BIOCHEMICAL ASPECTS OF BETEL NUT CARCINOGENESIS

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CERTIFIED that the thesis titled "BIOCHEMICAL ASPECTS OF BETEL NUT CARCINOGENESIS" submitted by Mr. Kishore Kumar Wary for the award of the degree of DOCTOR OF PHILOSOPHY in Biochemistry of the North-Eastern Hill University, Shillong embodies the record of original investigations carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the Ph. D. degree. This work has not been submitted for any degree of any University.

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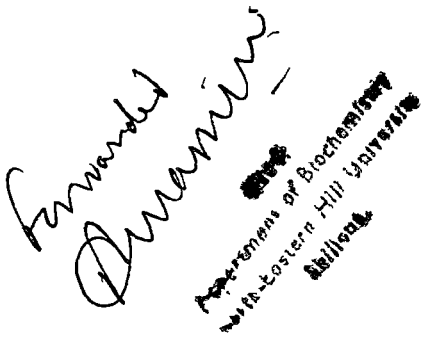

DEDICATED

TO

`Aai'  and  `Aafa'.

CONTENTS

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Kishore Kumar Wary

(Kishore Kumar Wary)

ABBREVIATIONS AND UNITS

AEBN	aqueous extract of betel nut
BSS	balanced salt solution
Ci	curie
cpm	counts per minute
DNA	deoxyribonucleic acid
ds-DNA	double strand DNA
GGT	gamma glutamyl transpeptidase
g	gram(s)
[^{3}H]-TdR	tritiated thymidine
HBSS	Hank's BSS
hr	hour(s)
l	liter(s)
M	molar(s)
min	minute(s)
ml	milliliter(s)
mM	millimolar
μ	micron
μg	microgram
μl	microliter
nm	nanometer
RNA	ribonucleic acid
UDS	unscheduled DNA synthesis

Temperatures expressed in degrees celsius (°C)

S-9 fraction: microsomal suspension prepared from rat liver homogenate

With the advancement of knowledge and better understanding of physiology it seems certain that many diseases hitherto regarded spontaneous may be caused by environmental factors, nutritional habits and social customs. The impact of these burdens on human health has become an universal concern. With the advent of modern civilization, human beings are subjected to more toxic chemical in the environment and in food materials. Looking back, it is evident that our ancestors ate pure, wholesome and natural food procured from their surroundings. This, however, is now limited to very small groups of people viz., Hunza valley in Pakistan today. Modern man has come to deviate from these norms and burdens his body with a host of unhealthy foods. Due to variety of reasons, man has picked up unhealthy habits like areca nut chewing, tobacco chewing, alcohol drinking and smoking, which exert now and sometimes undesired biochemical reactions in the living system at cellular, subcellular and at the molecular levels. These interactions may bring about diseases which have no definite cure as yet. A dreadful disease, cancer, is one of them. These changes may not be visible at early stage of life. After substantial period of

incubation but at some point of our lives, some or many of us become engrossed by the terrible disease known as cancer.

In the last two decades there has been a sharp increase in the awareness that some naturally occurring compounds in food may cause or at least contribute to human diseases, notably, cancer. However, the chewing of areca nut wrapped betel leaf stains the teeth and saliva red, which is a disquieting accompaniment, continues unabated in this region of North East India and elsewhere. "Kwai" (local name for raw areca nut) is consumed in large quantities in this region whereas "Pan", another form of consumption of betel nut, remains popular in many other parts of India. In Meghalaya, areca nut is grown profusely and the "Khasi" tribe employ this nut as masticatory. Some theists believe that chewing of betel nut is more or a form of courtesy rather than a habit and some people are of the opinion that chewing of betel nut is indeed a noble act. However, this chewing habit is considered as a part of social custom of theists and also other tribes. The raw areca nut is consumed along with betel leaf (Piper betle L.) and variable quantity of slaked lime. It shows an immediate thermogenic physiological response, lasting (2-3 minutes), with significant perspiration on forehead. This effect markedly differs from the effect of betel nut consumed

elsewhere in the world. Some preliminary work has been carried out by Shah et al (1986a) in Meghalaya and was found to have tannic acid in the saliva of a population of betel nut chewers.

Areca nut contains a number of chemical components (Prabhavan and Bastian, 1970) and much work has been done to calculate the chemical, pharmacological and therapeutical properties of areca nut and its chemical constituents (Jadhav, 1980). Similarly animal experimentation and in vitro tests have so far given a limited knowledge about biochemical events preceding/leading to carcinogenesis associated with areca nut chewing. Nonetheless, the knowledge gained so far clearly points to the fact that chewing of betel nut is apparently a major factor in the etiology of oral and associated cancers. The betel nut chewing has been shown to induce oropharyngeal cancer in India which accounts for over 50% of total cancer incidence in India (Gangadhar, 1981).

Areca nut and its chemical constituents have been tested for mutagenicity, carcinogenicity and for cytogenetic (Shivnagar et al, 1985; Sinha, 1984). Various extracts of betel nut have been tested for their ability to bring about changes in the genome. The saliva from areca nut chewers have been found to

be mutagenic (Stich et al, 1980). Elevated frequency of micronucleated cells in the buccal mucosa of individuals as a result of chewing betel nut and tobacco use have been reported (Stich et al, 1983b). So far these data have given a limited understanding of the mechanism of carcinogenesis associated with betel nut chewing (IARC, 1985). Our knowledge in connection with raw (unprocessed) betel nut, "kwai", is further limited.

Formation of N-Nitrosocompounds from arecoline (a major alkaloid from areca nut) upon mild nitrosation condition have been reported (Wenke and Hoffmann, [illegible]). N-Nitrosoguvacoline has been found to occur in the saliva of betel nut chewers; tobacco specific nitrosamines have also been detected in the saliva sample of betel quid chewers (Wenke et al, [illegible]). From existing data it is apparent that betel nut can undergo N-nitrosamine formation. The nitrosation condition may be offered by various food and drinks which contain significant amounts of nitrates which occurs freely in fruits and vegetables. Formation of nitrosamines and occurrence of nitrosamines in food and food-additives are mainly due to the metabolism of nitrites in in vivo which in turn are responsible for development of cancer ([illegible], [illegible]). Therefore, in areca nut chewers the formation of nitrosamine(s) is not unexpected.

The human body is complex organization of biological macromolecules. These are adapted to this complex system since time immemorial. Various proteins, enzymes and metabolite are involved in the metabolic processes. The metabolic processes include detoxification of harmful substances which may either be produced within the body or ingested from outside as foreign substances. One of such enzyme involved in detoxification is Cytochrome P-450, which are actively involved in activation and deactivation of xenobiotics (Gwenger et al., 1988). There has been no report on the possible detoxification or deactivation of arecaidine alkaloids (IARC, 1986). This study, however, seem relevant due to the fact that all betel nut chewers or all betel quid chewers do not automatically suffer from cancer.

Epidemiology and Use of Betel nut

In early 1900's the cancer associated with betel nut and betel quid chewing were recorded by Mahidol (1950) in Thailand and Travancore. The inhabitants of Travancore combined betel nut and betel quid chewing. Among this population three risk factors Vitamin A deficiency were found to be prominent in causing cancer among the inhabitants of Malabar coast. The carcinoma in cheek was prevalent among those who consumed betel nut and tobacco. But in 1953, in the report from Mysore hospital in Travancore, found prevalence of oral cancer, predominantly in the population of betel nut and betel quid chewers. The risk of oral cancer was found to increase when the population used smoke and lime in mouth. The habit of chewing and swallowing betel nut covers a wide spectrum of age groups.

A survey carried out in Maldives among 5 to 15 years age groups of 454 children showed 76% chewed betel nuts daily. The prevalence rates for the ages 5-6, 11 and 15 years of age was 1%, 27 and 51% respectively (Knudsen et al, 1980). A study on dental caries and betel nut chewing in Java and Bali showed higher prevalence among women than men. The habit was generally acquired between the ages of 15 and 19 years (Bhattacharya et al, 1977). Vacuolization of buccal epithelial cells, similar to

the ballooning cells was observed in the upper half of the spinal cell layer in 10 biopsies from leukoplakic lesions of Papua New Guineans all of whom were betel nut chewers and heavy smokers (Gorlin et al., 1965). Betel nut chewing and heavy smoking in Papua New Guinea reportedly increased leukoplakic lesions (Lindberg et al., 1968). Another survey in Papua New Guinea showed that raw or uncured areca nut chewing was very common. In a population of 1776, 57.7% were betel nut chewers and 7% were smokers. Of these, some had mixed habit chewing betel nut and smoking (Lindberg et al., 1984a). Similar habits are also seen among the Khasi tribes of North East India (Gupta et al., 1986a).

Betel nuts are grown profusely in this region and seeds are obtained from local market. The natives of this region (Khasi tribe) employ areca nut seed as masticatory. They consume raw betel nut with lime wrapped in betel leaves and call it "Kwai". The size of nut varies from 6 grams to 18 grams. These are cut into four or more pieces to obtain pieces of 2 to 5 grams. While chewing, it induces profuse salivation which soon turns red. The red saliva is not usually spat. The chewing is stimulative and exerts immediate thermogenic action (perspiration etc.) lasting 2 to 3 minutes. This characteristic response differs markedly from other types of betel nut consumed elsewhere in India. The reason could be the presence of high quantity of

alkaloids in this native areca nut. The chewing time of one betel nut varies from 5 to 20 minutes depending upon the size of betel nut chewed. The habit may be acquired at an early age varying from 5 to 15 years in both sexes. However, children are usually forbidden to chew betel nut at home as well as in schools; some children are only forbidden during school hours. The first piece of betel nut is likely to be consumed by habituated chewers after a cup of tea in the morning or after breakfast. Then throughout the day process of chewing of betel nut continues.

During off seasons, betel nuts are dumped in underground pits to facilitate fermentation. The time required for fermentation varies from 2 to 4 months. These betel nuts are then cut and the seeds are used for chewing with lime and betel leaves. Old people usually carry pounding appliances to the ground the betel nut finely with lime and betel leaves while they go for work. The betel nut is not only employed as a masticatory but also as meat tenderiser by Khasi people.

The cancer of oral cavity is one of the commonest sites of cancer which accounts for over 50% of total cancer incidence in India (Sanghvi, 1981). There has been a long thought that high rate of oral cancer incidence might be due to chewing

of betel and tobacco; this has been partly [illegible] observations and some experimental evidence (UICC, 1976). The National Registry of India (1984-85) indicated [illegible] in North East India and cases of cancer [illegible] are directly related. The habit of chewing of betel nut [illegible] not restricted to India, but it has also become a [illegible] and parcel of day to day life in countries like Pakistan, Burma, China, Papua New Guinea, Malaysia, Thailand, Sri Lanka, Bangladesh and some other parts of the world (IARC, 1985).

Epidemiological data from India and from elsewhere [illegible] oral and oropharyngeal cancer incidence due to betel quid and betel nut chewing habits (Hirayama, 1966). The hospital cancer registries and population based registries brought out by ICMR 1984 and 1985 (Annual Report, 1984; 1985) and Bulletin of WHO (1984) show mixed habit of betel nut and tobacco are significantly associated with cancers in all sites of upper alimentary tract.

The addition of tobacco with betel quid has been found to induce oral submucous fibrosis in populations in Bihar, Gujarat, Kerala and Andhra Pradesh (Mehta et al. 1971). Malignant transformation was reported in one out of 44 cases of oral submucous fibrosis in Ernakulam (Kerala) in 1966 (Pindborg et al.,

1980). Follow up study on the same population and same determined showed overall rate of 4.5% oral submucous fibrosis (Pindborg, 1980b).

The epidemiological studies and heterogeneous nature of data on cancer associated with betel nut chewing demands for scrupulous epidemiological survey to monitor the population at risk (Cartwright, 1984). Their habits, including dietary intake, are important because diet and nutrition may play significant modifying roles (Notani and Sanghvi, 1976; [illegible] et al., 1980). The necessity of detection and control of such lesions become highly important since the symptoms are precancerous in nature (Sanghvi, 1981).

Chemical Constituents of Betel nuts

Betel nuts contain various chemicals besides the pyridinium alkaloids. The earliest study on areca nut alkaloids came from Sharp, T.M. (1934). However, the major chemicals which are present in areca nut are tabulated by Raghavan & Baruah (1958) as shown below. The chemicals obtained from raw or unprocessed betel nut is subject to variation.

Table I

Chemical constituents	Approximate Quantity
Alkaloids	0.15-0.67%
Arecoline	0.07-0.50%
Arecaidine	small quantity
Guvacine	small quantity
Isoguvacine	trace quantity
Arecolidine	minute quantity
Guvacoline	minute quantity
Carotene	5 International Vitamin units/100g
Fats	1.5-1[illegible].0%
Tannins	11.4-26.0%
Gallotannic acid	18.0%
D-Catechol	5g/800g (0.4%)
Proteins	4.9-9.3%
Non protein nitrogen	0.[illegible]2-1.6%
Minerals	
Calcium	0.018-0.05%
Phosphorous	0.13-0.35%
Iron	1.5-11.6 mg/100g (0.002-0.01%)

[Based on Raghavan and Baruah, 1958.]

Variation in chemical constituents of processed or cured areca nuts have been reported. The occurrence of alkaloids, polyphenols, carbohydrates, crude fibre, fats vary in

cured or uncured (processed or unprocessed) variety of areca seeds (Shivashankar et al., 1969).

<u>Alkaloids</u>

The chief alkaloids of areca nuts, arecoline, arecaine, arecaidine and guvacine (Figure 1) were examined by Jahns (see Sharp, 1980). Arecoline, 1,2,5,6-tetrahydro-1-methyl-3-pyridinecarboxylic acid methyl ester, is a colorless, odorless substance and is soluble in water, alcohol, ether and chloroform. The molecular weight of this compound is 155.19 and lethal dose in mice is 100 mg/kg. Arecaine has been found to have same structure as of arecaidine. Arecaidine, which is insoluble in chloroform, ether and benzene, has a molecular weight of 141.1. Arecaidine is 1,2,5,6-Tetrahydro-1-methyl-3-pyridinecarboxylic acid. It is highly toxic and has similar pharmacological and physiological action to arecoline. Guvacine is methyl ester of arecaidine. On further methylation, guvacine forms guvacoline, another trace alkaloid (see Figure 1). The amount of alkaloids in betel nut supposedly vary from 0.15 to 0.67% (Goswami and Ahmed, 1956; Raghavan and Baruah, 1958). Trace amounts of other related alkaloids are also reported.

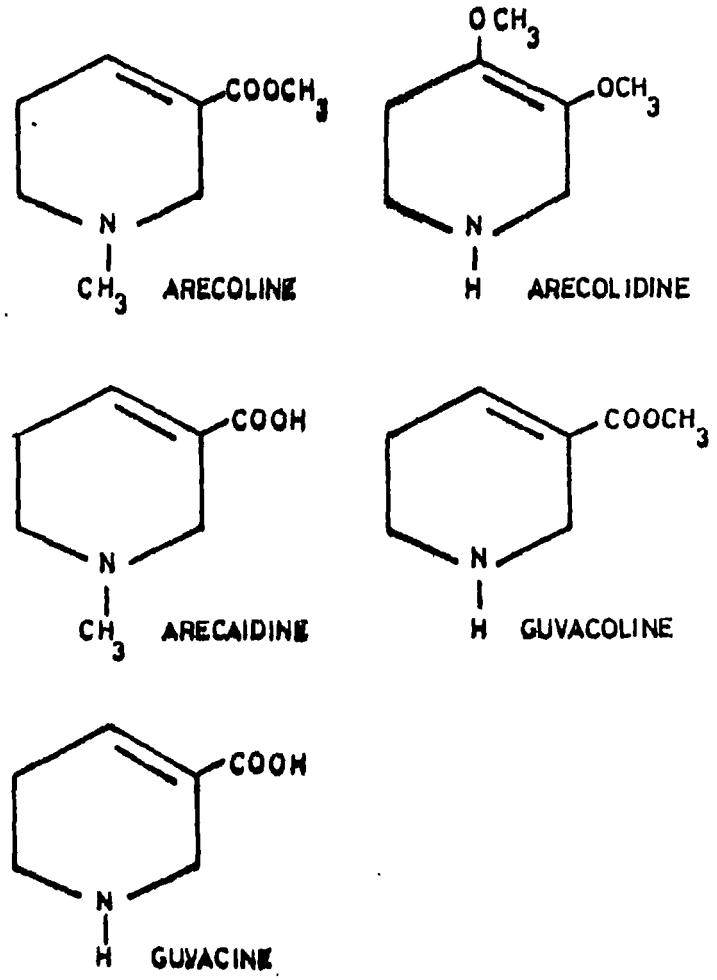

Figure I :

MAJOR ALKALOIDS PRESENT IN BETEL NUT

Carbohydrates

Polysaccharides or carbohydrates of young betel nut consequently increases when ripe. The carbohydrates in betel nuts are saccharose, reducing sugars, galactan and mannan (Raghavan and Baruah, 1958). The husk or peel of betel nut contains mainly cellulose and lignin (Ali and Chowdhury, 19[illegible]).

Carotene

Carotene occurs in betel nut in very low amounts. Other Vitamins of betel nuts are not reported (Raghavan and Baruah, 1958).

Fats and Fatty Acids

The areca nut when extracted with ether yields 14 to 15% fats. Of these, the main saturated fatty acids present in betel nuts are: myristic (46.2%), lauric (19.5%), palmitic (1[illegible]%), stearic (1.6%) and decanoic (0.3%) acids. The unsaturated fatty acids in betel nuts are: oleic (6.[illegible]%), linoleic ([illegible]%), dodecenoic (0.[illegible]%), tetradecenoic (0.5%) and hexadecenoic ([illegible]%) acids (Majumdar et al., 1979). The total fat is composed of 56% saturated and 44% of unsaturated glycerides (Rahman and Nathan, 1964).

Tannins

Tannins of betel nuts decrease when the nut ripens (Bravo, 1979 & Deas, 1941). The chief tannins of betel nuts are gallotannic acid (16.6%) and D-catechol (0.4%).

Polyphenols

Polyphenols of betel nuts are mostly flavanol and flavanol diols. The content of polyphenol is found to decrease with the maturity of areca nut (Mathew and Lewendowski, 1968). Polyphenols when oxidized in presence of lime gives red color to betel.

Apart from above mentioned components of areca nut, it also contains minerals and free amino acids in traces (Balakrishnan and Gireesan, 1965).

Betel Nut Carcinogenesis

Betel nut chewing habit has been thought to be one of the reasons for causing cancer in India and its neighbouring countries. On chewing, this makes the salivated and stains the teeth. Chronic chewers can easily be identified by checking their teeth which bears red deposits. There have been clinical studies on the carcinogenicity of betel nut (IARC, 1985). Some work has reviewed by Ahmad (1976) on the extracts of betel nut, but no conclusive report is available as yet (IARC, 1985).

the dimethyl sulphoxide (DMSO) extracts of betel nut when treated to buccal pouch of hamster produced tumor (Sunil et al., 1976). Transplantable fibrosarcomas were found to develope at the site of application when they were treated to extract of betel nuts (Ranadive et al., 1976 ; Lopez-Cepero et al., 1978). Following treatment of 20% betel nut diet with calcium hydroxide reportedly induced epidermal thickening with hyperkeratosis in mouth cavity in a mammalian test system (Hori et al., 1979). Aqueous extracts of betel nut fed to mice ad libitum produced gastrointestinal tumors (Bhide et al., 1979). Tumorigenic effect of aqueous and polyphenolic fractions of betel nut extracts have been obtained in mice (Shivapurkar et al., 1980a). Treatment of ethylacetate extract of areca nut, however, did not induce ouabain-resistant mutants in Chinese hamster V79 cells or sister chromatid exchanges in human lymphoblastoid cell line and also in Syrian hamster embryo cells (Umezawa et al., 1981).

Aqueous extracts of betel nut in Ames test gave increased rate of mutation in Salmonella typhimurium TA 1535 and TA 100 (Shirname et al., 1983), and in Chinese hamster V79 cells an enhanced rate of mutation was found following addition of microsomal S-9 mixture 1 (Shirname et al, 1984). Exposure of

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1See details in "Abbreviations and Notes".

aqueous, ethyl acetate or n-butanol extracts of cured areca nut in absence of S-9 mixture in Chinese hamster ovary cells induced chromatid breaks, exchanges and chromosomal aberration was significantly enhanced in presence of 0.1 ml (0.5 µg/ml) (Shirsath et al., 1983a). Treatment of mice with whole aqueous extract of betel nut induced sister chromatid exchanges in mouse bone marrow cells in vivo. The whole aqueous extract of betel nut showed genotoxic effects whether animals were given long or short exposure with test chemicals (Panigrahi and Rao, 1986).

Another essential ingredient of chew is betel leaf (Piper betle L.). Exposure of human lymphocytes to aqueous extract of betel leaf in absence of S-9 mixture reportedly produced chromosomal aberration (Sadasivan et al., 1978). Experiments on Chinese hamster ovary cells with extracts of betel leaf also showed chromosomal aberrations; ethyl acetate extract showed aberration upon addition of 0.1 ml (0.5 µg/ml) (Shirsath et al 1983a). In Ames test, aqueous extract of betel leaf failed to induce mutagenic effect on S.typhimurium TA 100, TA 98, TA 1535 or TA 1538 in presence or in absence of S-9 mixture; this extract when given in combination with aqueous extract of betel nut in strain TA 100, reduced mutagenicity of aqueous extract of betel nut (Shirname et al., 1985). Similarly, betel leaf has been

shown to suppress the mutagenicity on betel quid in Chinese hamster ovary cells (Padmanabhan et al., [illegible]). Betel leaf ([illegible] mg/pouch) given twice a day for six months in Syrian golden hamster cheek pouch did not develope any cheek pouch tumor in [illegible] animals while benz(a)pyrene plus betel nut treatment for [illegible] months induced tumor (Rao, 1984). Inhibition of initiation of 7,12-dimethyl benz(a)anthracene induced mammary gland carcinoma without body weight gain was observed when rats were treated with high dose of aqueous extract of betel leaf at 1 mg/gm. Following 5 weeks treatment with the same extract there was no significant suppression on DMBA-induced mammary carcinogenesis during [illegible] phase (Rao et al., 1985). A phenolic compound, hydroxychavicol, was extracted from betel leaf and it is thought to be antimutagenic (Amonkar et al., 1986).

Following repeated application of slaked lime (calcium hydroxide) in cheek pouch of hamster epithelial atypia was produced (Dunham et al., 1966). Slaked lime painting on palate and buccal mucosa of Wistar rats for a period of [illegible] months showed moderate to severe form of hyperplasia, hyperkeratosis, cytoplasmic vacuolation and invagination of the rete peg into the papillary layer (Sirsat and Kandarkar, 1968).

Aqueous extract of betel nut in combination with betel leaf and lime but without tobacco administered in cheek pouch of hamster developed forestomach carcinoma in 5 out of 20 animals (Ranadive et al., 1979). Subcutaneous administration of aqueous extract of betel nut and betel leaf produced local sarcomas in 7 out of 20 mice (Shivapurkar et al., 1980). However aqueous extract of betel quid without tobacco was not mutagenic in Chinese hamster V79 cells, with or without S9 mixture. The same extract also did not induce micronuclei in bone marrow cells of Swiss albino pregnant mice following administration of total extracts of ripe betel nuts or unprocessed and processed varieties yielded falsepositively principle of betel nuts (Sinha and Rao, 1985). Different preparation of areca nuts were fed orally to Swiss albino mice which, however, showed that only unprocessed areca nuts at high doses induced weak carcinogenecity especially in oral cavity including oesophageal and forestomach epithelia (Rao and Das, 1987).

Mutagenecity & Carcinogenecity Studies on Arecoline & Arecaidine

The short-term in vitro and in vivo assays which are generally used to monitor mutagenic and carcinogenic principles of xenobiotics help in predicting the genotoxicity of

, chromic [illegible] or mixture of chromates, bromides, arecoline undergoes deesterification in liver, both arecoline and arecaidine are excreted as the mercapturic acid N-acetyl-S-(2-carboxy-1-methyl-2-oxo)ethyl cysteine (Boyland and Bray, 1960). Hepatocarcinogenicity has been reported from time to time. It was found that arecoline and arecaidine can bring about cell transformation (Ashby et al., 1979). Arecaidine was reported to be more potent transformation (Ashby et al., 1979). However, due to the limited content of arecoline in betel nut to arecaidine, arecoline becomes a carcinogen of serious concern. Arecoline supposedly interferes with the macromolecular biosynthesis. Arecoline has been reported to decrease [3H]-thymidine incorporation in muscle and kidney, meaning inhibition of DNA synthesis (Shivapurkar and Gilde, 1979). On the other hand, increased incorporation of [3H]-thymidine indicating DNA synthesis was observed in liver and lung upon arecoline administration; concomitantly there was significant decrease in hepatic DNA and protein content in mice (Shivapurkar et al., 1979). Chromatid breaks and exchange in Chinese hamster ovary cells in absence of S-9 mix were observed following arecoline treatment (Ishidate et al., 1981). Arecoline was found to damage DNA in mouse bone marrow cells in vivo (Panigrahi and Rao, 1982) and caffeine and arecoline were found to have additive effect on sister

chromatid exchanges in mouse bone marrow cells in vivo (Panigrahi and Rao, 1983). Arecaidine was mutagenic in Ames test in presence of S-9 mixture, however, mutagenicity was lower than arecoline (Shirname et al., 1983). Arecoline, in Ames test, gave increased rate of mutation in S.typhimurium (Shirname et al., 1983) and in Chinese hamster V79 cells with or without S-9 mixture (Shirname et al., 1984). Induction of 8-azaguanine resistance in Chinese hamster V79 cells were obtained following arecaidine treatment in presence of S-9 mixture (Shirname et al., 1984). Dose dependent increased frequencies of sister chromatid exchanges were observed in Swiss albino mice bone marrow cells on intraperitoneal treatment to arecaidine which was not dependent on the duration of exposure (Panigrahi and Rao, 1986).

