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Effects of Pesticides on Occupationally Exposed Humans

Stylianos M. Piperakis[1,2,*], Konstantina Kontogianni[1,2], Michael M. Piperakis[3], Ricardo Marcos[4], and Smaragdi Tsilimigaki[2]

[1]Biology Unit, Department of Pre-School, Education, University of Thessaly, Argonafton and Filellinon Streets, Volos 38221, Greece; [2]DNA Repair Laboratory, Institute of Biology, National Center for Scientific Research "Demokritos", 153 10 Aghia Paraskevi, Athens, Greece; [3]Department of Chemistry, University of Sheffield, England; [4]Grup de Mutagenesi, Departament de Genetica i de Microbiologia, Facultat de Ciencies, Edifici Cn, Universitat Autonoma de Barcelona, 08193 Bellaterra, Spain

E-mail: piper@ece.uth.gr; nkontogianni@yahoo.gr; iraklissimo@hotmail.com ; ricard.marcos@uab.es; biomara2000@yahoo.com

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Pesticides are known to contain numerous genotoxic compounds; however, genotoxicity biomonitoring studies of workers occupationally exposed to pesticides have produced variable results. In this study, we employed the comet assay to examine DNA damage in peripheral blood lymphocytes (PBLs) from 64 greenhouse workers from Almería in southeastern Spain in comparison to PBLs from 50 men from the same area, but not engaged in any agricultural work. The results indicated that there were no differences in the basal levels of DNA damage in the two study groups. In addition, exposure of PBL from the workers and controls to hydrogen peroxide or γ-irradiation led to similar levels of DNA damage; the subsequent repair of the induced DNA damage was also similar for both study populations. Smoking had no impact on any of the responses. The results of this study indicate that the greenhouse workers had no detectable increase in DNA damage or alteration in the cellular response to DNA damage compared to our control population.

KEYWORDS: comet assay, DNA damage, DNA repair, greenhouses, pesticides

INTRODUCTION

Farmers are occupationally exposed to mixtures of pesticides. Beside acute injuries, long-term effects are suspected to result from these exposures. Over 1,000 chemicals have been classified as pesticides, while a number of them are potentially genotoxic[1,2,3,4]. Some studies have shown significantly increased risks of lung cancer[5], bladder cancer[6,7], and leukemia[8,9,10] in workers exposed to pesticides.

Among the many *in vitro* and *in vivo* assays that have been used to evaluate the induction of DNA damage and mutation by pesticides, the lymphocyte micronucleus assay perhaps has been the most widely used[11,12,13,14]. Genotoxic compounds have been found among all the major types of pesticides, including fungicides, insecticides, and herbicides. A number of epidemiological studies also have been

*Corresponding author.

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conducted to ascertain genetic risk, at least at the somatic cell level, for human populations[6,14,15,16,17,18].

Several studies have reported an association between occupational exposure to pesticides and increased levels of chromosome aberrations and/or sister chromatid exchange (SCE) in peripheral blood lymphocytes (PBLs)[19,20]. The single cell gel electrophoresis (SCGE) assay, or comet assay (CA), also has been used in biomonitoring studies of pesticide-exposed workers[13,21,22]. This assay has been used for a number of different types of toxicology studies, ranging from mechanistic studies and clinical investigations to biomonitoring and molecular epidemiology investigations[23].

In the present study the CA was used to compare long-term DNA damage in PBL from 64 pesticide-exposed greenhouse workers from Almería in southeastern Spain and in PBL from a nonexposed reference group living in the same area and with similar general characteristics. The CA data were also analyzed in relation to smoking habits to determine whether this potential confounding factor influenced levels of DNA damage. Finally, experiments were conducted to measure the levels of DNA damage induced by hydrogen peroxide and γ-irradiation in PBLs from worker and control populations, and the extent of repair of that damage, in order to investigate whether occupational exposure results in DNA repair efficiency alterations.

MATERIALS AND METHODS

Chemicals and Media

Plastics were from Corning (Corning, NY). RPMI 1640 medium with phenol red, fetal calf serum, phytohemagglutinin (PHA), and trypan blue were obtained from Biochrom KG (Berlin, Germany). Lymphoprep was supplied by Nycomed (Oslo, Norway). L-Glutamine, penicillin, and streptomycin were from ICN Flow (Irvine, CA). Low and normal melting point agarose were supplied by Gibco BRL (U.K.). Phosphate-buffered saline tablets (PBS), dextrose, hydrogen peroxide (H_2O_2), and 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI) were obtained from Sigma (St. Louis, MO).

