hrs	12.64±0.662*	8-20	-	28	56	16
3 hrs	13.84±0.603*	7-21	-	12	64	24
MMC 0.03 µg	21.92±0.585*	17-28	-	-	-	100

* $p<0.001$

Table-14

SCE induction by arecoline in CHO cells

Conc./ml.	Mean SCE/Cell±S.E.	Range	% Metaphases with SCE,			
			0-5	6-10	11-15	15
Control	6.48±0.392	3-11	32	64	4	-
12.5 µg	8.32±0.403**	5-15	4	92	4	-
25 µg	10.38±0.452*	7-14	-	56	44	-
50 µg	12.24±0.432*	9-16	-	32	64	4
75 µg	13.84±0.556*	10-20	-	12	60	28
100 µg	15.40±0.612*	10-22	-	4	48	48
MMC 0.007 µg	36.56±0.895*	20-49	-	-	-	100

* $p<0.001$ ** $p<0.01$

(with 250 µg arecoline followed by recovery in fresh GM) to 12.64 after 2 hours' treatment and 13.84 after 3 hours' treatment. Elevations in all the three values were statistically significant when compared to the control value of 6.88 SCE per cell and also when compared to each other.

The SCE frequencies were found to be directly proportional to the increase in the concentration of arecoline. This, as detailed in Table-14, could be observed in a continuous treatment of arecoline for 48 hours. All the concentrations utilized, induced a statistically significant increase in the SCE per cell values when compared to the controls. From the percent distribution of metaphases according to number of SCEs, as given in the table, it became evident that higher SCE values were due to more number of cells with higher SCE frequency and not because of a uniform SCE increase in all the cells.

Cellular kinetics:

In a short duration of 3 hours, arecoline treatment increased the AGT from 18.90 hours in controls to 19.67, 20.00, 20.78 and 21.24 in cells treated with different doses of arecoline. The AGT in positive controls was 27.12 hours (Table-15). With a single concentration i.e. 250 µg arecoline per ml GM. the duration of treatment also played a role in increasing the AGT from 20.43 hours in the controls to 21.62, 22.22 and 23.65 hours after 1 hour, 2 hours and 3 hours treatment, respectively.

As evidenced in Table-16 the cytostatic nature of arecoline could best be observed when the cells were continuously treated with various doses of arecoline. The gradual increase in the number of MI cells with a concurrent decline in MIII, deciphered the effect of arecoline on cell kinetics. The AGT increased by about 15 hours i.e. from 21.95 hours in controls to 36.92 hours in 100 µg arecoline per ml GM.

Table-15

Cellular kinetics in CHO following a 3 hour treatment with arecoline and recovery in fresh growth medium

Conc./ml.	MI	MII	MIII	PRI	AGT(hr.)
Control	00	46	54	2.54	18.90
100 µg	00	56	44	2.44	19.67
150 µg	00	60	40	2.40	20.00
200 µg	03	63	34	2.31	20.78
250 µg	05	64	31	2.26	21.24
MMC 0.03 µg	23	77	00	1.77	27.12

Table-16

Retardation in cell cycle progression in CHO on treatment with arecoline

Conc./ml.	MI	MII	MIII	PRI	AGT(hr.)
Control	04	65	31	2.27	21.95
12.5 µg	12	88	00	1.88	25.53
25 µg	27	73	00	1.73	27.75
50 µg	42	58	00	1.58	30.38
75 µg	53	47	00	1.47	32.65
100 µg	70	30	00	1.30	36.92
MMC 0.007 µg	12	88	00	1.88	25.53

DNA synthesis:

The observations after treatment of 12 hours and 24 hours with four doses of arecoline, are presented in Fig. 9. A dose and duration dependent decrease in the DNA synthesizing cells was observed. The control samples provided a labelling index of 65.15 which reduced to 57.85, 34.30, 27.05 and 7.15 in cells treated for 24 hours with four different concentrations of arecoline, respectively. The labelling index of positive control (MMC - 0.015 µg per ml GM.) was 8.0 after 24 hours' treatment.

3. *IN VITRO EFFECTS OF COMBINING ARECOLINE AND NICOTINE (A + N):*

Cell viability:

The cytotoxicity of a combination of two alkaloids, arecoline and nicotine, are recorded in Fig. 10. The durations at which the cell kill was studied was 24 hours and 48 hours. All the three combinations with various concentrations of arecoline and nicotine reduced the cell viability. The control samples had 94.60% viable cells at 0 hour and 89.26% at 24 hours. The treated cultures had reduced cell viability rate of 78.05%, 54.22% and 42.86%, respectively. This was further reduced after 48 hours' treatment, as observed in the Figure.

Chromosome aberrations:

Table-17 details the types and number of CAs observed after two hours' treatment with three different combinations of arecoline and nicotine, followed by recovery in fresh, alkaloid free GM for 24 hours. Although the number of aberrant metaphases was found to increase in all the concentrations, viz. 50 µg A + 90 µg N, 100 µg A + 200 µg N and 150 µg A + 300 µg N per ml GM the last two concentrations, utilized, gave a statistically significant increase in aberrations, even excluding the number of gaps observed.

Fig. 9

Effects of various doses and durations of arecoline treatments on ^{3}H-thymidine incorporation in CHO cell nuclei

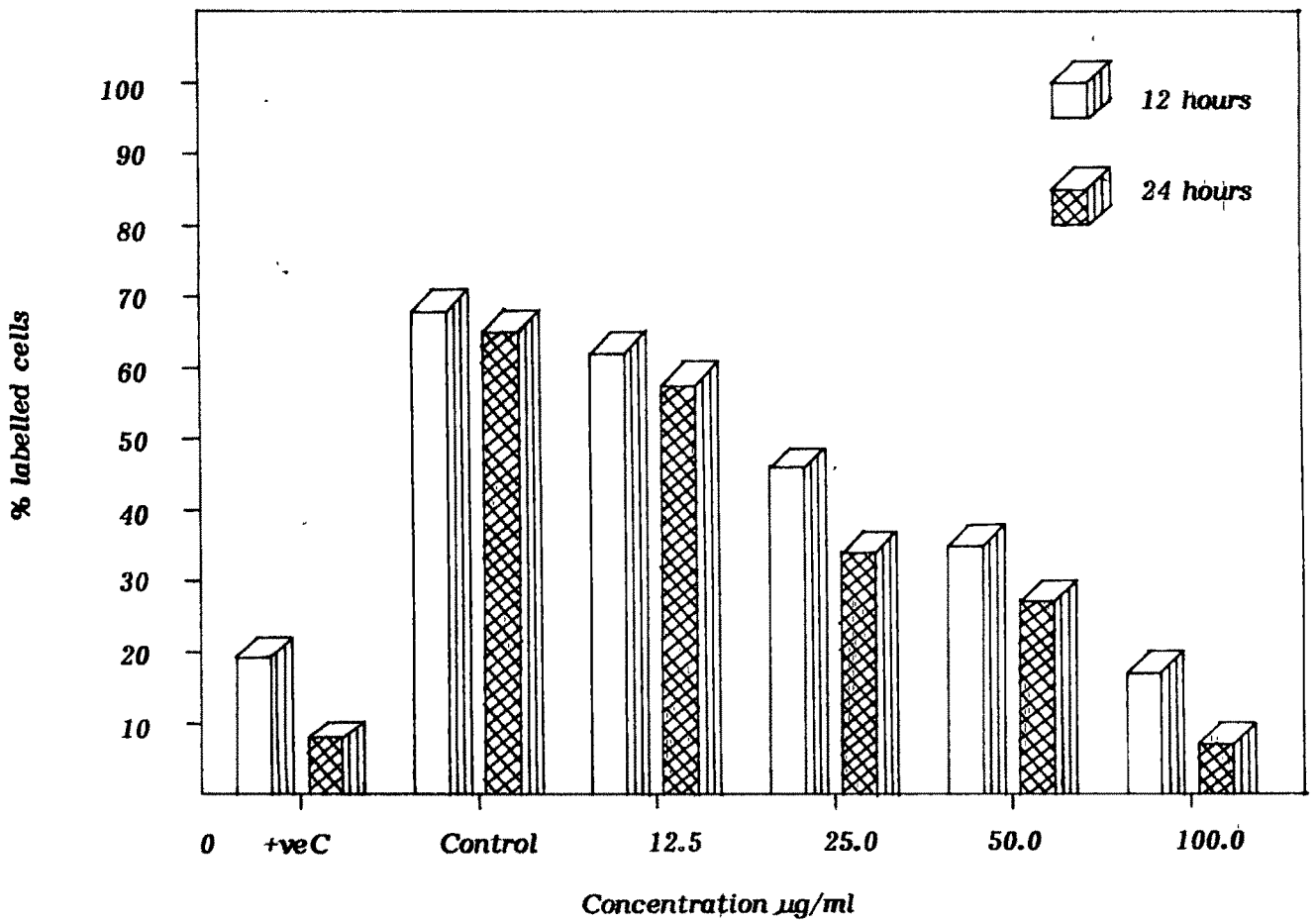


Fig. 10

Reduction in CHO cell viability on treatments with different concentrations of arecoline plus nicotine