Experimental evidences supporting squamous cell carcinoma in pouch epithelium, papillomas and advanced atypias of oesophagus in hamster following arecoline treatment have been reported (Dunham et al., 1974). Arecaidine is found to develop papillomas in cheek pouch of hamster (MacDonald, 1987).

Nitrosamine Carcinogenesis

It has been shown that areca nut alkaloid, arecoline, under mild nitrosation condition forms N-Nitrosamines (Figure II), notably, N-Nitrosoguvacoline, 3-(methylnitrosamino)-

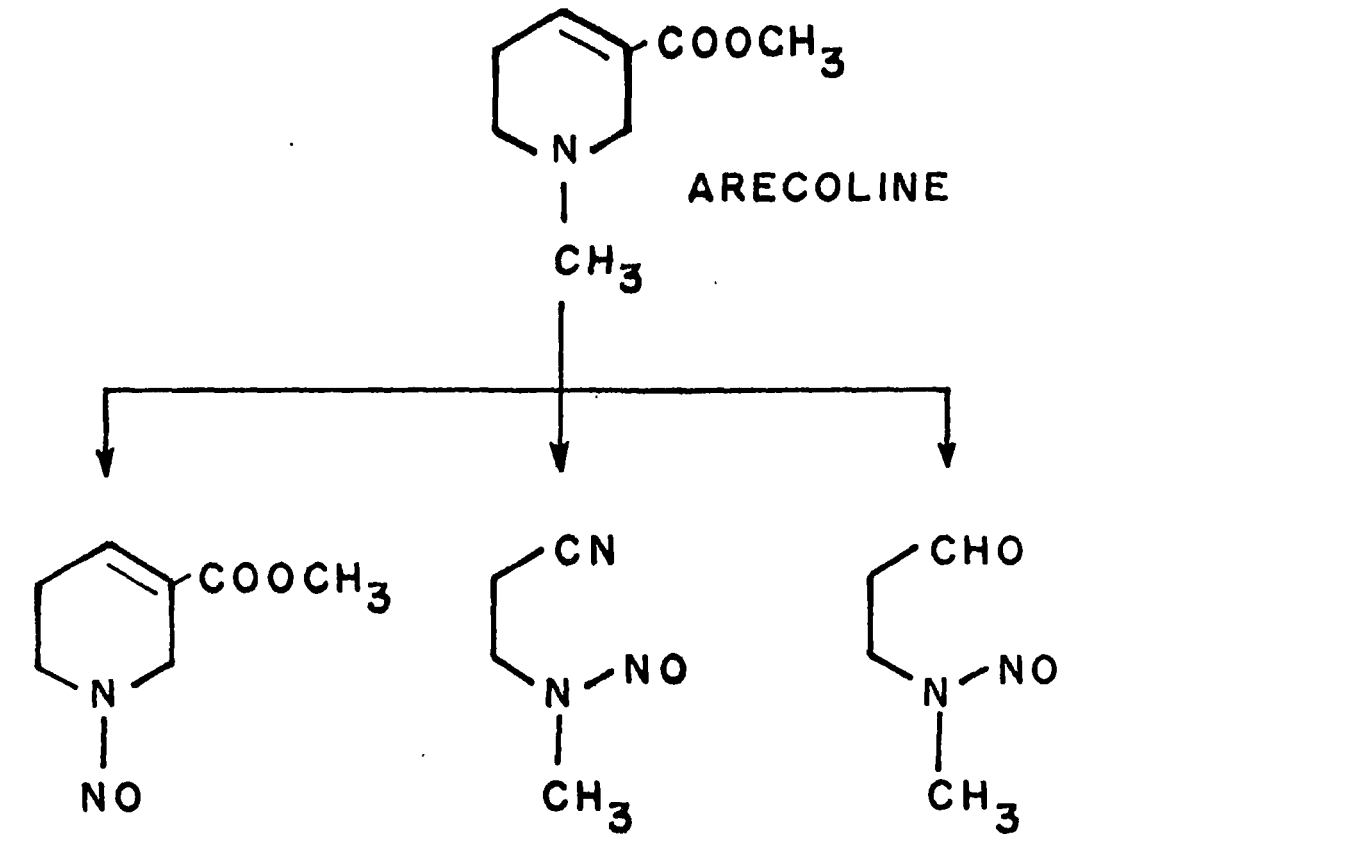

Figure : II

NITROSATION PRODUCT OF ARECOLINE

propionitrile, 3-(methylnitrosamino) propionaldehyde (Wenker & Hoffmann, 1985) and N-Nitrosoguvacoline (Nair et al., 1980) in vitro. Saliva sample analysis showed appreciable concentration of N-Nitrosoguvacoline and reduced specific N-Nitrosamine (Wenker et al., 1984; Nair et al., 1985). Urine sample analysis showed *presence of N-Nitrosoarecoline, N-Nitrosoguvacoline and N*-Nitrosothiazolidine-4-carboxylic acid in betel nut chewers. Upon ingestion of proline and nitrate with extract of areca nut, endogenous nitrosation is found to be inhibited which was indicated by N-Nitrosoproline excretion in urine (Stich et al., 1983b). Increased endogenous N-Nitrosamine formation was indicated by increased excretion of N-Nitrosothiazolidine-4-carboxylic acid and N-Nitrosoproline (Stich et al., 1983b).

N-methylnitrosaminopropionitrile (MNPN) was detected in the saliva of betel quid chewers. Upon treatment of MNPN in fed rats showed DNA methylation; a higher DNA methylation was found in nasal cavity (Prokopczyk et al., 1987). The addition of slaked lime to areca nut reportedly enhanced the formation of N-nitrosoguvacoline, during chewing session indicated by level of nitrosothiocyanate in betel quid chewers (Stich et al., 1986). Some polyphenolic compounds, depending on their chemical structure and pH of reaction media

and presence of nitrite, either inhibited or enhanced nitrosation of proline in vivo (Birch et al., 1980). Apart from these there are many sources of genotoxins in the human diet. Natural mutagens are contributed by flavonoids, pyrrolizidine alkaloids, hydrazines, alkylating agents and natural amines (Watson, 1987).

Nitrosamines are potent carcinogen and are organ specific. Most nitrosamines directly interact with DNA (Preussmann and Stewart, 1984). The food and drinks also contain exogenous amounts of nitrites and nitrates. In addition to that, nitrites have been found to occur in gastrointestinal tract, which indicates that the gastrointestinal tract may offer suitable reaction sites for N-nitrosamine formation. N-Nitrosamines may bind or cross link macromolecular genetic molecules, directly alkylate nucleic acid bases (somatic mutation) or produce other N-Nitroso compounds which eventually may cause damage to the cells (Ljungsby, 1987).

On the other hand, mammalian system possesses couple of metabolic pathways of detoxification. There are significant species differences encountered in metabolic fate of foreign compounds, but it is also important to note that similar type of detoxification mechanisms occur in all species. Many

other processes such as absorption, distribution and excretion participate in the modification of biological activity of xenobiotics, but metabolic fate of such compounds appear to be important in this regard. Among detoxification enzymes, the enzymes of the glutathione metabolic pathway plays important role. Glutathione is found to conjugate enzymatically or non-enzymatically with potentially harmful electrophilic compound, (Meister, 1970), such as those produced by the Cytochrome P-450 system. Many mutagens, on the other hand, require metabolic activation in order to be converted to ultimate bacterial mutagens in Ames test. On activation by enzymes, metabolites become reactive to DNA and proteins (Preussmann & Stewart, 1980). Some mutagens or potent carcinogens are degraded to less toxic substance(s). The enzymes carrying out these reactions sometimes may generate reactive metabolites but usually produce less toxic derivatives. Detoxifying enzymes eventually convert a foreign compounds to a more polar molecule and thus facilitate its excretion through urine and feces (Guengerich, 1980). Multiple forms of Cytochrome P-450 are one of the chief factories in the metabolism of xenobiotics such as drugs, carcinogens, and environmental chemicals as well as endobiotics such as fatty acids, prostaglandins, and steroids (Guengerich, 1980). No studies, whatsoever, has been reported regarding detoxification of areca nut or its chemical constituents (IARC, 1985).

Aim and Scope

Much work have been done to evaluate the pharmacological, clinical and mutagenic property of betel nut and its alkaloids in vitro and in vivo. The findings are, however, conflicting. There has been no conclusive report which can be strictly attributed to cancer due to betel nut chewing. Moreover, there has been a lack of literature which could indicate probable detoxification of betel nut or any of its chemical constituents in living system, because any betel nut chewer automatically does not develope cancer. Apart from these, studies on biochemical markers and interaction with DNA should give an understanding of betel nut carcinogenesis. So it was desired to take **Swiss albino mice** as a model system for the experiments. The choice was made mainly because of (i) the ease with which it was available, (ii) the convenience of maintenance and most importantly, (iii) the short gestation period (21 days) and moderately large litter size which were added advantage for raising generations. **DNA damage** in cell was monitored following exposure to test chemicals because: (i) many carcinogens are directly or indirectly reactive to DNA, and (ii) interaction of DNA with mutagen supposedly brings about changes in metabolic functions. **DNA strand break** analysis following treatment with alkali was taken for obvious reason because it is a sensitive and

quick protocol for DNA strand break analysis. Unscheduled DNA synthesis is a measure of extent of DNA damage and repair following mutagen insult to the cells. Analysis of marker enzyme is a valuable protocol to see early changes, if any, due to induction of neoplasia and preneoplastic lesions following mutagen treatment. Study of drug metabolizing enzymes cytochrome P-450, a multiple forms of isozymes from mouse liver, have been taken to monitor the activation or deactivation of areca nut alkaloid.

The results from analyses of areca nut, epidemiology and experimental system that are taken in the study are expected to give an understanding of betel nut induced cancer in mouse and man.

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MATERIALS AND METHODS

Animals

Specific pathogen free Swiss albino mice were obtained from Haffkine Veterinary Biological, Parel, Mumbai. Three to five week old, age matched, syngenic male and female mice were selected for the experiments. The animals were housed in polycarbonate cages with husk bedding. The room was kept approximately at 25° C temperature with 12 hours dark and 12 hours light arrangements. The room was provided with proper ventilation. Feed and water were made available at all times so that they consume as much as they need. Diet was stored under room temperature and humidity. Areas where diets were stored were protected from any possible entry of pests or rodents. Hygiene was maintained to avoid any contamination by microbes, antibiotics or fungicides into diets.

Chemicals

Phenol red, streptidine, coomassie brilliant blue G-250, calf thymus deoxyribonucleic acid (DNA), glycylglycine, L-gamma glutamyl-p-nitroanilide, bovine serum albumin (BSA), sodium dodecyl sulphate (SDS), Tris base, ethylenediamine tetraacetic acid (EDTA), fetal calf serum, phosphate buffered saline (PBS), trypsin, penicillin, streptomycin, neomycin sulphate were

obtained from Sigma chemical company (USA). 2,5 Diphenyl oxazole (PPO), 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP) and recrystallized toluene based scintillation cocktail was procured from Beckman Instruments, Inc., USA. High performance liquid chromatography (HPLC) grade solvents - acetonitrile, acetic acid and distilled water were procured from Merck chemical company, USA. Isoquinone (Amphotericin B) was obtained from Bristol Laboratories, USA. All other tissue culture media and chemicals were purchased from Hi-Media Laboratories Private Limited, India. Glass microfibre filter GF/C and GF/D were supplied by Whatman Limited, England. Sodium-dithionite was obtained from Riedel de Haen, Weinheim. Carbon monoxide (CO) gas was obtained from Bellhaven Gas Products, USA. Radiochemicals, L-(H) Thymidine (specific activity 5 mCi/mmol), L14-C)-Thymidine (specific activity 56 mCi/mmol) and L14 C)-Leucine (specific activity 5.5 mCi/mmol) were bought from Bhabha Atomic Research Centre (India). All other chemicals used in this study were of analytical grade and were used without further purification unless indicated.

Betel nut

Fresh betel nuts were obtained locally from Darbhanga market. These betel nuts came from West Bihar Hills,

Sahara and Far Areas of Rajasthan.

Preparation of extracts of Betel nut

i) After shelling the tribal's seeds, the nuts were ground and suspended in 250 ml. of distilled water and kept at room temperature for 24 hours. The suspension was filtered through a 0.45 µm millipore filter and the filtrate was lyophilized in a Secfroid Lyolab 811 lyophilizer. The lyophilized mass was kept at 4° C until use. This product is henceforth called aqueous extract of betel nut (AEBN).

ii) 100 grams of fresh areca nut after shelling the tribals seeds, were crushed finely in a mortar and extracted thrice with 0.1 N hydrochloric acid at room temperature. The reddish solution was filtered through Whatman No. 1 filter paper and rotary evaporated followed by freeze drying. The dry mass was stored at 4° C until use.

iii) Alcohol extracts of betel nut was prepared by suspending the powdered betel nut in alcohol at room temperature for 24 hours. The reddish solution was filtered and rotary evaporated to reduce the volume. The concentrated solution was freeze dried and the dry mass kept at 4° C until use.

ver 1 % acetic acid extract of boiled nuts prepared by degrading the powdered boiled nut in 1% acetic acid for 24 hours. The solution was filtered and rotary evaporated to reduce the volume followed by freeze drying. The dry mass was stored at 4° C until use.

2. Proximal analysis of boiled nut

1. Preparation of samples: The shiny coat on seed was removed and weight of the whole seed was taken. Non edible part of the seed was removed. The seed was then oven dried finely into powder and the following analyses were carried out using Association of official analytical chemists (AOAC, 1983).

2. Moisture content: 2 grams of powdered boiled nut was kept at 70° C for 16 hours or more. The process of heating and cooling continued till a constant weight was obtained. The difference in wet weight and dry weight was noted and the percentage of moisture content in boiled nut was calculated.

Moisture = Wet weight - Dry weight

3. Protein content: Water extracts of powdered boiled nut were subjected to estimation of total protein according

to the method of Bradford (1976). The boiled nut powder was soaked in water overnight. The suspension was filtered through cotton paper and the supernatant was used for determining the protein content. BSA was used as standard protein.

4. **Fat content:** Fat was estimated as crude ether extract on the moisture free powdered boiled nut samples. 10 grams of dry powdered samples were soaked in 100 ml of ether overnight at room temperature. The undissolved particles were removed and clear supernatant was retained. Then the ether was evaporated to dryness and the deposits collected in reaction vessel was carefully scrapped and the weight of the remaining crude fat content was taken.

5. **Crude Fibre:** 10 grams of moisture free powdered boiled nut were boiled in 200 ml of 0.125 N sulphuric acid for 30 minutes keeping the volume constant by adding water from time to time. Following boiling in sulphuric acid the residue was again boiled in 200 ml of 0.313 N sodium hydroxide for 30 minutes and the volume was maintained at 200 ml by adding water. The insoluble residue thus obtained was repeatedly washed with hot water to remove alkali. This was followed by two washes in ethanol and one in ether. Then the mass was dried at 60° C overnight and weighed

(W) was noted down. This was then heated to ashes till a constant weight (W') was obtained. The crude fiber content was then calculated by

$$\text{Crude fiber} = \frac{W - W'}{W} \times 100$$

6. **Ash content:** 1g grams of powdered material was in a porcelain crucible (which has been heated to about 600° C and cooled) was first heated in a low flame for about 5 minutes followed by heating it to about 600° C till ashing was complete and allowed to cool down before weighing. This process was repeated till a constant weight of ash was obtained.

7. **Carbohydrate content:** Water soluble carbohydrate content of powdered was measured by anthrone reagent. Anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 ml of concentrated sulphuric acid. In a set of test tubes, various concentrations of standard starch solution were pipetted. By adding water to each tube the final volume was made upto 1 ml. Again to each test tube 5 ml of reagent was added and mixed thoroughly by vortexing. The whole test tubes were transferred to a water bath and boiled for 10 minutes. After the test tubes were allowed to cool down and absorbance were recorded

at 620 nm. The concentration of total carbohydrate content was calculated by least squares method on Shimadzu UV visible spectrophotometer UV 240 model (Japan).

Test Sample Preparation for TLC/HPLC

Water extractible lyophilized mass (0.1 gm) was boiled and was dissolved in 10 mL of water containing 0.5 ml of 1 N sodium hydroxide. To this 20 ml of ethanol was added and was kept at 4° C for 16 hours. The precipitate was filtered off and filtrate was rotary evaporated to reduce the volume to 1 ml.

Thin Layer Chromatography

Thirty grams of silica gel G and 60 ml of water were stirred and five TLC plates were coated with the slurry to a thickness of 0.25 mm. The plates were allowed to dry in room temperature; just prior to use, the plates were heated at 70° C for 1 hours. The test chemicals were spotted about 1 inch above from the bottom of the plates and lower edge of the plates were dipped into methanol and ammonia solvent (70:30, v/v) in a closed chamber such that the spot remained above the solvent level. After 60 minutes, the plates were dried and the position of the test chemicals were developed by Dragendorff's reagent

according to Lerner and Gort (1977). This preserving reagent was prepared by dissolving 0.8 gram of bismuth subnitrate in 2 ml concentrated hydrochloric acid and 10 ml water solution no. 1 gram of potassium iodide was dissolved in 10 ml of water solution 10. These two solutions were mixed and diluted with hydrochloric acid (7 ml. of concentrated hydrochloric acid and 10 ml of water) and final volume was made upto 444 ml with distilled water.

High Performance Liquid Chromatography(HPLC)

Detection and quantification of alkaloids were carried out in a Shimadzu HPLC (Shimadzu Corporation, Japan) model LC-6A with ternary gradient system. The sample was injected to a Zorbax ODS (4.6 mm x 25 cm) reverse phase analytical column (Dupont, USA). The column was equilibrated with 1% acetic acid. A linear gradient of 25% acetonitrile in 1% acetic acid at a flow rate of 0.5 ml/minute for 16 minutes at 37° C was used for elution.

10 µL of test samples, prepared as described in "Test sample preparation for TLC/HPLC" (page 34), were analyzed by HPLC. The absorbance was read by Shimadzu SPD-6A UV spectrophotometer detector at 254 nm and recorded with a Shimadzu CR-6A integrator.

Primary Mouse Kidney Cell Culture

Three week old Swiss albino mice (male and female) were sacrificed for kidney cell culture. The kidneys were aseptically removed, minced and resuspended in saline solution. After thorough washing the tissue was trypsinized at 37° C in 0.25% trypsin solution in Ca++ and Mg++ free phosphate buffered saline. Sterile Eagle's Minimum Essential Medium buffered with sodium bicarbonate (2.2%), containing 100 units/ml antibiotics (viz., Streptomycin, Penicillin and Fungizone) and 10% fetal calf serum was used as growth medium. 2.0 x 10^5 cells were seeded into each cultured tube and incubated at 37° (± 0.5°) C in a CO2 incubator.

Hep 2 Cell Culture

The Hep 2 cell line, came as a gift from Dr. M.L. Prasad, Head, Department of Respiratory Virology, Walter Reed Institute, University of Delhi at passage 76. The cells were maintained in minimum essential media (MEM) supplemented with 5% of fetal calf serum and penicillin (100 units/ml) and streptomycin sulphate (100 µg/ml). The cells were subcultured at 1:4 splits every 5th day. The cells were maintained at 37° (± 0.5° C under 5% CO2 and 90% humidity. The plating efficiency was approximately 60%. Stocks of cells were routinely frozen and stored in liquid nitrogen.

Enumeration of Cells in Culture

Enumeration of viable cells in the culture tubes was done by dye exclusion technique (Phillips, 1973) and 0.4% trypan blue was added to 20 ml water containing 0.8% sodium chloride, 0.02% potassium hydrogen phosphate and 0.2% methyl p-hydroxybenzoate. The solution was thoroughly mixed and heated to boiling and cooled. The pH of this solution was adjusted to 7.2 with 1 N sodium hydroxide. The cell suspension was prepared in balanced salt solution (BSS). 0.9 ml of this cell suspension was put in a test tube, to which 0.1 ml of trypan blue stain was added. After 5 minutes of incubation at 37° C the number of blue stained nonviable cells were scored from hemocytometer.

Total Cell Number Count

0.2% crystal violet (Gentian violet) was dissolved in 0.1 M citric acid, mixed thoroughly and filtered through Whatman # 1 filter paper. Cell suspension was prepared in balanced salt solution. To 0.1 ml of cell suspension 0.1 ml of crystal violet was added. After 10 minutes of incubation at 37° C, the cell number was scored in a hemocytometer. This gave the total cell population.

Total Protein Estimation

The total protein estimation was carried out according to Bradford (1976) using BSA as standard. Bradford reagent was prepared by dissolving 100 mg of Brilliant Blue G (Sigma) in 50 ml of 95% ethanol. To this 100 ml of 85% (w/v) orthophosphoric acid was added. The working solution was prepared from 1 mL of above stock solution diluted to 100 ml with distilled water followed by filtration through Whatman # 1 filter paper.

Assay procedure

A known volume of sample was taken and final volume was made to 1 ml with distilled water. To this 5 ml of reagent was added and mixed thoroughly by vortexing the tube. The reaction time was 5 minutes and absorbance was read in Shimadzu UV 160 spectrophotometer (Japan) at 595 nm wave length. Each assay was repeated at least 5 to 6 times.

DNA Unwinding Technique

The essential features of this technique was described by Rydberg (1976) which was used with minor modifications.

(a) Preparation of solutions

i) Potassium phosphate buffer (PBS) (pH 7.4) was 0.2 M mono and dibasic potassium solutions were mixed in the ratio of 1:4 to get desired pH. Serial dilution was prepared from 0.5 M PBS to obtain 0.25 M and 0.125 M and 0.01 M PBS.

ii) Alkaline solution (1 0.5% pH 11.5) was prepared by thoroughly mixing 0.05 M NaOH, 0.01 M NaH_2PO_4 and 0.9 % NaCl solutions.

iii) Neutralizing solution: 0.039 M hydrochloric acid was used.

iv) Sodium lauryl sulphate solution (10%) : 100 µl of this in final volume of 2.5 ml would result in a SDS concentration approximately equal to 0.4%.

(b) Preparation of primary mouse kidney cell culture

The suspension of primary mouse kidney cells were prepared in PBS (pH 7.2). The number of cells were 6×10^6 for each test.

(c) Alkaline treatment to cells

1.5 ml of alkaline solution was taken in a two ml

[illegible] test tubes, 500 µl of the cell suspension to 10^6 cells/ml was drawn in an Eppendorf micropipette and forcefully ejected into the solution from a height of 2.00 cm. Mixing was carried out gently in dark at room temperature. After 30 minutes, 1.00 ml of neutralizing solution was added slowly. The content was centrifuged for 30 seconds at 12 µ amplitude by an ultramicrofuge in an ultracentrifuge tube. Following centrifugation, 100 µl of clear supernatant was added. Control as well as cells experimented with were prepared together in triplicate sets and tubes were kept at 4° C until used in chromatography.

(b) Hydroxyapatite column chromatography

All the steps of various concentrations were allowed to stand at 60° C in an oven. The experiment was carried out at 60° ± 2° C temperature. Hydroxyapatite was prepared in 10 mM PP$_i$. Fines were decanted after 10 minutes. Columns (5 ml) were set vertically and a small amount of 10 mM PP$_i$ was injected into each of them through the outlet to remove air bubbles from the dead-space. A small amount of buffer was left in the column. Now, a thick slurry of hydroxyapatite suspension was poured in. After allowing 10 minutes for packing without flow, the outlet was opened and finally a hydrostatic pressure of 2.0 cm column height was applied. The following steps were carried out in sequence:

[illegible] ml of [illegible] ml TE 8 was eluted through the columns.

The 1.5 ml of 10 mM TE 8 was then passed through each of them.

Two samples (200 µl) which were already at 60°C to be loaded on to the columns and allowed to run in without any pressure.

(d) 1.5 ml of 10 mM TE 8 was passed.

(e) The buffer was changed to 1.5 mM. For this, first the flow was stopped by closing the outlet. Then the remaining 10 mM buffer on top of the bed was fully removed with the help of a syringe. It was refilled with 1.5 ml TE 8 and connected to the 2.5 cm hydrostatic pressure. Then the flow was started. After discarding the void volume, 1.0 ml of 1.5 mM fraction was collected.

