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Mutagenic potential of Indian tobacco products

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The mutagenic potential of aqueous extracts of masheri (ME), chewing tobacco alone (CTE) and a mixture of chewing tobacco plus lime (CTLE) was tested using the Ames assay. ME exhibited mutagenicity in *Salmonella typhimurium* TA98 upon metabolic activation with aroclor-1254-induced rat liver S9, while nitrosation rendered it mutagenic in TA100 and TA102. CTE exhibited borderline mutagenicity in the absence or presence of S9 in TA98 and TA100 and after nitrosation in TA102, while nitrosation led to doubling of TA98 and TA100 revertants. In contrast, CTLE exhibited direct mutagenicity in TA98, TA100 and TA102, was mutagenic to TA98 upon S9 addition and induced mutagenic responses in all three tester strains after nitrosation. Experiments using scavengers of reactive oxygen species (ROS) suggested that CTLE-induced oxidative damage in TA102 was mediated by a variety of ROS. The high mutagenic potency of CTLE *vis à vis* that of CTE may be attributed to changes in the pH leading to differences in the amount and nature of compounds extracted from tobacco. Thus, exposure to a wide spectrum of tobacco-derived mutagens and promutagens may play a critical role in the development of oral cancer among users of tobacco plus lime.

Introduction

Several epidemiological studies have confirmed the high risk for cancer at various sites among tobacco *habitués* (Zaridze and Peto, 1986). Oral cancer, which is the most common type of cancer in India, is clearly associated with smokeless tobacco habits (Sanghvi, 1989). Chewing of tobacco with lime is common among males as well as females, while application of a pyrolysed tobacco product termed 'masheri' as a dentifrice to the gums and teeth is practised widely by females. Chemical analysis has revealed the presence of mutagens and carcinogens in chewing tobacco as well as masheri (Bhide *et al.*, 1984, 1987; Nair *et al.*, 1987, 1989). In the present investigation, the mutagenic potential of three commonly used Indian smokeless tobacco products was compared in a bid to ascertain the nature and extent of mutagenic exposure sustained by users of masheri, chewing tobacco alone and chewing tobacco mixed with lime.

Materials and methods

Chemicals

β-Nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, histidine, biotin, superoxide dismutase and catalase were from Sigma (St Louis, MO) while bacto agar and bacto nutrient broth were purchased from Difco (Detroit, MI, USA). Aroclor-1254 was a gift from Dr S.K.Nigam (National Institute of Occupational Health, Ahmedabad, India). All the other reagents used were of analytical grade.

Bacterial strains

Salmonella typhimurium tester strains TA98, TA100 and TA102 were generously provided by Prof. B.N.Ames (University of California, Berkeley, CA, USA).

Extraction procedures

Masheri. Sun-dried, uncured bidi tobacco (*Nicotiana tabacum*) was used to prepare masheri since bidi industry workers commonly use masheri prepared from this source. The tobacco was roasted on a hot iron griddle (180–200°C/10 min) with frequent turning until it was pyrolysed and blackened masheri was obtained. It was then ground to a fine powder and extracted in 10 mM phosphate buffer, pH 7, as described previously (Guttenplan, 1987a; Bagwe and Bhisey, 1991). The extract (ME) was passed through a millipore filter and stored at –20°C until use.

Chewing tobacco. A 10 g quantity of 'Pandharpuri' brand chewing tobacco (*N.tabacum*), alone or after mixing with 1 g of lime, was extracted in 100 ml of glass distilled water for 24 h at 22°C on a rotary shaker. The extracts were centrifuged at 12 000 *g* for 15 min, and the pH of each supernatant was recorded prior to lyophilization. The lyophilized powder was ground thoroughly and stored at –20°C. Just before mutagenicity testing, required amounts of the chewing tobacco extract (CTE) or chewing tobacco plus lime extract (CTLE) were reconstituted in glass distilled water (250 mg/ml) and passed through a millipore filter.

Metabolic activation system

A liver microsomal fraction from aroclor-1254-induced male Sprague–Dawley rats was used at 10% concentration in the S9 mix (Maron and Ames, 1983).

