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Comparison of Pure Nicotine- and Smokeless Tobacco Extract-Induced Toxicities and Oxidative Stress

D. Yildiz, Y.-S. Liu, N. Ercal, D. W. Armstrong

Department of Chemistry, University of Missouri–Rolla, Rolla, Missouri 65401, USA

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Abstract. The toxicities and oxidative stress-inducing actions of (−)-nicotine and smokeless tobacco extract (STE), containing equivalent amounts of nicotine, were studied. Toxicities were determined by colony formation assays using Chinese hamster ovary (CHO) cells. Results indicated that nicotine is less toxic than smokeless tobacco extract that contained the same amount of nicotine. The generation of reactive oxygen species, following treatment with smokeless tobacco extract and nicotine, was assessed by measurement of changes in glutathione (GSH) and malondialdehyde (MDA) levels. CHO cells (5×10^5 cells/5 ml media) were incubated with 4, 0.8, and 0.08 mg of nicotine and STE containing the same amounts of nicotine. All preparations of smokeless tobacco extract significantly decreased GSH levels and increased MDA generation. However, 0.08 mg of nicotine treatment did not result in a significant change in GSH level, and only 4 mg of nicotine were sufficient to increase MDA generation. Addition of free radical scavenging enzymes, superoxide dismutase (SOD) and catalase (CAT), and an intracellular GSH precursor, N-acetyl-L-cysteine (NAC), replenished the GSH levels in nicotine-treated cells. GSH levels in cells exposed to smokeless tobacco extract containing 4 and 0.8 mg nicotine remained significantly lower than the control with the addition of SOD and CAT. However, co-addition of NAC with smokeless tobacco extract preparations returned the GSH levels to the control level. Lactate dehydrogenase (LDH) activities were measured in the media to establish the membrane damage following exposure to smokeless tobacco extract and nicotine. Treatment of cells with 4 mg nicotine caused a significant increase in LDH activity, which was returned to control level in the presence of the antioxidant enzymes and NAC. Smokeless tobacco extract did not change the LDH activity.

The effects of an aqueous extract of smokeless tobacco and nicotine have been extensively studied in separately designed *in vivo* and *in vitro* experimental systems using either nicotine or smokeless tobacco extract (Schievelbein 1982; Christen *et al.* 1990; Gross *et al.* 1995). Alteration of heat shock protein production (Bagchi M *et al.* 1995; Hahn *et al.* 1991), inhibition of cell metabolism and proliferation (Konno *et al.* 1991; Waggoner and Wang 1994), and induction of nuclear aberrations (Livingstone *et al.* 1990; Doolittle *et al.* 1995) are some of the common results of smokeless tobacco extract and nicotine administration. In addition, it has been demonstrated that both nicotine and smokeless tobacco administration result in generation of reactive oxygen species in *in vitro* experimental systems (Bagchi D *et al.* 1995; Wetscher *et al.* 1995a). Treatment of rat peritoneal macrophage and J774.1 macrophage cells in a culture with smokeless tobacco extract has been shown to induce nitric oxide production (Hassoun *et al.* 1995) and generation of free radicals in other experimental systems (Bagchi *et al.* 1996). In similar sets of experiments, nicotine has also been shown to induce free radical generation, as evidenced by changes in intracellular oxidative stress parameters such as glutatione (GSH) and malondialdehyde (MDA) (Wetscher *et al.* 1995b; Ashakumary and Vijayamal 1996; Yildiz *et al.* 1998).

Some of the biological and physiological endpoints of tobacco consumption have been attributed to its major alkaloid, nicotine (Connolly *et al.* 1986; Benowitz 1988). However, there has not been any study directly comparing the extent of the effects of nicotine and smokeless tobacco extract on the same biological parameters. Therefore, the objective of this study was to compare the effects of pure nicotine and smokeless tobacco extract, containing the same amount of nicotine, induced toxicities and oxidative stress. The study also investigated the effects of antioxidant enzymes and of N-acetyl-L-cysteine (NAC) on nicotine- and smokeless tobacco extract-induced toxicity and oxidative stress.

