

Prologue

Cancer is one of the leading causes of morbidity and mortality. It is believed to have a multifactorial etiology, however, the global disparities in the incidence of different cancers have mostly been interpreted as reflection of differences in exposure to environmental risk factors. Human population is inevitably exposed to a number of chemical mutagens/carcinogens either accidentally, occupationally or by lifestyle. Almost 80 to 90 % of cancers are attributable to such factors. Nevertheless, cancers, which are linked with the exposure to carcinogen by lifestyle, can be largely avoided by restricting the exposure.

In the group of risk factors associated with personal habits, tobacco consumption seems to be the most important. It accounts for approximately 2.5 million premature deaths every year (Mahler, 1988). A wide spectrum of tobacco products are manufactured and marketed for human consumption. They can be identified into two fundamental patterns i.e. tobacco smoking and oral use of unburnt tobacco (smokeless tobacco). Irrespective of its mode of consumption, tobacco is carcinogenic. A close correlation between the manner of its consumption and the development of site specific cancers has also been observed (Jussawalla and Deshpande, 1971; Sanghvi, 1981). In the United States, tobacco smoking is more common and around 30 % of the deaths due to cancer may be attributed to this habit (U.S. Public health service, 1982). Likewise, oral use of smokeless tobacco is predominantly associated with the causation of oral cavity cancers (ICD

140-145). In India, these cancers account for almost 40 % of total cancer deaths (Segi and Kurihara, 1972). Moreover, if the habit of chewing tobacco starts before the age of 14 years, chances of developing oral cancer are ten times higher (Wahi, 1976). In India, the habit begins at 10-12 years of age and continues until old age (IARC, 1985). Thus, the consumption of various smokeless tobacco products and the magnitude of oral cancers in India, call for the in-depth analysis of carcinogenic potential of smokeless tobacco.

The smokeless tobacco is practised in a variety of ways ranging from chewing tobacco alone, tobacco with lime, tobacco with areca nut and lime, tobacco as an ingredient of betel quid (colloquially termed as 'pan'), snuff (powdered tobacco), masher (pyrolysed tobacco product) etc. As a result of several reports, pointing towards the harmful effects of passive smoking, a strong public opinion against tobacco smoking has been generated. Partly under the pretext of being a safer alternative and partly because of opinion against tobacco smoking, the smokeless tobacco has gained the acceptance (Chassin et al., 1985; Schaefer et al., 1985). Behavioral differences, which include convenience, detectability, duration and frequency of use, have also been reported to play important role in the adoption of this mode of tobacco consumption instead of tobacco smoking (Evans, 1988). Sun-dried and coarsely cut tobacco leaves, from Nicotiana rustica and Nicotiana tabacum, are used for chewing purposes in India. 85 % of tobacco is consumed in its raw form i.e. with-

out further processing (IARC, 1985). Tobacco consumption is increasing at a rate of more than 2 % per year (Stanely and Stjensward, 1986). Like tobacco smoking, oral uses of smokeless tobacco, mainly snuff and chewing tobacco, also bring about the change in mood and feelings in a dose-related manner. The advertisements by print and electronic media have coupled the use of smokeless tobacco with fun, popularity, success etc. Peer influence, emulating the elders and desire to reach the aim, imitating successful personalities in the field etc. are the reasons for its popularity among the urban literate upper and middle class. In urban slums, the 'dada' image, social defiance and feeling of being adult are main reasons of its usage. Among the socio-economically backward population, many chew tobacco to ward off hunger and thirst. The early medicinal uses of tobacco on wounds, animal bites, toothache etc. have still place in illiterate rural population.