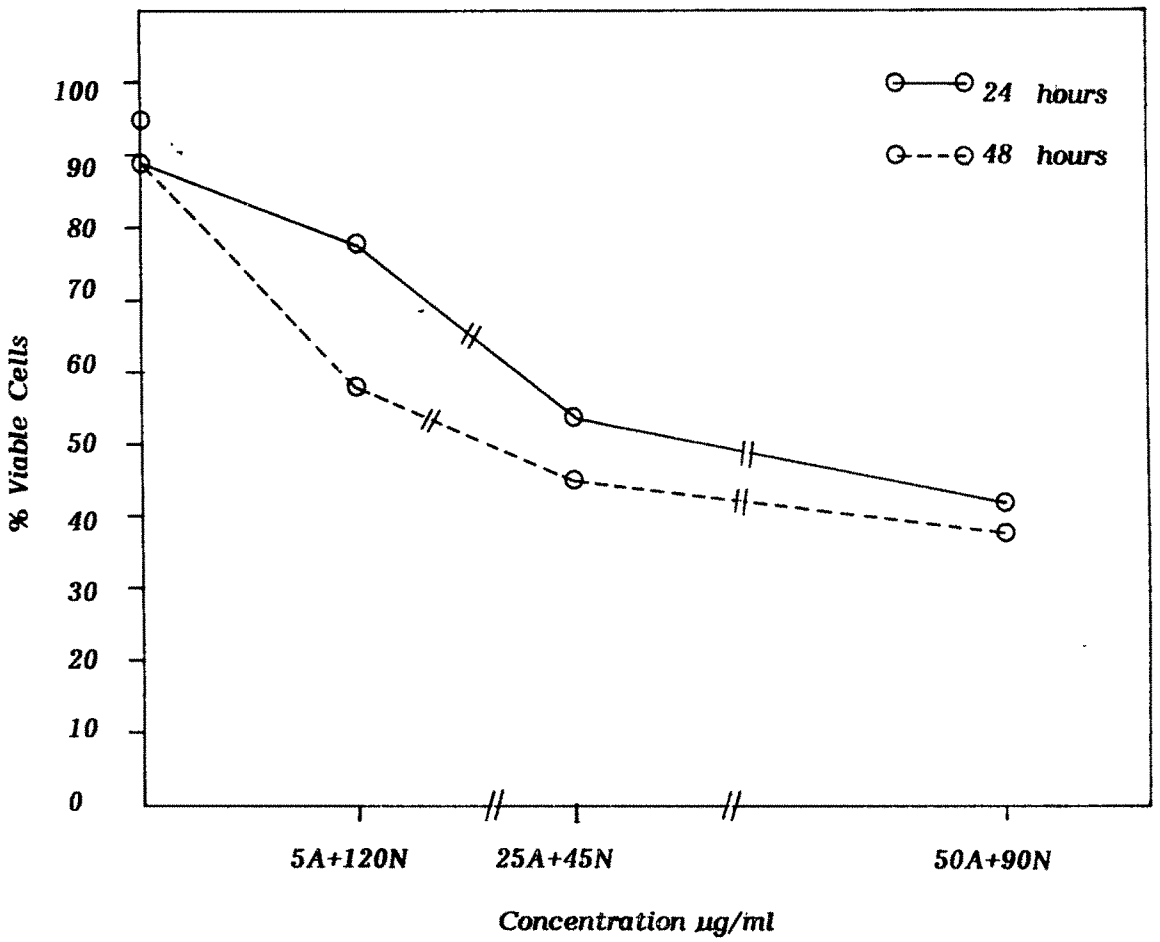


Table-17

Various CAs observed on treating CHO cells with arecoline plus nicotine (2 hours treatment and 24 hour recovery)

Conc. µg/ml.	%Aberrant Metaphase	Chromatid				Chromosome					CA/Cell±S.E.
		G	B	I	Af	GI	BI	DM	R	Dic	
Control	8	4	2	-	2	1	1	-	-	-	0.10±0.031
50 A + 90 N	18	12	1	1	1	2	-	1	-	3	0.21±0.048
100 A + 200 N	29	8	4	7	2	8	5	2	3	3	^{b}0.42±0.075*
150 A + 300 N	31	14	6	4	4	3	3	-	4	10	^{a}0.48±0.083 *
MMC 0.03 µg	24	8	6	1	2	2	4	-	2	1	^{c}0.26±0.048**

* $p<0.0.01$ ** $p<0.01$
a - $p<0.001$ b - $p<0.01$ c - $p<0.05$ - excluding gaps.

Table-18

Particulars of CAs in CHO cells on treatment with arecoline plus nicotine (A+N)

Conc. µg/ml.	%Aberrant Metaphase	Chromatid				Chromosome					CA/Cell±S.E.
		G	B	I	Af	GI	BI	DM	R	Dic	
Control	7	3	1	-	1	2	-	-	-	-	0.07±0.026
5 A	7	3	3	-	3	-	1	-	-	-	0.10±0.041
5 A + 60 N	9	3	1	-	8	-	-	-	1	-	^{a}0.13±0.046
5 A + 90 N	10	8	-	-	2	1	-	1	-	-	0.12±0.038
5 A + 120 N	11	4	2	-	2	1	1	3	-	-	^{b}0.13±0.039
25 A + 45 N	12	5	2	-	6	1	1	-	1	-	^{a}0.16±0.048
50 A + 90 A	15	8	7	3	4	1	-	-	-	1	^{b}0.24±0.063**
MMC 0.007	20	11	3	2	3	1	1	-	1	1	^{c}0.23±0.049*

* $p<0.01$ compared to controls. ** $p<0.02$ compared to controls.

a - $p<0.05$ b - $p<0.02$ c - $p<0.01$ - excluding gaps

G - Gap, B - Break, I - Interchange, Af - Acentric fragment,
GI - Gap isochromatid, BI - Break isochromatid, DM - Double minutes,
R - Ring, Dic - Dicentric

When the cultures were continuously subjected to a very low concentration of arecoline, with three increasing doses of nicotine, i.e. 5 µg A + 60 µg N, 5 µg A + 90 µg N and 5 µg A + 120 µg N, the aberration yield remained almost constant. However, at a concentration of 25 µg A + 45 µg N and 50 µg A + 90 µg N, the aberrant metaphases were 12% and 15%, respectively. The details are furnished in Table-18.

Sister chromatid exchanges:

The same combinations of arecoline and nicotine, as those mentioned in the CA assay, were utilized for SCE analysis. In a short duration of two hours, a twofold increase in SCE per cell frequency was observed. The control SCE per cell value was found to be 5.72 and in the cultures treated with three increasing concentrations, the values were 7.80, 9.44 and 11.96. With a difference of about 2, 4 and 6 SCEs per cell, the elevations were highly significant statistically ($p < 0.001$). The results are detailed in Table-19.

A continuous treatment, keeping the arecoline concentration constant and concurrently increasing the nicotine concentrations, elevated the average SCE per cell frequency. As summarized in Table-20, the control mean SCE per cell frequency was 5.72 and that of 5 µg arecoline per ml GM was 6.08. When nicotine was added the values were noted to be 6.96, 7.16 and 7.56, respectively for the three increasing concentrations of nicotine. The SCE per cell values in cultures containing 25 µg A + 45 µg N as well as 50 µg A + 90 µg N, were highly elevated i.e. 11.36 and 13.24, respectively. (The values were higher than those observed at 25 µg and 50 µg arecoline alone, Table-14). In the positive controls (MMC - 0.007 µg/ml) the SCE per cell value was observed to be 36.08.

Table-19

Values for SCE induced as a consequence of arecoline plus nicotine treatment for 2 hours followed by recovery for 48 hours

Conc.µg/ml	Mean SCE/Cell±S.E.	Range	% Metaphases with SCE.			
			0-5	6-10	11-15	15
Control	5.72±0.351	3-9	40	60	-	-
50 A + 90 N	7.80±0.353*	5-12	4	88	8	-
100 A + 200 N	9.44±0.315*	7-13	-	76	24	-
150 A + 300 N	11.96±0.468*	9-17	-	36	56	8
MMC 0.03	20.81±0.512*	17-28	-	-	-	100

* $p<0.001$

Table-20

Arecoline plus nicotine treatments resulting in SCE induction in CHO cells

Conc.µg/ml	Mean SCE/Cell±S.E.	Range	% Metaphases with SCE.			
			0-5	6-10	11-15	15
Control	5.72±0.369	2-9	44	56	-	-
5 A	6.08±0.392	3-11	44	52	4	-
5 A + 60 N	6.96±0.478	3-12	32	60	8	-
5 A + 90 N	7.16±0.382	4-12	24	72	4	-
5 A + 120 N	7.56±0.427**	4-12	20	68	12	-
25 A + 45 N	11.36±0.463*	8-15	-	44	56	-
50 A + 90 N	13.24±0.537*	9-20	-	16	64	20
MMC 0.007	36.08±0.880 *	20-48	-	-	-	100

* $p<0.001$ ** $p<0.02$ when compared to 5 µg A.

Cellular kinetics:

The treatment of arecoline plus nicotine for two hours, did not greatly increase the AGT as demonstrated in Table-21. Also a continuous treatment of 48 hours with a constant arecoline and an increasing nicotine concentration, did not induce any change in the generation time (Table-22). However, a definite retardation in the cell cycle progression was observed in the final combinations utilized. In control samples the AGT was 23.53 hours, which prolonged to 26.52 hours following 50 µg A + 90 µg N treatment. The treatment of MMC (positive control) adversely affected generation time, the AGT being 25.26 hours.

DNA synthesis:

On combining two different doses of arecoline with two different concentrations of nicotine (25 A + 150 N, 25 A + 250 N, 50 A + 150 N and 50 A + 250 N) the DNA synthesis was observed to reduce greatly. Even after 12 hours' treatment the additive effect of arecoline and nicotine was observed. The labelling index being 25.6, 20.7, 20.1 and 17.3 for the four combinations utilized. The control cultures had an index of 68.0 and in the positive controls it was found to be 19.6. The effect on inhibition of DNA synthesis was found to increase at 24 hours. Thus the combination of arecoline and nicotine affected the DNA synthesis in a dose and duration dependent manner. The increase in nicotine concentration also reduced the synthesis and the same was observed with the rise in arecoline concentration (Fig. 11).

