CARCINOGENICITY

Cancers of the upper alimentary and respiratory tracts (oral cavity, pharynx, larynx, oesophagus and lung) constitute more than half of the cancers in men and about a quarter of the cancers in women in India (Sanghvi, 1981). Among these cancers, two distinctly different patterns are found to prevail in different parts of the country. Cancers of the oral cavity are very common in some parts of the country and that of the pharynx, larynx and lung in others.

For centuries, numerous plant products have been chewed by many different population groups of the world. Indigenous habits of chewing and smoking seem to be primarily responsible for the high incidence of cancers of the upper alimentary tract and respiratory tracts in India like that of many other South-east Asian Countries.

Carcinogenicity of Betel Quid

In the early 20th century, some epidemiological reports first started coming to light revealing that the high frequency of oral and oesophageal cancer
Among Indians are associated with betel quid chewing (Niblock, 1902; Orr, 1933). It has also been estimated in terms of figure by now that among Indian population, about 70% of cancers of the oral cavity, 75% of cancers of the hypopharynx and larynx, and 50% of the oesophageal cancers appear to be due to betel quid chewing and/or tobacco smoking (Jayant et al., 1977).

The betel quid or popularly called 'Pan' contains certain ingredients like betel nut (Areca catechu), betel leaf (Piper betle), slaked lime, liquified catechu, an aqueous extract of heart wood (Acacia catechu) and tobacco (Nicotiana tabacum). Besides these, some other spicy ingredients are optional depending on local custom, socio-economic level and individual preference. Sometimes cardamom, cloves and anissed are added to the quid for additional flavour (Sanghvi, Rao and Khanolkar, 1955).

The chew as a whole is inserted into the buccal cavity and in some cases is kept there for several hours. Many people also sleep keeping the chew inside the mouth (Cooke, 1976) thereby lengthening the exposure period of the oral and oesophageal mucosa to various chemicals which are released from the quid.
Among betel quid chewing Indians, the risk of developing oral cancer is 12 to 18 times higher than among non-chewers and more than 60 times higher in individuals who retain the quid in the mouth throughout the sleeping period (Hirayama, 1966; Wahi, 1976).

Attempts to confirm the carcinogenic activities of the betel quid using experimental animals have also been made. Muir and Kirk (1960) painted the ears of Swiss mice with the aqueous extracts of typical Singapore betel quid containing tobacco and observed squamous cell carcinoma and benign squamous papilloma. Similarly, the extract of betel nut, tobacco and lime was found to produce carcinomatous changes in the vaginal mucous membrane and metastasis of some other tissues like kidney and lungs (Reddy and Anguli, 1967).

Ranadive et al. (1979) reported appearance of precancerous lesions in the hamster buccal pouches receiving betel nut, lime and tobacco combinations.

Hamner and Reed (1972) observed that the betel quid with tobacco produced carcinoma in situ in the surgically created buccal mucosal pouch of baboon after 34 months of treatment.
However, Dunham and Herrold (1962) could not induce tumors in the cheek pouch of hamsters by exposure to the ingredients of betel quid. Similarly, Ahluwalia and Ponnampalam (1968) could not correlate the betel chewing and the incidence of oral cancer but strongly suggested that it definitely causes lesions varying from leukoplakia to melanoplakia. Thus, carcinogenicity studies of betel quid have been carried out entirely by local administration of the extract and the results obtained were positive in some cases and negative in others.

Among the various ingredients of betel quid tobacco has been considered to be the most important constituent responsible for carcinogenicity of betel quid and to play a major role in the process of oral carcinogenesis (Wahi, 1976). However, Atkinson et al. (1964) strongly emphasized on the high incidence of oral cancer in New Guinea due to the habit of chewing betel quid, even though the betel quid is never mixed with tobacco. Jussawalla and Deshpande (1971) stated on the basis of their epidemiologic study that betel chewers who do not include tobacco in the quid often swallow the juice which comes into direct contact with
the oropharynx and oesophagus thereby leading to a higher risk of cancer at these sites.

Also, it has been observed that among Natal Indians, the frequency of oral cancer is prevalent in the non-tobacco quid chewing population, thereby indicating the importance of betel nuts and other ingredients of the betel quid in the etiology of oral cancer (Schonland and Bradshaw, 1969). Therefore, betel nut and lime, with or without **Piper betel** leaf seem to have a carcinogenic effect when chewed without tobacco.

**Carcinogenicity of Betel Nut**

It has been estimated that 10% of the world's population indulges in the habit of chewing betel nuts, especially in oriental countries (Fendell and Smith, 1970). Though several clinical studies have been carried out on betel quid, with or without tobacco, and its relationship with carcinogenesis has been established (Chin and Lee, 1970; Lee and Chin, 1970; Ramanathan *et al.*, 1973), no clinical report is available as yet on only betel nut induced carcinogenicity. This may be primarily because the nut is not chewed alone and is usually taken along with other ingredients wrapped in **Piper betel** leaf forming the quid. However,
a good number of experimental studies have been carried out with the total extracts of betel nut to investigate their possible role in the genesis of cancer and the earlier works on this have been reviewed by Arzungi (1976).

Woelfel et al. (1941) tested the ether, alcohol and insaponifiable fractions of betel nuts on mice but failed to produce tumors. But later on Suri et al. (1971) applied the dimethyl sulphoxide-extract of betel nut to the mucosa of the hamster buccal pouches and were able to induce 38% tumor incidence.

Ranadive et al. (1976) and Kapadia et al. (1978) observed that following subcutaneous administration of betel nut extract to the mice and rats, transplantable fibrosarcomas developed at the site of injection, and thus qualitatively supported the work of Suri et al. (1971). In the year 1979, Mori et al. examined the carcinogenicity of betel nuts, betel leaves and lime separately or in combination on rats. Although malignant tumors were not induced in any group of their experiment, epidermal thickening with hyperkeratosis was frequently observed in the tongue, oesophagus, or forestomach of rats in groups fed with the 20% betel nut diet mixed with calcium hydroxide. In the same year, Bhide et al. (1979), also reported that
the aqueous extract of betel nut was able to induce tumors of the gastrointestinal tract in 58% Swiss mice and 25% C17 mice. More recently, Shivapurkar et al. (1980) also from their studies on mice reported about the tumorigenic principles present in the betel nut.

Several biochemical parameters that may be relevant to the induction of carcinogenesis were also studied by administering whole extracts of betel nut to the animals. The aqueous whole extract of betel nut was found to increase the DNA and RNA contents in liver and muscle but to decrease the protein content in kidney (Shivapurkar et al. 1978). On the other hand, Yang et al. (1979) reported that betel nut extract had an inhibitory effect on the DNA synthesis in the culture of lymphocytes and tumor cells.

Carcinogenicity of Arecoline, a Major Betel Nut Alkaloid

Apart from phenolics, the betel nut contains the alkaloids. Alkaloids from many plants have been found to play considerable role in the process of development of carcinogenesis (Hirono et al., 1977).

The literature is scanty regarding the biological actions of betel nut alkaloids, and only a few
works have been recorded with arecoline and arecaidine, the two major alkaloids of betel nut. Arecoline has been reported to induce 20% lung tumors in mice (ICMR Bull., 1978). Also, Dunham et al. (1974) observed a superficial spreading carcinoma of pouch epithelium and papillomas and advanced atypias of oesophagus in hamsters due to the action of arecoline.

