Review of Literature

A brief survey of literature related to the carcinogenicity of smokeless tobacco and other relevant information was done. Topics included here are: (1) Botany and chemistry of tobacco, (2) Tobacco usages, (3) Tobacco and diseases (epidemiological aspect with special reference to oral cancers), (4) Pharmacological effects, (5) Prenatal toxicity and effects on reproduction and (6) Experimental studies - long term carcinogenicity studies in animals, short term tests of mutagenicity/genotoxicity and human studies.

BOTANY AND CHEMISTRY OF TOBACCO :

The cultivation of the plant dates back to atleast 7000 years. It was initially grown for its ornamental and medicinal values. Currently it is grown all over the world in appreciable amounts for its commercial values. Major tobacco growing states in India are Bihar, Bengal, Uttar Pradesh, Gujarat, Maharastra, Tamilnadu, Karnataka and Andhra Pradesh.

The tobacco plant belongs to the family Solanaceae. Out of 50 different species of tobacco, *N. tabacum* and *N. rustica* are commercially important. *N. tabacum* is a small annual herb with large, oval and sessile leaves clasping at their base and with pink flowers. *N. rustica*, probably originated in Mexico and is still grown in north America, is a hardier plant with yellow flowers.

In India tobacco is chiefly grown as a winter crop, however, different seasons are chosen for the cultivation in different provinces. After harvesting when leaves are ripe

the required texture, aroma and colour to the final product are provided by curing. It usually takes 3 to 6 months. Air-curing and flue-curing are the two principal methods of tobacco curing. Light air-cured tobacco is used for the production of cigarette, pipe and chewing tobacco, whereas, dark air-cured tobacco is used for the production of snuff and chewing tobacco.

N. tabacum has been analysed to understand the chemical composition of tobacco. About 2549 individual constituents have been identified in tobacco (Dube and Green, 1982). The alkaloids **(Fig.1)** comprise the commercially valuable class of chemicals in tobacco.

Nicotine accounts for about 95 % of the total alkaloids and the remainder being made up of nornicotine, myosmine, cotinine, anabasine, anatabine etc., in various proportions. Nicotine is present in L(-) form, which is the pharmacologically active substance. It also occurs as nicotine-N'-oxide in chewing tobacco. The concentration of nicotine in Indian chewing tobacco has been reported to vary between 2.05 % and 3.87 % (Brunnemann et al., 1985). Compared to N.tabacum, higher concentrations of nicotine, anabasine and nornicotine have been detected in N.rustica.

During curing and fermentation of tobacco four tobacco-specific nitrosamines (TSNAs), viz. N'-nitrosonornicotine **(NNN)**, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone **(NNK)**, N'-nitrosoanatabine **(NAT)** and N'-nitrosoanabasine **(NAB)**

Figure-1

MAJOR TOBACCO ALKALOIDS

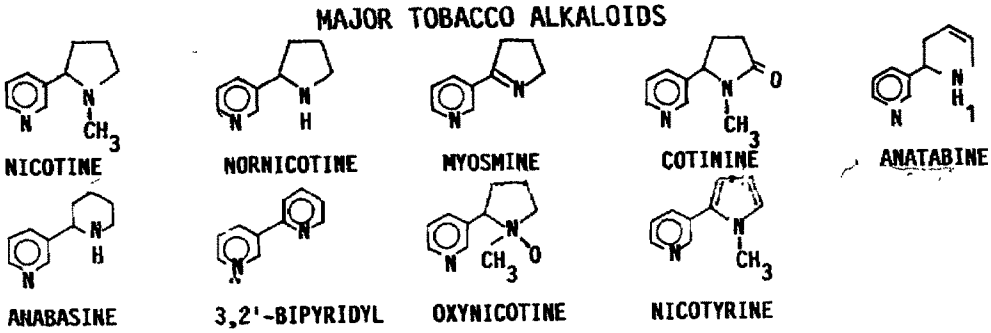
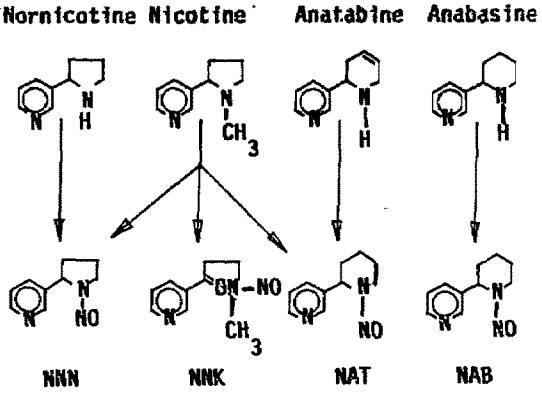