Study Population

A group of 64 male agricultural workers from the province of Almería (Spain), occupationally exposed to different mixtures of pesticides, was recruited for this study. In the group, 73% of the individuals worked on ornament plants, 17% on vegetables, and 10% on both. Table 1 gives the main pesticides used in this region, classified as fungicides, bactericides, and insecticides, with an indication of their frequency of use.

The control group consisted of 50 healthy men from the same area, without previous occupational exposure to pesticides (civil servants). Table 2 presents the main demographic characteristics and exposure information (i.e., age, smoking habits, years of pesticide exposure for both groups). They differ with respect to their average age, but this can be easily accommodated in the statistical analysis by introducing the age as a covariate.

Prior to the study, each individual provided informed consent. Blood samples were collected and further manipulated in accordance with the ethical standards. All volunteers were healthy individuals living in rural environment. At the time of drawing blood samples, a personal history questionnaire was completed. The questionnaire covered standard demographic questions (age, living area, etc.) as well as medical (genetic disorders, number of X-rays diagnoses, vaccinations, medications, etc.), lifestyle (smoking, 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 to farming: kind of crops, pesticide application, use of protective measures, etc.. The questionnaires used were similar to that published by the International Commission for Protection against Environmental Mutagens and Carcinogens[24].

TABLE 1
Pesticides used for spraying by the studied group, with indication of their frequencies of use, WHO classification by hazard, and mutagenicity (M) and carcinogenicity (C) experimental data

Type	Product	Use	Class (WHO)	M	C	Type	Product	Use (%)	Class (WHO)	M	C
Fungicides	Carbendazim	3.1	*	+	-	Insecticides	Abamectine	35.9	*	-	-
	Cymoxanil	14.1	*	*	-		Acrinathrin	17.2	*	*	*
	Diethofencarb	3.1	*	*	*		Buprofezin	4.7	IV	-	+
	Mancozeb	12.5	IV	+	-		Cyromazine	12.5	IV	*	-
	Nuarimol	3.1	*	+	*		Dichlorvos	3.2	Ib	+	+
	Fosetyl-aluminium	6.2	V	-	-		Endosulfan	20.3	II	+	-
	Procymidone	10.9	V	-	-		Formetanate	9.4	I	-	-
	Propamocarb	3.1	*	-	*		Imidacloprid	50	*	+	-
	Propineb	7.8	V	-	-		Malathion	12.5	III	+	-
							Methamidophos	34.4	Ib	+	-
							Methomyl	50	II	+	-
							Oxamyl	14.1	Ib	-	-
Bactericides	Kasugamycin	4.7	V	-	-		Permethrin	4.7	II	-	-
							Pyriproxyfen	14.1	*	*	*
							Tebufenozide	4.7	*	*	*
							Tralomethrin	15.6	*	*	*

*, Not available: -, no observed effects; +, adverse effects in at least one experiment (See M.L.Richardson, 1992, for more detailed information).

TABLE 2
Characteristics of the Studied Groups

	Control	Exposed	t
No. subjects	50	64	
Age (years)[a]	38.56 ± 1.35	32.83 ± 1.13	3.235*
Years of exposure	—	9.82 ± 1.04	
Smoking habits			
No. nonsmokers	20	29	
No. smokers	30	35	
Cigarettes/day[a]	14.70 ± 1.57	12.54 ± 1.65	0.927

[a]Mean ± S.E.; *p = 0.001.

Lymphocyte Isolation, Cryopreservation, and Thawing

A 5-ml blood sample was obtained from each volunteer by venipuncture using heparinized vacutainers with diluted 1:1 with RPMI 1640 (pH 7.3) and kept on ice for 15 min. The blood was layered onto 5-ml Lymphoprep and the PBLs were isolated by centrifugation at 200 × g for 30 min. The buffy coats were removed and washed twice with RPMI 1640. PBLs suspended in RPMI were counted in a hemocytometer and then cryopreserved. For the cryopreservation, the cell suspension was centrifuged at 200 × g for 5 min, and the cell pellet was resuspended at a concentration of 10×10^6 cells/ml in a freezing medium consisting of 10% dimethylsulfoxide, 40% RPMI 1640, and 50% fetal calf serum. Aliquots of 2×10^6 cell suspension were transferred to plastic freezing vials. The vials were placed in a Cryo 1°C freezing

container, then directly in a –70°C freezer so as to achieve a –1°C/min cooling rate; frozen cell stocks were stored at –70°C[25].

