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Cytogenetic analysis of Greek farmers using the micronucleus assay in peripheral lymphocytes and buccal cells

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The potential cytogenetic damage associated with pesticide use in Greek agricultural workers was evaluated using micronuclei (MN) as biomarkers in lymphocytes of peripheral blood and exfoliated cells of the buccal mucosa. In addition, the effects of pesticide exposure and other variables on the cytokinesis block proliferation index (CBPI) in lymphocytes were also evaluated. Both the exposed and control individuals were selected from Nea Makri, a village near Athens (Greece). This location was selected for its high greenhouse density. Micronuclei were analysed in 50 agricultural workers exposed to pesticides (30 men and 20 women) and in 66 non-exposed individuals that constituted the control group (41 men and 25 women). The comparison between workers and controls did not reveal any statistical significant difference in the MN frequency for either lymphocytes or buccal cells. Nevertheless, the multiple regression analysis revealed that the age and the interaction between gender and the number of X-ray examinations during the last 3 years preceding the sampling increased the number of MN in lymphocytes. Moreover, the results of the negative binomial regression analysis suggested that the level of MN in buccal cells could be reduced by the intake of fish, whilst being increased by olive oil consumption. Regarding CBPI, the value found in the exposed group was lower than in controls, the difference being statistically significant. On the other hand, CBPI was inversely associated with both age and X-ray exposure.

Introduction

Although pesticides are useful in enhancing crop productivity, their extensive use may have adverse health effects in humans. Some studies have found a relationship between exposure to pesticides and an extensive number of symptoms and diseases, including increase in the incidence of some cancers. In this context, it has been reported that exposure to pesticides can enhance the incidence of leukaemia and non-Hodgkin lymphoma (Hardell and Eriksson, 1999; Meinert *et al*., 2000), bladder and pancreatic cancer (Viel and Chalier, 1995; Ji *et al*., 2001), reproductive problems (Petrelli *et al*., 2000; Rojas *et al*., 2000) and, more recently, the incidence of Parkinson disease (Lockwood, 2000; Woodward, 2001). It must be pointed out that pesticides not only have a negative effect on human health, but also on other organisms and systems.

Populations occupationally exposed to pesticides, which are in direct contact almost daily with these chemicals, constitute one of the human groups at genotoxic risk. Many biomonitoring studies have evaluated cytogenetic effects in pesticide-exposed workers from different countries. Although some papers have found increases of cytogenetic damage in the exposed groups (Dulout *et al*., 1985; De Ferrari *et al*., 1991; Amr, 1999; Antonucci and De Syllos, 2000; Garaj-Vrhorac and Zeljezic, 2000; Gómez-Arroyo *et al*., 2000; Lander *et al*., 2000), others did not detect any effects (Carbonell *et al*., 1990; Hoyos *et al*., 1996; Scarpato *et al*., 1996a; Gregorio d'Arce and Colus, 2000). In this respect, it must be noted that the results from these kind of studies are difficult to extrapolate and generalize, because different pesticide formulations are used and complex combinations are applied depending on the regions, crops, seasons, etc. Taking that into account, in general, farmers mix different pesticides, the information on the particular adverse effects of a defined compound is not enough to adequately evaluate the real genotoxic risk related to complex mixtures.

In biomonitoring studies, the use of the cytokinesis-block micronucleus assay in peripheral lymphocytes is increasing as a useful technique to evaluate cytogenetic damage. The analysis of micronuclei (MN) may be considered a useful biomarker of genotoxic effects in populations occupationally exposed to genotoxicants. Micronuclei can be formed both from whole and fragmented chromosomes lagging behind the cell division; thus, the MN assay, in principle, allows the detection of both clastogenic and aneugenic agents. In addition, and in comparison with other cytogenetic techniques, the MN assay, when using the cytokinesis-block method, is a relatively rapid and simple test that permits the identification of cells that have divided once by adding cytochalasin-B (Fenech, 1993). For more detailed information on the advantages/disadvantages of the human lymphocytes MN assay, see Surrallés and Natarajan (1997).

