Mutagenesis vol. 21 no. 5 pp. 343–350, 2006
Advance Access Publication 15 September 2006
doi:10.1093/mutage/gel039

Cytogenetic and molecular biomonitoring of a Portuguese population exposed to pesticides

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Widespread use of pesticides in agriculture represents a threat not only to the environment but also to human populations exposed to them. Many of these compounds are capable of inducing mutations in DNA and lead to several diseases including cancer. In the present study, cytogenetic damage in peripheral lymphocytes from 33 farmers of Oporto district (Portugal) exposed to pesticides was evaluated by means of micronuclei (MN), sister chromatid exchange (SCE) and chromosomal aberrations (CA). In addition, effect of polymorphic genes of xenobiotic metabolizing enzymes (GSTM1, GSTT1, GSTP1, CYP2E1 and EPHX1) was also evaluated. A non-exposed group from the same area and with same demographic characteristics without exposure to genotoxic compounds was studied and data obtained from both groups was compared. MN and SCE frequencies were significantly higher in the exposed group ($P < 0.005$). In what concerns CA results, no significant differences were observed. It was possible to relate a specific working environment (greenhouses) with higher levels of genetic damage. Use of personal protective equipment revealed to be important to prevent exposure and diminish genetic damage inflicted by pesticides. Allele frequencies of studied polymorphic genes obtained in this study are similar to the ones described by other authors for Caucasian populations. Despite the low number of subjects, results suggest that low mEH (microsomal epoxide hydrolase) activity as well as GSTT1 positive genotype are associated with increased cytogenetic damage.

Introduction

Pesticides are among the most widely used chemicals throughout the world. They include a great variety of substances different both in composition and properties with the purpose to kill, destroy or repel undesirable living organisms. In Portugal these products have a higher use (3.74 kg/ha) than in the average European countries (2.10 kg/ha) (1). Pesticides are responsible for several adverse effects in human health other than acute intoxications. Effects in immune, nervous, endocrine and reproductive systems have been reported in many studies (2–4). DNA damage has also been related to pesticide exposure (5–10). These compounds can interact with DNA leading to abortions (11), degenerative diseases (12–14) and ultimately to cancer (15) in the absence of reparation processes.

Many of these compounds are classified by International Agency for Research on Cancer (IARC) as carcinogenic (16). Therefore it is important to evaluate occupational exposure to these products. This is particularly relevant as it is recognized that occupational exposure together with other environmental factors (tobacco smoke, contaminants in air, water and food, radiation, dietary constituents and infectious agents) are key contributors to human cancer (80–90% of cases) (17).

Human biological monitoring is a tool of great interest in cancer risk assessment once it allows estimating genetic risk deriving from environmental exposure to chemicals (18). Biomarkers such as micronucleus test (MN), sister chromatid exchange (SCE) and chromosomal aberrations (CA) provide information about DNA damage. The role of MN as an intermediate endpoint of carcinogenesis has received much support in the literature, but stronger evidence is available concerning the association between rate of structural CA and cancer risk (19).

Although difficult to establish a connection between pesticide exposure and cancer prevalence, especially because of the high number of involved compounds, some authors evidence a greater prevalence of certain types of cancer in pesticide exposed populations (15). According to Buckley *et al*. (20) and Meinert *et al*. (21) leukaemia and non-Hodgkin lymphomas incidence is higher in individuals exposed to pesticides. Conclusions of genotoxic damage studies are conflicting. Some indicate a significant increase in MN, SCE and CA frequencies (8,22–24) while others do not show significant differences (25–27).

Recent findings suggest that inherited differences in metabolic capacity may modify individual responses to genotoxic compounds and thereby susceptibility to cancer risk. Genetic polymorphisms exist in some of phase I (activating) and phase II (inactivating) enzymes. Phase I enzyme cytochrome P450 2E1 (CYP2E1) is involved in the metabolism of many indirect carcinogens. A number of environmental factors, including pesticides, may modify cancer risk through altered CYP2E1 enzyme activity (28). Among phase II enzymes, glutathione *S*-transferases (GST) are the most important group of detoxifying enzymes followed by microsomal epoxide hydrolase (mEH). The former presents in human populations genetic polymorphisms responsible for the glutathione conjugation with various reactive species of many chemicals including pesticides. Null genotypes for *GSTT1* and *GSTM1* genes as well as ^{105}Val homozygotes for *GSTP1* have been identified to be associated with an increase of cancer risk (28,29).

mEH is involved in the metabolism of toxic, highly reactive intermediates formed by cytochrome P450 mediated reactions

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to less toxic metabolites (30). Polymorphic sites identified within *mEH* gene result in variation of amine acid residues 113 (Tyr/His) and 139 (Arg/His). These genetic polymorphisms may be in part responsible for observed inter-individual variations in mEH activity (31). Clinical studies have demonstrated associations between low mEH enzyme activity and adverse drug responses or conditions of illness (30).

