



Genotoxicity of *Nicotiana tabacum* leaves on *Helix aspersa*

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Abstract

Tobacco farmers are routinely exposed to complex mixtures of inorganic and organic chemicals present in tobacco leaves. In this study, we examined the genotoxicity of tobacco leaves in the snail *Helix aspersa* as a measure of the risk to human health. DNA damage was evaluated using the micronucleus test and the Comet assay and the concentration of cytochrome P450 enzymes was estimated. Two groups of snails were studied: one fed on tobacco leaves and one fed on lettuce (*Lactuca sativa* L) leaves (control group). All of the snails received leaves (tobacco and lettuce leaves were the only food provided) and water *ad libitum*. Hemolymph cells were collected after 0, 24, 48 and 72 h. The Comet assay and micronucleus test showed that exposure to tobacco leaves for different periods of time caused significant DNA damage. Inhibition of cytochrome P450 enzymes occurred only in the tobacco group. Chemical analysis indicated the presence of the alkaloid nicotine, coumarins, saponins, flavonoids and various metals. These results show that tobacco leaves are genotoxic in *H. aspersa* and inhibit cytochrome P450 activity, probably through the action of the complex chemical mixture present in the plant.

Keywords: comet assay, genotoxicity, micronucleus test, *Helix aspersa*, *Nicotiana tabacum*, tobacco leaves.

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Introduction

Brazil is the world's second largest producer of tobacco (*Nicotiana tabacum*) leaves after China and accounts for 14.1% of the global production. The southern Brazilian state of Rio Grande do Sul (RS) is the largest tobacco producer in the country. More than 320,000 tons are produced annually, with over 906,000 workers employed in direct farming and 40,000 jobs in cigarette factories (AFUBRA, 2012).

Tobacco farmers are routinely exposed to the complex mixture of chemicals present in tobacco leaves, in addition to organic and inorganic pesticides used to treat tobacco plants. Tobacco leaves contain an unusually high number of different chemical compounds, such as nicotine,

nicotianine and malic acid (Hinds, 2012). These farmers are at risk of Green Tobacco Sickness (GTS), a disease caused by the dermal absorption of nicotine from wet tobacco leaves. The signs and symptoms of GTS include nausea, pallor, chills, vomiting, headache, difficulty in breathing, abdominal pain, diarrhea, loss of appetite, runny eyes, blurred vision, weakness, prostration and dizziness, and, occasionally, fluctuations in blood pressure or heart rate (Onuki *et al.*, 2003; Parikh *et al.*, 2005).

Nicotine is the most important alkaloid present in tobacco. The quantity of nicotine in dried tobacco leaves varies from 4% to 8% (Hinds, 2012; IPCS-INCHEM, 2012). During harvest time, farm workers may be exposed to up to 600 mL of dew or rain on the tobacco plants, which roughly corresponds to the nicotine content of 36 cigarettes (NIOSH, 2012). There have been few studies on the genotoxicity of nicotine and the findings are contradictory. Some authors have reported that nicotine and its four major metabolites are not genotoxic when assayed in the Ames

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test or other bacterial indicator assays (Doolittle *et al.*, 1995), whereas others have observed that tobacco induces DNA alterations in mammalian cells (Munzner and Renner, 1989; Trivedi *et al.*, 1990).

In contrast to other techniques, the study of bioindicator organisms can reveal the biologic impact of xenobiotics. Among terrestrial invertebrates, the snail *Helix aspersa* can accumulate different classes of chemicals and therefore serve as a pertinent species for monitoring trace metals and agrochemicals (Snyman *et al.*, 2000; Beeby and Richmond, 2002) as well as plant extracts (Pereira *et al.*, 2009). Xenobiotics accumulated through different routes are transported by blood cells to the digestive gland, which is also the main target organ for metabolic and detoxification processes (Beeby and Richmond, 2002).

In this study, we adapted the micronucleus test to investigate the response of *H. aspersa* to exposure to tobacco leaves as an indicator of the risk to human health. DNA damage was assessed with the micronucleus test (MN) and the Comet assay in hemolymph cells, and the inhibition of cytochrome P450 enzyme activity was also examined. In addition, trace element analysis, phytochemical screening and nicotine quantification were also done.

