LONG-TERM CARCINOGENICITY OF PAN MASALA IN SWISS MICE

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Carcinogenicity of pan masala, a dry powdered chewing mixture of areca nut, catechu, lime, spices and flavoring agents was evaluated by means of the long-term animal bio-assay 6- to 7-week old male and female S/RVCri mice were divided randomly into intermediate and lifetime exposure groups and fed normal diet without pan masala—(zero dose) or diet containing 2.5% and 5% pan masala. Animals in the intermediate-exposure group (n = 10/gender/dose group) were killed after 6, 12 or 18 months of treatment, while those in the lifetime-exposure group (n = 54/gender/dose group) were killed when moribund or at the termination of the experiment at 24 months. Several tissues were processed for histopathological examination. The body weight and survival rate of mice fed pan masala were lower than that of the controls. Histopathological observations of tissues from control animals did not reveal any neoplastic alterations. However, lifetime feeding of pan masala induced adenoma of the liver, stomach, prostate and sebaceous glands, also forestomach papilloma, liver hamartoma, hepatoma and hemangioma, carcinoma of the forestomach, adenocarcinoma of the lung and liver, and testicular lymphoma. Neoplastic lesions appeared mainly in the liver (n = 13), stomach (n = 3) and lung (n = 8). Lung adenocarcinoma, the most frequent malignant tumor type, was observed in 2/120 mice in the intermediate-exposure group and in 8/216 animals in the lifetime-exposure group. Statistical analysis of tumor-induction data revealed a significant dose-related increase in lung adenocarcinomas but not in liver and stomach neoplasms indicating that lung is the major target tissue for the carcinogenic action of pan masala. *Int. J. Cancer 83:679–684, 1999.*
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Nearly 2 decades ago, pan masala, a dry powdered chewing mixture of areca nut, catechu, lime, unspecified spices and flavoring agents, was introduced into the Indian market. Presently, 2 types of chewing products are available, *i.e.,* ''pan masala'' without tobacco, and ''gutkha,'' which is a mixture of pan masala and tobacco. Aggressive product promotion through the mass media has contributed to the immense popularity of the ready-to-use chewing products in the Indian subcontinent. Pan masala is popular not only with males but also among women and children who generally refrain from tobacco use in any form.

Areca nut, the major constituent of pan masala, has been shown to exhibit clastogenic, mutagenic, genotoxic and carcinogenic properties in different experimental systems (Suri *et al.,* 1971; Ranadive *et al.,* 1976; IARC, 1985; Panigrahi and Rao, 1986; Dave *et al.,* 1992). Habitual use of areca nut is causally associated with increased risk of human oral pre-cancer and cancer (Chin and Lee, 1970; IARC, 1985; Sinor *et al.,* 1990). Catechu, another constituent of pan masala, was found to possess strong mutagenic (Stich *et al.,* 1983) and clastogenic (Giri *et al.,* 1987) activity, while lime is known to cause irritation and hyperplasia of the oral mucosa (Dunham *et al.,* 1966).

In other studies, pan masala *per se* was reported to exhibit genotoxic and mutagenic properties in several short-term assays (Adhvaryu *et al.,* 1989; Bagwe *et al.,* 1990; Mukherjee and Giri, 1991; Polasa *et al.,* 1993). Chronic feeding of pan masala was shown to impair liver function in rats and to affect the mouse germinal cells by inducing sperm-head abnormalities (Mukherjee *et al.,* 1991; Sarma *et al.,* 1992). Increased cytogenetic damage was also reported in the peripheral-blood lymphocytes and exfoliated buccal mucosal cells of pan masala consumers (Dave *et al.,* 1991). Moreover, pan-masala chewing has been linked to the development

of oral sub-mucous fibrosis, a condition known to pre-dispose to cancer (Anuradha and Devi, 1993). These observations provided compelling evidence for evaluation of the carcinogenicity of pan masala. We have reported that an extract of pan masala exerts tumor promoting and progressor activities in the skin, stomach and esophagus of 2 different mouse models (Ramchandani *et al.,* 1998). In the present study, the implications of chronic pan-masala usage were elucidated in the long-term carcinogenicity assay using another mouse model.

