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Mutagenic activity of gastric fluid from chewers of tobacco with lime

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Although tobacco chewing is strongly associated with a high risk of oral and upper alimentary tract cancers, the nature of mutagenic exposure among users has not been clearly defined. In this study, tobacco-specific and mutagenic exposure of chewers of tobacco with lime was evaluated by analysis of gastric fluid (GF). The pH, nitrite and cotinine levels of GF samples from chewers and non-chewers were determined and the samples were tested for mutagenicity in the Ames *Salmonella*/microsome assay using *Salmonella typhimurium* strains TA98, TA100 and TA102. Cotinine was not detected in GF from non-chewers while the levels ranged between 0.4–13.64 μg/ml in samples from chewers; however, the mean pH values (3.8 ± 0.4 versus 2.8 ± 0.3) and nitrite levels (29.40 ± 1.51 versus 27.39 ± 0.83 μM) were similar in both groups. While all GF samples from non-chewers were non-mutagenic, samples from chewers were directly mutagenic or upon nitrosation to all the three tester strains and to TA102 strain in the presence of S9. Experiments using scavengers of reactive oxygen species (ROS) showed that mannitol and benzoate abolished the mutagenic response of TA102, indicating that ROS are principally responsible for oxidative damage. The findings provide specific information regarding the mutagenic exposure among tobacco chewers and suggest that tobacco chewing may be an important risk factor in the development of gastric cancer.

Introduction

Tobacco habits such as chewing of tobacco with lime or with betel quid are widely practiced in several Asian countries, including India, and are responsible for the high risk of oral, upper alimentary tract (1) and possibly gastric cancers (2). Tobacco contains a number of carcinogens (3) and smokeless tobacco extracts are found to be mutagenic in the bacterial reverse and forward mutation assays (4,5) as well as in the TK–human cell system (6).

Human exposure to mutagenic chemicals can be ascertained by monitoring the concentration of the chemical in question or its metabolites in body fluids (7) and by evaluating urinary mutagenicity. Although urinary mutagenicity testing detects exposure to mutagens, the assay is not specific to the chemicals under consideration since dietary and other mutagens are also excreted in urine (8). The presence of nicotine, cotinine and tobacco-specific nitrosamines (TSNA*) in the saliva of tobacco chewers (9) indicates that saliva-extractable constituents, including mutagens and fine tobacco particles, are ingested during tobacco chewing. Moreover, gastric conditions being favourable for the formation for *N*-nitroso compounds (NOC) (10), the gastric fluid (GF) is likely to contain mutagenic NOC produced from tobacco-derived nitrosatable precursors (11). Therefore in this investigation, GF was used to evaluate tobacco-specific and mutagenic exposure caused by tobacco chewing. Our findings on the mutagenic activity of GF samples from chewers of tobacco with lime are presented in this communication.

*Abbreviations: TSNA, tobacco-specific nitrosamines; NOC, *N*-nitroso compounds; ROS, reactive oxygen species; GF, gastric fluid; G-6-P, glucose-6-phosphate; DMSO, dimethylsulphoxide; SOD, superoxide dismutase; DETB, diethylthiobarbituric acid; GFC, gastric fluid concentrate.

Materials and methods

Chemicals

Chloramine T, cotinine, NADP, biotin, histidine, glucose-6-phosphate (G-6-P), dimethylsulphoxide (DMSO), superoxide dismutase (SOD) and catalase from Sigma (USA), diethylthiobarbituric acid (DETB) and potassium cyanide from Aldrich (USA) and Bacto agar from Difco (USA) were used. Sulphanilamide, ammonium sulphamate and *N*-(1-naphthyl)-1-ethylenediamine dihydrochloride were obtained from Sisco Research Laboratories (India). All other reagents used were of analytical grade.

Sample collection and processing

GF samples were obtained with prior consent (n = 15) from patients undergoing routine endoscopic examination after overnight fasting. Nine individuals who had a chewing tobacco habit, voluntarily chewed 50–300 mg of 'Pandharpuri' brand of chewing tobacco mixed with lime 1–1.5 h prior to endoscopy. Control samples were provided by six individuals, who had no tobacco chewing habits. A catheter was passed through the suction/biopsy channel for aspirating GF (3–6 ml), which was collected in sterile tubes and stored in ice until transportation to the laboratory. Each GF sample was coded by an independent worker not connected with this work. Samples were centrifuged at 3000 r.p.m. for 15 min and the supernatants were stored at −20°C until further use. Prior to storage, the pH of each GF sample was recorded and aliquots of 1 and 0.5 ml were processed for estimation of cotinine and nitrite respectively.