Daily exposure to several mutagenic/carcinogenic constituents of tobacco poses a serious threat to the health of its consumers. About 90 % of more than 100,000 new cases of oral cancers, are caused by tobacco consumption in South-East Asia every year (Stanley and Stjernsward, 1986). It comprises almost one third of the total cancer cases in India (WHO, 1984). In India, it is the commonest cancer among the males and third commonest among the females (Annual Reports of National Cancer Registry Project of India, 1982-1985; Sankarnarayanan et al., 1989). Using data from various

national and international registries, Notani (1990) has reviewed the incidence rates of cancers of upper alimentary tract and respiratory tract in males and indicated the highest prevalence of cancers of oral cavity in Gujarat, India. In the United States, oral and pharyngeal cancers account for only 3 % of all cancers (Silverberg and Lubera, 1989). This explains the severity of the problem in India and in specific for the state of Gujarat.

Epidemiological findings have been supported by various laboratory studies. Highly carcinogenic tobacco specific nitrosamines (TSNA) have been detected in various smokeless tobacco products as well as in the saliva of the tobacco chewers (Bhide et al., 1986; 1989; Hoffmann and Adams, 1981; Nair et al., 1985; 1989). The carcinogenicity of tobacco and its extracts have also been assessed in long-term animal studies (Bhide et al., 1979; 1984; Shah et al., 1985; van Duuren, 1968; Wynder and Hoffmann, 1968). However, it has been observed by the working group of International Agency for Research on Cancer (IARC) that all of these studies were suffering from certain deficiencies regarding the experimental protocol, and hence, IARC (1985; 1987) concluded that there was inadequate evidence to ascertain the carcinogenicity of chewing tobacco or snuff to the experimental animals. Thus, as a first step, it was important to have laboratory research for determining the carcinogenic potential of these forms of tobacco consumptions.

The identification of carcinogens by long term animal experiments has always been the system of choice. However, the shortcomings of this assay are the cost and the time consumed. These have led to search for alternative ways. Numerous short-term tests have been developed and are being successfully used for detecting the ability of chemicals to cause alterations in genetic material and thereby to play a crucial role in causing cancer. Compound which exhibit carcinogenic activity, generally also exhibit genotoxic activity (Tennant et al., 1987). Thus, genetic toxicology has major application in determining the exposure to genotoxic carcinogens of tobacco. It has also been suggested that in genetic toxicology studies, the *in vitro* cytogenetic assays should take a central role in a test battery (Natarajan and Obe, 1982). The *in vitro* assays are sensitive, reliable, reproducible and require small amount of the test substance. The established cell lines, rather than whole mammal, provide an ideal test system with homogeneous cell population, growing in chemically defined media and under controlled conditions. Cytogenetic methodologies offer some of the most sensitive approaches for detecting genotoxic effects of mutagenic carcinogens (Perry and Evans, 1975). Microscopic examination of chromosomes is an established procedure for investigating adverse effects of chemical and physical agents on mammalian cells.

Hence, for analysing possible genotoxic effects of smokeless tobacco, various cytogenetic endpoints were used in *in*

vitro experiments and simultaneously, some of these markers were employed for studying the possible genotoxic effects on cells of human beings consuming smokeless tobacco.

PARAMETERS used for measuring the *in vitro* effects of tobacco and its major alkaloid, nicotine were: (1) Cell viability (2) Mitotic index (3) Micronucleus assay (4) Chromosome aberration frequency (5) Sister chromatid exchange frequency and (6) DNA synthesis.

(1) Cell viability : "Dye exclusion test" is widely used technique for deciding the lethality of the test substance *in vitro*. It is a simple and rapid method. It does not require additional treatment that may affect the results (Tsudeka et al., 1978). The test is carried out to determine lethal/sublethal concentrations and thereby, is useful in selecting suitable doses for further experiments.

(2) Mitotic index (M.I.) : It expresses the frequency of mitotic figures in the cell population and thereby helps in understanding the factors affecting the normal cell division. The process of cell division depends upon successful completion of many macromolecule synthesizing events. Any alteration in these sequenced processes results into abnormal cell division, which might have more consequences than the point mutations (Au and Hsu, 1982). The chemicals that interfere with mitotic apparatus are called mitotic poisons and might escape from being detected by mutation assays. The capacity of the test substances to interfere with normal cell division has

been checked by this assay.