MATERIAL AND METHODS

Test substance

A market survey on the rate of purchase of different brands of pan masala was conducted in different parts of Mumbai, India. The brand with the highest sale was then used for carcinogenicity testing. The product was purchased in bulk, so as to avoid batch-to-batch variation, and was stored at $-20°C$ until use.

Diet preparation

Dry, finely powdered pan masala was mixed with standard animal feed at concentrations of 10%, 5% and 2.5%. The feed containing pan masala was pelleted, sealed in air-tight plastic bags and stored at 0 to 4°C until use. Fresh diet was prepared once every 8 weeks.

Animals and maintenance

Inbred Swiss mice of the S/RVCri strain used in the present study were initially obtained from the Rockefeller Institute, New York, and were random bred at the Virus Research Institute, Pune, India. The breeding stock was transferred to the animal colony of the Cancer Research Institute, Mumbai, India, in 1953. A total of 554 mice of both sexes, aged 6 to 7 weeks, were provided by the animal house of the Cancer Research Institute, Mumbai, India. Animals of the same sex were randomly assigned to different dose groups, and were housed 5 to a cage in an air-conditioned animal house (temperature $21 \pm 1°C$, humidity 55 to 60%, constant 12-hr light/dark cycle) and were fed normal diet without pan masala or mouse feed containing pan masala. Water was given *ad libitum.*

Carcinogenicity testing of pan masala

Dose selection. Determination of maximum tolerated dose (MTD) has to be undertaken before commencement of long-term carcinogenicity study, in order to select a non-toxic dose. The choice of the high dose should be such that it elicits minimal signs of toxicity but ensures that the test animals have been sufficiently challenged. In the present study, pan masala was mixed at 5% and 10% dose levels and fed to 10 mice of each sex per dose group for a period of 8 weeks. Control mice (5 of each sex) were fed normal diet for the same period. Food consumption was recorded at 24-hr intervals, 5 days a week, and expressed as mean $\pm$ S.E. For assessing gross adverse effects, animals were observed weekly for behavioral and macroscopic changes. Differential leucocyte count

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and estimation of hemoglobin were carried out in each mouse at the end of the experimental period, when animals were killed by ether anesthesia. Lung, heart, liver, stomach, esophagus, spleen, pancreas, kidney, intestine, urinary bladder and ovaries were routinely excised, fixed in Bouin's fluid and processed for histopathology.

No macroscopic, behavioral or clinical abnormalities were observed in control or in pan-masala-treated animals. The mean body weight of mice of both sexes fed pan masala at 5% and 10% levels was similar to that of controls. Food consumption of mice on 5% or 10% pan-masala diet was similar, but significantly lower than that of controls ($p < 0.001$). Thus the mean food consumption in male control animals and in those receiving 5% and 10% pan-masala diet was 4.9, 3.8 and 3.6 g/day, while the respective values in females were 4.4, 3.7 and 3.6 g/day. In spite of the reduced dietary intake, the mean body weight of mice of both sexes fed pan masala at 5% and 10% levels for 8 weeks was similar to that of the respective controls. Histopathologically, all the organs were essentially normal, although a mild increase in the number of squamous-cell layers, marked increase in rugosity and keratinization, were observed in the forestomach epithelium of pan-masala-treated animals. These changes were equally marked in animals feeding on both the doses of pan masala. Mild to moderate interstitial inflammation of the kidney was noted in a few cases.

Long-term studies using putative carcinogens are generally conducted at a relatively high exposure level, to maximize the probability of observing an effect in a relatively small sample of experimental animals. In this study, the food consumption and other responses of animals feeding on 5% and 10% pan-masala diet were similar. However, it was felt that in lifetime-exposure studies, the higher dose may interfere with nutrition (IARC, 1986). To avoid such a possibility, instead of MTD (10% dose), ½ MTD and ¼ MTD were used for carcinogenicity testing.