Several studies in populations exposed to pesticides have showed that the MN assay is a good method of detecting increases of cytogenetic damage in the exposed individuals (Bolognesi *et al*., 1993; Scarpato *et al*., 1996a,b; da Silva Augusto *et al*., 1997; Joksic *et al*., 1997; Meng and Zhang, 1997; Calvert *et al*., 1998; Gómez-Arroyo *et al*., 2000), although a lack of effect in the same assay was found in other investigations (Titenko-Holland *et al*., 1997; Davies *et al*., 1998; Venegas *et al*., 1998; Lucero *et al*., 2000; Pastor *et al*., 2001). To add further knowledge to the genetic risk related to pesticide exposure, we applied the MN assay in peripheral blood lymphocytes and epithelial buccal cells to evaluate possible cytogenetic damage in a group of Greek farmers. Exfoliated epithelial cells, which are continuously in contact with the environment, can be easily collected and rapidly analysed and are therefore a very appropriate cell system for the study of the effects of mutagenic pollutants (Titenko-Holland *et al*., 1996; Moore *et al*., 1997).

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Table I. Pesticides used by the studied group, with indication of their frequency of use, WHO classification by hazard and mutagenicity (M) and carcinogenicity (C) experimental data

Type	Product	Use (%)	Class (WHO)	M	C
Insecticides	Buprofezin	4.7	IV	−	+
	Cyromazine	12.5	IV	NA	−
	Dichlorvos	8.0	Ib	+	+
	Endosulfan	20.3	II	+	−
	Imidacloprid	50.0	NA	+	–
	Malathion	8.0	III	+	−
	Methamidophos	25.0	Ib	+	−
	Methomyl	30.0	II	+	−
	Oxamyl	14.1	Ib	−	−
	Permethrin	10.0	II	−	−
	Pyriproxifen	14.1	NA	NA	NA
	Tralomethrin	15.6	NA	NA	
Bactericides	Kasugamycin	2.0	V	−	−
Fungicides	Carbendazim	3.1	NA	+	−
	Cymoxanil	14.1	NA	NA	−
	Diethofencarb	3.1	NA	NA	NA
	Mancozeb	20.0	IV	+	−
	Fosetyl-aluminium	6.2	V	−	−
	Procymidone	10.9	V	−	–
	Propamocarb	3.1	NA	−	NA
	Propineb	7.8	V	−	−

NA, Not available; –, no observed effects; +, adverse effects in at least one experiment (see Richardson, 1992).

This group was occupationally exposed to complex mixtures of pesticides and the observed results were compared with those from a control group from the same area and with similar general characteristics. Cytogenetic data were statistically analysed with relation to some confounding factors such as age, diet, alcohol, etc., that may influence the expression of the cytogenetic parameters evaluated.

Materials and methods

Population

A total of 116 individuals (50 exposed to pesticides and 66 controls) were analysed in this study. All of them came from an area outside Athens (Greece), called Nea Makri. This is a village surrounded by cultivated land, with many greenhouses. The exposed group was selected from the village farmers and was composed of 30 men and 20 women who were regularly exposed to complex mixtures of pesticides. Both the women and the men did the same work in the greenhouses. Table I gives the main pesticides used, with an indication of their frequency of use and hazard classification. Most of them belong to the carbamate, nicotinoid and organophosphorus families. Forty-one men and 25 women who carried out clerical jobs in the same village composed the control group. They had no previous occupational exposure to pesticides or any particular environmental agent.

At the time of drawing samples for cytogenetic determination, a personal history questionnaire was filled-in. The questionnaire covered standard demographic questions (age, gender, etc.), as well as medical (genetic disorders, number of X-ray diagnoses, vaccinations, medication, etc.), lifestyle (smoking, coffee, alcohol, diet, etc.) and occupational questions (working hours/day, years of exposure, etc.). For the exposed group, a further questionnaire was completed including specific questions related with farming: kind of crops, pesticide application, use of protective measures, etc. All the individuals included in the study were non- or ex-smokers. Table II shows the main characteristics of both groups.

