Toxicological evaluation of pan masala in pure inbred Swiss mice: A preliminary report on long-term exposure study

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Pan masala, a product similar to betel quid in powder form, is stated to be a combination of ingredients like betel nut, catechu, lime, sandal oil, menthol, cardamom, flavour spices, fennel seeds, sugar, waxes, til seeds, colours, etc. Pan masalas are consumed abundantly even by those who generally refrain from smoking or with other tobacco addictions. Use of pan masala is considered to be harmless. In this study, the carcinogenic potential of pan masala was determined by exposing pure inbred Swiss mice of both sexes to pan masala mixed in feed in 2% concentration for eighty weeks in total, but the schedule of sacrifice of 6 animals from each group was started after 16 and 56 weeks of exposure, in order to find out the earlier tumour recurrence, if any, in these groups. Pan masala in 2% concentration in feed fed for 16 weeks could not produce any tumour, but after 56 weeks of exposure in sada pan masala group, 4 out of 12 animals were observed with tumours. But, in the group having tobacco as one of the constituents, 7 out of 12 animals showed tumours primarily affecting lungs, stomach, liver, testis, ovary and adrenal. In the control group, 1 out of 12 animals developed tumours, suggesting that the pan masala may be carcinogenic and habitual pan masala use in humans may exert carcinogenic influence.

USING traditional Indian technology, the tobacco industry has developed a product similar to betel quid ready for immediate consumption, packed in small, beautiful convenient sachets. This product is known as 'pan masala'. This is consumed abundantly by the people, even by those who do not have the habit of smoking or any other form of tobacco addiction. As printed on the packets, pan masala consists of arecanut, catechu, lime, cardamom and unspecified flavouring agents with or without tobacco. Even though tobacco with betel-nut chewing is not a common practice among the school children, consumption of pan masala is gaining tremendous popularity even among children at the secondary school level. Both boys and girls are equally attracted to the pan masala consumption.

Arecanut, a major constituent of pan masala, contains several alkaloids which are found to be clastogenic, genotoxic and carcinogenic in nature as is evident from different experimental systems[1–7]. Extract of pan masala increased the frequency of sister chromatid exchange and chromosomal aberrations in both mouse bone marrow cells[8] and CHO cell lines[9]. Bagwe *et al.*[10] and Polasa *et al.*[11] reported mutagenic potential of ethanolic and aqueous extracts of different brands of pan masala.

Similarly, catechu has been shown to cause dominant lethal mutation and chromosomal abnormality in mouse bone marrow cells[12]. Epidemiological studies have shown an association between the habit of chewing betel-nut and risk of oral cancer in the Indian population[13].

However, so far no chronic toxicity studies have been initiated with regard to neoplastic potential of pan masala. Therefore, the present investigation – a 80-week long exposure study – has been initiated with the animals sacrificed in 16-weeks gap and at the end of 56 weeks. Finally, to find out the sequential changes, the rest of the animals will be sacrificed after 80 weeks and histopathological alterations will be studied in lung, liver, kidney, testis, ovary, oesophagus and fore stomach, the target tissues.

Pure inbred Swiss mice raised in the Animal House at our institute were used for these experiments. Sada pan masala and pan masala with tobacco (*Gutkha*) of a very popular brand were obtained from the market and used throughout the experiment. A total of 180 mice with an average age of 6 weeks were used. Thirty animals each of both sexes were exposed to sada pan masala and pan masala with tobacco, and an equal number of controls were provided with normal diet. Pan masala in powdered form was mixed in the diet (2%) after grinding properly in an electric mixer. The diet consisted of cracked wheat 70%, cracked Bengal gram 20%, fish meal 5%, yeast powder 4%, groundnut oil 0.5% and shark liver oil 1% in the form of dry mash. Pan masala was fed for 80 weeks. Meanwhile, 6 animals from each group were sacrificed after 16 weeks, followed by 56 weeks, and the rest were left for observation for another 24 weeks. During the observation period, all the animals were periodically weighed and toxicological signs and symptoms were recorded. Six animals from each group were sacrificed after 16 weeks and 56 weeks of exposure to pan masala mixed in feed. Autopsy was performed and lungs, heart, kidney, liver, spleen, brain, gonads, oesophagus and stomach were examined histologically. The incidence of tumour in the experimental group compared to the control group was evaluated statistically.