The aim of this study was to evaluate potential genotoxic damage in individuals occupationally exposed to pesticides by means of MN, CA and SCE. We also intended to determine if different forms in genes involved in metabolic processes can affect prevalence of genetic damage in cells. Influence of the personal protective equipment (PPE) usage behaviours and of practices that may enhance exposure were also considered.

Material and methods

Study population and sample collection

The study population consisted of 33 workers exposed to pesticides (17 men and 16 women) in agricultural explorations and greenhouses located in Oporto district, Portugal. The control group comprised 33 non-exposed control employees working mainly in administrative offices in the same area matched by age, sex, lifestyle and smoking habits. Each subject signed an informed consent and filled a detailed questionnaire to determine confounding factors and none of them was exposed to any known mutagen (e.g. X-rays) during 6 months prior to the venipuncture. Subjects of the exposed group also gave information concerning work practices such as usage of PPE and years of employment. Characteristics of the studied groups are presented in Table I.

With respect to work environment, 18 of the exposed individuals worked exclusively in open-field, 8 exclusively in greenhouses and 7 split their time equally between both spaces. In addition, 8 of the 33 individuals of the exposed group did not apply pesticides (non-applicators) and only one had previously applied them (no longer an applicator for the past 12 years). Six of the non-applicators worked exclusively in open-field, one exclusively in greenhouses and one in both environments. Analysis of questionnaires revealed that only 32% of the applicators wore gloves during all pesticide applications. Mask, appropriate clothes and goggles were used even less often. None of them wore protective equipment at all times.

Venous heparinized blood samples (5 ml) were collected from each donor in a ~4 month period (March to June). Most exposed individuals were in contact with pesticides few days before sample collection. All samples were coded and analysed under blind conditions. Blood cultures for cytogenetic analysis were set up few hours after sampling. For genotype analysis blood samples of all subjects were stored at −20°C until use.

Micronucleus analysis

Aliquots of 0.5 ml of heparinized whole blood were cultured and treated as described elsewhere (32). To determine the total number of MN in binucleated cells, a total of 1000 binucleated cells with well preserved cytoplasm (500 per replicate) were scored for each subject. MN were scored blindly by the same reader and identified according to the criteria of Caria *et al*. (33) using ×500 magnification.

Chromosomal aberrations and aneuploidies

For chromosomal aberrations assay, 0.5 ml aliquots of whole blood were cultured as described in Roma-Torres *et al*. (34). One hundred metaphases (50 per replicate) were scored for each individual. Cells with 46 chromosomes were scored for structural chromosome type aberrations that included chromatid and chromosome breaks and gaps, dicentric and ring chromosomes. Aneuploidies (An)–cells with 45 and 47 chromosomes–were also scored. All alterations were scored blindly by the same reader using ×1250 magnification.

Sister chromatid exchange

Heparinized venous blood samples were treated as described by Teixeira *et al*. (32). In order to score SCE, 50 metaphases were observed for each subject (25 per replicate). Each point of discontinuous staining was enumerated as an exchange. Slides were scored blindly by the same reader using ×500 magnification. The procedure proposed by Carrano and Moore (35) was used to identify cells with high frequency of SCE (HFC), based on a threshold level corresponding to the 95th percentile of the pooled distribution of the number of SCE per cell in a reference population. Cells were classified as HFC if their number of SCE was above the threshold level.

Genotype analysis

Genomic DNA was obtained from 250 µl of whole blood using a commercially available kit according to the manufacturer instructions (QIAamp DNA extraction kit; Qiagen, Hilden, Germany). Each DNA sample was stored at −20°C until analysis. *CYP2E1, GSTT1. GSTP1, GSTM1* and *EPHX1* polymorphisms were all determined as described in Teixeira *et al*. (32).

All genotype determinations were carried out twice in independent experiments and inconclusive samples were reanalysed.

