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ARTICLE

Effects of Dermal Exposure to *Nicotiana tabacum* (Jean Nicot, 1560) Leaves in Mouse Evaluated by Multiple Methods and Tissues

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Tobacco farmers are routinely exposed to complex mixtures of the compounds present in tobacco leaves, including organic and inorganic pesticides. Penetration through skin is the most significant route of uptake in occupational exposure to chemicals, including dust and liquids containing toxic and carcinogenic substances. This study evaluates the genotoxic effect of tobacco leaves with and without dermal exposure to flumetralin in *Mus musculus*, determining cell damage by the micronucleus test and the Comet assay as well as antioxidant enzyme activities and hematologic parameters. Nicotine was used as positive control. Blood samples were collected for 0, 3, 24 and 48 h exposure periods, and DNA damage by Comet assay and micronucleus test was evaluated for all these periods. Bone marrow and liver cells were also evaluated for the 48 h exposure period. Significant differences between Comet assay results in blood cells from animals exposed to tobacco leaves with and without pesticide were found in 24 and 48 h exposure periods in relation to negative control. Bone marrow cells from the group exposed to leaves with pesticide (48 h) also demonstrated significant increase in DNA damage. Concerning the micronucleus test, only animals exposed to tobacco leaves without pesticide (24 h) showed increase in frequency of micronuclei when compared to the negative control. Oxidative stress activities also were demonstrated for different groups. The results demonstrate the injury effect caused by tobacco leaves in different *Mus musculus* tissues, suggesting that the effects of dermal exposure to tobacco leaves are caused by complex mixtures present in the plant, but mainly by nicotine.

KEYWORDS: *Nicotiana tabacum*; Comet assay; micronucleus test; *Mus musculus*; nicotine

1. INTRODUCTION

The tobacco plant (*Nicotiana tabacum*) is an herbaceous annual of the order Solanaceae, genus *Nicotiana*. Chemical analysis has revealed that tobacco leaves contain an unusual number of constituents: nicotine, nicotianine, malic acid (*1*), tobacco-specific nitrosamines, nitrate, nitrite (*2*), and coumarin (*3*) are typically present; however, nicotine is the major alkaloid present in tobacco (*1*).

Concerning nicotine, classical genotoxicity tests have produced some controversial results. Some of these results revealed slight positive effects of nicotine on sister chromatid exchange in mammalian cells (*4*) and on chromosome aberrations in the bone marrow cells of Chinese hamsters (*5*). However, no nicotine effects were detected in the *Salmonella*/microsome assay or in other bacterial indicator assays (*6*). Similarly, no chromosome aberrations have ever been reported in mice (*7*).

Acute toxicity and long-term hazards have been reported to result from skin contamination by dust and liquids containing toxic and carcinogenic substances that manage to penetrate the organism effectively. Penetration through skin and subsequent systemic absorption is the most significant extrapulmonary uptake route in occupational exposure to chemicals (*8*). Some characteristics exhibited by nicotine theoretically increase the alkaloid's absorption potential, like its high solubility in both polar and nonpolar solvents ($\log K_w = 1.17$), its low molecular weight (162.2 g/mol) (*8*) and the presence of nicotinic receptors in the skin, on keratinocytes, fibroblasts and blood vessels (*9*).

Tobacco farm workers are at risk of developing green tobacco sickness (GTS), a disease caused by dermal absorption of nicotine from wet tobacco leaves. Nausea, pallor, chills, vomiting, headache, difficulty to breathe, abdominal pain, diarrhea, loss of appetite,

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runny eyes, blurred vision, weakness, prostration and dizziness, and occasionally oscillation in blood pressure or heart rate are typical signs and GTS symptoms (*10*, *11*). Also, GTS has been linked to nicotine, and these symptoms have been attributed to acute nicotine poisoning following dermal contact with mature tobacco plants.

To date, no information is available on any long-term health effects of chronic exposure to dermally absorbed nicotine. For farm workers who are already at risk of a number of work-related health problems and who suffer from other morbidity and mortality risks at rates higher than the general population, nicotine exposure and GTS may add to this burden of disease and contribute to long-term health problems (*10*, *11*).

Research has shown that tobacco farmers are routinely exposed to complex mixtures of the compounds present in tobacco leaves, including organic and inorganic pesticides. Considering these pesticides, tobacco farmers are mostly exposed to flumetralin, a compound widely used as a shoot inhibitor applied on the bottom leaves of the plant. This procedure aims at increasing root growth and promoting higher leaf weight and nicotine content at harvest. To do this, workers walk down rows of 4−6-foot-tall tobacco plants, break off the flowers at the top and apply flumetralin, directly exposing the skin to tobacco leaves (*10*). According to Johnson and Connell (*12*), residues of this nitropesticide are present on untreated leaves and may persist there for over a year.

Due to the lack of knowledge about DNA damage caused by dermal absorption of nicotine and other substances present on the surface of tobacco leaves, biomonitors have gained increasing importance as analytic tools to assess genotoxicity. This study investigates the genotoxic effect of tobacco leaves by dermal absorption in *Mus musculus*. Genotoxicity was evaluated using the Comet assay and the micronucleus test. The results were compared to the data of hematocrit and plasma protein concentration, cytochrome P450 activity and antioxidant enzyme activities in *Mus musculus* liver.

2. MATERIALS AND METHODS

2.1. Plant Material. *Nicotiana tabacum* leaves were collected in Crystal Farm in October 2005. The farm is located in the south-central region of the Rio Grande do Sul State, Brazil. The tobacco plants were grown in the same farm; however its growth was planned: tobacco was seeded into trays with a soil-less potting mix. The trays were then floated on a bed of water (Float System) with and without pesticide. When plants reached transplant size, they were transplanted to the field. Tobacco plants without pesticide were transplanted distant from the farm work tobacco. Tobacco leaves with and without pesticides were sampled about 60 days after transplanting. Tobacco leaves were harvested from the bottom up. The only pesticide utilized in the tobacco farm work was flumetralin (2-chloro-*N*-(2,6-dinitro-4-(trifluoromethyl)phenyl)-*N*-ethyl-6-fluorobenzenemethanamine), which inhibits axillary shoot growth in the terminal bud of the plant. Fresh leaves were packaged and stored in freezer at −30 °C.

