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A follow-up study on micronucleus frequency in Spanish agricultural workers exposed to pesticides

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To determine whether occupational exposure to a complex mixture of pesticides results in a significant increase in the level of cytogenetic damage, a follow-up study was planned on 39 greenhouse workers from Almería (southeastern Spain). Taking into account that pesticide exposure can be season-related, two blood samples were taken from each individual at different times: one in a period of high exposure (sample A, spring–summer) and the other in a period of lower exposure (sample B, autumn–winter). Using the cytokinesis block micronucleus technique the frequency of binucleated cells with micronuclei (BNMN) and the cytokinesis blocked proliferation index (CBPI) were determined in peripheral blood lymphocytes. The results obtained indicate that there were no statistically significant differences in BNMN frequencies between the two sampling periods nor between exposed and controls. ANCOVA analysis of repeated measures revealed that the age of the individuals showed a direct relation with BNMN in the first study period. With regard to CBPI, a significant and season-related effect was found.

Introduction

Undesirable effects of pesticides have been reported in farmers, including genotoxic effects, such as cancer and other genetic diseases (IARC, 1991; Dich *et al.*, 1997), including leukemia (Blair and Zahm, 1995), bladder cancer (Viel and Chalier, 1995), non-Hodgkin's lymphoma (Waddell *et al.*, 2001) and pancreatic cancer (Ji *et al.*, 2001), among others.

Biomonitoring studies using somatic cells have been extensively used to evaluate the possible genotoxic risk of a defined exposure and some indicators, such as chromosome aberrations, have been shown to be a relevant biomarker for further cancer incidence (Hagmar *et al*., 1994, 1998). In addition, the use of appropriate biomarkers in these biomonitoring studies can provide useful tools to elucidate the mechanisms of action of the exposure. In this context, different studies have been conducted in human populations occupationally exposed to pesticides, although the results obtained are not conclusive and conflicting (Scarpato *et al.*, 1996a; Venegas *et al.*, 1998; Gómez-Arroyo *et al.*, 2000; Gregorio d'Arce and Colus, 2000; Lucero *et al*., 2000). One of the possible causes of these divergent data, among others, could be seasonal variations in the levels of pesticide exposure, as shown by Carbonell *et al.* (1995). Thus, depending on the occupational levels of exposure at the time of sampling, the detected effect can be more or less noteworthy. The best way to detect the effects of seasonal variation is to use a follow-up study approach, which allows a comparison of genetic damage levels over time (Steenland *et al.*, 1985; Carbonell *et al.*, 1995; Scarpato *et al.*, 1996b; Lander *et al.*, 2000). This approach also permits one of the most problematical aspects of human biomonitoring studies, selection of the matched control group, to be overcome. Nevertheless, and in spite of the advantages of this type of approach (Gutiérrez *et al.*, 1999), follow-up studies are rarely conducted, mainly due to the difficulty of obtaining repeated blood samples from the same individual over time.

Therefore, to investigate a potential seasonal effect of pesticide exposure, a follow-up biomonitoring study was carried out by analysing the variation in frequency of micronuclei in peripheral blood lymphocytes in a group of agricultural workers from Almería (southeastern Spain). This population was previously studied (Lucero *et al.*, 2000), without any indication of cytogenetic differences between the exposed and control groups. Nevertheless, and due to the intensive greenhouse agricultural activity in the region, we considered that it would be advisable to carry out a more complete study of this exposed population with a follow-up design and in this manner rule out a season-related effect of pesticide exposure.

It must be pointed out that both peripheral blood cells and micronuclei are widely used for this type of study (Carrano and Natarajan, 1988; Surrallés *et al.*, 1992; Fenech, 1993; Gutiérrez *et al.*, 1997). In particular, the simplicity of scoring micronuclei as well as the ability to detect exposure to both clastogenic and aneugenic agents make micronuclei a good biomarker in human biomonitoring studies (Kirsch-Volders *et al.*, 1997).