In what concerns *EPHX1* genotypes once the individuals were classified for codon 113 and 139 polymorphisms they were included in one of the following groups according to their expected enzymatic activity (36):

Low activity: His/His–His/His; His/His–His/Arg; Tyr/His–His/His; His/His–Arg/Arg;

Medium activity: Tyr/Tyr–His/His; Tyr/His–His/Arg; Tyr/His–Arg/Arg;

High activity: Tyr/Tyr–Arg/Arg; Tyr/Tyr–His/Arg.

Statistical analysis

Distribution of every variable obtained in this work was compared with the normal distribution (Kolmogorov–Smirnov goodness-of-fit test). Since they all departed significantly from normality, they were normalized by means of square root transformation. Analysis of variance (ANOVA), followed by Bonferroni's correction for multiple comparisons among groups when the overall *F*-test was significant, was used to detect significant differences among exposure groups, and the contribution of lifestyle factors and genotype characteristics to the analysed biomarkers. Then, the interactions among factors and covariates were evaluated by means of the multifactor analysis of variance, by introducing into the model only variables that showed significant differences in the previous univariate model. For GSTP1 polymorphisms, the homozygous and heterozygous carriers of the variant alleles were combined in the statistical analysis, due to the low number of variant homozygotes. The associations between two variables were analysed by Pearson's correlation. All analyses were conducted using the SPSS for Windows statistical package, version 11.0.0.

Table I. Characteristics of the study population

	Control group	Exposed group
No. of subjects	33	33
Age (in years) [mean ± SD; (range)]	41 ± 9; (22–56)	43 ± 10; (24–77)
Gender	17 males; 16 females	17 males; 16 females
Years of employment [mean ± SD; (range)]		15 ± 13; (0.5–48)
Smokers *n*(%)	11 (33)	10 (30)
Non-smokers *n*(%)	22 (67)	23 (70)

Results

Features of control and exposed groups are shown in Table I. Groups are similar regarding age and smoking habits. Duration of exposure in exposed group ranged from 0.5 to 48 years.

The effect of exposure status on analysed cytogenetic biomarkers is shown in Table II. CA include breaks and gaps all together. Two quadriradial configurations and three dicentric chromosomes were found in the exposed group and were scored as representing two breaks each (37). Both SCE and MN frequencies were significantly higher ($P < 0.005$) among farmers compared with control group. No significant differences were observed for CA, Breaks and An frequencies. The percentage of high frequency cells (HFC) was also determined in both groups and no significant difference was observed.

In order to highlight possible gender-related differences, data are expressed separately for males and females in Table III. The effect of gender was only observed in MN frequency within control group, with females presenting a significant increase ($P < 0.005$) when compared with males. No significant differences were observed for other cytogenetic tests.

Table II. MN, SCE, HFC, CA, Breaks and An frequencies in control and exposed groups

Group	MN (‰)	No.	SCE (per cell)	No.	HFC (%)	No.	CA (%)	No.	Breaks (%)	No.	An (%)	No.
Control [mean ± SE (range)]	3.27 ± 0.37 (0–9)	33	4.33 ± 0.19 (2.52–6.40)	29	18.9	29	2.90 ± 0.40 (0–6)	21	1.71± 0.30 (0–5)	21	2.38 ± 0.36 (0–6)	21
Exposed [mean ± SE (range)]	9.03 ± 1.04* (0–27)	33	5.19 ± 0.20* (3.26–7.71)	25	19.2	25	2.52 ± 0.34 (0–7)	31	1.68 ± 0.34 (0–6)	31	2.58 ± 0.37 (0–8)	31

*Significantly different from control group ($P < 0.005$).

Table III. Effect of gender on frequencies of analysed biomarkers

Biomarkers	Control group [mean ± SE (range)]				Exposed group [mean ± SE (range)]			
	Males	No.	Females	No.	Males	No.	Females	No.
MN (‰)	2.12 ± 0.19 (0–3)	17	4.50 ± 0.61* (0–9)	16	7.29 ± 1.07 (2–17)	17	10.88 ± 1.73 (0–27)	16
SCE (per cell)	4.65 ± 0.27 (3.01–6.40)	17	3.87 ± 0.17 (2.52–5.10)	12	4.93 ± 0.35 (3.26–7.71)	12	5.43 ± 0.20 (4.40–6.66)	13
CA (%)	3.38 ± 0.45 (2–6)	13	2.13 ± 0.72 (0–6)	8	2.63 ± 0.52 (0–7)	16	2.40 ± 0.45 (0–6)	15
Breaks (%)	1.85 ± 0.36 (0–4)	13	1.50 ± 0.57 (0–5)	8	2.00 ± 0.51 (0–6)	16	1.33 ± 0.45 (0–6)	15
An (%)	2.62 ± 0.49 (0–6)	13	2.00 ± 0.53 (0–4)	8	3.13 ± 0.55 (0–8)	16	2.00 ± 0.46 (0–6)	15

*Significantly different from males of control group ($P < 0.005$).