Materials and Methods

Chinese hamster ovary (CHO) K1 cells were obtained from American Type Culture Collection (Rockville, MD). The items required for the maintenance of cell cultures, Ham's F-12 media, fetal calf serum (FCS), and glutamine were obtained from Sigma Chemical Company (St. Louis, MO). Catalase (CAT), superoxide dismutase (SOD), lactate dehydrogenase (LDH), and (−)-nicotine were also purchased from Sigma. N-(1-pyrenyl)-maleimide (NPM) was purchased from Aldrich Chemical Company (St. Louis, MO). Bradford reagent was obtained from BioRad (Melville, NY). The HPLC-grade reagents were purchased from Fisher Scientific (St. Louis, MO). Smokeless tobacco was

Correspondence to: N. Ercal

obtained from University of Kentucky, Tobacco and Health Research Institute (Lexington, KY).

Preparation of Smokeless Tobacco Extract and Quantitation of Nicotine Content

Extraction: Chewing tobacco (80 g) was cut into small pieces and stirred in distilled water (300 ml) for 24 h in the dark at room temperature. The mixture was filtered and the filtrate was centrifuged at 4,000 *g* for 1 h. The supernatant fraction was filtered through a millipore filter (0.45 μm) and frozen, then put onto a freeze-drier. The sample was not taken to dryness, but was stopped and then thawed to give 60 ml of solution. NaOH (1 ml of 1 N solution) was added to 1 ml of the above solution. The mixture was extracted with ether (4 ml) until additional extraction did not show any nicotine peak by gas chromatography (usually this occurred by five extractions). An internal standard of 1 ml (50 mM anthranilamide in methanol) was added to the combined etheral extract, and the mixture was concentrated on a water bath at 45°C to about 2 ml (Raisi *et al.* 1986).

Equipment: Analyses were carried out on a Hewlett Packard (Corvallis, OR) model 5890 series II gas chromatograph equipped with a flame ionization detector and HP 3396 series II integrator. The column was a DB5 (J&W Scientific, 30 m × 0.25 mm ID, 0.25 μm film thickness). Helium was used as the carrier gas. The temperatures for injector and detector were set at 220°C and 250°C, respectively. A split ratio of about 100/1 was used for all the analyses. Nicotine and the internal standard in the sample extract were eluted from the column as sharp and symmetrical peaks within 12 and 20 min, respectively.

Calibration Curve: A calibration curve for nicotine was constructed over a range of 20–100 mM of nicotine in water. The data were subjected to linear regression analysis to give the appropriate calibration factor. The calibration curve was found to be linear over the range of concentrations 20–100 mM (correlation coefficient 0.996).

Cells and Culture Conditions

CHO cells were propagated in Ham's F-12 culture media supplemented with 10% FCS and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Colony Formation Assay

For colony formation assay, exponentially growing cultures were detached from the surface by trypsinization, and the cell suspension was centrifuged at 2,000 *g* for 5 min. The resulting cell pellets were resuspended in fresh media and counted on a hemocytometer. Between 100 and 1,000 cells were seeded into small (60-mm) petri dishes in 5 ml media and incubated for 4 h to allow cell attachment to the surface. Nicotine and smokeless tobacco extract were then added to the petri dishes. The cells were then incubated for 7 days in the presence of nicotine and smokeless tobacco extract. At the end of incubation, colonies were stained and counted.

Staining, Counting of Colonies, and Construction of Cell Survival Curve

After the media was decanted carefully, crystal violet (1 g crystal violet dissolved in 400 ml methanol prepared 50× staining solution) was added for 10 min to stain the colonies. The plates were washed with distilled water, allowed to air dry, and the number of colonies were then counted. The plating efficiency was calculated as follows: plating efficiency = colonies counted/cells seeded × 100. The survival fraction was calculated as follows: survival fraction = colonies counted / cells seeded × (plating efficiency$_{control}$ / 100) (Hall 1988).

Oxidative Stress Studies

Cells from exponentially growing cells were established (5×10^5 / 5 ml media) in culture flasks. After 4 h of incubation to allow cell attachment, various concentrations of nicotine (0.08 mg, 0.8 mg, and 4 mg) and smokeless tobacco extract containing the same amounts of nicotine were added to the media. Cells were then further incubated for 24 h in the presence of (−)-nicotine or smokeless tobacco extract. SOD (10 units/ml) and CAT (10 units/ml) or NAC (2 mM) were added along with nicotine and smokeless tobacco extract. At the end of the incubation, LDH activity was assayed in the media. The cells were trypsinized, collected, and homogenized for the determination of GSH and MDA levels.