Materials and methods

Study population

The study was performed in a group of 39 healthy men working in greenhouses in the province of Almería (Spain), exposed to different mixtures of pesticides, principally carbamates, organophosphates and pyrethroids. For more detailed information see Lucero *et al.* (2000). In addition, a control group constituting 22 healthy men from the same area, working as clerks, without previous occupational exposure to pesticides or any particular environmental agent, was also studied.

Blood samples were obtained at two different times: in a period of high exposure (March–April, sample A) and in a period of lower exposure (November–December, sample B), after a break for the summer holidays followed by a pause of 3 weeks in the application of pesticides.

Prior to the study all the individuals gave informed consent and blood samples were collected and further manipulated in accordance with ethical standards. All participants completed a questionnaire. See Lucero *et al.* (2000) for more details.

Lymphocyte cultures and MN analysis

Blood was obtained from each subject by venipuncture using heparinized vacutainers and immediately sent to Barcelona for the establishment of

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Table I. Characteristics of the groups studied (means ± SE)

	Control	Exposed
Number of subjects	22	39
Age	37.55 ± 2.18[a]	31.00 ± 1.37
Years of pesticide exposure		8.31 ± 1.12
Smoking habits		
No. of non-smokers	8	19
No. of ex-smokers	1	0
No. of smokers	13	20
Cigarettes/day	10.04 ± 2.29	7.94 ± 1.71
Coffee consumption (cups/day)	1.79 ± 0.32[a]	0.69 ± 0.15
Alcohol consumption (g/week)	98.29 ± 13.93	82.00 ± 14.15

[a]Significantly different from exposed (*U*-test).

lymphocyte cultures. Lymphocyte cultures were set up by adding 0.5 ml of whole blood to 4.5 ml of RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 1% antibiotics (penicillin and streptomycin) and 1% L-glutamine (all provided by Gibco Life Technologies, Paisley, UK). Lymphocytes were stimulated by addition of 1% phytohaemagglutinin (Gibco) and incubated for 72 h at 37°C.

A cytochalasin B (Cyt-B) (Sigma, St Louis, MO) solution was prepared in dimethylsulphoxide at a final concentration of 6 µg/ml (Surrallés *et al.*, 1994) and added to the cultures after 44 h incubation to arrest cytokinesis. At 72 h incubation the cultures were harvested by centrifugation at 800 r.p.m. for 8 min. Next, in order to eliminate red cells and to preserve cytoplasm, the cell pellet was treated with a hypotonic solution (2–3 min in 0.075 M KCl at 4°C). Cells were then centrifuged and a methanol/acetic acid (3:1 v/v) 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, they were stained with 10% Giemsa (Merck, Darmstadt, Germany) in phosphate buffer, pH 6.8, for 10 min.

To determine the frequency of binucleated cells with micronuclei (BNMN) and the total number of micronuclei, a total of 1000 binucleated cells with well preserved cytoplasm (500 per replicate) were scored for each subject. In addition, 500 lymphocytes were scored to evaluate the percentage of cells with one to four nuclei and the cytokinesis block proliferation index (CBPI) was calculated (Surrallés *et al.*, 1995). Microscopic scoring was performed on coded slides and, to minimize variability, the same expert performed all the microscopic analyses.

Statistical methods

To analyse possible differences between the control and exposed groups, with regard to age and lifestyle habits such as smoking and coffee and alcohol consumption, a Mann–Whitney *U*-test was performed.

An analysis of covariance (ANCOVA) of repeated measures was carried out to detect differences over time (BNMN and CBPI), as well as to evaluate the effects of covariates (age, alcohol and coffee). BNMN data were transformed to square roots in order to homogenize the variances and make them independent of the means (Draper and Smith, 1981).

A logistic binary regression analysis was used to evaluate differences in the incidence of miscarriages, considering the possible effects of age, alcohol, coffee and exposure to pesticides. Owing to the lack of normality of some haematological and biochemical data, logarithmic transformation was used. Differences between groups were evaluated by the *t*-test for independent samples, while the *t*-test for dependent samples was used to compare the variation in the frequency of each selected variable during and after the period of highest exposure. In addition, the χ^2 test was used to compare the frequencies between the two groups for other variables, such as hygiene, illnesses, intoxication symptoms, etc.