Arecoline and arecaidine have shown cell transforming (Ashby et al., 1979), and genotoxic (Stich et al., 1981; Panigrahi and Rao, 1982, 1983) properties. Shirname et al. (1982) also obtained positive results by Salmonella/microsome assay, both arecoline and arecaidine inducing cell transformations.

Coming to effects at the macromolecular synthesis level, arecoline has been found to interfere with their biosynthesis. It decreased the $^3$H-thymidine incorporation into muscle and kidney tissues which may finally inhibit DNA synthesis. However, DNA synthesis has been observed to increase in liver and lung tissues after arecoline administration (Shivapurkar et al., 1979). The same authors reported the decrease in tissue RNA and protein content by arecoline treatment in mice and assumed that reduction in RNA content
would be due to significant increase of the RNAse activity.

Chemical Constituents and Properties of Betel Nuts

The betel nut, a principal component of betel quid, contains predominantly polyphenols, carbohydrates (polysaccharides), fats and alkaloids. Mathew et al. (1964) reported the chemical composition of betel nut and husk (Table 1).

Table 1: Composition of tender and ripe betel nuts

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Tender nut (%)</th>
<th>Ripe nut (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water extractives</td>
<td>75</td>
<td>20-30</td>
</tr>
<tr>
<td>Polyphenols*</td>
<td>40</td>
<td>11-18</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>0.0 - 0.1</td>
<td>0.20-0.24</td>
</tr>
<tr>
<td>Fat</td>
<td>1 - 4</td>
<td>10-15</td>
</tr>
<tr>
<td>Free fatty acid</td>
<td>0.5 - 6.7</td>
<td>Decrease</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>1 - 2</td>
<td>15</td>
</tr>
<tr>
<td>N, mineral matter, water soluble, water insoluble ash</td>
<td>1.1 - 3.8</td>
<td>Decrease</td>
</tr>
</tbody>
</table>

* Per cent of polyphenols in water extractive remained same in all stages.
According to their study, it was shown that on a dry weight basis high concentration of polyphenols and low concentration of alkaloids are present at the young stage of nuts. At the ripe stage the polyphenol content decreases to a third or quarter of the initial value, while the alkaloid content increases.

**Fats and Fatty Acids (Ether Extract)**

Ripe betel nut contains 10-15 per cent fats. The saturated fatty acid components of betel nut fat are: lauric - 19.5%; myristic - 46.2%, palmitic - 12.7%; stearic - 1.6% and decanoic - 0.3%. The component unsaturated fatty acids are: oleic - 6.2%; linoleic - 5.4%; dodecanoic - 0.3%, tetradecanoic - 0.6% and hexadecanoic - 7.2% (Majumdar et al., 1979). The fat contains 56% completely saturated, 30% mono-unsaturated and 14% diunsaturated glycerides (Pathak and Mathur, 1954).

**Polysaccharides**

The betel nut husk contains 47.6% cellulose on a dry weight basis whereas the lignin content of it varies from 13.5 to 27.5% depending upon the method of isolation employed (Ali and Khundkar, 1953).
Tannins and Polyphenols

Grasser (1929) estimated the tannin content in betel nut as 11.4%. However, Beans (1941) showed that the relative proportion of tannin content decreased as the nut ripened.

The predominant polyphenols of betel nut at all maturity stages are flavonoids, flavan-3:4-diols (all yielding cyanidins) and their concentration decreases with maturity on a dry weight basis. The pattern of changes with maturation and ripening is due to insolubilization of higher polymers together with the formation of fresh monomers and intermediate polymers (Mathew and Govindarajan, 1964).

Govindarajan and Mathew (1963) studied the structure of betel nut. Their studies showed that tannins and polyphenols are the derivatives of (+)-d catechin and monomeric and polymeric leucocyanidins.

Alkaloids

Betel nut contains six major alkaloids (1%), viz. arecoline, arecaidine, arecolidine, guvacine, guvacoline, and isoguvacine, which are hydrogenated pyridine derivatives. The parent member of these alkaloids is an amino acid guvacine (C₆H₉O₂N), and Freudenberg (1918) showed
Structures of Betel Nut Alkaloids

- Arecoline
- Arecaidine
- Guvacine
- Guvacoline
- Arecolidine
that guvacine is identical with tetrahydronicotinic acid. The corresponding N-methyl derivative of guvacine is arecaidine. Arecoline is the methyl ester of arecaidine; similarly guvacoline is the methyl ester of guvacine. The structure of arecolidine was given by Emde (1915) and was shown to be identical with 1,2-dihydro-3,4-methoxy-1-methyl pyridine.

Arecoline, the main and physiologically most active alkaloid of betel nut has been reported to be present to the extent of 0.8% in some ripe nuts (Goswami and Ahmed, 1956). Arecaidine and guvacine occur in smaller quantities while guvacoline and arecolidine are found only in trace amounts. The chemical structures of betel nut alkaloids are shown in Figure 1.

Miscellaneous Constituents

Betel nut contains several other compounds in small quantities. These include sapogenin in trace amount (Marker et al., 1947), fluorine, also in trace amount (Nanda and Kapoor, 1971), mineral matters (Ca - 0.05%, P - 0.13%, Fe - 0.001-0.005%) and free amino acids, proline - 15%, tyrosine, phenyl alanine and arginine - 10% each, tryptophan and methionine - trace amounts (Lalitha Kumari and Sirsi, 1963).
Pharmacological and Physiological Properties of Betel Nut

Betel nuts have been known for at least 400 years in China as a remedy for intestinal tapeworm (Liu, 1936) and ringworm (McCurrah, 1960). Infact, the use of these nuts as a vermifuge was known to Chinese as early as in the sixth century and is still in practice there for the same purpose (Goodman and Gilman, 1975). Lalitha Kumari et al. (1965) reported antimicrobial activity of betel nut while antifertility effect of various extracts of betel nut was observed by Garg and Garg (1971).

The nuts are highly acidic and astringent to the taste. The addition of lime not only neutralizes the acidity to a large extent but also promotes the appearance of a red dye (Muir and Kirk, 1960). The nuts also increase salivation (Eisenn, 1946) and their extract was found to have an effect on cardiovascular system, isolated ileum and uterus of mouse (Dhawan et al., 1980).

Pharmacological and Biological Properties of Arecoline and Arecaidine

Vanrossum (1962) studied the structure activity relationship of arecoline. Arecoline bears a certain
structural resemblance to nicotine and has been found to be pharmacologically active in protonated form.

Arecoline administration to rats and mice has shown various effects on central nervous system, e.g., increase and decrease of spontaneous motor activity, water and food consumption at low and high doses respectively, its action being muscarinic and biphasic in nature (Majumdar et al., 1979).

Inhibition of conditioned avoidance responses (Herz and Yocoud, 1964), activation of electro-encephalogram response (Reiehl et al., 1962), analgesic action (Herz, 1962) and cataleptogenic potency (Green et al., 1979) of arecoline are all in records. Arecoline has been shown to stimulate autonomic effector cells which are acted upon by cholinergic post-ganglionic nerve impulses resulting in bradycardia, hypotension, salivation, sweating and also increase of the tone and rhythm of the smooth muscles of alimentary canal (Majumdar et al., 1979).

The cardiovascular effects of arecoline in the form of cardiac depression through vagal stimulation have been observed by Heymans (1922). These effects of arecoline are mediated through the cholinergic system, since these are blocked by atropine.
Williams and Carter (1965) showed that subcutaneous injection of arecoline hydrochloride (1.25-3.00 mg/kg) to the hydrated rats produced marked natriuresis and chlorouresis, while Avrunin and Carter (1970) observed that arecoline increased Na and K, and osmolarity of urine without altering the urine volume.