Estimation of cotinine and nitrite

Cotinine and related metabolites of nicotine (hereafter referred to as 'cotinine') were estimated by the method of Peach *et al.* (12) as previously described (13). The generation of a pink colour was taken as a positive reaction. The concentration of cotinine in test samples was calculated from the absorbance at 532 nm using cotinine as standard.

For the determination of nitrite levels (14), 0.5 ml GF was diluted to 5 ml with glass-distilled water and 0.1 ml of 5 N HCl, 1 ml of 0.2% aqueous sulphanilamide, 0.2 ml of ammonium sulphamate and 1 ml of *N*-(1-naphthyl)-1-ethylenediamine dihydrochloride were sequentially added to the tube. The absorbance was measured at 546 nm in a Shimadzu double-beam spectrophotometer. The concentration of nitrite was calculated using a standard curve for sodium nitrite.

Sample processing and mutagenicity testing

Preliminary studies revealed that mutagens were retained more strongly on XAD-2 resin columns at pH 2 than at neutral pH (results not shown). Hence, all GF samples were adjusted to pH 2 with 1 N HCl prior to passing through XAD-2 column for removal of histidine (15). The column was eluted with acetone, the eluate was lyophilized and the residue was dissolved in DMSO in order to obtain a 5-fold GF concentrate (GFC).

The protocol of Maron and Ames (16) was employed. GFC were tested at six different doses (0–5 μl), at increments of 1 μl in a liquid preincubation assay (17) without any modification, after metabolic activation with Aroclor 1254-induced rat liver microsomal fraction (18) or upon nitrosation at pH 2 for 20 min at 37°C with 150 μg sodium nitrite, a dose reported to be non-mutagenic in previous studies (19). In some cases, 0.5 μl GFC was also tested. S9 mix (1 ml) contained 100 μl of 80 mM KCl–$MgCl_2$, 8 mM NADP, 50 mM G-6-P; 300 μl 0.25 M

Sorenson's phosphate buffer (pH 7.4) and 50 μl of S9. After incubation for 90 min at 37°C, 2 ml of histidine-poor top agar was added to the tubes and the mixture was superimposed onto minimal glucose agar plates. Duplicate plates were incubated at 37°C for 48 h and the number of revertants was counted.

Scavenging studies

SOD and catalase were used as scavengers of superoxide anions and H_2O_2 respectively, while mannitol and sodium benzoate served as scavengers of hydroxyl radicals (20). All the scavengers were used at non-toxic concentrations, the final concentration of SOD and catalase being 25 μg/plate while that of mannitol and benzoate was 50 μg/plate. For each sample the dose of GFC that elicited maximum mutagenic response in TA102 was used to study the effect of ROS scavengers. The overnight culture of *S.typhimurium* was spun down and resuspended in PBS (pH 7.2) to achieve a 2-fold concentration. After mixing with 2× concentration of scavengers, the culture was equilibrated on ice for 30 min and used for mutagenicity testing by the protocol described above.

Statistical analysis

The samples were decoded prior to data analysis. The correlation matrix of the software 'Statgraphics' was used to determine the correlation between the amount of tobacco chewed and cotinine in GF. Cotinine and nitrite levels in GF of chewers and non-chewers were compared using the Mann–Whitney Wilcoxon test. A GF sample was considered mutagenic if (i) it yielded a clear dose-related increase in the revertant number, and (ii) the maximum revertant number was significantly higher ($P < 0.05$) than the spontaneous revertant number, when compared with the simple chi-square test. In each case the maximum revertant number was found to be beyond the upper 99% confidence interval limit, i.e. >3 SD of the respective spontaneous revertant number.

Table I. Amount of tobacco chewed, pH, nitrite and cotinine levels in GF

Sample no.	Tobacco chewed (mg)	pH	Nitrite (μM)	Cotinine (μg/ml)
Non-chewers				
1	–	4.3	27.23	ND
2	–	4.0	28.41	ND
3	–	3.8	32.94	ND
4	–	3.6	25.83	ND
5	–	2.0	25.50	ND
6	–	5.2	35.52	ND
Chewers				
1	50	2.4	27.13	0.40
2	50	2.0	25.83	4.30
3	100	3.9	26.48	1.70
4	200	4.0	33.59	2.15
5	200	2.0	25.83	5.50
6	200	2.5	28.41	4.80
7	200	2.0	29.07	13.64
8	300	2.3	27.13	1.10
9	300	3.7	25.50	0.40

ND, not detectable.

