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SHORT COMMUNICATION

Smokeless tobacco extracts mutate human cells

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Two commercial brands of smokeless tobacco were extracted with water and these extracts were tested in human cell mutation assays. Using the human cell line TK-6 which expresses no cytochrome P450, the two extracts tested were found to be detectably mutagenic in the range 1–3 mg/ml extractable solids. In AHH-1 cells which constitutively express cytochrome P450IAI, a similar result was found for both brands tested. The two extracts were treated with neutral nitrite solutions to mimic physiologic oral conditions or acidic conditions or acidic conditions with nitrite to mimic physiologic gastric conditions. The mutagenicity of both extracts for both TK-6 and AHH-1 cells was markedly decreased by treatment at neutral pH with sodium nitrite (0.25 mM) and by acidic treatment (2 h, pH 3.0). Treatment of extracts with sodium nitrite at pH 3.0 did not have any effect on the mutagenicity of the untreated extracts for TK-6 cells. The mutagenicity of both the extracts destroyed by acidic treatment however, seemed to be restored to a level equivalent to the mutagenicity of the untreated extracts for the TK-6 cells. The same series of experiments with P450-proficient AHH-1 cells showed uniform reduction of mutagenic activity. Since the two cell lines are equally sensitive to mutation by aqueous tobacco extracts it is concluded that mutagenicity is not cytochrome P450 mediated. It would further appear that the extract mutagen(s) is acid and neutral nitrite labile.

Chronic use of smokeless tobacco (chewing tobacco and snuff) is associated with increased incidence of oral cancer in man (1–6). Experimental studies have revealed carcinogenic chemicals both in snuff and chewing tobacco. These include benzo[*a*]pyrene (B[*a*]P*), radioactive polonium 210 and tobacco-specific *N*-nitrosamines (7–10). Tobacco-specific nitrosamines have been shown to be carcinogenic and mutagenic in rodent and bacterial cells respectively (11–13). Smokeless tobacco, both chewing tobacco and snuff, has been shown to be carcinogenic in different animal systems (14–19). Other studies have also reported that extracts of smokeless tobacco are mutagenic in bacterial assays (20–22). All these findings indicate the genotoxic potential of smokeless tobacco. However, evidence for the genotoxic effect of smokeless tobacco in human systems is limited. Induction of sister chromatid exchanges (23) and inhibition of lymphokine-activated killer (LAK) activity in the human peripheral blood lymphocytes by snuff extracts (24) was observed. Elevated frequency of micronuclei were reported in the exfoliated oral epithelial cells of smokeless tobacco users (25). Recently, cytologic and cytogenic studies in smokeless tobacco users revealed exposure-dependent nuclear alteration and significant induction of micronuclei in the oral epithelium (26).

In the present study the effect of smokeless tobacco on two human cell lines, namely TK-6 (which does not express metabolizing system) and AHH-1 (which constitutively expresses cytochrome P450IAI) was studied. Since smokeless tobacco contains nitrosable substances (8) which can be nitrosated by salivary nitrite (27–29), the effect of nitrite at neutral pH and acid alone and nitrite at acidic pH to simulate physiologic oral and gastric conditions respectively was also studied.

Two leading brands of American smokeless tobaccos (moist snuff) used in the study were purchased from the supermarket.

Aqueous extracts of smokeless tobacco were prepared by mixing 35 g of tobacco with three times its weight of double-distilled water, shaking it overnight at room temperature and filtering the extract under vacuum. All extracts were stored at −70°C.

Samples of extract (0.1 ml) were applied to pre-weighed planchets and allowed to dry in a vacuum dessicator overnight. The weight of the residual dark-brown powder was determined on a microbalance. A range of 40–45 mg 'solids'/ml extract was found for the two commercial brands used. The yields of the two water extracts were 12–12.7%.