2.2. Animals. The animals used were 5−7-week-old outbreed line CF1 mice, weighing 20.49 ± 2.3 g (mean ± SD) provided by IPB/LACEN (Institute of Biological Research and Central Laboratory of State), Porto Alegre, RS, Brazil. The temperature in the experimental room was about 24 °C, and relative humidity was roughly 60%. The light cycle was 12 h light/12 h dark. All animals received commercial standard mouse cube diet (Nuvilab, CR1, Moinho Nuvipal Ltd.a, Curitiba, PR, Brazil) and water *ad libitum*. All experimental procedures were performed in accordance with the local Ethics Committee guidelines.

2.3. Treatment. The animal groups consisted of a negative control group, which was exposed to gauze patch; a positive control, exposed to a nicotine patch (Niquitin, Alza Corporation) (0.015 mg/g body weight); and exposed test groups, which were exposed to tobacco leaves with pesticide (TLP) (3 mg/g body weight) and tobacco leaves without pesticide (TL) (3 mg/g body weight). Mice were divided into experimental groups of 10 animals each (5 males and 5 females) and treated by dermal exposure according to the method acute dermal toxicity (OECD) (*13*). Healthy animals were acclimated to the laboratory conditions for at least 7 days prior to the test. Before the test, animals were randomized and assigned to the treatment groups. Approximately 24 h before the test, fur was removed from the dorsal area of the body of the test animals by clipping and shaving. Care was taken to avoid abrading the skin, which could alter its permeability. At least 10% of the body surface area was thus prepared for the application of samples. The weight of the animal was taken into account when deciding on the area to be prepared and on the dimensions of the covering. Prior to application, tobacco leaves were sufficiently moistened with distilled water to ensure good contact with the skin. In the exposed group, tobacco leaves were applied to a small area of skin and covered with a gauze patch, which was held in place with nonirritating tape. Animals of all groups were observed for a 48 h period in total. One drop of peripheral blood was taken from the tip of the tail of each mouse in the TLP, TL, negative and positive groups at 0, 3, 24 and 48 h (T0, T3, T24 and T48) exposure periods, for the Comet assay and the micronucleus test. The animals were sacrificed by cervical dislocation at the 48 h exposure period. The micronucleus test was carried out with bone marrow cells and the Comet assay with liver and bone marrow cells. Liver was stored in freezer at −70 °C for enzyme analysis. Blood was collected by cardiac punction only in the 48 h exposure period and stored in tubes containing EDTA for plasma protein concentrations and hematocrit analysis. The same rodent groups were utilized for all assays.

2.4. Comet Assay. The alkaline Comet assay was performed as described by Singh et al. (*14*) and as modified in Da Silva et al. (*15*). Cells from different tissues were obtained according to the method described by Tice (*16*), where heparinized whole blood was utilized, and bone marrow perfusions of femur and liver were homogenized with fetal bovine serum and RPMI, respectively. Cells isolated from tissues (10 μL) were embedded in 90 μL of 0.75% (w/v) low melting point agarose, and the mixture was added to a microscope slide precoated with 1.5% (w/v) of normal melting point agarose and topped with a coverslip. The slide was briefly placed on ice for agarose to solidify and the coverslip carefully removed. Next, the slide was immersed in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0−10.5) containing freshly added 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO) for at least 1 h at 4 °C. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min for DNA unwinding, and electrophoresis was performed in the same buffer. The electrophoresis conditions were 15 min at 300 mA and 25 V (0.7 V/cm). All these steps were carried out under dim indirect light. Following electrophoresis, slides were neutralized in 400 mM Tris (pH 7.5) and fixed (15% w/v trichloroacetic acid, 5% w/v zinc sulfate, 5% glycerol), washed in distilled water and dried overnight. The gels were rehydrated for 5 min in distilled water, and then stained for 15 min (37 °C) with a solution containing the following sequence: 34 mL of solution B (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5% w/v tungstosilicic acid, 0.15% v/v formaldehyde, 5% w/v sodium carbonate) and 66 mL of solution A (5% sodium carbonate). The staining was stopped with 1% acetic acid, and the gels were air-dried (*17*). To calculate a damage index (DI), cells were visually allocated into 5 classes according to tail size (0 = no tails, and 4 = maximum-length tails), which resulted in a single DNA damage score for each sample and consequently for each group studied. Thus, the damage index (DI) of the group could range from 0 (completely undamaged = 100 cells ×0) to 400 (maximum damage = 100 cells × 4). The damage frequency (DF in %) was calculated for each sample based on the number of cells with tail versus those without. All slides were coded for blind analysis.

2.5. Micronucleus Test. Each complete test was made according to a report by the US Environmental Protection Agency Gene-Tox program (*18*) and Da Silva et al. (*15*) recommendations. Whole blood smears were prepared on slides for the 0, 3, 24 and 48 h (T0, T3, T24 and T48) exposure samples, and bone marrow smears were prepared for the 48 h exposure sample, when animals were killed by cervical dislocation. The bone marrow was extracted from the two femurs. Smears were prepared directly on slides with bone marrow and blood, two per animal and per tissue. Bone marrow smear was prepared with a drop of fetal calf serum. The slides were stained with 5% Giemsa, air-dried and coded for blind analysis. To avoid false negative results and as a measure of toxicity in bone marrow, the polychromatic erythrocyte:normochromatic erythrocyte (PCE/NCE) ratio was scored in 1,000 cells. The incidence of micronuclei (MN) was observed in 2,000 PCE and 2,000 peripheral blood reticulocytes (RET) for each animal (i.e., 1,000 from each of the two slides prepared from the duplicate), using bright-field optical microscopy at a

magnification of 200−1000×. All slides were coded to blind analysis. The test groups were compared to the respective negative controls by gender, separately and in combination.