Arecoline gets metabolically converted into arecaidine in liver, which is devoid of any parasympathetic action. Arecaidine acts as a sedative only in higher doses (Nieschulz and Schmersahl, 1967). However, arecaidine has been found to be an inhibitor of GABA uptake in rats and psychic effects of betel nut consumption may, therefore, be the result of inhibition of GABA uptake by this component (Johnston, 1975).

Short-term Genotoxicity Tests as Tools for Carcinogen Screening

The mutagenic, clastogenic, recombinogenic and DNA-breaking capacities of a compound are being widely used now-a-days as indicator for its carcinogenic potential.

The sequence of events leading from an initial lesion to cancer is considered to be a multistage
process including initiation, promotion and progression. Some chemicals appear to act primarily as initiators and others primarily as promoters, although few carcinogenic chemicals appear to lack an element of both activities.

According to Bridges and Fry (1978), current dogma favours the view that initiating event in carcinogenesis is the direct interaction of a chemical or more frequently its active metabolite(s) with the DNA of the cell's genetic apparatus in a manner which causes a permanent modification in gene expression (somatic mutation theory). The short-term genotoxicity tests for carcinogens is based on the rationale that carcinogens cause a cell mutation as the primary event in a sequence of reactions leading to the ultimate development of cancer, and that this type of compound-DNA interaction does not occur with non-carcinogens.

Therefore, the guiding principle adopted in favour of such short-term tests conducted in vivo using 'mutation' as an endpoint is primarily to detect the initiator and also to assess the potential of eliciting cancer by the test compound as it becomes expressed phenotypically in vivo. Transplacental
micronucleus test, sperm morphology assay and unscheduled DNA synthesis study are a few of such short-term genotoxicity tests.

Transplacental Micronucleus Test

The micronucleus test employed by several investigators (Matter and Schmid, 1971; Von Ledebur and Schmid, 1973; Heddle, 1973) as in vivo cytogenetic screen is an efficient alternative to metaphase analysis for rapid screening of potential clastogenic chemicals (Kliesch et al., 1981). Chemicals can be screened for chromosome-breaking ability by measuring the frequency of polychromatic erythrocytes with micronuclei formed from the acentric chromosomal fragments in the bone marrow cells in vivo (Heddle and Carrano, 1977). Owing to its wide range applicability, the test has been used extensively as one of the major short-term tests for evaluating mutagenicity and carcinogenicity (Wild, 1978; Bruce and Heddle, 1979; Tsuchimoto and Matter, 1979; Jenssen and Ramel, 1980).

Induction of micronuclei is evidently one of the useful indicators of the impact of DNA-damaging chemicals on individual cells of adult animals (Kliesch et al., 1981; Jenssen and Ramel, 1980), which can also
be applied to assessment of risks associated with transplacental exposure (Cole et al., 1981).

The transplacental micronucleus test has been proved to be sensitive to agents which are even negative in bone marrow tests since the production of erythroid cells in the fetal liver persists till late gestation, by which time the fetal hepatic cells develop greater capacity for metabolic activation qualitatively and quantitatively than the adult bone marrow (Cole et al., 1979, 1981, 1982).

Different flavonoids and alkaloids obtained from various plant species used as an important source of food stuff additives and drugs have been tested for the genotoxicity by their micronuclei yielding response in the bone marrow erythrocytes.

A significant increase in the induction of micronuclei after a single treatment with vincristine (Hart and Hartley-Asp, 1983) and with the flavonoid 5,3',4'- trihydroxy-3,6,7,8-tetramethoxy flavone (Cea et al., 1983) has been observed in mice.

Stich et al. (1982) observed an elevated frequency of micronuclei also in the buccal mucosal cells of betel quid chewers.

Sperm Morphology Assay

Sperm morphology assay, another cytogenetic screen, has also been applied to a wide variety of
industrial carcinogens (Wyrobek and Bruce, 1975; Wyrobek et al., 1981; Topham, 1980a,b). The sperm morphology assay was investigated as a possible adjunct for assessing potential testicular toxicity since treatment of male mice with some carcinogens and mutagens has been shown to lead to an increase in the production of abnormally shaped sperm heads (Wyrobek and Bruce, 1975; Heddle and Bruce, 1977).

The sperm abnormality has the advantage of identifying those carcinogens that damage the mammalian testis and such agents are of major concern as they may also induce heritable genetic abnormality.

Twenty carcinogens and thirteen non-carcinogens were tested by Topham (1981) to induce abnormal sperm morphology in (CBA x BALB)F1 mice. As 4 of the carcinogens tested gave a positive response and none of the 13 non-carcinogens tested gave any positive response, the incidence of false positives has been proved to be zero.

More recently, Wyrobek et al. (1983) reviewed the results of mouse sperm morphology test with 154 chemical agents and observed that a positive response in this test was highly specific for carcinogenic
potential (100% for the agents surveyed) and overall, 50% of carcinogens were positive in the test. Thus, in the context of the prediction of carcinogenicity, positive results in the sperm abnormality test are of considerable value.

Unscheduled DNA Synthesis Study (UDS)

It is possible to study repair of damaged DNA by its unscheduled synthesis in vivo due to the action of mutagenic or carcinogenic agents in meiotic and post-meiotic germ cell stages of male mice by making use of well-investigated sequence of events that occur during spermatogenesis and spermiogenesis (Monesi, 1962; Oakberg, 1956a,b). In developing male germ cells, the last scheduled DNA synthesis takes place during a 14-hr period (Monesi, 1962) in pre-leptotene spermatocytes. After this scheduled DNA synthesis phase, the spermatocytes continue to develop through a series of germ-cell stages for about 28-30 days (Oakberg, 1956a,b) before the spermatids finally leave the testis and enter the caput epididymis. The developing sperms reach the caudal epididymis 2-3 days later, and finally enter the vasa deferentia after an additional 2-3 days (Sefa and Sotomayor, 1982). If at any meiotic or post-meiotic germ-cell stage, the
DNA is damaged and repair occurs, the repair can be detected by an unscheduled incorporation of $^3$H-thymidine which is measured after the developing cells leave the testes and enter the rest part of the reproductive tract.

Any alteration produced by any chemical agents in the normal DNA repair process of mammalian germ cells has the potential for increasing the genetic risk from it, be it a mutagen or carcinogen. Thus, the in vivo UDS assay in the germ cells of male mice is a very sensitive method to determine the potential of the test chemical to produce lesions in the DNA and also to elicit its repair synthesis in the genetically important cell targets, i.e., the germ cells, which is one of the main characteristics accounted for the carcinogens.

All the direct acting carcinogens have been found to initiate unscheduled DNA repair, whereas unscheduled $^3$H-thymidine incorporation has not been detected using the non-carcinogens (Bridges and Fry, 1978).
In vivo DNA repair occurring in early spermatid stages of mouse has been studied with many alkylating agents that have been proved to be both potent mutagens and carcinogens (Sega et al., 1976; Sotomayor et al., 1982) and a linear increase in the unscheduled DNA synthesis has been observed with increasing doses of these test chemicals in almost all the cases.