The statistical software used for the data analyses was STATISTICA (StatSoft, Tulsa, OK) and SPSS version 10.0 (SPSS Inc., Chicago, IL).

Results and discussion

Table I shows the main characteristics (age, lifestyle and exposure) for both groups (exposed and control). The groups differed significantly only with respect to average age ($P = 0.041$, *U*-test) and coffee consumption ($P = 0.004$, *U*-test), which were higher in the control group. Another aspect to note in this population is that the average period working in agriculture was ~8 years, which corresponds with a great increase in the number of greenhouses in Almería in the last 10–15 years.

Table II. Mean values (‰ ± SE) of the cytogenetic parameters analysed in the groups studied

	Control		Exposed	
	Sample A	Sample B	Sample A	Sample B
Total MN	8.95 ± 1.15	11.81 ± 1.07	9.43 ± 1.21	8.10 ± 0.79
BNMN	8.45 ± 1.04	11.59 ± 1.07	8.49 ± 1.01	7.69 ± 0.75
CBPI	1.75 ± 0.04	1.97 ± 0.08	1.78 ± 0.03	1.93 ± 0.02

MN, micronuclei in 1000 binucleated cells; BMMN, binucleated cells with micronuclei in 1000 binucleated cells; CBPI, cytokinesis block proliferation index. Sample A, high exposure (March–April); sample B, lower exposure (November–December).

Table III. Summary of the effects for the cytogenetics variables (ANCOVA of repeated measures) over time

	Mean square effect	Mean square error	*F*	*P*
BNMN				
Exposure	0.08	0.82	0.99	0.75
Repeated measures	1.19	0.67	1.67	0.20
Interaction 1–2	2.52	0.67	3.7	0.06
CBPI				
Exposure	0.05	0.03	1.32	0.26
Repeated measures	0.83	0.02	39.26	0.00
Interaction 1–2	0.01	0.02	0.71	0.40

BNMN, binucleated cells with micronuclei; CBPI, cytokinesis block proliferation index.

Table II shows the mean values (± SE) for the cytogenetic variables evaluated in both groups and samples. Although the MN values are higher in control sample B than in control sample A, the difference was not statistically significant. Comparing the results reported here with other data from our laboratory corresponding to biomonitoring studies of agricultural workers from Poland and Greece (Pastor *et al.*, 2001a,b), it appears that the MN values found in the population from Almería are lower in both control and exposed individuals.

In the case of BNMN levels, ANCOVA showed that neither exposure nor the differences with season had any effect on BNMN (Table III). Of the covariates introduced in the analysis only the age of individuals in sample A had a significant influence on micronucleus frequency (Table IV), in agreement with other authors (Migliore *et al.*, 1991), whilst a lack of effect has also been indicated (da Silva *et al.*, 1997). With regard to CBPI, the ANCOVA analysis revealed a significant effect only with season (Table III). Thus, in both groups the CBPI value was slightly higher in sample B when compared with sample A, suggesting enhanced proliferation of lymphocytes *in vitro* during the period autumn–winter. Sample B was drawn after a period of low application of pesticides in the winter. Therefore, the increase in proliferation index may reflect a decrease in the cytotoxic effects of pesticides in agricultural workers. Nevertheless, taking into account that an increased level of CBPI was also observed in the control group, the possibility of seasonal fluctuations that induced variations in CBPI over time cannot be ruled out.

Thus, the results described above indicate that in the group of agricultural workers no significant induction of cytogenetic damage was observed at the two sampling times under these

Table IV. Regression coefficients for the covariates introduced in the ANCOVA analysis

	β ± SE	*t*	*P*
BNMN (A)			
Age	0.32 ± 0.013	2.19	0.03
Alcohol	−0.28 ± 0.001	−1.95	0.06
Coffee	0.06 ± 0.098	0.42	0.67
BNMN (B)			
Age	0.11 ± 0.014	0.74	0.46
Alcohol	0.12 ± 0.002	0.81	0.42
Coffee	0.24 ± 0.109	1.76	0.08
CBPI (A)			
Age	−0.28 ± 0.003	−1.44	0.16
Alcohol	0.13 ± 0.000	0.88	0.38
Coffee	−0.03 ± 0.026	−0.22	0.82
CBPI (B)			
Age	−0.15 ± 0.002	−1.00	0.32
Alcohol	−0.08 ± 0.000	−0.53	0.60
Coffee	−0.09 ± 0.013	−0.65	0.52