Sample size. To obtain meaningful biological results, a sufficient number of animals is needed for the experiment. In determining the carcinogenicity of a test substance, 2 types of statistical errors are possible: false-positive (type I) and false-negative (type II), which need to be minimized. Based on tests of linear trend for 3 equally spaced doses and fixing the probability values at 0.05 and 0.10 for type-I and type-II errors respectively, it was estimated that from 70 to a little under 50 animals per group would be needed to detect an increase in the incidence rate of 0.2 or more in the high-dose group to a background rate of 0.1 or less. Hence, to allow for logistic and budgetary constraints on the one hand and meaningful statistical evaluation of the results on the other, 54 mice of each sex per dose group were used in the present study. This number would be quite adequate if the background tumor rate is lower than 5%.

Carcinogenicity experiments. A total of 504 mice (252 of each sex) were distributed into different cages (5 mice/cage) and randomly assigned into control (normal diet) and experimental groups, *i.e.,* diet containing 2.5% and 5% pan masala. In order to study the progressive pathogenesis of the lesions of interest, 10 mice of each sex/dose group were killed at pre-determined time points of 6, 12 and 18 months; these are referred to as the intermediate-exposure group. Blood was collected from the tail vein of 3 mice of each sex in each of the intermediate-exposure groups for hematological examination, before the animals were killed. For the long-term experiments, 54 mice of each sex were fed normal diet (zero dose) or diet containing pan masala at 2 dose levels (2.5% and 5%) for the entire lifespan of about 24 months, and are hereafter referred to as the lifetime-exposure group.

All the animals were observed daily for any abnormalities. Individual body weight was recorded weekly for the first 8 weeks and thereafter once every 4 weeks. In the lifetime-exposure group, mice were killed when grossly moribund or at the termination of the experiment at 24 months. Necropsy was performed on all the animals and the tissues (mentioned in the section on dose selection) were excised, fixed in Bouin's fluid and processed for routine histopathology.

Statistical analysis

The log-rank test and Kaplan-Meier graphical output were used for survival analysis. Since all tumors were observed in the fatal context, the ''death-rate'' method was used for comparing site-specific tumor occurrence in the 3 groups, *i.e.,* controls, 2.5% dose and 5% dose groups, and tested for positive trend. These trends were adjusted for sex and intercurrent mortality (Peto *et al.,* 1980).

RESULTS

Intermediate-exposure group

To study progressive pathogenesis of the lesions, 10 male and 10 female mice each belonging to control, 2.5% and 5% dose groups, *i.e.,* 60 mice per dose group, were killed at 6, 12 and 18 months. The mean hemoglobin levels ± S.E. of control mice were 14.2 ± 0.9 g% in males and 12.8 ± 0.6 g% in females. Hemoglobin levels and differential leucocyte count were similar in the control and experimental animals. Non-neoplastic lesions observed in this group included forestomach hyperplasia in 10 out of 60 mice in the 2.5% dose group and in 5 out of 60 animals in the 5% dose group. Cystic changes in the ovary were also seen in 2 out of 30 control females and in 6 out of 60 female mice exposed to pan masala and killed at 12 and 18 months. No benign or malignant tumors were detected in the controls or in the tissues of mice exposed to 2.5% pan masala. However, in the 5% dose group, adenocarcinoma of the lung was noted in 1 male and 1 female mouse killed at 12 and 18 months respectively.

Lifetime-exposure group

Clinical manifestations such as weakness and lethargy were observed in older mice of all 3 groups. Data on the body weight for the control and experimental groups are presented in Figure 1. The mean body weight of male and female animals fed pan masala at both the doses was consistently lower than that of the controls, and the difference was particularly marked from month 6 onwards.

Survival of animals. The survival curves of male and female mice fed normal and pan-masala-containing diet at both the doses are shown in Figure 2. The mean survival time for all the animals in the control, the 2.5% and the 5% pan-masala groups was 18, 17 and 16 months respectively. The log-rank test revealed a significant decrease in the overall survival rate of mice receiving 2.5% and 5% pan masala, as compared with the controls whether analyzed with ($p = 0.02$) or without ($p = 0.02$) adjustment for sex. In males, the mean survival time for both the dose groups was 17 months, as compared with 19 months in the control group. Similarly, in females in both the dose groups, the mean survival time was lower (16 months) than in the controls (18 months).