Figure-2

FORMATION OF TOBACCO-SPECIFIC NITROSAMINES (TSNA)



(Fig.2) are formed from nicotine, nornicotine, anatabine and anabasine (Hoffmann et al., 1984). Bhide et al.(1987b) have shown the presence of TSNAs in mature green leaves of N.tabacum and N.rustica. Moreover, they have also reported elevated concentrations of TSNAs in sun-dried tobacco than green tobacco and the highest concentration was found in the processed variety. The effects of post-harvesting processing on TSNA content of tobacco have been described in detail by Anderson and Kemp (1985). Experimental studies on a variety of chewing tobacco used commonly in India have determined total TSNAs in the range of 1000-1450 ppb (Brunnemann et al., 1985). NNN and NNK have been estimated in µg. quantities per gm. of chewing tobacco samples from the Western India (Bhide et al., 1984).

Tobacco contains 0.65 % to 0.80 % nitrate which is reduced to nitrite during processing. The possible role of nitrite in the formation of TSNAs has been discussed (Andersen et al., 1987; Hecht et al., 1978). The wax constituents of the leaf belong to the aliphatic hydrocarbon groups of chemicals. Formaldehyde ranged from 1.6-7.4 µg/gm of snuff (Hoffmann et al., 1987). Isoprenoids formed during post-harvest period contribute aroma to the leaf. These isoprenoids have one or more alcoholic functional groups. Phytosterols, like free alcohols, esters and glycosides, have been reported in µg/gm quantity of tobacco. Coumarins, caffetannins and flavonoids are the major phenols present in the leaf. The two major polyphenols namely, chlorogenic acid (0.0 - 3.2 %) and rutin

(0.08 - 1.29 %) have been detected in processed tobacco (Hausermann and Waltz, 1962). More than 80 organic acids have been identified in tobacco. High levels of malic acid and citric acid have been detected in the processed tobacco. The leaf also contains free amino acids. 27 volatile amines, 11 aromatic amines and more than 50 N-heterocyclic compounds have been detected in processed tobacco. Polycyclic aromatic hydrocarbons (PAHs), an important class of carcinogens, have also been identified in trace amount in the processed tobacco only. It has been suggested that presence of PAHs may be due to contamination with ambient air pollutants during curing process. Lower levels of PAHs have been observed in air-cured tobacco than flue-cured tobacco (Passey et al., 1971). Moreover, the type of soil, aging, curing, fermentation, harvesting period, position of leaf on plant and different parts of the leaf (stems, stalk, veins etc.) are the factors affecting the ultimate chemical composition of final tobacco product.

TOBACCO USAGES:

Historical Perspective:

As early as 1493, the smokeless tobacco was used by Europeans in the form of dry snuff, i.e. unscented powdered tobacco. It was inhaled to clear the nasal passage and was also used as analgesic. In early 1600s, use of snuff became popular in South America, Japan, Africa, Sweden and England. It reached a peak in England during 1702-1714. Tobacco chewing has not been described until 1704 in U.S.A. (Gottsegen,

1940). Initially it was used against hunger, thirst, fatigue and for teeth cleaning. By 1800s, chewing of loose tobacco leaf and plug tobacco became popular and reached a peak in the U.S.A. in the last decade of 19th century (Heimann, 1960).

During late 1800s, chewing and spitting were severely criticized by Koch, Pasture and Lister. As the "germ theory of infection" gained recognition, the habit of public spitting, formerly viewed as merely disgusting, was considered as a public health menace. With the advent of antispitting laws and loss of social acceptability, tobacco chewing decreased greatly during the 20th century (Cullen et al., 1986). After the first world war, the use of manufactured cigarettes became common. Only after a couple of decades, the phenomenal rise in lung cancer was observed. Now with intense antismoking programmes, smoking is on decline and the use of snuff and chewing tobacco is once again increasing, especially among the youth (Guggenheimer et al., 1986; Hunter et al., 1986; Marty et al., 1986). Even after realizing the adverse effects of tobacco consumption on health, tobacco chewing has been considered less of a social evil (Glover et al., 1984) and erroneously, a less harmful or safer alternative to smoking (Binnie et al., 1983; Chassine et al., 1985; Schaefer et al., 1985).

