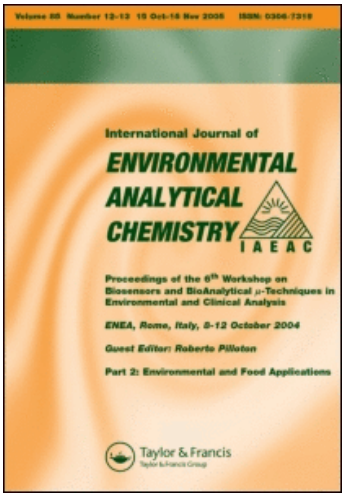

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Evaluation of genetic damage in tobacco-growing farmers occupationally exposed to a mixture of metalaxyl and imidacloprid

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EVALUATION OF GENETIC DAMAGE IN TOBACCO-GROWING FARMERS OCCUPATIONALLY EXPOSED TO A MIXTURE OF METALAXYL AND IMIDACLOPRID

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The use of pesticides, to which humans are exposed, will possibly be increased in the near future. Exposure occurs via inhalation or skin contact during the preparation of the solutions to be sprayed or during spraying. The aim of this study was to evaluate the potent genetic damage in tobacco-growing farmers occupationally exposed to a mixture of metalaxyl and imidacloprid before and after spraying. This evaluation was performed by the micronucleus assay. The results obtained show that there are no statistically significant differences in the micronuclei frequencies of our studied groups before and after spraying. Analysis on the basis of smokers *versus* non-smokers did not show statistically significant differences. Analysis between control and exposed farmers revealed a significant difference in the smoking group after exposure to these pesticides. Further studies are required in order to estimate the potential effects of pesticides.

Keywords: Pesticides; Metalaxyl–Imidacloprid; Micronucleus assay

INTRODUCTION

Pesticides are chemical compounds that are widely used in agriculture, thus invading the environment in great quantities. They represent an important group of environmental pollutants to which people are daily exposed, mainly as a consequence of their wide use, which is likely to continue and possibly increase in the near future [1].

Exposure to pesticides occurs via inhalation or skin contact during the preparation of the solutions to be sprayed or during spraying. Because human exposure does not occur under controlled conditions it is very difficult to estimate the exposure level to chemicals *in vivo*.

In the Aitoloakarnania area of Western Greece, tobacco growing and processing is the main farming activity. Thus farmers are widely exposed to particular pesticides,

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such as metalaxyl and imidacloprid. Their potential genotoxic effect on farmers' health is of concern to the authorities.

Metalaxyl, *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-alaninate, is the active ingredient of the fungicide Ridomil. Since its introduction in 1977, it has been widely used for the control of plant diseases caused by Oomycetous fungi of the order Peronosporales. Examples of use include control of late blight of potatoes, downy mildew on tobacco, hops, vines and red core on strawberries [2].

Imidacloprid, 1-(6-chloro-3-pyridinylmethyl)-*N*-nitroimidazolidin-2-ylideneamine, is a broad-spectrum systemic insecticide, introduced by Bayer as an ingredient of two formulations: Confidor and Gaucho. It acts as an antagonist of acetylcholine and it is effective against sucking insects, soil insects, termites and some species of chewing insects. It is used as seed dressing, soil treatment and foliar treatment in different crops [3].

A great number of tests have been developed to evaluate genetic damage. The micronucleus test in human peripheral blood lymphocytes, first reported by Fenech and Morley [4], has been widely applied in studies of genetic toxicology. Micronuclei (MN) contain acentric chromosome fragments or whole chromosomes and they are recognized as distinct formations that exist in daughter cells separated from the main nucleus [5,6]. They are the result of chromosome breakage and/or chromosome loss due to abnormal chromosome distribution during mitosis. The main characteristic of the test is the use of Cytochalasin-B, an inhibitor of actin polymerization, which prevents cytokinesis while permitting nuclear division [4,7]. As a result, binucleated (BN) cells are produced. These cells are scored for the presence of MN. The frequency of micronuclei in peripheral blood lymphocytes was used as a biomarker of genotoxic effects resulting from occupational exposure to pesticides [8–10].