GSH and MDA Determination

Details of the GSH and MDA measurements and of the HPLC systems were described in a previous study (Neal R *et al.* 1997). MDA levels were determined as described by Draper *et al.* (1993) and Esterbauer *et al.* (1991). The GSH numerical values for the different experiments varied due to GSH fluctuations. For this reason, we converted the numbers to percent control to represent the data more clearly.

LDH Activity Assay

The LDH activity assay was performed as described previously (Tietz 1986). For the LDH measurements in a cell free system, 3 μl of LDH stock solution (1,000 U/ml) were added to the media (5 ml) containing either nicotine or smokeless tobacco extract. Samples were then removed at 0-, 4-, and 24-h time intervals for the determination of LDH activities. For time 0, samples were taken as soon as LDH was added to the media containing either nicotine or smokeless tobacco extract. LDH activities were then determined.

Protein Determination

The Bradford (1976) method was used to determine the protein content of the cell samples using coomassie blue and optical density determinations at 595 nm.

Statistical Analysis

InStat by GraphPad software (San Diego, CA) was used to conduct a statistical analysis. One-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparison tests were applied. Values of p less than 0.05 were considered to be significant.

Results

Colony Formation

Table 1 shows the survival fractions of nicotine- and smokeless tobacco extract–exposed cells in the presence and absence of

Table 1. Survival fractions of CHO cells following exposure to nicotine and smokeless tobacco extract containing equivalent concentrations of nicotine

Groups[a]	Survival Fractions		
	No Antioxidant	SOD and CAT	NAC
Control	1 ± 0	1 ± 0	1 ± 0
0.08 mg nicotine	0.90 ± 0.09	1.00 ± 0.06	0.80 ± 0.04
Smokeless tobacco extract containing 0.08 mg nicotine	0.40 ± 0.04	0.80 ± 0.07	1.00 ± 0.03
0.8 mg nicotine	0.80 ± 0.10	0.70 ± 0.04	0.60 ± 0.03
Smokeless tobacco extract containing 0.8 mg nicotine	0	0	0.50 ± 0.04
4 mg nicotine	0.03 ± 0.01	0.04 ± 0.00	0.04 ± 0.00
Smokeless tobacco extract containing 4 mg nicotine	0	0	0

Details of the procedure are explained in the Materials and Methods section

Values represent the mean ± SD of three separate experiments

[a] The final volume of cell culture media for control, nicotine, and smokeless tobacco extract groups was 5 ml

Table 2. GSH and MDA levels in CHO cells following exposure to nicotine and smokeless tobacco extract containing equivalent concentrations of nicotine

Groups[a]	GSH (% Control)	GSH with SOD and CAT (% Control)	GSH With NAC (% Control)	MDA
Control	100 ± 29	100 ± 18	100 ± 12	9.0 ± 0.4
0.08 mg nicotine	76 ± 19[b]	87 ± 5	85 ± 5	10 ± 2[b]
Smokeless tobacco extract containing 0.08 mg nicotine	38 ± 14[c]	98 ± 11	91 ± 6	12 ± 1[c]
0.8 mg nicotine	61 ± 11[b,c]	103.0 ± 0.4[b]	84 ± 2	8 ± 1[b]
Smokeless tobacco extract containing 0.8 mg nicotine	24 ± 1[c]	40 ± 2[c]	89 ± 3	12 ± 1[c]
4 mg nicotine	64 ± 12[b,c]	90 ± 5[b]	70 ± 6[c]	12 ± 1[b,c]
Smokeless tobacco extract containing 4 mg nicotine	5 ± 1[c]	22 ± 10[c]	88 ± 7	19 ± 1[c]

GSH levels are reported as % control (nmol/mg protein). MDA levels are reported as nmol/100 mg protein. GSH was measured by NPM derivatization followed by HPLC. The thiobarbutiric acid derivative of MDA was identified by HPLC. Details of the procedure are explained in the Materials and Methods section. Values represent the mean ± SD of three separate experiments

[a] The final volume of cell culture media for control, nicotine, and smokeless tobacco extract groups was 5 ml

[b] Significantly different from smokeless tobacco extract containing the same amount of nicotine

[c] Significantly different from the corresponding control value

antioxidant enzymes and NAC. Incubation of CHO cells with nicotine and smokeless tobacco extract inhibited colony formation. Nicotine inhibited the colony formation 97% at 4 mg.