Pan or betel chewing presumably originated in India. However, it is not clear when the addition of tobacco to the

betel quid began. It might perhaps be around the 14th century, when tobacco was introduced to Africa and Asia by the Turks (Jayant and Deo, 1986).

Current Practices:

The use of smokeless tobacco, predominantly in the form of chewing tobacco and snuff, is a worldwide practice. Several distinct combinations of unburnt tobacco with other ingredients have been reported to be prevalent in different countries. Some are presented in the following table.

Country	Name of the product	Composition
India	Tobacco alone	per se
	Snuff (dry)	Powdered tobacco
	Masheri/Misheri	Roasted/half burnt tobacco powder
	Khaini	Tobacco + lime
	Zarda	Tobacco + lime + spices etc.
	Kiwam	Tobacco + rose water + saffron/cardamom/musk etc.
	Granules	Dry pellets of Kiwam
	Gudakhu	Tobacco + molasses + other unspecified ingradients
	Mainpuri	Tobacco + areca nut + lime (a ready-made mixture)
	Betel quid with tobacco	Betel leaf + areca nut + lime + catechu + Tobacco etc.
	Mava/Masala	Tobacco + areca nut + lime (taken freshly mixed)
Pakistan & Afghanistan	Naswar	Powdered Tobacco + lime + indigo
Soudi Arabia	Shammah	Powdered Tobacco + lime + ash + other substances
Iran & U.S.S.R.	Nass	Tobacco + lime + ash + cotton oil/sesame oil
U.S.A. & Europe	Fine-cut tobacco/ Moist snuff/ Tobacco sachets	Powdered Tobacco + wintergreen oil + licorice + other flavouring agents

Plug tobacco/ Pressed-leaf tobacco	Tobacco leaf fragments + honey + sugar + syrup and/or licorice
Loose-leaf/ Scrap tobacco	Fermented tobacco leaf + sugar + syrup + licorice + flavouring agents
Twist tobacco/ Roll tobacco	Powdered tobacco + sugar + molasses and/or syrup

TOBACCO AND DISEASE (EPIDEMIOLOGICAL ASPECT):

Tobacco has been universally accepted as the major cause of human morbidity and mortality. About 2.5 million people die every year from diseases associated with tobacco (Mahler, 1988). Out of an estimated five million deaths in the adult population in India, a minimum of 6,30,000 deaths can be attributed to health hazards related to use of tobacco (Luthra et al., 1990). Of all other diseases, cancer of oral cavity, upper aerodigestive tract and lungs have been doubtlessly documented as a direct consequence of either chewing or smoking tobacco.

TOBACCO AND ORAL CANCER:

All over the world, tobacco related cancers are the major health problems and constitute one, two or three leading cancer sites out of the first five. Oral cavity cancers (ICD 140-145) are the only well-established, life-threatening chronic diseases associated with the use of smokeless tobacco, with squamous cell carcinoma being the most common histological type.

The custom of chewing 'pan', composed of betel leaf, areca nut, slaked lime, tobacco and other ingredients, has been

linked to the excessive rate of oral cancers observed in India. Niblock (1902) has attributed the development of oral cancer to the habit of chewing pan and tobacco. Bentall (1908) noted habit of chewing betel quid with tobacco among the oral cancer patients. Fells (1908) was the first to observe that cancer developes at the site where the quid was habitually placed. Orr (1933) has reported an analytical study on the role of tobacco chewing habit together with poor nutrition in the occurrence of oral cancer. He has grouped the cancers of alveolus, tongue, floor of mouth, cheek, palate and lip as oral cancer. The work of Khanolkar (1944) , a major landmark in the field of cancer epidemiology in India, dealt with different lifestyles and concluded that it was the addition of tobacco to the areca nut which made it carcinogenic. The report also highlighted the importance of statistical methods in establishing associations between cancer and causative factors. A supportive study from Madras has pointed out that chewing of betel leaf, areca nut and tobacco is probably the dominant etiological factor in causation of oral cancers (Shanta and Krishnamurthy, 1963). The working group of International Agency for Research on Cancer (1985) reported prevalence of oral cancer among tobacco chewers from the data obtained during a cross-sectional survey in Kerala, Gujarat, Bihar and Andhra Pradesh (Mehta et al., 1971). Comparative analysis of relative risk of oral cancer by The Advisory Committee to the Surgeon General, U.S.A. (1986), from the case-control studies at Calcutta, Madras and Bombay,

has revealed considerably higher risk of oral cancer for the users of betel quid with tobacco compared to those chewing the betel quid without tobacco. Smokers have not been included in these data. A critical review of studies of betel quid chewing, with and without tobacco, has clearly mentioned that in absence of tobacco, the quids may be very weakly carcinogenic, implying that either tobacco is the active agent or that other ingredients need to be combined with tobacco for producing oral cancer (Gupta et al., 1982).