Previous to the study, all individuals gave informed consent, and blood and buccal cell samples were obtained following the procedure described below and manipulated according to the ethical standards. The samples were collected in late winter/early spring of 1997.

Lymphocyte cultures and MN analysis

Blood samples were obtained from each subject by venipuncture in heparinized vacutainers, coded and sent within 24 h to the laboratory where they were processed (Universitat Autònoma de Barcelona, Spain). Lymphocyte cultures were set up by adding 0.5 ml whole blood to 4.5 ml RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 1% antibiotics (penicillin and streptomycin) and L-glutamine (all obtained from Gibco, Paisley, UK). Lymphocytes were stimulated by 1% phytohaemagglutinin (PHA; Gibco) and incubated for 72 h at 37°C. Two cultures per subject were established. A final concentration of 6 μg/ml cytochalasin B (Sigma, St Louis, MO) was added to the cultures 44 h later to arrest cytokinesis (Surrallés *et al*., 1994). At 72 h of incubation, the cultures were harvested by centrifugation at 800 r.p.m. for 8 min and treated with an hypotonic solution (2–3 min in 0.075 M KCl at 4°C). Cells were centrifuged thereafter and a 3:1 (v/v) methanol:acetic acid solution was gently added. This fixation step was repeated twice and the resulting cells were resuspended in a small volume of fixative solution and dropped onto clean slides. Finally the slides were stained with 10% Giemsa (Merck, Darmstadt, Germany) in phosphate buffer (pH 6.8) for 10 min and scored.

To determine the frequency of binucleated cells with micronuclei (BNMN) and the total number of MN in lymphocytes (MNL), a total of 1000 binucleated cells with well preserved cytoplasm (500 per replicate) were scored per subject on coded slides. This is the number of cells usually scored in most laboratories (Surrallés and Natarajan, 1997). In addition, 500 lymphocytes were scored to determine the percentage of cells with one to four nuclei and the cytokinesis-block proliferation index (CBPI) was calculated according to Surrallés *et al*. (1995). To minimize variability, the same expert carried out all the microscopic analysis.

MN analysis in buccal cells

Buccal cell samples were obtained by rubbing the inside of the cheeks with a toothbrush. Cells were collected in a conical tube containing 20 ml buffer solution (0.1 M EDTA, 0.01 M Tris–HCl and 0.02 M NaCl, pH 7). After three steps of washes in this solution followed by centrifugation at 1500 r.p.m. for 10 min, 50 μl of an adequate cell suspension density was dropped onto preheated (55°C) slides and allowed to air-dry for 15 min on a slide-warmer. The slides were fixed in 80% cold methanol for 30 min, air-dried overnight at room temperature and stored at –20°C until use. Then coded slides were sent to the laboratory of mutagenesis, Department of Genetics and Microbiology, University of Barcelona, where they were stained with a final concentration of 1 μg/ml 4′,6-di-amidino-2-phenylindole dihydrochloride (DAPI) solution (Sigma), a DNA-specific fluorochrome that avoids possible artefacts. According to the criteria of Tolbert *et al.* (1992), a total of 2000 cells/donor were scored by one observer under an Olympus BX50 fluorescent microscope. The criteria for MN evaluation were those suggested by Titenko-Holland *et al.* (1998). The frequency of mononucleated buccal cells with micronuclei (BCMN) and the total number of micronuclei in buccal cells (MNBC) were determined for each studied subject.

Statistical analysis

The statistical computations were performed using the SPSS, version 10.0 (SPSS, Chicago, IL) and the SAS system for windows, version 8.0 (SAS, Cary, NC).

To detect differences between groups with regard to the mean value of confounding factors (age, alcohol, etc.), the Mann–Whitney *U*-test was applied due to the observed departure from normality. The models for main variable adjustment took into account all the continuous and dichotomized variables, as well as the interactions between the most important variables studied.