However, smokeless tobacco extract containing 0.08 mg nicotine was sufficient to reduce the colony formation by more than half. At higher nicotine contents (0.8 and 4 mg), smokeless tobacco extract completely inhibited the colony forming ability of CHO cells. These results indicate that nicotine alone is less toxic than smokeless tobacco extract containing an equivalent amount of nicotine.

The administration of antioxidant enzymes and NAC along with nicotine improved the rate of colony formation for both nicotine and smokeless tobacco extract. NAC completely returned the survival fractions to the control level with smokeless tobacco extract containing 0.08 mg nicotine. NAC also restored the colony-forming ability of the cells exposed to smokeless tobacco extract containing 0.8 mg nicotine. However, it did not improve the colony formation with smokeless tobacco extract containing 4 mg nicotine or analogous smokeless tobacco extract.

GSH and MDA Measurement

GSH levels in the presence and absence of antioxidant enzymes and NAC were determined after exposure for 24 h to nicotine and smokeless tobacco extract containing the same amount of nicotine. As shown in Table 2, all preparations of smokeless tobacco extract induced significant decreases in GSH levels. Nicotine exposure of the cells also resulted in a decrease in GSH levels. However, significant decreases were observed only with 0.8 and 4 mg nicotine exposures. Although both nicotine and smokeless tobacco extract lowered the intracellular GSH contents of the cells, their efficiencies were considerably different with smokeless tobacco extract being more potent than the corresponding nicotine concentration. Treatment of the cells with 4 mg of nicotine resulted in a 36% decrease in GSH level as compared to control. However, there was a 95% decrease in GSH levels in cells exposed to smokeless tobacco extract containing 4 mg of nicotine.

Coaddition of antioxidant enzymes returned the GSH levels to the control level in nicotine-exposed cells. These enzymes also increased the GSH levels in cells treated with smokeless tobacco extract containing 0.08 mg nicotine. Although GSH levels in cells exposed to smokeless tobacco extract with higher nicotine contents remained significantly lower than the control in the presence of SOD and CAT, small increases in GSH levels were still observed. In contrast to SOD and CAT, coaddition of NAC with all preparations of smokeless tobacco extract returned the GSH levels to the control level. The GSH levels in cells treated with 4 mg of nicotine remained significantly decreased in the presence of NAC. However, NAC returned the GSH levels to the control level in 0.8 mg of nicotine-treated cells.

To determine if the decreases in GSH levels were associated with an increase in lipid peroxidation, the MDA contents were measured following exposure to nicotine and to smokeless tobacco extract. As shown in Table 2, significantly increased MDA levels were observed for both nicotine and smokeless tobacco extract that corresponded to the decreased GSH levels. Since employment of NAC and antioxidant enzymes already demonstrated that the effects observed were due to free radical generation, we did not evaluate the effects of NAC and antioxidant enzymes on MDA generation.

Table 3. LDH activities in the media following exposure to nicotine and smokeless tobacco extract containing equivalent concentrations of nicotine

Groups[a]	LDH Activity	LDH Activity with SOD and CAT	LDH Activity with NAC
Control	47 ± 2	47 ± 2	54 ± 6
0.08 mg nicotine	58 ± 6	52 ± 4	48 ± 4
Smokeless tobacco extract containing 0.08 mg nicotine	52 ± 8	51 ± 1	62 ± 5
0.8 mg nicotine	56 ± 8	55 ± 1	50 ± 6
Smokeless tobacco extract containing 0.8 mg nicotine	55 ± 5	50 ± 1	55 ± 4
4 mg nicotine	67 ± 5[b]	54 ± 1	57 ± 5
Smokeless tobacco extract containing 4 mg nicotine	43 ± 2	43 ± 7	63 ± 3

LDH activity is reported as unit/L. Details of the procedure are explained in the Materials and Methods section. Results represent the mean ± SD of three separate experiments

[a] The final volume of cell culture media for control, nicotine, and smokeless tobacco extract groups was 5 ml