DISCUSSION

A wide variation can occur in the composition of processed areca nut (Shivshankar et al., 1969). It is also known that the alkaloid content increases with the maturation of the nut (Mathew et al., 1964). In the present study, the variety of areca

Table-21

Slowing down of CHO cellular kinetics following a 2 hour treatment with arecoline plus nicotine and recovery in fresh growth medium

Conc.µg/ml.	MI	MII	MIII	PRI	AGT(hr.)
Control	02	87	11	2.09	22.97
50 A + 90 N	04	82	14	2.10	22.86
100 A + 200 N	05	85	10	2.05	23.41
150 A + 300 N	08	81	11	2.03	23.65
MMC 0.03	25	75	00	1.75	27.43

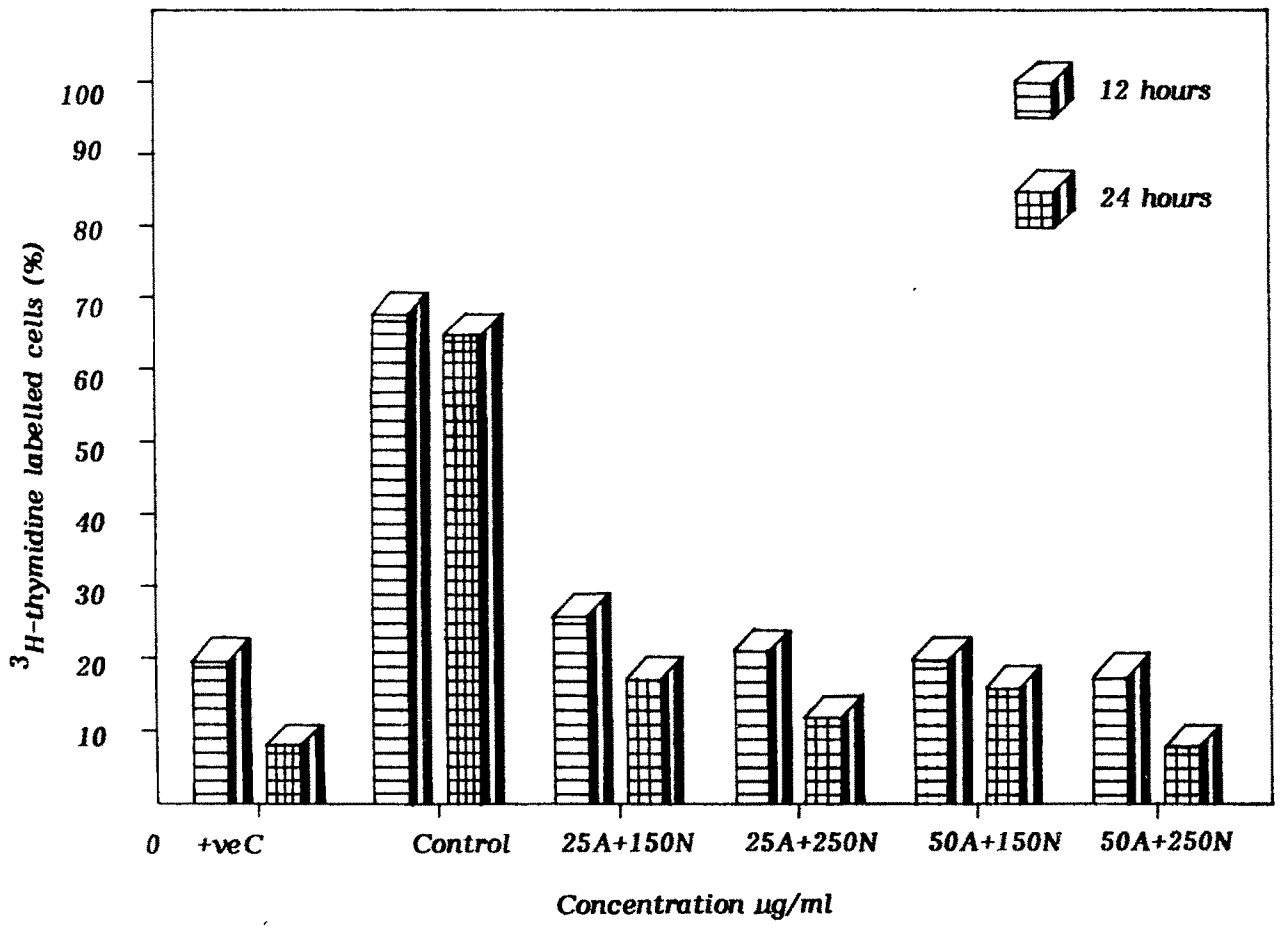
Table-22

CHO cell propagation on treatment with arecoline plus nicotine

Conc.µg/ml.	MI	MII	MIII	PRI	AGT(hr.)
Control	04	88	08	2.04	23.53
5 A	04	89	07	2.03	23.65
5 A + 60 N	03	93	04	1.98	24.24
5 A + 90 N	05	93	02	1.97	24.37
5 A + 120 N	04	96	00	1.96	24.49
25 A + 45 N	11	89	00	1.89	25.40
50 A + 90 N	19	81	00	2.03	26.52
MMC 0.007	10	90	00	1.90	25.26

Fig. 11

Effect of treatments with different combinations of arecoline plus nicotine on DNA synthesizing CHO cells

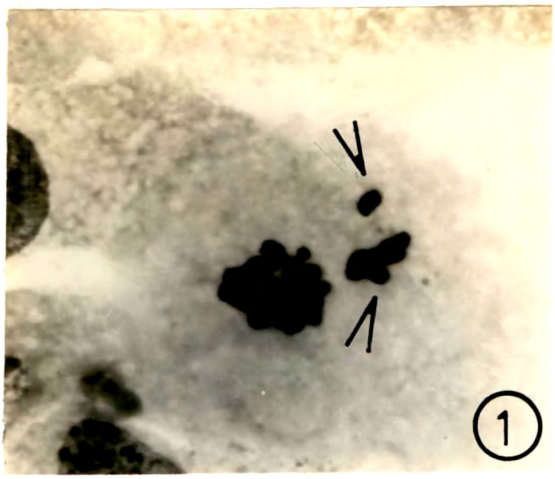


Illustrations 1-11 *Photomicrographs of some abnormalities observed in CHO cells after treatment with arecoline*

1 *Grouping of chromosomes in metaphase*

2-6 *Anaphases showing chromatin bridges and lagging chromosomes.*

Magnification: *1400x.*



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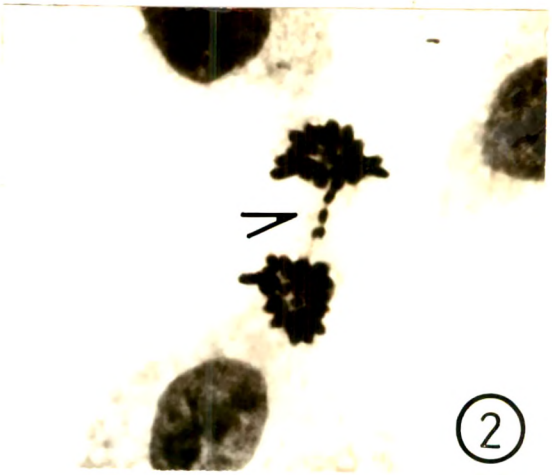

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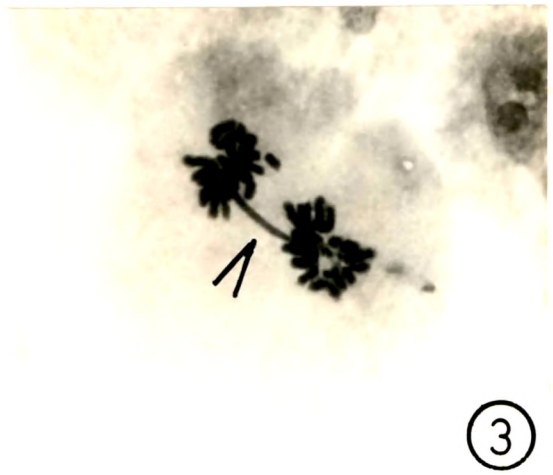

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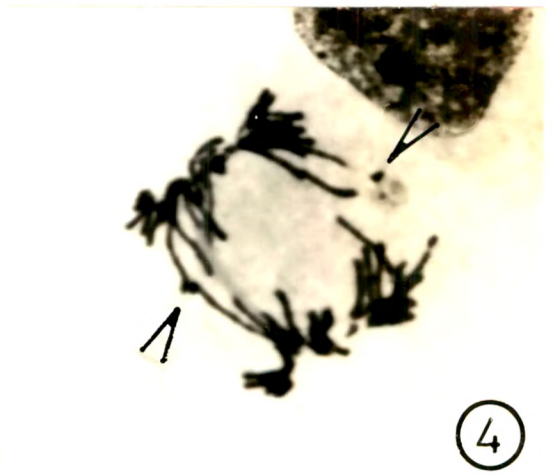

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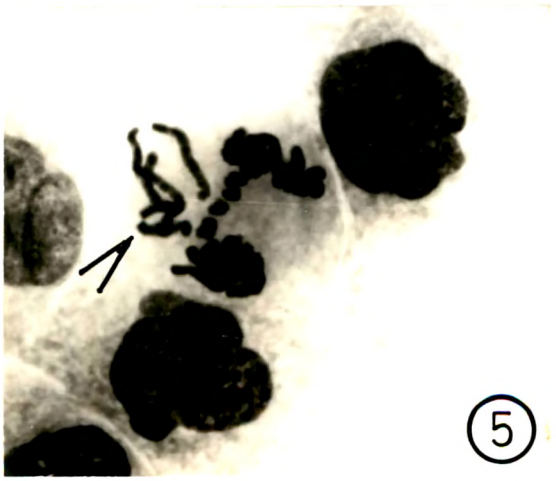

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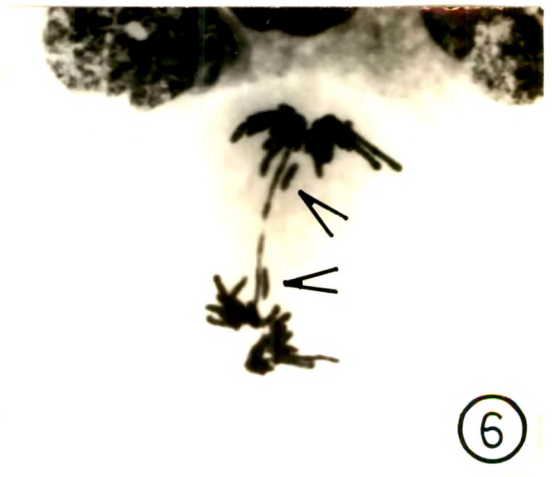

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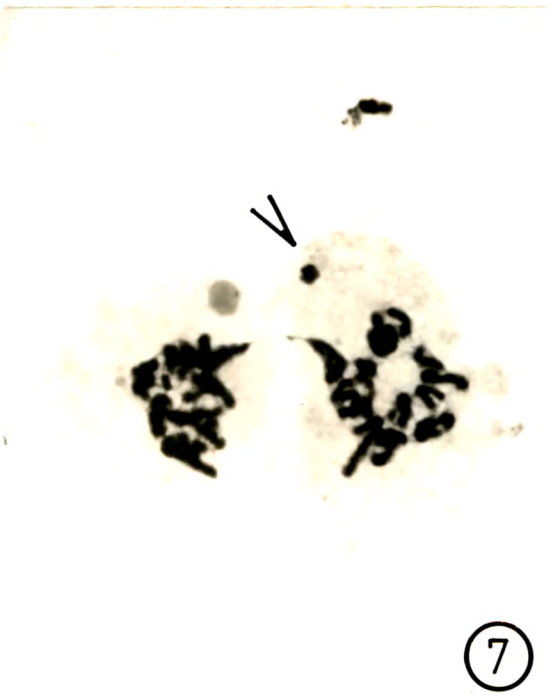
Illustration 7 *Early telophase showing the formation of micronucleated cell.*