(f) Similarly, the buffer was changed to 1.0 mM and 1.0 ml of this fraction was also collected.

(g) Following this, 1.0 ml fraction of 200 mM TE 8 was collected, after which column was again equilibrated with 10 mM TE 8 and second set of samples were loaded. The first eluate was discarded after 1.0 hours.

(h) The 1.5 ml and 2.0 ml fractions contained single (ss) and double (ds) stranded DNA respectively.

(F) Estimation of DNA

The fractions obtained from above chromatography were subjected to quantitative test for DNA according to Burton (1956) with minor modifications. The test was carried out after perchloric acid extraction of DNA at 70° C for 20 minutes.

The diphenylamine reagent containing aqueous acetaldehyde was used to develope color reaction. After incubation at 30° C. The absorbance was read at 600 nm and concentration of DNA was directly calculated by local square method on Shimadzu UV Visible spectrophotometer, UV 160 Diagram.

Assay of Marker Enzyme

The assay of gamma-glutamyl transpeptidase (GGT) activity in mouse kidney homogenate (10% W/V in 0.1 M Tris-HCl, pH 8.0) were carried out according to Meister et al. (1981). The assay time was five minutes. Each assay was repeated 3 to 6 times in a thermostated (37° C) UV-Vis Uvikon (Kontron) spectrophotometer. The enzyme activity is expressed as units/100 milligram protein.

The unit (I.U.) of enzyme activity is defined as the amount of enzyme which transforms one micro mole of substrate per minute under these assay conditions.

Unscheduled DNA synthesis (UDS) Assay

1) Hep 2 cells at confluence (8×10^6) in culture vessels in 5 ml of MEM (without arginine) were exposed to various doses of test chemicals. Methyl-[^{3}H]-thymidine (5 µCi/ml) and test chemicals were added and incubated for 14 hours. The monolayers were washed with HBSS and removed by shaking vigorously followed by centrifugation at 400 * g. The cell pellet was carefully preserved for DNA precipitation.

ii) UDS in Hep 2 cells was followed after treatment with test chemicals for 30 minutes at pH 4.7 in presence of S 9 mixture. The cells were pulsed with 1 µl Thymidine (5 µCi/ml) at termination of incubation, cells were washed with cold 5% trichloroacetic acid (TCA) and DNA was collected on GF/C glass microfibre filters. After washing with 10 ml. of cold TCA and drying, the incorporation of H3 Thymidine was recorded as DPM in a Beckman LS-7000 liquid scintillation counter using toluene based scintillation cocktail. Each data point represents mean of five to seven observations.

DNA Precipitation

The cells were lysed in ice cold water. The lysate was collected by washing thrice in cold water and precipitated on ice in a final concentration of 10% TCA. This solution was centrifuged at 12,000 * g for 40 minutes. The acid soluble fraction was ignored. The precipitate was collected on GF/C filters. The precipitate was washed with 20 ml of 5% TCA and 20 ml of ethanol and ether (1:1) and air dried. The papers were placed in scintillation vial for counting.

Detection of Radiolabelled DNA by Scintillation Counter

Scintillation cocktail containing 0.1 gm PPO[3] and 0.0 gm POPOP in sulphur free toluene was prepared. The radiolabelled DNA was detected by Backman liquid scintillation counter LS 7000 (USA). The radioactivity was recorded as count per minute (CPM) showing incorporation of radiolabelled thymidine and hence synthesis of DNA.

Preparation of S-9 Mixture

Microsomal S-9 mixture was prepared from Swiss albino mice liver according to McCann et al (1975). Briefly, a 10% liver homogenate was prepared in 0.15 M potassium chloride and 50 mM Tris-HCl buffer (pH 7.4) by giving ten strokes of 15 seconds each in a PCU-Ultramatrica (Switzerland) homogenizer. The homogenate was centrifuged at 9,000 * g in a Backman centrifuge at 4 °C for 15 minutes. The resulting supernatant was used as S-9 mixture. The components of the S-9 mixture were 8 mM $MgCl_2$, 5 mM glucose-6 phosphate, 4 mM NADP and 100 mM sodium phosphate. The protein concentration was 24 mg/ml as determined by Bradford (1976) method.

Macromolecular Synthesis Assay

The macromolecular synthesis assays were carried out immediately or at various time intervals after removal of test chemicals. The cells were pulsed with 1 µCi [^{3}H]-Thymidine (1 µCi/ml) for DNA synthesis and 1 µCi [^{3}H]-Leucine (1 µCi/ml) for protein synthesis. The labelling of the cells were terminated by washing with HBSS, scrapped into 5% (0.1 N sodium chloride and 0.01 M sodium citrate) and filtered through 0.20 µm microfibre filters moistened with cold 5% TCA. The filters were washed three with cold 5% TCA (3 mL) and dried. They were then placed in scintillation vial and their radioactivity was counted in a Packman Liquid Scintillation Counter LS-7000 using toluene based cocktail. The results were recorded as counts per minute (CPM). There were at least three determinants for each test.

DNA Synthesis Inhibition Test

The rate of synthesis, measured as percentage of control of incorporation of [5-H3]-IdR into DNA of Hep 2 cells following treatments with test chemicals at pH 4.5 in presence of 5% CO_2 mixture, was carried out according to Painter (1977) with minor modifications. The cells were prelabelled with [^{3}H]-I IdR (0.02 µCi/mL) for 24 hours. The cells were then exposed to test

chemicals for 30 minutes and the cells were pulsed with 1 %-Hi-IdR (5 µCi/ml) in MEM for 10 minutes. Incubation continued upto 180 minutes. The amount of radiolabelled DNA was determined in a Packard Liquid Scintillation Counter (TR-/000). Each data point represents mean of five to seven determinants.

Microsomal Cytochrome P-450 Preparation

A 15% (w/v) mice liver homogenate was prepared in 0.15 M potassium chloride and 10 mM Iris-HCl buffer (pH 7.4) in EYELA homogenizer (Switzerland) homogenizer by giving 10 to 12 strokes of 15 seconds each. The microsomal suspension of mouse liver was prepared by centrifuging at 15,000 x g for 15 minutes in a Beckman refrigerated centrifuge at 4° C and the resulting supernatant was centrifuged at 105,000 x g in an ultracentrifuge (52P-85H Hitachi, Japan) for 60 minutes at 4° C. The pellets obtained were resuspended in 0.15 M potassium chloride and 10 mM Iris-HCl buffer (pH 7.4) so that the final concentration of protein remained at 30 mg/ml. Protein estimation was done according to Bradford, 1976.

Assay of cytochrome P-450

Microsomal suspension (1 mg/ml) in Iris HCl buffer

pH 7.4) containing 10 mM $MgCl_2$ and 1 mM NADPH were treated with a few grains of sodium dithionite. After obtaining the baseline, the contents of the sample cuvette was transferred into a tube and bubbled with CO gas for 60 seconds. Following CO treatment the content was transferred back to sample cuvette and the difference spectrum was recorded from 400 nm to 500 nm as described by Omura and Sato (1964). Similarly, arecoline (50 µg/ml) at 37° C was treated with reduced and oxidized form of microsomal cytochrome P 450 suspension and difference spectrum was recorded. This was repeated 10 to 15 times.

EXPERIMENTAL PROTOCOLS

A. Treatment of cell culture with arecoline and AEBN:

Sterile arecoline (1 mg/mL) and AEBN (1 mg/mL) stock solutions were prepared in Hank's balanced salt solution (HBSS). Cultures were exposed to these two chemicals on the day of seeding. A single dose of arecoline (10 µg/mL) and two doses of AEBN (100 µg/mL and 250 µg/mL) were used in these experiments.

B. Study of marker enzymes:

i) Areca nut and arecoline diet -

Fresh and raw areca nut, obtained locally, were finely crushed and fed to mice along with regular food and normal drinking water. At the same time, arecoline (1 mM or 3 mM) in drinking water were given to separate batches of mice to serve as positive controls. These groups of mice received normal food.

ii) Animals were divided into four different sets (each with 5 male mice) as follows

i) mice fed with regular food and normal drinking water, ii) mice fed with ground raw areca nut with normal diet and normal drinking water, iii) mice on normal diet and water containing 1 mM arecoline and iv) mice on normal diet and water containing 3 mM arecoline.

iii) Sacrifice -

Mice were maintained on areca nut and arecoline substituted diet were killed on the 10th, 30th, 60th, 90th, 120th, 150th and on the 180th day. The kidneys were washed with phosphate buffered saline and weighed.

Gamma glutamyl transpeptidase (GGT) enzyme activity was assayed as described above (page 4).

C. Effect of arecoline and various extracts of betel nut on Hep 2 cells:

i) On Hep 2 cell Survival -

Hep 2 cells at confluence (2×10^6 were exposed to arecoline and various extracts of betel nut of varying concentration for 2 hours. The test chemicals were (a) arecoline, (b) aqueous extract of betel nut, (c) 0.1 N hydrochloric acid extract of betel nut, (d) alcohol extract of betel nut and (e) 1% acetic acid extract of betel nut. The number of cells were scored in a hemocytometer using dye exclusion technique as described previously (page 32).

ii) Unscheduled DNA Synthesis (UDS) assay -

Unscheduled DNA synthesis as a result of exposure of Hep 2 cells to arecoline and various extracts of betel nut were measured. Hep 2 cells of confluence were treated with various doses of test chemicals in 5 ml of MEM (without arginine). [^{3}H]-TdR and test chemicals were added and incubated for 14 hours. On termination the monolayer were washed with HBSS and DNA was precipitated as described previously (page 43). The

radiolabelled thymidine into DNA was determined by liquid scintillation counter as described previously (para 4(c)).

D. Treatment of Hep 2 cells to test chemicals:

(i) Appropriate concentrations of stock solutions of test chemicals were prepared in PBS. The concentration of sodium nitrite was 10, 50 and 100 µg/ml per culture vessel. The concentrations of arecoline in culture vessels were 10, 20 and 30 µg/ml. For monitoring the combined effect of these two test chemicals, three combinations were taken after 50 to 90 pilot experiments. These were - (a) set of cultures treated with 10 µg sodium nitrite and 10 µg of arecoline, (b) set of cultures treated with 10 µg sodium nitrite and 20 µg arecoline and (c) set of cultures treated with 10 µg sodium nitrite and 30 µg of arecoline.

(ii) Hep 2 cells (10^5) were exposed to the test chemicals at pH 4.2 for 30 minutes in presence of 0.2 ml of S-9 mixture. Following exposure to test chemicals, media were removed from culture vessels including that of controls (controls were at pH 7.2 and 4.2 in presence of S-9 mixture without test chemicals) and washed with HBSS.

iii) following exposure of cells to test chemicals, the cell survival was monitored after end of treatment of cells with test chemicals using labelled precursors. The rate of synthesis of DNA in cells following test chemicals treatment were measured. Mutagenicity or carcinogenicity of arecoline and sodium nitrite was also studied using unscheduled DNA synthesis assay protocol.

E. Activation/deactivation of arecoline by Cytochrome P-450 enzyme:

In view of the metabolic activation or deactivation and chemical structure of arecoline it seems likely that cytochrome P 450 dependent nitrosation might be necessary. The effect of incubation of cytochrome P 450 preparation with arecoline was investigated as described above (page 42). Concentration of cytochrome P 450 was 4.6 pM/ml protein, the temperature of the cuvette was maintained at 37° C and path length of cuvette was 10 mm.

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A. GENERAL OBSERVATIONS

All animals which were treated with arecaidine or arecoline did not show any visually distinguishable sign of abnormality throughout the period of experimentation. Food and water consumption remained normal. All sacrifice of animals were carried out at the same time (sacrifice time). Increase in body weight of all treated animals as well as control animals seemed related to age and growth.

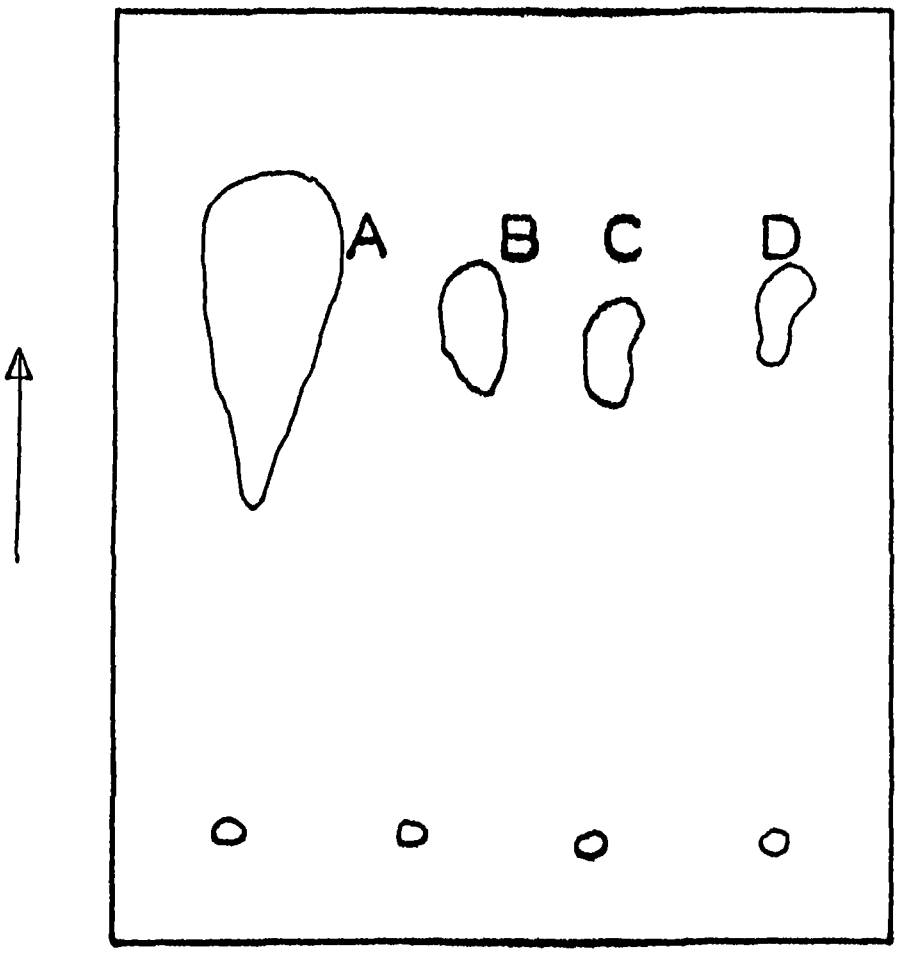
Arecaidine was found to be highly toxic when administered to mice in drinking water at 0.1 mg/ml concentration whereas arecoline in drinking water did not cause death to mice beginning 6 hours to 28 hours depending upon the concentration of arecaidine consumed. Normal mice consumed 5.5 mL of water per day. Mice exposed to arecaidine showed vigorous muscular movement which eventually ended with death of mice.

B. ANALYSIS OF BETEL NUT CONTENTS

Table I : analysis of betel nut contents.

Constituents	Quantity (mean±SD-M)	n
1. Moisture content	12.67 ± 0.11 %	8
2. Protein (water extractible)	15.9% ± 1.44 %	10
3. Fat	2.31 ± 0.1? %	11
4. Crude fibre	18.94 ± 0.69 %	5
5. Ash	20.62 ± 0.1? %	5
6. Carbohydrates (water extractible)	14.45 ± 1.12? %	7
7. Alkaloids	0.38 ± 0.12 %	
Arecoline	0.21 ± 0.09 %	15
Arecaidine	0.15 ± 0.11 %	15

Figure 1 shows the profile of thin layer chromatography of AEBN and standard alkaloids (A. arecoline and B. arecaidine). Arecoline and arecaidine concentration was 10 µg/ml whereas AEBN was 200 µg/mL. The spots were developed by spraying Dragendorff's reagent, which reacts with pyridinium ring. On reaction with pyridinium ring, gives red



Thin layer chromatography of AEBN

Figure 1. Thin layer chromatography of aqueous extract of betel nut (AEBN) for detection of presence of alkaloids. A. arecoline, B. arecaidine and C & D AEBN following use of Dragendorff's reagent (see Materials and Methods).

color on TLC plates. The alkaloid which was spotted by this reagent was scrapped and was dissolved in water and silica gel was removed by filtration. However, recovery was too small to analyze it by high performance liquid chromatography (HPLC).

Figure 2 depicts the high performance liquid chromatography profile of alkaloid analysis of nBIN. Figure 2(A) and (B) show standard arecoline and arecaidine elution profile. Arecoline retention time of 4 minutes was followed by a broad peak. Arecaidine was eluted at 7 minutes preceded by a minor peak at 6 minutes. nBIN gave relatively higher peaks. There were two major peaks and a minor peak in between the two peaks, Figure 2(C) and (D). The first peak of Figure 2(C) and (D) corresponds to the peak of arecoline (A) elution profile whereas the second major peak corresponds to the elution profile of arecaidine (B). This was repeated 20 times which did not show any major variation in the elution profile in both standard and alkaloids and nBIN. By this analysis, amounts of arecoline and arecaidine were calculated to be 0.71 ± 0.09% and 0.15 ± 0.11% (mean ± SEM) respectively (minimum confidence level 95%).

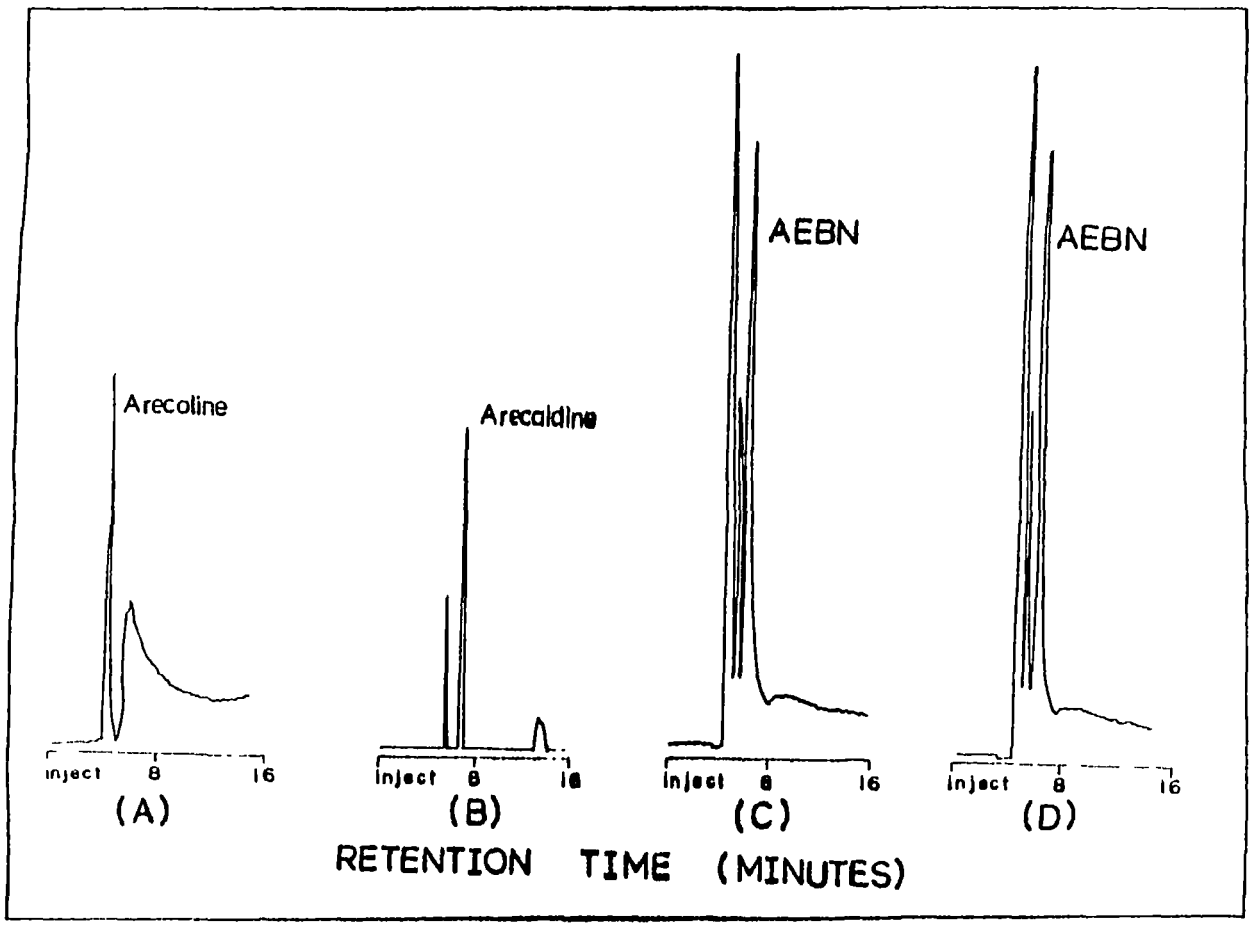


Figure 2. High performance liquid chromatography profile of A. arecoline, B. arecaidine, C & D AEBN. 10 µl of test sample were injected (see Materials and Methods for details) to a Zorbax ODS_{18} reverse phase analytical column. The column was equilibrated with 1% acetic acid. A linear gradient of 25% acetonitrile in 1% acetic acid at a flow rate of 0.5 ml/minute for 16 minutes at 37°C was used for elution.

C. SHORT TERM IN VITRO TESTS

(i) Cell survival and Rate of cell proliferation

Figure 3 depicts the viable cell growth with respect to time for control (untreated), aflatoxin treated and fdUN-treated cultures. The control tubes showed approximately 100% increase in population 18 to 19 hours with confluent monolayer formation on the 3rd day after seeding. The aflatoxin (10 μg/ml) treated cells showed a delayed cell duplication time and monolayer formation. While 200 μg/ml of fd-UN was found to be drastically toxic to the cells, the culture tubes with 100 μg/ml fdUN exhibited attainment of confluence a day earlier than control. However, it showed an approximately 10% lower viable cell count. Owing to acute toxicity of 1.50 μg/ml AFUN, this dose has not been used in the following experimental protocol.

(ii) DNA strand break analysis

Figure 4 shows the results of DNA strand break analysis. Damage to DNA has been expressed as the ratio of ssDNA/dsDNA recovered. Aflatoxin-treated cells show a very

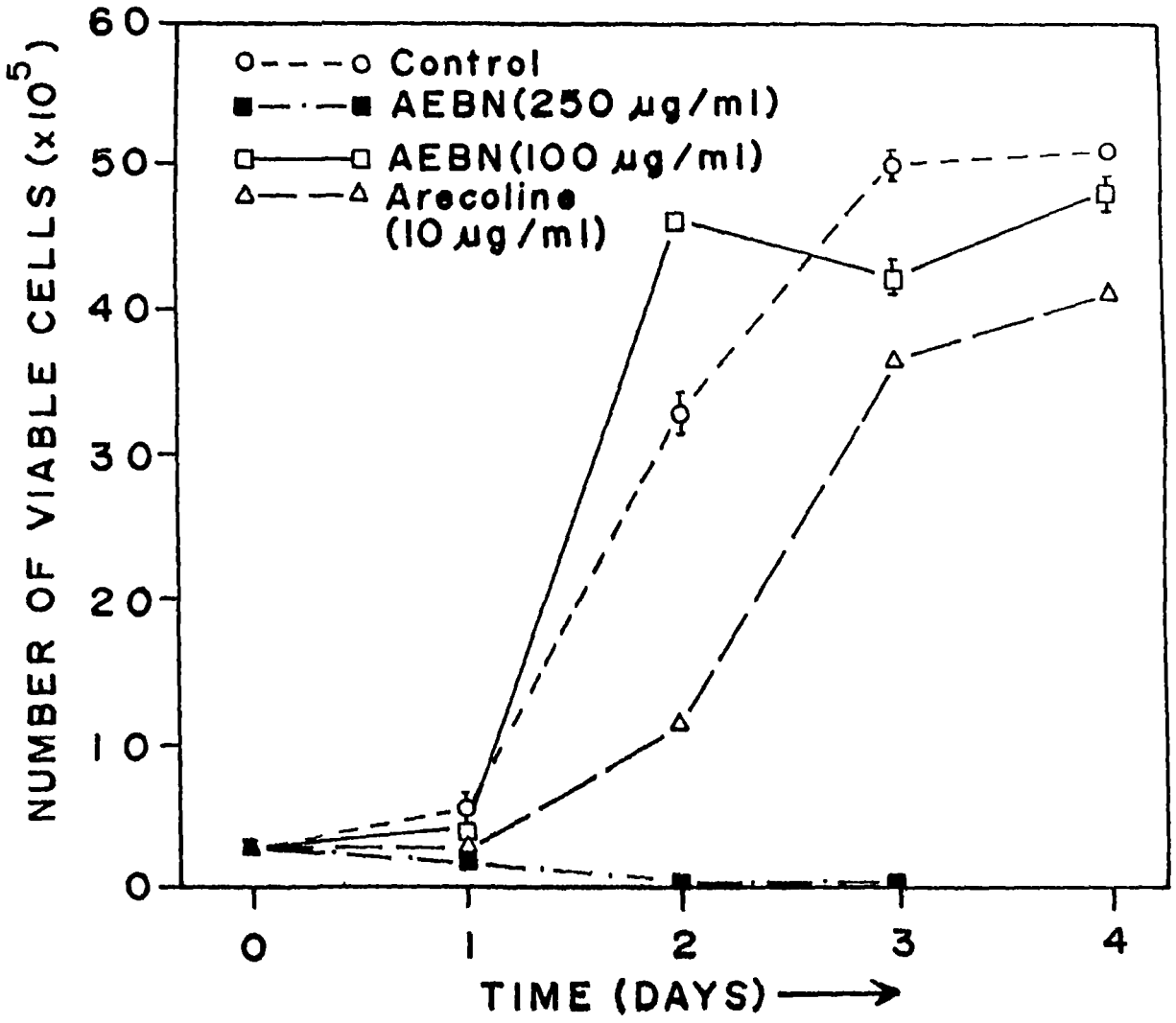


Figure 3. Count of viable cells as a function of time for control, arecoline and AEBN treated mouse kidney cells in culture (also see Materials and Methods). Each data point is mean ± S.D. (shown by bars) of 5-7 independent experiments. No bar means S.D. is smaller than the thickness of the point.