Materials and Methods

Plant material

Nicotiana tabacum leaves were collected on a farm in the south-central region of Rio Grande do Sul State, Brazil. Only tobacco leaves without pesticides were sampled. The leaves were packed and stored in a freezer at -30 °C.

Snails

Adult individuals (n = 20) of the land snail *H. aspersa* (8.96 + 1.62 g; mean + SD) were obtained from a snail breeder at the Lutheran University of Brazil, Canoas, RS, Brazil. The snails were acclimatized in a cage at 22 + 3 °C for seven days, during which period they received leaves from organically grown lettuce (*Lactuca sativa* L.) and water *ad libitum*. After acclimatization, the snails were assigned to control and test groups.

Treatments and hemolymph sampling

The study involved two groups of snails: the first was fed tobacco leaves and the second was fed lettuce leaves (control group) (n = 10 each). All snails received tobacco or lettuce leaves (the only food provided) and water *ad libitum*. Hemolymph samples for the Comet assay were obtained from the snails 0 h, 24 h, 48 h and 72 h after the beginning of feeding each type of leaf, and after 72 h for the micronucleus test. The snails were weighed every day throughout the experiment. The hemolymph samples were collected using a syringe containing heparin and processed within 30 min of collection. At the end of the 72 h period, all snails were killed and stored in a freezer at -30 °C for

trace element analysis and determination of cytochrome P450 activity.

Comet assay

The alkaline Comet assay was done as described by Singh *et al.* (1998) with several modifications for hemolymph (Janistcki *et al.*, 2009). Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed for each individual. To calculate a damage index (DI), cells were visually classified into one of five classes based on tail size (0 = no tails and 4 = maximum-length tails) as assessed by light microscopy. This classification resulted in a single DNA damage score for each individual and consequently for each group studied. The DI for each individual could range from 0 (completely undamaged = 100 cells x 0) to 400 (maximum damage = 100 cells x 4). The damage frequency (DF, in %) was calculated for each sample based on the number of cells with tails vs. those without tails (Heuser *et al.*, 2002). All sides were coded for blind analysis.

Micronucleus test (MN)

The MN test was done on hemolymph as a second monitoring system for comparison with the alkaline Comet assay. The MN test was adapted to the characteristics of the test organism based on a protocol described for *Limnoperna fortunei*, a mussel species (Villela *et al.*, 2007). A syringe containing 0.5 mL of freshly prepared methanol:acetic acid (1:1, v/v) was used to collect the same volume (0.5 mL) of hemolymph. The syringe was briefly placed on ice (7-10 min) and the hemolymph samples then smeared onto microscope slides, fixed in methanol for 10 min, air-dried and stained for 7 min with a mixture of 10 mL Giemsa and 90 mL 0.2 M phosphate buffer (pH 5.8) prior to examination by light microscopy. Two thousand cells in two hemolymph smears from each snail were examined for the presence or absence of micronuclei. All sides were coded for blind analysis.

Enzyme analysis

For microsome preparation the tissue (digestive gland) was homogenized in an Ultra-Turrax homogenizer for 15 s in a tissue:buffer (0.1 M potassium phosphate, pH 7.5, 0.15 M KCl, 10 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride) ratio of 1:5 (w/v). The homogenate was centrifuged at 12,000 x g for 20 min and the supernatant then centrifuged twice at 105,000 x g (60 min each). The resulting microsome fraction was resuspended in buffer (0.2 M potassium phosphate, pH 7.5, 0.15 M KCl, 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 20% glycerol) at a tissue:buffer ratio of 1:3 and then stored at -70 °C (Vrolijk *et al.*, 1994).

The microsomal protein concentrations (expressed in mg/mL) were determined as described by Lowry *et al.* (1951) using bovine serum albumin as the standard. The

samples were assayed in duplicate and the final absorbance was read at 625 nm in a spectrophotometer.