2.6. Antioxidant Enzyme Activities. The livers were excised, weighed, and immediately frozen at −70 °C. Frozen tissue from each rat was homogenized in ice-cold phosphate buffer (KCl 140 mM, phosphate 20 mM, pH 7.4) and centrifuged at 3,000 rpm for 10 min. Cytosolic superoxide dismutase (SOD) was assayed according to Misra and Fridovich (*19*) at 30 °C. Oxygen radicals, generated by the interaction of Fe^{2+} and H_2O_2, were proposed as the species responsible for the oxidation of epinephrine to adrenochrome in Fenton's reagent. The observation that superoxide dismutase acted as a potent inhibitor of the spontaneous oxidation of epinephrine at pH 10.2 also provided a convenient and sensitive assay for this enzyme. The rate of epinephrine autoxidation, which is progressively inhibited by increasing amounts of SOD in the homogenate, was monitored spectrophotometrically at 560 nm. The amount of enzyme that inhibits epinephrine autoxidation at 50% of the maximum inhibition is defined as 1 U of SOD activity. Catalase (CAT) activity was determined by measuring the exponential disappearance of H_2O_2 at 240 nm, and was expressed as picomoles per milligram of protein (*20*). Oxidative stress was determined by measuring the concentration of thiobarbituric acid reactive substances (TBARS). The amount of aldehydic products generated by lipid peroxidation was quantified by thiobarbituric acid reaction using 3 mg of protein for sample. The samples were incubated at 90 °C for 30 min after adding 500 μL of 0.37% thiobarbituric acid in 15% trichloroacetic acid, and then centrifuged at 4 °C at 2000*g* for 15 min. Spectrophotometric absorbance was determined in the supernatant at 535 nm (*21*). Microsomes were prepared in the standard procedure: tissues were homogenized in ice-cold 0.1 M potassium phosphate, pH 7.5, containing 0.15 M KCl and 0.1 mM EDTA (1:5 w/v). Crude homogenate was centrifuged at 12000*g* for 20 min and the supernatant centrifuged at 105000*g* for 60 min. The pellet was resuspended in potassium phosphate buffer and recentrifuged at 105000*g* for 60 min. Microsomes were isolated in the pellet. The microsomal fraction was resuspended in the same buffer solution containing 20% glycerol and frozen at −70 °C (*22*). Proteins were determined according to the Lowry method (*23*). The cytochrome P450 content was assayed in microsomes according to the method of Omura and Sato (*24*), and expressed as nmol/mg protein on the basis of the millimolar extinction coefficient of 91 mM cm^{-1}. The measurements were realized in a spectrophotometer (Beckman DU70) at 400−700 nm absorption.

2.7. Plasma Protein Concentrations and Hematocrit Analysis. For hematological analysis, blood was collected by cardiac puncture and stored in tubes containing EDTA. The determination of erythroid cell volume, expressed in percentage, was carried out according to the microhematocrit method (*25*). Next, the samples of the total blood with anticoagulant were transferred to capillary tubes and microcentrifuged at 10,000 rpm for 5 min. The total plasma protein (TPP) concentration was measured by refractometry. This concentration has been analyzed in association with hematocrit.

2.8. Statistical Analysis. The normality of variables was evaluated using the Kolmogorov−Smirnov test. The statistical differences between the four groups (nicotine, TLP, TL and negative control) were analyzed using the nonparametric two-tailed Kruskal−Wallis test with the Dunn correction for multiple comparisons for Comet assay and micronucleus test results. The statistical differences between the four groups (nicotine, TLP, TL and negative control) were analyzed using the one-way analysis of variance (ANOVA) with Student−Newman−Keuls test for multiple comparisons to hematological parameters and antioxidant enzyme activity analysis. Difference between genders was tested using the Wilcoxon−Mann−Whitney test. The critical level for rejection of the null hypothesis was considered to be a *P* value of 5%.

3. RESULTS

3.1. DNA Damage by Comet Assay. Table 1 summarizes the Comet assay data expressed as damage index (DI) and damage frequency (DF) for blood cells of *Mus musculus* exposed to tobacco leaves (TL and TLP) and for the controls groups animals, at different exposure times (T0, T3, T24 and T48). The animals' cells from negative control group did not show statistically significant differences when different exposure times were used. No difference was found in mean DI and DF values for blood cells across all different exposure groups (negative and positive controls, TL and TLP) in T0. The positive control (nicotine patch) group showed significant values in mean DI in T24 and T48 and DF in T3, T24 and T48, as compared to the samples collected at T0 as well as when compared to negative control of same exposure time. Concerning TL, a significant increase was observed for both DI and DF values, when comparing T24 and T48 in relation to T0 and to negative control of same exposure time. The TLP group showed values significantly higher for DI and DF (T3, T24 and T48) when compared to T0, as well as when compared to negative control in the same exposure time. In T3 the DF in TL was significantly higher than in TLP. High values of DI and DF were observed similarly to the groups: positive control, TL and TLP. **Table 2** presents results for bone marrow cell. The table shows higher DI and DF means for the TLP group than negative control. **Table 3** presents data about liver cells, by which it is possible to observe that only positive control (nicotine patch) shows a significant increase of DI in relation to negative control. No difference was observed between genders for the different tissues.

3.2. Micronucleus Test. Tables 4 and **5** show the results of the micronucleus test for peripheral blood samples collected at T0, T3, T24 and T48 and for bone marrow collected at T48. The Kruskal−Wallis test revealed that the mean micronucleus frequency in peripheral blood was significantly higher in the positive control group at T3 when compared to the negative control. Samples collected from the group treated with TL at T24 showed higher values as compared to those collected at T0 and to the negative control group samples (**Table 4**). No difference in micronucleus frequency was observed between groups in the bone marrow tissue, neither in PCE/NCE ratio (**Table 5**). No difference was observed between genders.

3.3. Hematological Parameters. Hematocrit and plasma protein concentration were analyzed (**Table 6**). No significant difference was found in hematocrit and total plasma protein between TLP, TL, negative and positive control groups when compared per group tested by ANOVA Test. No difference was observed between females and males.

3.4. Antioxidant Enzyme. Figure 1 show the results of different antioxidant enzymes: TBARS, SOD, cytochrome P450 and CAT. Animals exposed to tobacco leaves without pesticide significantly increased the TBARS concentration in the liver, when compared with negative control group. In relation to SOD, individuals exposed to tobacco leaves without pesticide significantly increased activity in the liver when compared to negative control and TLP. No difference was detected between the groups in terms of enzyme Cytochrome P450 activity. In relation to the CAT activity, TLP, TL and positive control (nicotine) groups significantly increased CAT activities when compared with negative control group.