In case of tobacco chewing habit and its association with the development of oral cancer, extensive epidemiological studies have strongly suggested:

(i) The close correlation between use of smokeless tobacco and the incidence of oral cancer in India,

(ii) Development of oral cancer at sites where the tobacco is usually placed,

(iii) A higher incidence of oral cancer among those who sleep with the tobacco in their mouth, and

(iv) Chances of developing oral cancer are ten times higher, if the habit of tobacco chewing starts under the age of 14 years (Wahi, 1976).

A possible link between smokeless tobacco use and oral cancers in north America and Europe was suggested for the first time by Abbe (1915). Later on, in Sweden, Ahblom (1937) observed that the use of snuff and chewing tobacco was more frequent among patients with buccal, gingival and mandibular

cancers, than with other cancers. In United States, case reports of oral cancer among users of snuff and/or chewing tobacco appeared in the early 1940's (Friedell and Rosenthal, 1941). For the first time, during the early 1950's (Moore et al., 1952), epidemiologic study was conducted in this relation. From the case-control studies carried out by Moore et al. (1953) in Minnesota; Peacock et al. (1960) in North Carolina; Vincent and Marchetta (1963) in New York and Williams and Horm (1977) on patients from the Third National Cancer Survey (1969 - 1971), U.S.A., a strong association between the use of chewing tobacco and/or snuff (unspecified) and cancer of oral cavity has been suggested. Wynder et al. (1957) have indicated a moderate correlation between tobacco chewing and cancer of the oral cavity and pharynx. Vogler et al. (1962) observed a significantly high percentage of tobacco chewers among the group of oral cancer patients. The working group of International Agency for Research on Cancer has noted that this relation was not confounded by smoking (IARC, 1985). A cohort of tobacco chewers or oral snuff users (not specified), studied by Schuman et al. (1982) in Norway, showed that for regular users of tobacco, the relative risk was 2.8 for buccal cavity cancers. The results of the study carried out among the women using oral snuff have shown a four fold increase in the risk of oral cancer. The risk for developing cancer of cheek and gum, where the tobacco was routinely placed, was far higher, reaching close to 50-fold, for 50 or more years of use (Winn et al., 1981).

International Agency for Research on Cancer (1985) has evaluated the available literature and concluded that:

(i) There is sufficient evidence that oral use of tobacco mixed with lime (Khaini) and chewing of betel quid with tobacco is carcinogenic to human beings,

(ii) There is inadequate evidence for the carcinogenic potential of oral use of other smokeless tobacco preparations, e.g. nass, naswar, mishri, gudakhu and shammah,

(iii) There is sufficient evidence that snuff of the types commonly used in North America and Western Europe, is carcinogenic to humans, however, there is limited evidence that chewing tobacco, of the types commonly used in these areas, is carcinogenic.

TOBACCO AND ORAL PRECANCER:

Oral cancer is almost always preceded by some oral lesions (WHO, 1984). The precancerous nature of oral submucous fibrosis (O-SMF), an insidious, chronic disease affecting the oral mucosa, was mentioned for the first time by Paymaster (1956). The disease appears almost exclusively among Indians and Pakistanis (Evenson, 1983). Pindborg et al. (1968) have observed the highest prevalence of the disease in Kerala and the lowest in Banglore. Paymaster (1956) observed the development of squamous cell carcinoma in about 1/3 of the patients with O-SMF. McGurk and Craig (1984) have also reported the malignant transformation of O-SMF in two Indian women living in U.K. Chewing of areca nut has been identified as the important etiologic factor in the causation of O-SMF

(Gupta et al., 1980; Mehta et al., 1972; Sirsat and Khanolkar, 1962). It has also been suggested that this condition might not be associated with smoking or with chewing only tobacco (Lucas, 1964), however, it could be a contributor to the development of O-SMF when used in combination with areca nut (Pindborg et al., 1968).