8-9 *Interphase nucleus with micronucleus.*

10 *Multinucleated cells seen after treatments with high concentrations of arecoline.*

11 *Necrotic cells with a metaphase depicting grouping of chromosomes.*

Magnification: *1400x.*



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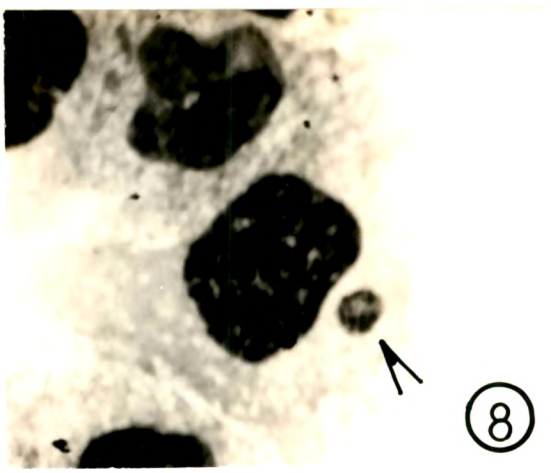

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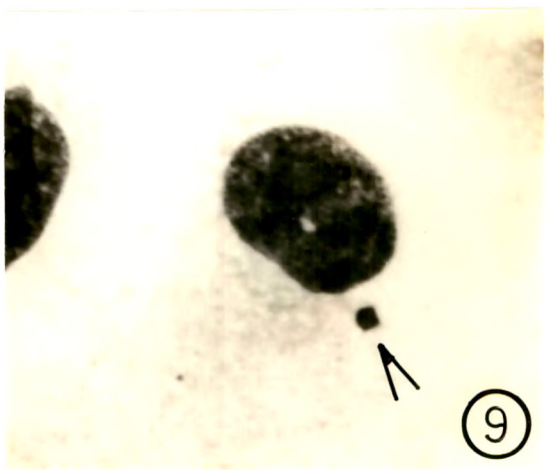

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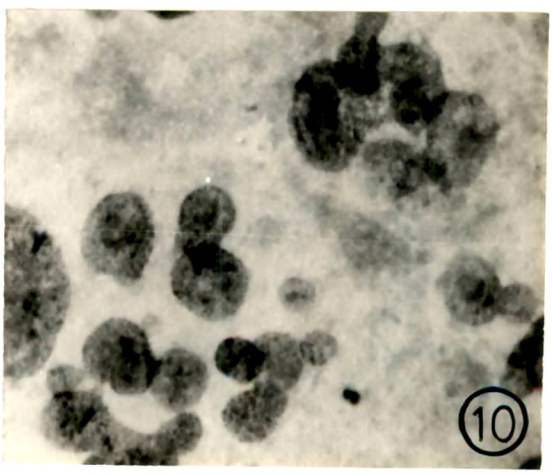
11



10

Illustrations 12-14 *CHO cells stained using FPG protocol to differentiate metaphases in MI, MII and MIII of the cell cycle.*

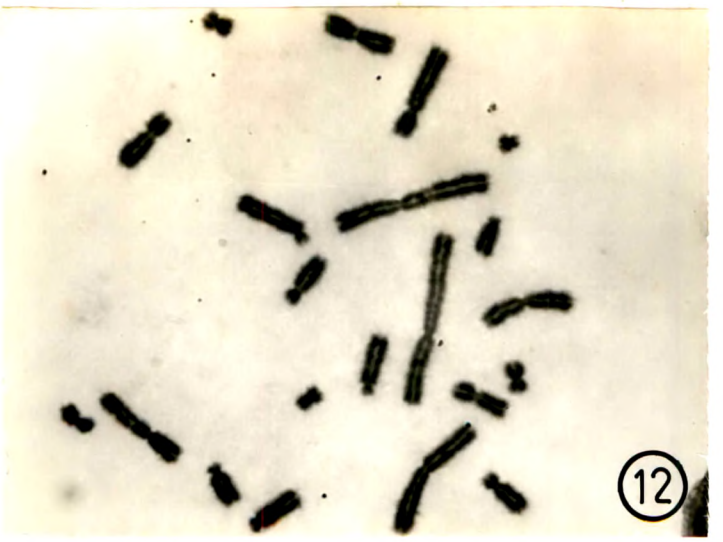
12 *A Metaphase in MI.*

13 *A metaphase in MII with differentially stained sister chromatids.*

14 *A metaphase in MIII showing some chromosomes with both the chromatids lightly stained.*

Magnifications: 12-13 *1400x*

14 *1600x*



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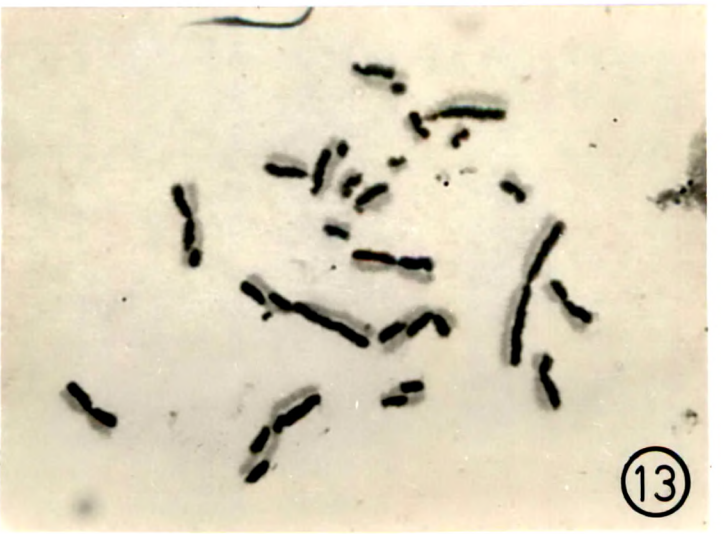

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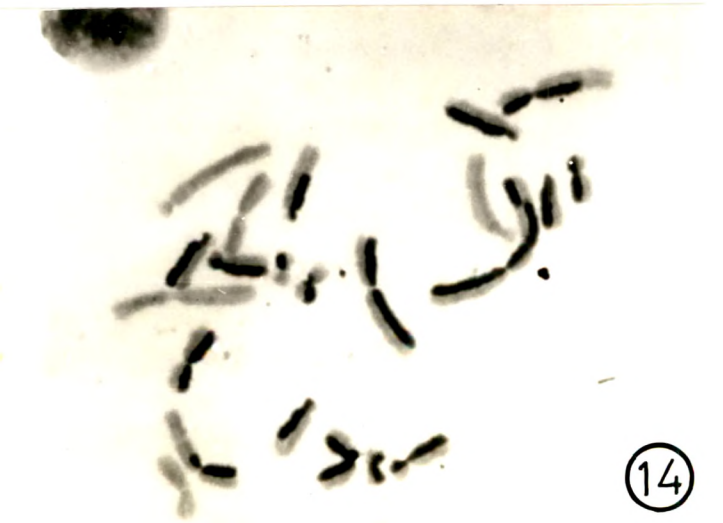


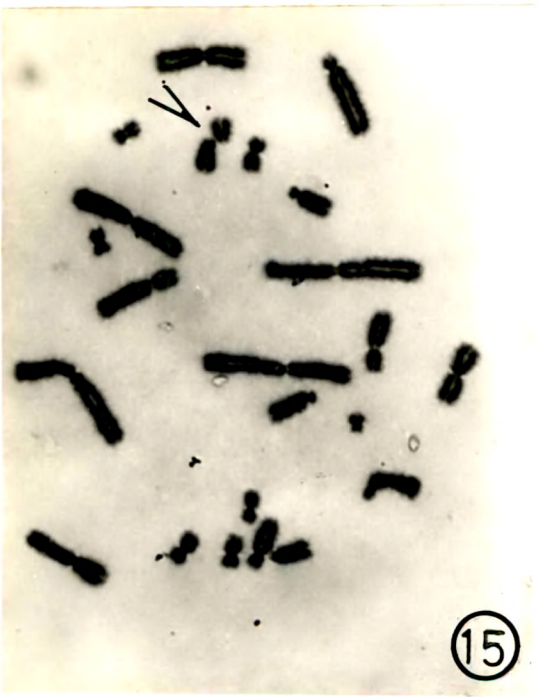
14

Illustrations 15-18 *Photomicrographs demonstrating chromosome breaks and gaps observed on treating CHO cells with various concentrations of AN ext.*