Cytogenetic studies in human lymphocytes exposed *in vitro* to between 300 and 1000 μg/mL metalaxyl in the absence of microsomal activation showed a significant induction of chromosomal aberrations and in the presence of S9 mix produced less cytogenetic damage. The micronuclei test may not by itself comprise direct evidence of cytotoxicity although it has been applied to the bone marrow of treated animals [11].

The transforming properties of metalaxyl were analyzed by the *in vitro* BALB/c 3T3 cell transformation test. Metalaxyl induced cell transformation at any assayed dosage, i.e. 500, 250 and 50 μg/mL in the presence of bioactivation and at the highest dosage (500 μg/mL) in the absence of bioactivation [12].

Several dependent reactions have been studied in Swiss Albino CD1 mice liver, kidney and lung microsomes treated intra-peritoneal with single [200 or 400 mg/kg of body weight (b.w.)] or repeated (200 mg/kg b.w. for three days) administrations of metalaxyl. The data seem to indicate the cotoxic and cocarcinogenic potential of metalaxyl [13].

The study of the resulting adducts on DNA, due to the action of chemicals, is a genotoxicity test. Shah *et al.* [14] observed an increase of adduct number on calf thymus DNA, owing to its reaction with enzymatically obtained imidacloprid metabolites, using an *in vitro* system.

The acute and chronic oral toxicity of imidacloprid was investigated in *Apis mellifera*. Acute toxicity (LD_{50}) values of imidacloprid were about 600 μg/kg at 72 and 96 h. In the chronic toxicity test, imidacloprid was toxic at doses 60–6000 times lower than those required to produce the same effects as in acute intoxication studies [15].

According to regulations established by EPA (40 CFR Part 180) imidacloprid has been classified as a classes II and III agent presenting low toxicity to mammals as they have resistant nicotinic receptor subtypes. When absorbed in the organism it is eliminated via urine and feces through its main metabolite 6-chloronicotinic acid, a compound that acts on the nervous system, which may be conjugated with glycine or reduced to guanidine [16]. The acute imidacloprid toxicity reported [17] appears to be an adverse effect of the solvent *N*-methyl pyrrolidone used and not of imidacloprid *per se*.

The aim of the present study was to evaluate the potential genetic damage in tobacco-growing farmers occupationally exposed to a mixture of metalaxyl and imidacloprid. This evaluation was performed by the use of the cytokinesis block micronucleus assay of peripheral blood lymphocytes *in vivo*.

EXPERIMENTAL

Healthy adults and age-matched healthy farmers were selected for the present study. All subjects were divided into smokers and non-smokers. According to information supplied by farmers, they did not receive any medical treatment and had no obvious health problems for the last two months before spraying. One half of the farmers (smokers and non-smokers) used gloves and masks during spraying while the other half did not. All farmers sprayed several acres, two or three times over a period of three months. The duration of their spraying varied between 4 and 6 h.

Blood samples were obtained under sterile conditions from control subjects and from farmers before and after a whole day's spraying in the field.

Lymphocyte Cultures

0.5 mL of whole blood obtained from each healthy adult subject was added to 8.2 mL of culture medium. The culture medium was made of Ham's F-10 growth medium (Gibco, NY), supplemented with 2% foetal calf serum (Gibco, NY). Phytohaemagglutinin (Gibco, NY) was added to culture medium to promote lymphocyte proliferation. Cultures were incubated at 37°C for 72 h.

Micronucleus Induction

For studying MN induction, 6 μg/mL Cytochalasin-B (Sigma) was added to cultures 44 h after their initiation. Cells were harvested by centrifugation at 1500 rpm for 10 min, 72 h post culture initiation. A mild hypotonic treatment with a 1 : 3 solution of Ham's medium and double-distilled H_2O supplemented with 2% serum was given for 3 min at room temperature followed by a 10 min fixation with fresh 5 : 1 solution of methanol–acetic acid. Finally, cells were stained with 7% Giemsa. For calculating the MN frequencies, at least 1000 BN cells were scored for each donor and for each case. Standard criteria [5,6] were used for scoring MN. 2000 cells were counted for calculation of the Cytokinesis Block Proliferation Index (CBPI), which is given by the equation: $\mathrm{CBPI} = [M_1 + 2M_2 + 3(M_3 + M_4)]/N$, to determine possible cytotoxic effects [18]. M_1, M_2, M_3 and M_4 correspond to the number of cells with one, two, three and four nuclei and N is the total number of cells. Micronucleus size is expressed

as the ratio of micronucleus diameter to the cell nucleus diameter. This ratio was determined as follows: small micronuclei $\leq 1/10$; medium micronuclei $1/9 < MN < 1/3$; large micronuclei $\approx 1/3$, of nuclear diameter [19].