TOBACCO AND NON-CANCEROUS DISEASES:

Besides the development of oral cancer and precancerous lesions, tobacco usage can also produce other unhygenic as well as pathological alterations in oral soft and hard tissues, namely, bad breath, decreased ability to smell and taste, discoloured teeth & gingival recession, periodontal bone destruction and tooth abrasion (Christen, 1985; Christen and Glover, 1981). The hemodynamic changes produced by oral smokeless tobacco use were similar to that of cigarette smoking, which include: increase in heart rate and systolic/diastolic blood pressure, decrease in blood flow through the coronary circumflex etc. (Squires et al., 1984). However, no direct epidemiological data are available on cardiovascular morbidity and mortality associated with smokeless tobacco usage.

PHARMACOLOGICAL EFFECTS:

Use of smokeless tobacco brings about various psychological effects like relaxation, arousal and euphoria. It also leads to a state of dependence in most regular users. All forms of tobacco deliver a centrally active substance, nicotine (Jaffe, 1985), which has been identified as the

habituating factor in tobacco. Studies in humans and animals have shown that it produces dose-related changes in mood and feelings which are mediated by central nicotine receptors i.e. nicotine is psychoactive (Henningfield and Goldberg, 1985). Despite the initial unpleasant side effects like tremulousness, dizziness and nausea, tolerance to smokeless tobacco has been observed among the addicted users. At higher doses nicotine was found to be extremely toxic and rapidly acting poison (Arena, 1979). The ingestion of nicotine can induce symptoms, like central nervous system depression, convulsions etc., in adults, and ~40 mg. i.e. 0.6 - 0.9 mg/kg body weight is reported to be lethal to adults (Hanson, 1984).

PHARMACOKINETICS:

In vivo **: (Absorption, Distribution, Metabolism, Excretion)**

Following 15 minutes of oral use of tobacco, nicotine and TSNAs have been detected in the saliva. Increased level of TSNAs and cotinine in saliva has been reported with increase in the period and frequency of snuff dipping (Brunnemann et al., 1987). The analysis of saliva of chewers, who use betel quid with tobacco, revealed the presence of areca nut specific nitrosamines (ASNAs), three TSNAs viz. NNN, NNK, NAT and volatile nitrosamines (VNAs) in a few cases, plus nitrate, thiocyanate, nicotine and arecoline (Nair et al., 1985; Shivapurkar et al., 1980; Sipahimalani et al., 1984; Wenke et al., 1984a). ***In vivo*** formation of TSNAs has also been suggested (Nair et al., 1987).

Nicotine is absorbed through the oral mucosa into blood stream. Blood nicotine level, measured within 5 minutes of oral use of smokeless tobacco, was comparable to that achieved during cigarette smoking by a dependent smoker (Russell et al., 1980). Since smokeless tobacco products are usually buffered to alkaline pH that facilitate absorption of nicotine, it has been reported that a smokeless tobacco consumer gets exposed to more nicotine than cigarette smoker. At pH 11 or more, an accelerated rate of its absorption, reaching to the central nervous system very quickly, has also been documented (Brunnemann et al., 1985). Moreover, Russell et al. (1985) have reported a more gradual increase in its level during oral use of smokeless tobacco, in comparison to that of smoking. The accumulation of nicotine in blood has been suggested with repeated long-duration smokeless tobacco use (Cullen et al., 1986). The metabolic conversion of nicotine to Nicotine-1'-N-oxide or cotinine has been discussed in detail by Gorrod and Jenner (1975).

Nicotine, cotinine and TSNAs have been detected in the urine samples from chewers of tobacco alone or of betel quid with tobacco (Nair et al., 1985; 1987). It has also been observed that urinary nicotine:cotinine ratio increases with the increase in period of tobacco consumption per day. Higher urinary recovery of more polar nornicotine and anabasine, in comparison to their tertiary amines (nicotine and methylanabasine), has indicated a high degree of absorption of these alkaloids (Gorrod and Jenner, 1975).

PRENATAL TOXICITY AND EFFECTS ON REPRODUCTION:

Tobacco chewing has been reported to increase the rate of still-births among Indian women. The lower mean birth weight in offsprings of tobacco chewers was associated with a decrease in the mean gestation period (Krishna, 1978; Verma et al., 1983).

N. tabacum was found to induce defects, like cleft palate, in the offspring (Crowe, 1978). Nicotine has also been reported to be teratogenic in rabbits (Vara and kinnunen, 1951), mice (Nishimura and Nakai, 1958) and chicks (Landauer, 1960), however, failed to induce defects in pigs, sheep or cows (Keeler, 1979).