Mutagenicity testing

The mutagenicity of the three tobacco extracts was determined using the Ames assay–liquid preincubation protocol (Maron and Ames, 1983; Yahagi *et al.*, 1975). Extracts were tested without any modification, upon metabolic activation with 100 μl of S9 mix or after nitrosation at pH 2 with 300 μg of acidified nitrite for 2 h at 37°C (Bagwe *et al.*, 1990). One hundred microlitres of a 16-h-old culture of *S.typhimurium* strains TA98/TA100/TA102 were added to the unmodified or chemically modified extracts and the tubes were incubated for 20 min at 37°C. To this, 2 ml of histidine-poor soft agar were added and the mixture was overlaid on minimal glucose agar plates. After 48 h incubation at 37°C, revertant colonies on these plates were enumerated.

Doses of all the extracts were based on the original dry weight of masheri/tobacco. Initially, a wide range of extract concentrations was tested to determine the toxic dose, and further experiments were carried out at doses below this level. A sparse background lawn was considered to be an indicator of toxicity. Results represent the mean of two independent experiments using triplicate plates. An extract was considered to be mutagenic if it elicited a clear dose response and induced at least a 2-fold increase over the spontaneous reversion (SR) at any of the doses tested. In cases where the first criterion was fulfilled and the revertant number was 1.5-fold higher than the control value, mutagenic response was considered as borderline.

Scavenging studies

Non-toxic and non-mutagenic doses of the antioxidant enzymes superoxide dismutase (SOD) and catalase (25 μg/plate), and of the hydroxyl/peroxyl radical scavengers mannitol and benzoate (50 μg/plate), were incorporated into the assay mix in order to determine the nature of reactive oxygen species (ROS) responsible for direct mutagenicity of CTLE in TA102 strain (Niphadkar *et al.*, 1994). The 2-fold concentrated culture was pre-equilibrated with each scavenger in chilled buffer for 30 min. Following this, CTLE was added, incubation was continued for 90 min at 37°C and the reaction mix was assayed for mutagenicity (Islam *et al.*, 1991).

Results

Initially, ME was tested in the concentration range 700 μg–140 mg/plate, CTE in the range 1–50 mg/plate and CTLE in the range 0.25–25 mg/plate. Under all the conditions, a toxic

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Table I. Mutagenicity[a] of ME

ME (mg/plate)	TA98			TA100			TA102		
	−S9	+S9	$+NO_2$	−S9	+S9	$+NO_2$	−S9	+S9	$+NO_2$
0	21 ± 1	24 ± 2	20 ± 1	106 ± 6	124 ± 4	112 ± 2	269 ± 16	294 ± 17	322 ± 2
10	22 ± 1	25 ± 1	23 ± 1	98 ± 5	117 ± 6	394 ± 15[b]	288 ± 11	345 ± 2	1370 ± 17
20	23 ± 1	33 ± 2	22 ± 1	102 ± 5	96 ± 5	254 ± 17	296 ± 27	312 ± 16	1736 ± 17[b]
40	23 ± 2	30 ± 2	21 ± 2	115 ± 2	101 ± 5	T	245 ± 21	294 ± 21	1050 ± 34
60	21 ± 1	36 ± 1	T	107 ± 3	97 ± 5	T	260 ± 18	289 ± 11	210 ± 26
80	28 ± 2	49 ± 2[b]	T	101 ± 4	106 ± 5	T	255 ± 23	315 ± 8	T

T = toxic.
[a]Results of two experiments using triplicate plates (mean ± SE).
[b]Maximum mutagenicity.

Table II. Mutagenicity[a] of CTE

CTE (mg/plate)	TA98			TA100			TA102		
	−S9	+S9	$+NO_2$	−S9	+S9	$+NO_2$	−S9	+S9	$+NO_2$
0	26 ± 3	43 ± 4	18 ± 1	126 ± 3	137 ± 3	101 ± 2	289 ± 5	253 ± 6	238 ± 5
1	33 ± 2	53 ± 5	22 ± 1	116 ± 3	217 ± 6[b]	100 ± 3	299 ± 4	255 ± 7	282 ± 4
2	38 ± 4	69 ± 6[b]	26 ± 2	229 ± 5[b]	183 ± 3	119 ± 2	297 ± 4	268 ± 4	317 ± 3
5	40 ± 6	51 ± 3	37 ± 2	193 ± 4	174 ± 6	189 ± 4	286 ± 5	251 ± 5	445 ± 8[b]
10	50 ± 4[b]	40 ± 3	49 ± 1[c]	146 ± 2	158 ± 3	214 ± 5[c]	299 ± 5	251 ± 6	T
20	27 ± 2	38 ± 6	31 ± 2	133 ± 4	101 ± 3	167 ± 3	280 ± 7	258 ± 6	T

T = toxic.
[a]Results of two experiments using triplicate plates (mean ± SE).
[b]Borderline mutagenicity.
[c]Maximum mutagenicity.