Comparison between the studied groups was performed by the one-way ANOVA test. The statistical software used for data analysis was Origin 7.0 (OriginLab Corporation, Northampton, USA).

RESULTS AND DISCUSSION

In the central area of Western Greece the main agricultural activity is tobacco growing and processing. During the year round farmers are exposed for various periods to pesticides. Occasionally, the hospitals of the area treat farmers with intoxication problems. It is well established that pesticides represent an important group of environmental pollutants. As such, intoxication is the least someone may suffer as a result of exposure to pesticides. A number of pesticides have been accused of having possible genotoxic effect on humans. The majority of tests carried out to study the possible effects of various pesticides have been performed either on experimental animals or on blood samples from healthy donors. The number of tests performed on farmers occupationally exposed to pesticides is restricted. We aimed to study the possible genotoxic effects of metalaxyl and imidacloprid on farmers exposed to them during the period they use them for protection of their crops. The characteristics of the studied groups are depicted in Table I.

Blood samples taken from healthy adults were analyzed for spontaneous micronuclei induction. MN induction and calculation of the Cytokinesis Block Proliferation Index (CBPI) were examined under standard criteria, in lymphocyte cultures. Table II presents the data of micronucleated binucleated cells (BNMN) and MN spontaneously induced in the healthy controls grouped into smokers and non-smokers. The values for BNMN cells and MN per 1000 BN cells, for each donor is presented. The smokers' BNMN frequencies were calculated as 8.67 ± 1.31, while the MN frequencies were calculated as 9.83 ± 1.40. Meanwhile, the corresponding values for non-smokers were 10.00 ± 1.64 and 10.40 ± 1.89, respectively. Data analysis revealed no statistically significant differences in the BNMN induction ($F = 0.41$, $p = 0.54$) as well as in the MN induction ($F = 0.06$, $p = 0.81$) in our control groups. CBPI was calculated to detect possible variations in the proliferative kinetics of lymphocytes due to pesticide exposure. Analysis of the observed CBPI between smokers (1.66 ± 0.13) and non-smokers (1.78 ± 0.06) in control subjects revealed no statistically significant differences ($F = 0.64$, $p = 0.45$). The present data are in accordance with those reported recently

TABLE I Characteristics of the studied groups

	Control	*Exposed*	
No. subjects	11	11	
Age (years)[a]	38.18 ± 4.05	43.82 ± 3.76	$F = 1.04$, $p = 0.32$*
Range	22–58	23–60	
Years of exposure	–	23.64 ± 4.13	
Smoking habit			
No. non-smokers	5	5	
No. smokers	6	6	
Cigarettes/day[a]	27.5 ± 5.44	40 ± 8.94	$F = 1.43$, $p = 0.26$*

[a]Mean ± S.E; *$p > 0.05$.

TABLE II Frequencies of micronucleated binucleated cells (BNMN) and micronuclei (MN), as revealed after Giemsa staining, in control human lymphocyte cultures

	Donors	*BNMN*	*MN*	*CBPI*
Smokers (A)	1	14	15	1.87
	2	9	11	1.47
	3	5	7	1.22
	4	6	6	1.70
	5	8	8	2.11
	6	10	12	1.57
Total – A		**52**	**59**	–
Non-smokers (B)	7	14	15	1.67
	8	14	15	1.75
	9	7	7	1.99
	10	7	7	1.70
	11	8	8	1.77
Total – B		**50**	**52**	–
Total – A + B		**102**[a]	**111**[a]	–

BNMN: micronucleated binucleated cells; MN: micronuclei; CBPI: Cytokinesis Block Proliferation Index. [a]11 000 binucleated cells (BN) cells were counted in total for each studied group.