For the spectral characterization of cytochrome b_5 , P450 and P420: the microsomal suspension was diluted to 1 mg/mL and sodium dithionite crystals were added for analysis of cytochrome b_5 . The samples were read against a suspension of microsomes. Cytochromes P450 and P420 were identified based on the compound formed by the interaction of reduced microsomes with sodium dithionite and 80 μ L of carbon monoxide. This mixture was analyzed against a microsomal suspension reduced with sodium dithionite (Omura and Sato, 1964). The measurements were done in a Beckman DU70 spectrophotometer at 400-700 nm.

Chemical analysis of tobacco leaves

Tobacco leaves were oven-dried for 4 h at 60 °C and then were ground to a fine powder. The tobacco leaf powder was used to prepare pellets for trace element analysis using the PIXE (Particle-Induced X-ray Emission) technique and phytochemical screening. Nicotine was quantified by HPLC.

For the PIXE analysis, the pellets were placed in the target holder inside the reaction chamber. During the experiments, the pressure inside the reaction chamber was $\sim 10^{-5}$ mbar. The experiments were carried out at the Ion Implantation Laboratory of the Institute of Physics, Federal University of Rio Grande do Sul (IF-UFRGS). A 3 MV Tandemron accelerator provided a 2 MeV proton beam with an average current of 5 nA at the target. The X-rays produced in the samples were detected by a germanium (Ge) detector with an energy resolution of about 180 eV in 5.9 keV with high efficiency between 3 and 100 keV. The spectra were analyzed with the GUPIX software package and the amounts were expressed in ppm (Campbell *et al.*, 2000).

The phytochemical profile of *N. tabacum* leaves was determined as described by Harborne (1998). The method consists of several procedures for the detection of flavonoids, tannins, anthraquinones, alkaloids, saponins, coumarins and cardiac glycosides. The confirmatory thin layer chromatography analysis was done using the systems and developers indicated by Wagner and Bladt (1996).

The nicotine dosage in tobacco leaves extracted with water was determined by HPLC. Standard nicotine was purchased from Sigma (lot no. 093K4121). Analysis was done on a C18 reverse-phase column (cartridge: 5 μ m, 250 x 4.6 mm). The separation was done at 35 °C. The isocratic mobile phase used was a mixture of aqueous phase (phosphate buffer, pH 6.8, and methanol at a ratio of 35:65, v/v). The solution was sonicated before use and the flow rate was 1.2 mL/min. The HPLC method used was based on work by Tambwekar *et al.* (2003). A stock solution of nicotine (1 mg/mL) was prepared by dissolving 100 mg in 100 mL of phosphate buffer (pH 6.8). Various dilutions

(2.5-50 μ g/mL; five points per curve) were prepared the areas under the peaks were determined for each concentration (assayed in quadruplicate) and used to construct the standard curve. The retention time of nicotine was ~ 3.93 min. The assay was linear within the expected concentration range, indicating its suitability for this analysis. The equation of the regression curve was $y = 0.02475 + 0.00005x$ and r^2 was 0.996. Tobacco leaves (65 g) were washed with 70 mL of distilled water and the resulting solution was lyophilized and yielded 705 mg of residue. The crude extract (100 mg; labeled as solution A) was dissolved in 100 mL of phosphate buffer (pH 6.8) and a 20 μ L aliquot was injected (in triplicate) and eluted with the mobile phase (10 mM phosphate buffer (pH 6.8):methanol, 35:65 v/v). No interfering substances were detected in the chromatogram.

Statistical analysis

The normality of the data was evaluated using the Kolmogorov-Smirnov test. Statistical comparisons between the groups were done using Student's two-tailed *t*-test. Differences between exposure times were analyzed using the non-parametric two-tailed Kruskal-Wallis test followed by Dunns test for multiple comparisons. The critical level for rejection of the null hypothesis was $p < 0.05$.