15 & 17 *Arrows indicate chromatid and isochromatid breaks*

16 & 18 *Metaphases with chromatid gaps (arrows).*

Magnification: *1400x*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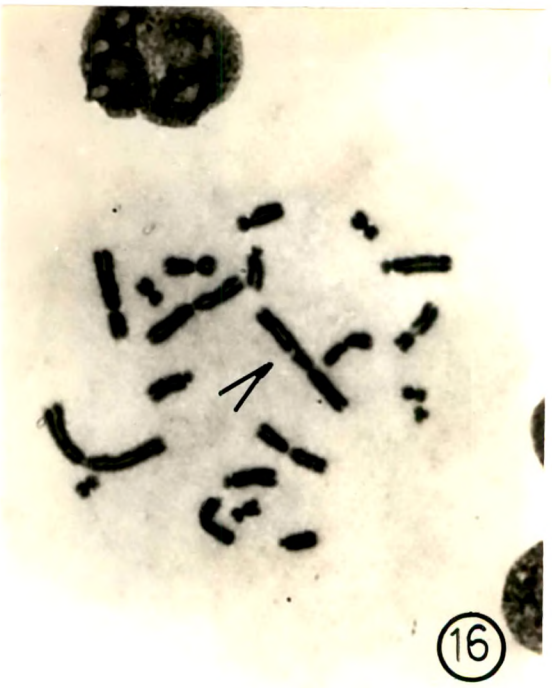
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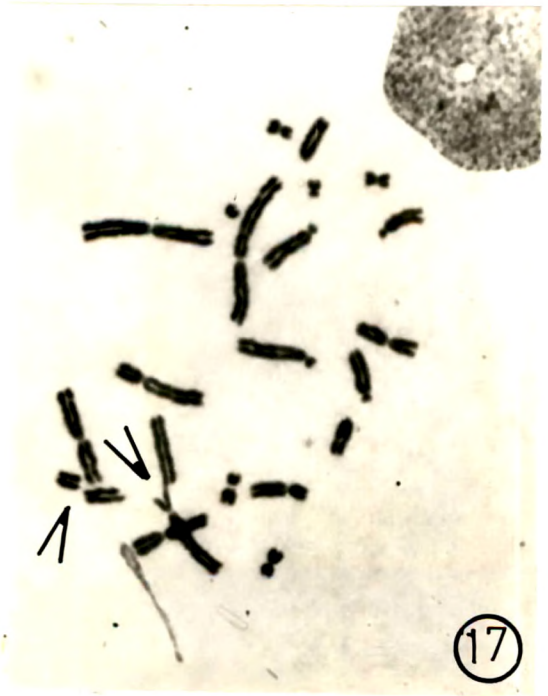
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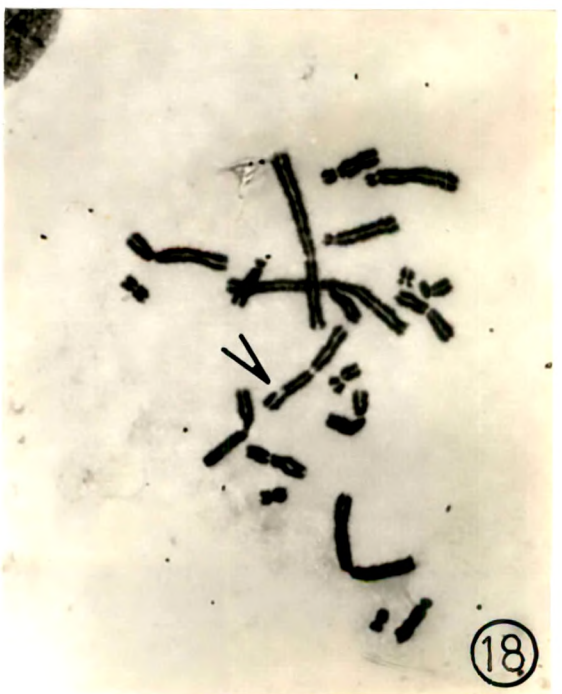

18

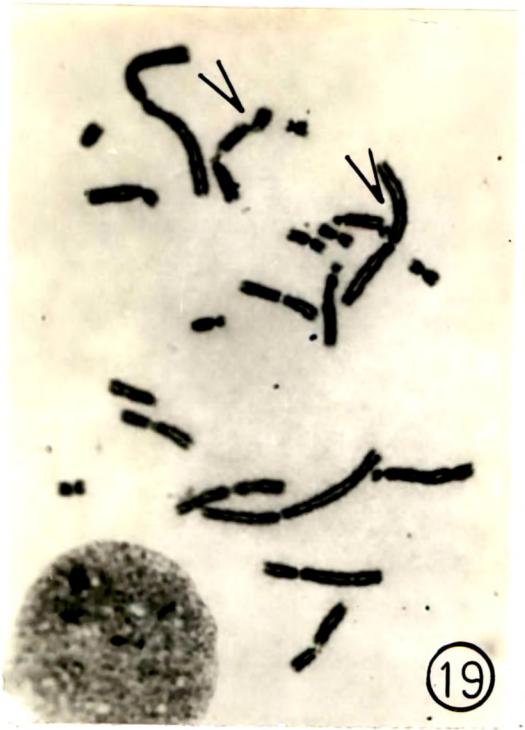
Illustrations 19-22 *CHO chromosomes exhibiting aberrations after a continuous treatment with 100 µg arecoline per ml GM.*

19 *Chromosomes showing isocromatid breaks (arrows)*

20 *A metaphase with ring chromosome.*

21-22 *Metaphases showing dicentric chromosomes.*

Magnification : *1400x*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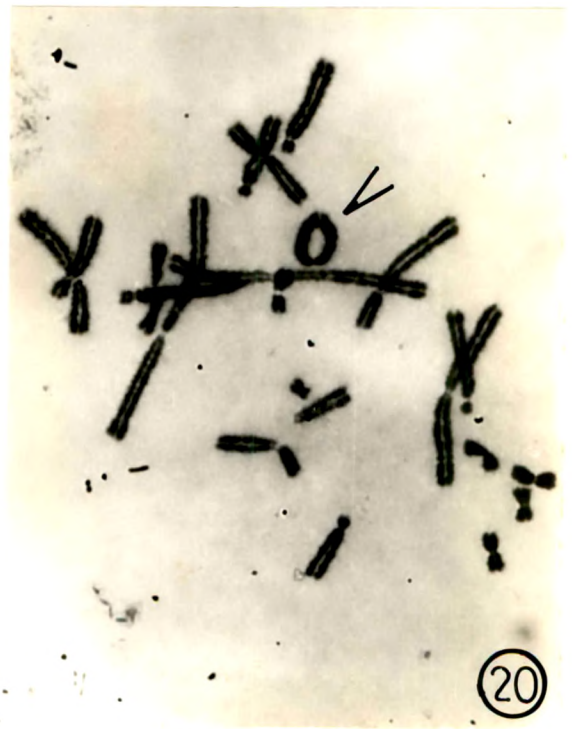
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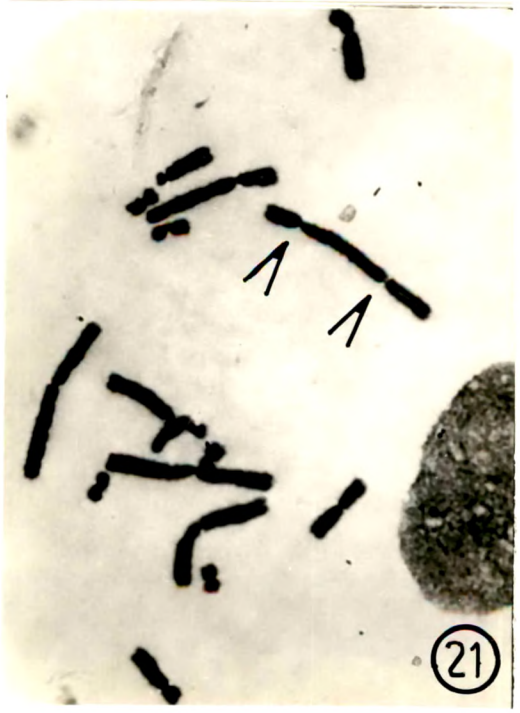
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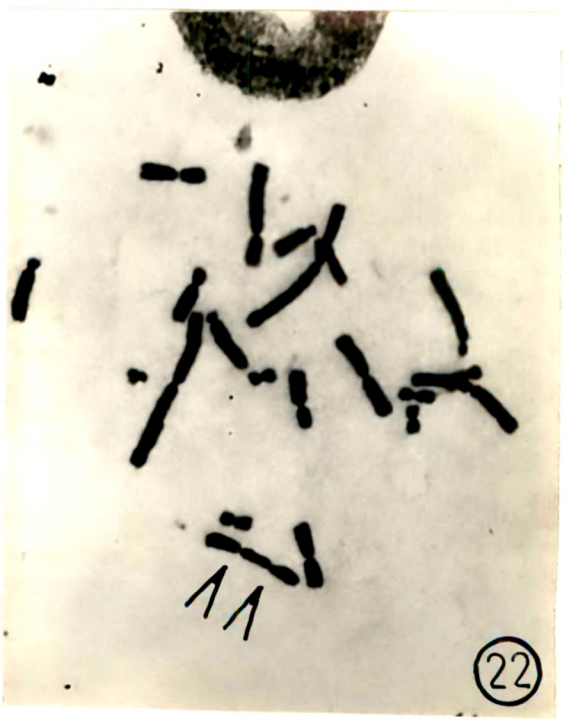

22

Illustrations 23-26 *Metaphases exhibiting the effects of arecoline + nicotine treatment for 2 hours followed by recovery in fresh GM.*

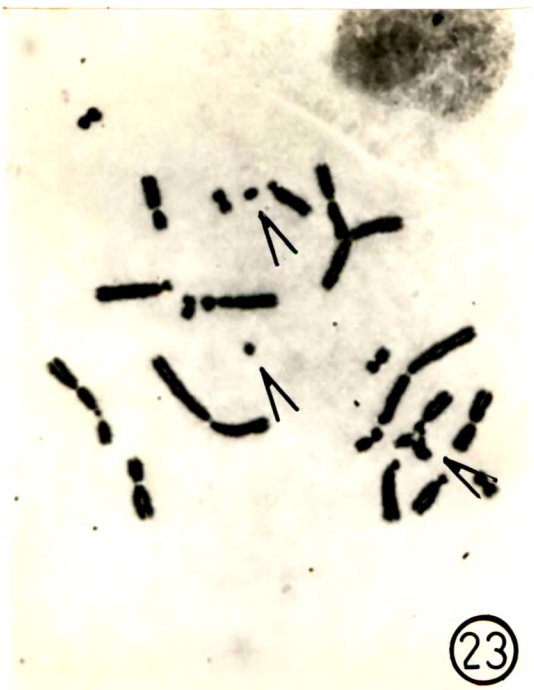
23 *Arrows pointing towards acentric fragment and formation of interchange.*

24 *A metaphase showing interchange and chromatid gaps.*

25 *A metaphase with a dicentric chromosome and interchange formation between nonhomologous chromosomes (arrows).*

26 *Triradial and quadriradial interchanges between nonhomologous chromosomes (arrows)*

Magnification: *1400x*



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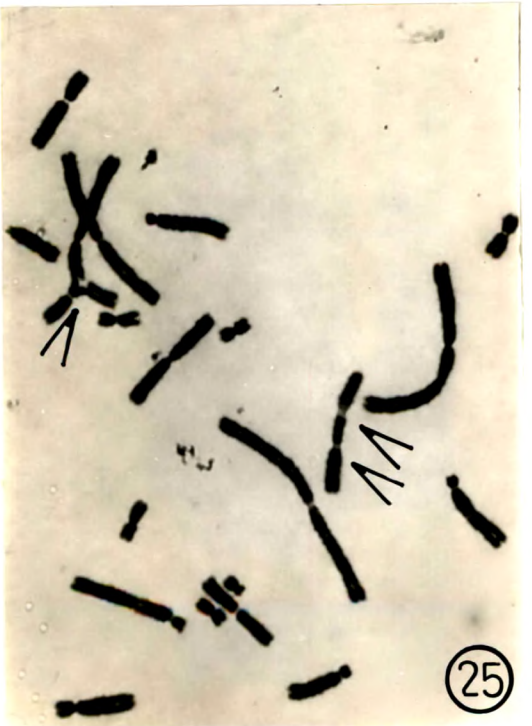