TABLE III Frequencies of micronucleated binucleated cells (BNMN) and micronuclei (MN), as revealed after Giemsa staining, in farmers' lymphocyte cultures before and after spraying

	Donors	*BNMN*		*MN*		*CBPI*	
		Before	*After*	*Before*	*After*	*Before*	*After*
Smokers (A)	1	11	16	12	16	1.82	2.17
	2	20	13	23	13	1.99	2.02
	3	7	15	7	16	1.76	2.12
	4	13	34	14	36	2.05	2.00
	5	8	18	8	18	2.07	2.12
	6	21	15	21	19	2.11	1.92
Total – A		**80**	**111**	**85**	**118**	–	–
Non-smokers (B)	7	5	9	7	10	1.65	1.41
	8	19	23	21	24	2.25	1.56
	9	14	16	14	17	1.28	1.92
	10	12	13	12	14	1.65	2.00
	11	11	15	13	15	1.70	2.05
Total – B		**61**	**76**	**67**	**80**	–	–
Total – A + B		**141**[a]	**187**[a]	**152**[a]	**198**[a]	–	–

BNMN: micronucleated binucleated cells; MN: micronuclei; CBPI: Cytokinesis Block Proliferation Index. [a]11 000 binucleated cells (BN) cells were counted in total for each studied group.

by the HUMN collaborative group studying the effect of smoking on micronuclei frequencies in human lymphocytes [20].

Blood samples were also taken under sterile conditions from healthy farmers prior to and after spraying. Table III depicts the data of BNMN and MN induction in lymphocyte cultures of smoking and non-smoking farmers before and after spraying. The values for BNMN cells and MN per 1000 BN cells for each donor and for each case are presented. A slight increase in the BNMN and MN frequencies after spraying was observed.

An inconsistency observed for the second donor could easily be attributed to the variable spraying conditions applied, i.e. overall sprayed area and use of protective devices. Data analysis revealed no statistically significant differences in the MN induction ($F=2.39$, $p=0.14$) before (13.82 ± 1.71) and after (18.00 ± 2.10) spraying as well as in the BNMN induction ($F=2.67$, $p=0.18$) before (12.82 ± 1.61) and after (17.00 ± 1.99) spraying.

In agreement with our results, recent studies on micronucleus frequencies in Spanish and Hungarian farmers, occupationally exposed to a complex mixture of pesticides, reported no significant differences between the high-exposure period (spring–summer) and the low-exposure period (autumn–winter), or between farmer moderately exposed and highly exposed to pesticides [9,10].

CBPI analysis revealed no cytotoxic effect from the studied pesticides in farmers (Table III). Reduced CBPI is an indication of a possible genotoxic effect to pesticide exposure. Analysis of the observed CBPI indicates that there is no effect due to pesticide exposure, 1.85 ± 0.08 before and 1.94 ± 0.07 after spraying ($F=0.62$, $p=0.44$). However, differences observed among the studied groups could be the result of variable exposure levels during spraying (Table III).

To elucidate the contribution of smoking to pesticide effects on MN frequencies, the farmers were grouped into smokers (Table III, A) and non-smokers (Table III, B). No statistically significant differences were observed for smokers in the MN induction ($F=1.62$, $p=0.23$) before (14.17 ± 2.70) and after (19.67 ± 3.37) spraying, as well as in the BNMN induction ($F=1.67$, $p=0.23$) before (13.33 ± 2.43) and after (18.50 ± 3.17) spraying. Similar results were obtained for the non-smokers for both MN induction ($F=0.65$, $p=0.44$) before (13.40 ± 2.25) and after (16.00 ± 2.30) spraying and BNMN induction ($F=0.87$, $p=0.38$) before (12.20 ± 2.27) and after (15.20 ± 2.29) spraying. Comparing the mean frequencies between smokers and non-smokers we observed no statistically significant differences in the induction of MN and BNMN before and after spraying (Table IV). The effect of smoking on MN frequency remains a controversial issue. The present results are in accordance with earlier studies that reported no significant correlation between MN frequency and smoking habit [20–22].