Aqueous extracts were reacted with sodium nitrite (final concentration 0.25 mM). For acidic nitrite treatment the pH was adjusted to 3.0 using concentrated HCl. The control tobacco extracts and nitrite solutions were also adjusted to appropriate pH under the same conditions. All extracts were incubated at 37°C in the dark for 2 h before testing for mutagenicity. Acid treated extracts were neutralized with 1 N NaOH before testing for mutagenicity in order to maintain the pH of the culture medium.

Two lines of human lymphoblasts were used, AHH-1 (expresses cytochrome P450IAI) and TK-6 (expresses no cytochrome P450). Isolation and use of AHH-1 and TK-6 cells in mutation assays is described elsewhere (30–32). Mutants resistant to the purine analog 6-thioguanine (6TG) were scored. To assay for mutagenic activity 4.0×10^7 TK-6 or AHH-1 lymphoblasts in 100 ml suspension culture were incubated with aqueous tobacco extracts for 28 h, cells were removed and resuspended in fresh medium. Each treatment was done in duplicate. The cells were then grown for eight generations to permit expression of the 6TG-resistant phenotype. Appropriate dilutions in media containing 6TG (1.0 μg/ml) were made and distributed to 96-well microtitre plates. Cells were also placed at low cell concentration in the absence of 6TG to determine plating efficiency. Following 2 weeks of incubation, wells with visible colonies were counted and the mutant fraction was determined according to the methods of Furth *et al.* (33).

Each treatment was done in duplicate and results of the duplicate experiments were averaged and expressed as mean ± standard deviation. The test substance was defined as positive if the mean mutant fraction was statistically greater than the concurrent control (Student's *t*-test, $P = 0.05$) and the historical,

*Abbreviations: B[*a*]P, benzo[*a*]pyrene; LAK, lymphokine-activated killer; 6TG, 6-thioguanine; 4NQO, 4-nitroquinoline oxide.

negative control observations (99% upper confidence limit based on Poisson statistics).

Exposure of simple aqueous extracts to the two cell lines showed that both brands were toxic and mutagenic to TK-6 and AHH-1 cells in the range 1–3 mg extractable solids/ml in a 28 h exposure as shown in Figure 1. In TK-6 cells extract A was very toxic at 3 mg/ml and there were insufficient live cells to give a mutant fraction with 95% confidence at that dose. Extract of brand B was somewhat less toxic and mutagenic than brand A in both cell lines.

Treatment of extracts A and B with nitrite (0.25 mM) at pH 7.0 for 2 h in the dark prior to reacting with the cells reduced the mutagenicity and toxicity (Figure 1) for both cells as shown in Table I. The reduction was statistically significant only for brand A ($P > 0.05$).

When extracts A and B were acidified in the dark for 2 h prior to introduction in cell culture the mutagenicity of both extracts (Figure 1) was reduced significantly for TK-6 cells ($P > 0.05$) as shown in Table I. In AHH-1 cells, although both the brands showed reduction in mutagenicity, the reduction was statistically significant only for brand A ($P > 0.05$). Toxicity was not significantly affected except for increased cell survival in the presence of sample A for TK-6 cells at the highest concentrations (Table I) when compared to untreated extract (Figure 1).

Treatment with nitrite (0.25 mM) at pH 3.0 for 2 h in the dark seemed to restore the mutagenicity of both the extracts destroyed by acid treatment (Table I) to levels equivalent to untreated extracts in TK-6 cells as illustrated in Figure 1. The toxicity of the extracts was not significantly affected for brand A which gave increased cell survival in TK-6 cells at the highest concentration when compared to untreated extract (Figure 1a). In AHH-1 cells acidic nitrite treatment reduced the mutagenicity of both the extracts (Figure 1d). The reduction of mutagenicity was statistically significant ($P > 0.05$) only for brand A. The toxicity was not altered by acidic nitrite treatment for both the extracts in this cell line (Table I).