[b] Significantly different from the control

Table 4. Effects of nicotine and smokeless tobacco extract on LDH activity in a cell-free *in vitro* system

Groups	LDH Activity (0 h)	LDH Activity (4 h)	LDH Activity (24 h)
Control	593 ± 32	583 ± 17	545 ± 70
0.08 mg nicotine	626 ± 10	572 ± 17	618 ± 39
Smokeless tobacco extract containing 0.08 mg nicotine	593 ± 45	565 ± 9	578 ± 54
0.8 mg nicotine	596 ± 38	579 ± 20	600 ± 8
Smokeless tobacco extract containing 0.8 mg nicotine	557 ± 40	571 ± 13	615 ± 20
4 mg nicotine	606 ± 16	570 ± 17	611 ± 10
Smokeless tobacco extract containing 4 mg nicotine	420 ± 12[a]	419 ± 15[a]	394 ± 46[a]

LDH activity is reported as units/L. Details of the procedure are explained in the Materials and Methods section. Values represent the mean ± SD of three separate experiments

[a] Significantly different from the control

LDH Activity Measurements

LDH activities in the media of nicotine- and smokeless tobacco extract-exposed cells were determined at the end of the 24-h incubation time. As shown in Table 3, only 4 mg of nicotine caused a significant increase in LDH activity. Treatment of cells with smokeless tobacco extract caused no statistically significant change in LDH activities in the media. LDH activities were also determined in the presence of the antioxidant enzymes and NAC. The presence of antioxidant enzymes and NAC restored the LDH activity to the control level in the media of 4 mg nicotine-treated cells. LDH activities in smokeless tobacco extract-treated cells, in the presence of the antioxidant enzymes and NAC, still remained in the range of control LDH activity. The numerical values were not statistically different.

To test if smokeless tobacco extract or nicotine has any direct effect on the LDH enzymatic activity, we incubated the enzyme in a cell-free media in the presence of nicotine and smokeless tobacco extract for 24 hs. As shown in Table 4, only smokeless tobacco extract containing 4 mg nicotine reduced the LDH activity significantly in a cell free environment. This inhibition occurred as soon as the enzyme was added to the media containing smokeless tobacco extract and the extent of inhibition did not change for 24 hs. The other preparations of smokeless tobacco extract and nicotine did not cause any change in LDH activities.

Discussion

The present results indicate that nicotine is considerably less toxic than smokeless tobacco extract containing the same amount of nicotine. These profound differences between nicotine and smokeless tobacco extract must result from, in addition to nicotine, the large numbers of other biologically active compounds that adversely affect the cell survival. It has been reported that tobacco contains, besides nicotine, tobacco-specific N-nitrosamines formed during curing and fermentation of tobacco from nicotine and other alkaloids such as nornicotine, anatabine, and anabasine (Brunnemann *et al.* 1996). Some of the tobacco-specific N-nitrosamines have been shown to be metabolically activated in mammalian cells, leading to the formation of highly reactive electrophiles that react with nucleophilic molecules of the cells (Hoffmann and Hecht 1988). The presence of tobacco-specific N-nitrosamines and various carcinogenic compounds such as benzo[a]pyrene in tobacco may explain the smokeless tobacco extract's greater inhibitory effect on colony formation compared to nicotine alone.

Our present GSH and MDA data suggest that generation of free radicals may contribute to both nicotine and smokeless tobacco extract toxicity. Both nicotine and smokeless tobacco extract significantly increased MDA generation and reduced intracellular GSH levels, which were either partially or completely returned to control levels in the presence of free radical-scavenging enzymes and NAC. However, neither NAC nor the antioxidant enzymes had a significant protective effect on nicotine-induced inhibition of colony formation. These results indicate the presence of other mechanism(s) contributing to nicotine toxicity. In addition to free radical generation, one possibility is that constant exposure of the cells to nicotine for a long time overrides protection by the antioxidant enzymes, and NAC and toxicity become a result of nicotine-induced genotoxic effects. NAC and antioxidants cannot protect the cells from nicotine-induced genotoxic effects that become effective with overextended time period. In contrast to nicotine, employment of the free radical-scavenging enzymes and, particularly, NAC, with smokeless tobacco extract displayed a more protective effect, suggesting an important role for GSH depletion and oxidative stress in smokeless tobacco extract toxicity.