Results

pH, cotinine and nitrite levels

The pH of GF samples from chewers and non-chewers ranged from 2–4 and 2–5.2 respectively. However, the mean pH values in the two groups were similar (2.8 ± 0.3 versus 3.8 ± 0.4) (Table I). Cotinine was present in the GF samples from chewers, while it was not detectable in the samples from non-chewers (Table I). Among chewers, cotinine levels ranged from 0.4 to 13.64 μg/ml, with a mean of 3.78 ± 1.39 μg/ml. In these individuals no correlation was obtained between the amount of tobacco chewed and cotinine levels in GF. As shown in Table I,

Table II. Mutagenicity of GF samples from tobacco chewers

Sample no.	Mean no. of revertants ± SE								
	−S9			+S9			$+NO_2$		
	SR	MR	*P* value	SR	MR	*P* value	SR	MR	*P* value
TA98									
1	29 ± 1	58 ± 2	0.002	28 ± 1	28 ± 5	NS	28 ± 4	61 ± 3	0.0005
2	32 ± 4	50 ± 3	0.047	24 ± 2	35 ± 1	NS	33 ± 5	80 ± 2	1×10^{-5}
3	24 ± 0	42 ± 2	0.015	22 ± 1	26 ± 1	NS	26 ± 1	39 ± 2	NS
4	23 ± 1	46 ± 3	0.006	26 ± 1	34 ± 2	NS	31 ± 2	45 ± 1	NS
5	39 ± 1	77 ± 4	0.0004	24 ± 2	34 ± 1	NS	38 ± 1	79 ± 6	0.0002
6	29 ± 1	56 ± 1	0.003	28 ± 1	45 ± 2	0.047	28 ± 4	68 ± 3	5×10^{-5}
7	29 ± 1	56 ± 2	0.003	24 ± 2	40 ± 3	0.046	31 ± 1	59 ± 1	0.003
8	29 ± 1	60 ± 1	0.001	24 ± 2	37 ± 2	NS	31 ± 1	56 ± 1	0.007
9	34 ± 5	43 ± 1	NS	24 ± 2	37 ± 2	NS	28 ± 1	59 ± 3	0.0008
TA100									
1	117 ± 4	184 ± 4	0.0001	116 ± 5	189 ± 2	3×10^{-5}	130 ± 4	218 ± 3	2×10^{-6}
2	141 ± 4	235 ± 5	1×10^{-6}	118 ± 5	159 ± 4	0.013	142 ± 5	231 ± 4	5×10^{-6}
3	111 ± 4	159 ± 4	0.004	124 ± 6	155 ± 2	NS	128 ± 3	224 ± 2	3×10^{-7}
4	119 ± 3	159 ± 1	0.016	124 ± 6	164 ± 2	0.018	128 ± 3	167 ± 2	0.023
5	119 ± 3	188 ± 0	8×10^{-5}	118 ± 5	140 ± 4	NS	128 ± 3	237 ± 2	1×10^{-8}
6	117 ± 4	193 ± 3	2×10^{-5}	116 ± 5	137 ± 3	NS	130 ± 4	228 ± 3	2×10^{-7}
7	119 ± 3	220 ± 4	4×10^{-8}	138 ± 4	222 ± 5	1×10^{-5}	132 ± 4	203 ± 2	0.0001
8	119 ± 3	190 ± 4	0.005	138 ± 4	181 ± 6	0.016	132 ± 4	231 ± 5	2×10^{-7}
9	135 ± 6	177 ± 4	0.017	124 ± 6	156 ± 2	NS	135 ± 5	212 ± 3	4×10^{-6}
TA102									
1	284 ± 6	375 ± 9	0.0004	265 ± 10	348 ± 15	8×10^{-4}	280 ± 15	465 ± 8	1×10^{-11}
2	255 ± 5	278 ± 3	NS	235 ± 5	265 ± 2	NS	253 ± 8	773 ± 13	0
3	388 ± 8	615 ± 9	3×10^{-7}	394 ± 14	1672 ± 28	0	266 ± 15	240 ± 10	NS
4	388 ± 8	524 ± 8	7×10^{-6}	394 ± 14	1030 ± 10	0	280 ± 15	420 ± 21	1×10^{-7}
5	256 ± 6	323 ± 8	5×10^{-5}	243 ± 4	299 ± 7	0.01	264 ± 4	394 ± 7	4×10^{-7}
6	284 ± 6	399 ± 5	1×10^{-5}	265 ± 10	305 ± 10	NS	280 ± 15	405 ± 9	2×10^{-6}
7	284 ± 6	344 ± 6	0.017	255 ± 10	617 ± 7	0	280 ± 15	644 ± 14	0
8	388 ± 8	788 ± 4	0	265 ± 10	660 ± 22	0	280 ± 15	585 ± 7	0
9	281 ± 6	352 ± 7	0.005	235 ± 5	255 ± 3	NS	287 ± 7	426 ± 11	5×10^{-7}

SR, spontaneous revertants; MR, maximum revertants; NS, not significant by chi-square test.

the range of nitrite levels in samples from non-chewers and chewers was 25.50–33.59 and 25.50–35.42 μM respectively, the mean nitrite level was similar in the two groups (29.40 ± 1.51 and 27.39 ± 0.83 μM).