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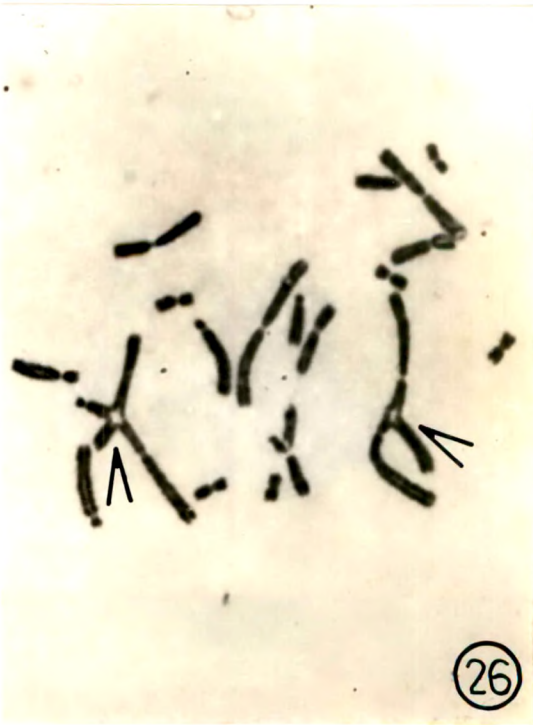


26

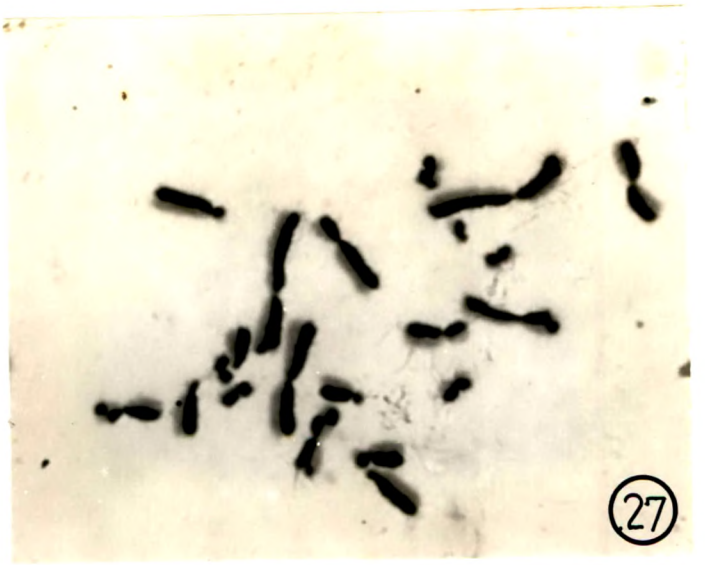
Illustrations 27-29 *Sister chromatid exchanges in CHO cells.*

27 *A metaphase from an untreated culture showing few SCEs.*

28-29 *Metaphases from treated cultures showing more number of SCEs.*

Magnifications: 27-28 *1600x*

29 *1400x*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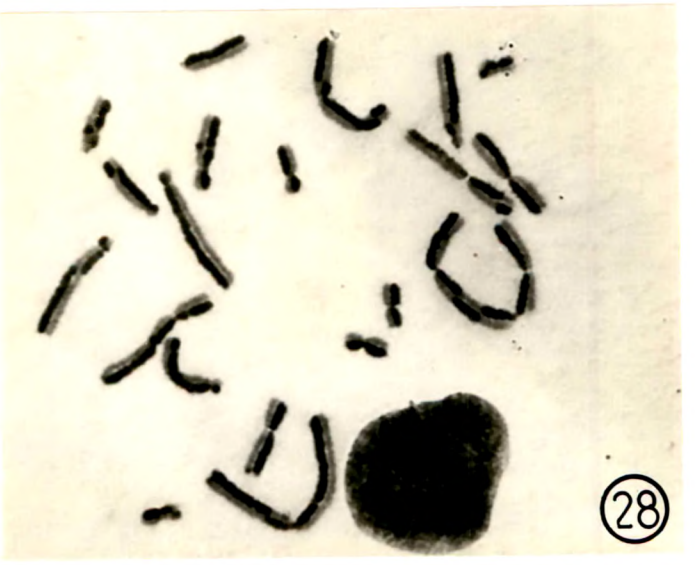
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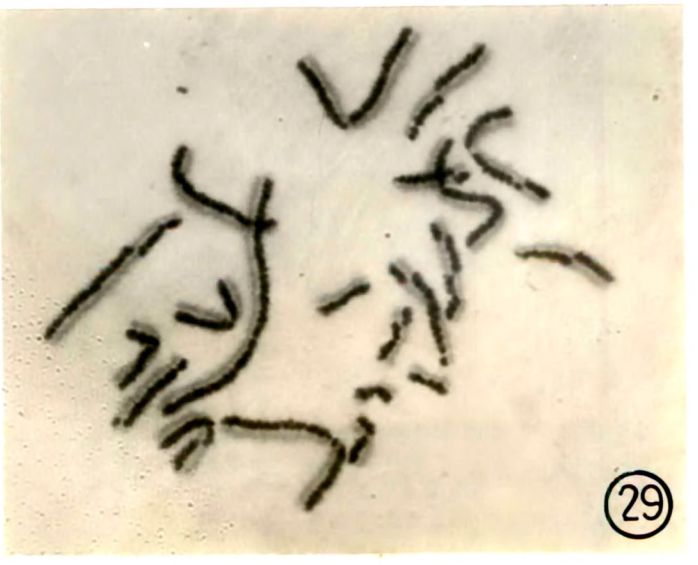
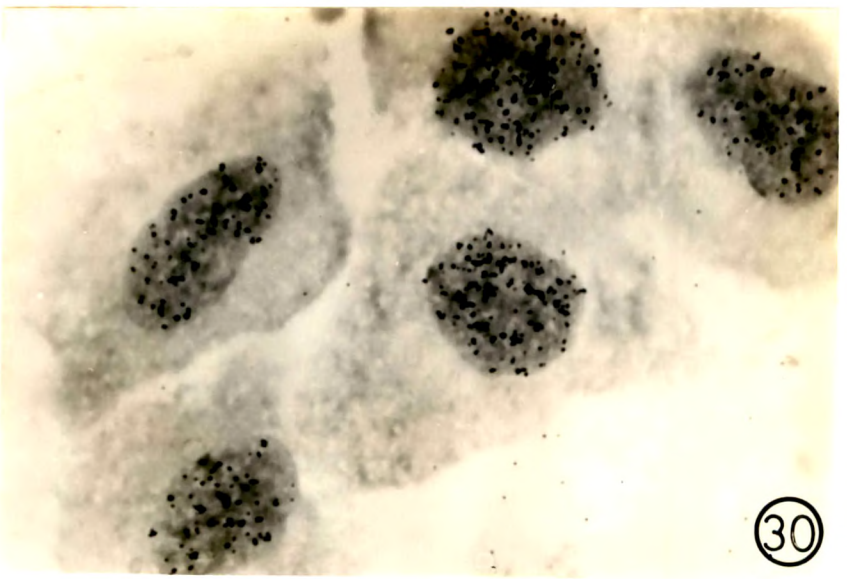

29

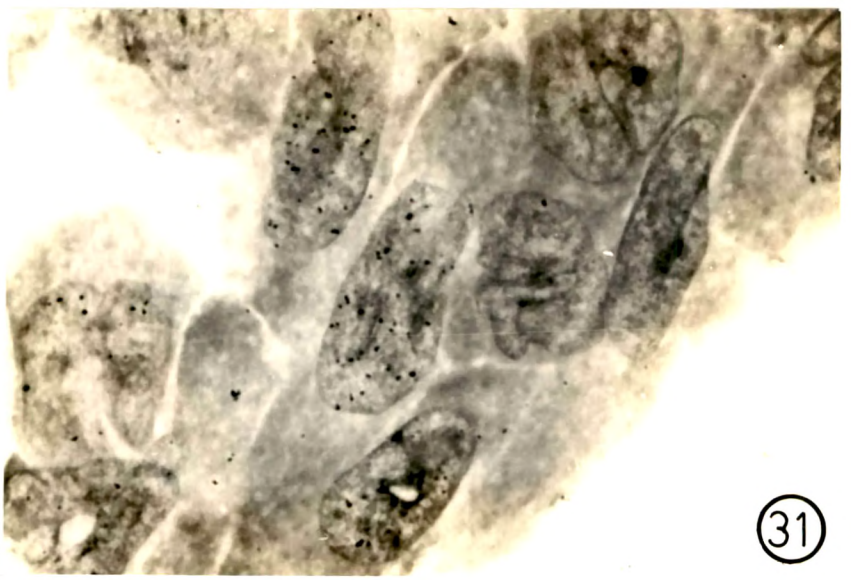
Illustrations 30-31 *Autoradiograms showing ^{3}H-thymidine incorporation in CHO cells.*

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

31 *CHO cells treated with AN ext. for 24 hours. Note the reduction in labelling intensity over nuclei.*

Magnification : *1400x*


30


31

nut used to prepare the aqueous extract was unprocessed, ripe and sundried nut which is usually consumed in this part of the country. Owing to the fact that they are not subjected to boiling, and are dried in sun before dehusking, the loss of chemical constituents is unlikely. Recently, Rao and Das (1989) have reported that the unprocessed nuts have greater carcinogenic potency than the processed ones. An aqueous extract of areca nut would more closely simulate the local action of areca nut in the oral cavity than an organic extract. Hence, an aqueous extract (10 µl ≡ 1.1 mg of areca nut) was preferred for the study.

The concentrations of arecoline selected for the study were comparable to the estimated saliva levels reached, when areca nut is chewed with or without tobacco. Nair et al. (1985) reported an average of 25 µg arecoline per ml of saliva (range 0-89 µg per ml saliva) when areca nut (betel quid) was masticated without tobacco. When it was chewed together with tobacco, an average of about 50 µg arecoline and 90 µg nicotine per ml of saliva was observed. In absence of metabolizing enzymes in oral cavity, the areca nut constituents would more often remain in their original state.