4. DISCUSSION

Substances present in tobacco form a complex mixture of organic and inorganic compounds that may interact to produce additive, synergistic or antagonistic effects (*10*). In the light of the paucity of data on DNA damage caused by tobacco leaves via dermal absorption, we investigated the genotoxic effect of substances present in tobacco leaves through that pathway in *Mus musculus*. The DNA damage was assessed by Comet assay (single cell gel electrophoresis assay) and by the micronucleus test. These tests were chose due to its simplicity of scoring and wide applicability in different cell types. Comet assay has been used in different studies as a rapid and sensitive tool for demonstrating chemically induced DNA damage, cells with damaged DNA displaying increased migration of DNA fragments from the nucleus and the formation of a Comet shape (*14*−*16*). Another cytogenetic test used to measure DNA damage to toxic agents is the micronucleus test, which assesses the micronuclei originating

Table 1. Damage Index and Damage Frequency (Comet Assay) (Mean ± Standard Deviation) in Peripheral Blood Cells of Exposed Animals to Tobacco Leaves without (TL) and with Pesticide (TLP), to Nicotine Patch (Positive Control) and to Gauze and Tape (Negative Control)

			damage index		damage frequency (%)	
treatment	time (h)	gender	per gender	per group	per gender	per group
negative control	T0	male	15.8 ± 14.7	19.5 ± 15.6	11.2 ± 6.6	14.3 ± 11.2
		female	23.2 ± 17.2		17.4 ± 14.6	
	T3	male	19.4 ± 11.9	25.2 ± 14.7	12.0 ± 6.1	16.5 ± 7.6
		female	31.0 ± 16.2		21.0 ± 6.6	
	T24	male	21.6 ± 19.9	31.1 ± 20.9	16.2 ± 4.4	18.3 ± 9.8
		female	40.6 ± 24.2		20.4 ± 13.4	
	T48	male	28.8 ± 16.2	38.7 ± 17.8	21.8 ± 9.1	24.0 ± 12.4
		female	48.6 ± 14.4		26.2 ± 15.9	
positive control	T0	male	11.6 ± 5.0	20.3 ± 18.8	10.8 ± 4.3	15.3 ± 10.8
		female	29.0 ± 24.1		19.8 ± 13.9	
	T3	male	94.4 ± 87.4	96.8 ± 81.4	51.2 ± 27.7	50.8 ± 29.8[f]
		female	99.2 ± 85.1		50.4 ± 34.9	
	T24	male	178.0 ± 83.0	174.8 ± 84.6[b,g]	65.0 ± 19.9	65.7 ± 19.0[b,f]
		female	171.6 ± 95.8		66.4 ± 20.5	
	T48	male	95.6 ± 60.9	112.9 ± 52.6[a,e]	49.0 ± 20.2	57.1 ± 18.6[a,e]
		female	130.2 ± 41.9		65.2 ± 14.4	
TL	T0	male	13.4 ± 6.4	23.3 ± 19.9	15.2 ± 9.3	16.8 ± 8.4
		female	33.2 ± 24.7		18.4 ± 8.1	
	T3	male	31.0 ± 18.6	49.9 ± 34.6	21.6 ± 9.9	27.7 ± 17.2
		female	68.8 ± 38.0		33.8 ± 21.8	
	T24	male	157.0 ± 50.2	165.5 ± 52.8[b,g]	69.0 ± 23.5	64.1 ± 18.3[b,c,g]
		female	174.0 ± 59.9		59.2 ± 26.9	
	T48	male	151.4 ± 24.9	162.4 ± 55.2[b,d,g]	73.2 ± 13.3	68.6 ± 16.1[b,d,g]
		female	173.4 ± 76.9		64.0 ± 18.9	
TLP	T0	male	17.2 ± 8.8	24.4 ± 15.2	11.4 ± 5.7	15.5 ± 11.3
		female	31.6 ± 17.7		19.6 ± 14.5	
	T3	male	149.0 ± 72.7	122.8 ± 69.9[a,g]	58.6 ± 18.6	54.4 ± 22.3[a,f,h]
		female	96.6 ± 63.4		50.2 ± 26.9	
	T24	male	159.8 ± 99.7	145.3 ± 65.3[b,f]	72.2 ± 15.6	66.6 ± 19.4[b,g]
		female	130.8 ± 24.8		64.2 ± 24.4	
	T48	male	179.9 ± 71.5	157.2 ± 62.9[b,g]	72.2 ± 11.4	73.2 ± 12.5[b,g]
		female	134.6 ± 50.4		74.2 ± 14.2	

[a] Significant in relation to T0 in same treatment group at $P < 0.01$. [b] Significant in relation to T0 in same treatment group at $P < 0.001$. [c] Significant in relation to T3 in same treatment group at $P < 0.05$. [d] Significant in relation to T3 in same treatment group at $P < 0.01$. [e] Significant in relation to negative control in same exposure time group at $P < 0.05$. [f] Significant in relation to negative control in same exposure time group at $P < 0.01$. [g] Significant in relation to negative control in same exposure time group at $P < 0.001$. [h] Significant in relation to TL in same exposure time group at $P < 0.05$. Tested by Kruskal−Wallis test.

Table 2. Damage Index and Damage Frequency (Comet Assay) (Mean ± Standard Deviation) in Bone Marrow Cells of Exposed Animals to Tobacco Leaves without (TL) and with Pesticide (TLP), to Nicotine Patch (Positive Control) and to Gauze and Tape (Negative Control) during 48 h (T48)

		damage index (DI)		damage frequency (DF%)	
treatment	gender	per gender	per group	per gender	per group
negative control	male	27.6 ± 33.2	34.8 ± 29.6	30.2 ± 19.9	22.6 ± 18.4
	female	42.0 ± 27.3		15.0 ± 14.8	
positive control	male	41.0 ± 37.5	66.5 ± 54.7	24.6 ± 17.4	35.8 ± 23.8
	female	92.0 ± 60.8		47.0 ± 25.6	
TL	male	38.2 ± 34.1	62.5 ± 58.9	26.6 ± 19.0	35.8 ± 26.6
	female	86.8 ± 71.8		45.0 ± 31.8	
TLP	male	71.2 ± 17.4	90.2 ± 46.5[a]	43.0 ± 5.8	52.0 ± 22.4[a]
	female	109.2 ± 60.6		61.0 ± 29.9	

[a] Significant in relation to negative control at $P < 0.05$. Tested by Kruskal−Wallis test.

from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. The micronucleus test provides a measure of both chromosome breakage and chromosome loss and has been shown to be at least as sensitive an indicator of chromosome damage as the classical metaphase chromosome analysis (*18*).