Results

Table 1 summarizes the Comet assay data expressed as Damage index and Damage frequency for *H. aspersa* hemolymph cells exposed to tobacco leaves compared to cells from control snails. Control snails (fed lettuce leaves) showed no significant variation throughout the experiment, although there was a slight increase in DNA damage after 48 h and 72 h. There was also no difference between the groups (control and tobacco leaves) at 0 h. However, snails fed tobacco leaves had higher values than the control group at all subsequent times. When analyzed by the Kruskal-Wallis test, the mean DI and DF for snail hemolymph cells after 24, 48 and 72 h of exposure were significantly higher than those of the corresponding controls and the values at 0 h (see Table 1 for p values).

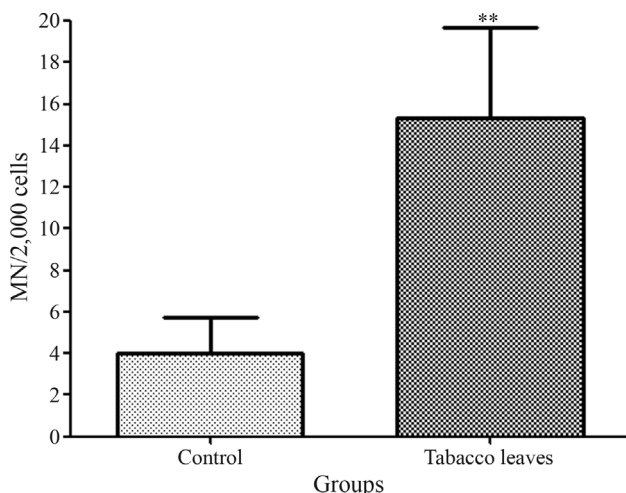
Figure 1 shows the micronucleus test (MN) results for hemolymph cells after 72 h. Cells from snails fed tobacco leaves had a significantly higher MN value than the control group (Student's *t*-test). Signs of toxicity such as swelling and death were observed in some snails at the end of the 72 h treatment period.

Table 2 shows that 11 trace elements were detected in the tobacco leaf samples analyzed using PIXE. There was no significant difference in the protein content of the digestive glands of snails in the two groups, but there was a significant reduction in the cytochrome b_5 , P450 and P420 activities in snails fed tobacco leaves (Table 3).

Table 1 - Damage index and damage frequency (Comet assay) in hemolymph of snails exposed to tobacco leaves or lettuce leaves (control group).

Exposure time (h)	Exposure group			
	Control		Tobacco leaves	
	Damage index	Damage frequency	Damage index	Damage frequency
0	56.80 + 34.53	29.90 + 14.16	60.00 + 22.98	29.00 + 10.88
24	53.60 + 36.76	28.30 + 17.34	166.80 + 45.29 ^{**} , ^a	73.90 + 14.22 ^{**} , ^a
48	96.60 + 41.18	44.50 + 18.11	207.60 + 48.52 ^{**} , ^a	83.00 + 16.05 ^{**} , ^a
72	97.50 + 38.55	45.60 + 17.78	224.10 + 42.95 ^{***} , ^a	88.40 + 7.32 ^{***} , ^a

The values are the mean + SD of 10 snails per group. ^{**} $p < 0.01$ and ^{*} $p < 0.001$ compared to the corresponding control parameter (Students *t*-test). ^a $p < 0.05$ (24 h) and $p < 0.001$ (48 h and 72 h) compared to 0 h (Kruskal-Wallis test).

**Figure 1** - Detection of micronuclei (MN) in hemolymph cells of *Helix aspersa* exposed to tobacco leaves. The columns represent the mean + SD determined in 10 snails of each group. ^{*} $p < 0.01$ compared to snails fed lettuce leaves (control) (Students *t*-test).

The nicotine concentration in the extract was 22.36 $\mu\text{g}/\text{mL}$ (solution A). The solution obtained from washed tobacco leaves was lyophilized and yielded 705 mg of residue. Extraction of 65 g of this residue with 70 mL of water yielded 15.76 mg of nicotine, which corresponded to $\sim 0.02\%$ of nicotine per leaf. Phytochemical analysis of tobacco leaves revealed the presence of coumarins, saponins traces, alkaloids and flavonoids.