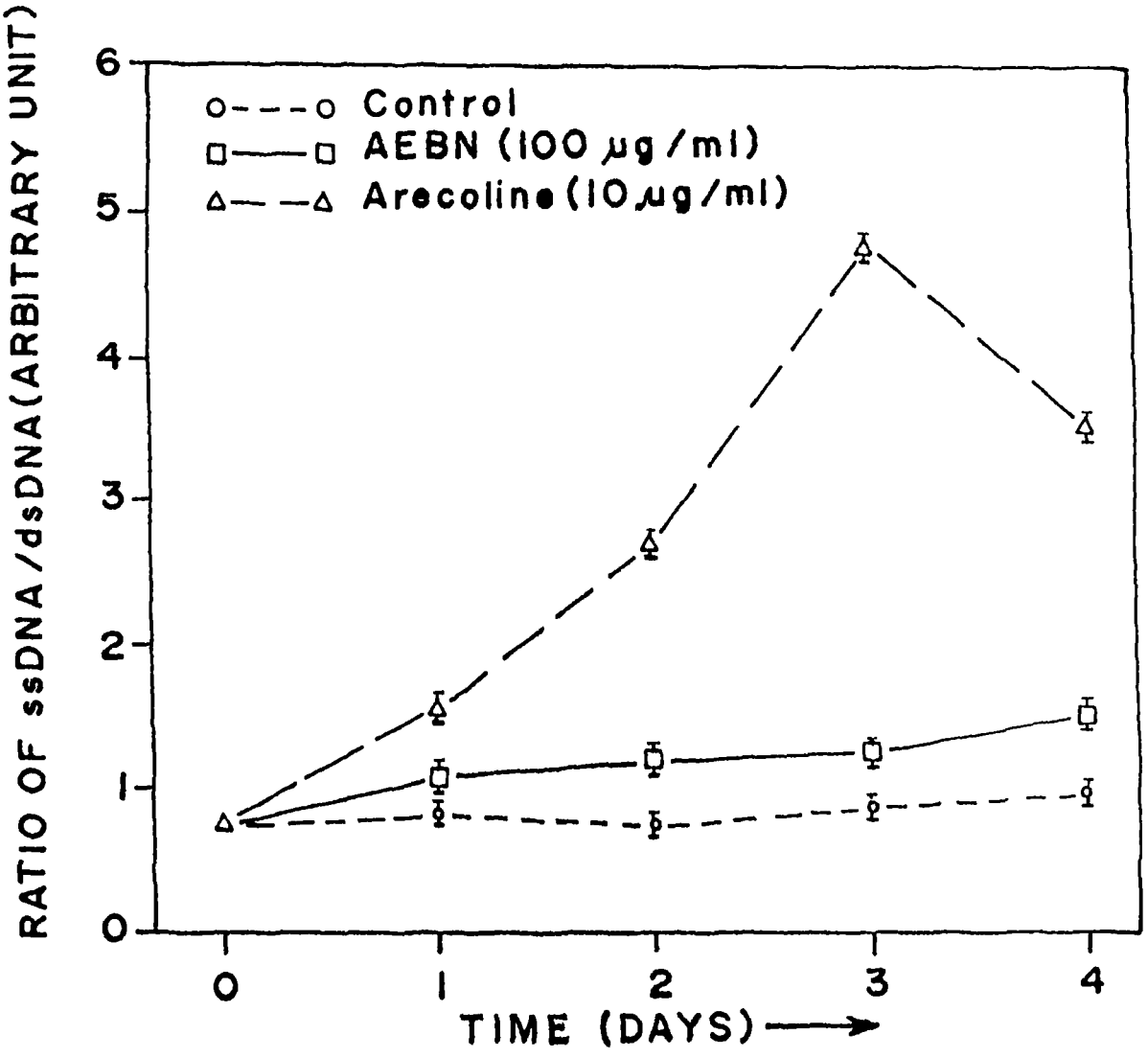


Figure 4. Damage to DNA (expressed as the ratio of ssDNA/dsDNA) as a function of time for control, arecoline and AEBN treated mouse kidney cells in culture (also see Materials and Methods and Srivastava, Sharan & Pozzi 1982). Each point is mean S.D. (S.D. is shown by bars) of at least 4 determinants.

significant increase in the ssDNA/dsDNA ratio which means DNA damages with time. The damage reduces slightly after 3rd day. The nA-IBN treated (100 µg/ml) cells show significant damage to DNA compared to controls, but this is not as drastic as that caused by urea alone. These data have been tabulated from the total amounts of ssDNA and dsDNA recovered from hydroxyapatite columns from different groups of cells. All experiments were carried out at least 4 times independently (minimum confidence level 95%).

D. LONG TERM IN VIVO TEST

(1) Gamma-glutamyl transpeptidase (GGT) activity

Figure 5 shows the changes in GGT activity over the entire span of study for various groups of animals. GGT activity substantially increased upto the 60th day as compared to the control regardless of the mode of treatment. This was followed by a gradual decline in activity in all groups till the 150th day. However, this decline was again succeeded by another peak in the GGT activity profile for all sets, whereby it reached a relatively higher value by 150th day followed by slower decline until the end of the experiment. GGT activity in the control set showed gradual increase with time. The inset of figure 5 shows

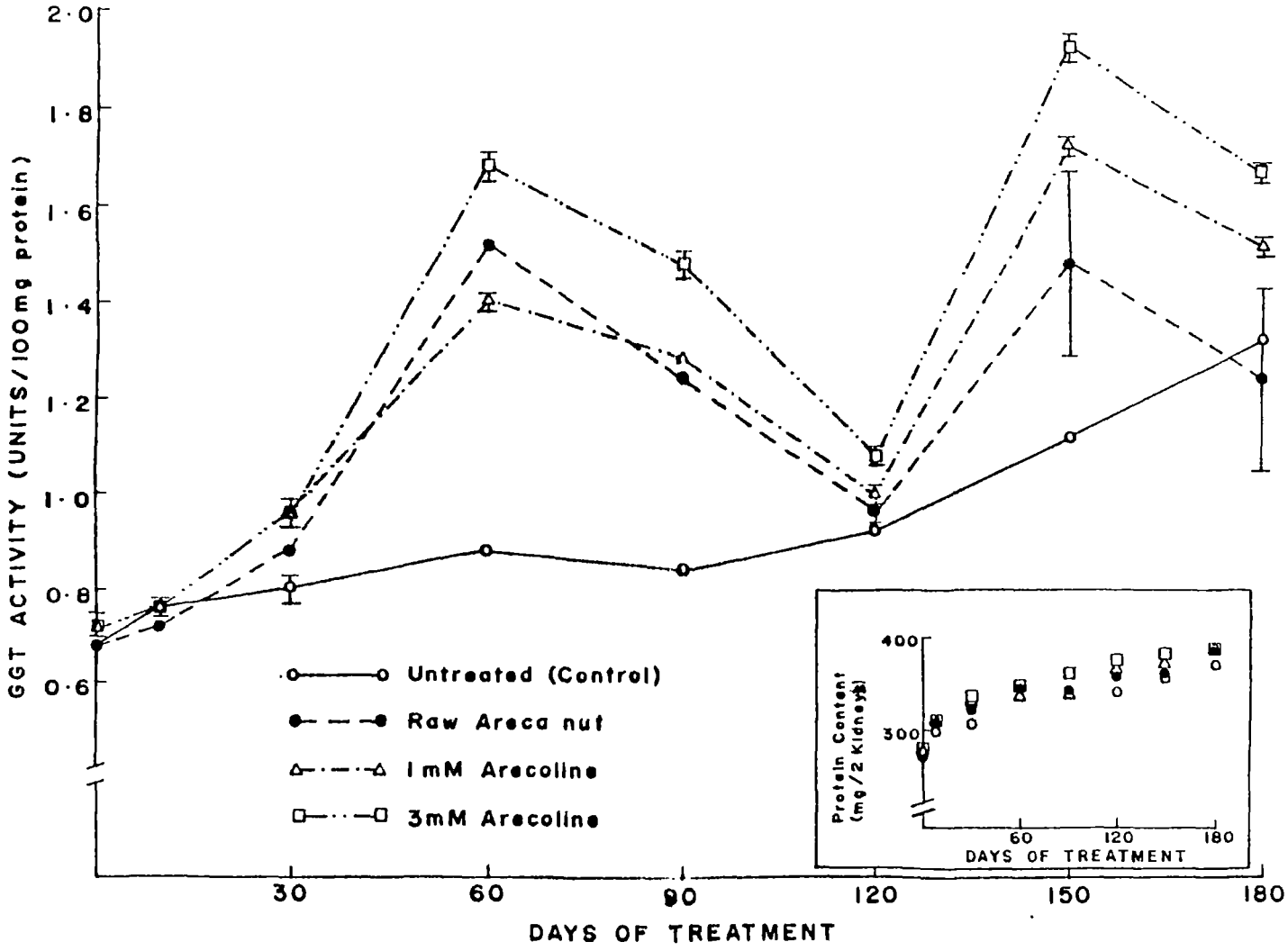


Figure 5. Gamma-glutamyl transpeptidase enzyme activity (units/100 mg protein) following areca nut or arecoline treatments over a period of 180 days (mean ± SEM). The bars indicate SEM; when there is no bar it means the deviation was smaller than the thickness of the line. The inset shows the average total protein in kidneys over the same protocol.

the total protein content in mice liver from these sets of experiments. Unlike the recurring periodicity in GGT activity, the total protein content was comparatively stable. There was no readily distinguishable morphological sign or symptom which could be attributed to malignant transformation in control and treated sets of experimental animals. Scrupulous dissection of all organs did not show anomaly of any organs. There was no body weight loss. However, slow gain in the body weight was indicative of age and growth in general.

E. IN VITRO TESTS IN HUMAN CELL LINE

(i) Cell survival

Figure 6 shows the viable cell culture growth with respect to concentration of various test chemicals. The cells at $0 * 10^6$ population (this population was obtained on the 3rd day after seeding) at confluence were treated to aceclidine, rdBB, alcohol extract, 0.1 N hydrochloric acid extract and 1 acetic acid extract of betel nut. Aceclidine showed toxicity to the Hep 2 cells from 50 µg onwards (approximately 50% cell death compared to control cells). 50 µg and 100 µg concentrations of aceclidine treated cell cultures resulted in approximately 50% and 60% death

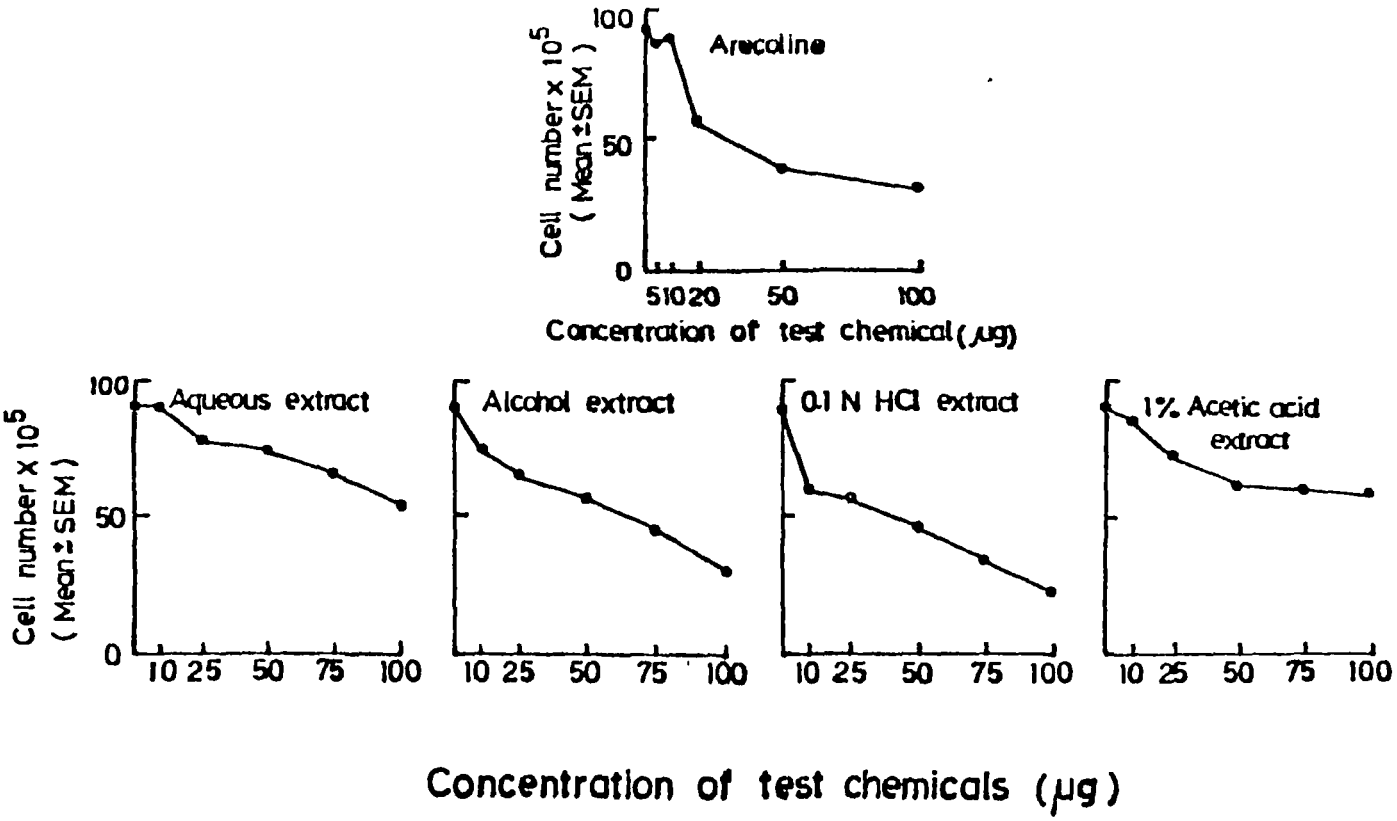


Figure 6. Count of viable cells as a function of concentration for arecoline, AEBN, alcohol extract of betel nut, 0.1 N HCl extract of betel nut and 1% acetic acid extract of betel nut treated Hep 2 cell culture (see also Materials and Methods). Each data point is mean ± S.E.M. of 7 independent experiments (S.E.M. is smaller than the thickness of the point).

of cells respectively. The toxicity of the cells was seemingly arecoline dose dependant. The 6AIBN treated cells did not show significant cell death as compared to arecoline. However, 100 µg 6AIBN showed approximately 40% reduction in cell population. Alcohol extract of betel nut showed dose dependent cell death of which 100 µg showed approximately 60% reduction of viable cell number.

(iii) Unscheduled DNA synthesis (UDS) assay

Figure 7 shows the unscheduled DNA synthesis (UDS) in Hep 2 cells following treatments with arecoline and various extracts of betel nut in vitro on the 3rd day after seeding at $8 * 10^6$ cell population. Exposure of cells to arecoline showed dose dependant ^{3}H-TdR incorporation. Aqueous extract of betel nut treated cells showed UDS which was lower than arecoline treated group. Alcohol extract of betel nut induced UDS which was also dose dependant, however, ^{3}H-TdR incorporation was significantly lower than 6AIBN treated or arecoline treated sets. 0.1 N HCl extract of betel nut induced UDS, which was significantly lower than other treated sets. Upon exposure of Hep 2 cells to 1% acetic acid extract of betel nut, however, induced higher and dose dependant UDS.

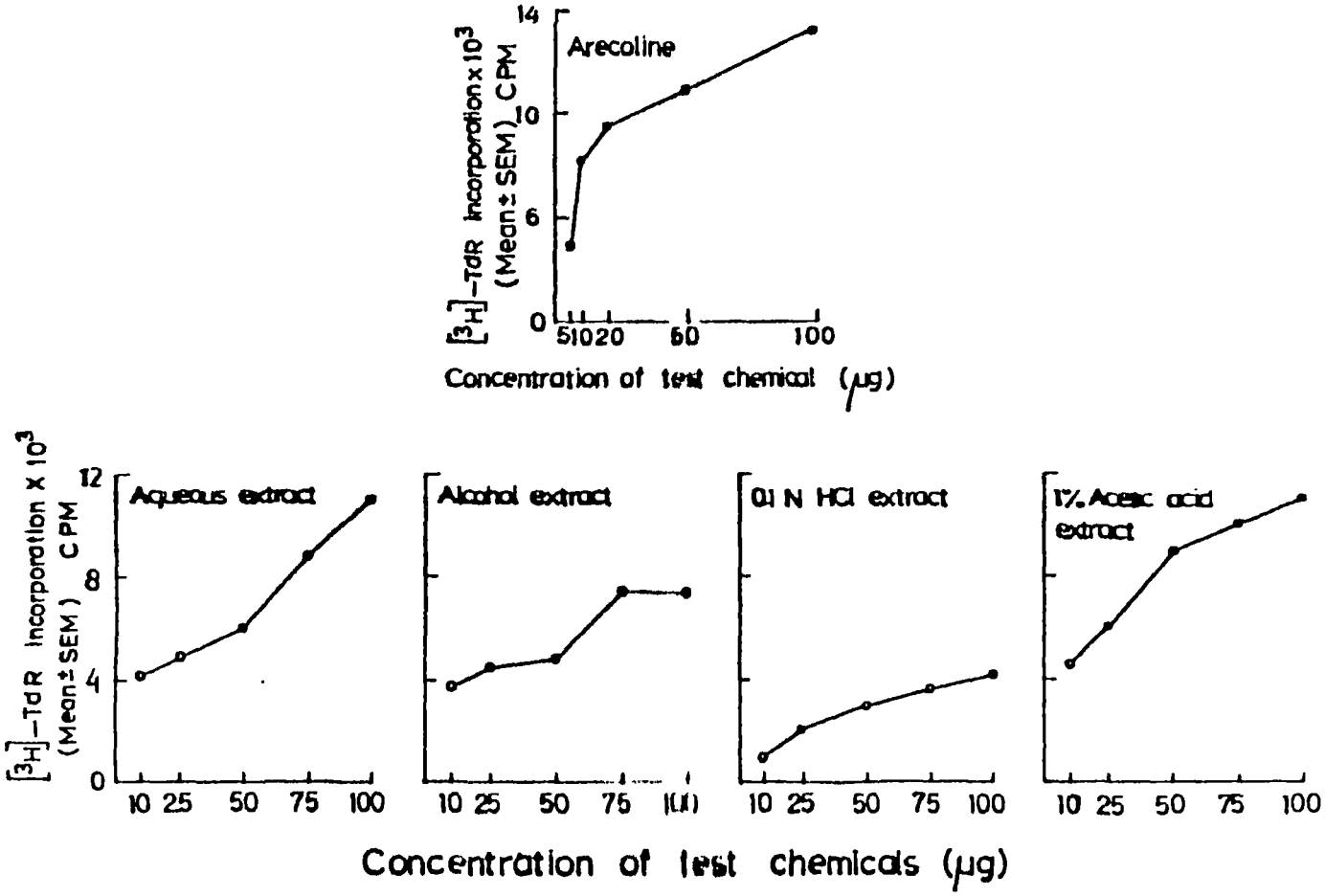


Figure 7. Unscheduled DNA synthesis following exposure of Hep 2 cell culture as a function of various concentration of arecoline, AEBN, 0.1 H HCl extract of betel nut and 1% acetic acid extract of betel nut. Each point represents mean ± S.E.M. obtained from at least 4 independent experiments (S.E.M. is smaller than the thickness of point).

F. EFFECTS OF ARECOLINE VIS-A-VIS SODIUM NITRITE

Sodium nitrite exerted its effect in acid environments when nitrous acid was formed or, nitrosation of arecoline took place resulting in the formation of host of nitrosocompounds of arecoline (when sodium nitrite and arecoline were given together) in presence of S-9 mixture. All experiments were carried out only after appropriate conditions were found to be suitable. S-9 mixture was used to enhance the process of nitrosation or denitrosation and pH 4.2 was found to be appropriate, which is near gastric pH to mimic the natural pH of human digestive system.

(i) Cell survival

Figure 8 shows the number of viable cells enumerated at intervals following 30 minutes of treatments with (10, 20 and 30 µg) or without arecoline at pH 4.2 in presence S-9 mixture. The number of cells reduced drastically when 30 µg arecoline dose was given. Similarly, in figure 9, sodium nitrite (10, 50 and 100 µg) showed reduced cell survival with extreme toxicity to the cells at 50 µg and 100 µg concentrations. In the subsequent experiments, therefore, 10 µg sodium nitrite was chosen. Since, our main emphasis was to enhance the formation of active metabolite from arecoline and monitor its effects on cells, the concentration of sodium nitrite was kept at 10 µg

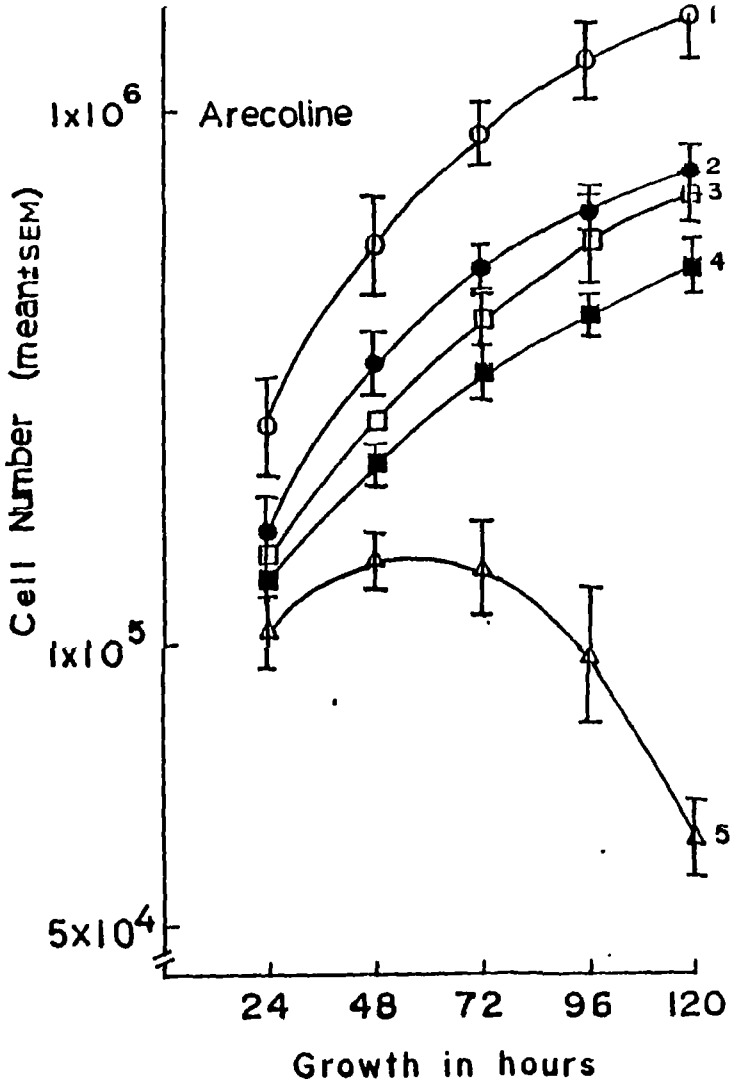


Figure 8. The number of cells were scored at intervals, after 30 minutes of treatment with or without arecoline at pH 4.2 in presence of S-9 mixture. 1. pH 7.2 (o——o); 2. pH 4.2 (●——●); 3. 10 µg arecoline (□——□); 4. 20 µg arecoline (■——■) and 5. 80 µg arecoline (Δ——Δ). Each data point represents mean ± S.E.M. (S.E.M. is shown by bars). No bar means S.E.M. is smaller than the thickness of point. Each data point was obtained from at least 4 independent experiments.