Similarly, in another study, pre-treatment of the mice with 100 mg caffeine/kg, a major alkaloid from coffee and tea, was found to enhance the unscheduled uptake of $^3$H-thymidine up to the extent of 60 per cent into the methyl methane sulphonate (MMS) damaged germ cells, compared to that with MMS treatment alone (Sega et al., 1983).

Promotion of Hyperplastic Liver Nodule Development in Mice Pre-treated with N-nitrosodiethylamine: A New Approach for an in vivo rapid screening test for Carcinogens

Extensive research has indicated that chemical carcinogenesis involves the sequential steps of initiation and promotion (Berenblum and Shubik, 1947; Marks et al., 1978; Marx, 1978; Scribner, 1978). There have been many biological molecular, genetic and chemical
analyses of the initiation step but little is known as yet about the promotion step. This is because the promotional phase of cancer is very complicated, takes a long time, and is influenced by many factors in in vivo conditions.

An increasing number of compounds in our environment including synthetic chemicals, drugs, pesticides, industrial pollutants, food additives, mycotoxins and constituents of vegetables, have been identified as chemical carcinogens (Sugimura et al., 1977; Wynder and Gori, 1977). Many of these chemicals have not been tested since long-term animal carcinogenicity studies are time-consuming and expensive.

Recently, new methods for the rapid induction of hyperplastic liver nodules by chemicals have been developed by several investigators as screening tests for carcinogens (Farber et al., 1976, 1977; Solt and Farber, 1977; Tatematsu et al., 1977; Tsuda and Farber, 1980). In other words, these tests were based on the rapid induction of preneoplastic changes in the carcinogen-initiated liver of experimental animals.
Craddock (1975) suggested that a single injection of either diethylnitrosamine (DEN) or dimethylnitrosamine (DMN) provides a good model for the initiation of carcinogenesis in liver. Solt and Farber (1976) showed the rapid formation of hyperplastic nodules in the liver of rats pre-treated with DEN, subsequently fed with N-2-fluorenylacetamide (2-FAA) and also partially (67%) hepatectomized.

Histochemically, γ-glutamyl transpeptidase (γ-GTPase) enzyme has been demonstrated in the hyperplastic nodules and the pre-neoplastic hepatic lesions but could not be detected in the non-hyperplastic areas of liver thereby identifying it to be a 'positive marker' for such changes induced by the carcinogenic chemicals (Tatematsu et al., 1977; Ito et al., 1980).

Biochemically also, rate of activity of γ-GTPase has been observed to be augmented to a great extent in the putative pre-malignant liver cell populations during the induction of hepatocarcinogenesis in rats or mice by azo dyes (Taniguchi et al., 1974, 1975), by 3'-methyl-4-dimethylaminoazobenzene (Fiala et al., 1972), by diethyl nitrosamine (Cameron et al., 1978) and even in the human hepatomas compared to the normal liver tissues (Yamamoto
et al., 1981) emphasizing its usefulness as a good marker for the preneoplastic hepatocytes.

TERATOGENICITY

It is a remarkable fact that prior to early 1940s, little credibility was given to the notion that during the developmental processes of the human embryos certain environmental factors might interfere and cause malformations. In 1941, when Gregg's classical demonstration first revealed that rubella (German measles) infection in the mother was teratogenic to the fetuses causing death, blindness and deafness among offspring of women exposed to rubella during pregnancy, the scientific community started to recognize the role of exogeneous environmental agents in adversely affecting the human fetuses in the long run. Then came to light, the episode of thalidomide syndrome. Thalidomide, a sedative-hypnotic was introduced in the late 1950s in West Germany, England and other countries. Shortly after introduction of thalidomide, there was an increase in the number of infants born to the mothers treated with this drug during pregnancy with phocomelia and shortening or complete absence of the ribs (McBride, 1961). Following this experience, investigation of the teratogenicity of drugs, both established
as well as new ones, and chemicals was intensified by routine testing.

Nevertheless, according to Wilson's (1973) estimates, the cause of fully 65% of malformations is still unknown, while some 25% may be due to genetic and chromosomal factors and about 10% to known environmental factor.

Regarding drugs and chemicals, various types of adverse effects on the human fetus have been reported for about 200 different drugs, while of the approximately 2,000 chemicals tested in mammals, about one third have demonstrated teratogenicity in various experiment designs (Harbison, 1980).

Teratogenicity of Alkaloids:

Plant alkaloids like many other cytotoxic agents, by their ability to block various biosynthetic processes may be expected to cause fetal death or a wide variety of developmental abnormalities, if administered during the pregnancy period.

The teratogenic effects of a few plant derived alkaloids and also antitumor agents, vincristine, colchicine, maytansine in pregnant Swiss albino mice have been reported by Sieber et al. (1978). All these
compounds, at their teratogenic doses, induced various cranial abnormalities including excencephaly, hydrocephalus, anophthalmia and microtia, as well as major skeletal malformations. Colchicine has been observed to be embryotoxic also in rabbits (Morris et al., 1967; Diddcock et al., 1965), hamsters (Ferm, 1963) and rats (Thiersch, 1958). Similarly, fetotoxic effects of vinblastine has also been reported in monkeys (Courtney and Valerio, 1968), hamsters (Ferm, 1963) and in rats (Demeyer, 1965; Tamaki et al., 1967). Vinblastine, another vinca alkaloid and an antitumor agent has been found to be teratogenic in rats (Cohlan and Kitay, 1965) and also in hamsters (Ferm, 1963), causing microphthalmia, anophthalmia, spina bifida and skeletal anomalies like rib fusions and vertebral arch deformities.

Malformations have been reported to be induced by pyrrolizidine alkaloid, heliotrine in rat fetuses (Green and Christie, 1961).

Mun et al. (1975) observed various abnormalities in early chick embryos including absence of the tail or trunk below the wing bed (rumplessness) after administration of various amounts of Solanine or PIPE, a preparation of mixed glycoalkaloids from potatoes.
Some of the alkaloids which act as spindle poisons may also behave like potent human teratogens. These include griseofulvin and podophyllotoxin (Harbison, 1980).

Elwazer et al. (1982) reported that caffeine in doses up to 250 mg/kg per day in drinking water or up to 150 mg/kg per day in the form of pellets induced cleft palates and reduction in fetal weights in mice.

As to the betel nut alkaloid induced teratogenicity, there is no report in the literature till date and the exact nature and magnitude of the effect of these alkaloids on fetuses, if any, has yet to be investigated.

Overlap of Teratogenesis and Carcinogenesis

Like the seemingly close correlation between somatic cell mutagenesis and carcinogenesis, the incidences of overlap of carcinogenic process with environmental teratogenesis have started coming to light very recently. Some environmental teratogens are also found to be carcinogens (Miller, 1977).

Herbst et al. (1975) observed that Diethylstilbestrol (DES), induces frequent malformations of the lower genital tract in human females and occasionally gives rise to cancer of the same site.
Teratogenesis of alkyl nitrosoureas, some of which are also potent carcinogens in adult animals of different species, has been investigated. When given to rats, Syrian golden hamsters or minipigs in the first half of pregnancy, a single, sufficiently high dose of methyl nitrosourea (MNU) or ethyl nitrosourea (ENU), has been found to induce malformations in the brain and bones respectively (Ivankovic, 1979).

Similarly, ethylmethane sulfonate (EMS), a directly acting monofunctional alkylating agent and carcinogen has been shown by Platzek et al. (1982) also to be a potent teratogen in NMRI mice inducing limb malformations after single intraperitoneal injections on days 9, 10, 11 or 12 of pregnancy.