4-Nitroquinoline oxide (4NQO) and B[*a*]P used as positive controls for TK-6 and AHH-1 cells respectively induced the following mean mutant fraction ($\times 10^{-6}$): 4NQO (0.25 μM) 70 ± 7.2; B[*a*]P (3 μM) 14.9 ± 1.5. In TK-6 cells B[*a*]P (3.0 μM) induced a mean mutant fraction ($\times 10^{-6}$) of 2.0 ± 0.5 indicating absence of any cytochrome P450 activity in this cell line.

The results of this study indicate that aqueous extracts of smokeless tobacco are mutagenic to two human cell lines, TK-6 which does not contain detectable levels of cytochrome P450 and AHH-1 cells which express cytochrome P450IAI at concentrations of 1–3 mg extractable solids/ml. This equal sensitivity of TK-6 and AHH-1 cells leads to the conclusion that the mutagenicity is not cytochrome P450 mediated. Further, the mutagen(s) is acid and neutral nitrite labile (Table I).

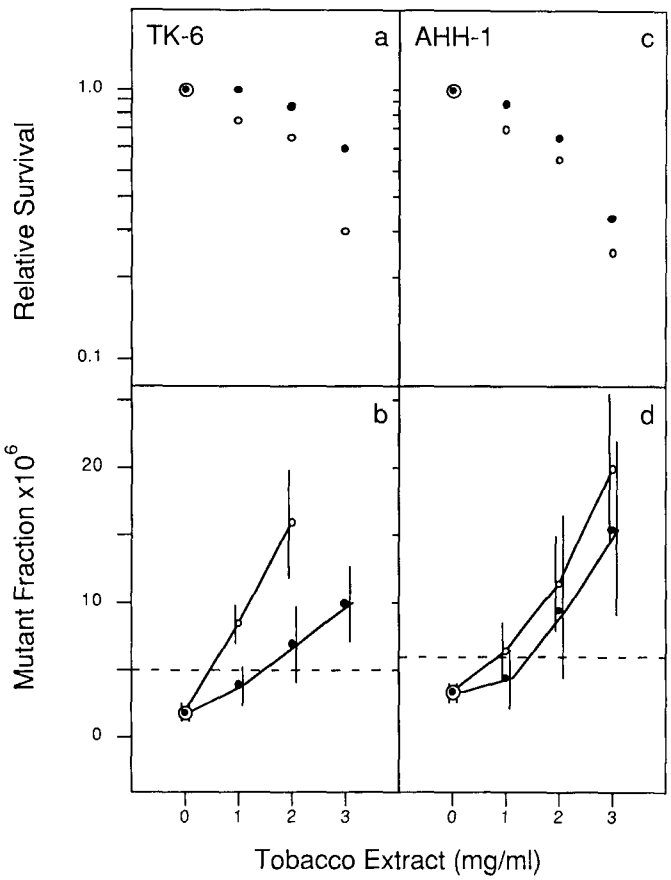

Fig. 1. Mutagenicity and toxicity of aqueous extracts of two smokeless tobaccos A (○) and B (●) using TK-6 cells (**a** and **b**) and AHH-1 cells (**c** and **d**). The error bars are mean ± standard deviation of four readings from duplicate experiments. The broken line represents the 99% upper confidence limit for the historical negative control ($n = 50$) and is calculated based on Poisson Statistics.* Statistically significant $P > 0.05$.

Table I. Mutagenicity and toxicity of aqueous extracts of smokeless tobacco brands A and B in TK-6 and AHH-1 cells in the presence of nitrite, acid and acidic nitrite