Table V presents data on the size ratio of MN. To distinguish between the clastogenic and the aneugenic activity due to exposure to the particular pesticides we compared the MN sizes calculated. MN of small size have a great possibility of containing acentric chromosome fragments, indicating a clastogenic effect. On the other hand MN of large size may possibly contain whole chromosomes, indicating an aneugenic effect [23–25].

TABLE IV Mean frequencies (‰) ± standard error of micronucleated binucleated cells (BNMN) and micronuclei (MN), as revealed after Giemsa staining, in farmers' lymphocyte cultures from smokers and non-smokers before and after spraying

	BNMN		*MN*	
	Before	*After*	*Before*	*After*
Smokers	13.33 ± 2.43	18.50 ± 3.17	14.17 ± 2.70	19.67 ± 3.37
Non-smokers	12.20 ± 2.27	15.20 ± 2.29	13.40 ± 2.25	16.00 ± 2.30
	$F=0.11$, $p=0.74$	$F=0.66$, $p=0.44$	$F=0.05$, $p=0.84$	$F=0.74$, $p=0.41$

BNMN: micronucleated binucleated cells; MN: micronuclei.

TABLE V Frequencies of micronuclei (MN) per size, as revealed after Giemsa staining, in farmers' lymphocyte cultures before and after spraying

	Donors	*Small MN*		*Medium MN*		*Large MN*	
		Before	*After*	*Before*	*After*	*Before*	*After*
Smokers (A)	1	4	8	6	4	2	4
	2	18	9	3	3	2	1
	3	4	6	1	10	2	0
	4	4	13	6	18	4	5
	5	5	8	2	7	1	3
	6	10	8	10	7	1	4
Total – A		**45**	**52**	**28**	**49**	**12**	**17**
Non-smokers (B)	7	5	3	2	5	0	2
	8	7	16	8	8	6	0
	9	6	4	5	6	3	7
	10	5	3	3	5	4	6
	11	7	6	4	6	2	3
Total – B		**30**	**32**	**22**	**30**	**15**	**18**
Total – A + B		**75**[a]	**84**[a]	**50**[a]	**79**[a]	**27**[a]	**35**[a]

MN: micronuclei. [a]11 000 binucleated cells (BN) cells were totally counted in each studied group.

TABLE VI Mean frequencies (‰) ± standard error of micronuclei (MN) per size, as revealed after Giemsa staining, in farmers' lymphocyte cultures from smokers and non-smokers before and after spraying

	Small MN		*Medium MN*		*Large MN*	
	Before	*After*	*Before*	*After*	*Before*	*After*
Smokers	7.50 ± 2.31	8.67 ± 0.95	4.67 ± 1.36	8.17 ± 2.21	2.00 ± 0.45	2.83 ± 0.79
Non-smokers	6.00 ± 0.45	6.40 ± 2.46	4.40 ± 1.03	6.00 ± 0.55	3.00 ± 1.00	3.60 ± 1.29
	$F = 0.34$	$F = 0.85$	$F = 0.02$	$F = 0.75$	$F = 0.94$	$F = 0.28$
	$p = 0.58$	$p = 0.38$	$p = 0.88$	$p = 0.41$	$p = 0.36$	$p = 0.61$

MN: micronuclei.

A slight increase of total values in small (75–84), medium (50–79) and large (27–35) size MN was observed after spraying. No statistically significant differences on the size ratio of small MN ($F = 0.22$, $p = 0.64$), medium MN ($F = 3.15$, $p = 0.09$) and large MN ($F = 0.71$, $p = 0.41$) before and after spraying were found. Similar results on the size ratio of MN were detected in smokers (Table V, A) and non-smokers (Table V, B).

In the group of smokers, a slight increase of total values in small (45–52), medium (28–49) and large (12–17) size MN was observed after spraying. No statistically significant differences on the size ratio of small MN ($F = 0.22$, $p = 0.65$), medium MN ($F = 1.82$, $p = 0.21$) and large MN ($F = 0.84$, $p = 0.38$) before and after spraying were calculated. Similarly in the non-smokers a slight increase of total values in small (30–32), medium (22–30) and large (15–18) size MN was observed after spraying. Again no statistically significant differences on the size ratio of small MN ($F = 0.02$, $p = 0.88$), medium MN ($F = 1.88$, $p = 0.21$) and large MN ($F = 0.14$, $p = 0.72$) before and after spraying were calculated.