Table IV. Influence of smoking habits in studied biomarkers

Biomarkers	Control group [mean ± SE (range)]				Exposed group [mean ± SE (range)]			
	Smokers	No.	Non-smokers	No.	Smokers	No.	Non-smokers	No.
MN (‰)	2.64 ± 0.43 (0–5)	11	3.59 ± 0.51 (0–9)	22	6.50 ± 1.17 (2–15)	10	10.13 ± 1.35 (0–27)	23
SCE (per cell)	4.57 ± 0.30 (3.12–6.05)	10	4.20 ± 0.24 (2.52–6.40)	19	5.46 ± 0.63 (4.40–7.71)	5	5.12 ± 0.20 (3.26–6.66)	20
CA (%)	3.43 ± 0.69 (2–6)	7	2.64 ± 0.50 (0–6)	14	3.67 ± 0.76* (0–7)	9	2.05 ± 0.32 (0–5)	22
Breaks (%)	2.00 ± 0.53 (1–4)	7	1.57 ± 0.37 (0-5)	14	3.22 ± 0.72** (0–6)	9	1.05 ± 0.30 (0–5)	22
An (%)	3.14 ± 0.55 (1–6)	7	2.00 ± 0.44 (0–5)	14	4.00 ± 0.80* (1–8)	9	2.00 ± 0.35 (0–6)	22

*Significantly different from non-smokers of the exposed group ($P < 0.05$).
**Significantly different from non-smokers of the exposed group ($P < 0.01$).

Table V. Frequencies of analysed biomarkers according to the type of exposure

Biomarkers	Exposed group [mean ± SE (range)]			
	Applicators	No.	Non-applicators	No.
MN (‰)	8.96 ± 1.33 (0–27)	25	9.25 ± 1.18 (4–15)	8
SCE (per cell)	5.16 ± 0.24 (3.26–7.71)	20	5.31 ± 0.34 (4.42–6.04)	5
CA (%)	2.13 ± 0.33 (0–5)	24	3.86 ± 0.83 (1–7)	7
Breaks (%)	1.38 ± 0.31 (0–5)	24	2.71 ± 1.06 (0–6)	7
An (%)	2.67 ± 0.38 (0–7)	24	2.29 ± 1.06 (0–8)	7

Table IV summarizes mean data of cytogenetic variables studied stratified by smoking habits. When smokers were split from non-smokers, smokers presented elevated frequencies in SCE, CA, An and Breaks as compared to non-smokers in both control and exposed group, but statistical significance was only obtained for CA, An ($P < 0.05$) and Breaks ($P < 0.01$) within the exposed group.

Possible differences between applicators and non-applicators were studied (Table V) and no significant differences were observed. Nevertheless it is worthy of note that for all studied endpoints (except for An) non-applicators presented a slight increase in frequencies when compared with applicators.

When the working environment was considered, a statistically significant increase ($P < 0.05$) in MN frequency (Figure 1a) was observed in farmers that worked exclusively in greenhouses when compared with those who worked exclusively in open-field. An increase in SCE frequency of greenhouse workers was also observed but did not reach significance (Figure 1b). No differences were observed in CA, Breaks and An frequencies.

Due to the absence of most PPE during work activities only the influence of gloves utilization was studied. Although none of the studied endpoints showed significant differences among workers who did not wear gloves when compared with the ones who wore this type of protection (data not shown), MN frequencies were higher in the former group (9.36 ± 1.30) when compared with the latter (8.00 ± 1.35).

Within this study a significant correlation was found between age and MN frequency ($P < 0.01$) as well as between age and An frequency ($P < 0.01$) in the whole population. When only the exposed population was considered a significant correlation was found between age and An frequency and also between An frequency and years of employment ($P < 0.05$).

Results of genotype analysis for control and exposed groups as well for the whole population are presented in Table VI. The distribution of *CYP2E1, EPHX1* and *GSTP1* genotypes was in Hardy–Weinberg equilibrium (χ^2-test).