The mortality and survival rates of animals after 56 weeks are presented in Table 1. There was no significant difference between the survival rates of the animals in control and experimental groups, although more animals died in exposed groups. Signs of pan masala intoxication such as ruffled skin, loss in weight, bleeding

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Table 1. Morbidity rate of animals during 56 weeks

Group	Sex	No. of animals at the beginning of experiment	No. of survivors					
			16-weeks			56-weeks		
			Before	Animals died before sacrifice	After sacrifice	Before	Animals died before sacrifice	After sacrifice
Control	Male	30	29	1	23	20	3	14
	Female	30	30	Nil	24	22	2	16
Sada pan masala	Male	30	28	2	22	18	4	12
	Female	30	29	1	23	20	3	14
Pan masala with tobacco	Male	30	28	2	22	17	5	11
	Female	30	28	2	22	18	4	12

Table 2. Incidence of tumour in control and experimental mice

Group	Sex	No. of animals killed	No. of animals with tumour		Lung tumour	Liver tumour	Haemangioma	Haemangio endothelioma	Testis tumour	Ovary tumour	Adrenal tumour	Stomach tumour
			16 weeks	56 weeks								
Control	Male	6	Nil	1	1	–	–	–	–	–	–	–
	Female	6	Nil	Nil	–	–	–	–	–	–	–	–
Sada pan masala	Male	6	Nil	2	1	–	–	1		–	–	–
	Female	6	Nil	2	1	–	1	–	–	–	–	–
Pan masala with tobacco	Male	6	Nil	4	1	1	–	–	1	–	1	–
	Female	6	Nil	3	–	–	–	1	–	1	–	1

from eyes with unilateral or bilateral opacity were observed in animals having 56 weeks of exposure. It was more pronounced in animals fed with pan masala with tobacco.

The occurrence of tumours in control and experimental mice is presented in Table 2. In the experimental group receiving sada pan masala and pan masala with tobacco, the number of tumours was significantly higher than the control group. In control group the only detected tumour was lung adenoma. But in the group exposed with sada masala, 4 tumours were observed out of 12 animals; thus 33% of the animals had tumours after 56 weeks of exposure. The lung tumour grew either as small foci confined to one lobe and histologically the tumour was papillary adenoma in females. In males, although the tumour has confined to one lobe, it almost replaced the whole lobe and infiltrated the different regions of the lung and histologically it was adenocarcinoma (Figure 1 *a*). The liver tumour grossly appeared pinkish confined to one lobe only and both the tumours in this group were histologically haemangioma and haemangio endotheliomas.

In pan masala with tobacco, out of 12 animals 7 developed tumour. This included histologically well-differentiated liver cell carcinoma (Figure 1 *b*), lung adenoma papillary type, adeno-carcinoma of the adrenal gland (Figure 1 *c*), testicular tumour (Leydig's cell tumour, Figure 1 *d*), cystadenoma in ovary (Figure 1 *e*) and papilloma in stomach (Figure 1 *f* and *g*).

Few attempts have been made to evaluate the carcinogenic effects of pan masala in animals, but none of the reports have described the long-term exposure of animals to pan masala. Sinha[14] observed dysplasia in 95% albino rats when pan masala paste with tobacco was applied in the buccal mucosa. Khirme *et al.*[15] observed mild leukoplakia and submucous fibrosis in the oral cavity of albino rats when painted with paste of pan masala. After six months of exposure to the paste of pan masala, mild to moderate loss of nuclear polarity and increase in keratosis, parakeratosis, inflammatory cell infiltration and vascularity were observed in excess compared to the control group.