Table III. Mutagenicity[a] of CTLE

CTLE (mg/plate)	TA98			TA100			TA102		
	−S9	+S9	$+NO_2$	−S9	+S9	$+NO_2$	−S9	+S9	$+NO_2$
0	27 ± 1	43 ± 2	24 ± 1	114 ± 2	137 ± 3	133 ± 3	295 ± 6	253 ± 5	235 ± 3
0.25	NT	NT	45 ± 2	NT	NT	202 ± 3	336 ± 4	NT	278 ± 5
0.5	NT	169 ± 3	61 ± 3	204 ± 5	121 ± 2	279 ± 3[b]	428 ± 6	254 ± 5	415 ± 7
1	32 ± 1	192 ± 3[b]	68 ± 2[b]	274 ± 4[b]	105 ± 4	242 ± 4	694 ± 4[b]	255 ± 7	519 ± 6[b]
2	39 ± 1	164 ± 3	50 ± 4	85 ± 3	103 ± 3	T	353 ± 6	251 ± 5	405 ± 6
5	55 ± 2[b]	88 ± 2	T	93 ± 2	90 ± 2	T	274 ± 4	249 ± 5	T

NT = not tested; T = toxic.
[a]Results of two experiments using triplicate plates (mean ± SE)
[b]Maximum mutagenicity.

response was observed at doses >80 mg/plate of ME, 20 mg/plate of CTE and 5 mg/plate of CTLE. Tables I, II and III show the results of mutagenicity testing of ME, CTE and CTLE, respectively, using tester strains TA98, TA100 and TA102.

Mutagenicity of ME

TA98. A dose-dependent increase in revertant number was obtained upon metabolic activation of ME, with maximum reversion induced at the 80 mg dose. It was non-mutagenic in the TA98 strain both in the absence of S9 and upon nitrosation (Table I).

TA100. ME was non-mutagenic in this strain in the absence and presence of S9. Upon nitrosation, mutagenicity was noted at the 10 mg dose.

TA102. Nitrosated ME was highly mutagenic in this strain, with a >5-fold increase in induced reversion at the 20 mg dose, while ME was non-mutagenic in the absence and presence of S9.

Mutagenicity of CTE

TA98. CTE was weakly mutagenic in the absence of S9 (10 mg/plate) as well as upon metabolic activation (2 mg dose). Nitrosation led to doubling of the revertant number over SR (37 ± 2 versus 18 ± 1) at the 5 mg dose, with maximum mutagenicity being observed at the 10 mg dose (Table II).

TA100. CTE was weakly mutagenic in this strain at the 2 and 1 mg doses in the absence and presence of S9, respectively. However, doubling over SR was obtained with nitrosated CTE at a dose of 10 mg.

TA102. Nitrosation rendered CTE weakly mutagenic at a dose of 5 mg, while the extract was non-mutagenic both in the absence and presence of metabolic activation.

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Table IV. Effect of ROS scavengers on oxidative mutagenicity[a] of CTLE

CTLE (mg/plate)	Modifier				
	None	SOD	Catalase	Mannitol	Benzoate
0	287 ± 5	272 ± 5	284 ± 4	270 ± 5	264 ± 3
0.5	459 ± 7	349 ± 12	355 ± 6	265 ± 5	267 ± 6
1	686 ± 5[b]	348 ± 16	352 ± 11	270 ± 5	273 ± 4

[a]Results of two experiments using triplicate plates (mean ± SE of TA102 revertants).
[b]Mutagenic.

Mutagenicity of CTLE

TA98. CTLE was directly mutagenic in strain TA98 at a dose of 5 mg. In the presence of S9, mutagenicity was noted at the low dose of 0.5 mg, and the maximum revertant number was observed at the 1 mg dose upon metabolic activation and also after nitrosation (Table III).