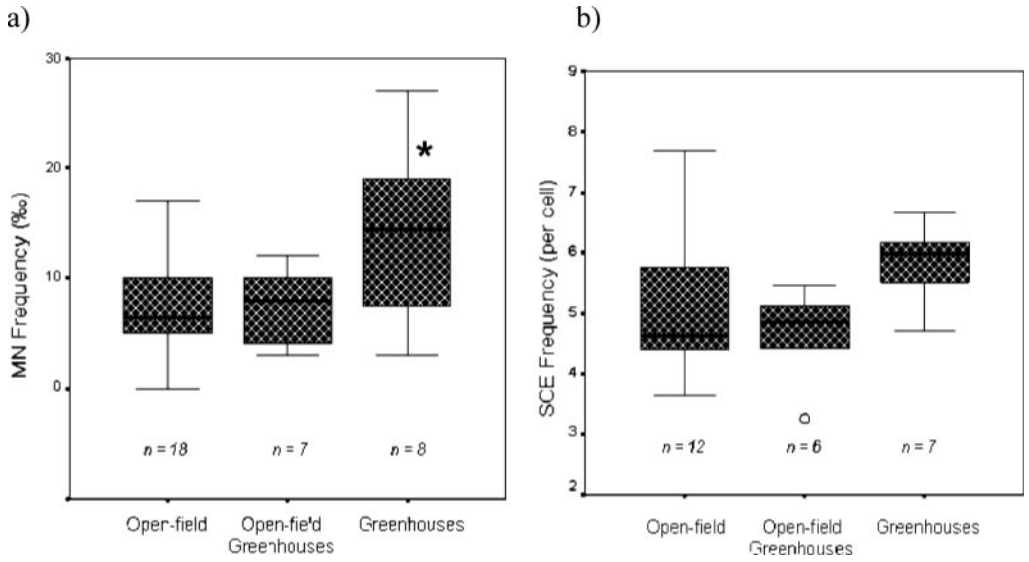

Fig. 1. Boxplots showing frequency distribution of (**a**) MN (‰); and (**b**) SCE (per cell) in different working environments within the exposed group. Boxes are limited by 1st and 3rd quartiles divided by median; thin vertical lines represent minimum and maximum values except when outliers (o) are present.

Table VI. Frequency of metabolic genotypes in study population

Genotypes	Total no. (%)	Controls no. (%)	Exposed no. (%)
CYP2E1 D/D	49 (77)	23 (70)	26 (84)
CYP2E1 D/C	15 (23)	10 (30)	5 (16)
CYP2E1 C/C	0	0	0
EPHX1—low activity	20 (31)	9 (27)	11 (35)
EPHX1—medium activity	36 (56)	20 (61)	16 (52)
EPHX1—high activity	8 (13)	4 (12)	4 (13)
GSTM1 positive	28 (44)	19 (58)	9 (29)
GSTM1 null	36 (56)	14 (42)	22 (71)
GSTT1 positive	55 (86)	30 (91)	25 (81)
GSTT1 null	9 (14)	3 (9)	6 (19)
GSTP1 Ile/Ile	33 (52)	18 (55)	15 (48)
GSTP1 Ile/Val	25 (39)	13 (39)	12 (39)
GSTP1 Val/Val	6 (9)	2 (6)	4 (13)

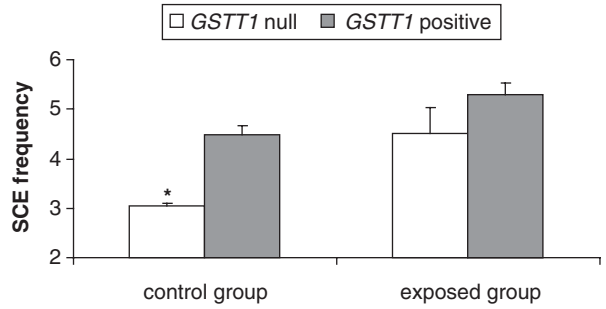

Fig. 2. SCE frequency in control and exposed groups according to their *GSTT1* genotype.

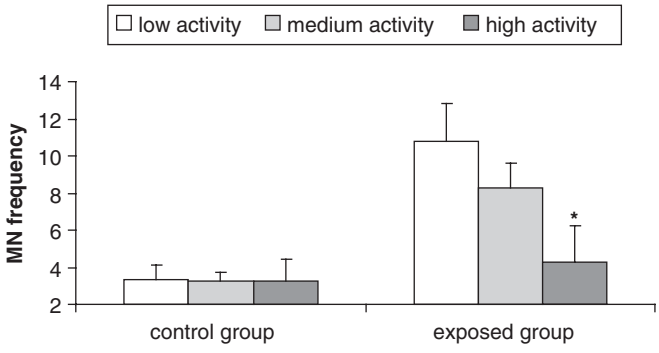

Fig. 3. MN frequency in control and exposed groups according to their expected mEH activity.