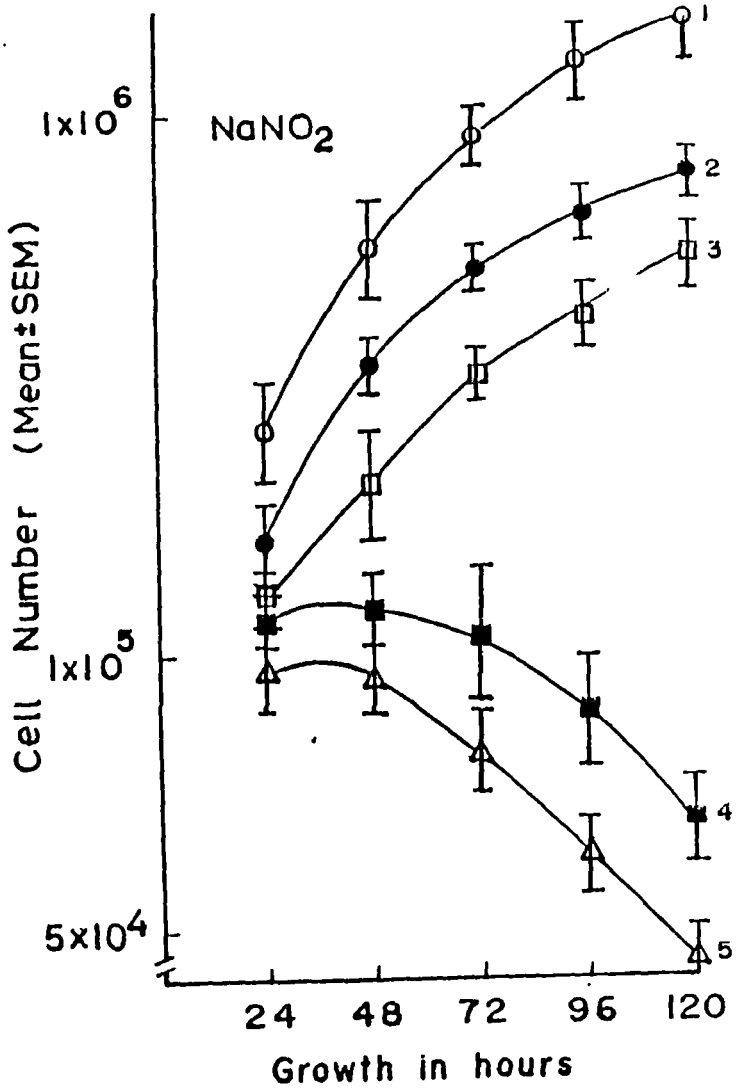


Figure 9. The number of cells were scored at intervals, after 30 minutes of treatment with or without sodium nitrite at pH 4.2 in presence of S-9 mixture. 1. pH 7.2 (o——o); 2. pH 4.2 (●——●); 3. 10 µg sodium nitrite (□——□); 4. 50 µg sodium nitrite (■——■) and 5. 100 µg sodium nitrite (Δ——Δ). Each data point represents mean ± S.E.M. (S.E.M. is shown by bars) obtained from at least 4 independent experiments. No bar means S.E.M. is smaller than the thickness of point.

and the concentration of arecoline varied i.e., 10 μg, 20 μg and 40 μg. In all cases, 30 minutes exposure to test chemicals was maintained. Figure 10 shows the effect of the combination of of sodium nitrite (10 μg) and varied concentration of arecoline (see figure legend) exerting extreme cytotoxicity. All enumeration of cells were carried out approximately at the same time. Each data point depicted in the figure 8, 9 and 10 were obtained from at least 4 independent experiments (minimum confidence level 90%).

(ii) Rate of DNA synthesis

Figure 11 shows the DNA synthetic activity as a function of time following exposure of cells to arecoline. Figure 12 shows the rate of DNA synthesis upon exposure of cells to sodium nitrite. It was found that the strong cytotoxic effect of sodium nitrite was accompanied by anomaly in the macromolecular synthesis; from figure 12 it is apparent that in Hep 2 cells, after 30 minutes of treatment, the total DNA synthesis was inhibited significantly due to amount of sodium nitrite administered in vitro. Arecoline showed less toxicity in this respect (see figure 11).

Figure 13 depicts the incorporation of 114-C-14-IdR into Hep 2 cells on treatment with sodium nitrite (10 μg) and

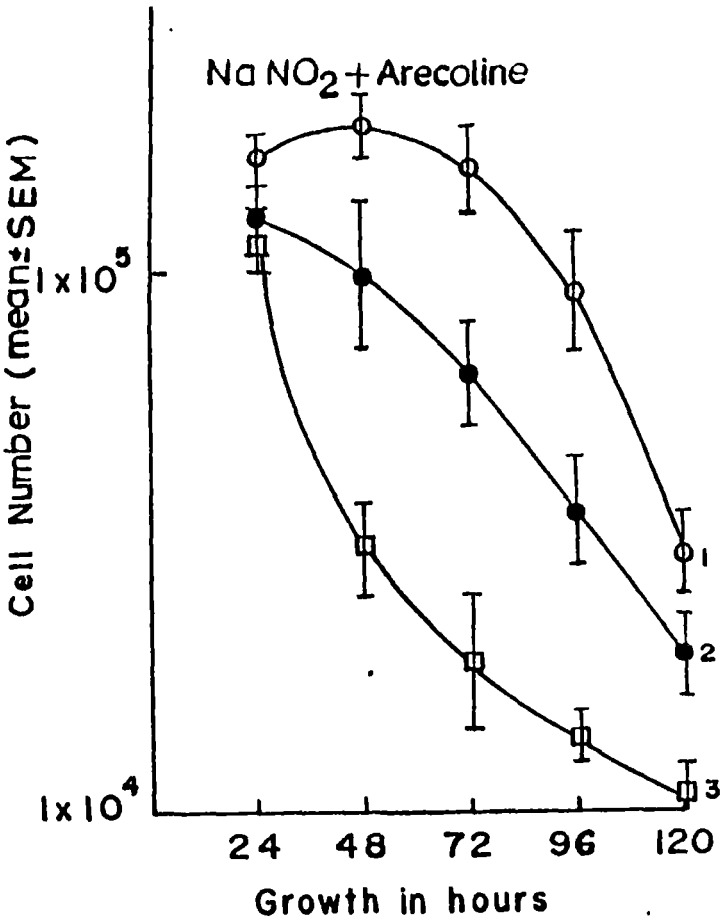


Figure 10. The number of cells were scored at intervals, after 30 minutes treatment with or without sodium nitrite and arecoline in combination at pH 4.2 in presence of S-9 mixture. 1. 10 µg sodium nitrite and 10 µg arecoline (o—o); 2. 10 µg sodium nitrite and 20 µg arecoline (●—●) and 3. 10 µg sodium nitrite and 80 µg arecoline (□—□). Each data point represents mean ± S.E.M. (S.E.M. is shown by bars) obtained from at least 4 independent experiments.

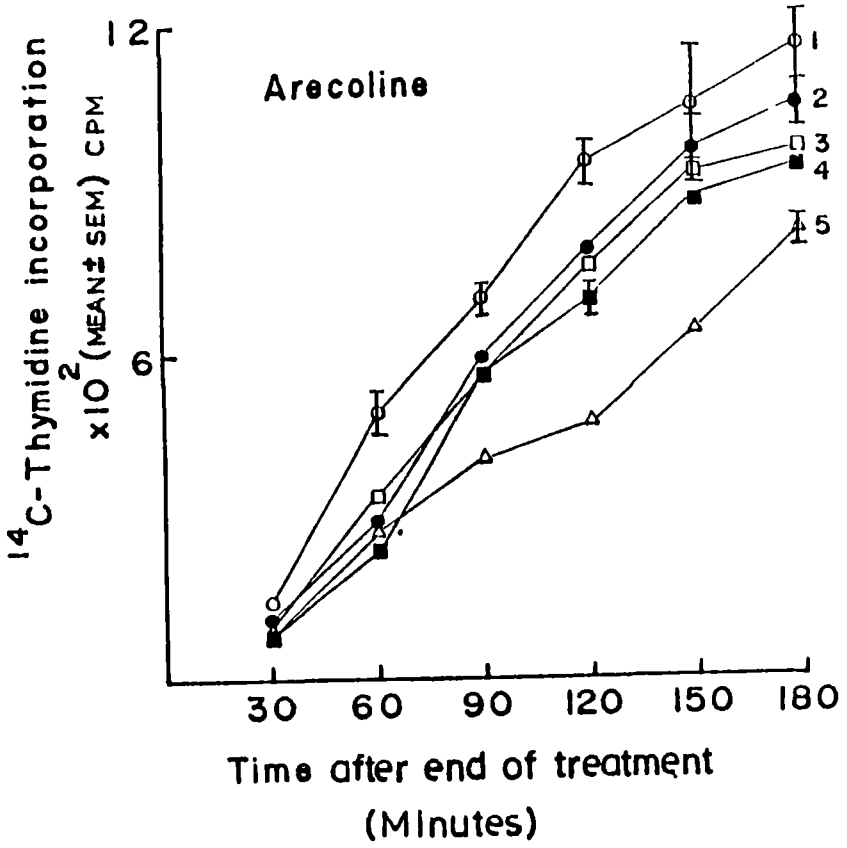


Figure 11. ^{14}C-Thymidine (1 µCi/ml) incorporation to the cells were studied at intervals following 30 minutes treatment with or without arecoline at pH 4.2 in presence of S-9 mixture. 1. pH 7.2 (o—o); 2. pH 4.2 (●—●); 3. 10 µg arecoline (□—□); 4. 20 µg arecoline (■—■) and 5. 80 µg arecoline (△—△). Each data point represents mean ± S.E.M. (S.E.M. is shown by bars) obtained from at least 5 independent experiments (no bar means S.E.M. is smaller than the thickness of point).

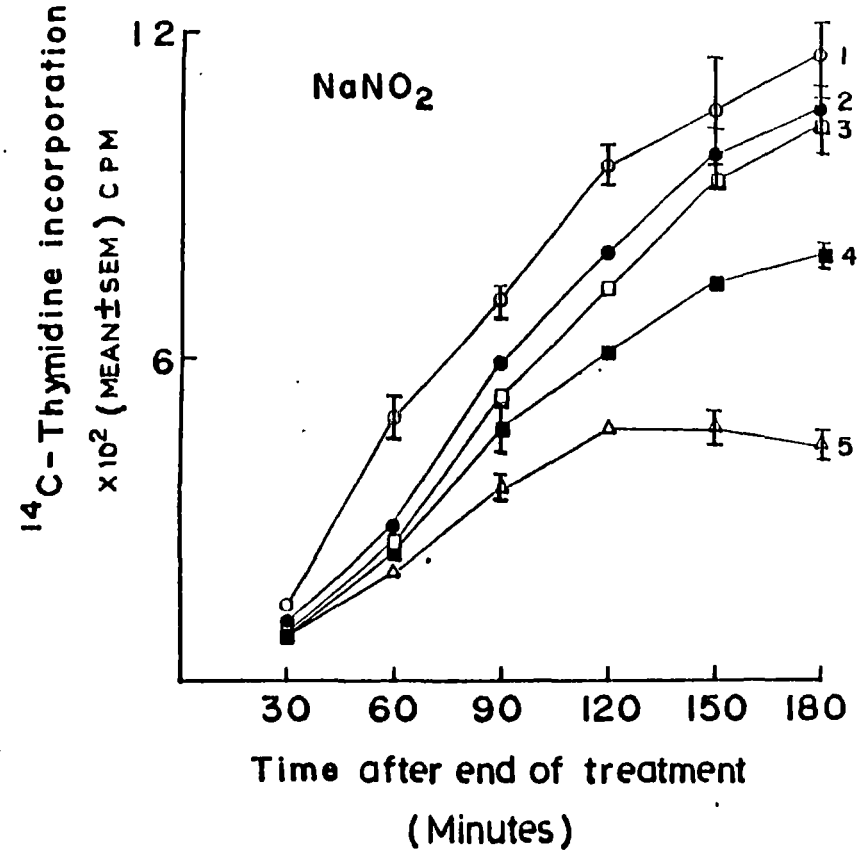


Figure 12. ^{14}C-Thymidine (1 μCi/ml) incorporation to the cells studied at intervals following 30 minutes treatment with or without sodium nitrite at pH 4.2 in presence of S-9 mixture. 1. pH 7.2 (o——o); 2. pH 4.2 (●——●); 3. 10 μg sodium nitrite (□——□); 4. 50 μg sodium nitrite (■——■) and 5. 100 μg sodium nitrite (△——△). Each data point represents mean ± S.E.M. (S.E.M. is shown by bars) obtained from at least 4 independent experiments (no bar means S.E.M. is smaller than the thickness of point).

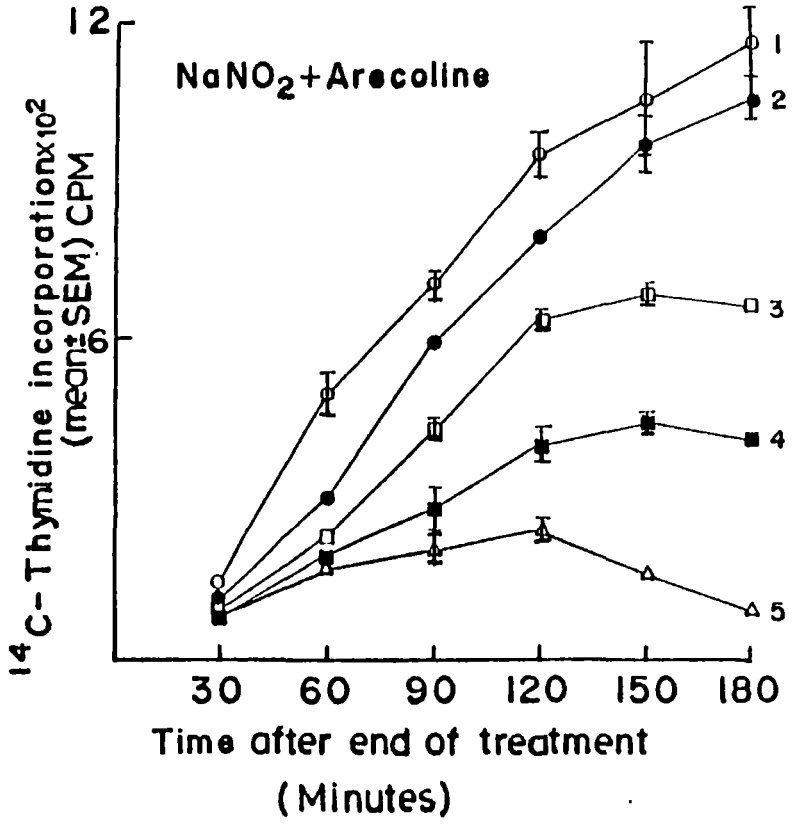


Figure 13. ^{14}C-Thymidine (1 µCi/ml) incorporation to the cells were studied at intervals following 30 minutes treatment with or without sodium nitrite and arecoline at pH 4.2 in presence of S-9 mixture. 1. pH 7.2 (o—o); 2. pH 4.2 (●—●); 3. 10 µg sodium nitrite and 10 µg arecoline (□ □); 4. 10 µg sodium nitrite and 20 µg arecoline (■—■) and 5. 10 µg sodium nitrite and 80 µg arecoline (△—△). Each data point represents mean ± S.E.M. (S.E.M. is shown by bars) obtained from at least 4 independent experiments (no bar means S.E.M. is smaller than the thickness of point).

varied concentration of arecoline. The effect was found to be additive in nature regardless of any concentration of arecoline used. There was positive delay in DNA synthetic activity in this set of experiment.

(iii) Rate of protein synthesis

Protein synthetic activity is depicted in figure 14 for Hep 2 cells. Sodium nitrite inhibited protein synthesis significantly compared to arecoline treated sets irrespective of dose of sodium nitrite used (see figure legend). The protein synthesis, which mainly occurs in the G-1 phase, are generally stimulated by DNA replication. However, there was positive delay in protein synthesis when sodium nitrite and arecoline were given together.

(iv) Unscheduled DNA synthesis (UDS)

Unscheduled DNA synthesis is depicted in figure 15 which was related to the proportion of cells capable of normal DNA synthesis. UDS occurred in Hep 2 cells following treatment with test chemicals i.e., sodium nitrite and arecoline (in combination). The test concentration at 10 µg sodium nitrite and

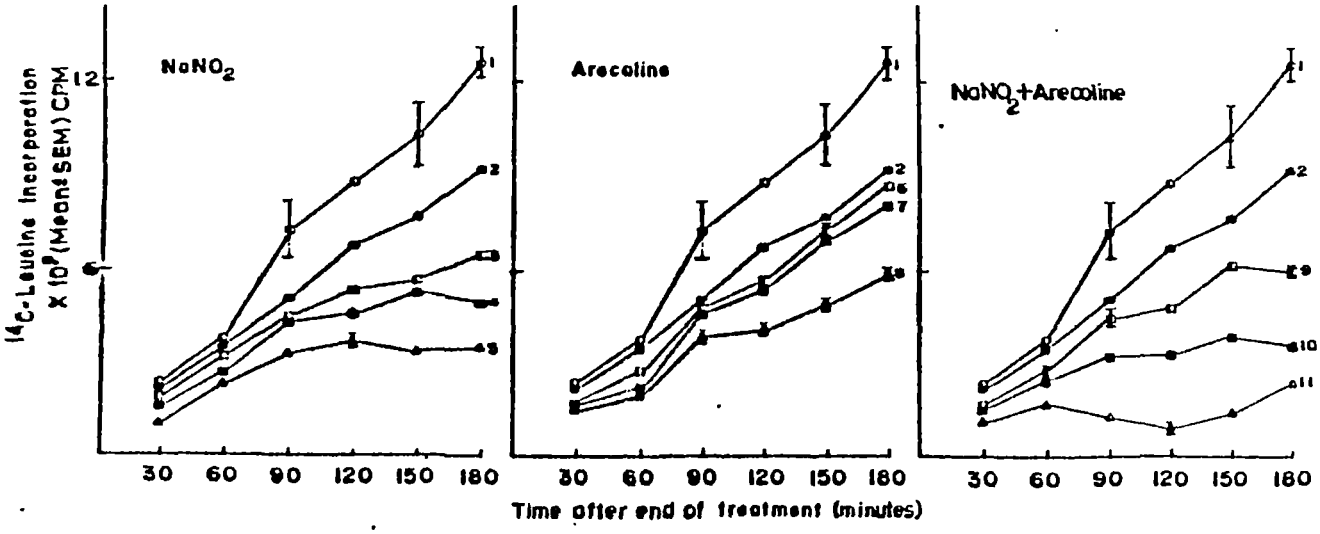


Figure 14. ^{14}C-Leucine (1 µCi/ml) incorporation to the cells were studied at intervals following 30 minutes treatment with or without sodium nitrite, arecoline, sodium nitrite and arecoline (in combination) at pH 4.2 in presence of S-9 mixture. 1. pH 7.2 (o——o); 2. pH 4.2 (●——●); 3. 10 µg sodium nitrite (□——□); 4. 50 µg sodium nitrite (■——■); 5. 100 µg sodium nitrite (▲——▲); 6. 10 µg arecoline (□——□); 7. 20 µg arecoline (□——□); 8. 80 µg arecoline (Δ——Δ); 9. 10 µg sodium nitrite and 10 µg arecoline (□——□); 10. 10 µg sodium nitrite and 20 µg arecoline (■——■); 11. 10 µg sodium nitrite and 80 µg arecoline (Δ——Δ). Each data point represents mean ± S.E.M. (S.E.M. is shown by bars) obtained from at least 4 independent experiments (no bar means S.E.M. is smaller than the thickness of the point).

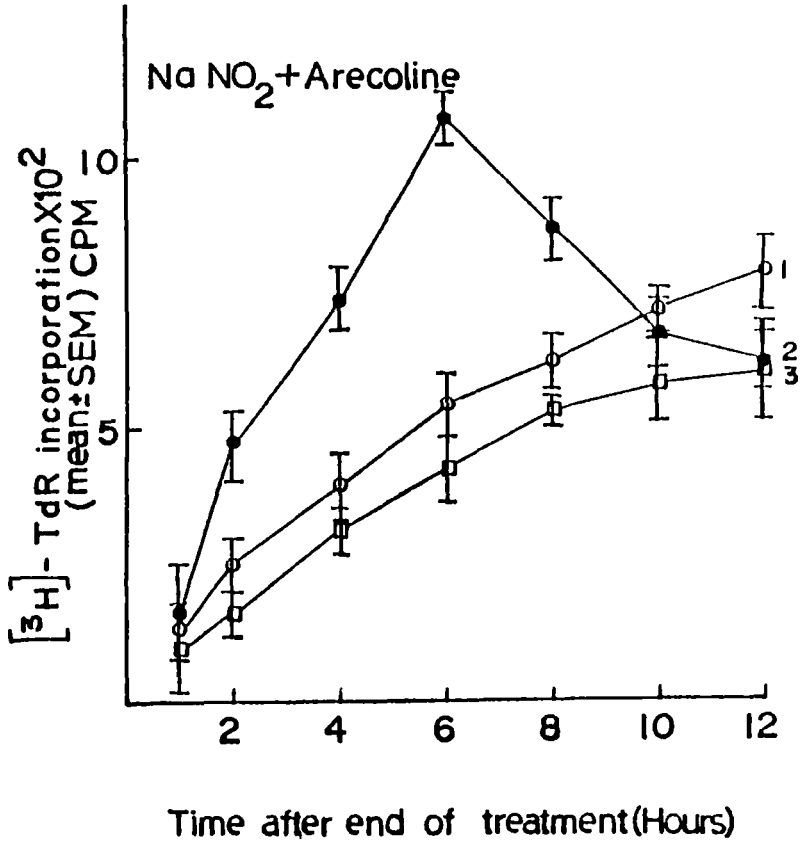


Figure 15. Unscheduled DNA synthesis in cells following treatment with sodium nitrite and arecoline (in combination) for 30 minutes at pH 4.2 in presence of S-9 mixture. 1. 10 µg sodium nitrite and 10 µg arecoline (o——o); 2. 10 µg sodium nitrite and 20 µg arecoline (●——●) and 3. 10 µg sodium nitrite and 80 µg arecoline (□——□). Each data point represents mean ± S.E.M. (S.E.M. is shown by bars) obtained from at least 4 independent experiments.

...per arecoline showed [figure 17(2)] a peak at 6 hours, which was absent in other two sets. The peak at 6 hours was succeeded by decline in the incorporation of ^{3}H-TdR into cells.

(v) DNA synthesis inhibition

The DNA synthesis inhibition test in Hep 2 cells as a result of exposure to various test chemicals (combination of sodium nitrite and arecoline) is depicted in figure 16. There was a rapid increase in the percentage of control of DNA synthesis. When test chemicals and S-9 mixture was removed at 60 minutes and the media was replenished, the DNA synthesis rate started to increase. At the end of 180 minutes the DNA synthesis recovery was 70% to 100%. The decline in the rate of DNA synthesis following exposure to test chemicals was seen in all cases irrespective of concentration of test chemicals. This DNA inhibition was reversible in nature, since on removal of test chemicals and S-9 mixture have resulted in DNA synthesis.

(vi) Cytochrome P-450

Figure 17 shows carbon monoxide binding to cytochrome P-450. From this the amount of cytochrome P-450 was

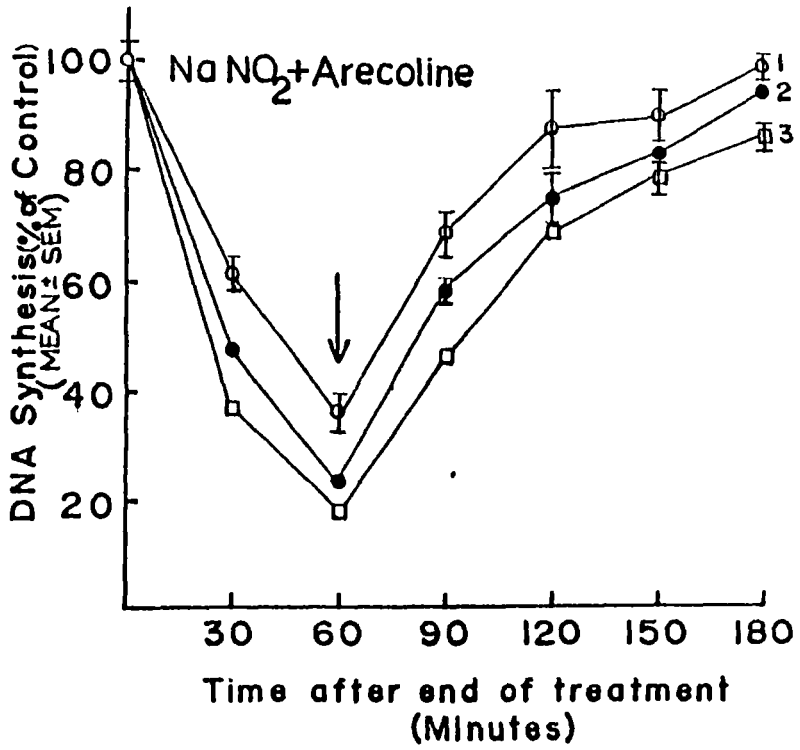


Figure 16. DNA inhibition test expressed as % of control in Hep 2 cells following treatment with sodium nitrite and arecoline (in combination) at pH 4.2 in presence of S-9 mixture. Arrow mark indicates removal of S-9 mixture (see Materials and Methods for details). The cells were pulsed for 10 minutes prior to addition of S-9 mixture and test chemicals and incubation continued upto 180 minutes. 1. 10 µg sodium nitrite and 10 µg arecoline (o—o); 2. 10 µg sodium nitrite and 20 µg arecoline (●——●); 3. 10 µg sodium nitrite and 80 µg arecoline (□—□). Each data point represents mean ± S.E.M. (S.E.M. is shown by bars) obtained at least 4 independent experiments (no bar means S.E.M. is smaller than the thickness of point).