The first experiment covered the effects of areca nut extract, its alkaloid arecoline and a combination of arecoline with nicotine on the viability of CHO cells. Such studies help in giving concentration versus cytotoxicity relationships and thereby in providing an index for the general biological reactivity of the substance. The availability of cytotoxicity data facilitates the design of cytogenetic studies. The dye exclusion test is widely used to decide the in vitro toxicity and was, therefore, applied to study the cytocidal action of areca nut extract and the areca nut alkaloid on mammalian cells. Boyland and Nery (1969) observed that an ip LD_{50} of arecoline was 40 mg/kg body weight. In our studies a dose dependent cell death was observed in cultures treated with AN ext. as well as arecoline. The cytocidal action observed at 24 hours was more pronounced at 48 hour sampling time, bringing down the survival rates considerably. Harvey et al (1986)

also observed the cytotoxic action of arecoline at 100 µg/ml. We observed that in the cultures treated with AN ext. the toxic effects were greater. As investigated by Sundquist et al. (1987), an aqueous extract of areca nut decreased colony forming efficiency and clonal growth rate of cultured human epithelial cells. Wary and Sharan (1988) observed that higher dose of areca nut extract were detrimental to cells in culture. According to the method of extract preparation and considering the arecoline content of areca nut (Arjungi, 1976), one ml of the aqueous extract contains approximately 555 µg of arecoline i.e. 5.55 µg arecoline per ml GM was added when the lowest concentration of the extract (10 µl) was utilized. Thus, despite the low dose of arecoline, a cytocidal action was exerted by the extract. Hence, it is likely that, in addition to arecoline, the extract contains other water soluble component(s) of areca nut which add to the cytocidal action of arecoline.

It was interestingly noted that arecoline in combination with nicotine was more toxic. At 25 µg arecoline concentration, the effects observed after 48 hours were seen within 24 hours when the same concentration was combined with 45 µg nicotine, which by itself is nontoxic at this concentration (our observation).

The second experiment dealt with the studies on the effects of arecoline on cell division. Abnormal cell division reflects inhibition or alteration in macromolecule syntheses of the cell. Our observations revealed that arecoline elicited a dose and duration dependent inhibition of the cell division.

The continuous presence of arecoline in the medium gradually reduced the mitotic index. This was observed for all the five concentrations utilized. There was an accumulation of cells in metaphase. With the increase in arecoline concentrations the inability of the dividing cells to reach the anaphase, led us to assume that arecoline might be preventing spindle contraction or bringing about a modification

in the spindle function, which ultimately results in metaphase accumulation. A similar speculation was made by Sinha and Rao (1985a; 1985b). Chromatin bridges during anaphase were also observed. McGill et al. (1974) and Pathak et al. (1975) have suggested that this may either result in a tetraploid nucleus or in two cells deficient of a particular set of genes. Thus the inhibitory effects of arecoline on the dividing capacity of cells was observed during continuous treatment.

An irreversible inhibition of cell division was encountered after 24 hours' treatment with all the three concentrations used. However, following a treatment of 6 hours with the same concentrations of arecoline, the cells were able to restore the rates of cell division, within 18 hours, in arecoline free medium. However, when the cells were treated for longer periods, they failed to recover. It can be presumed that when the duration of treatment exceeds a period of 24 hours, extensive damage to mitotic apparatus might lead to the inability in restoring normal cell division. Abnormal cell divisions frequently figured in the recovery samples. These were encountered as grouping of chromosomes in metaphase, lagging of chromosome in anaphase, multinucleated cells, necrotic cells etc. The multinucleated cells may result due to the destortion of spindle apparatus. Alkaloids from various plants, consumed as food or drugs are known to act as spindle poisons (Yamanaka et al., 1979; Takanashi et al., 1980). Although arecoline is known to function as a monofunctional alkylating agent (Boyland and Nery, 1969), the probability that it may also function as a spindle poison, cannot be ruled out.

The third experiment dealt with the induction of micronuclei in CHO cultures by the presence of arecoline. Micronuclei originate from chromosomal material that has lagged during anaphase. In course of mitosis, this material is distributed

to only one of the daughter cells and it may be included in the main nucleus or form one or more separate micronuclei. In the previous experiment of cell division, we found a number of anaphases with chromatin bridges or lagging chromosomes and it was felt worthwhile to analyse the number of micronuclei formed due to arecoline treatments. Micronuclei consist of acentric fragments as demonstrated by DNA content measurements (Heddle and Carrano, 1977). They may also consist of entire chromosomes and may result from nondisjunction due to malfunction of the spindle apparatus. These larger micronuclei are formed by spindle poisons (Yamamoto and Kikuchi, 1980; Högstedt and Karlsson, 1985). In our observations, at higher concentrations of arecoline, both types of micronuclei could be seen, however, only those smaller than $1/5^{th}$ of the size of the main nucleus, have been considered for the present study. Shirname et al. (1984) reported that an aqueous extract of areca nut as well as arecoline induced MN in mouse bone marrow cells. Our results manifested a dose dependent increase in the number of MNC, indicating the clastogenic action of arecoline on CHO cells. Sinha and Rao (1985b) found that transplacental exposure to arecoline induced MN in fetal erythrocytes in mice, which indicated that placenta did not form an efficient barrier to arecoline.

Most of the mutagens/carcinogens are clastogenic and induce chromosomal aberrations. An extensive body of literature exists on the association between CA and carcinogenesis. CA assay has been used as an effective screen for evaluating possible carcinogenic potentials. A monofunctional alkylating agent like arecoline, may induce several types of DNA lesions including DNA single strand breaks. These lesions are subjected to cellular repair mechanisms, and the unrepaired or misrepaired lesions lead to CA. Several inhibitors of DNA synthesis/repair have been studied for their ability to potentiate the frequencies of induced CA

(Natarajan and Obe, 1984; Kihlman and Natarajan, 1984). Our previous experiment revealed the clastogenic capacity of arecoline, which was expressed by increased MNC frequency. A quantitative and qualitative analysis of CAs further confirmed these findings. The continuous and recovery experiments with AN ext., arecoline and also its combination with nicotine, yielded a dose related increase in the frequency of CAs. Chromatid type of aberrations were more frequently observed than chromosome type. It is known that the aberrations induced in G1 and early S phase are chromosome type, whereas those induced in late S and G2 phase are chromatid type. The inhibitors of DNA synthesis usually induce a very high frequency of gaps when the cells are treated in late S or G2 phase (Natarajan and Obe, 1982). As presented in our data, the occurrence of gaps, breaks and exchanges was more common compared to other aberrations.

The aqueous extract of areca nut and arecoline have been tested for their clastogenicity by other investigators and they also have observed a positive effect (Stich et al., 1981; 1983a; Panigrahi and Rao, 1982; Stich and Tsang, 1989). DNA strand breaks produced by areca nut extract have recently been reported by Wary and Sharan (1988). It could be perceived from our findings that arecoline by itself, possesses chromosome damaging ability, nonetheless, looking to the frequency of CAs, the aqueous extract appears to contain other clastogen(s) which potentiate the chromosome damaging effects. Stich et al. (1983a) have observed that the tannic acid fractions derived from areca nut have been reported to induce chromatid breaks and exchanges in mammalian cells and gene conversion in yeast.

A more pronounced chromosome damage was recorded when arecoline was coupled with nicotine. The frequency of CAs observed on treating the cells with 250

µg arecoline per ml GM, was comparable to the effects produced by 50 µg A + 90 µg N per ml GM. Nicotine at 90 µg per ml does not induce CA (our observation). The average saliva level of arecoline and nicotine in individuals chewing areca nut with tobacco is 50 µg and 90 µg per ml saliva respectively (Nair et al., 1985). It has been manifested in many carcinogenicity studies in animals that areca nut powder or aqueous/DMSO extracts when combined with tobacco gave a more pronounced effect (Ranadive et al., 1976; 1979; Suri et al., 1971). It was also interesting to note that statistically significant CAs were induced by concentrations at which neither arecoline nor nicotine, per se, were clastogenic. Similar effects have been observed by Stich et al. (1981) when compounds found in betel quid, viz. arecoline, eugenol, quercetin, chlorogenic acid and Mn^{++} were studied to search possible potentiation of the effects by combining these compounds.

SCE, a cytological revelation of DNA breakage and misrepair, can be observed in cells that have completed two cell cycles in presence of BrdU. The Flourescence plus Giemsa (FPG) technique developed by Perry and Wolff (1974) has given the assay a more simpler form. Our fifth experiment covers the details of SCE frequency induced by AN ext., arecoline and a combination of arecoline with nicotine. It is well known that SCEs reflect a direct interaction of the substance with DNA, and represent a useful system for the detection of genotoxic substances. The mechanisms involved in gross chromosome aberration and SCE formation are not the same and hence, the two assays may not always exhibit a correlation. Thus, while examining a mutagenic carcinogen, a combination of both assays serves more effectively (Abe and Sasaki, 1977; Latt and Schreck, 1980; Gebhart, 1981). The CAs induced by arecoline and AN ext. are mostly chromatid type. These type of agents may be efficient inducers of SCE (Carrano and Natarajan, 1988).

A dose dependent increase in the number of SCEs, with a high degree of statistical significance, observed after the treatment of AN ext. and arecoline, suggest that they are potent inducers of SCEs. Arecoline increases SCE frequency in human peripheral blood lymphocytes in vitro, in a dose related manner (our observation). The chromatid aberrations are predominantly induced by DNA cross-linking agents. It was proposed that intrastrand cross-links give rise to CA and interstrand corss-links give rise to SCEs (Scott, 1980). The increase in CAs and SCEs suggest that areca nut induces intrastrand as well as interstrand crosslinks in DNA.

Umezawa et al. (1981) found that the ethyl acetate extract of areca nut could not induce SCEs in virally transformed human lymphocytes. Panigrahi and Rao (1986) detected a dose related increase in the frequency of SCEs in mouse bone marrow cells induced by aqueous extract of areca nut. Our results are in agreement with those of Panigrahi and Rao (1986). They found that the whole aqueous extract of areca nut was more genotoxic than the tannin component, and attributed the SCE induction to the areca nut alkaloid, arecoline. The tannin fraction on longer exposure did induce significant SCE increase. In our experiments, arecoline showed a dose and duration dependent increase in SCEs. The duration dependence was not observed in CA assay. The AN ext. resulted in higher number of SCEs than arecoline. It can be speculated that other water extractable fraction(s) in the areca nut extract add to the genotoxic potency of arecoline.