Vials were retrieved as needed and submerged in a 37°C water bath until the last trace of ice was melted. The thawed PBLs were quickly transferred to conical centrifuge tubes containing 15 ml of prechilled thawing medium consisting of 50% fetal calf serum, 40% RPMI, and 10% dextrose (one tube/vial). The cells were centrifuged at 200 × *g* for 10 min at 4°C and the cell pellet was resuspended in ice-cold PBS (pH 7.3) for use in the CA. Cell viability, using trypan blue, was found to be over 95% for both untreated and treated cells[26].

Hydrogen Peroxide (H_2O_2) and γ-Irradiation Treatments and Repair Studies

Thawed PBL were suspended in RPMI 1640 medium and placed in microcentrifuge tubes at a concentration of 1×10^5 cells/tube. The cells then were exposed to 150 μ*M* H_2O_2 on ice for 5 min or 4.2 Gy of γ-irradiation from a ^{60}Co source.

To examine repair of the DNA damage caused by exposure of the PBL to H_2O_2 or γ-irradiation, the cells were resuspended in 1-ml RPMI 1640 medium supplemented with 20% fetal calf serum, 2 m*M* L-glutamine, and penicillin, and transferred into a well of a 24-well plate (Corning). PHA was added to the wells to produce a final concentration of 2.4 μg/ml, and the plates were incubated for 2 h at 37°C in a humidified atmosphere of 5% CO_2. Further incubation of up to 24 h did not increase the extent of repair measured after 2 h[26].

Single Cell Gel Electrophoresis

The SCGE assay was performed under alkaline conditions using an adaptation of previously described methods[27,28,29,30]. Cells with or without H_2O_2 and γ-irradiation treatment were suspended in molten 1% low melting point agarose in PBS (pH 7.4) at 37°C, and 100 μl were spread onto a microscope slide precoated with 100 μl of 1% agarose. The agarose was solidified on ice for 10 min, followed by immersion of the slides in a lysis solution (2.5 *M* NaCl, 100 m*M* Na_2EDTA, 10 m*M* Tris, NaOH to pH 10.0, and 1% Triton-X-100) for 1 h at 4°C, in order to remove cellular proteins. The slides then were placed in an electrophoresis tank containing 0.3 *M* NaOH and 1 m*M* Na_2EDTA (pH >13) for 40 min. Subsequently, electrophoresis was performed using the same buffer at 25 V (1 V/cm, 300 mA) for 30 min at an ambient temperature of 4°C. The slides then were washed three times for 5 min each, with 0.4 *M* Tris-HCl (pH 7.5), at 4°C before staining with 5 μg/ml DAPI[31,32].

Evaluation of DNA Damage

DAPI-stained nucleoids were examined at 400× magnification with a WANG epi-fluorescence microscope (WANG BioMedical, Amsterdam, The Netherlands), equipped with a 350-nm excitation filter and a 420-nm barrier filter. One hundred comets on each slide were scored visually as belonging to one of five predefined classes according to tail intensity and were given a value of 0, 1, 2, 3, or 4 (from undamaged, 0, to maximally damaged, 4). Thus, the total score for 100 comets could range from 0 (all undamaged) to 400 (all maximally damaged) in arbitrary units[22]. To confirm the visually scored result, the percentage of DNA in comet tails was estimated using an image analysis system (Kinetic Analysis, Wirral, U.K.) connected to a computer with a suitable program.

Statistical Analysis

For each donor, 300 comets per treatment condition (100 comets/slide, triplicate slides/treatment) were used to evaluate DNA damage and repair. Mean scores, in arbitrary units (±SD), were calculated from the respective values, as well from the image analysis estimates. A multivariate analysis of variance (GLM-Repeated Measures) was used to evaluate differences in the distribution of DNA damage and repair in the group of 64 male agricultural workers and the 50 healthy men who served as controls. A level of at least 0.05 was used to determine significance. To detect differences between groups with regard to the mean value of confounding factors (age, smoking, etc.), the Mann-Whitney U-test was applied due to the observed departure from normality.