Histopathological observations. As mentioned earlier, all animals were subjected to necropsy, and tissues were examined by histopathology.

Non-neoplastic alterations. A few animals in control and experimental groups developed pneumonitis (3–9%) and interstitial inflammation of the kidney (4–7%). Ovarian cyst with chronic inflammatory reaction was noted in 5 (9.2%) of 54 control females and in 15 (13.9%) of 108 female mice exposed to pan masala. Cystic change was also observed in the stomach, urinary bladder and skin of one mouse each receiving lifetime exposure to pan masala. These lesions were absent in the control animals. Hyperplasia of the forestomach epithelium, the commonest alteration characterized by basal-cell proliferation and excessive keratinization (Fig. 3), was noted in 22 out of 216 pan-masala-treated mice (3 out of 108 animals in the 2.5%-dose group and 19 out of 108 mice in the 5%-dose group) and none in the controls. Statistical analysis revealed a significant dose-response relationship in the incidence of forestomach hyperplasia, ($z = 4.82$, $p < 0.001$ adjusted for sex and intercurrent mortality).

Neoplastic lesions. Data on benign and malignant tumors induced in animals receiving lifetime exposure to 2.5% and 5% pan masala are shown in Table I. No neoplastic lesions were observed in any of the tissues from control animals, while 15 benign lesions
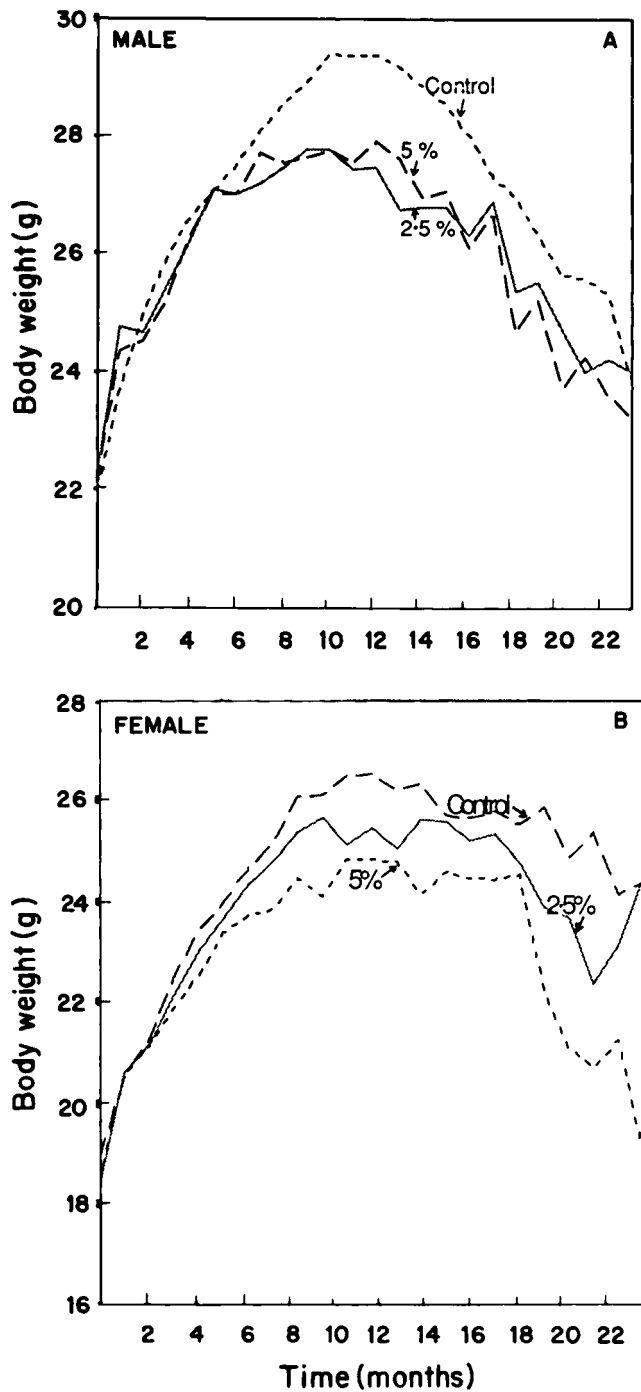
FIGURE 1 – Mean body weight of male *(a)* and female *(b)* S/RVCri mice fed normal and 2.5%- or 5%-pan-masala-containing diet over a period of 24 months.