Tobacco	Dose (mg/ml)	TK-6		AHH-1	
		Survival	Mutant fraction ($\times 10^{-6}$) ± SD	Survival	Mutant fraction ($\times 10^{-6}$) ± SD
Nitrite					
A	0	1.0	1.5 ± 1.2	1.0	3.5 ± 0.8
	1	1.0	3.5 ± 0.5*	0.6	7.0 ± 0.9
	2	0.8	4.0 ± 1.0*	0.5	10.0 ± 0.5
	3	0.5	7.5 ± 2.0	0.3	12.5 ± 1.7*
B	0	1.0	2.0 ± 0.8	1.0	3.0 ± 0.8
	1	0.8	2.5 ± 0.8	0.9	4.0 ± 0.4
	2	0.6	5.0 ± 1.3	0.7	7.0 ± 1.0
	3	0.4	6.5 ± 1.8	0.5	9.0 ± 1.9
Acid					
A	0	1.0	1.5 ± 0.7	1.0	3.0 ± 0.8
	1	0.8	1.5 ± 0.7*	0.7	3.5 ± 0.8*
	2	0.6	3.0 ± 1.4*	0.3	9.0 ± 1.4
	3	0.5	5.0 ± 1.4	0.2	11.5 ± 2.1*
B	0	1.0	2.0 ± 0.7	1.0	3.0 ± 0.5
	1	0.9	2.5 ± 0.7	0.9	4.0 ± 0.5
	2	0.7	4.0 ± 0.8*	0.6	7.0 ± 1.0
	3	0.3	5.5 ± 1.6*	0.2	9.0 ± 1.5
Nitrite+acid					
A	0	1.0	2.5 ± 0.5	1.0	2.5 ± 0.5
	1	0.5	5.5 ± 1.2	0.7	5.0 ± 1.0
	2	0.3	14.5 ± 2.3	0.5	5.5 ± 1.9*
	3	0.2	20.0 ± 4.1	0.2	8.5 ± 1.4
B	0	1.0	2.5 ± 0.5	1.0	2.5 ± 0.5
	1	0.7	2.5 ± 0.7	0.9	6.0 ± 1.4
	2	0.6	8.5 ± 3.1	0.6	6.0 ± 1.5
	3	0.4	15.0 ± 3.5	0.5	9.0 ± 2.5

Results are mean ± SD.
*Statistically significant compared to untreated extracts ($P > 0.05$) as shown in Figure 1.

Treatment of the extracts with acidic nitrite solution did not affect the mutagenicity produced by untreated extracts in TK-6 cells (Table I) however, it significantly restored the mutagenicity destroyed by acidic treatment in the same cell line to levels equivalent to untreated extracts in TK-6 cells. In AHH-1 cells the mutagenicity produced by untreated extracts was reduced significantly by acidic nitrite treatment, once again leading to the conclusion that the mutagenicity is not cytochrome P450 dependent. Brand A seemed to be more sensitive to the mutagenic or antimutagenic effects of the different treatments as seen in Figure 1 and Table I although both brands were the same type of tobacco.

Tobacco is a complex mixture and over 2550 compounds have been isolated from the mixture (1). Polyphenols, amines and amides are among the many compounds present in smokeless tobacco. Polyphenols have revealed a spectrum of activities in bacterial and yeast cells in the absence of metabolic activation (34–36). The reduction of mutagenicity of the extracts in the presence of nitrite at neutral pH is surprising since formation of *N*-nitroso compounds in the neutral environment of the mouth has been found in betel quid chewers and snuff dippers (37,38). The marked decrease in mutagenicity of the acidic nitrite treated extracts in AHH-1 cells is also surprising since rapid nitrosation of nitrosable substances present in tobacco can take place at acidic pH (28) to form mutagenic *N*-nitroso compounds (8). It is possible that the cytochrome P450 present in the AHH-1 cells is unable to metabolize the nitroso compounds that modify the genetic material of these human cells. Similar studies carried out in a bacterial system using a forward mutation assay showed only acidic nitrite mediated mutagenicity in the absence of metabolic activation (39).

It thus appears that the tobacco contains precursors for the formation of mutagen(s) whose activity is not cytochrome P450 mediated. Since tobacco is a complex mixture and since no chemical analysis of the extracts has been done in the present study, the underlying chemistry of the mutagenicity observed here is not understood. Nevertheless, the mutagenicity of the water extracts of smokeless tobacco in human cell lines is important considering the association of smokeless tobacco usage and oral cancer in humans.

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