Nicotine also was found to influence arecoline induced SCEs. Nicotine, by itself, could induce SCEs in CHO cells only at high concentrations (Riebe and Westphal, 1983). We observed that a continuous treatment of arecoline with nicotine raised the frequency of SCEs, at a concentration at which neither of them alone could produce this effect. The additive effect was clearly evident by the increase in SCEs with the concurrent increase in the concentration of nicotine. Even after

a brief treatment of two hours with 50 µg A + 90 µg N, (concentrations comparable to those found in saliva of chewers) a statistically significant induction of SCEs was witnessed.

The sixth experiment explains the effects of AN ext. and arecoline on cellular proliferation. The BrdU labelling technique easily identifies the number of DNA replications i.e. MI, MII and MIII, the cell has undergone in presence of BrdU. AN ext. as well as arecoline suppressed cell cycle progression when the treatment was given for longer durations. The reason underlying this can be the efficiency of areca nut to inhibit DNA synthesis. The inhibition of DNA synthesis would ultimately lead to the failure to synthesize proteins. The cytostatic nature of areca nut can be attributed to the alkaloids. Caniff and Harvey (1981) found such effects of arecoline and arecaidine on human fibroblasts in vitro. Nicotine, the tobacco alkaloid, causes a dose and time dependent inhibition of cell growth in human promyelocytic leukemic cells. It inhibits cellular proliferation and affects cell growth, mainly de novo synthesis of proteins, as reported by Konno et al. (1986). These effects of nicotine might augment those produced by arecoline at higher concentrations, as observed in our results. Wary and Sharan (1988) recently reported that at higher doses the aqueous extract of areca nut was extremely toxic. The overall impact of AN ext., arecoline as well as arecoline with nicotine on mitotic machinery was reflected by increased AGT values.

The statistically significant increase in SCE and CA frequencies, the large number of necrotic nuclei observed during cell division experiment and the retardation in cell proliferation prompted the experiment on DNA synthesizing capacity in treated cells. In most organisms DNA damage leads to a rapid and dose dependent inhibition of DNA synthesis due to actual blockage of replication. Arecoline reacts with cysteine to form β-alkylation adduct (Boyland and Nery, 1969) and can thus

interfere with the synthesis of DNA. Different concentrations of AN ext., arecoline and a combination of arecoline with nicotine added 24 hours prior to ^{3}H thymidine pulse, reduced ^{3}H-thymidine uptake upto almost 90%, indicating highly efficient inhibition of DNA synthesis. Yang et al. (1979) reported similar results with aqueous extract of areca nut in human lymphocytes and in monolayer cells in vitro. They pointed to a possibility that the inhibitory factors might be the tannins and/or the alkaloids. Arecoline is known to decrease the incorporation of ^{3}H-thymidine in muscle and kidney tissues (Shivapurkar and Bhide, 1979). It binds with nucleic acids and proteins (Nery, 1971) and has been found to inhibit the nucleic acid and protein syntheses in mouse fetuses (unpublished data referred by Sinha and Rao, 1985b). Our results, on the decrease in ^{3}H-thymidine incorporation in presence of arecoline, are in accordance with these data. A dose dependent inhibition was observed. A 50% reduction in ^{3}H-thymidine incorporating cells was observed following a 24 hours' treatment with 25 µg arecoline or 2.5 µl of AN ext. per ml GM. There was a marked reduction in the number of grains per nucleus. This indicates that the inhibition of DNA synthesis is at both levels, i.e. units synthesizing DNA at a given time, as well as the amount of DNA being synthesized by a given cell at a given time. It should be noted that the decrease in ^{3}H-thymidine uptake was more pronounced in the samples treated with areca nut extract than the ones treated with arecoline in pure form. This implies that besides arecoline, other water soluble component(s) present in the extract might have some implications in causing greater inhibition of DNA synthesis. The additive effect of combining arecoline with nicotine in DNA synthesizing cells was observed. Both, arecoline as well as nicotine, were found to affect the DNA synthesis. Even on increasing the concentrations of any one of them, and keeping the other constant, a concurrent decrease in DNA synthesis was observed.

The carcinogenicity of areca nut has been attributed by some investigators to its tannin enriched fractions (Kirby, 1960; Kapadia et al., 1978). The areca nut tannins inhibit collagenase activity which may result in collagen accumulation in oral submucous fibrosis (Meghji et al., 1982). A number of investigators postulate the possibility that the tannis/polyphenol fraction present in areca nut play an important role in its genotoxic effects (Ranadive et al., 1979; Panigrahi and Rao, 1986; Bhide et al., 1979; Stich et al., 1983a). Arecaidine, which constitutes a small fraction of areca nut alkaloids, has been reported to induce mutations in salmonella strains (Shirname et al., 1983) and induce 8-azaguanine resistance in chinese hamster V79 cells (Shirname et al., 1984). It also increased the frequency of SCE in mouse bone marrow cells (Panigrahi and Rao, 1984). Ashby et al. (1979) observed that arecoline and arecaidine produced comparable positive response in cell transformation assay. At high concentrations, arecaidine elevated the SCE frequency and inhibited DNA synthesis in CHO cells (Our observation). The in vitro nitrosation of arecoline may lead to the formation of several nitrosamines (Fig. 2, page 6). All these factors might contribute to the genotoxic effects of areca nut. The present findings confirm that arecoline elicits genotoxic effects and also an inhibitory action on DNA synthesis in vitro, which is farthered by other constituents of areca nut, as demonstrated by the AN ext.

SUMMARY

1. *The AN ext. was found to be cytotoxic, arecoline also exhibited a similar effect.*

2. *The clastogenic action of arecoline was evidenced by the increase in MNC and CAs with the rise in the concentrations. The AN ext. also induced a dose dependent increase in CAs. The high rate of chromatid aberrations explain that the effect was during late S or G2 phase of the cell division.*

3. *A dose and duration dependent elevation in SCE frequencies by AN ext. and arecoline, with a high level of statistical significance observed in the induction, proved the assay to be an extremely sensitive endpoint in the assessment of the genotoxic potentials of AN ext. and arecoline.*

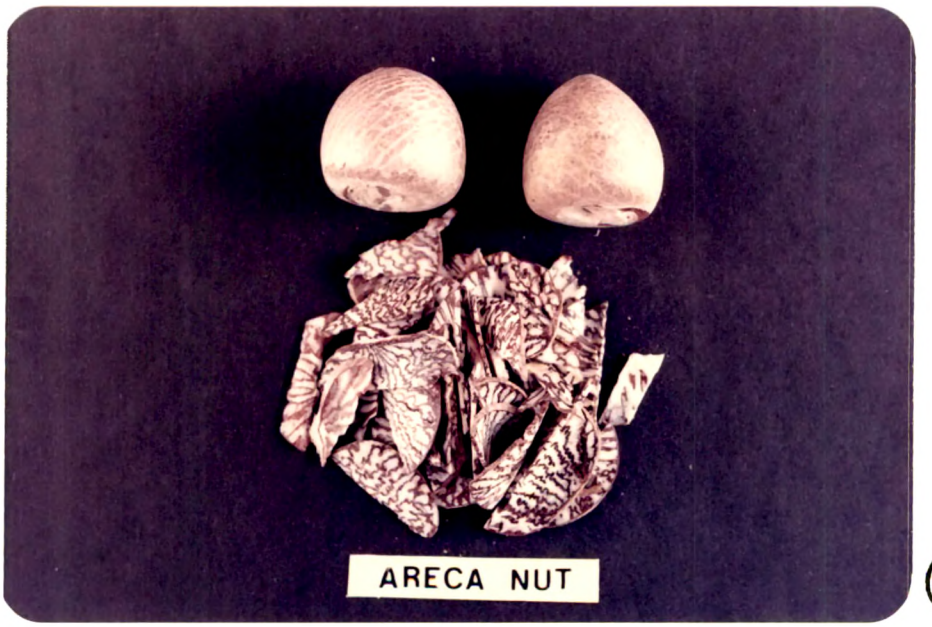
4. *Arecoline brought about a dose and duration dependent decrease in the cellular turnover arresting the cells in metaphase, which led to a speculation that arecoline might be acting even as a spindle poison.*

5. *The AN ext. and arecoline reduced the cell proliferation rate and inhibited DNA synthesis, the effects being dose dependent.*

6. *Considering the arecoline content of areca nut, and the observed genotoxic effects on AN ext., it can be presumed that in addition to arecoline, it contains other water soluble component(s) increasing the ultimate effect.*

7. *Besides being cytotoxic, clastogenic and an inhibitory to DNA synthesis in its own merit, areca nut can produce more severe effects when combined with tobacco, as evidenced by the studies combining arecoline and nicotine.*

8. *The positive genotoxic effects observed in all the experiments even in absence of metabolic activation indicate that areca nut alkaloids and other constituents are reactive in their native form.*



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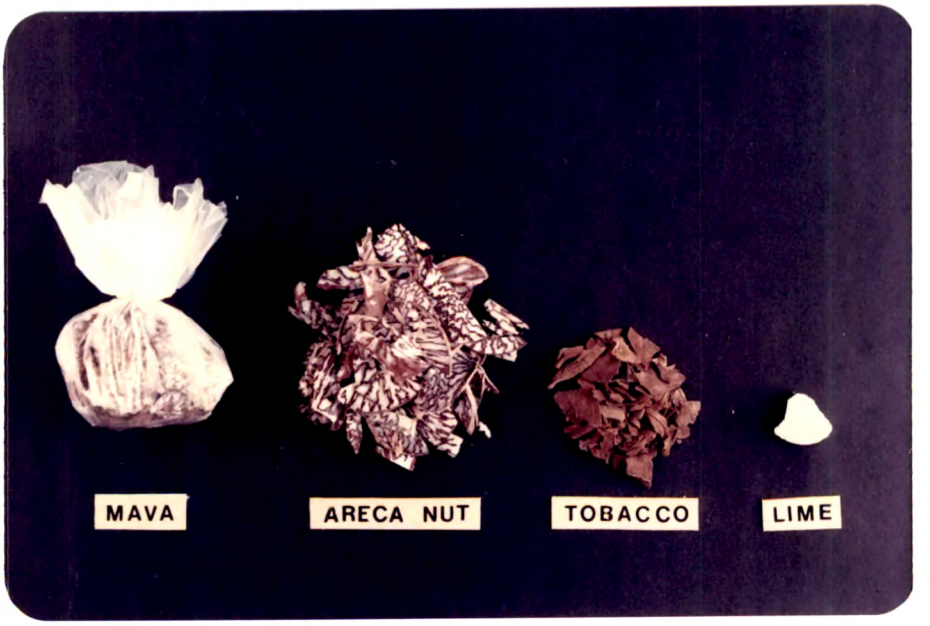

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