RESULTS

Fig. 1 presents DNA migration data for PBL from the worker and control populations stratified by their smoking habits (basal damage). DNA damage was measured by visual scoring of the comets. DNA damage was also measured following a 5-min treatment with 150 μ*M* H_2O_2. Analysis of variance indicated that there were no significant differences in the level of basal DNA damage between the nonsmoker controls and the nonsmoker greenhouse workers ($p > 0.05$) and also between smoker controls and smoker greenhouse workers ($p > 0.05$). Although the smoker groups had somewhat increased levels of DNA damage, the observations indicated that neither smoking nor exposure to pesticides had a significant effect on the basal levels of DNA damage.

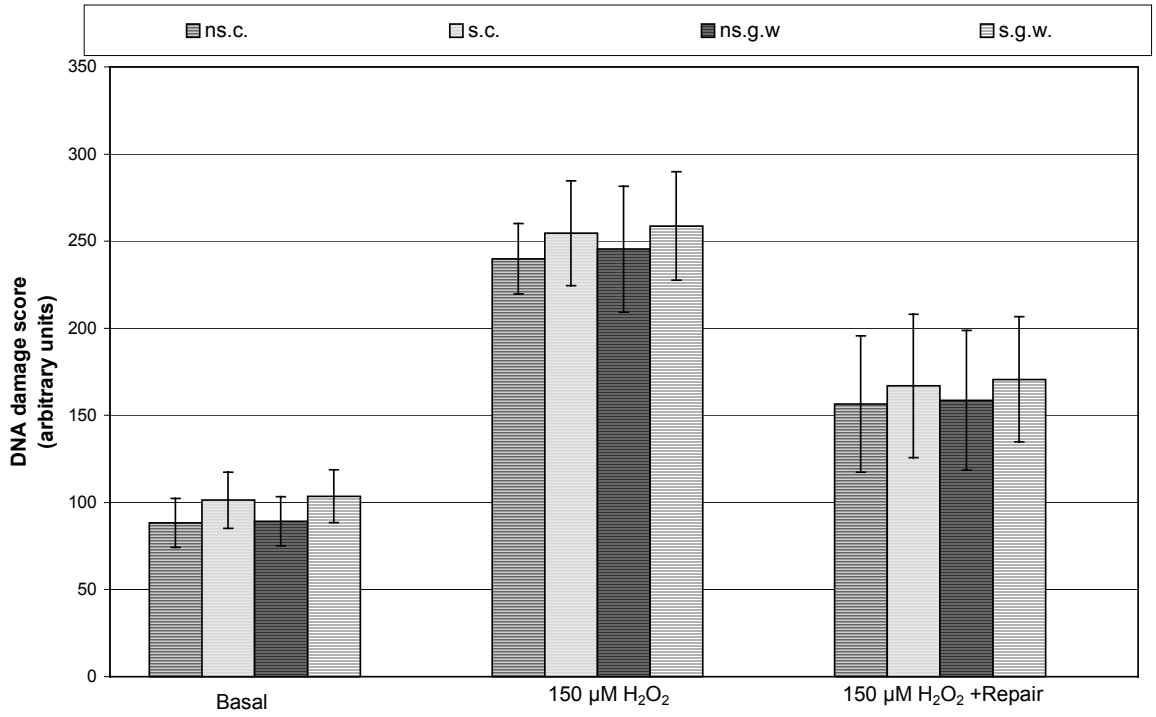

FIGURE 1. Response of lymphocytes of the groups [nonsmokers control (ns.c); smokers control (s.c.); nonsmokers greenhouse workers (ns.g.w.); smokers greenhouse workers (s.g.w.)] after exposure to 150 μ*M* H_2O_2 and after repair. Error bars represent the standard deviation of the mean among the donors.

Treatment with 150 μ*M* H_2O_2 produced a significant increase in DNA damage in comparison to the level of DNA damage in the corresponding untreated group ($p < 0.05$). This increase in DNA damage, however, was similar for the workers and controls and was not influenced by smoking, indicating that the working conditions had no effect on the oxidative DNA damage induced by the H_2O_2 treatment.