Therefore, keeping in view the findings of investigations of recent years in the field of transplacental carcinogenesis and teratogenesis, that cancer can be caused by many exogenous chemical compounds which can act on the fetuses too and can produce malformations in them after resorption through the placenta, an investigation about the possible carcinogenic as well as teratogenic manifestations of betel nuts has been undertaken for the present study.
AIM AND SCOPE OF THE PRESENT WORK

The present work was undertaken with the following few broad objectives:

A. Teratogenicity Aspects

a) To study the overall teratogenic or embryotoxic effects of betel nuts of two different varieties and also of two major alkaloids of betel nuts, viz. arecoline and arecaidine.

b) To study the *in vivo* biosynthesis of DNA and protein by mouse embryos following exposure to the embryotoxic dose of arecoline which is the main active principle present in the betel nuts and acts as a monofunctional alkylating agent. This was done with a desire to observe whether any correlation exists between possible embryotoxic effects in terms of growth retardation or cell death and the macromolecular syntheses due to arecoline treatment.

c) To explore the mechanism of probable DNA damage due to the action of arecoline on the embryos by estimating the alkylation at different sites of purine bases of DNA isolated from the embryonic tissue.
B. Carcinogenicity aspects

a) To assess and evaluate the carcinogenic potential of betel nuts by studying the effects of long-term and short-term exposures respectively on the induction of direct neoplasms as well as rapid promotion of pre-neoplastic lesions, measured indirectly by a marker enzyme, $\gamma$-Glutamyltranspeptidase.

b) To study the carcinogenic potential of arecoline, a major alkaloid of betel nuts by means of a few in vivo genotoxicity tests, keeping 'mutation' as end point.

The results of all these studies will not only be useful in predicting the probable teratogenic risk for the pregnant women from the betel nuts, so habitually and popularly taken with the betel quid throughout a large portion of the globe, but also in determining the carcinogenic responses that may be initiated or promoted within the human systems due to the cumulative effects of betel nut chewing for years together.
MATERIALS AND METHODS

Animals

Swiss albino mice obtained from Disease Free Small animal House, Haryana Agricultural University, Hissar, India, were used for the present study. Young, virgin females (8-9 weeks old) were employed for all the teratogenic and also for the transplacental carcinogenic investigations whereas both young (5-6 weeks old) and adult males (14-16 weeks old) were employed for rest of the carcinogenic investigations depending on the nature of the experiments. The animals were housed in cages with husk bedding, maintained in an airconditioned animal facility and provided with standard mouse pellets obtained from Hindusthan Lever Co., India and water ad libitum. During the teratogenic experiments 12 hr light and dark cycle was maintained in the animal room.

Chemicals

Arecoline, Arecaidine, N-diethylnitrosamine (DEN), Aspirin (Acetyl salicyclic acid), Alizarin red-S, Alcian Blue, Coomassie Brilliant Blue (GS-250), Calf Thymus Deoxyribonucleic Acid (DNA), Bovine Serum Albumin, Sodium-p-amino salicylate, Ethylenediaminetetraacetic acid (EDTA), 7-methylguanine, Sodium
Dodecyl Sulphate (SDS), Bovine pancreatic Ribonuclease (RNase), γ-Glutamyl-p-nitroanilide, Glycyl glycine, Trizma base, Ficoll-400, Giemsa, and 2,5-Di-phenyloxazole (PPO) were obtained from Sigma Chemical Company, USA, while 8-hydroxyquinoline, Malachite Green, May-Grunwald and Ethylene glycol were obtained from Merck, Germany. Protosol was purchased from New England Nuclear, Boston, USA, while 4-bis-2-(4-Methyl-5-phenyloxazole)-benzene (Dimethyl POPOP) was purchased from Packard Instrument Company Inc, Illinois, USA. Napthaline was procured from Reidel, Germany and Diphenylamine was procured from May and Baker, England. Trichloro acetic acid (TCA) was supplied by British Drug House, England. 06-Methyl guanine was a gift from the Baylor College of Medicine, USA. Cellulose coated chromatogram sheets with fluorescent indicator (No. 6065) from Eastman Kodak Co. USA, also came as gifts. The two radiochemicals, 3H-Thymidine (specific activity, 15,800 mCi/m mole) and 14C-Leucine (Specific activity 102 mCi/m mole) were supplied by Bhabha Atomic Research Centre, Bombay, India. All other chemicals used for the present study were either from Sarabhai M-Chemicals, India (Guaranteed Reagent grade) or from British Drug House, India (AnalaR grade).
Betel nuts

Ripe, sundried betel nuts of unprocessed variety were purchased from the local markets while the nuts of processed variety were procured from the markets of Bangalore, South India.

Betel nut extract preparation

Fifty grams of freshly prepared powder from unprocessed or processed variety of betel nuts was suspended in 200 ml of distilled water and ground into a smooth paste. After keeping at 4°C overnight the paste was placed on an automatic shaker for 3 hrs and then filtered through Buchner's funnel under vacuum. The residue was resuspended in distilled water and made into paste again. The whole process was repeated twice more and all the extracts in the form of filtrates were pooled together. The combined extracts were finally lyophilized at -42°C by a Toshniwal Lyophilizer into a dry amorphous powder. The lyophilized extract was stored and redissolved in distilled water at different concentrations only before use.

A. Teratogenicity Studies

Nulliparous female mice (8-9 weeks old and 25-28 g body weight) were used for all the teratogenic studies. Two females and one male mice were housed together in each cage overnight for mating. Females were checked for vaginal plugs on the following morning and the day '0' of gestation
was marked. Those animals which showed the plugs were separated and caged together. On day 6 of gestation, the dams were assorted into experimental and control groups. Depending on the nature of the experiment to be performed, the aqueous extract of betel nuts or the two major alkaloids of betel nuts, viz., arecoline and arecaidine were administered to the pregnant dams by gastric intubation either only on 11th day of gestation or continuously through days 6-15 of gestation. At the end of different treatment schedules, the animals were killed by the overdose of ether anaesthesia and autopsies were performed in order to study the overall embryotoxic effects of the different treatments on the fetuses and also on the macromolecular biosynthesis levels of the fetuses as well as a maternal tissue (placenta) in vivo.

1. Administration of betel-nut extracts, arecoline, arecaidine and teratogenicity: The effects of betel nut total extracts or betel nut alkaloids, arecoline and arecaidine on fetuses were studied by the external, visceral and skeletal examinations. For this, the experimental animals were treated either with a daily dose of 1, 3 or 5 mg/0.05 ml of lyophilized extract of unprocessed or processed betel nuts or with a daily dose of 0.5, 1.0 or 2.0 mg/0.05 ml of the alkaloids of betel nuts, arecoline or arecaidine. The positive
control animals received a daily dose of 250 mg/kg of aspirin suspended in 0.05 ml of distilled water while the negative control animals received only 0.05 ml of distilled water (vehicle) per day. All the treatments were administered to the females once a day at approximately 10 A.M. from day 6 to day 15 of gestation by gavage.