The Comet assay (**Tables 1**, **2** and **3**) revealed an increase in DNA damage during the exposure to tobacco leaves with and without pesticide (flumetralin). The same was observed for mice treated with nicotine patches (positive control). TL group presented increase in DNA damage only for blood cells, TLP for blood and bone marrow cells, and nicotine group for blood and liver cells.

Our previous study (*26*) shows in phytochemical analysis the presence of coumarins, saponins traces, alkaloids and flavonoids. This complex mixture is being responsible for the induction of damage to the group TL. It was described that nicotine is the main constituent of the tobacco leaves (*1*), easily soluble in water and absorbed through the skin. Significant direct genotoxic effects have also been shown in human gingival fibroblasts (*27*), in spermatozoa (*28*), in human peripheral lymphocytes and lymphatic tissue of the palatine tonsils (*29*) and in mini-organ cultures of human upper aerodigestive tract epithelia (*30*) exposed to nicotine. In our study, although females showed mean DI and DF values higher than males in the majority of the samples, no significant difference between genders was found across exposure groups, exposure times and tissues. Kyerematen et al. (*31*) affirm that nicotine bioavailability is gender-dependent, and other studies showed that male rats eliminate nicotine faster than females.

It was also shown that the group exposed to nicotine only (positive control) showed increased damage to the peripheral blood and liver. The CYP enzymes involved in nicotine metabolism are expressed primarily in the liver and to a lesser extent in other extrahepatic tissues (*32*). One possibility for this result is that nicotine metabolites showed a genotoxic effect in liver cells and that the nicotine present in tobacco leaves might have its

Table 3. Damage Index and Damage Frequency (Comet Assay) (Mean ± Standard Deviation) in Liver Cells of Exposed Animals to Tobacco Leaves without (TL) and with Pesticide (TLP), to Nicotine Patch (Positive Control) and to Gauze and Tape (Negative Control) during 48 h (T48)

		damage index (DI)		damage frequency (DF%)	
treatment	gender	per gender	per group	per gender	per group
negative control	male	37.6 ± 17.4	43.5 ± 24.3	26.8 ± 16.4	27.1 ± 13.2
	female	49.4 ± 30.7		27.4 ± 11.1	
positive control	male	116.4 ± 41.8	104.8 ± 36.4[a]	56.6 ± 26.0	50.7 ± 21.7
	female	93.2 ± 29.9		41.8 ± 13.6	
TL	male	101.4 ± 78.9	70.5 ± 65.5	49.6 ± 23.8	37.6 ± 23.5
	female	39.6 ± 32.4		25.6 ± 17.8	
TLP	male	67.2 ± 34.2	87.1 ± 41.8	36.2 ± 20.7	44.6 ± 20.9
	female	107.0 ± 42.2		53.0 ± 19.6	

[a] Significant in relation to negative control at $P < 0.05$. Tested by Kruskal−Wallis Test.

Table 4. Detection of Micronuclei Mean (± Standard Deviation) in Peripheral Blood Reticulocytes (MNRET) of Mice Exposed to Tobacco Leaves, without Pesticide (TL) and with Pesticide (TLP), Nicotine Patch (Positive Control) and Only Tape and Gauze (Negative Control)

			MNRET (2,000 cells/animal)	
treatment	time (T*h*)	gender	per gender	per group
negative control	T0	male	0.2 ± 0.4	0.9 ± 1.3
		female	1.4 ± 1.1	
	T3	male	1.0 ± 1.2	0.9 ± 0.9
		female	0.8 ± 0.4	
	T24	male	1.0 ± 0.7	0.9 ± 0.7
		female	0.8 ± 0.8	
	T48	male	0.2 ± 0.4	0.7 ± 0.8
		female	1.2 ± 0.8	
positive control	T0	male	1.0 ± 1.0	1.0 ± 0.9
		female	1.0 ± 1.0	
	T3	male	3.8 ± 1.8	2.9 ± 1.9[a]
		female	2.0 ± 1.7	
	T24	male	2.0 ± 1.2	1.8 ± 1.2
		female	1.6 ± 1.3	
	T48	male	1.2 ± 0.4	1.2 ± 0.9
		female	1.2 ± 1.3	
TL	T0	male	0.8 ± 0.8	0.6 ± 0.7
		female	0.4 ± 0.5	
	T3	male	2.0 ± 2.5	2.0 ± 2.1
		female	2.0 ± 1.7	
	T24	male	1.8 ± 0.8	2.6 ± 1.3[a,b]
		female	3.4 ± 1.1	
	T48	male	1.2 ± 1.1	1.2 ± 1 0.0
		female	1.2 ± 1.1	
TLP	T0	male	1.2 ± 0.84	1.2 ± 0.6
		female	1.2 ± 0.45	
	T3	male	1.4 ± 1.14	1.4 ± 0.8
		female	1.4 ± 0.5	
	T24	male	3.4 ± 2.5	2.8 ± 2.1
		female	2.2 ± 1.6	
	T48	male	0.6 ± 0.5	1.5 ± 1.7
		female	2.0 ± 1.2	

[a] Significant in relation to negative control in same exposure time group at $P < 0.05$. [b] Significant in relation to 0 h in same treatment group at $P < 0.05$. Tested by Kruskal−Wallis test.

metabolism altered by other leaf constituents. Another possibility is that nicotine quantity in tobacco leaves (0.02% of nicotine per leaf; data not shown) is not sufficient to cause DNA damage in liver cells; therefore, only animals exposed to pure nicotine showed a genotoxic effect significantly higher in these cells.