Similar results were found when lymphocytes from greenhouse workers and controls were treated with 4.2 Gy of γ-rays (Fig. 2). The treatment resulted in a significant increase in DNA damage compared with the level of DNA damage detected in the corresponding untreated PBL. The increase in DNA damage was similar for the worker and control PBLs, and was not affected by prior exposure to tobacco smoke.

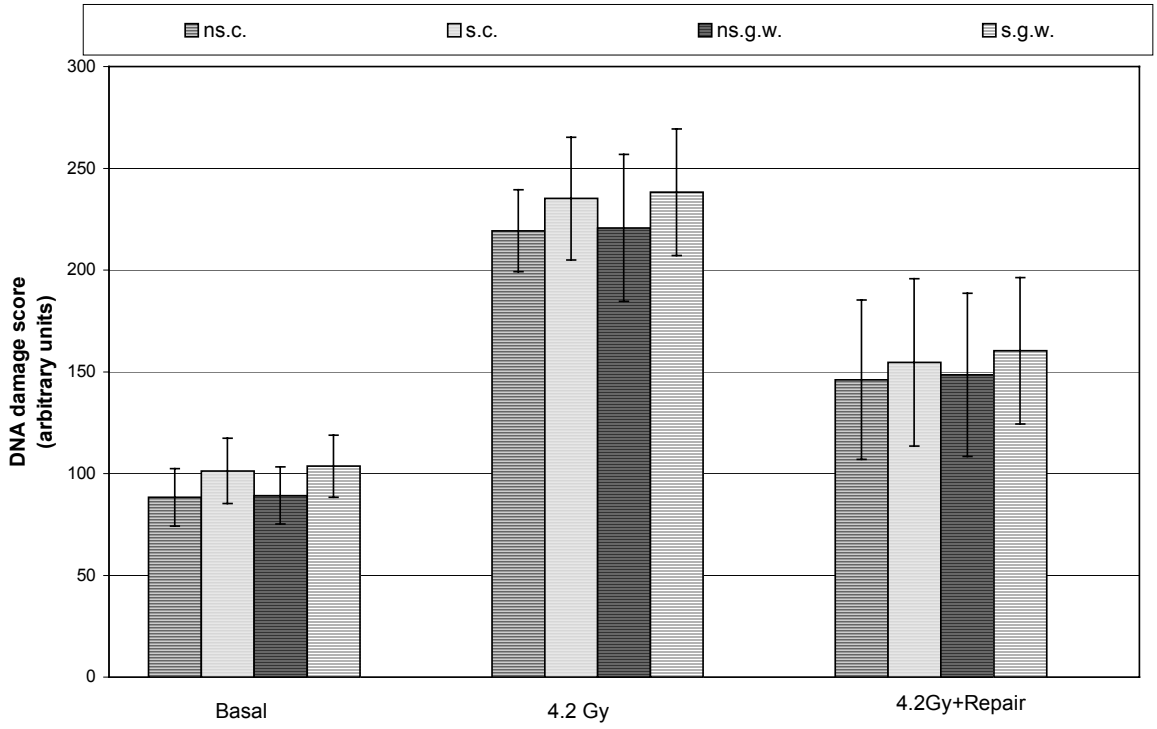

FIGURE 2. Response of lymphocytes of the groups [nonsmokers control (ns.c); smokers control (s.c.); nonsmokers greenhouse workers (ns.g.w.); smokers greenhouse workers (s.g.w.)] after exposure to 4.2 Gy ^{60}Co and after repair. Error bars represent the standard deviation of the mean among the donors.

A 2-h repair period resulted in a significant ($p < 0.05$) decrease in the initial level of damage produced by the H_2O_2 treatment. The degree of repair of the H_2O_2-induced damage was similar for PBLs from greenhouse workers and the controls, and was unaffected by smoking (Fig. 1). Repair had a similar effect on the DNA damage induced by γ-rays; the 2-h incubation resulted a significant ($p < 0.05$) decrease in the initial level of damage, and the degree of the decrease was similar for smoker and nonsmoker workers and controls.

DNA damage was also measured by the percent DNA in the comet tail using the Kinetic Analysis image analysis system (Table 3). The results using this metric of DNA damage were similar to those obtained by microscopic classification of comets (Figs. 1 and 2).