Cytogenetic variables BNMN, MNL and CBPI, scored in lymphocytes, were first investigated by using multiple linear regression analysis. Due to the lack of adjustment to the model requirements, MNL and BNMN were suitably transformed to normalize distributions and homogenize variances. Thus, the square root transformation was performed to analyse the MNL data and a Box–Cox transformation ($\lambda = 0.166$) was used for BNMN. The CBPI values did not require transformation. Different methods for variable selection (stepwise, backward and forward) were used. After these analyses, all relevant variables were analysed by a final multiple regression analysis. Each model was checked for adequacy of the fit by the analysis of residuals, tolerance limits and homogeneity of variances.

The cytological variables BCMN and MNBC were studied first by Poisson regression. Due to the high over-dispersion found, a binomial regression analysis was performed. A backward selection method was used.

P values correspond to two-sided tests, with a type I error $\alpha < 0.05$ as the significance level.

Results

Table II shows the main characteristics of the population studied. Age and sex ratio were similar in both groups. Regarding nutritional habits, it was observed that the controls drank more wine than the exposed.

Table II. Characteristics of the population studied

	Controls		Exposed	
	n (%)	Mean ± SE	*n* (%)	Mean ± SE
Age (years)				
Total	66	43.94 ± 1.11	50	42.98 ± 1.60
Men	41 (62)	45.88 ± 1.23	30 (60)	42.47 ± 2.31
Women	25 (38)	40.76 ± 2.01	20 (40)	43.75 ± 2.06
Diet				
Red meat (times/week)	66	1.63 ± 0.13	50	1.66 ± 0.13
White meat (times/week)	66	1.31 ± 0.11	50	1.30 ± 0.15
Fish (times/week)	66	1.31 ± 0.11	50	1.23 ± 0.12
Raw vegetables (times/week)	65	4.86 ± 0.40	50	4.26 ± 0.37
Cooked vegetables(times/week)	66	1.91 ± 0.20	50	2.72 ± 0.32
Fruit (g/day)	64	308.59 ± 34.94	49	243.88 ± 24.12
Drinking habits				
Wine (glasses/week)	66	4.64 ± 0.77	50	2.00 ± 0.41
Beer (glasses/week)	66	1.06 ± 0.55	50	0.64 ± 0.22
Spirits (glasses/week)	66	2.42 ± 0.77	50	0.98 ± 0.43
Coffee (cups/day)	66	1.85 ± 0.13	50	2.04 ± 0.17
Tea (cups/day)	66	0.24 ± 0.06	50	0.26 ± 0.08
Smoking habits				
Non-smokers	35 (54)	–	29 (59)	–
Ex-smokers	21 (32)	–	19 (39)	–
Passive smokers	9 (14)	–	1 (2)	–

Table III. Characteristics of the group of greenhouse farmers

	Mean ± SE
Years at current job	8.62 ± 1.13
Last pesticide application (days)	7.29 ± 1.05
Pesticides application (h/week)[a]	
Spring	2.50 ± 0.31
Summer	3.06 ± 0.31
Autumn	2.42 ± 0.32
Winter	1.84 ± 0.36
Average	2.45 ± 0.31

Data from 50 individuals.
[a]Hours of mixing and spraying pesticides.

As indicated above, only non- and ex-smokers were included in this study to avoid any possible interference of tobacco in the results. Donors were classified as non-smokers when they had never smoked or had quit smoking for more than 5 years, and as ex-smokers when they had quit smoking between 1 and 5 years ago.

An elevated number of individuals received X-rays (as diagnostic) in the past 3 years: 40% of the controls and 28% of the exposed.

With regard to the working activity of the farmer group, the majority carried out more than one kind of activity (farming, applying, harvesting, packing, etc.); the crop types were mainly ornamental plants (78%), vegetables (8%) or both (8%). The pesticide application was usually carried out from above the head with motor and manual sprayers, which increased the probability of both inhalation and dermal contact. The hours of pesticide application were similar between seasons. Relating to the protection measures used, 62% of the farmers asserted to use some kind of protection during the preparation and application of pesticides (52% used gloves, 38% impermeable boots, 42% breathing masks). Three individuals (6%) had suffered pesticide intoxication, two of which required hospitalization. Table III shows some of the characteristics of the farmers with reference to the exposure levels, measured as the average of hours directly involved in the use of pesticides.