FIGURE 2 – Kaplan-Meier survival curves for male *(a)* and female *(b)* S/RVCri mice in control and experimental groups.

FIGURE 3 – Forestomach hyperplasia. The squamous epithelium shows basal-cell proliferation and is thrown into folds. Keratinization is excessive. Scale bar = 156 μm.

and 12 malignant tumors appeared in mice fed pan masala in the diet. The commonest benign lesion was liver hemangioma, observed in 7 out of 108 mice fed 2.5% pan-masala dose and in 1 out of 108 animals receiving the 5% dose. The lesion was characterized by multiple dilated vascular spaces lined by endothelial cells with normal hepatocytes at the periphery of the lesion (Fig. 4). Other benign lesions (forestomach papilloma, stomach adenoma, liver hamartoma and adenoma, and prostate adenoma) appeared in one mouse each in the 2.5%-dose group, while a liver hamartoma and a sebaceous-gland skin adenoma were observed in the 5%-dose group.
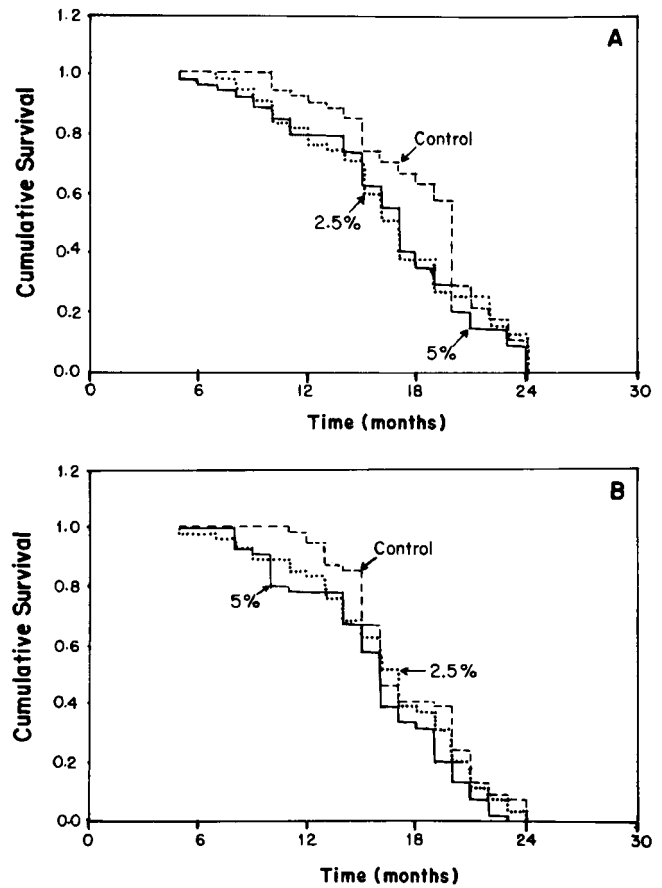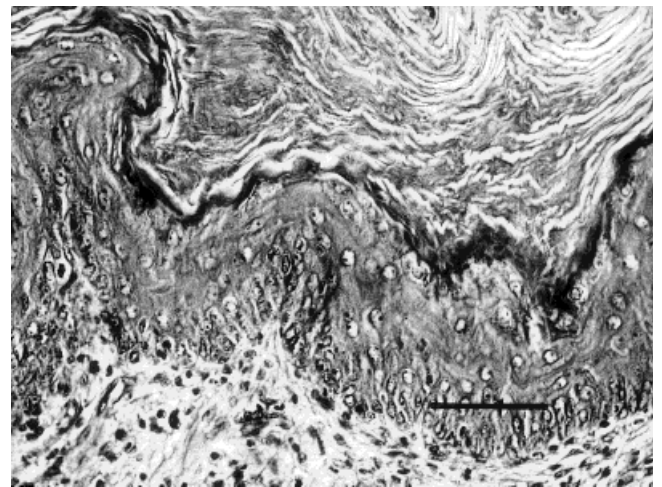
Malignant tumors were noted in 5 out of 108 mice fed diet containing 2.5% pan masala and 7 out of 108 animals receiving 5% pan masala dose. In the 2.5%-dose group, 1 liver adenocarcinoma, 1 hepatoma and 3 lung adenocarcinomas were seen, while in the 5%-dose group there were 5 adenocarcinomas of the lung and 1 each of forestomach carcinoma and testicular lymphoma. Lung adenocarcinoma, the most frequent malignant tumor type, was