No association was found between *GSTM1*, *GSTP1* or *CYP2E1* and cytogenetic damage. A significant increase in SCE frequency was observed in *GSTT1* positive individuals of control group ($P < 0.05$) (Figure 2). MN frequency was found to be significantly higher in exposed individuals presenting low mEH activity as compared with the ones with high activity (Figure 3).

Multifactor analysis of variance has only evidenced a significant interaction between mEH activity and gender for MN frequency. No interaction could be found for other studied endpoints.

Discussion

Cytogenetic damage in individuals occupationally exposed to pesticides has received the attention of investigators in several countries but no definitive conclusions could yet be made. Reviews on this matter (28,38) evidence that most studies found an increase in biomonitoring indices of genotoxicity in pesticide applicators. Work environment, PPE usage, time of exposure and exposure conditions are described in literature as factors capable of affecting cytogenetic damage levels (28). Another factor that complicates the comparison of different studies performed to date is the high number and variety of chemicals generally used. Agricultural workers included in this study were also exposed to a great number of pesticides (61% of the subjects were exposed to more than ten different pesticides) some of them classified as being carcinogenic by US Environmental Protection Agency (US-EPA) (39) and

Table VII. List of pesticides used by the exposed subjects

Pesticides	Compound	Chemical class	IARC	US EPA	WHO
Fungicides	Benomyl	Benzimidazole	NL	C	U
	Bitertanol	Azole	NL	—	U
	Bupirimate	Pyrimidine	NL	—	U
	Captan	Thiophthalimide	3	B2	U
	Carbendazim	Benzimidazole	NL	C	U
	Cymoxanil-propineb	Dithiocarbamate	NL	Not likely	—
	Kresoxim-methyl	Strobin	NL	Likely	—
	Sulfur	Inorganic	NL	—	U
	Fenarimol	Pyrimidine	NL	Not likely	U
	Mancozeb	Dithiocarbamate	NL	B2	U
	Mancozeb-metalaxyl M	Dithiocarbamate	NL	B2	U
	Myclobutanil	Azole	NL	Not likely	III
	Propineb	Dithiocarbamate	NL	—	U
	Copper sulfate	Inorganic-copper	NL	—	II
	Tebuconazole	Azole	NL	C	III
	Thiram	Dithiocarbamate	3	Not likely	III
	Tolylfluanid	Phenylsulfamide	NL	Likely	U
Insecticides	Buprofezin	Triazine	NL	Suggestive	U
	Butocarboxim	N-Methyl Carbamate	NL	—	Ib
	Deltamethrin	Pyretroid	3	Not likely	II
	Dimethoate	Organophosphorus	NL	C	II
	Endosulfan	Organochlorine	NL	Not likely	II
	Fosmethilan	Organophosphorus	NL	—	—
	Imidacloprid	Chloro-nicotinyl	NL	E	II
	Lambda-cyhalothrin	Pyretroid	NL	D	II
	Methiocarb	N-Methyl Carbamate	NL	D	Ib
	Methomyl	N-Methyl Carbamate	NL	E	Ib
Rodenticide	Acrinathrin	Pyretroid	NL	D	—
Acaricides	Cyromazine	Triazine	NL	E	U
	Formetanate hydrochloride	N-Methyl Carbamate	NL	E	Ib
	Pirimicarb	N-Methyl Carbamate	NL	—	II
Herbicides	Glyphosate	Phosphonoglycine	NL	E	U
	Linuron	Urea	NL	C	U

IARC classification: 3 = Not classifiable as to carcinogenicity to humans; **NL** = Not listed.
US EPA classification: Group B = Probable Human Carcinogen; **B2** = sufficient evidence of carcinogenicity from animal studies; **Group C** = Possible Human Carcinogen; **Group D** = Not classifiable as to human carcinogenicity; **Group E** = Evidence of non-carcinogenicity to humans.
WHO hazard classification: Ib = Highly hazardous; **II** = Moderately hazardous; **III** = Slightly hazardous; **U** = Unlikely to pose an acute hazard in normal use.

hazardous by World Health Organization (WHO) (40) but not yet listed by IARC (Table VII) (41). This fact does not allow the establishment of an association between a single compound and the damage that it inflicts.