Table IV. Summary of the cytogenetic variables[a]

	Controls		Exposed	
	n	Mean ± SE	*n*	Mean ± SE
BNMN	66	14.42 ± 1.29	50	11.12 ± 0.82
Men	41	12.90 ± 1.20	30	10.90 ± 0.98
Women	25	16.92 ± 2.77	20	11.45 ± 1.45
MNL	66	16.38 ± 1.50	50	12.22 ± 0.93
Men	41	14.68 ± 1.44	30	12.33 ± 1.20
Women	25	19.16 ± 3.16	20	12.05 ± 1.51
BCMN	56	1.73 ± 0.19	47	1.45 ± 0.23
Men	34	1.56 ± 0.23	28	1.39 ± 0.33
Women	22	2.00 ± 0.34	19	1.53 ± 0.33
MNBC	56	2.00 ± 0.25	47	1.55 ± 0.26
Men	34	1.74 ± 0.29	28	1.50 ± 0.36
Women	22	2.41 ± 0.43	19	1.63 ± 0.36
CBPI	66	1.88 ± 0.02	50	1.76 ± 0.02
Men	41	1.92 ± 0.02	30	1.75 ± 0.03
Women	25	1.84 ± 0.03	20	1.78 ± 0.03

Abbreviations: BNMN, binucleated lymphocytes with micronucleus; MNL, total number of micronuclei in binucleated lymphocytes; BCMN, mononucleated buccal cells with MN; MNBC, total number of micronuclei in mononucleated exfoliated buccal cells; CBPI, cell blocking proliferation index.
[a]Data corresponding to the scoring of 1000 cells per donor for BNMN and MNL, and 2000 for BCMN and MNBC.

A summary of the mean data of the cytogenetic variables studied and the cell proliferation index (CBPI) are indicated in Table IV. The data are expressed separately for both men and women to highlight possible gender-related differences. Although control women presented higher MN values, the differences were not statistically significant.

The results obtained in the multiple linear regression analysis do not show any effect of exposure on the lymphocyte cytogenetic variables studied (BNMN and MNL). However,

Table V. Summary of the results obtained in the multiple linear regression analysis

	n	B	Beta	Significance	Tolerance	R^2 model
BNMN						0.209
Intercept	–	1.196	–	0.000	–	
Age	116	0.006	0.405	0.000	0.997	
Gender*X-rays	116	0.013	0.235	0.006	0.997	
MNL						0.235
Intercept	–	1.220	–	0.008	–	
Age	116	0.052	0.428	0.000	0.997	
Gender*X-rays	116	0.106	0.252	0.003	0.997	
CBPI						0.260
Intercept	–	2.212	–	0.000	–	
Exposure	116	−0.138	−0.422	0.000	0.982	
Age	116	−0.003	−0.226	0.007	0.982	
X-Rays	116	−0.028	−0.278	0.001	0.982	

Abbreviations: B, no standardized coefficient; Beta, standardized coefficient.

Table VI. Summary of the results obtained in the negative binomial analysis

	n	B	P	Scale deviance	Value/DF
MNBC	–	–	–	115.045	1.150
Intercept	–	−0.967	0.0001	–	–
Fish consumption	103	−0.303	0.0177	–	–
Olive oil intake	103	0.663	0.0117	–	–
BCMN	–	–	–	117.462	1.174
Intercept	–	−0.954	0.2136	–	–
Fish consumption	103	−0.265	0.0275	–	–
Olive oil intake	103	0.605	0.0186	–	–

Abbreviations: B, no standardized coefficient; DF, degrees of freedom.

from the list of potential confounding factors included in the analysis (age, gender, X-irradiation, coffee, alcohol, diet, etc.), it can be observed that age and the interaction gender–X-irradiation have a direct and significant effect on the lymphocyte micronuclei frequency. In the case of ageing effect, the significant association indicated that BNMN and MNL values also increased with increasing age (Table V). In the case of gender-X-rays interaction, women showed higher BNMN and MNL frequencies than men, when both were equally exposed to X-rays (Table V). In the multiple linear regression analysis of the CBPI, the exposure as well as the age and the number of X-rays received in the last 3 years affects the proliferation index. Thus, the group exposed to pesticides showed lower CBPI levels than the control group and the increase of both age and number of X-rays taken produced a decrease in the CBPI values (Table V).