TA100. CTLE elicited maximum mutagenic response at the 1 mg dose in the absence of S9 and nitrosation rendered it mutagenic to TA100 at 0.5 mg, while it was non-mutagenic in the presence of S9.

TA102. CTLE evoked a mutagenic response in the absence of S9 and upon nitrosation at a dose of 1 mg/plate, while addition of S9 abolished its mutagenicity.

Effect of ROS scavengers on the oxidative mutagenicity of CTLE

Incorporation of superoxide dismutase and catalase reduced the number of revertants at 0.5 and 1 mg doses of CTLE, while mannitol and benzoate completely abolished the mutagenicity of CTLE at all the doses tested (Table IV).

Discussion

The black masheri used in this study was prepared from processed tobacco used for the manufacture of bidis—Indian substitutes for cigarettes. As in the case of the aqueous extract of unburnt bidi tobacco (Bagwe and Bhisey, 1991), ME was mutagenic to TA100 upon nitrosation. However, the minimum mutagenic dose of nitrosated ME was 2.5-fold lower than that of similarly treated bidi tobacco extract. Nitrosated ME also exhibited mutagenic activity in TA102. In addition, ME was mutagenic to TA98 upon metabolic activation. It is well known that while unburnt tobacco contains only traces (parts per billion) of polycyclic aromatic hydrocarbons (PAH), masheri contains substantial amounts (µg/g level) of PAH (IARC, 1985; Nair *et al.*, 1987). While PAH are known to require metabolic activation to exert a mutagenic effect, formation of directly mutagenic nitro-PAH has been reported upon nitrosation of PAH (Wang *et al.*, 1978). Thus, the mutagenicity of masheri in TA98 after S9 treatment could be partially attributed to PAH, while direct mutagenicity of nitrosated ME could implicate the formation of nitro-PAH.

In keeping with the reported mutagenicity of Western chewing tobacco products and snuff (Whong *et al.*, 1984, 1985; Shirname-More, 1991), both CTE and CTLE were rendered mutagenic upon nitrosation at acidic pH. However, marked differences were noted in the mutagenic potential of CTE and CTLE. While CTE was weakly mutagenic in TA98 and TA100 in the presence and absence of S9, CTLE was directly mutagenic in all three strains and caused a 4-fold higher induction of TA98 revertants in the presence of S9. Further studies using scavengers of ROS showed that direct mutagenicity of CTLE to TA102 is mediated by ROS such as hydroxyl and peroxyl radicals, superoxide anions and hydrogen peroxide (Brawn and Fridovich, 1981). The mutagenic responses induced by CTLE are similar to those obtained earlier using gastric fluid from users of chewing tobacco mixed with lime (Niphadkar *et al.*, 1994). Although the same brand of chewing tobacco was used for preparing the extracts, addition of lime increased the pH of CTLE to 9.5 while that of CTE was 6.6. It is likely that, in case of CTLE, alkaline conditions may not only have resulted in more efficient extraction but the chemical nature of extracted compounds, too, may be different from those present in CTE.

Tobacco is known to contain several compounds such as aliphatic and aromatic hydrocarbons, phenols, alkaloids, amines, amides, *N*- and *O*-heterocyclic compounds, etc. (IARC, 1985). Of these, several nitrosamines and PAH are converted to mutagens after S9 treatment or upon nitrosation (Guttenplan, 1987b ; Hecht and Hoffmann, 1988; Jung *et al.*, 1991). In a recent study, the *O*-acetyl transferase-overproducing strain YG1024 was found to be highly sensitive to cigarette smoke mutagens (De Flora *et al.*, 1995). Thus, the use of this strain and nitroreductase-overproducing derivatives may throw further light on the mutagenicity of tobacco products tested in the present study. In view of the high extent of endogenous nitrosation reported among tobacco *habitués* (Nair *et al.*, 1986), users of all three of these tobacco products would run the risk of exposure to mutagenic *N*-nitroso compounds. However, clear-cut evidence regarding human carcinogenicity exists in the case of oral use of tobacco plus lime, betel quid containing tobacco and snuff (IARC, 1985). Thus it appears that, in users of tobacco plus lime, cumulative exposure to direct-acting mutagens and promutagens that act via frame shift, base pair substitution and oxidative DNA damage may play a critical role in the development of oral cancers.

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