**Determination of maternal reproductive status and fetal abnormalities:** On day 17 of pregnancy, the animals were sacrificed, laparatomy was done and the reproductive status of the dams was determined followed by the observations of the conceptuses. Implantation sites in each uterine horn were counted and the general condition of each conceptus was recorded. The dead and resorbed fetuses (identified as the black resorbed bodies) were counted. The live fetuses were then removed, counted, blotted dry, weighed individually and also examined externally for the gross abnormalities. At least two-third of the fetuses from each litter, selected at random, were fixed in Bouin's solution for two weeks and then examined for visceral abnormalities by the razor blade sectioning method (Wilson, 1965). The remaining one-third of the fetuses
were fixed in 95% ethanol, cleared in 2% potassium hydroxide solution and stained differentially for bones and cartillages with alizarin red S and alcian blue as described by McLeod (1980), prior to the examination of skeletal abnormalities.

**Statistical analysis:** The litter was considered to be the experimental unit for statistical analysis. Mann-Whitney U-test was employed for determining the statistical significance of the differences in the mean fetal body weight between experimental and control groups.

**II. Administration of arecoline and incorporation of labeled precursors of DNA and Protein:** The effects of arecoline on the rate of DNA and protein synthesis by embryo and placenta were studied by measuring the rate of incorporation of labeled thymidine and leucine into these tissues. For this, pregnant animals were administered a single dose of 2.0 mg arecoline per 0.1 ml saline by gastric intubation on day 11 of gestation. At selected time points (2, 22 and 46 hr)
after this treatment, the individual animals were again intraperitoneally injected with $^3$H-thymidine and $^{14}$C leucine at the dose of 0.5 $\mu$Ci/gm. Control animals were given only 0.1 ml saline (0.9%) and 0.5 $\mu$Ci/gm $^3$H-thymidine and $^{14}$C-leucine. At least three animals were used for each time point studied and for both arecoline treated and untreated groups.

**TCA precipitation and GFC filtration:** Animals were killed 2 hrs after the precursor administration (4, 24, and 48 hr post-treatment with arecoline) and three embryos along with the placentae were dissected free from the uterus and placed in cold 0.9% NaCl. The tissue wet weights were taken and an estimation of the incorporation of labelled thymidine and leucine into DNA and protein respectively was done by Trichloroacetic acid (TCA) precipitation method (Kennel, 1967). Tissues were homogenized in chilled Saline-EDTA (0.15 M NaCl, 0.1 M EDTA) at 4°C and a 2.5% homogenate was prepared for each tissue. The homogenates were centrifuged at 1000g and 4°C for 15 min in a K-24 Centrifuge. The pellets containing cell debris and unbroken cells were discarded and the supernatants were collected. To each supernatant equal volume of cold 20%
TCA was added and the mixture was kept at 4°C for overnight. The precipitate containing DNA and protein was collected on Whatman GFC filters (2.5 mm dia) fitted on a millipore sampling manifold and washed thoroughly by cold 10% TCA. Filters were further washed with 70% cold ethanol. The filter papers with washed precipitates were carefully transferred to the scintillation vials and dried in an oven at 60°C for 1 hr.

**Scintillation counting:** Ten ml of scintillation cocktail (4 gm PPO + 0.1 gm POPOP in a litre of sulphur-free toluene) was added to each vial and radioactivity was determined in a Beckman Liquid Scintillation Spectrometer. Counts per minute (cpm) in TCA insoluble fractions were converted into disintegrations per min (dpm) which provided an estimate of the rate of incorporation of thymidine and leucine and therefore, the rate of DNA and protein synthesis. DNA and protein synthesis rate was finally expressed in dpm per gm of tissue.

**Statistical analysis:** The results were analysed statistically by means of Student's t-test and differences were regarded to be significant only if P < 0.05.
III. **Administration of arecoline and extraction of DNA and Protein:** The effects of arecoline on total DNA and protein contents of the embryo and a maternal tissue, placenta, were studied by quantitative estimation after extracting the same from the tissues.

**Extraction procedure:** The pregnant females were treated with a single dose of 2.0 mg arecoline by gavage on day 11 of gestation as in the case of incorporation study. The animals were killed also at the same selected time points (4, 24 and 48 hr) after the administration of arecoline. Embryos and placentae were removed from the uteri and washed with chilled 0.9% NaCl. The tissue wet weights were determined, and each tissue was homogenized separately by 0.5 M perchloric acid (PCA) in cold so as to give 40% (w/v) homogenate. The acid insoluble fractions containing DNA and protein were then further processed for extraction by the method of Packard *et al.* (1973).

Following the initial centrifugation, at 3000g and 4°C for 15 minutes in K-24 Centrifuge, of the homogenate, the resulting pellets were washed twice with cold 0.2 M PCA. The acid washed tissue pellets
were further washed with 95% cold ethanol and chloroform: methanol (2:1) successively, suspended in 4 ml of 0.3 M KOH and then allowed to stand overnight at room temperature. The resulting alkaline hydrolysates were cooled to 0°C, brought to neutrality with cold 6N HCl and DNA-Protein fraction was precipitated by the additions of cold 0.5 M PCA. The pellets were spun out and again hydrolysed in 0.5 M PCA at 90°C for 20 minutes. The supernatant was designated the DNA fraction while the pellet was solubilized in 5 volumes of 1 M NaOH at 60°C for 1 hr which gave the protein fraction.

**Estimation of DNA:** DNA extracted from the tissues was estimated quantitatively following the modified method described by Burton (1968) based on the color reaction between deoxyribose of the DNA and the diphenylamine reagent.

**Preparation of Diphenylamine reagent:** 1.5 gm of re-crystallized diphenylamine was dissolved in 100 ml of glacial acetic acid and 1.5 ml of conc. H₂SO₄ was then added to it. Just before use, 0.1 ml of aqueous acetaldehyde (1.6%) was added per 20 ml of reagent.

**Assay procedure:** 200 µl of the extract was made up to a final volume of 1 ml by the addition of 0.5 M PCA.
2 ml of diphenyl amine reagent was added to each tube which was then incubated for 17 hrs at 30°C in a waterbath. Absorbance was measured at 600 nm in a Shimadzu Recording Spectrophotometer. The DNA concentration was expressed as μg per gm of tissue.

Estimation of Protein: Protein extracted from the tissues was estimated quantitatively following the method of Bradford (1976) with little modifications. The reaction was based on the binding of the dye Coomassie Brilliant Blue G-250 to tissue proteins.

Preparation of Protein reagent: 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol. To this solution 100 ml of 85% (w/v) phosphoric acid was added. One part of the stock solution was diluted with 4 parts of distilled water, filtered with Whatman #1 filter paper and stored in room temperature.

Assay procedure: 100 μl of the extract was mixed with 1 M NaOH so as to make the final volume 0.1 ml. 5 ml of protein reagent was added to each test tube and the contents were mixed by vortexing. The absorbance was read at 595 nm within an hour of color development in a Shimadzu Recording Spectrophotometer.
The protein concentration was expressed as μg per gm of tissue.

IV. Administration of arecoline and isolation of DNA:
The effects of arecoline on DNA at some of its specific base sites were studied by isolating it from the arecoline treated embryos. For this, the pregnant mice on day 11 of gestation were treated with 2.0 mg of arecoline dissolved in 0.1 ml saline (0.9%) by intraperitoneal injection. Animals were killed by overdose of ether anaesthesia after 4 hrs of the treatment. Embryos from the mother were removed, frozen in liquid Nitrogen immediately and stored at -70°C in an ultra low temperature-cabinet. DNA was isolated from the pooled embryos by phenolic extraction procedure of Montesano et al. (1979) with some minor modifications.