(3) Micronucleus (MN) assay : Chromatid or chromosome fragments and aberrant chromosomes, which are not included in the main nucleus and remain separate in the cytoplasm during mitotic cycle, form the micronucleus. Its presence indicate chromosome breakage and/or malfunction of the spindle apparatus. Micronuclei formed by spindle poisons are, as a rule, larger than those originating as a consequence of chromosomal aberrations (Hogstedt and Karlsson, 1985; Schmid, 1976; Yamamoto and Kikuchi, 1980).

The usefulness of the MN test for detecting and quantitating the genotoxic effects of a compound has been well established. The *in vitro* and *in vivo* induction of MN by carcinogens and mutagens is a result of the genomic damage (Blakey et al., 1985; Bruce and Heddle, 1979; Heddle, 1973; Heddle et al., 1981; Ronen and Heddle, 1984). Stich and co-workers (1982-1986) have widely applied MN assay at population level, for studying the effects of tobacco/areca nut chewing habits on the buccal mucosa. The test permits a non-invasive assess to human tissues as well as repeated samplings appear ethically and emotionally acceptable. An in-depth knowledge about the origin of MN, a positive relationship between MN induction and carcinogenicity of chemicals, and the ease of scoring have influenced the inclusion of this test in the present evaluation programme.

(4) Chromosome aberration (C.A.) frequency : Chemical or physical mutagen-induced DNA damage has been considered as a primary event in cancer development. Hence, clastogens, i.e. chemicals capable of inducing CA, have been viewed as potential mutagens/carcinogens. Compared to MN test, metaphase analysis for CA frequency has been considered as a more sensitive method for identifying clastogenic compounds (Kliesch and Adler, 1983). The induction of CA is a property common to many carcinogenic chemicals (De Serres and Ashby, 1981), and recent studies have shown that some carcinogens, that do not induce mutations in bacterial systems, are capable of causing chromosomal damage in cultured mammalian cells (Dean, 1985). Thus, CA frequency in cultured cells is the most commonly used cytogenetic endpoint for mutagenicity testing.

CA assay is also one of the most valid approaches for monitoring human exposure to genotoxic agents. Genomic damage, caused by exposure to harmful environmental agents, which may ultimately increase the risk of cancer, can be assessed in terms of CA frequency in the peripheral blood lymphocytes (Carrano and Natarajan, 1988). This has been reported in cigarette smokers (Obe and Herha, 1978), in alcoholics (Obe et al., 1980) and in bone marrow cells & peripheral blood lymphocytes of human beings exposed either to petroleum vapours (Hogstedt et al., 1981) or to ethylene oxide (Hogstedt et al., 1983). Hence, the parameter was included in present work.

(5) Sister chromatid exchange (S.C.E.) frequency : The misrepaired chromosomal lesions, which escape being detected as CA, can be detected effectively by studying SCE frequencies. SCEs are reciprocal exchanges between sister chromatids. It occurs at homologous loci which presumably involve the DNA breakage and aberrant rejoining, however, the exact molecular mechanisms for SCE formation are largely unknown. Cells with SCEs are capable of subsequent growth, and hence, SCE elevation may thus be a more valid indicator of heritable genomic damage. However, SCE assay should be regarded as a complement to, rather than a substitute for, chromosome aberration analysis (Perry et al., 1984).

SCE frequency has been considered to be one of the sensitive markers for assessing genotoxic effects of carcinogenic and/or mutagenic agents. It can be induced *in vitro* or *in vivo* by a wide variety of genomic lesions (Gebhart, 1981; Latt, 1981; Wolff, 1977). The most efficient SCE inducers appear to be the agents that can form adducts or otherwise distort the DNA backbone. Thus, the assay is a useful method for detecting chemicals that interact with and damage DNA. Induction of SCE correlate well with mutation frequency and cell transformation in several cell lines (Carrano et al., 1978; Evans and Vijayalaxmi, 1981; IARC, 1982; Popescu et al., 1981; 1984). Increased SCE rates have been reported in cells of individuals exposed to various environmental mutagens/carcinogens, e.g. in smokers (Ghosh and Ghosh, 1987; Hopkin and Evans, 1980; Husum et al., 1986; Lambert et al.,