EXPERIMENTAL STUDIES: (CARCINOGENESIS)

Carcinogenicity in Animals:

The long-term carcinogenicity testings have been recommended strongly for the confirmation of epidemiological observations. The tumourigenicity/carcinogenicity of tobacco and its extracts have been reported in various animal models by administering them through different routes and for different durations **(Table-1)**. However, repeated experimental studies have failed to provide adequate evidence that chewing tobacco, snuff or extracts derived from them can induce cancer in animals (IARC, 1985; 1987). Moreover, testing of nicotine for carcinogenicity is difficult because of the high toxicity (Boyland, 1968).

Table-1
CARCINOGENICITY STUDIES IN ANIMALS
(Reports published after 1975)

Species	Route	Extraction	Effect	Reference
TOBACCO				
Rat	Cheek pouch	Acetone	No tumour at site of application Hyperkeratinization and patches in oral mucosa	Ghothoskar et al.,1975.
Mouse	Skin	DMSO	No skin tumour	Ranadive et al.,1976.
Hamster	Cheek pouch	DMSO	No tumour, only moderate hyperkara-tosis.	Ranadive et al.,1976.
Rat	Oral mucosa	Acetone	No frank carcinoma	Ranadive and Gothoskar,1978.
Mouse	Oral intubation	Ethanol	lung adenocarcinoma (8/15); hepatocell-lar carcinoma,4/10	Bhide et al., al.,1984.
Hamster	Cheek pouch	Aqueous	Squamous cell papilloma/carcinoma 3/17	Rao,1984.
Mouse	Oral	Alcoholic	42-53% tumour incidence	Shah et al., 1985.
Mouse	Oral, in diet	Alcoholic	44% tumour incidence	Shah et al., 1985.
Mouse	Oral, in diet	per se	41% lung tumour	Bhide et al., 1989.

TOBACCO + LIME				
Hamster	Cheek pouch Beeswax pellet	per se	No tumour at site of implantation	Dunham and Harrold,1962.
Rat	Oral mocosa	Acetone ext. + lime	Marked keratiniz-ation & epithelial hyperplasia	Ranadive and Gothoskar,1978.
SNUFF				
Hamster	Oral, in diet	per se	No increase in tumour incidence	Homberger et al.,1976.
Rat	surgically created canal in lower lip	per se	No local tumour	Hirsch and Johansson, 1983.
Rat	Surgically created canal in lower lip	per se	No local tumour	Hirsch et al.,1984.
Rat	Oral	Aqueous	No tumour	Hecht et al., 1986.
SNUFF + LIME				
Hamster	Cheek pouch depositon	per se	No tumour, only inflammatory lesion	Dunham and Harrold,1962.
Hamster	Cheek pouch	per se	No tumour	Dunham et al., 1966.
SNUFF + NNN/NNK				
Rat	Oral	Aqueous	No tumour	Hecht et al., 1986.

SNUFF + HSV-1				
Rat	Surgically created canal in lower lip	snuff-per se HSV-1-injection	Squamous cell carcinoma of oral cavity; statistically insignificant	Hirsch et al., 1984.
MASHERI				
Mouse	Midscapular region	Acetone	20 % papilloma	Bhide et al., 1987a.
Rat	Oral, in diet	per se	37% forestomach papilloma	Kulkarni et al.,1988.
Mouse	Oral, in diet	per se	42-47% forestomach papilloma	Kulkarni et al.,1988.
Hamster	Oral, in diet	per se	25-43% forestomach papilloma	Kulkarni et al.,1988.
Mouse	Oral, in diet	per se	53-56% stomach papilloma	Bhide et al., 1989.
Mouse	Skin	Toluene	No tumour, only hyperplasia	Kulkarni et al.,1989.
Mouse (bare)	Skin	Toluene	30-35% tumour incidence by black masheri	Kulkarni et al.,1989.
ZARDA				
Hamster	Cheek pouch	per se	No malignant tumour only keratotic and dysplastic changes	Kandarkar et al.,1981.

NASS

Hamster	Skin	Aqueous	No local tumour	Kiseleva et al.,1976.
Hamster	Cheek pouch	per se	No local tumour	Milievskaja and Kiseleva, 1976.