Isolation procedure: Tissues were allowed to thaw in 10 volumes of a solution containing 6% (w/v) sodium p-aminosalicylate and 1% (w/v) sodium chloride, and subsequently homogenized by hand in a loose fitting Teflon-glass homogenizer. The homogenate was shaken vigorously for 30 min with an equal volume of phenol reagent (phenol-m-cresol-8-hydroxyquinoline-water, 100:14:0.1:11, w/v/w/v/w/v) at a temperature of 24°C. After centrifugation
at 4°C and 16000g in a K-24 Centrifuge for 30 min, the upper aqueous and viscous layer was removed and 30 mg of NaCl were added per ml. The mixture was re-extracted with 0.5 volume of the phenol reagent and centrifuged as described above. The upper layer was again removed and 2 volumes of cold ethanol-m-cresol (9:1, v/v) were added to it with 5-6 times repetitive inversions and kept in cold for 30 min. The precipitate formed was spooled out to a glass rod and removed. It was next washed thrice with 20 ml of cold 70% (v/v) ethanol containing 2% (w/v) sodium acetate, redissolved in 3 ml of 3.0 M NaCl solution and then left at 4°C overnight. The precipitated RNA was removed by centrifugation at 13000g and 4°C for 20 min in a K-24 centrifuge and the supernatant was preserved. The pellet was extracted thrice more by washing with 10 ml of 3 M sodium acetate. To the combined supernatants were added 2 volumes of cold 2-ethoxy ethanol and the precipitated crude DNA was again washed with 70% ethanol containing 2% sodium acetate followed by its resuspension in 3 ml of distilled water. 0.4 ml of Bovine pancreatic ribonuclease solution (2 mg/ml) and 0.15 ml of 3 M sodium acetate solution were next added to the
DNA solution, and the mixture was incubated overnight at 4°C. Glycogen was removed by centrifugation at 60,000g for 60 min at 4°C in a Beckman Ultracentrifuge and the purified DNA was precipitated from the supernatant with 2 volumes of cold 2-ethoxy-ethanol and spooled out as before. This DNA was washed with ethanol: diethylether (1:1 v/v) once, and ether also once and finally dried under vacuum and stored at -20°C.

**Analysis of Purine bases:** Purine bases were released from the isolated DNA samples by hydrolysis in 0.1 M of HCl (0.2 ml/mg of DNA) at 70°C for 30 min as described by Lawley and Thatcher (1970). The hydrolysed sample containing purine bases were analysed for possible site-specific reactions due to arecoline treatment by thin-layer chromatography.

**Thin-layer chromatography:** Ascending separations of bases were carried out on cellulose thin layers, pre-coated on plastic sheets, following the methods of Randerath and Randerath (1968).

**Application of the sample and run of chromatogram:** The samples (10-20 μl) containing purine bases were applied on the cellulose coated sheets by a micro-pipette 3 cm from the edges. Aliquots (10μl) of
standard 7-methyl guanine, 6-methyl guanine and guanine (1-2 mg/ml) were also then applied to the sheets in the same way. The chromatogram was developed at 30°C in a closed rectangular chromatotank without chamber saturation by a solvent system consisting of Isopropanol-NH₄OH-H₂O (7:1:2).

Quantitation by UV absorption: The developed spots were exposed under UV lamp and their boundaries were marked. Distances travelled by the solvent front and the individual solutes were recorded for calculation of Rf values. The compounds within the marked boundaries were then removed by scraping and eluted with 1.0 ml of 0.1 M HCl for 16 hours at room temperature. An adjacent blank circle of the same size was processed in exactly the same way. After centrifugation at 2000 rpm for 5 minutes, the supernatant solutions were transferred to 1 cm quartz cells and were evaluated in a Shimadzu Recording Spectrophotometer against the corresponding blank solutions at 260 and 280 μ. The UV absorption spectra of these solutions were also scanned in the range of 210-350 μ and the individual products present in the solution were characterized by their absorption maxima (λmax) at pH 1. Finally, the quantities of the bases were calculated by the obtained absorbance values and the molar extinction coefficients.
B. Carcinogenicity Studies

I. Long-term betel nut diet administration and tumor induction: 5-6 weeks old male and female mice and betel nuts of both unprocessed and processed varieties were used for this study. The animals were fed with betel nuts mixed diet for a long-time period, and its cumulative effects were studied in terms of induction of tumors.

Preparation and administration of betel nut diet: The food pellets and betel nuts were ground into fine powders separately. Betel nut powder was next mixed with the food pellet powder in an electric grinder-cum-mixer in 1:9 ratio by weight. The betel nut diet prepared in this way was given to the mice continuously for 14-16 months. Control animals were maintained on normal basal diet in parallel to the diet fed animals for the same time period.

Evaluation of tumor induction: At the end of the long-term schedule of betel nut diet administration or whenever found moribund, the animals were sacrificed and dissected open. All the organs were first grossly observed for any visible morphological changes. In case of any tumor formation, it was carefully removed
from the surrounding tissues. Its diameter was measured using a slide callipers with vernier scale. It was next cut into small pieces with the help of sharp razor blade or scalpel and put into Bouin's fixative for at-least one week. From the fixed tumor pieces tissue blocks were prepared in paraplast and 5 µm sections were prepared following the standard histological procedures (Humason, 1979). The slides containing the sections were stained with eosin-hematoxylin and observed under the immersion oil objectives of a Leitz Aristophot microscope.

II. Short-term betel nut diet administration and tumor promotion study by a marker enzyme, γ-Glutamyl-transpeptidase: In order to study the effects of short-term administration of betel nuts as a promoter on the induction of preneoplastic lesions in the mice liver, pre-initiated with diethyl nitrosamine (DEN) and hepatectomized subsequently, activities of a marker enzyme γ-Glutamyl transpeptidase from liver was estimated.

Exposure regimens and experimental protocol: Male mice (5-6 weeks old, 16-22 g body weight) were divided into 8 groups. Groups 1 to 4: Mice were injected intraperitoneally with DEN at a dose 0.06 mg/g body weight and fed on basal diet for 2 weeks. Then Groups 3-6 were
given diet containing betel nuts (1:9) for 2 weeks. At the end of third week, mice in groups 2, 4, 5 and 7 were partially hepatectomized as described by Higgins and Anderson (1931). Group 8: Mice were given only basal diet. Neither betel nut was administered in diet, nor partial hepatectomy was performed in this group. Mice in all the groups were killed after 4 weeks.

Preparation of enzyme extract: Livers from each mice were quickly removed and washed with 0.9% chilled saline. The tissue was weighed and homogenized in Sucrose-Tris-EDTA buffer (pH 7.4) at 4°C using 10 strokes of a Potter-Elvehjem homogenizer equipped with a teflon pestle at approximately 2500 rpm. A 1:10 homogenate (w/v) was prepared and 5.0 ml of the whole homogenate were centrifuged at 12,000g for 10 min in a K-24 centrifuge. The pellet containing nuclei and cellular debris was discarded, while the supernatant was further spinned at 105,000g for 1 hour in vac 601 or Beckman ultracentrifuge. Pellet fraction was resuspended in 1 ml of Sucrose-Tris-EDTA buffer and it being the enzyme source, was used for the assay.