These differences in relation to exposure response to tobacco leaves and to nicotine found in our study can be due to several biological factors. These differences must be due to different types

Table 5. Detection of Micronuclei Mean (± Standard Deviation) in Bone Marrow Polychromatic Erythrocytes (MNPCE) of Mice Exposed to Tobacco Leaves, without Pesticide (TL) and with Pesticide (TLP), Nicotine Patch (Positive Control) and Only Tape and Gauze (Negative Control) during 48 h (T48)

		MNPCE (2,000 cells/animal)		ratio (PCE:NCE[a])	
treatment	gender	per gender	per group	per gender	per group
negative control	male	1.2 ± 1.1	1.2 ± 1.0	1.3 ± 0.3	1.3 ± 0.3
	female	1.2 ± 1.1		1.2 ± 0.3	
positive control	male	3.0 ± 1.9	2.7 ± 1.6	1.3 ± 0.1	1.2 ± 0.3
	female	2.4 ± 1.5		1.0 ± 0.3	
TL	male	1.8 ± 1.3	2.3 ± 1.3	1.5 ± 0.2	1.4 ± 0.2
	female	2.8 ± 1.3		1.4 ± 0.2	
TLP	male	2.2 ± 2.2	3.0 ± 1.9	1.3 ± 0.2	1.1 ± 0.3
	female	3.8 ± 1.3		0.9 ± 0.3	

[a] PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes.

Table 6. Hematological Parameters of Mice Exposed to Tobacco Leaves, without Pesticide (TL) and with Pesticide (TLP), Nicotine Patch (Positive Control) and Only Tape and Gauze (Negative Control) during 48 h (T48)

		hematocrit (± SD)		plasma protein concentration (± SD)	
treatment	gender	per gender	per group	per gender	per group
negative control	male	25.5 ± 6.6	24.8 ± 7.2	7.8 ± 1.7	8.1 ± 1.4
	female	24.2 ± 8.4		8.4 ± 1.3	
positive control	male	17.3 ± 8.7	19.1 ± 6.5	10.1 ± 2.7	9.1 ± 1.8
	female	20.2 ± 5.7		8.4 ± 0.8	
TLP	male	18.2 ± 5.1	18.1 ± 8.8	9.1 ± 0.5	4.0 ± 1.4
	female	18.0 ± 2.7		8.5 ± 2.2	
TL	male	15.0 ± 4.8	17.0 ± 7.5	8.7 ± 0.6	7.9 ± 1.3
	female	21.0 ± 12.7		6.4 ± 0.0	

of nicotine receptor(s) present on target cell nicotine (*32*, *33*) and cotinine (*33*), difference in the doses, exposure route (*32*, *34*) and interindividual variability (*33*).

When the tobacco leaves are exposed to flumetralin, it is observed that both peripheral blood and bone marrow cells show increased damage indices. According to Johnson and Connell (*12*), residues of this nitropesticide are present on untreated leaves and may persist there for over a year. Although flumetralin showed no genotoxic potential in mutagenicity studies that investigated concentrations of up to 200 mg/kg, the maximum tolerated dose was not fetotoxic nor teratogenic in rats or rabbits (*35*).

The Comet assay detected recent lesions that can be repaired, such as breaks and alkali-labile sites, while the observed micronuclei detected nonrepairable damage, such as clastogenic and aneugenic lesions (*15*, *36*). Looking at our results for the micronucleus test (**Tables 4** and **5**) we can see that only the TL group at T24 and positive control at T3 showed micronucleus frequency increases significantly. It can be seen in our results that there is increased damage observed in the Comet assay in all groups and that it is not seen in the micronucleus test, which leads us to conclude that the damage probably is being repaired. Usually the damage caused by oxidative stress is more easily repaired (*36*). DNA damage induced by nicotine is triggered by free radical production and oxidative stress (*37*).

Our studies showed that SOD activity, CAT activity and lipid peroxidation were higher in the TL group as compared to negative control (**Figure 1**). The TLP group showed increased activity only to CAT activity. The increase in lipid peroxidation indicates that exposure to tobacco leaves causes oxidative damage in these animals. The increase in activity of antioxidant enzymes suggests that the TL group has a higher antioxidant defense capability than the TLP group. The DNA damage caused by nicotine is related to the generation of reactive oxygen species (ROS). Wetscher et al. (*38*)