Discussion

Substances present in tobacco form a complex mixture of organic and inorganic compounds that may interact to produce additive, synergistic or antagonistic effects (Fent, 2003). In the present study, the results of the Comet assay and MN test (Table 1 and Figure 1) of hemolymph from snails (*H. aspersa*) fed tobacco leaves were significantly higher than those of the control group (fed lettuce). The application of the Comet assay in these snails has been shown to be an inexpensive, effective and sensitive method for assessing the genotoxicity of chemical mixtures

Table 2 - Content of trace elements (ppm) in tobacco leaves.

Trace element	Tobacco leaves	Lettuce leaves ^a
Na	458965 + 38828	995.70
K	33449 + 508	0.11
Mg	4138 + 224	15.31
P	1659 + 94	0.03
S	1822 + 75	0.01
Cl	3425 + 112	0.01
Al	1272 + 342	0.90
Si	1470 + 90	1.95
Ti	140 + 10	2.47×10^{-4}
Ca	ND	0.07
Fe	ND	1.32×10^{-3}
Cu	ND	7.25×10^{-5}
Mn	85 + 5	4.60×10^{-5}
Zn	31 + 4	1.35×10^{-4}

Values are the mean + SD of 3 determinations. ^aData from Leffa *et al.* (2010). ND = not detected (samples with content below the limit of detection). Limits of detection: Ca = 2.60×10^{-3} , Cu = 4.03×10^{-5} and Fe = 1.02×10^{-4} .

(Ianiestcki *et al.*, 2009). The Comet assay detects recent lesions that can be repaired, such as breaks and alkali-labile sites, while the MN test detects non-repairable damage, such as clastogenic and aneugenic lesions (Villela *et al.*, 2007). Our findings show that the compounds present in tobacco leaves were genotoxic and mutagenic in *H. aspersa*. The slight time-dependent increase in DNA damage (seen in the Comet assay) in untreated snails can be explained by animal stress. Other reports have described similar results for controls (Villela *et al.*, 2007). Since the frequency of MN seen here in the control group was within an acceptable range, our results can be considered reliable.

The phytochemical analysis revealed the presence of alkaloids in tobacco leaves. Hinds (2012) stated that nicotine is the main tobacco alkaloid. Using HPLC, we found a nicotine content of $\sim 0.02\%$ per leaf. This lower value of nicotine compared to that reported by others (IPCS-

Table 3 - Protein content and cytochrome activities of digestive glands from *Helix aspersa* individuals exposed to lettuce leaves (control) or tobacco leaves.

Parameters	Control (n = 6)	Tobacco leaves (n = 6)
Protein (mg/mL)	8.33 + 0.49	6.59 + 2.23
Cytochrome b ₅ (nmol/mg)	35.47 + 4.26	26.92 + 2.86**
Cytochrome P450 (nmol/mg)	126.09 + 6.99	84.6 + 15.48***
Cytochrome P420 (nmol/mg)	231.72 + 12.41	149.55 + 31.98***

The values are the mean + SD. *p < 0.01 and **p < 0.001 compared to the control group (Students *t*-test).

INCHEM, 2012) can be explained by the extraction method used. The highest frequency of damage was observed after 72 h, although a similar extent of damage was observed after 24 h. Argentin and Cichetti (2004) applied the MN test to cultured mammalian cells treated with nicotine and observed rapid induction of DNA damage in samples treated for up to 72 h, with maximum levels observed after 24 h of exposure. Nicotine caused a concentration-dependent increase in DNA fragmentation, as assessed by the Comet assay, in mini-organ cultures of human nasal epithelium, indicating a direct genotoxic effect (Sassen *et al.*, 2005); similar findings have been reported for lymphatic tissue of palatine tonsils and peripheral lymphocytes (Kleinsasser *et al.*, 2005). Nicotine has also been implicated in free radical generation in various types of rodent and human cells, with a direct relationship between reactive oxygen species (ROS) induction and DNA damage (Yildiz *et al.*, 1999; Argentin and Cichetti, 2004; Da Silva *et al.*, 2010).