The negative binomial analysis of buccal cells, evaluating both BCMN and MNBC parameters, also shows a lack of increase of micronuclei in the exposed group. Regarding the role of the different confounding variables, the analysis indicates that buccal cell parameters were inversely influenced by fish consumption and directly affected by olive oil intake (Table VI).

Discussion

The main objective of this study was to evaluate if the exposure to complex mixtures of pesticides, primarily in greenhouses, induced increases in the levels of cytogenetic damage. The study was carried out in parallel with an exposed and a control group, both from the same area and with similar individual characteristics. To evaluate the chromosome damage, two different types of cells were chosen covering a wide range of exposure routes: peripheral lymphocytes and epithelial buccal cells, which are the most common cell targets used for human biomonitoring purposes. Peripheral lymphocytes have been classically used for detecting genotoxic effects in a great number of studies, since they are considered to be adequate for detecting general exposure. In addition, these cells are in a non-proliferative stage (G_0) and have a long half-life (about 3 years) (Murray and Edwards, 1999; Amorin *et al*., 2000; Thierens *et al*., 2000). Exfoliated cells from the buccal mucosa are representative epithelial cells. It must be recalled that epithelial cells are highly proliferative and they are the origin of more than 90% of cancers (Rosin and Gilbert, 1990), for which their use in biomonitoring studies is increasing (Casartelli *et al*., 2000; Dietz *et al*., 2000).

The results obtained indicate that, under the particular conditions of this study, there is no exposure-related induction of chromosome damage, as measured by the MN assay, in neither lymphocytes (BNMN, MNL) nor buccal epithelial cells (BCMN, MNBC). However, these results are of interest when considering that the exposed group had a decreasing CBPI value, which could be related to the exposure to pesticides, mainly carried out in greenhouses. The negative results, indicating lack of chromosome damage related to pesticide exposure agree with recent studies (Hoyos *et al*., 1996; Scarpato *et al*., 1996b; Gregorio d'Arce and Colus, 2000; Lucero *et al*., 2000). In contrast, other studies have revealed the induction of cytogenetic damage after pesticide exposure (Carbonell *et al*., 1993; Falck *et al*., 1999; Antonucci and De Syllos, 2000; Garaj-Vrhorac and Zeljezic, 2000; Gómez-Arroyo *et al*., 2000). In this context, it must be recalled that every biomonitoring study on populations exposed to chemical pesticides is different from the other(s), since in each area different groups of pesticides are used, depending on the crop type and on environmental factors. In addition, working conditions are generally different, the weather can influence chemical absorption, etc. Furthermore, it is obvious that real pesticide exposure is highly influenced by the protective measures used by the agricultural workers. Thus, in the population from Nea Makri, 62% of the farmers indicated the use of some kind of protective measure, which would reduce the exposure level. Nevertheless, the results for those farmers who used protection are similar to the findings for those who did not use protective measures. The multiple linear regression analysis reveals that some of the confounding factors have a significant relationship with the cytogenetic variables analysed. Thus, regarding lymphocytes, BNMN and MNL show a significant positive relationship with age. This strengthens the results obtained by other authors who established that spontaneous frequencies of MN in lymphocytes grow in a linear way with age (Fenech and Morley, 1986; Ramsey *et al*., 1995; Barale *et al*., 1998; Vaglenov and Carbonell, 1998). This effect could be attributed to the increase of aneuploidy with age, mainly in women. In fact, generally higher levels of MN were observed in women when compared with men, as described in Table III. BNMN and MNL also show a significant positive relationship with the interaction gender–radiographs. The positive effect of this interaction is due to women exhibiting higher values of BNMN and MNL, when they received the same amount of X-ray radiographs as men. The literature regarding gender and MN shows that the frequencies of MN are greater in females

