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 arco-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 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, USA).

Extraction procedures

Masheri. Sun-dried, unceded tobacco (Nicotiana tabacum) was used to prepare mashers since bidi industry workers commonly use mashers 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 ‘Pandharupuri’ 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.

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.

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...
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.
Mutagenicity of CTE

TA98. CTE 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. CTE 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. CTE 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 CTE

Incorporation of superoxide dismutase and catalase reduced the number of revertants at 0.5 and 1 mg doses of CTE, while mannitol and benzoate completely abolished the mutagenicity of CTE 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; Shimame-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 alfiphatic 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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References


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