Induction of oxidative stress by both nicotine and smokeless tobacco extract has been demonstrated previously in separate experimental systems (Ashakumary and Vijayammal 1996;

Bagchi *et al.* 1996). One of the main objectives of this study was to compare nicotine- and smokeless tobacco extract-induced oxidative stress by measuring GSH and MDA levels. The lower GSH and higher MDA levels in smokeless tobacco extract-exposed cells, as compared to nicotine-exposed cells, suggest that nicotine alone is less efficient in induction of oxidative stress than smokeless tobacco extract containing the same amount of nicotine. This indicates the presence of other compounds besides nicotine in smokeless tobacco extract that are involved in free radical generation. Addition of antioxidant enzymes and NAC increased the GSH levels in the presence of smokeless tobacco extract and nicotine. However, the responses of nicotine and smokeless tobacco extract-treated cells to antioxidant enzymes or NAC were different. In nicotine-exposed cells, GSH levels were more efficiently restored in the presence of antioxidant enzymes as compared to the presence of NAC. In contrast, in smokeless tobacco extract-exposed cells, GSH levels were more efficiently restored in the presence of NAC as compared to the presence of antioxidant enzymes. These results suggest that the nature of free radicals generated or the mechanism of GSH depletion in nicotine and smokeless tobacco extract-treated cells is different, which further indicates that some of the nicotine-specific actions could be blocked in smokeless tobacco extract due to complex interactions among the constituents of smokeless tobacco extract.

In contrast to NAC, SOD and CAT cannot cross the cell membranes. Therefore, increases in GSH levels by SOD and CAT of nicotine-treated cells suggest that free radicals are generated or are present outside of the cells and are more efficiently scavenged by these enzymes rather than NAC. In contrast, the increase in GSH levels by NAC in smokeless tobacco extract-exposed cells suggests that free radicals are mainly present inside the cells and are more efficiently scavenged by the presence of NAC rather than SOD and CAT. Another possibility is that the GSH of the smokeless tobacco extract-exposed cells is also depleted by conjugation reactions with components and/or metabolites of smokeless tobacco extract. In this respect, NAC may replace GSH in conjugation reactions and directly activate GSH synthesis or directly scavenge the free radicals formed following exposure to smokeless tobacco extract, thereby replenishing the GSH levels to the control level.

Lipid peroxidation is known to disturb the integrity of cellular membranes, leading to the leakage of cytoplasmic enzymes, such as LDH, into the media (Bagchi D *et al.* 1995). To show the extent of membrane damage by nicotine and smokeless tobacco extract, the LDH activities were measured in the media. LDH activity was increased only in the media of 4 mg nicotine-treated cells. Although 4-mg-nicotine-containing smokeless tobacco extract resulted in generation of higher MDA levels, it did not increase the LDH activity in the media. This indicates that smokeless tobacco extract either prevents LDH leakage or contains an inhibitor of the enzyme. To test the possibility that smokeless tobacco extract may contain an inhibitor for the LDH enzymatic activity, we determined the changes in LDH activities in the presence of smokeless tobacco extract in a cell free system. Results showed that smokeless tobacco extract containing 4 mg nicotine significantly inhibits the LDH activity. This may be sufficient to explain the absence of LDH activity in the media of cells treated with smokeless tobacco extract containing 4 mg nicotine. However, smokeless tobacco extract containing 0.8 mg nicotine, which had no effect on LDH activity in a cell-free system, generated the same amount of MDA as 4 mg nicotine. Therefore, it is still not possible to completely attribute the LDH leakage in nicotine treated cell to the level of lipid peroxidation. These results indicate that LDH release by nicotine may involve another mechanism, in addition to the increase in MDA generation.

The present study concludes that the toxicity and oxidative stress-inducing actions of smokeless tobacco extract are not due entirely to its nicotine content. The mechanisms of toxicities and free radical generation are different in cells treated with nicotine and smokeless tobacco extract containing the same amounts of nicotine. In addition to nicotine, smokeless tobacco extract may constitute other mechanisms for toxicity. Some of the nicotine specific effects could be blocked when present in smokeless tobacco extract. NAC may have an important role in protecting against smokeless tobacco extract-induced cellular damage due to GSH depletion and oxidative stress.

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