BETEL QUID WITH TOBACCO

Hamster	Cheek pouch	Aqueous	Forestomach carcinoma 4/13	Ranadive et al.,1979.
Hamster	Cheek pouch wax pellet	per se	Cheek pouch carcinoma 3/21; Forestomach carcinoma 6/21	Ranadive et al.,1979.
Mouse	s.c.	Aqueous	Local sarcoma 2/20	Shivapurkar et al.,1980.
Mouse	Oral intubation	Aqueous	Statistically insignificant increase in lung tumour incidence	Shirname et al.,1983.

TABLE-2
DETAILS OF SHORT TERM STUDIES
(Reports published after 1975)

Cell type	Extraction	Assay	Effect	Reference
TOBACCO				
Syrian hamster embryo cells	Ethanol	Transformation (- S9)	Positive	Umezawa et al.,1978
Syrian hamster embryo cells	Ethyl acetate	Transformation (- S9)	Positive	Umezawa et al.,1978.
Larvae of Drosophila	per se	Sex chromosome loss Sex-linked recessive lethal mutation Autosomal translocation	Negative	Abraham et al.,1979.
Hamster embryo cells	Ethyl acetate	Transformation (- S9)	Positive	Umezawa et al.,1981.
Lymphocytes	Ethyl acetate	SCE (- S9)	Positive	Umezawa et al.,1981.
Lymphoblastoid cell line	Ethyl acetate	SCE (+/- S9)	Positive	Umezawa et al.,1981.
V79	Ethyl acetate	Mutation (- S9)	Negative	Umezawa et al.,1981.
CHO	Salivary-Indian type tobacco	CA (+/- S9)	Positive	Stich and Stich,1982.
CHO	Salivary-Western type tobacco	CA (+/- S9)	Negative	Stich and Stich,1982.

S.typhimurium TA98, TA100, TA1535, TA1538	Ethanol	Mutation (+/- S9)	Positive for TA98 with S9	Bhide et al.,1984.
V79	Ethanol	Mutation (+/- S9)	Positive	Shirname et al.,1984.
Mouse Bone-marrow	Ethanol	MN	Positive	Shirname et al.,1984.
S.typhimurium TA98, TA100	Organic	Mutation (- S9) (- nitrite)	Negative	Whong et al.,1985.
S.typhimurium TA98, TA100	Aqueous	Mutation (- S9) (- nitrite)	Negative	Whong et al.,1985.
S.typhimurium TA98, TA100	Organic	Mutation (- S9) (+ nitrite)	Positive	Whong et al.,1985.
S.typhimurium TA98, TA100	Aqueous	Mutation (- S9) (+ nitrite)	Positive	Whong et al.,1985.
S.typhimurium TA98, TA100, TA1535, TA1538	Ethanol	Mutation (+/- S9)	Positive for TA98	Shah et al.,1985.
V79	Ethanol	Mutation (+/- S9)	Positive	Shah et al.,1985.
C3H/10T1/2	Aqueous	Transformation (- S9)	Negative	Stich and Tsang,1989.
CHO	Aqueous	CA (- S9)	Positive	Stich and Tsang,1989.

S.typhimurium TA98	Ethanol	Mutation (+ S9)	Positive	Bhide et al.,1989.
Mouse bone-marrow	per se	MN	Positive	Bhide et al.,1989.
SNUFF				
S.typhimurium TA98, TA100 TA1535	Organic	Mutation (- S9)	Negative at neutral pH, Positive at acidic pH	Whong et al.,1984.
S.typhimurium TA98, TA100 TA1535	Aqueous	Mutation (- S9)	Negative at neutral pH, Positive at acidic pH	Whong et al.,1984.
MASHERI				
S.typhimurium TA98	Toluene	Mutation (+ S9)	Positive	Bhide et al.,1987a.
V79	Toluene	Mutation	Positive	Bhide et al.,1987a.
Mouse bone-marrow	per se	MN	Positive	Bhide et al.,1987a.
NASS				
CHO	Aqueous	CA (+/- S9, + catalase, + Superoxide dismutase)	Positive	Zaridze et al.,1985.

BETEL QUID WITH TOBACCO

Mouse bone-marrow	per se	MN	Positive	Shirname et al.,1984.

CHEWING TOBACCO, SNUFF, KHAINI, ZARDA, NASS, GORATHIN, TOBACCO TOOTHPASTE

CHO	Aqueous	CA, MN	Positive	Stich,1986.