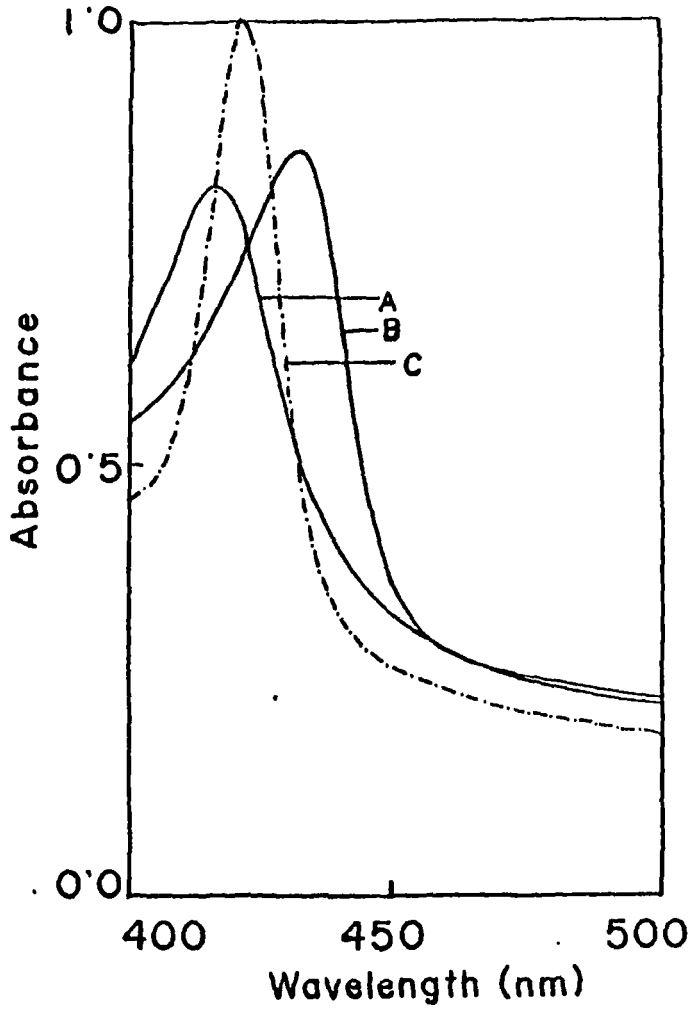


Figure 17. Optical absorbance spectra of Cytochrome P-450 from microsomes of mice liver (A). Curve (B) represents reduced spectra of cytochrome P-450 following reduction with sodium dithionite and curve (C) represents carbon monoxide binding spectra of Cytochrome P-450. (30 mg protein/ml, Cytochrome P-450 4.6 µM/mg protein, at 37°C, path-length 10 mm).

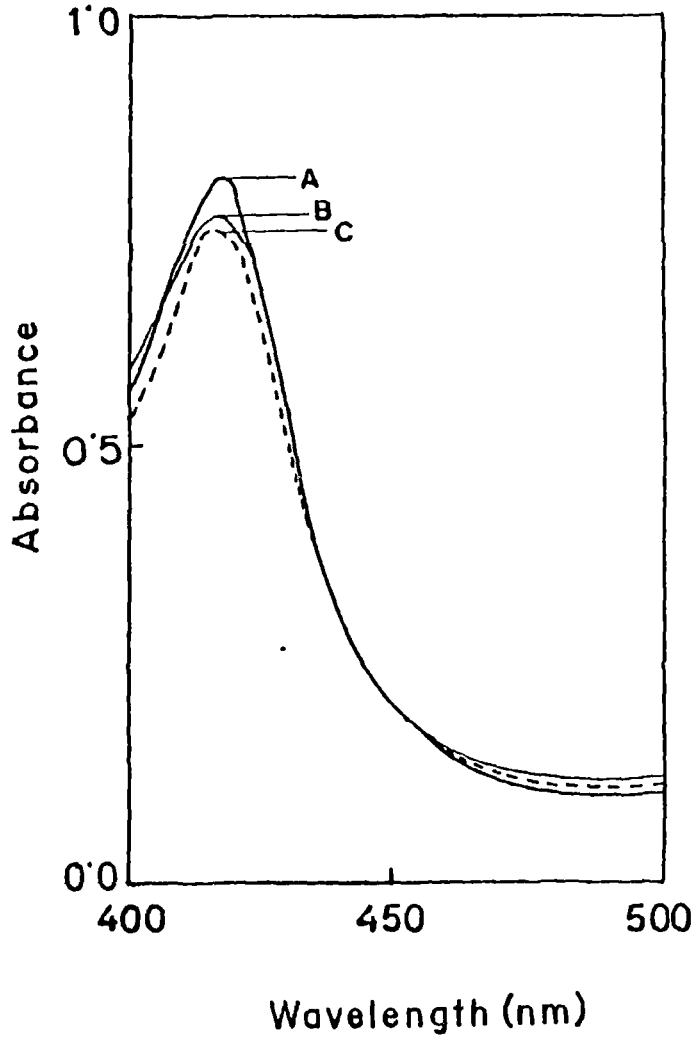


Figure 18. Optical absorbance spectra of Cytochrome P-450 (Curve A). Following 30 minutes of incubation of Cytochrome P-450 with 10 µg arecoline curve B was obtained. On further incubation (10 minutes) curve C was obtained. The concentration of Cytochrome P-450 was 4.6 µM/mg protein at 37°C and 10 mm pathlength.

calculated and was found to be [illegible] μM in presence of [illegible]° C and [illegible] nm [illegible]. Figure 18 shows the optical difference spectra of cytochrome P-450 (A) in presence of [illegible] μM [illegible]ecadine, [illegible] [illegible] minutes incubation there was [illegible] nm of blue shift upto (B). Upon further [illegible] minutes incubation curve C was obtained which showed further blue shift of [illegible] nm compared to curve B. There was no reaction at [illegible]° C, therefore, [illegible]° C was chosen, which mimics the physiological temperature.

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DISCUSSIONS

There have been a large number of reports on association of habit of betel nut chewing and cancer ([illegible]), areca nut and their pharmacological and mutagenic properties have been catalogued in detail ([illegible]), but no conclusive report is so far available which can give biochemical insight into the carcinogenic process associated with this habit of betel nut chewing. For the unprocessed betel nut ("Kwai") of this area, there is hardly any information.

This work was carried out in vitro and in vivo systems using mice as model experimental animal. The results that obtained in animal system, were intended to be extrapolated to human conditions. Since, tests could not be carried out in human being for obvious reasons, it was desired to use established human cell lines (Hep 2) for further investigations. These cells are derived from human carcinoma of larynx. These cells were metabolically active and their metabolic behaviour remained uniform.

In our experimental protocol the results related to DNA synthesis, protein synthesis, cytotoxicity, DNA repair

process and study of preneoplastic indicators/markers is monitored. It was borne in mind that animals can develop compensatory mechanisms for converting carcinogenic intermediates into substances which could be excreted readily. The process may involve several steps of metabolism before it could be finally excreted. Poor metabolic systems may reduce the metabolic rate of excreting them outside the body, which in turn may interact with DNA or proteins. This will apparently initiate the process of initiation of carcinogenesis. There are many which are responsible for such events as described in text. The enzyme family Cytochrome P-450 has been implicated in both activation or deactivation of chemical carcinogen in particular (Brenneisch, 1988). Although many enzyme systems are uniquely involved in the process of activation and deactivation of carcinogens. The Cytochrome P-450 system is an inducible system which offers suitable tool for study involvement of metabolic activation processes. Glutathione metabolism has also been associated with impairment of cellular systems due to carcinogenesis (Meister, 1979). There are instances in chemical carcinogenesis where metabolites undergo multiple modifications to form active electrophiles. Thus, reduction due to deactivation of activation are also expected in the process of chemical carcinogenesis.

Processed betel nut has been reported to have various chemical components viz., alkaloids, carbohydrates, proteins, fats, tannins, fibres, polyphenols and traces of vitamins (IARC, 1985). It is difficult to single out which of these chemicals are really involved with the etiology of cancer. Many of these chemicals alone or together may act as mutagen/carcinogen and may exert mutagenic or carcinogenic effect (Rao and Das, 1989). Of these chemicals, alkaloids, mainly arecoline and arecaidine, have been thought to be major carcinogenic principles of betel nut (IARC, 1985). Alkaloids present in betel nut are derivatives of 1,2,5,6 tetrahydro pyridine, containing Δ^3-ethylenic bond at 1-4 position of pyridinium ring. This has been implicated in biological allylating process (Boyland and Nery, 1967); during metabolism one methyl group ($-CH_3$) has been found to disappear. The allylating agents are reported to have multiple effects on growing cells and mostly interact with DNA double helix (Wheeler 1967). In light of this, this study becomes relevant because a sizeable population of the world is accustomed to the chewing of betel nut.

Analysis of unprocessed betel nut by thin layer chromatography followed by high performance liquid chromatography

showed (see Figures 1 and 2) contents of alkaloids (see also Table 1) which are relatively higher than the reported literature values of processed betel nut (Raghavan and Bartual, 1967; IARC, 1985). The contents of other chemicals have been reported to vary depending upon the maturity of betel nut (ripe or unripe) analyzed (Mathew and Govindrajan, 1964). Other chemical constituents, however, seem to be almost similar in both unprocessed and processed varieties of betel nut.

Aqueous extract of betel nut (AEBN) was chosen for this experimental protocol to equate the extraction of betel nut components by saliva in natural condition of human consumption of betel nut. The saliva samples were not analyzed because there are large variations in the saliva samples from person to person. The saliva from the same individual may vary at different times owing to variation in physiological condition(s). Since saliva is water based, water extract of betel nut was prepared to mimic this condition and to maintain uniformity in experimentation.

In the short-term in vitro tests, mice kidney cells were cultured and exposed to AEBN and their survival, rate of proliferation and DNA strand break analysis were carried out (Figures 3 and 4). The dose of AEBN to be administered in our

experiment was determined such that we have a dose comparable to that to which the population in this area is exposed following customary consumption of betel nuts. On the basis of surveys, a dose of 100 μg/ml AEBN was found appropriate. In addition, a higher dose of 500 μg/ml AEBN was also chosen. Similarly, the dose of arecoline was adjusted to have approximately the same amount of the alkaloid present as the average total alkaloid in 100 μg/ml AEBN (see [illegible], 1980).

We observed no morphological signs of transformation until the end of our experimental protocol (9 days) with either arecoline or AEBN, except for a reduced cell cycle time (see below). Reddy et al (1979) have reported induction of cell transformation by both arecoline and arecaidine from betel nut. The difference in our results could be due to the doses of arecoline (10 μg/ml) and AEBN (100 μg/ml); probably both are below the threshold dose for showing morphological signs of transformation in just 9 days. However, we made a significant observation that 100 μg/ml AEBN is able to reduce the duration of the cell-cycle such that monolayer formation occurs a day earlier as compared to the control (Figure 5). It is interesting to note that while 100 μg/ml AEBN does not cause drastic damage to DNA through strand breakage (Figure 4) it hastens the process of

monolayer formation by 1 day (Figure 5). This could be taken as an important sign of initiation of carcinogenesis or transformation. As against this, 10 µg/ml arecoline does cause drastic damage to DNA but fails to accelerate the monolayer process of formation. The cells of this group, instead, had a slower rate of proliferation, as the monolayer was formed on the 11th day after seeding (Figure 5). Both AEBN (100 µg/ml) and arecoline (10 µg/ml) showed respectively 5%, 7.5% and 1[illegible]% [illegible] level (Figure 5). Panigrahi and Rao (1982) have also shown the chromosome breaking ability of arecoline in bone marrow cells. Our results conform with their results, but in addition, we observe an important phenomenon of higher DNA damage and lower rate of cell proliferation after 10 µg/ml arecoline and relatively low DNA damage and enhanced rate of cell proliferation after 100 µg/ml AEBN. This, therefore, suggests that in addition to the ability to break DNA strands, the AEBN contains some additional factor(s) that can initiate and/or promote cell proliferation, which could eventually lead to carcinogenic transformation. The additional factor(s) may be present in the AEBN or may be metabolic product(s) of the constituents of AEBN in the biological system. Apparently, optimal (or specific ?) DNA damage and the presence of this (these) additional factor(s) seem necessary to enhance the cell proliferation rate because higher dose of AEBN (250 µg/ml) was lethal to the cells in

culture (Figure 5). This may mean that a higher dose of AEBN causes very severe damage or more non-specific damage to DNA so that it leads to cell death rather than transformation. Failure of arecoline (10 µg/ml) to enhance monolayer formation could be due to the lack of other "additional" factor(s) that may have been present in AEBN. Alternatively, arecoline may have caused cytotoxicity and at the same time directly interacted with DNA, which blocked the biosynthetic processes necessary for cellular replication and thereby inhibited the cell proliferation. AEBN has also been found to have more genotoxic effects than tannin component of betel nut (Parigrahi and Rao, 1986). It means that most water soluble components of betel nut have genotoxic potentials in vitro as well as in vivo. Exposure of virally transformed human lymphocyte to ethyl acetate extract of betel nut was unable to induce sister chromatid exchange and failed to show any transformation in hamster ovary cells (Umezawa et al., 1981). Aqueous and alcohol extract of betel nut have been found to be bactericidal. The bactericidal effect is reported to have been associated with tannin components, rather than alkaloids (Kalithakumari and Sirsi, 1965; Majumdar et al., 1980). Treatment of AEBN to mice by oral intubation, dietary administration, skin application and intraperitoneal and subcutaneous injection, produced a number of tumors depending upon the route of application (IARC, 1985). Therefore, it seems that AEBN is

genotoxic and is able to show preneoplastic lesions in our test system due to presence of alkaloids and other components not only polyphenols, tannins etc.; polyphenols and tannins from other plants have also been reported to be genotoxic (Stich and Powers, 1979).

Preneoplastic lesion as a result of carcinogen insult(s) have been reported and gamma-glutamyl transpeptidase (GGT) has been widely used as marker enzyme for preneoplastic lesion indicator in several studies (Richardson, 1985). Gamma-glutamyl transpeptidase (GGT) activity have been found to increase in many growing tissues, ageing cells and in differentiating tissues (Richardson, 1985). Thus increase in activity of GGT probably indicates that amino acid requirements have increased in response to growth as this enzyme is implicated in the transport across the membrane (Meister, 1976). Our result (Figure 5) shows that arecoline and arecoline (1 mM and 5 mM) feeding exerted many fold increase in the GGT activity over the control; for the two doses of arecoline (1 mM and 5 mM) the induction of GGT was dose dependant. The increase in the GGT activity has been shown to increase significantly in 1,2-DMH feeding rodent in GGT positive rat hepatocyte foci (Pugh and Roberts, 1978). Our earlier result (Figure 3) showed induction of rapid cell proliferation upon exposure of primary kidney cells

in culture by AEBN, whereas arecoline failed to do so. This may mean that the present observation of enhanced GGT activity both by areca nut and arecoline need not necessarily indicate enhanced cell proliferation as also suggested by Richards (1985). Since GGT plays a major role in the gamma-glutamyl cycle which metabolizes glutathione, the increased activity may indicate increased local concentration of glutathione following areca nut and arecoline treatments. This may mean involvement of glutathione in the process of detoxification of these carcinogens. The increased amount of glutathione, on the other hand, enhances the absorption of amino acid by renal cells probably to account for the higher rate of protein synthesis including that of GGT. This study probably indicates specific enzyme activation which is possible by many ways including by gene activation. The cellular mechanisms which are responsible for the observed enzyme alterations (Figure 5) following areca nut or arecoline treatments are not clear. Since the genotoxic effects of areca nut and arecoline have been observed in our study, mutational events in structural or regulatory genes of GGT may effect the regulation and expression of GGT. This, either directly or indirectly, may result in enhancement or repression of enzyme synthesis which could account for the variability observed in our study. Kunz et al (1987) and Buchmann et al (1987) support possibility of regulatory system getting

affected rather than mutational events in structural genes for nitrosamine induced hepatocarcinogenesis in rats. For our study the increase and decrease probably indicates 'pull and push' between normal and neoplastic gene expression in mice following areca nut and arecoline treatments. The increase may be indicative of the onset of neoplasma in activated (*) cells, but due to the normal cell which are in close contact with these activated cell(s) (*) the GGT activity declined after attaining a peak on the 60th day (Figure 5). Since the feeding of areca nut and arecoline continued, a second peak of GGT activity appeared which attained a higher level on the 150th day followed by decline in the activity on the 180th day. This probably means that the cells are on the progressional stage of carcinogenesis, the periodicity of increase and decrease is indicative of partial reversible mechanism of chemical carcinogenesis, at least during initial stages. In addition, the variable GGT activity also indicates the variability of glutathione metabolism in mouse kidney after exposure to areca nut and arecoline. Glutathione metabolism anomalies may induce mitotic anomalies as had been suggested by Meister (1975) leading to preneoplastic changes. The concentration of arecoline (1 mM and 3mM) in this experiment was clinically below the toxic level (IARC, 1986) and dose burden to mice is comparable to the amount of betel nut consumed by the inhabitants of Khasi hills in Meghalaya (India).

Conflicting reports exist as regard to effects of various extracts of betel nut *in vivo* and no uniformity in studies can be seen (IARC, 1985). No cytotoxicity study and study of onset of unscheduled DNA synthesis after areca nut treatment to cells have been done so far. Our present study, therefore, include exposure of cells to various extracts of betel nut *in vitro*. The idea of preparing alcohol extract of betel nut was to see the effect of alcohol soluble extract against water soluble part. At the same time, extraction of betel nut components either by 0.1 N HCl or by 1% acetic acid was to mimic the condition of extraction in acid pH as in buccal cavity and the digestive system in humans. We observed dose dependant Hep 2 cell death *in vitro* regardless of any type of extract used (Figure 6). We also found a positive unscheduled DNA synthesis in Hep 2 cells induced by arecoline, AEBN, alcohol extract of betel nut, 0.1 N HCl extract of betel nut and 1% acetic acid extract of betel nut (Figure 7). The hydrochloric acid extract of betel nut on did not show profound UDS in Hep 2 cells meaning, thereby, that the extract is a weak carcinogen. The UDS in Hep 2 cells by these extracts would indicate DNA damage in the cells. We observe that arecoline or extracts of betel nut interfere with the biosynthetic processes required for the cell duplication. Our results are statistically significant which has been further confirmed by other observations (see below).

DNA is the primary cellular target for chemical carcinogens. These carcinogen(s) exhibit mutagenic activity when suitably activated by a metabolizing system in a living organism (Ames et al, 1973) which suggests correlation of mutagenecity with carcinogenecity. In our study we employed sodium nitrite in food additive in order to enhance the process of nitrosation of arecoline in presence of S-9 mixture at a low pH of 4.2. It has been reported that under mild nitrosation condition arecoline forms several nitroso compounds viz., 3-(methylnitrosamino)-propionitrile, 3-(methylnitrosamino)-propionaldehyde, N-nitrosoguvacoline) and N-(nitrosoguvacine) (Wenke and Hoffmann, 1985). Of these, N-(nitrosoguvacoline) has been detected in the saliva sample of betel nut chewers and other nitrosamine in betel quid chewers (Wenke and Hoffmann, 1985; Nair et al , 1986). It has also been shown that aqueous extract of betel nut administered along with proline and nitrite in rats result in production of N-nitrosoproline in urine sample of treated rats (Stich et al, 1984).

Our results show that both arecoline or sodium nitrite or their combination is cytotoxic and cytostatic in Hep 2 cells in presence of S-9 mixture at pH 4.2. We observe inhibition

of DNA synthesis (Figures 11, 12 and 13) and in protein synthesis (Figure 14). The results suggest that metabolic activation of test chemicals has brought out a reactive chemical species in vitro which interfere with DNA replicative activity. The delay in DNA synthesis may have caused delayed protein synthesis. This chemical species may be nitrosocompound(s) (!) of arecoline as suggested in the literature (IARC, 1985). The interaction of nitrosocompounds thus formed in this test system may have triggered off many biochemical events which expressed as cytotoxic effect, cytostatic effect and eventual cell death. In the present study we have not identified the reactive chemical species. Unscheduled DNA synthesis study (Figure 7) indicated DNA damaging capability of arecoline in Hep 2 cells. The interesting observation which was made in this experiment was reversible nature of DNA synthesis inhibition by this chemicals (Figure 16). Upon removal of these chemicals the DNA synthetic activity was restored.

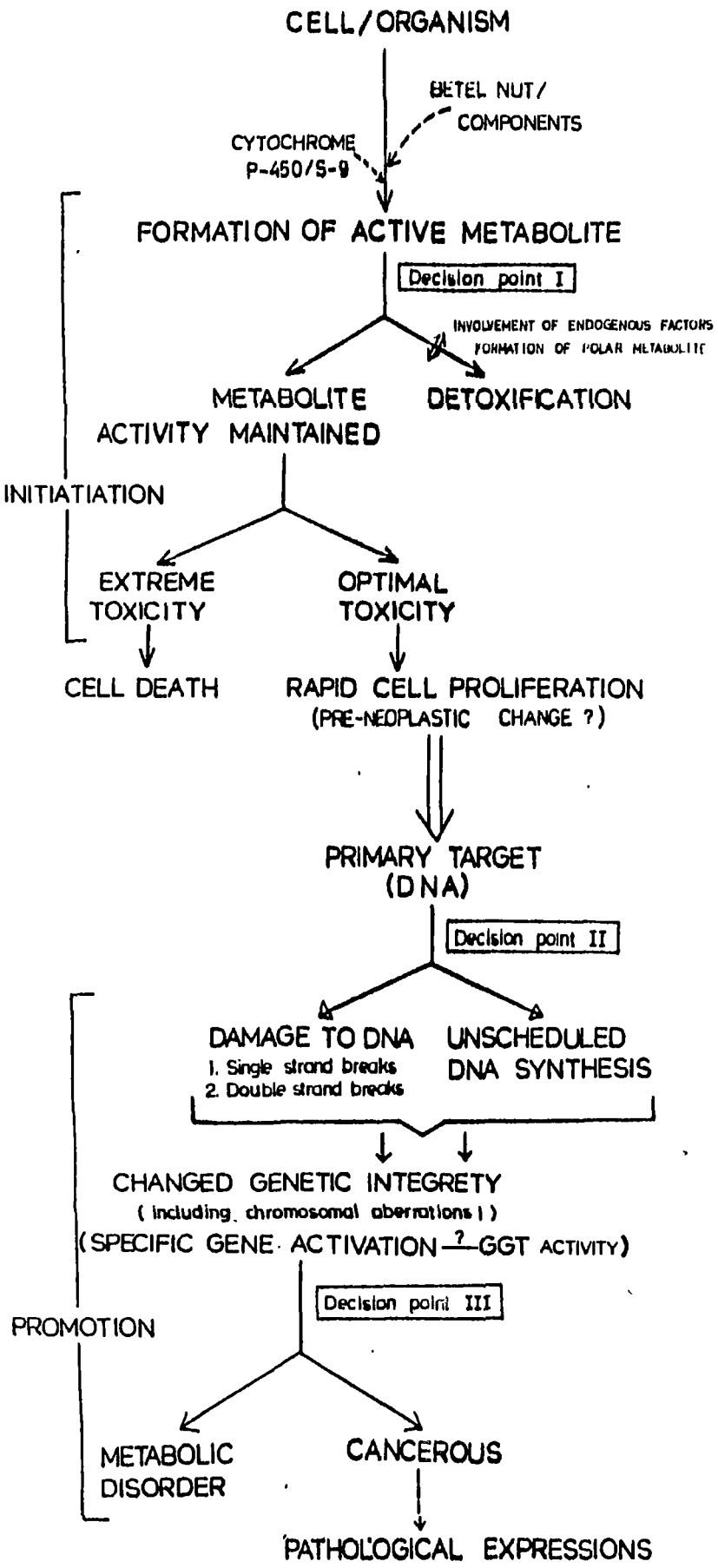
One of the enzymes which present in S-9 mixture is cytochrome P-450. Since, it is known that cytochrome P-450 can activate or deactivate many carcinogen, we monitored the interaction of arecoline with this enzyme spectrophotometrically (Figure 18). From our spectral analysis, we have found that the arecoline molecule underwent formation of nitrosocompound(s);

presumably these nitrosocompounds are the same as reported by Wenke and Hoffmann (1983). However, the actual chemical species formed following incubation of arecoline in microsomal fractions containing cytochrome P-450 has not been investigated. Nevertheless, we propose that cytochrome P-450 and metabolic activation system are closely associated with the process of initiation of betel nut induced carcinogenesis which have been observed in short-term and long-term, in vitro as well as in vivo studies.