TABLE I – SITE-SPECIFIC NEOPLASTIC LESIONS* IN S/RVCri MICE EXPOSED TO PAN MASALA IN DIET FOR LIFETIME

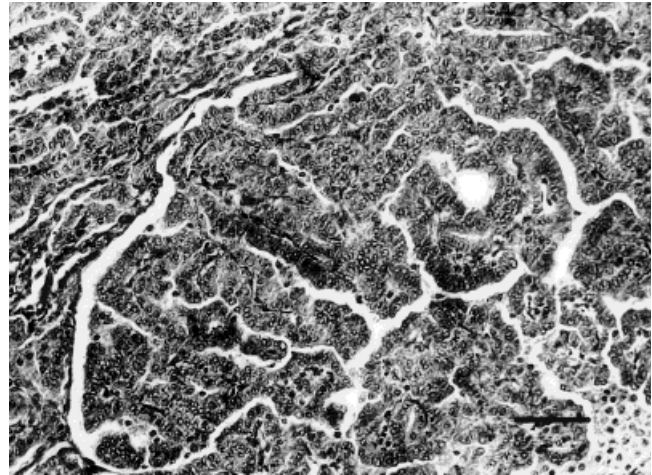|	Treatment			
	2.5% pan masala		5% pan masala	
	Male (n = 54)	Female (n = 54)	Male (n = 54)	Female (n = 54)
Site and type of lesions				
Lung				
Adenocarcinoma	3	0	3	2
Liver				
Hamartoma	0	1	0	1
Hepatoma	0	1	0	0
Hemangioma	6	1	0	1
Adenoma	1	0	0	0
Adenocarcinoma	0	1	0	0
Forestomach				
Papilloma	1	0	0	0
Carcinoma	0	0	1	0
Glandular stomach				
Adenoma	0	1	0	0
Testis				
Lymphoma	0	0	1	0
Prostate				
Adenoma	1	0	0	0
Skin				
Sebaceous adenoma	0	0	1	0

*No neoplastic lesions were observed in the control group comprising 54 male and 54 female mice.
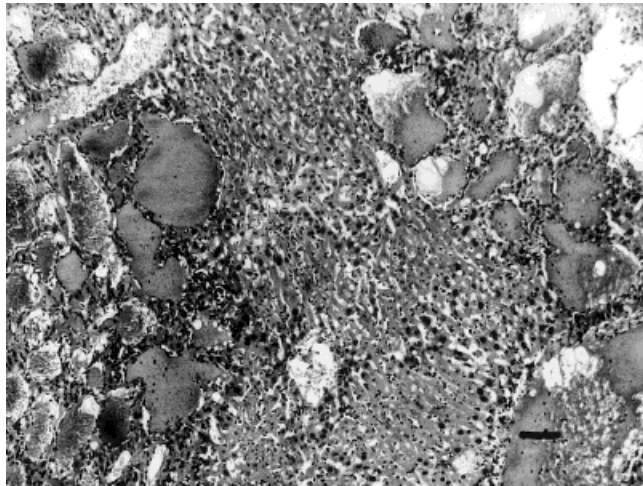
FIGURE 4 – Liver hemangioma. Multiple dilated, endothelial-lined vascular spaces are seen ramifying between normal hepatocytes. Scale bar = 50 μm.