A comparison of the mean frequencies between smokers and non-smokers reveals that there are no statistically significant differences on the size ratio in small, medium and large MN before and after spraying (Table VI). The reported data on the size ratio of MN would indicate a lack of possible clastogenic and/or aneugenic

TABLE VII A comparison of micronucleated binucleated cells (BNMN) and micronuclei (MN) mean frequencies (‰) ± standard error, as revealed after Giemsa staining, in control and exposed (before and after spraying) human lymphocyte cultures from smokers and non-smokers

	BNMN	*MN*
Control/smokers	8.67 ± 1.31	9.83 ± 1.40
Exposed/smokers (before)*	13.33 ± 2.43	14.17 ± 2.70
Exposed/smokers (after)**	18.50 ± 3.17	19.67 ± 3.37
	$*F = 2.86$, $p = 0.12$	$*F = 2.03$, $p = 0.18$
	$**F = 8.22$, $p = 0.02$[a]	$**F = 7.25$, $p = 0.02$[a]
Control/non-smokers	10.00 ± 1.64	10.40 ± 1.89
Exposed/non-smokers (before)*	12.20 ± 2.27	13.40 ± 2.25
Exposed/non-smokers (after)**	15.20 ± 2.29	16.00 ± 2.30
	$*F = 0.29$, $p = 0.60$	$*F = 1.04$, $p = 0.34$
	$**F = 3.40$, $p = 0.10$	$**F = 3.54$, $p = 0.10$
Total – control	9.27 ± 1.00	10.09 ± 1.89
Total – exposed (before)*	12.82 ± 1.61	13.82 ± 1.71
Total – exposed (after)**	17.00 ± 1.99	18.00 ± 2.10
	$*F = 3.51$, $p = 0.08$	$*F = 3.38$, $p = 0.08$
	$**F = 12.03$, $p = 0.002$[b]	$** F = 11.19$, $p = 0.003$[b]

BNMN: micronucleated binucleated cells; MN: micronuclei; CBPI: Cytokinesis Block Proliferation Index. [a]$p < 0.05$; [b]$p < 0.01$.

effect associated with the exposure of the studied farmers to metalaxyl and imidacloprid.

Table VII presents statistical analysis results from our studied groups. Analysis was carried out between control and exposed (before and after spraying) smokers as well as non-smokers and of total control and exposed (before and after spraying) subjects. The analysis revealed that among smokers there is a statistically significant difference after spraying in BNMN induction ($F = 8.22$, $p = 0.02$) as well as in MN induction ($F = 7.25$, $p = 0.02$). In the non-smoking group the analysis did not reveal statistically significant differences. In the overall group analysis we observed statistically significant differences in BNMN induction ($F = 12.03$, $p = 0.002$) as well as in MN induction ($F = 11.19$, $p = 0.003$) after spraying.

CONCLUSIONS

The main results of our study could be summarized as follows:

- Metalaxyl and imidacloprid are capable of inducing increased MN frequencies in farmers after spraying.
- A comparison of MN frequencies in farmers before and after spraying did not reveal statistically significant differences.
- Regarding CBPI, no statistically significant differences were observed on farmers before and after spraying.
- Data on MN size ratio indicate a lack of possible clastogenic and/or aneugenic effect associated to metalaxyl and imidacloprid exposure.
- A comparison of MN frequencies on control and exposed (after spraying) smokers revealed statistically significant differences.

- A comparison of MN frequencies on total control subjects and exposed (after spraying) farmers revealed statistically significant differences.

These results revealed no cytotoxic effect of metalaxyl and imidacloprid. It should be noted that it is difficult to calculate the dose received by farmers during spraying. This dose is naturally related to the sprayed area and the particular precautions taken by individual farmers. It is quite possible that the doses to which the farmers were exposed were below the toxicological dose calculated by EPA for human risk assessment. Further studies have to be carried out in order to elucidate possible effects of these pesticides on farmers' health.

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