Herein gaps were included in statistical analysis together with breaks (referred as CA) once Paz-y-Miño *et al*. (42) stated the importance of gaps as indicative of DNA damage. In the former study authors only found a significant correlation between comet assay and CA if both gaps and breaks were included in CA number. However, since this remains a controversial question, Breaks data are also presented in this study and considered in further statistical analysis.

Results obtained in this study show increased MN and SCE frequencies in farmers when compared to controls. Garaj-Vrhovac *et al*. (43) also observed the occurrence of DNA damage assessed by MN and SCE in pesticide exposed individuals. Comparisons of CA, Breaks and An frequencies among exposed and control groups showed no statistically significant differences. This result agrees with those of Scarpato *et al*. (44) and D'Arce *et al*. (45), who analysed CA and showed that there was no significant differences between control and exposed groups. On the other hand, Joksic *et al*. (46) reported increased CA frequencies in populations occupationally exposed to pesticides. Concerning An frequencies, no comparable data are available in literature. In contrast to SCE results, HFC did not reveal any statistically significant diference between the two studied groups. This was unexpected since findings suggest that HFC sample can contain a subpopulation of more sensitive cells or long living lymphocytes with DNA damage accumulated over the years being a more sensitive tool in detecting differences in SCEs among individuals (47). Landi *et al*. (48) found the same contradicting result between SCE and HFC frequencies.

Throughout the years authors (49–51) have reported an association between sex and cytogenetic damage. In a review that included 2131 subjects, Bonassi *et al*. (52) confirmed the influence of sex in MN frequency; MN frequencies in females were found to be 20–30% higher than in males and no significant differences were found between genders for SCE and CA frequencies. Generally the increase in MN frequencies observed in women is attributed to aneuploidogenic events involving the X-chromosome. This chromosome is represented in MN more often than expected if equal probability between this sexual chromosome and autosomes is assumed. The reason for decreased inclusion of autosomes and increased inclusion of X-chromosome in MN of binucleate cells remains unclear (49). Results obtained also showed the influence of gender only in MN frequency within control group. None of the other analysed cytogenetic endpoints presented statistically significant differences for males and females. The effect of gender was not found in MN frequencies within the exposed group.

The influence of smoking habits in cytogenetic damage was studied and an increase in CA, An and Breaks (but not MN frequency) was observed in smokers within exposed group. It is acknowledged that many substances contained in cigarette smoke are genotoxic and therefore cytogenetic biomarkers are probably the better biomarkers for determining the effect of exposure to genotoxicants present in tobacco smoke (53). There is clear evidence of molecular changes, such as DNA damage induced by tobacco smoke (54). The lack of association observed between MN frequencies and tobacco exposure was also observed by other authors (27,55). Moreover a review on this matter confirmed that smokers do not experience an overall increase in MN frequency that can be ascribed to the lower effective concentration of cigarette smoke chemicals in the blood than in other organs, such as the lung, and therefore insufficient to cause recognizable cytogenetic damage in blood cells analysed by means of MN assay (53).

In our study non-applicators were included in the exposed group since they were present during all working activities, including pesticide applications. Although most studies (46,56–58) report that pesticide sprayers (applicators) represent the most exposed group of agricultural workers, in this study similar frequencies for all cytogenetic tests were observed in applicators and non-applicators. This can be due to the misconception that non-applicators are not as exposed as applicators, leading to a diminished use of PPE by the former group.

Different working environments within the exposed group allowed comparisons between them and a significant increase was found in MN frequencies in those who worked exclusively in greenhouses when compared with the ones who worked exclusively in open-field. The same trend was observed for SCE frequency but differences were not significant. Bolognesi *et al*. (5) also found a 28% increase in MN frequency of greenhouse workers. Increase in MN frequency can be due to the closed environment of greenhouses with high temperatures and humidity that favours a more acute and intense exposure to pesticides (6). Working environment showed no influence in CA, Breaks and An frequencies.

In a recent review, Bull *et al*. (38) referred to genotoxicity in pesticide applicators and highlighted the importance of PPE usage. In four studies where the majority of workers (>60%) allegedly took protective measures, no increases in CA, SCE and MN frequencies were found. Conversely, seven of eight studies in which little or no PPE were used reported significant increases in cytogenetic damage. Although not significant, we noticed an increase in MN frequency among applicators that do not wear gloves during working activity. Lander *et al*. (59) also reported that cytogenetic effects are observed primarily in workers who did not wear gloves. The increase observed herein may be due not only to the absence of gloves during labour activities but also the lack of other PPEs and safe practices.