than in males (Barale *et al*., 1998; Fenech, 1998; Thierens *et al*., 2000) and this gender effect was attributed to the high X-chromosome micronucleation (Surrallés *et al*., 1996; Catalán *et al*., 1998). The increase of cytogenetic damage in lymphocytes due to the exposure to ionizing radiation is also well-known (da Cruz *et al*., 1994; He *et al*., 2000; Thierens *et al*., 2000). Consequently, the finding in our study of X-ray-related increase of micronuclei in women would agree with previous data indicating that exposure to ionizing radiation induces age-dependent aneugenic effects in thyroid cancer women treated with radioactive iodine (Ramírez *et al*., 1997). Nevertheless, the observed aneugenic effect of ionizing radiation indicated that the X-chromosome was not preferentially involved in the effects of radioactive iodine (Ramírez *et al*., 1997) and that this X-independent aneugenic activity is mainly induced in older women. On the other hand, although women showed more micronuclei than men when they were exposed to the same number of X-radiographs, the absorbed dose by women could have also been higher than that absorbed by men.

With respect to the buccal cells results, although no effects related to exposure were found, some dietary factors such as olive oil and fish intake seem to influence the frequency of micronuclei. Olive oil and fish consumption was determined by the questionnaire in a semi-quantitative way and the results indicate a decrease in MN with increasing fish intake. Some studies have found evidence that the omega-3 polyunsaturated fatty acids (derived from fish) may play a protective role in coronary diseases through a variety of actions, including effects on lipids, blood pressure, cardiac and vascular function, coagulation and immune response (Cho *et al*., 2001; Iso *et al*., 2001; Mori and Beilin, 2001). These protective effects of polyunsaturated acids might also exert cell protection against genetic damage.

The results from consumption of olive oil indicated a direct relationship between its intake and MN level. This finding is difficult to explain since a positive and protective effect has been generally reported in relation to cancer and other diseases, being attributed to the antioxidant role of monounsaturated fatty acids (Yaqoob, 1998; Norrish *et al*., 2000; Stoneham *et al*., 2000). It is possible that the way in which the olive oil is consumed (pure or refined, raw or fried, etc.) affects the results, for which a specifically well-designed study would be needed to elucidate the eventual role of olive oil intake in modulating genetic damage.

Concerning the proliferation index (CBPI), a reduction related to age, exposure and X-irradiation was observed. The decrease observed in CBPI induced by age can be interpreted as an indicator of cell cycle delay due to physiological reasons. In this way, studies on cell proliferation kinetics have also found a negative correlation of the replication index and cell proliferation rate with age (Lazutka *et al*., 1994), which would reflect an age-related decline in the rate of blastogenesis (Lucivero *et al*., 1988). In addition, the observed reduction in the proliferation index of lymphocytes of the group exposed to pesticides suggests that the studied farmers were exposed to chemicals with cytotoxic properties, which would affect the cell proliferation kinetics (Rupa *et al*., 1991; Pasquini *et al*., 1996). The fact that the exposed group shows a decrease in CBPI and a lack of increase in cytogenetic damage could be because the exposure level is not high enough to induce chromosome breakage and/or due to the weakness of the biomarker used for detecting this kind of genetic damage. Another explanation could be that chronic low level exposure to pesticides induces an adaptive response related to an increase in apoptosis sensitivity, or a more extended cell cycle delay that enables appropriate repair (Kirsch-Volders *et al*., 2001).

In summary, apart from the CBPI, no differences in cytogenetic damage were observed between the control and the exposed group when using the micronucleus assay. This indicates that, under the particular conditions of exposure, the agricultural tasks related to the use of several pesticides (mainly carbamates, nicotinoids and organophosphorus) was not associated with detectable chromosomal damage when measured by the incidence of micronuclei. Nevertheless, it must be pointed out that the age of the individuals, as well as gender and medical X-ray exposure, are factors affecting MN expression and therefore must be taken into account in biomonitoring studies, as they can influence the results.

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