BNMN, binucleated cells with micronuclei; CBPI, cytokinesis block proliferation index; A, sample A (March–April); B, sample B (November–December).

particular conditions of exposure. Our findings agree with other follow-up studies on exposure to pesticides showing no significant changes in micronuclei, chromosomal aberrations (CA) and sister chromatid exchanges (SCE) (Steenland *et al.*, 1985; Scarpato *et al.*, 1996b). However, in other studies pesticide sprayers sampled at two different times were found to show an increase in the level of CA after the pesticide spraying season (Carbonell *et al.*, 1995; Lander *et al.*, 2000).

Up to now, many biomonitoring studies have been performed in agricultural workers from different regions and under a variety of exposure conditions. In this context, it is not surprising that the results obtained have shown high variability. The usual explanation for this has been, mainly, different levels of exposure (Scarpato *et al.*, 1996a). Nevertheless, the agricultural workers in this study were selected because of the particular characteristics of their working area, where agricultural activity is intensive and exclusively inside greenhouses, leading to a high level of exposure. In addition, the climatic conditions of the area allow three or four crops per year, which implies continual use and application of pesticides throughout the year. The continued exposure to pesticides would also suggest that this kind of exposure could induce an adaptive response. This possible adaptive response would induce an increase in apoptosis of the damaged cells *in vitro* or a delay in nuclear division, allowing repair of the damage, preventing the detection of exposure effects as BNMN cells (Kirsch-Volders and Fenech, 2001).

On the other hand, another possible explanation for the lack of genotoxic damage could be the protective measures taken by the workers. It is assumed that all the workers who participated in this study were aware that they carried out a job with a potential risk. In accordance with the information obtained from the questionnaires, ~93% of them normally used some protective measures. Nevertheless, given the temperature conditions inside the greenhouses, these protective measures may not be followed all year round.

Concerning the adverse health problems associated with pesticide exposure (Weisenburger, 1993), it is interesting to note that from the answers to the questionnaire the percentage of miscarriages in the exposed group (50%) was higher than in the controls (20%), although no significant differences were found in the logistic regression analysis, neither with respect to exposure ($P = 0.093$) nor for the rest of the variables included as covariates (age, $P = 0.186$; alcohol, $P = 0.522$; coffee, $P = 0.261$). Reproductive dysfunctions, such as spontaneous abortions, infant prematurity, congenital malformations and reduced fertility, have been associated with pesticide exposure in previous investigations (Czeizel *et al.*, 1993; Garry *et al.*, 1996); thus, the high percentage of miscarriages found in the exposed group could be a consequence of pesticide exposure.

In addition, the values obtained for the biochemical and haematological parameters revealed that there were no statistically significant differences between the exposed group and the control group for the parameters analysed (data not shown). Although the plasma cholinesterase (PChE) levels in greenhouse workers were lower in the period of major exposure (sample A) with respect to the period of minor exposure (sample B), both values (12 059.83 ± 369.13 versus 10 501.51 ± 348.28) are within the normal range (<13 200 U/l). Nevertheless, this difference could be attributed to the fact that in the March–April period higher exposure to pesticides produced a reduction in the concentration of PChE. In this context it should be noted that several pesticides, such as organophosphates and carbamates (most used by these workers), are reported to be the major cause of a depression of serum cholinesterases (Yeary *et al.*, 1993) and, as consequence, this is used as a good biomarker of exposure.

In addition, the χ^2 analysis did not reveal any significant differences between groups for the list of symptoms with high probability of being induced by pesticide exposure (asthenia, dermatitis, cramps, paresthesia, etc.). However, a higher percentage of asthenia (34.5%) and conjunctivitis (13.8%) was observed in the agricultural workers when compared with the controls (20% asthenia and 6.6% conjunctivitis).

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