FIGURE 5 – Lung adenocarcinoma. Papillary structures lined by columnar cells are seen infiltrating the pulmonary parenchyma. Scale bar = 98 μm.

TABLE II – LUNG ADENOCARCINOMAS*: OBSERVED NUMBERS AND EXPECTED NUMBERS ADJUSTED FOR SEX AND INTERCURRENT MORTALITY

Treatment group	Observed (O)	Expected (E)	O/E
Control	0	3.2183	0
2.5% dose	3	2.6258	1.14
5% dose	5	2.1559	2.32

*All tumors observed in the fatal context.

characterized by columnar cells arranged in acini, with papillary formations infiltrating the pulmonary parenchyma (Fig. 5). The number of animals with lung adenocarcinoma exhibited a statistically significant positive trend with respect to dose (z = 2.68, 1-tailed $p = 0.004$). As seen in Table II, the higher-dose group showed a 2-fold increase in lung neoplasms as compared with the low-dose group (ratio of O/E 2.03). Similar analyses of animals with liver tumors did not yield a significant trend with dose (z = 1.38, 1-sided $p = 0.08$), nor was there a significant trend for stomach neoplasms (z = 1.06, 1-sided $p = 0.15$).

DISCUSSION

The essence of the long-term animal bio-assay is to simulate the human situation wherein neoplastic development due to chronic exposure to environmental agents is preceded by a long latency period. In the present study, Swiss mice of S/RVCri strain were used to evaluate the carcinogenic influence of lifetime exposure to

a popular brand of pan masala which does not contain tobacco, mainly because these inbred mice are susceptible to tumor development by chemical carcinogens (Bhisey *et al.,* 1987). An important consideration was that spontaneous tumors have not been observed in this strain during their life span of over 2 years, according to the records of the Animal Sciences Division at our Institute. This fact helped to maintain the sample size at a manageable level, while permitting statistical evaluation of small increases in tumor number in pan-masala-treated animals.

In order that the route of exposure corresponds to and is as close as possible to that in the human situation, pan masala was mixed in the pelleted diet at 2.5% and 5% dose levels. The body weight and survival rate of animals fed pan masala for their lifetime were found to be lower than that of the control mice. These changes may be related to chronic nutritional insufficiency and possibly to liver toxicity, reported to occur after long-term pan-masala consumption (Mukherjee *et al.,* 1991). Similar observations have also been reported by Tanaka *et al.* (1983) and Rao and Das (1989) in animals fed diet containing powdered areca nut, the major ingredient of pan masala. A reduction in body weight was also observed in F344 rats injected 3-(methylnitrosamino) proprionitrile (MNPN), the areca-nut-specific carcinogenic nitrosamine (Prokopczyk *et al.,* 1987). Besides these changes, a dose-dependent increase in the incidence of focal hyperplasia of the forestomach epithelium was observed in this study, in mice receiving lifetime exposure to pan masala. This finding correlates with its forestomach-tumor-promoting activity noted in our earlier study (Ramchandani *et al.,* 1998). Thus, lowered body weight and survival rate of S/RVCri mice as well as induction of forestomach hyperplasia appear to be related to the consumption of areca nut present in the pan-masala mixture.

Swiss mice used in the present study are occasionally found to develop cystic ovary and interstitial inflammation in the kidney. However, no spontaneous tumors have been reported in this mouse strain to date (Randelia *et al.,* 1983; and data not shown). In this study too, ovarian cysts were observed both in control and in pan-masala-treated mice, while no tumors were noted in any of the tissues from control animals. However, a variety of benign and

malignant tumors were induced in different tissues of animals given 2.5% and 5% doses of pan masala throughout their life span.