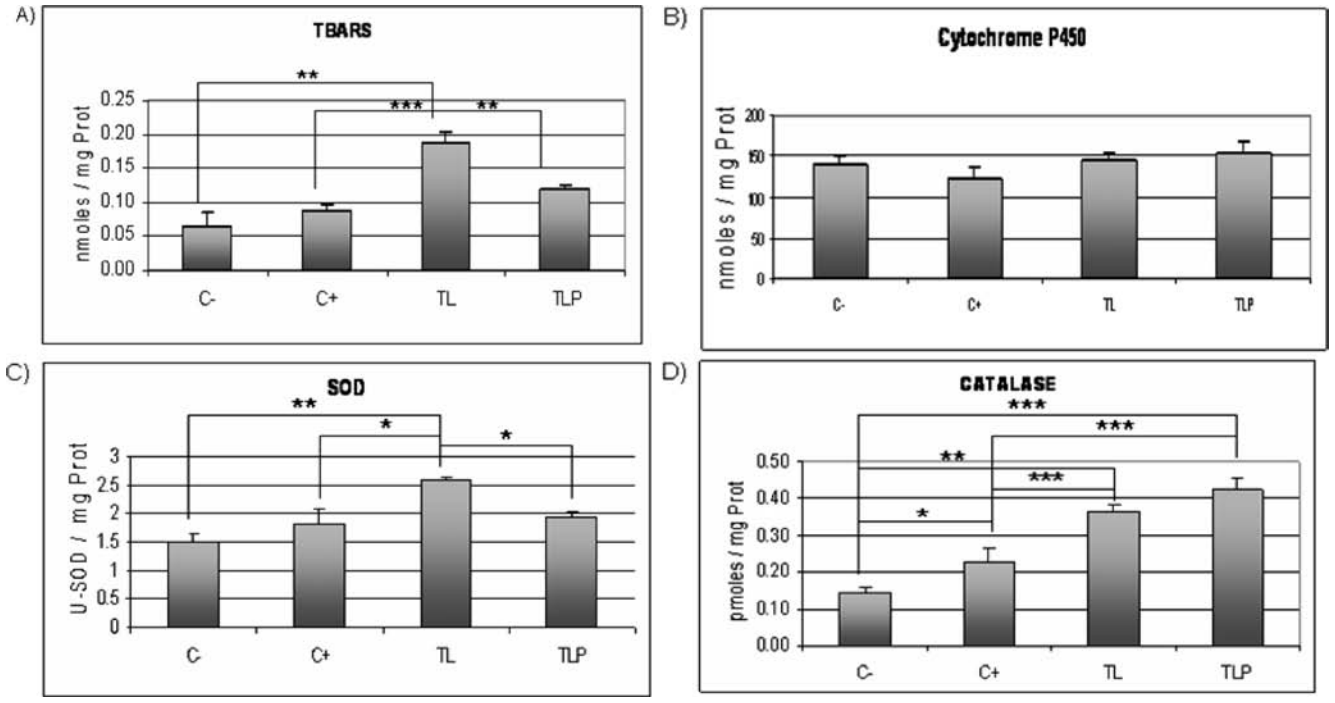

Figure 1. Antioxidant enzyme activities in liver of the animals exposed at gauze patch (C^-), nicotine patch (C^+), tobacco leaves without pesticide (TL) and tobacco leaves with pesticide (TLP). (**A**) Lipid peroxidation measured by thiobarbituric acid reactive substances (TBARS). (**B**) Cytochrome P450 activity. (**C**) Superoxide dismutase (SOD) activity. (**D**) Catalase activity. Values are expressed as mean ± standard error tested by one-way analysis of variance (ANOVA). *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$.

demonstrated that nicotine caused oxidative stress to the pancreatic tissue of the rats positively correlated to the duration of nicotine treatment, and that it was also dose-dependent. Nicotine has also been implicated in free radical generation in rodent and human cells of various types, directly addressing the relationship between ROS induction and the observed DNA damage (*26*, *37*, *38*).

Hematological parameters can reveal the presence of hemopoietic disorders (*39*). Fish exposed to tobacco leaf dust presented reduction in blood parameters, became anemia and the severity of this condition were directly proportional to the tobacco dust concentrations (*40*). However, no effect was found in hematocrit and plasma protein concentration among TLP, TL, negative and positive groups (**Table 6**).

In conclusion, our study demonstrates the presence of genotoxic effects and slight mutagenic effects in mice caused by tobacco leaves. This induction of DNA damage by dermal exposure to tobacco leaves must be caused by a complex mixture present in the leaves, probably by interaction with compounds such as nicotine, coumarin, cotinine and flavonoids. It was also shown that similarly to pesticide exposure flumetralin also led to increased DNA damage.

ABBREVIATIONS USED

DNA, deoxyribonucleic acid; GTS, green tobacco sickness; TLP, tobacco leaves with pesticide; TL, tobacco leaves without pesticide; MN, micronucleus test; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; pH, potential hidrogenionic; RPMI, Roswell Park Memorial Institute (culture media); ROS: reactive oxygen species; SD, standard deviation; DI, damage index; DF, damage frequency; SSB, single-strand break; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; RET, peripheral blood reticulocytes; SOD, superoxide dismutase; CAT, catalase; TBARS, thiobarbituric acid reactive substances; TPP, total plasma protein.

LITERATURE CITED

(1) Hinds, J. I. D. *The use of tobacco*; Cumberland Presbyterian Publishing House: Nashville, TN, 1882; pp 1−138.

(2) Burton, H. R.; Dye, N. K.; Bush, L. P. Distribution of tobacco constituents in tobacco leaf tissue. 1. Tobacco-specific nitrosamines, nitrate, nitrite, and alkaloids. *J. Agric. Food Chem.* **1992**, *40*, 1050–1055.

(3) Christakopoulos, A.; Feldhusen, K.; Norin, H.; Palmqvist, A.; Wahlberg, I. Determination of natural levels of coumarin in different types of tobacco using a mass fragmentographic method. *J. Agric. Food Chem.* **1992**, *40*, 1358–1361.

(4) Trivedi, A. H.; Dave, B. J.; Adhvaryu, S. G. Assessment of genotoxicity of nicotine employing in vitro mammalian test systems. *Cancer Lett.* **1990**, *54*, 89–94.

(5) Munzner, R.; Renner, H. W. Genotoxic investigations of tobacco protein using microbial and mammalian test systems. *Z. Ernaehrungswiss.* **1989**, *28*, 300–309.

(6) Doolittle, D. J.; Winegar, R.; Lee, C. K. The genotoxic potential of nicotine and its major metabolites. *Mutat. Res.* **1995**, *344*, 95–102.

(7) Adler, I. D.; Attia, S. M. Nicotine is not clastogenic at doses of 1 or 2 mg/kg body weight given orally to male mice. *Mutat. Res.* **2003**, *542*, 139–142.

(8) Zorin, S.; Kuylenstierna, F.; Thulin, H. In vitro test of nicotine's permeability through human skin. Risk evaluation and safety aspects. *Ann. Occup. Hyg.* **1999**, *43*, 405–13.

(9) Misery, L. Nicotine effects on skin: Are they positive or negative? *Exp. Dermatol.* **2004**, *13*, 665–670.

(10) Onuki, M.; Yokoyama, K.; Kimura, K.; Sato, H.; Nordin, R. B.; Naing, L.; Morita, Y.; Sakai, T.; Kobayashi, Y.; Araki, S. Assessment of urinary cotinine as a marker of nicotine absorption from tobacco leaves: a study on tobacco farmers in Malaysia. *J. Occup. Health.* **2003**, *45*, 140–145.

(11) Arcury, T. A.; Quandt, S. A.; Preisser, J. S.; Bernert, J. T.; Norton, D.; Wang, J. High levels of transdermal nicotine exposure produce green tobacco sickness in Latino farmworkers. *Nicotine Tob. Res.* **2003**, *5*, 315–321.