Mutagenicity of GFC

All samples from non-chewers (controls) were uniformly non-mutagenic to all tester strains under the test conditions employed in this study. Table II summarizes data on the mutagenic potential of GFC from chewers to *S.typhimurium* strains TA98, TA100 and TA102. The mutagenic response was accompanied by a good dose–response relationship. Representative dose–response curves are depicted in Figure 1.

TA98

As shown in Table II, 8/9 GF samples were directly mutagenic. S9 treatment abolished the mutagenic response of six samples.

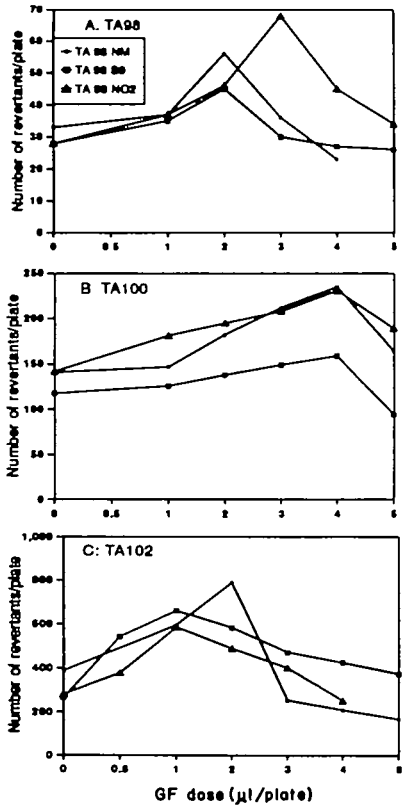

Fig. 1. Typical dose–response curves of GF mutagenicity. (A) TA98; (B) TA100; (C) TA102.

Upon nitrosation, 7/9 samples were mutagenic. In five of these, the minimum mutagenic dose was lower than that of the respective unmodified sample (Table III).

TA100

In the absence of metabolic activation, all the GF samples were mutagenic. S9 addition rendered four samples non-mutagenic, while the maximum revertant number was significantly higher than the spontaneous revertant number in the rest. Upon nitrosation, a significant increase in revertant number was observed in all the samples (Table II). Furthermore, in four nitrosated samples, the minimum mutagenic dose was lower than that of the respective unmodified sample (Table III).

TA102

As seen in Table II, 8/9 and 6/9 samples were mutagenic, without and with metabolic activation respectively. In the presence of S9, the minimum mutagenic dose was lower than that of the unmodified samples. Upon nitrosation 8/9 samples were mutagenic and the minimum dose was lower than that of the corresponding unmodified sample (Table III).

Table IV shows that at the doses employed, SOD, catalase, mannitol and benzoate were non-mutagenic. Incorporation of SOD in the assay completely abolished mutagenicity in 3/8 samples, while catalase abolished the mutagenic potential of 5/8 samples. However, the mutagenic potential of all the eight samples was completely abolished when mannitol or benzoate were added in the assay.

Discussion

That tobacco constituents find their way into the gastric cavity was evident from the presence of cotinine at a range of 0.4–13.4 μg/ml in GF samples from chewers, while it was not detected in the non-chewers' samples. Inter-individual variation in cotinine levels and lack of correlation between the amount of tobacco chewed and cotinine levels observed in this study are consistent with the reported differences in individual cotinine levels in other body fluids of tobacco chewers (9,21). In a study of residents from an area with a high gastric cancer rate, the mutagenic activity of GF was found to correlate with high nitrite levels (22). In the present study, nitrite levels were familiar in the two groups and did not correlate with the mutagenic activity of GF, while the pH of GF samples from chewers was lower than that of non-chewers.