TABLE 3
Mean Values of DNA Damage as Measured by Image Analysis System in the Four Groups

Samples	Mean ± SD			
	Men, Nonsmokers Control	Men, Smokers Control	Men, Nonsmokers Pesticides	Men, Smokers Pesticides
Control	16.73 ± 5.3	19.30 ± 5.9	16.91 ± 7.8	19.76 ± 3.5
150 μM H_2O_2	46.76 ± 4.4	49.69 ± 2.3	47.87 ± 2.7	50.50 ± 3.3
150 μM H2O2 + Repair	30.23 ± 3.1	32.31 ± 2.8	30.68 ± 2.9	33.06 ± 2.1
4.2 Gy ^{60}Co	42.69 ± 1.1	45.82 ± 1.9	42.97 ± 3.1	46.46 ± 3.1
4.2 Gy ^{60}Co + Repair	28.18 ± 2.1	29.87 ± 3.5	28.66 ± 1.6	31.02 ± 3.7

[a]As measured by image system analysis ± SD.

DISCUSSION

By definition, pesticides are toxicants intended to control pest populations. Although the benefits associated with their use in agriculture are unquestionable, many of their active substances have potentially adverse effects on human health. A number of cytogenetic biomonitoring studies have been performed on agricultural workers from different regions who were subjected to a variety of exposure conditions. Yoder et al.[33] noted a marked increase in chromatid lesions in lymphocyte cultures prepared from individuals exposed to pesticides during heavy spraying periods. Carbonell et al.[34,35] found that the frequency of chromosome aberrations is related to the degree of the pesticides exposure. Crossen et al.[36] and Dulout et al.[37,38] found significantly elevated frequency of SCE in lymphocytes from subjects occupationally exposed to pesticides. Kourakis et al.[39,40] also observed increased clastogenicity in human somatic cells from workers occupationally exposed to pesticides. However, several similar studies did not detect increased DNA damage in pesticide-exposed workers[41,42,43,44]. Several biomonitoring studies found an increase in micronucleus frequency in workers with occupational exposure to pesticides[18,45,46], although negative results also have been reported[14,17,44]. Finally, conflicting results have been found using the CA[13,21,22]. Some of the variability in the results of these studies may be explained by exposure of the various study populations to different pesticides, and by the use of assays having different sensitivities and specificities[44].

Cigarette smoking is a well-documented source of a variety of potentially mutagenic and carcinogenic compounds. Smoking, however, has produced conflicting results in human biomonitoring studies that measured micronuclei and DNA damage using the CA[47,48,49,50]. H_2O_2 readily penetrates the cell membrane and initiates the generation of highly reactive species through the transition metal-catalyzed Haber-Weiss reaction[51,52]. In order to produce DNA damage, a concentration of H_2O_2 must be present that is sufficient to overwhelm the cellular antioxidant capacity. Antioxidant defences include antioxidant enzymes (catalase), scavenger molecules, and the ability of cells to remove altered molecules by turnover[53]. γ-Irradiation is able to break DNA directly by deposition of energy in the deoxyribosephosphate backbone; single-strand breaks and double-strand breaks are produced. However, most of the energy is deposited in water leading to hydroxyl radicals that can subsequently react with bases and sugars in DNA producing base modifications, sites of base loss (abasic sites), and strand breaks[26].

The agricultural workers were selected for the present study because of the particular characteristics of their working area; the agricultural activity is intensive and exclusively in greenhouses, suggesting the potential for a high level of pesticide exposure. In addition, the climatic conditions of the area allow 3–4 crops/year, which implies a constant use and application of pesticides throughout the year. The results of

our study were in agreement with the results of a previous study carried in the same population using micronuclei as the endpoint[14]. This previous study also failed to find significant differences between the pesticide-exposed population and control populations. Taken together, the results of these studies indicate that this specific group of agricultural workers, under their particular exposure conditions, do not have significantly increased levels of DNA damage. Our results also indicate that PBLs from these workers are damaged by H_2O_2 and γ-irradiation similarly to PBLs from controls, and that the repair of the DNA damage produced by H_2O_2 and γ-irradiation is similar to that of the controls. As far as smoking habits are concerned, our CA data indicated that the smokers had slightly more DNA damage than the nonsmokers; however, this difference was not significant.

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