NICOTINE

Ascites sarcoma BP8	per se	Toxicity	Negative	Pilotti et al.,1975.
Human embryonic lung fibroblast (MRC-5)	per se	Plasma membrane porosity (- S9)	Negative	Thelestam et al.,1980
S.typhimurium TA98, TA100 TA1537	per se	Mutation (+/- S9)	Negative	Riebe et al.,1982.
E.coli 343/113 pol A+ 1787 pol A-	per se	pol A+/pol A- (- S9)	Positive	Riebe et al.,1982.
CHO	per se	SCE (- S9)	Positive	Riebe and Westphal, 1983.
CHO	per se	SCE (+ S9)	Negative	Riebe and Westphal, 1983.

HL-60	per se	Cytotoxicity	Positive	Konno et al.,1986.
		Cell cycle distribution	Positive-block in G1 phase	
		DNA synthesis	Positive	
		RNA synthesis	Positive	
		Protein synthesis	Positive	

Short-term Tests:

The results of various short-term assays, conducted to search the carcinogenic potential of tobacco, have been reviewed in **Table-2.**

Human Studies:

Various cell types and body fluids have been screened to study the genotoxic effect of tobacco chewing. The most extensively studied are the cells of the exfoliated buccal mucosa. Elevated frequencies of micronucleated cells (MNC) in buccal mucosa have been reported among khaini chewers or chewers of betel quid with tobacco (Stich et al., 1982). Similar results have been documented among snuff users or nass users. However, compared to snuff users and khaini tobacco chewers, the MNC frequencies in the oral mucosa of nass chewers or chewers of betel quid with tobacco were significantly higher (Stich, 1986).

Exfoliated buccal mucosa cells have also been analysed for the presence of aromatic carcinogen-DNA adducts. No specific adduct spots were detected in samples from tobacco chewers that were not present in the controls (Chacko and Gupta, 1988; Dunn and Stich, 1986). This has been explained on the basis of (i) the presence of specific inhibitors in the saliva capable of preventing the formation of tobacco specific DNA adducts or (ii) since only 10-50 % cell viability has been reported for oral mucosal cells (Rubio et al., 1973), the occurrence of little or no metabolic activation of the tobacco specific precarcinogens during the ***in vivo*** exposure

(Chacko and Gupta, 1988). However, the possibility of TSNA-related adducts, which are more polar in nature, has not been ruled out in the buccal mucosa. ^{32}P-postlabelling assays for other adducts are being developed and appear to hold promise for detecting NNK- or NNN-DNA adducts *in vivo*.

Lymphocytes, another widely used test system, have also been studied to analyse the genomic damage caused by the habit of tobacco chewing. Ghosh and Ghosh (1984) have reported increased lymphocytic SCE frequencies in individuals consuming tobacco containing betel quids. They have also reported higher frequency of SCE in betel quid chewing pregnant women and in women who were using oral contraceptives compared to control women (Ghosh and Ghosh, 1988). Adhvaryu et al. (1986; 1988a) have reported higher lymphocytic SCEs in tobacco/areca nut chewers.

Saliva of tobacco chewers has been found to induce CA frequency in CHO cells. Strong clastogenicity has been reported for the saliva of chewers of Indian tobacco, whereas, the saliva of the chewers of Western type of tobacco failed to produce any clastogenic effect (Stich and Stich, 1982). Menon and Bhide (1984) have shown the mutagenic activity in urine of tobacco chewers, which was comparable to that observed in cigarette smokers' urine. Curvall et al. (1987) have reported comparable mutagenic activity in urine samples from snuff users and from tobacco non-users, whereas, compared to both, the activity was significantly higher in cigarette smokers.

Evaluating genotoxic burden imposed by occupational tobacco exposure, Govekar (1991) has reported mutagenicity in the urine samples from bidi rollers.

Thus, information so far gathered clearly showed that the use of smokeless tobacco is a major health hazard in India. Epidemiological studies have identified it as a principal etiological factor for oral cavity cancers. However, long term animal experiments failed to provide adequate evidence for its carcinogenicity. Various extracts of tobacco and nicotine, per se, elicited mutagenic/genotoxic effects in bacterial as well as mammalian test systems, however, the information on aqueous extract of tobacco, which would be more comparable to human consumption, is sparse. Moreover, reports regarding the possible genotoxic effects of smokeless tobacco consumption on human beings are also few. All these facts bring into sharp focus the need for experimental research on smokeless tobacco consumption.