Following this study we proposed a mechanism which could eventually initiate carcinogenic process (see Scheme 1). We proposed that DNA is the primary target for betel nut or its alkaloid result. In case of involvement of endogenous factors there could be detoxification to harmless derivatives which would be readily excreted. When metabolite activity is maintained it could react with DNA to initiate the carcinogenic process. This interaction of metabolite with DNA was demonstrated by DNA strand breaks and unscheduled DNA synthesis assays. The damage to DNA could result in alteration or activation of specific gene(s), for example, gamma-glutamyl transpeptidase gene. Our assay of GGT reflects the anomaly of genetic integrity.

It may be noted that many human tumors are caused

SCHEME 1


CELL/ORGANISM
BETEL NUT/
COMPONENTS
CYTOCHROME
P-450/S-9
FORMATION OF ACTIVE METABOLITE
Decision point I
INVOLVEMENT OF ENDOGENOUS FACTORS
FORMATION OF POLAR METABOLITE
METABOLITE
ACTIVITY MAINTAINED
DETOXIFICATION
INITIATIATION
EXTREME
TOXICITY
OPTIMAL
TOXICITY
CELL DEATH
RAPID CELL PROLIFERATION
(PRE-NEOPLASTIC CHANGE ?)
PRIMARY TARGET
(DNA)
Decision point II
DAMAGE TO DNA
1. Single strand breaks
2. Double strand breaks
UNSCHEDULED
DNA SYNTHESIS
CHANGED GENETIC INTEGRETY
(including chromosomal aberrations)
(SPECIFIC GENE ACTIVATION —?— GGT ACTIVITY)
Decision point III
PROMOTION
METABOLIC
DISORDER
CANCEROUS
PATHOLOGICAL EXPRESSIONS

by exogenous factors viz., chemicals, radiations etc. It appears that human cancers are caused by a complex interaction between multiple factors including the combined actions of chemical and viral agents. The process of carcinogenesis proceeds through multiple discernible stages, which could be roughly divided into two steps i.e., initiation and promotion. The process of initiation appears to be rapid one and is usually reversible (Weinstein, 1983). This initiation step involves interaction(s) of chemical(s) (epigenetic or genotoxic). The next step involved in the process of carcinogenesis is promotion, which often has quite a long incubation period. There is no pathological expressions and this step may be reversible in some cases (Weinstein, 1988).

Many carcinogens can interact with DNA directly; some carcinogen(s) require metabolic activation. This was also observed in our experiments. Most carcinogen(s) are electrophilic in nature to be able to interact with DNA. Formation of DNA adduct(s), DNA-protein cross links, double strand breaks, single strand breaks, thymine dimers, hydration (-OH) and allylation are the major lesions in DNA which may bring about covalent modifications of informational molecules in cells. This results in the distortion of DNA duplex and may initiate the process of carcinogenesis.

Our present study was aimed at understanding the biochemical events leading to carcinogenesis associated with the use of betel nut ('kwai') which is a popular masticatory in South-East Asia, India in particular. Epidemiological studies have revealed that use of betel nut is associated with high incidence of oral cavity cancer in India which may account for over 50% of total cancer incidence in India (Sanghvi, 1961).

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CONCLUSIONS

A comprehensive idea has been generated from present study which demonstrates the biochemical events involved in betel nut carcinogenecity.

1. The betel nut from this part of the country (Meghalaya, India) contains higher amounts of alkaloids and was found to have carcinogenic potency.

2. Short-term *in vitro* tests have shown genotoxicity of this unprocessed variety of betel nut. The genotoxicity studies included induction of rapid cell proliferation, strand breaks in DNA and unscheduled DNA synthesis which are important signs of onset of carcinogenesis. "Additional factor(s)" of unknown nature present in the unprocessed variety of betel nut ("Iwai") help in initiation of transformation.

3. Treatment of human cell line with various extracts of betel nut induced cytotoxic effects and unscheduled DNA synthesis demonstrating the DNA damaging capability of these extracts.

4. Long-term study *in vivo* in mice have shown increased level and recurring periodicity of marker enzyme GGT, which has been thought to be due to alteration in specific gene activation under the influence of betel nut or its alkaloid, arecoline. This indicates that betel nut or its alkaloid are capable of influencing the control mechanism of gene expression.

5. Areca nut and its active alkaloid i.e., arecoline, have cytotoxic and cytostatic effects in mouse primary cells as well as in human cell lines *in vitro*. There were positive interferences of constituents of betel nut with the cells resulting in the anomaly of macromolecular syntheses.

6. The enzyme cytochrome P-450 has been implicated in the production of active metabolite (nitrosocompounds ?) from areca nut and arecoline which could finally react with biomolecules, DNA in particular, resulting in alteration in cellular and sub-cellular activities.

Our present study indicates that chewing of betel-nut is a health hazard of serious concern. The betel nut chewing may not only result in cancer of oral cavity but also cancers of other sites. However, food and nutrition, environment and genetic factors may play modifying roles in carcinogenesis associated with the use of betel nut.

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Cancer Research
Clinical Oncology

Aqueous extract of betel-nut of North-East India induces DNA-strand breaks and enhances rate of cell proliferation in vitro*

Effects of betel-nut extract in vitro

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Summary. An aqueous extract of betel-nut has been found to be able to induce strand breaks in DNA of mouse kidney cells in vitro. It has been also found to be able to enhance the rate of cell division at a dose of 100 μg/ml while a higher dose of 250 μg/ml was extremely toxic to the cells. Compared with arecoline (10 μg/ml), the aqueous extract of betel-nut seems to be a more potent carcinogen to mouse kidney cells in vitro.

Key words: Betel-nut – DNA-strand breaks – Carcinogenesis – In vitro

Introduction

In the North-Eastern part of India, betel-nut (*Areca catechu* L.) is normally chewed with betel leaf (*Piper betle* L.) and a little slaked lime. This results in characteristically red saliva and teeth. The habit of betel-nut chewing and occasional swallowing is associated with oropharyngeal cancers in humans (Muir and Kirk 1960; Ashby et al. 1979; IARC 1985) and accounts for over 50% of the total cancer incidence in India (Sanghvi 1981). The Khasi tribe of North-Eastern India chew raw and unprocessed betel-nut (locally known as *"Kwai"*) as part of the normal social custom. This betel-nut consumption shows an immediate thermogenic physiological response, lasting 2–3 min, with significant perspiration on the forehead. This is markedly different from the effect of processed betel-nut consumed elsewhere in the world, especially in Southeast Asian countries (see IARC 1985). Various constituents of betel-nut have been shown to induce mutation, sister chromatid exchange and transformation (Umezawa et al. 1981; for review see IARC 1985). From this region a higher frequency of occurrence of micronucleated cells in buccal mucosa of people who chew betel-nuts has been reported (Stich et al. 1982; Stich et al. 1983). Chromosome-damaging activity has also been reported in the saliva of people who chew betel-nuts (Stich and Stich 1982). It has also been found that betel-nut can induce an increased rate of mutation in *Salmonella typhimurium* (Shirname et al. 1983).

In the process of carcinogenesis, DNA is the prime target for various chemicals and agents. Betel-nut, which is rich in alkaloids (Arjungi 1976; Ashby et al. 1979; Sharp 1948), becomes an important subject of investigation because of its customary large consumption in Southeast Asian countries and the fact that the major alkaloids of betel-nut, arecoline and arecaidine, are capable of inducing cell transformation (Ashby et al. 1979); arecoline is also capable of breaking DNA strands in the bone marrow cells in vitro (Panigrahi and Rao 1982). These reports of work using purified alkaloids of the betel-nut (arecoline, arecaidine etc.) provide valuable information about their role in the process of carcinogenesis. But the results may not be the same when a complex mixture of alkaloids and other organic materials, such as those in betel-nut, is administered. This information, however, will be very relevant to the human condition where betel-nut chewers are exposed to such a complex mixture of alkaloids and other organic materials. The behaviour of a purified alkaloid and that of the same alkaloid in combination with other chemicals may not be the same. Furthermore, in a biological environment, these chemicals, especially alkaloids, are likely to be metabolized to produce an array of metabolites that may also influence the process of carcinogenesis. In addition, we have no information on damage to DNA

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caused by betel-nut products (IARC 1985). With this in mind, we have attempted to shed more light on the situation. We report the ability of aqueous extracts of betel-nut (AEBN) to break DNA strands of mouse kidney cells in vitro. We relate this event to the process of transformation.

Materials and methods

Chemicals. All chemicals for tissue culture were of tissue-culture grade. Other chemicals were of analytical grade and were used without further purification. Deionized double-distilled water was used to prepare reagents and solutions.

Preparation of betel-nut extract. For these tests, 250 g ripe betel-nuts from the West Khasi Hills area were obtained from the local market. After removal of the fibrous coats, the nuts were ground and suspended in 250 ml distilled water and kept at room temperature for 24 h. The suspension was filtered through a 0.45 μm Millipore filter and the filtrate was lyophilized in a Seefroid Lyolab BII lyophilizer. The lyophilized mass was kept at 4 °C until use. This procedure is henceforth called aqueous extract of betel-nut (AEBN).

Tissue culture. Three-week-old Swiss albino mice (male and female) were sacrificed for kidney cell culture. The kidneys were aspetically removed, minced and suspended in saline solution. After thorough washing the tissue was trypsinized at 37 °C in 0.25% trypsin solution in Ca^{2+}- and MG^{2+}-free-phosphate-buffered saline. Sterile Eagle's minimal essential medium, buffered with sodium bicarbonate (2.8%), containing 100 units/ml antibiotics (streptomycin, penicillin and fungizone) and 10% fetal calf serum was used as growth medium. Cells (2.85×10^5) were dispensed into each culture tube and incubated at 37 ± 0.2 °C in a sterile incubator.

Enumeration of cells in culture. Enumeration of viable cells in the culture tubes was done by a dye-exclusion technique (Phillips 1973). In brief, a drop of the content of each tissue-culture tube was stained with 1% trypan blue. Five minutes later this was counted in a light microscope using a haemocytometer. From this, the population doubling time and the toxicities of AEBN or arecoline treatments were calculated by counting the viable and dead cell populations.

Treatment of cell culture with arecoline and AEBN. Sterile arecoline (1 mg/ml) and AEBN (5 mg/ml) stock solutions were prepared in Hanks' balanced salt solution. Cultures were exposed to these two chemicals on the day of seeding. A single dose of arecoline (10 μg/ml) and two doses of AEBN (100 μg/ml and 250 μg/ml) were used in these experiments. A dose of 100 μg/ml AEBN contains approximately 2%–3.5% arecoline and 2%–3% arecaidine as analysed in our laboratory, higher values than that reported in the literature (see IARC 1985).

Chromatography. The alkaline DNA-unwinding technique of Rydberg (1975) was employed with minor modifications to analyse DNA strand breaks on hydroxyapatite columns. The exact method has been described in our earlier publication (Srivastava et al. 1982). DNA estimation was carried out according to Burton (1968) with minor modifications.

Results and discussion

Figure 1 shows the viable cell growth with respect to time for control (untreated), arecoline-treated and

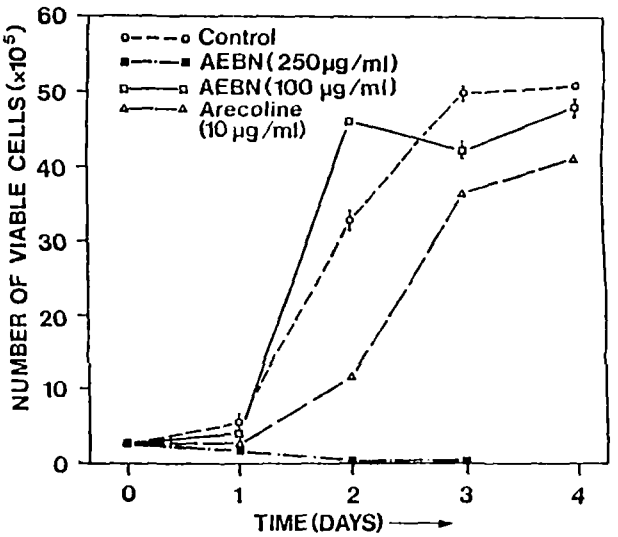

Fig. 1. Count of viable cells as a function of time for control, arecoline and AEBN-treated mouse kidney cells in culture (see also Materials and methods). Each *point* is mean ± SD (shown by *bars*) of 5–7 independent experiments. No bar means that SD is smaller than the thickness of the point

AEBN-treated cultures. The control tubes showed approximately 100% increase in population after 18–19 h with confluent monolayer formation on the 3rd day after seeding. The arecoline (10 μg/ml)-treated cells showed a delayed cell duplication time and monolayer formation. While 250 μg/ml AEBN was drastically toxic to the cells, the culture tubes with 100 μg/ml AEBN exhibited attainment of confluency a day earlier than the control. However, it showed an approximately 10% lower viable cell count. Owing to the acute toxicity of 250 μg/ml AEBN, this dose has not been used in the following experimental protocol.

Figure 2 shows the results of DNA strand break analysis. Damage to DNA has been expressed as the ratio of ssDNA/dsDNA recovered. Arecoline-treated cells show a very significant increase in the ssDNA/dsDNA ratio (which means DNA damage) with time. The damage reduces slightly after 3rd day. The AEBN-(100 μg/ml)-treated cells show significant damage to DNA compared with the controls, but this is not as drastic as that caused by arecoline. These data have been tabulated from the total amounts of ssDNA and dsDNA recovered from hydroxyapatite columns from different groups of cells (data not shown here).

The dose of AEBN to be administered in our experiment was determined such that we have a dose comparable to that to which the population in this area is exposed following the customary consumption of betel-nuts. On the basis of our surveys (to be communicated separately), a dose of 100 μg/ml AEBN was found appropriate. In addition, a higher dose of 250 μg/ml AEBN was also chosen. Similarly, the dose

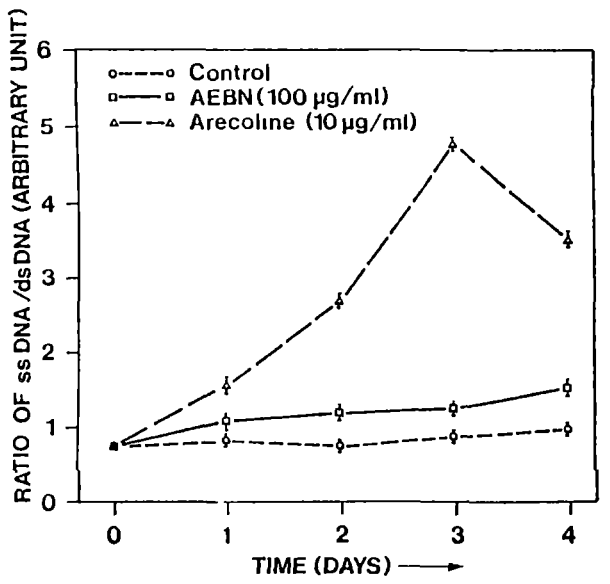

Fig. 2. Damage to DNA (expressed as the ratio of ssDNA to dsDNA) as function of time for control, arecoline and AEBN-treated mouse kidney cells in culture (see also Materials and methods and Srivastava et al. 1982) Each *point* is the mean ± SD (shown by *bars*) of at least four determinations

of arecoline was adjusted to have approximately the same amount of this alkaloid present as the average total alkaloids in 100 µg/ml AEBN (see IARC 1985).

We observe no morphological signs of transformation until the end of 4 days with either arecoline or AEBN, except for a reduced cell-cycle time (see below) Ashby et al. (1979) have reported induction of cell transformation by both arecoline and arecaidine from betel-nut. The difference in our results could be due to the doses of arecoline (10 µg/ml) and AEBN (100 µg/ml); probably both are below the threshold dose for showing morphological signs of transformation in just 4 days. However, we make a significant observation that 100 µg/ml AEBN is able to reduce the duration of the cell cycle such that monolayer formation occurs a day earlier as compared to the control tubes (Fig. 2). It is interesting to note that while 100 µg/ml AEBN does not cause drastic damage to DNA through strand breakage (Fig. 2) it hastens the process of monolayer formation by 1 day (which could be taken as an important sign of initiation of carcinogenesis or transformation). As against this, 10 µg/ml arecoline does cause drastic damage to DNA through strand breakage but fails to accelerate the monolayer formation. The cells of this group, instead, show a slower rate of proliferation, as the monolayer was formed on the 4th day after seeding. Both AEBN (100 µg/ml) and arecoline (10 µg/ml) showed respectively 5%–7.5% and 10%–12% toxicity Panigrahi and Rao (1982) have also shown the chromosome-breaking ability of arecoline in bone marrow cells. Our results conform with their results, but in addition we observe an important phenomenon of higher DNA damage and a lower rate of cell proliferation after 10 µg/ml arecoline, and relatively low DNA damage and an enhanced rate of cell proliferation after 100 µg/ml AEBN. This, therefore, suggests that in additional to an ability to break DNA strands, the AEBN has some additional factor(s) that can initiate and/or promote cell proliferation, which could eventually lead to carcinogenic transformation. These additional factors may be present in the AEBN or may be a metabolic product of the constituents of AEBN in the biological system. Apparently, optimal (or specific?) DNA damage and the presence of this (these) additional factor(s) seem necessary to enhance the cell proliferation rate because a higher dose of AEBN (250 µg/ml) was lethal to the cells in culture (Fig. 1) This may mean that a higher dose of AEBN may cause very severe damage or more nonspecific damage to DNA so that it leads to cell death rather than transformation. Failure of arecoline (10 µg/ml) to enhance monolayer formation could be due to lack of additional factor(s) that may have been present in AEBN We are in the process of analysing the components of these unprocessed betel-nuts and monitoring unscheduled DNA synthesis (UDS) under these experimental conditions

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OTHER PUBLICATIONS

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Effect of the Radioprotector 2-Mercaptopropionylglycine (MPG) on the Radiation Inactivation of Catalase *In Vitro*

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MPG/Radiation protection/Radiolysis/Catalase

Studies were performed to provide mechanistic insights into the action of the radioprotector drug, 2-mercaptopropionylglycine (MPG) following the radiolysis of catalase, a detoxifying enzyme The enzyme solution was γ-irradiated in the presence and absence of MPG The enzyme activity was monitored *in vitro* using H_2O_2 as a substrate MPG behaved primarily as a radioprotective drug However, due to the presence of Fe^{++}/Fe^{+++}, and the in homogeneity of catalase, under certain conditions there was circumstancial interaction of Fe^{++}/Fe^{+++} with MPG, resulting in the formation of an unstable catalase Fe^{++}/Fe^{+++}-MPG chelate/complex This resulted in the radiosensitizing effect of MPG on enzyme catalase.

INTRODUCTION

2-mercaptopropionylglycine (MPG) has been widely used as a potent radioprotector *in vivo* and *in vitro*[1–3]. The proposed radioprotective mechanism varies from a release of endogenous protectors[4], to a high redox potential[5], to free radical scavenging[6] However, MPG did not exhibit the protection characteristic of spontaneous or chemically induced lipid peroxidation[7], or certain conditions of radiation-induced microsomal lipid peroxidation[2] From these view points, we have strived to reason the protection/non-protection offered by MPG We have chosen an enzyme catalase because, (i) it acts as an oxidizing agent to ethanol, with the consumption of 1 mole of peroxide, that is, it decomposes hydrogen peroxide (detoxification), providing a convenient assay procedure, and (ii) it has been used extensively to decipher the mechanism of radiolysis of protein[8]. Our experimental goal was to procure insight into the mechanism of radioprotection or lack of protection by MPG in the radiation induced inactivation of catalase

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EXPERIMENTAL

Chemicals

All chemicals used were of analytical grade and were used without further purification. Water was deionized before glass-double distillation.

Catalase (EC.1 11 1.6)

Catalase was obtained from Sigma Chemical Co., USA. Freshly prepared catalase solution in 50 mM phosphate buffer, pH 7.0, was used The following concentrations of catalase were used in this work (in μg/ml) 0.44, 1.32, 2.20, 3.10, 3.70, and 4.80 One unit of enzyme decomposes 1 0 μmole of H_2O_2 per minute, at pH 7.0 and 25°C.

Catalase Assay System

Immediately after irradiation at room temperature (23°C), assay was performed according to the method of Aebi (1984)[9], with minor modifications. The duration of assay was 30 sec. The enzyme activity is expressed as a difference in absorbance (ΔA_{240}) per unit time. Each data point represents a mean ± S.E. for 10 to 15 assays in a Hitachi spectrophotometer.

2-mercaptopropionylglycine (MPG)

MPG was obtained from Prof. Tsutomu Sugahara, Kyoto (Japan), under the trade name, Tiopronin, marketed by Santen Pharmaceutical Co. Ltd., Japan. MPG, 0.002 M, was freshly prepared in 50 mM phosphate buffer, pH 7.0. The concentration of MPG in the experiments was 0 001 M.

Gamma-irradiation

A ^{60}Co-source (Model-Gamma Chamber-900, BARC, India) delivering γ-radiation at a dose rate of 1 Gy/sec, was used. 1 ml enzyme solutions of appropriate concentrations were mixed with 1 ml phosphate buffer or 1 ml MPG, as required, were allowed to stand for 15 minutes at room temperature (23°C), then irradiated under normal atmospheric conditions with doses of 10, 20, 40 and 80 Gy.

RESULTS AND DISCUSSIONS

Changes in enzyme activity in terms of change in absorbance at 240 nm following various doses ^{60}Co irradiation, with and without MPG, are shown in Figures 1 and 2 A relatively sharp reduction in catalase activity (50 to 68%) was observed in all cases, when it was supplemented with MPG. At low enzyme concentrations (0.44 and 1.32 μg/ml), the initial depression in activity at 10 Gy γ-ray shows increased activity up to 40 Gy (Figures 1A and 1B). At higher concentrations of enzyme (2.20, 3.10, 3.90 and 4.80 μg/ml), however, the observed activity at 10 Gy is followed by a gradual decline in activity with increasing γ-ray doses (Fig 2A, B, C and D). While the radiation dose remains the same for the low enzyme concentration group (Fig. 1A and B) and the high enzyme concentration group (Fig. 2A, B, C and D), only the former shows a trend of reversibility of effect after a dose of 10 Gy. The damage seems to be severe,

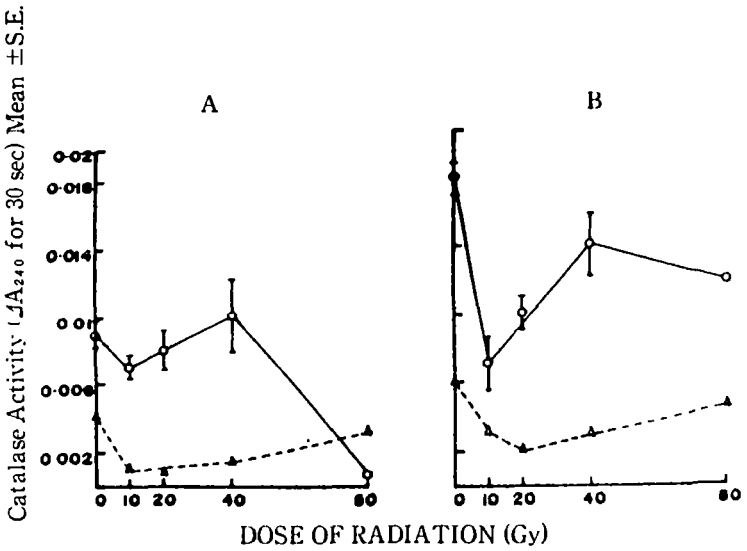

Fig. 1. Catalase activity following various doses of gamma-irradiation with (Δ - - - - - - - - - - Δ) and without (o———————o) MPG (0.002 M). Concentration of Catalase is 0.44 μg/ml in A and 1.32 μg/ml in B.