Data on GF mutagenicity showed that all GFCs from control individuals were non-mutagenic, while those from chewers exhibited direct mutagenicity to TA98, TA100 and TA102. Our findings on the direct mutagenicity of an aqueous extract of a

Table III. Minimum mutagenic dose (μl)[a] of GFC from tobacco chewers

Sample no.	TA98			TA100			TA102		
	−S9	+S9	$+NO_2$	−S9	+S9	$+NO_2$	−S9	+S9	$+NO_2$
1	4.0	NM	3.0	3.0	3.0	2.0	2.0	1.0	0.5
2	2.0	NM	1.0	2.0	4.0	1.0	NM	NM	1.0
3	2.0	NM	NM	2.0	NM	0.5	1.0	1.0	NM
4	2.0	NM	NM	1.0	1.0	1.0	2.0	1.0	0.5
5	3.0	NM	4.0	1.0	NM	1.0	3.0	2.0	1.0
6	3.0	2.0	2.0	2.0	NM	2.0	2.0	NM	0.5
7	3.0	1.0	3.0	0.5	1.0	1.0	3.0	1.0	0.5
8	4.0	NM	2.0	1.0	2.0	2.0	1.0	0.5	0.5
9	NM	NM	3.0	2.0	NM	1.0	2.0	NM	1.0

[a]1 μl GFC corresponds to 5 μl of original GF. NM, non-mutagenic.

Table IV. Effects of ROS scavengers on mutagenicity of GF to TA102

Expt. no.	Sample no.	Mean no. of revertants ± SE				
		None	SOD	Catalase	Mannitol	Benzoate
1	SR	272 ± 4	309 ± 18	272 ± 8	282 ± 1	278 ± 4
	7	330 ± 6	265 ± 3[b]	299 ± 17[b]	196 ± 10[b]	231 ± 14[b]
2	SR	258 ± 8	257 ± 12	270 ± 5	27 ± 15	251 ± 6
	1	359 ± 7	316 ± 1	310 ± 2[b]	285 ± 10[b]	283 ± 3[b]
	2[a]	301 ± 1	250 ± 4	262 ± 4	255 ± 15	281 ± 1
	3	493 ± 6	TOXIC	443 ± 11	257 ± 5[b]	267 ± 5[b]
	4	459 ± 4	364 ± 2	345 ± 8	260 ± 13[b]	251 ± 6[b]
	5	355 ± 8	285 ± 10[b]	277 ± 6[b]	280 ± 2[b]	278 ± 18[b]
	6	376 ± 6	307 ± 2	303 ± 8[b]	273 ± 2[b]	267 ± 7[b]
	8	464 ± 9	374 ± 2	367 ± 12	288 ± 7[b]	277 ± 9[b]
	9	346 ± 6	300 ± 8[b]	287 ± 5[b]	271 ± 4[b]	241 ± 9[b]

[a]Sample no. 2 was uniformly non-mutagenic.
[b]Abolition of mutagenicity, SR, spontaneous revertants.

mixture of tobacco and lime to the three tester strains (manuscript under preparation) suggest that direct-acting mutagens in GF may be derived from the chewing mixture. On the other hand, endogenous nitrosation in the oral cavity and in the acidic gastric environment could play a role in the production of directly mutagenic NOC such as nitrosamides (23). Tobacco is known to contain TSNA that reportedly exhibit mutagenic activity in the strain TA100 upon metabolic activation with S9 (24,25). In the absence of a similar response in TA100, evidence for GF mutagenicity attributable to TSNA is lacking in the present study. However, a reduction in the minimum mutagenic dose and an elevated mutagenic potential of nitrosated GFC to all the three tester strains indicated that GF contained nitrosatable precursors of mutagenic NOC. The presence of other promutagenic moieties is also evident from an increase in TA102 revertant number upon treatment of samples with S9.

Scavengers of ROS were used in order to determine the nature of free radicals involved in GF mutagenicity to TA102. The results showed that oxidative damage was caused by superoxide radicals, H_2O_2 and hydroxyl radicals. However, superoxide radicals are known to be less reactive in aqueous solutions and can interact with H_2O_2 to produce hydroxyl radicals (26). Thus it is likely that hydroxyl radicals may be the actual mutagenic agent.

Among smokers the mutagenic activity of urine samples to the TA98 strain is attributed to smoking (17,27). However, similar studies on smokeless tobacco users have failed to provide clear-cut results regarding the nature of mutagenic exposure (13,28). In contrast, our findings on the presence in GF of mutagens that act by causing frameshifts, base pair substitutions and oxidative damage provide for the first time, unequivocal evidence regarding the mutagenic exposure that results from chewing of tobacco with lime. The presence of mutagens and promutagens in the GF of chewers suggests that tobacco chewing may be an important risk factor in the development of gastric cancer.

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