(12) Johnson, G. C. C. Connell, J. F. *Shallow ground-water quality adjacent to burley tobacco fields in Northeastern Tennessee and*

Southwestern Virginia, Spring 1997; US Geological Survey, Water Resources Investigations Report 01-4009; Tennessee, 2001; p 37.
(13) OECD Guideline for testing of chemicals. Acute dermal toxicity. 402. Adopted: 24 Feb **1987**.
(14) Singh, N. P.; McCoy, M. T.; Tice, R. R.; Schneider, E. L. A simple technique for quantification of low levels of DNA damage in individual cells. *Exp. Cell Res.* **1998**, *175*, 184–191.
(15) Da Silva, J.; Freitas, T. R. O.; Marinho, J. R.; Speit, G.; Erdtmann, B. Effects of chronic exposure to coal in wild rodents (*Ctenomys torquatus*) evaluated by multiple methods and tissues. *Mutat. Res.* **2000**, *470*, 39–51.
(16) Tice, R. R. Applications of the single cell gel assay to environmental biomonitoring for genotoxic pollutants. In *Biomonitors and Biomarkers as Indicators of Environmental Change*; Butterworth, B. E., Corkum, L. D., Guzmán-Rincón, J., Eds.; Plenum Press: New York, 1995; pp 69−79.
(17) Nadin, S. B.; Vargas-Roig, L. M.; Ciocca, D. R. A silver staining method for single-cell gel assay. *J. Histochem. Cytochem.* **2001**, *49*, 1183–1186.
(18) Mavournin, K. H.; Blakey, D. H.; Cimino, M. C.; Salamone, M. F.; Hedle, J. A. The in vivo Micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency. Gene-Tox Program. *Mutat. Res.* **1990**, *239*, 29–80.
(19) Misra, H. P.; Fridovich, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* **1972**, *247*, 3170–3175.
(20) Boveris, A.; Chance, B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem. J.* **1973**, *134*, 707–716.
(21) Buege, J. Á.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol.* **1978**, *52*, 302–309.
(22) Vrolijk, N. H.; Targett, N. M.; Woodin, B. R.; Stegeman, J. J. Toxicological and ecological implication of biotransformation enzymes in tropical teleost *Chaetodon capistratus*. *Mar. Biol.* **1994**, *119*, 151–158.
(23) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
(24) Omura, T.; Sato, R. The carbon Monoxide-binding pigment of liver microsomes. I -Evidence for its heme protein nature. *J. Biol. Chem.* **1964**, *239*, 2370–2378.
(25) Omura, T.; Sato, R. The carbon Monoxide-binding pigment of liver microsomes. II − Solubilization, purification and properties. *J. Biol. Chem.* **1964**, *239*, 2379–2385.
(26) Da Silva, F. R.; Da Silva, J.; Dalpiaz, T.; Nunes, E.; Ferraz, A.; Martins, T.; Dias, J. D.; Rosa, D.; Porawskie, M.; Bona, S.; Erdtmann, B. Genotoxicity effect of *Nicotiana tabacum* leaves on *Helix aspersa*. In preparation.
(27) Argentin, G.; Cichetti, R. Genotoxic and antiapoptotic effect of nicotine on human gingival fibroblasts. *Toxicol. Sci.* **2004**, *79*, 75–81.
(28) Arabi, M. Nicotinic infertility: Assessing DNA and plasma membrane integrity of human spermatozoa. *Andrologia* **2004**, *36*, 305–310.
(29) Kleinsasser, N. H.; Sassen, A. W.; Semmler, M. P.; Harréus, U. A.; Licht, A.-K.; Richter, E. The tobacco alkaloid nicotine demosntrates genotoxicity in human tonsillar tissue and lymphocytes. *Toxicol. Sci.* **2005**, *86* (2), 309–317.
(30) Sassen, A. W.; Richter, E.; Semmler, M. P.; Harréus, U. A.; Gamarra, F.; Kleinsasser, N. H. Genotoxicity of nicotine in mini-organ cultures of human upper aerodigestive tract epithelia. *Toxicol. Sci.* **2005**, *88* (1), 134–141.
(31) Kyerematen, G. A.; Owens, G. F.; Chattopadhyay, B.; Bethizy, J. D.; Vesell, E. S. Sexual dimorphism of nicotine metabolism and distribution in the rat: studies in vivo and in vitro. *Drug Metab. Dispos.* **1988**, *16*, 823–828.
(32) Tricker, A. R. Nicotine metabolism, human drug metabolism polymorphisms, and smoking behaviour. *Toxicology* **2003**, *183*, 151–173.
(33) Benowitz, N. L.; Zevin, S.; Jacob, P. Sources of variability in nicotine and cotinine levels with use of nicotine nasal spray, transdermal nicotine, and cigarette smoking. *Br. J. Clin. Pharmacol.* **1997**, *43*, 259–267.
(34) Sipe, R. V., III; Buck, D. C.; Hollinger, J. O. Nicotine administration in rabbits using Habitrol® nicotine patches and nicotine nasal spray. *Clin. Exp. Pharmacol. Physiol.* **2000**, *27*, 480–482.
(35) Sherertz, P. C. Virginia Department of Health: Division of Health Hazards Control. http://www.vdh.virginia.gov/epi/publichealthtoxicology/Prime.pdf (accessed in March 2010), Richmond 804, 1995, 786−1763.
(36) Groff, A.; Da Silva, J.; Nunes, E.; Ianistcki, M.; Guecheva, T.; Oliveira, A.; Oliveira, C.; Val, A.; Henriques, J. UVA/UVB-induced genotoxicity and lesion repair in Colossoma macropomum and Arapaima gigas Amazonian fish. *J. Photochem. Photobiol., B* **2010**, *99* (2), 93–99.
(37) Yildiz, D.; Liu, Y. S.; Ercal, N.; Armstrong, D. W. Comparison of pure nicotine- and smokeless tobacco extract-induced toxicities and oxidative stress. *Arch. Environ. Contam. Toxicol.* **1999**, *37*, 434–439.
(38) Wetscher, G.; Bagchi, M.; Bagchi, D.; Perdikis, G.; Hinder, P.; Glaser, K.; Hinder, R. A. Free Radical Production in Nicotine Treated Pancreatic Tissue. *Free Radical Biol. Med.* **1995**, *18* (5), 877–882.
(39) Maffei, F.; Hrelia, P.; Angelini, S.; Carbone, F.; Forti, G. C.; Barbieri, A.; Sanguinetti, G.; Mattioli, S.; Violante, F. S. Effects of environmental benzene: micronucleus frequencies and haematological values in traffic police working in an urban area. *Mutat. Res.* **2005**, *583*, 1–11.
(40) Omoniyi, I.; Agbon, A. O.; Sodunke, S. A. Effect of lethal and sublethal concentrations of tobacco (*Nicotiana tabaccum*) leaf dust extract on weight and hematological changes in *Clarias gariepinus* (Burchell). *J. Appl. Sci. Environ. Manage.* **2002**, *6*, 37–41.

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