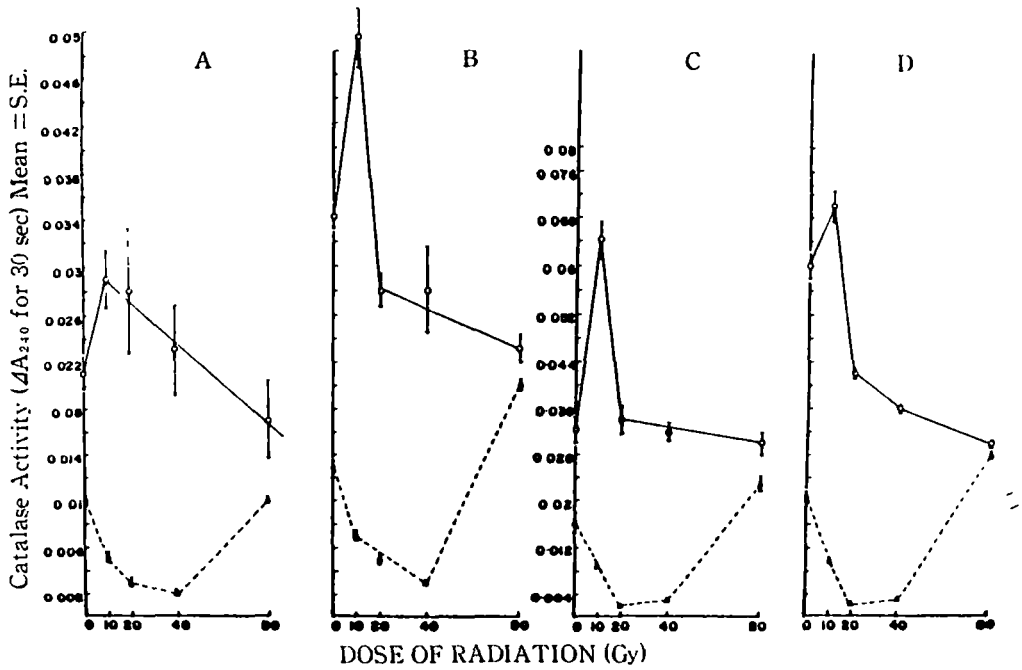

Fig. 2. Catalase activity following various doses of gamma-irradiation with (Δ - - - - - - - - - - Δ) and without (o———————o) MPG (0.002 M). Concentration of Catalase in 2.20 μg/ml in A, 3.10 μg/ml in B, 3.90 μg/ml in C and 4.80 μg/ml in D.

and to some extent dose-dependent, beyond a radiation dose of 10 Gy for higher concentrations of enzyme (Fig. 2A, B, C and D). These results indicate that the concentration of the enzyme may influence radiation damage after a dose of 10 Gy.

In the presence of MPG (0.002 M added to the enzyme preparation 15 min prior to irradiation), from 20 to 40 Gy radiation for various concentrations of catalase induced further reduction in enzyme activity (meaning sensitization of catalase) instead of the expected increase in enzyme activity towards the normal value (which would have meant the protection of catalase). This was followed by an insignificant increase in enzyme activity (protection) in the low protein concentration group (Fig. 1) and a noticeable increase in enzyme activity (protection) in the high protein concentration group (Fig. 2).

The initial decrease in enzyme activity in the controls with the mere addition of MPG can be explained by the formation of MPG-Fe^{++} chelate compound, as suggested by Ayene and Srivastava (1985)[2]; Fe^{++} being provided by the four haematinic groups of the enzyme. We believe that Fe^{++} may not be fully released from the enzyme, but rather, that it remains partially exposed for this chelate formation (see below). This has also been suggested by Ayene and Srivastava (1985)[2]. This may possibly result in the formation of a complex like MPG-Fe^{++}-catalase. This presumption is advanced in an attempt to explain some of the observations in this experiment, cited. To determine whether of this complex involving Fe^{++} was formed, we attempted to supply Fe^{++} and Fe^{+++} ions exogenously to the catalase-MPG solution in order to check the competition of the exogenously supplied Fe^{++}/Fe^{+++} and the haematinic groups of the catalase for MPG. Our efforts were unsuccessful due to the drastic lowering of pH, and problems in solubility and denaturation. Further work to ascertain this is in progress in this laboratory. The possibility of an interaction of MPG with the substrate of catalase (i.e., H_2O_2) has been experimentally ruled out in our laboratory, but the results are not included here.

Hence, three factors may be operating on catalase in the presence only of MPG, or MPG and radiation:

i) Formation of MPG-Fe^{++} chelate/complex, partially disturbing the structural entity of catalase in the haematinic unit, may be the reason for the decrease in enzyme activity in controls;

ii) Radiation-mediated formation of the chelate/complex leading to the same factor as cited above;

iii) Interaction of free radicals produced as a result of radiation, with the amino acid moieties of the enzyme causing deactivation.

The results indicate that radiation probably enhances the MPG-Fe^{++}/Fe^{+++} chelate/complex formation which apparently induces the sensitization effect of MPG for up to 20 Gy (Fig. 1) or 40 Gy (Fig. 2) radiation. The free radical population may not be scavenged by MPG in the form of MPG-Fe^{++}/Fe^{+++} chelate/complex. With doses higher than 20–40 Gy, probably the MPG-Fe^{++}/Fe^{+++} chelate/complex is broken down to MPG and Fe^{++}/Fe^{+++} components. In this condition, while MPG can scavenge free radicals, Fe^{++}/Fe^{+++} can freely restore the structural entity of the haematinic groups by interaction with apocatalase for the restoration of enzyme activity, thereby showing protective effect.

The differences in the pattern of MPG-mediated protective action in Fig. 1 and Fig. 2 needs further clarification. In the case of the high enzyme concentration group (Fig. 2), the

free Fe^{++}/Fe^{+++} could probably restore catalase activity to the MPG treated control level (i.e. without radiation) indicating that the apocatalase portion was not irreversibly damaged by radiation. In the low enzyme concentration group (Fig. 1), since there was only partial MPG-mediated restoration of activity (below MPG treated control level), apparently, the apocatalase portion was damaged more significantly. This may be due to the concentration of free radicals available to react with unit amino acid residues of the apocatalase is higher in the case of low enzyme concentration group, and vice versa.

Thus, our results seem to indicate that the radioprotective effect of MPG is primarily due to free radical scavenging. This is supported by Mishra and Srivastava (1981)[6], and Ambanelli et al. (1981)[10]. The apparent sensitization by MPG for lower doses of radiation was due to circumstantial interaction of MPG with Fe^{++}/Fe^{+++}. However, this dual behaviour of MPG (protection as well as sensitization) must be carefully considered in radiotherapy involving MPG, for the most effective use of this drug.

Additional work is in progress in this laboratory to study the interaction of MPG with Fe^{++}/Fe^{+++} using stopped flow spectroscopy to elucidate the chemical nature of this interaction.

ACKNOWLEDGEMENT

The authors are thankful to Prof. Tsutomu Sugahara, Japan for his donating the MPG, and to Prof. R.R. Mishra for allowing us to use his laboratory facility. They are grateful to Mr. R. Prakasham for his assistance with the experimental work, and to Miss D. Beaujean for typing the manuscript.

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Radiosensitization Newsletter

Vol.8, No.2, April, 1989

A - 2

This paper was published in the last issue (Vol.8 No.1). But since it included some serious misprint, the same paper is published again in its complete form. The editor apologizes for the mistake to the authors.

2-Mercaptoproplonyl Glycine (MPG) Induced Protection of DNA Damage in Gamma-Irradiated Human Lymphocytes: A Preliminary Report

K.K. Wary
Laltanpuia
R.N. Sharan
(Shillong)

2-Mercaptopropionyl glycine (MPG) is an aminothiol which has been found to be a promising radioprotective compound with the effective dose (20 mg/kg body weight) far below the toxic dose (2100 mg/kg body weight) in mice (1-4). The influence of MPG, Thioproline, WR 2721 and Cysteamine on radiation induced lipid peroxidation of erythrocytes show that MPG renders the most effective radioprotection (5).

Lymphocytes are a vulnerable radiation target. It has been shown that lymphocyte count did not increase in cervical carcinoma patients following radiotherapy when the patients received MPG (250 mg/patient) 15 to 30 min prior to irradiation (6). In DNA, ionizing radiations induce predominantly single strand breaks (SSB) but double strand breaks (DSB) and altered DNA bases are also produced (7). The formations of SSB, DSB and intermolecularly linked DNA due to exposure to radiation have been studied for many years. However, no clear understanding about the influence of radioprotective drug, such as MPG, is available. Due to therapeutic importance in radiotherapy it would be desirable to see the influence of MPG on DNA damage. It is further necessitated because our recent study on MPG affordable protection to the enzyme catalase has shown that under specific circumstances MPG may apparently behave as a radiosensitizer rather than radioprotector (4).

Materials and Methods

Chemical:

All chemicals were of analytical grade and were used without further purification. Deionized double distilled water was used to prepare reagents and solutions.

Lymphocyte culture:

From 20.00 ml aseptically drawn human venous blood, lymphocytes were obtained by use of histopaque. Cell population was enumerated in a hemocytometer and adjusted to 2 x 10^6 cells/ml in a growth medium (RPMI 1640, Difco Labs., USA) containing 0.2% phytohemagglutinin (Gibco Labs., USA) and 2.8% sodium bicarbonate at pH 7.2 and supplemented with 15% fetal calf serum (Sigma Chem. Co., USA). The culture tubes were incubated in a CO_2 incubator at 37°C.

Treatment of cells with MPG:

Appropriate amounts of MPG were added to the 58 hour-old lymphocytes in culture tubes to get desired 0.02 mM, 0.05 mM and 0.1 mM concentrations 15 min prior to irradiation. MPG was a gift from Professor T.Sugahara, Japan marketed by Santen Pharmaceutical Co., Japan.

Irradiation:

The lymphocytes (6 - 7 x 10^6 in 3.00 ml of PBS) were irradiated in cold with a ^{60}Co source (Gamma Chamber 900, BARC, India) delivering gamma-radiation at a dose rate of 0.6 Gy/sec.

Hydroxylapatite column chromatography:

Alkaline unwinding technique was employed to analyze DNA strand breaks by use of hydroxylapatite as described by Rydberg (8). The details of the methodology has been described in our previous publication (9). DNA was estimated by the method of Burton (10) with minor modifications.

The damage of DNA has been calculated as ratio of SSB to DSB obtained using the following relation:

$$\frac{\text{amount of DNA eluted by 125 mM phosphate buffer (SSB)}}{\text{amount of DNA eluted by 250 mM phosphate buffer (DSB)}}$$

Each data point in the graph represents mean ± SEM of 4 to 5 independent experiments.

Results and Discussion

In **figure 1**, the control and MPG treated lymphocytes show a dose dependent increase in the damage to DNA following gamma-irradiation. A significant increase in the SSB to DSB ratio was observed in all MPG treated groups, as compared to the control for the dose between 2 Gy and 4 Gy of gamma radiation. But beyond 4 Gy of gamma radiation 0.02 mM and 0.05 mM MPG appeared to induce no significant protection. On the contrary, the group with 0.1 mM MPG continued to show protection to DNA beyond 4 Gy gamma dose also. Hence, our results show that the optimal concentration of MPG necessary to provide protection to DNA for the dose range used in our experiments is 0.1 mM. Apparently, as the dose increases, the amount of MPG should also increase in order to cope up with the increase in damage to DNA or, perhaps, to protect the repair enzymes necessary to repair the damage to DNA. In order to check the influence of 0.1 mM MPG on lymphocyte survival following gamma-irradiation in this dose range, another experiment was conducted (**Fig. 2**). Progressively increasing survival was seen as the dose increased indicating that this dose of MPG was effectively protecting the lymphocyte population without any apparent toxicity.

Our results show that treatment of human lymphocytes with MPG can modify gamma-ray induced DNA damage and repair. The effective dose of MPG to show protection to DNA against Gamma-ray induced strand breaks of MPG (0.02mM and 0.05 mM) show protection only up to 4 Gy dose. For significant protection beyond 4 Gy dose, a higher dose of MPG (0.1 mM) seem necessary. We, therefore, conclude that depending on the dose of radiation required in radiotherapy it may be necessary to adjust the quantity of MPG to be administered for most optimal radioprotection.

Acknowledgement

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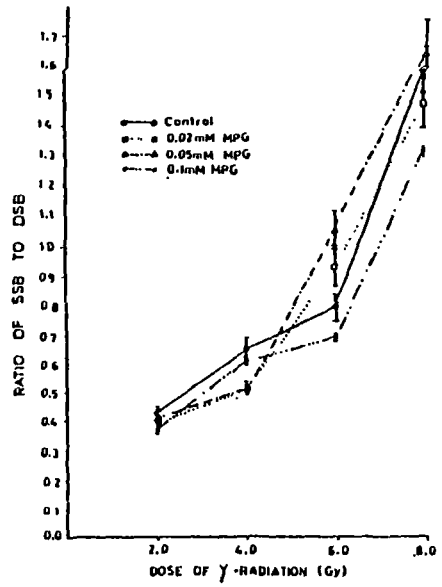

Fig. 1
SSB to DSB ratio following various doses of Gamma radiation to human lymphocytes without (○——○) and with 0.02 mM MPG (□·····□), 0.05 mM MPG (△·····△) and 0.1 mM MPG (●·····●).

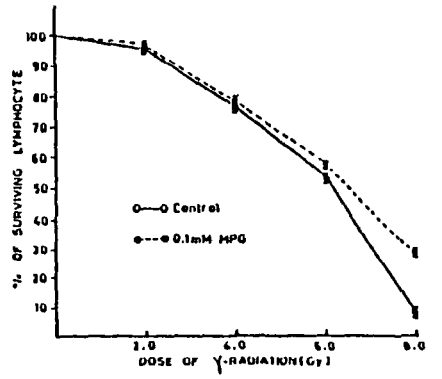

Fig. 2
Effect of 0.1 mM MPG on the survival of human lymphocytes following gamma-irradiation. The cells were incubated with or without MPG (0.1 mM) 15 min before irradiation. The survival count was done 15 min post irradiation using dye-exclusion technique. The survival in absence of MPG is shown by ○——○ and that in presence is shown by ●·····● .

COMMUNICATED PAPERS

PERIODICITY OF GAMMA-GLUTAMYL TRANSPEPTIDASE ACTIVITY IN MICE KIDNEY FOLLOWING ARECA-NUT AND ARECOLINE FEEDING *ad libitum*.

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Running title: Areca nut and arecoline induced GGT activity in mice.

Key words: Areca-nut, Arecoline, Gamma-glutamyl transpeptidase, preneoplastic changes.

SUMMARY:

To evaluate the carcinogenic potency of areca nut (*Areca catechu L.*) mice were fed with crushed areca nut or arecoline, with their normal diet and gamma-glutamyl transpeptidase (GGT) activity was monitored in kidney cells for various time intervals up to 180 days. A periodicity in GGT activity was observed; the peak of enzyme activity repeating every 60 days post-treatment. This report discusses implication of GGT in clinical evaluation of preneoplastic changes following areca nut and its alkaloid treatment.

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INTRODUCTION:

Gamma-glutamyl transpeptidase (GGT) is an enzyme, predominantly membrane bound, occurring in both normal and preneoplastic tissues. This enzyme plays important roles in glutathione, S-substituted glutathione derivatives and gamma-glutamyl compound metabolisms and transport of amino acids and peptides across the membrane [Richards 1983]. Even though the role of GGT in normal cellular metabolism or with respect to tumorigenesis is not absolutely clear, this has been employed as the most widely used indicator of preneoplastic transformation [Richards 1983; Hatayagi et al 1975] because elevated levels of GGT has been reported in neoplasm of several tissues of various animal species [see Richards 1983]. Areca nut (Areca catechu L) contains several alkaloids, notably arecoline, arecaidine, guvacoline, and guvacine [IARC 1985]. Arecoline has been reported as mutagenic and carcinogenic in vivo and in vitro [IARC 1985]. Earlier we have reported that aqueous extract of betel (areca) nut induced significant DNA strand breaks in primary kidney cells in vitro and appreciably enhanced the rate of cell proliferation which was interpreted as preneoplastic change [Wary and Sharan 1988]. In this report we have used GGT as marker of preneoplastic change in vivo in mouse kidney cells to reconfirm the carcinogenic potency of raw areca nut of North-East India because this raw areca nut consumed here is remarkably different from the processed betel nut consumed elsewhere in the world [for detailed account see Wary and Sharan 1988].

MATERIALS AND METHODS :

Chemicals: Arecoline, glycylglycine, L-gamma-glutamyl-p-nitroanilide and Bovine serum albumin (BSA) were purchased from Sigma Chemical Co (USA). All chemicals used in these experiments were of analytical grade and were used without further purification.

Mice: Specific pathogen free Swiss albino mice were obtained from Assam Veterinary Biologicals, Gauhati (India). Five weeks old age matched male mice were selected for this experiment.

Areca nut and arecoline diet: Fresh, raw areca nut, obtained locally, were finely crushed and fed to mice along with regular food and normal drinking water. At the same time, arecoline (1 mM or 3 mM) in drinking water were given to separate batches of mice to serve as positive controls. These groups of mice received normal food.

Sacrifice: Mice were maintained on areca nut and arecoline substituted diet and were killed on the 10th, 30th, 60th, 90th, 120th, 150th and on the 180th day. Kidneys were removed and weighed. The tissues were washed with phosphate buffered saline.

Assay system: The assay of gamma-glutamyl transpeptidase activity in mouse kidney homogenate (15% w/v in 0.1 M Tris-HCl, pH 8.0) were carried out according to Meister et al. [1981]. The assay time was five minutes. Each assay was repeated 5 to 6 times in a thermostated (37°C) UV-150 JASCO spectrophotometer. The enzyme activity is expressed as units/100 milligram protein.

Total protein estimation: The total protein estimation was

carried out according to Bradford (1976) using BSA as standard.

RESULTS AND DISCUSSION :

Animals were divided into four different set (each with 5 male mice) as follows (i) mice maintained on normal diet and water (control), (ii) mice fed with ground raw areca nut with normal diet and normal water, (iii) mice on normal diet and water containing 1 mM arecoline and (iv) mice on normal diet and water containing 3 mM arecoline. Figure 1 shows the changes in GGT activity over the entire span of study for various groups of animals. GGT activity substantially increased up to the 60th day as compared to the control regardless of the mode of treatment. This was followed by a gradual decline in GGT activity in all groups till the 120th day. However, this decline was again succeeded by another peak in the GGT activity profile for all sets, whereby it reached a relatively higher value by the 150th day followed by a slower decline until the end of the experiment. GGT activity in the control set showed gradual increase with time. The inset of figure 1 shows the total protein content in mice kidney for these sets of experiments. Unlike the recurring periodicity in GGT activity, the total protein content was comparatively stable; the increase probably depicts the normal growth of the mice.

GGT enzyme activity have been found to increase in many growing tissues, ageing cells and in differentiating tissues [Richards

1983]. This increase in activity of GGT probably indicates that amino acid requirements have increased in response to growth, as this enzyme is implicated in the transport of amino acids across the membrane [Meister 1975]. It can be seen from the figure that areca nut and both doses of arecoline feeding exert many fold increase in the GGT activity over the control; for the two doses of arecoline (1 and 3 mM) the induction of GGT was dose dependent. The increase in GGT activity had been shown to significantly increase ^{3}H-TdR labelling index in GGT positive rat hepatocyte foci [Pugh and Goldfarb 1978]. Our own study, to be published separately, using Hep 2 cells *in vitro* show an arecoline dose dependent enhanced ^{3}H-TdR labelling apparently indicating enhanced cell proliferating activity. But, our earlier report on direct observation of cell proliferation [Wary and Sharan 1988] shows that while aqueous extract of betel nut can enhance the rate of cell proliferation, arecoline fails to do so. This may mean that the present observation of enhanced GGT activity both by areca nut and arecoline need not necessarily indicate enhanced cell proliferation as also suggested by Richards [1983]. Since GGT plays a major role in the gamma-glutamyl cycle which metabolizes glutathione, the increased activity of GGT may indicate increased local concentration of glutathione following areca nut and arecoline treatments. This may mean involvement of glutathione in the process of detoxification of these carcinogens. The increased amount of glutathione, on the other hand, enhances the absorption of

amino acids by the renal cells probably to account for the higher rate of protein synthesis, including that of GGT. The significance of the periodicity of increase and decrease in activity of GGT remains obscure at the moment but is probably indicative of the alteration of glutathione metabolism in mouse kidney after exposure to areca nut and arecoline. Thus it is apparent that areca nut and its alkaloids may be able to directly influence the normal glutathione metabolism. The peak of GGT activity probably signifies that some carcinogen may be metabolized or detoxified by gamma-glutamyl cycle with the help of GGT. However, glutathione metabolism anamolies may induce mitotic anamolies as had been suggested by Meister earlier [1975] leading to preneoplastic changes. Work is under progress to examine the significance of periodicity of GGT following areca nut treatment. Nonetheless, our experimental data provide a clinical basis of evaluation of areca nut and arecoline induced preneoplastic changes. The concentration of arecoline used in our experiments is clinically below the toxic level [1980]. Our results, thus, show that chronic exposure of areca nut and arecoline in vivo below toxic levels can also alter the normal GGT related metabolic functions of the kidney cells.

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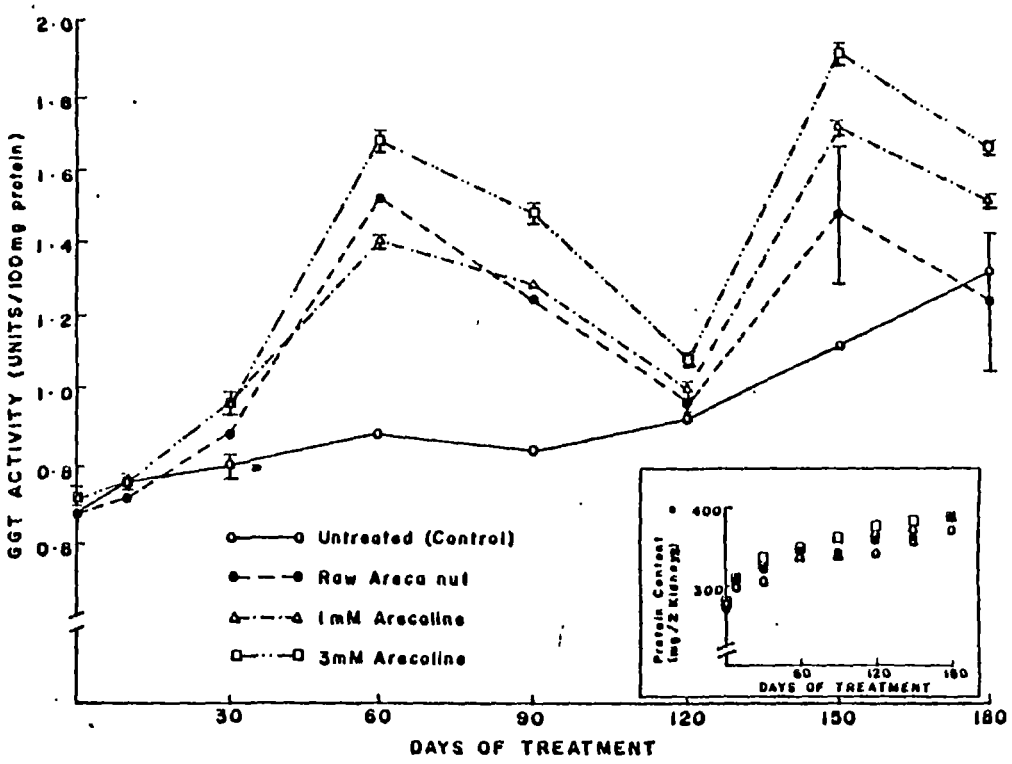
FIGURE LEGENDS :

Figure 1. Gamma-glutamyl transpeptidase enzyme activity [Units/100 mg protein] following area nut or arecoline treatments over a periods of 180 days [mean ± SEM]. The bars indicate SEM; when there is no bar it means the deviation was smaller than the thickness of the line. The inset shows the average total protein in kidneys over the same protocol.


GGT ACTIVITY (UNITS/100mg protein)
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
30
60
90
120
150
180
DAYS OF TREATMENT
Untreated (Control)
Raw Areca nut
1mM Arecoline
3mM Arecoline
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DAYS OF TREATMENT

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EUROPEAN SOCIETY FOR RADIATION BIOLOGY 22nd ANNUAL MEETING
BRUSSELS, SEPTEMBER 11-16, 1989

EFFECTS OF ARECOLINE AND SODIUM NITRITE ON IN VITRO CULTURED HUMAN CELLS

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Arecoline, an active alkaloid of Areca catechu L and sodium nitrite, a food additive, have been shown to be cytotoxic and cytostatic in Hep 2 human cell line if administered in an acid environment [pH 4.2] in presence of S-9 mixture. We have found that their effects were additive in nature. On treatment of Hep 2 cells to this combination, the decreased cell survival and de novo DNA synthesis indicates mutagenecity of these chemicals to Hep 2 cells. Mutagenecity was further substantiated by the fact that on ^{3}H-Thymidine labelling showed synthesis of DNA outside the S-phase. The observation of temporary inhibition of DNA synthesis by these chemicals seemed due to metabolic block, because upon removal of these chemicals DNA synthesis was restored. We also observed delay in protein synthesis. The cytotoxicity seemed related to the replicative activity of DNA.The role of chemically reactive species formed by arecoline and sodium nitrite will be discussed in relation to cytotoxicity.