The correlation of increased DNA damage with age has been documented over the years (60). Jacobs (61) was the first to report an increase in An with advancing age. It is possible that lagging chromosomes give rise to micronuclei. Therefore, as lagging chromosome can be incorporated into MN, the increase in An with increasing age should correlate with the increase in MN formation (62). This is exactly what has been observed in our data: a significant correlation between age and An as well as between MN and age. The increase in spontaneous chromosomal instability with age is associated with an accumulation of DNA damage due to a progressive impairment of overall DNA repair capacity and increase of highly reactive free radicals in cells (63).

Duration of exposure (years of employment) has been positively correlated with cytogenetic damage. Apparently, clastogenic effects seem to be cumulative for continuous exposure to pesticide mixtures (28). Herein, chromosomal damage mesasured as An frequency was also found to be higher in individuals exposed for longer to pesticides. Since age did not show a positive correlation with duration of exposure the increase in An here described can only be attributed to duration of exposure with no influence of age.

Concerning the prevalence of genetic polymorphisms of *GSTM1* and *GSTT1*, null genotypes in the whole population are in accordance with previous studies carried out in the Portuguese population (64–66). The frequencies of *GSTP1* and *CYP2E1* alleles are similar to the ones reported for a north-western Mediterranean population (67) and for a Czech population (36), respectively. The frequency of expected enzymatic activity according to EPHX1 genotypes is slightly different from the one published for French Caucasians (13% high, 38% medium and 49% low mEH activity) (68), but similar to that previously described for another Portuguese population (20% high, 50% medium and 30% low mEH activity) (32).

Genetic polymorphisms in metabolic enzymes have been studied in the last years in order to understand the importance of genetic determinants in DNA damage and some enzymatic isoforms have been associated with individual cancer susceptibility (30,69,70). The results obtained herein suggest that mEH activity can modulate the levels of MN frequency and GSTT1 can influence SCE frequencies.

mEH is a detoxifying enzyme that catalyses the addition of a molecule of H_2O to an epoxide, which is generated by cytochrome P450 and other Phase I enzymes to produce a metabolite more water-soluble and less reactive that can be readily conjugated and excreted (71). Thus, an increased activity of mEH results in higher detoxifying potential and therefore lower levels of cytogenetic damage are expected in these individuals (72). Our results are in accordance with this theory since lower MN frequencies were found in individuals presenting high activity of mEH. Laffon *et al*. (73) also reports increased DNA damage (comet assay) with decreasing mEH activity.

GSTT1 null genotype has been associated with an increased baseline level of SCE in lymphocytes (74). Contrarily to this, in our study an increase in SCE levels was found in *GSTT1* positive individuals. Laffon *et al*. (75) found the same when studying DNA damage (comet assay) induced by styrene. In fact, GSTT1 has both detoxifying and activating properties in many environmental pollutants being able to activate some alkylating agents (71). This can partially explain the result found herein. Nevertheless, the low frequency of *GSTT1* null genotype among Caucasians raises difficulties in the evaluation of *GSTT1* influence in DNA damage (74). Therefore, to better characterize the effect of genetic polymorphisms in cytogenetic damage it would be necessary to study a larger population. Up to date, few studies have referred to the effect of genetic polymorphisms in cytogenetic damage caused by pesticide exposure and their results remain inconclusive (76,77).

Interactions between variables included in this work have been only found in MN test. According to data presented here women (gender) presenting low mEH activity can be considered at higher risk for DNA damage (MN) related to

exposure. However, cautious interpretations must be taken because of the small sample size.

In conclusion, the present study showed an elevated rate of MN and SCE in pesticide exposed individuals. The relatively low number of individuals precluded concrete conclusions on the contribution of polymorphisms of xenobiotic metabolizing enzymes in cytogenetic damage. Nevertheless we can say that conditions at the workplace should be improved to minimize exposure to these products. This study emphasizes the need to aware those who work with pesticides about the potential hazard of occupational exposure and the importance of PPEs usage.

Acknowledgements

Our current research is supported by Fundação Calouste Gulbenkian and Fundação da Ciência e Tecnologia (POCTI/BIO/38922/2000 and PCDT/SAU-OBS/59821/2004).

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Received on May 23, 2006; revised and accepted on August 2, 2006