Another chemical group present in tobacco leaves is coumarins. Gasparotto Jr *et al.* (2005) showed that coumarins have significant molluscicidal activity against *Biomphalaria glabrata*. Our results confirmed the presence of coumarins and we believe that this chemical group accounts for the toxicity in *H. aspersa* after 72 h, when 50% of the snails exposed to tobacco leaves died (data not shown). Overall, coumarins are not genotoxic, and both negative and positive responses have been reported for these compounds in the Ames test, sister chromatid exchange (SCE), MN and chromosome aberration tests in mammalian cells (Lake, 1999).

Flavonoids are phenolic compounds that are widely distributed in all foods of plant origin. Several beneficial properties have been attributed to these compounds, including antioxidant, anti-inflammatory and anticarcinogenic effects (Da Silva *et al.*, 2002; Galati and O'Brien, 2004; Nunes *et al.*, 2011). Flavonoids can be potentially harmful since some are mutagenic in bacterial and mammalian test systems (MacGregor, 1986; Skibola and Smith, 2000; Da Silva *et al.*, 2002). Nevertheless, the data on flavonoid genotoxicity are incomplete and misleading results have been obtained with respect to mutagenicity in tests using mammalian cells (Skibola and Smith, 2000).

Our phytochemical analysis revealed only traces of saponins in tobacco leaves. This chemical group has anti-

genotoxic and anti-mutagenic (Scarpato *et al.*, 1998), anti-tumoral (Lee *et al.*, 1999) and anti-inflammatory (Navarro *et al.*, 2001) activities, and probably did not increase the DNA damage observed in snails fed tobacco leaves.

PIXE analysis revealed different levels of inorganic elements in tobacco leaves when compared with lettuce leaves (Table 2). The levels of trace elements in lettuce were obtained in a previous study by our group. All of the inorganic elements detected were present in much higher levels in tobacco leaves than in lettuce leaves. This difference reflects the fact that the two plants are different species. Studies in humans and animals have demonstrated that a wide variety of metals act as mutagenic and carcinogenic agents (Leonard *et al.*, 2004). In general, metal genotoxicity is caused by indirect mechanisms, a major one of which involves interference with cellular redox regulation and the induction of oxidative stress (ROS generation), which may cause oxidative DNA damage (Beyersmann and Hartwig, 2008).

When digestive gland proteins and enzymes were analyzed (Table 2), the cytochrome P450 and P420 activities were lower in snails fed tobacco leaves compared with the control group. This decrease probably reflected the fact that nicotine and flavonoids inhibit cytochrome P450 activity, as observed by Shao *et al.* (2009) and Galati and O'Brien (2004). Galati and O'Brien (2004) also demonstrated that flavonoids inhibit drug-metabolizing enzymes. In addition, P450 isoforms are responsive to induction and inhibition by xenobiotics, including plant constituents (Ioannides, 2002; Ueng and Chen, 2004). Thus, inhibition of cytochrome enzymes in *H. aspersa* fed tobacco leaves may indicate that these snails can accumulate genotoxic agents capable of causing DNA damage. As indicated by Kim *et al.* (2002), the inhibition of cytochrome activity may disturb endocrine systems and lead to adverse effects such as carcinogenicity, immunological dysfunction and reproductive abnormality.

In conclusion, our results demonstrate that tobacco leaves are genotoxic in *H. aspersa*. The genotoxicity, mutagenicity and enzymatic inhibition caused by exposure to tobacco leaves was probably mediated by the complex mixture of substances (nicotine, coumarins, traces of saponins, flavonoids and different inorganic elements) present in these leaves. Our findings confirm the sensitivity of the Comet assay and MN test in detecting damage caused by

complex mixtures such as that present in tobacco leaves. Several biological markers have been proposed for assessing exposure to tobacco or tobacco smoke in order to elucidate the mechanisms of DNA damage. In this work, we attempted to interpret the biological action of complex mixtures of components present in tobacco leaves. This approach may be useful for studying complex mixtures in other plant species.

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