1978; Watanabe and Endo, 1984), in betel and tobacco chewers (Ghosh and Ghosh, 1984), in alcoholics (Hoeft and Obe, 1983), and in individuals occupationally exposed either to styrene (Barale, 1991) or to toluene (Bauchinger et al., 1982). Taking all the above facts into consideration, the assay has been included to evaluate the possible genotoxic effects of smokeless tobacco.

(6) DNA synthesis : The ability of genotoxic agents to interfere with DNA replication has long been recognized. Frequently, cells exhibit a progressive depression of the rate of nascent DNA synthesis following a short treatment with various genotoxic agents (Roberts, 1984). The accumulation of chromosomal aberrations by DNA synthesis inhibitors, is a finding of relevance (Preston, 1982). Any change in DNA replication can be analyzed by estimating ³H-thymidine incorporation in the nuclei with the help of autoradiography. The technique has been used for studying the effects of tobacco on DNA synthesis.

PLAN OF WORK :

The present work was an effort to evaluate the possible carcinogenic/genotoxic impact of smokeless tobacco on mammalian cells *in vitro* as well as on somatic cells of human beings consuming it. The study revolved around the tobacco varieties consumed commonly in India and various modes of smokeless tobacco consumption encountered locally.

The FIRST PART of the thesis highlights the effects of aqueous extract of tobacco (Nicotiana tabacum), as well as of its alkaloid, nicotine, in pure form, employing the *in vitro* short-term studies. Since tobacco is often chewed with areca nut, an analysis of effect of combining nicotine and arecoline -the major alkaloid of areca nut- was thought to provide a better insight. The data has been generated with the help of established cell line and the parameters detailed earlier. However, Human response to the substance is not always comparable to that elicited in *in vitro* test system.

The SECOND PART encompasses *in vivo* effects of smokeless tobacco consumption. The studies were carried out in persons having a habit of consuming only one kind of tobacco throughout their life, with no other tobacco-areca nut habit. Here, three different types of tobacco habits were considered, viz. I. Snuff (powdered tobacco) rubbing on teeth and gums, II. Chewing tobacco with lime and III. Chewing tobacco with areca nut and lime, locally called as 'mava' (the most common form of tobacco chewing in Gujarat, India). The individuals included in the study were further categorized into three groups, namely, **Controls**- individuals not consuming tobacco and/or areca nut in any form, **Normal chewers**- individuals who consume tobacco and/or areca nut for atleast last two years, however, have not suffered the consequences of the habit i.e. they have normal oral mucosa and **Patients** suffering from premalignant or malignant diseases of the oral cavity. The genotoxic effect of the habit on target tissue was assessed

in terms of frequency of the micronucleated cells (MNC) in the exfoliated buccal mucosa, whereas, the analysis of chromosome aberrations (CA) and sister chromatid exchanges (SCE) frequencies in phytohaemagglutinin (PHA-M) stimulated peripheral blood lymphocytes (PBLs) was carried out to assess the genomic damage to the somatic cells of body. Such an experimental design was expected to provide useful information about the possible genotoxic effects of smokeless tobacco consumption on **target as well as non-target tissues**.

The THIRD PART of the thesis embodies the studies on tobacco containing 'pan masalas', a new substitute to the prevailing for tobacco habits. As stated on its packing, it is a mixture of tobacco, areca nut, catechu, lime and unspecified spices and flavouring agents. To evaluate the genotoxic potential of this complex mixture, *in vitro* short-term tests, as described in First part, were carried out using an aqueous extract of tobacco containing 'pan masalas'. The findings were further substantiated by studying the individuals consuming the same brand of tobacco containing pan masala, with no other concurrent tobacco or areca nut consumption habit.