Enzyme assay procedure: The method of Szasz (1976) was followed with minor modifications. In Principle,
following reaction is catalyzed by $\gamma$-Glutamyl transpeptidase:

$$L\gamma-Glutamyl-p\text{-}nitroanilide + Glycylglycine \xrightarrow{\gamma\text{-}GTPase} MgCl_2$$

$$L\gamma-Glutamyl\text{-}glycylglycide + p\text{-}nitroaniline.$$

Consequently, the amount of p-nitroaniline formed becomes the measure of $\gamma$ GTPase activity and it was therefore, assayed with $L\gamma$-Glutamyl-p-nitroanilide as substrate and Glycylglycine as $\gamma$-Glutamyl acceptor. The final assay mixture contained 4.2 mM $L\gamma$-Glutamyl-p-nitroanilide, 52.5 mM Glycylglycine, 10.5 mM MgCl$_2\cdot6$H$_2$O and 100 mM Tris-HCl buffer (pH 7.6).

**Determination of enzyme activity:** 2.0 ml of assay mixture (the substrate solution) was taken in a cuvette, brought to 37°C by keeping in a constant temperature cuvette holder and incubating for 3 minutes. 100 $\mu$l of the enzyme preparation was added and mixed quickly by inverting the cuvette twice. The change in absorbance was measured in an Eppendorf photometer at 405 nm and the reaction rate followed every 20 seconds up to 1 min. A blank was prepared (substrate solution without enzyme) for each series of measurements. The activity of enzyme expressed in terms of $\mu$m moles of p-nitroaniline formed per minute per gm tissue at 37°C was
computed by the following formula:

\[ \text{my moles p-nitroaniline formed/min/g tissue} = \frac{A_{405 \text{ nm}} \times \text{dilution factor} \times \text{total assay vol (ml)}}{\text{Vol of enzyme ext. (ml)} \times e \times \text{time (min)}} \]

Here, \( A \) = change in absorbance

\[ e = \text{extinction coefficient of p-nitroaniline at 405 nm and 37°C and is 10.82 micromoles per cm}^2 \]

**Unit of enzyme activity:** The unit of enzyme activity (I.U.) is equivalent to the amount of enzyme which transforms one micro mole of substrate per minute under the assay conditions.

**III. Genotoxicity Studies**

Three *in vivo* genotoxicity studies, viz. transplacental micro-nucleus test, sperm abnormality test and unscheduled DNA synthesis test were carried out in mice after treating them with arecoline, an alkaloid of betel nut. Arecoline was administered at 3 dose levels (0.5 mg, 1.0 mg and 2.0 mg per 0.1 ml saline per mouse) and a minimum of 3 mice were used per dose level of arecoline for all the three tests. Positive controls were treated with 0.8 mg/kg of Mitomycin C and negative controls were treated with the vehicle (either 0.9% saline or Hank's balanced solution) alone.
a) Transplacental micronucleus test: 8-9 weeks old virgin female mice were used for this study. Animals (25-30 g) were mated overnight and the morning after mating was designated day 0, based on 'vaginal plug' formation. Arecoline was administered by intraperitoneal injection in saline on the 17th day of gestation for the induction of micronuclei in neonatal erythrocytes.

Blood films were prepared directly from dissected fetuses as described by Cole et al. (1981) with minor modifications.

Preparation of erythrocytes: After 45 hr of the arecoline treatment, either the already delivered newborn fetuses or after removal of the same from the mother by laparotomy under ether anaesthesia, were taken for the preparation of erythrocytes. The fetuses were killed by decapitation and the peripheral blood was collected from the cervical blood vessels in microhaematocrit tubes rinsed with 1% sodium citrate. Blood smears were drawn on clean glass slides which were air-dried, fixed in absolute methanol for 5 min and stained with freshly filtered May-Grunwald and Giemsa stains respectively for 3 and 12 minutes following the method of Clark (1981).
Analysis of Polychromatic erythrocytes containing micronuclei: For determination of micronucleated polychromatic erythrocyte (MN/PCE) frequency, 2 fetuses per litter were used and 2000 polychromatic erythrocytes (PCE) per pregnant animal evaluated under oil-immersion (100x) objectives. Statistical significance was determined by using Student's t-test.

b) Sperm abnormality test: 14-16 weeks old male mice were used for this study. For 5 consecutive days, the animals (27-30 g body weight) were treated with arecoline by intra-peritoneal injection in saline for the induction of abnormality in the sperm morphology.

Preparation of sperm smears: On day 35, following the first injection, the mice were killed and abdomen was opened immediately. Sperm smears were prepared and stained following the method of Taylor (1982) with minor changes. Cauda epididymes were removed, placed in petri-dishes containing 4 ml of warm phosphate buffered saline at 37°C and minced with scissors. A sperm suspension was prepared and mixed by repipetting and then filtered through 80 μm wire-mesh. 10 μl of suspensions were pipetted onto clean glass slides and were air-dried. The slides were next stained with eosin-fast green stain for one minute.
Analysis of sperms for shape abnormality: For determination of abnormal sperms, the morphology of 1000 sperms per mouse was assessed using an oil immersion objective (100x). Statistical significance was assessed by the Student's t-test.

c) Unscheduled DNA Synthesis (UDS) test: 14-16 weeks old male mice (27-30 g) were used for this study. Animals were injected intraperitoneally with arecoline dissolved in Hank's balanced solution in order to observe UDS response in their germ cells (early spermatids).

Testicular injections of \(^3\)HThymidine: The mice were given testicular injections of tritiated thymidine (\(^3\)HdThd) under ether anaesthesia immediately after the treatment with arecoline following the procedures of Sega et al. (1976). Each testis, after being exposed by small dermal incisions in the scrotal regions, was injected with 36 µl of \(^3\)HdThd containing 36 µCi of activity using a Hamilton Syringe fitted with a repeating dispenser. No post-injection suturing of the scrotum was performed to the animals.

Recovery of sperms: 16 days after the treatment with arecoline the males were sacrificed and abdomen was
opened immediately. Cauda epididymes were removed and pooled together for each dose point. The sperm heads were recovered and purified from the Cauda epididymes by the use of procedures described by Sotomayor et al. (1982) with little changes. For this, Cauda epididymes were minced with scissors in a 10% Ficoll-0.2% sodium dodecyl sulfate (SDS) solution and then sonicated for 1 min, in order to shear off sperm tails and midpieces by using exponential probe (amplitude 12\lambda). The sonicated suspension was filtered through 80 μm wire mesh to remove cellular debris and then centrifuged at 3500g for 15 min at 4°C. The pellet was washed several times in additional 10% Ficoll - 0.2% SDS solution until a purified population of sperm heads completely free of tails, midpieces and other cellular debris was obtained and confirmed under Leitz phase-contrast microscope. These sperm heads were washed with 1 ml of 0.2% Ficoll - 0.2% SDS-saline solution and centrifuged at 2000g for 20 min. The final sperm pellets were resuspended in 0.5 ml of a 0.2% SDS-saline solution. An aliquote of each suspension was diluted 50-fold and counted in a hemocytometer at least thrice.
Scintillation counting: For assay of the unscheduled presence of $^3$HdThd, 100 µl samples of sperm suspension per dose point were digested with 0.5 ml of protosol, a tissue solubilizer in scintillation vials by warming for 2 hr at 60°C. 15 ml of Bray's scintillation Cocktail (Napthalene - 60 g; PPO - 4 g; POPOP - 0.2 g; Methanol - 100 ml; Ethylene glycol - 20 ml and Dioxane - 1000 ml) was added to each vial and the $^3$H-radioactivity present in the sperm head samples was determined by a Beckman Liquid Scintillation Counter. The unscheduled uptake of $^3$HdThd was finally expressed as disintegrations per minute (dpm) per million sperm heads.