Sarma *et al.*[16] reported that chronic feeding of pan masala impaired liver function and decreased relative weight of the gonad and brain. Kashyap *et al.*[17] while evaluating the toxicological effect of pan masala using pure inbred Swiss mice observed significant increase in chromosomal abnormalities in bone marrow cells in comparison to control group. Oral administration also caused somatic chromosome aberrations as well as sperm-head abnormalities. Ramchandani *et al.*[7] evaluated carcinogenic activity of pan masala by painting the

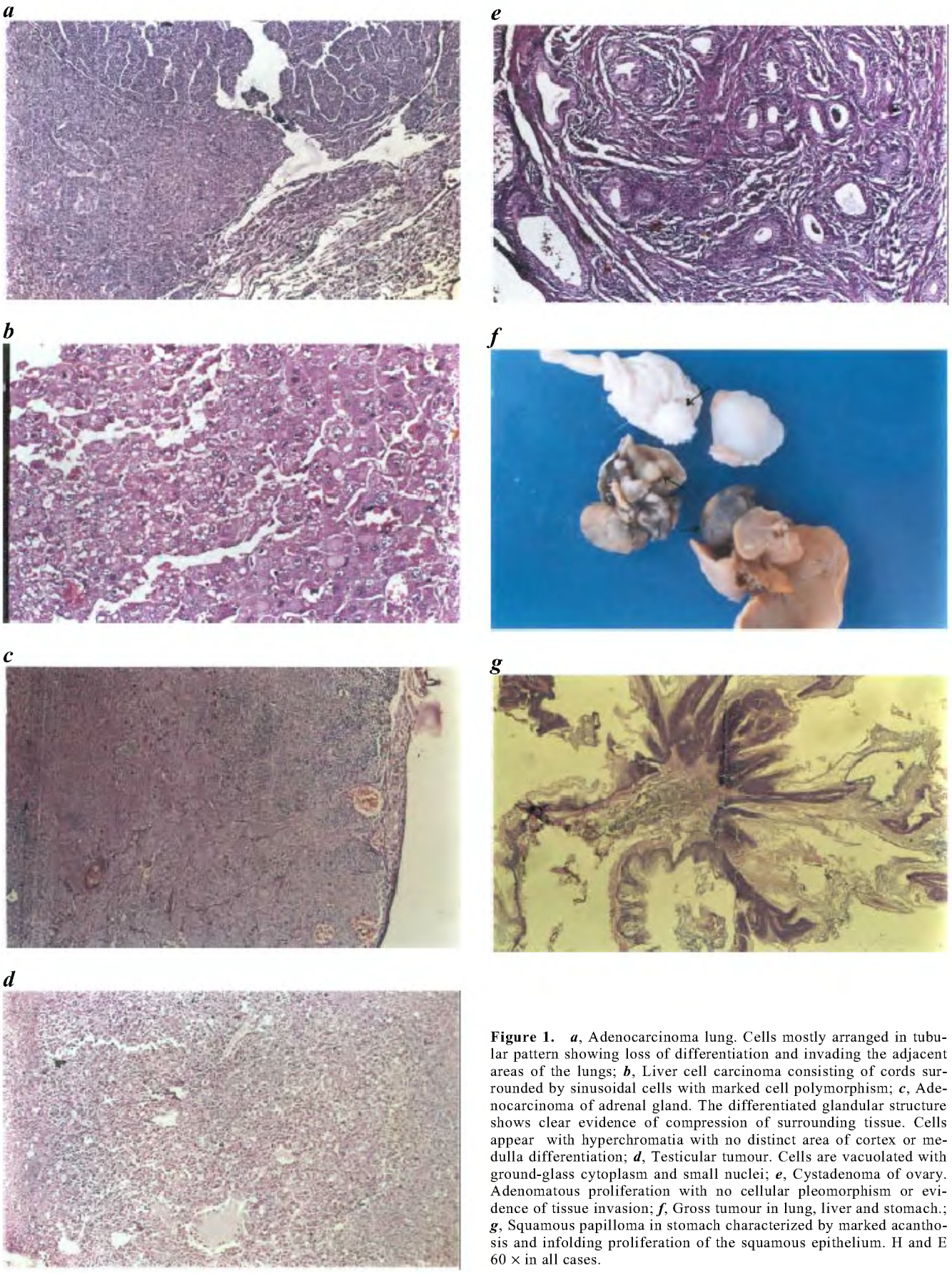

Figure 1. ***a***, Adenocarcinoma lung. Cells mostly arranged in tubular pattern showing loss of differentiation and invading the adjacent areas of the lungs; ***b***, Liver cell carcinoma consisting of cords surrounded by sinusoidal cells with marked cell polymorphism; ***c***, Adenocarcinoma of adrenal gland. The differentiated glandular structure shows clear evidence of compression of surrounding tissue. Cells appear with hyperchromatia with no distinct area of cortex or medulla differentiation; ***d***, Testicular tumour. Cells are vacuolated with ground-glass cytoplasm and small nuclei; ***e***, Cystadenoma of ovary. Adenomatous proliferation with no cellular pleomorphism or evidence of tissue invasion; ***f***, Gross tumour in lung, liver and stomach.; ***g***, Squamous papilloma in stomach characterized by marked acanthosis and infolding proliferation of the squamous epithelium. H and E 60 × in all cases.

mouse skin for 40 weeks with EPME (ethanolic pan masala extract) or by gavage-feeding for six months. Following initiation with DMBA (9,10-dimethylbenz (a) anthracene), carcinogenesis of mouse skin was promoted with different doses of EPME, while gastric and oesophageal tumour-promoting activity was evaluated by administering EPME by gavage to animals initiated with DEN (diethylnitrosomine). EPME at 25 mg per dose promoted skin papilloma formation between 30 and 40 weeks of treatment and enhanced the rate of conversion of papilloma to carcinoma. EPME was inactive as a complete carcinogen, but effectively promoted the development of fore stomach and oesophageal papilloma and carcinoma in a concentration-dependent manner.