Neoplastic lesions were induced mainly in the liver (n = 13), lung (n = 8) and stomach (n = 3), and 1 neoplasm each also appeared in the prostate, testes and skin of mice in the lifetime-exposure group. Of these lesions, 12 were malignant, the most frequent being lung adenocarcinoma, observed in 8 mice followed by one each of liver adenocarcinoma, hepatoma stomach carcinoma and testicular lymphoma. These findings indicate that liver, lung and stomach are the target tissues for carcinogenic action of pan masala. However, unlike the lung, where a dose-response relationship was observed, such an increase with dose was not observed in the case of stomach tumors and liver neoplasms. In earlier studies, induction of liver hemangioma, hepatoma, forestomach carcinoma and lung adenocarcinoma was reported in Swiss mice given s.c. injections of an aqueous areca nut extract or polyphenolic fraction of areca nut (Bhide *et al.,* 1979; Shivapurkar *et al.,* 1980). Forestomach papillomas were also observed in Syrian hamsters and ACI rats fed powdered areca nut or areca nut and lime respectively in the diet, for most of their life span (Tanaka *et al.,* 1983; Ernst *et al.,* 1987). However, in these studies, tumor-induction data were not analyzed statistically presumably due to small sample size. A dose-dependent increase in lung adenocarcinoma observed in the present study indicates that pan masala exerts maximum carcinogenic influence in the lung tissue. This inference is strengthened by the appearance of 2 lung adenocarcinomas in the intermediate-exposure group in mice fed high dose (5%) of pan masala and killed at pre-determined time points of 12 and 18 months.

In humans, oral tumors generally develop at sites in direct contact with tobacco/areca nut containing chewing products. Differences in the mode of pan-masala exposure may contribute to differences in the target site of humans and experimental animals. However, differences in the expression of catalytic activities and substrate specificities of cytochrome P450 enzymes in rodents and man (Nebert *et al.,* 1991) may also be responsible for tumor occurrence at sites different from those in man.

Chemical analysis has revealed that pan-masala mixture contains polycyclic aromatic hydrocarbons, nitrosamines, toxic metals and residual pesticides (Kashyap *et al.,* 1989). Similar analysis from our laboratory has revealed the presence of volatile aldehydes including formaldehyde, acrolein, crotonaldehyde, propionaldehyde and isobutaraldehyde in pan masala without tobacco (data not shown). Some of these aldehydes are known to be carcinogenic in

rats, producing tumors in the liver and nasal mucosa (IARC, 1987). Areca nut, the major component of pan masala, is composed of tannins, fats, free fatty acids, polysaccharides, crude fibers and minerals, and has at least 6 reduced pyridine alkaloids, namely, arecoline, arecaidine, arecolidine, guvacoline, guvacine and isoguvacine (Ashby *et al.,* 1979; Majumdar *et al.,* 1979). Some of these alkaloids are converted to carcinogenic nitrosamines in mild nitrosation conditions (Nair *et al.,* 1985) and have been detected in the saliva of betel-quid chewers. Prokopczyk *et al.* (1987) found that MNPN is metabolically activated to diazomethane and acrylonitrile, which are capable of alkylating DNA. In addition, arecaidine may undergo epoxidation at alkaline pH to give rise to carcinogenic 3,4-epoxide (Panigrahi and Rao, 1984). Lime, another component of pan masala, could conceivably act by generation of reactive oxygen species, which are implicated in the carcinogenic process (Nair *et al.,* 1992), while catechu is known to liberate tannins which are genotoxic (IARC, 1985). Thus, it appears that the induction of histogenetically distinct lesions in different organs of mice in the present study can be attributed to various carcinogens and their derivatives present in the pan-masala mixture. However, it is not possible to specify individual carcinogens that may be responsible for the development of different tumors.

A causal association appears to exist between the habit of chewing pan masala and early onset of oral submucous fibrosis, a condition known to predispose to malignancy (Babu *et al.,* 1996). We have reported that pan masala exhibits tumor-promotor and -progressor activity in mouse skin, stomach and esophagus (Ramchandani *et al.,* 1998). In the present study, we have provided unequivocal evidence regarding carcinogenicity of pan masala in different tissues, and particularly in the lung of S/RVCri mice. Taken together, our findings strongly indicate that pan masala, a chewing product without tobacco, should be considered as a potential human carcinogen.

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