In the present study, oral feeding for 56 weeks was continued simulating the condition that exists in human beings. Further, this was a report of oral feeding in Swiss inbred mice for a long duration, which induced variable types of cancer in different organs.

Considering human exposure to pan masala, unlike tobacco chewers who spit out the juice, pan masala users often swallow the saliva extract, thus increasing the possibility of severe carcinogenic effects of pan masala at sites other than the oral cavity. In the present experimental set-up a similar situation existed, which resulted in variable types of cancer in different organs. An earlier report on[17] chemical and toxicological evaluation of pan masala revealed the presence of polyaromatic hydrocarbons, nitrosamine, toxic metals such as lead, cadmium and nickel and residual pesticides like DDT and BHC and their isomers which are known to be carcinogenic. Besides this, various types of fungi, including *Aspergillus* sp. were isolated from pan masala[18]. Aflatoxigenic strain of *Aspergillus* is known to produce aflatoxin, a potent liver carcinogen. Further, arecanut, the main component of pan masala, contains several alkaloids which are converted to carcinogenic nitrosamines in mild nitrosation conditions[19]. Arecoidine and its methyl ester, arecoline have been suspected to exhibit carcinogenic/mutagenic properties since they are capable of reacting with cysteine *in vivo* and *in vitro* to produce cysteine/3-alkylation adducts[20]. In addition, super oxide ions may be associated with multi-step process of carcinogenesis which may be generated due to auto-oxidation of polyphenols and interaction of catechin with lime[21]. Thus, it is reasonable to presume that some of these compounds as well as tanin in catechu[3] may be responsible for the carcinogenic properties of pan masala (sada or with tobacco) observed in the present study.

The findings on increased induction of benign and malignant tumours in different organs suggest that pan masala use, whether it is sada or with tobacco, may exert carcinogenic or even co-carcinogenic influence in habitual users of such products.

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