Evaluation of genetic damage in workers employed in pesticide production utilizing the Comet assay

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The use of pesticides has been increasing in recent years, resulting in the need for increased production of pesticides. However, some pesticides may represent a hazard to human health, especially by causing cancer. Genotoxicity tests form an important part of cancer research and risk assessment of potential carcinogens. Therefore, in the current study the potential DNA damage associated with exposure to pesticides of Indian pesticide production workers was assessed using the single cell gel electrophoresis assay or Comet assay. Blood leukocytes of a group of 54 pesticide workers and an equal number of control subjects were examined for genotoxicity in this study. The two groups had similar mean ages and smoking prevalences. The mean comet tail length was used to measure DNA damage. The exposed workers had significantly greater mean comet tail lengths than those of controls (mean ± SD 19.17 ± 2.467 versus 8.938 ± 2.889, P < 0.001). Smokers had significantly larger mean tail lengths than non-smokers (19.75 ± 2.52 versus 18.26 ± 2.13, P = 0.024). Analysis of covariance showed that occupational exposure (P < 0.05) and smoking (P < 0.05) had significant effects on mean tail length, whereas age and gender had no effect on DNA damage. The present study suggests that occupational exposure to pesticides and smoking can cause DNA damage. This investigation confirms the sensitivity of the Comet assay.

Introduction
Pesticides have been a boon, especially to the developing nations in their efforts to eradicate insect-borne endemic diseases, to produce adequate food and to protect forests, plantations and fibers. Nevertheless, many pesticides are a source of potential hazard to the environment and non-target organisms. A large number of epidemiological studies on cancer risk in farmers have produced conflicting results. However, meta-analyses and reviews are available in the scientific literature reporting increased risk of tumors such as leukemia and multiple myeloma (Daniels et al., 1997; Khuder and Mutgi, 1997; Zahm et al., 1997; Viel et al., 1998). Hence, the undesirable health effects caused by pesticides in humans are of special concern. Among these are their genotoxic effects, including cancer and several other genetic diseases (IARC, 1991). Genetic biomonitoring of populations exposed to potential carcinogens is an early warning system for genetic diseases or cancer. It also allows identification of risk factors at a time when control measures could still be implemented (Kassie et al., 2000). Many approaches and techniques have been developed for monitoring human populations that have been exposed to environmental genotoxins (Hulka et al., 1990). Human biomonitoring can be performed using different genetic markers (Legator and Au, 1994). Cytogenetic markers such as chromosomal aberrations (CA), micronuclei (MN) and sister chromatid exchanges (SCEs) are among the most extensively used markers of genotoxic effects of pesticides.

Several researchers have used cytogenetic assays to evaluate the potential genotoxicity of pesticide exposures in occupationally exposed populations from various countries (Nehez et al., 1981; Dulout et al., 1985; Rupa et al., 1988; Carbonell et al., 1993; Joksic et al., 1997; Davies et al., 1998; Gomez-Arroyo et al., 2000; Zeljezic and Garaj-Vrhovac, 2002). However, there are reports on positive genotoxic effects in populations exposed to pesticides (Paldy et al., 1987; De Ferrari et al., 1991; Bolognesi et al., 1993; Kourakis et al., 1996; Garaj-Vrhovac and Zeljezic, 1999, Antonucci and Colus, 2000) as well as negative findings (Carbonell et al., 1990; Gomez Arroyo et al., 1992; Hoyos et al., 1996; Scarpati et al., 1996; Lucero et al., 2000; Pastor et al., 2001a,b). These conflicting results obtained in the biomonitoring of pesticide-exposed groups could reflect heterogeneity of pesticide exposure (e.g. farm workers, floriculturists, pesticide applicators and pesticide manufacturing workers). The contradictory data may also be due to differences in the pesticides used, in the protective measures employed or in the cytogenetic endpoint evaluated. Thus, data from one study in one particular occupational setting cannot be used to draw conclusions on genetic risk in another occupational setting. This justifies this study (and other studies as well), despite the availability in the literature of a large number of studies of this kind (but on different populations, with different exposures).

During the last few years the alkaline single cell gel electrophoresis (SCGE) assay, also known as the Comet assay, has increasingly been used in human biomonitoring studies. This assay is a rapid and sensitive tool to demonstrate the damaging effects of different compounds on DNA at the individual cell level. Cells with damaged DNA display increased migration of DNA fragments from the nucleus, generating a comet shape (Singh et al., 1988; Fairbairn et al., 1995). Despite the fact that the Comet assay has been used in several occupational studies, only a limited number of molecular epidemiological studies have applied this assay to evaluate the genotoxic effect of pesticides in human populations (Lebailly et al., 1998a,b; Moretti et al., 2000; Garaj-Vrhovac and Zeljezic, 2000, 2001; Zeljezic and Garaj-Vrhovac, 2001).

All populations have a degree of risk in relation to pesticide exposures from agricultural and non-agricultural use and through residues in food. However, workers employed in pesticide production and farmers who use pesticides have a higher risk of exposure and hence are more prone to the potential health effects of pesticides. Investigations that have
examined the genotoxic effects in pesticide industry workers are scarce (Kiraly et al., 1979; Amr, 1999; Padmavathi et al., 2000; Garaj-Vrhovac and Zeljezic, 1999, 2000, 2001; Zeljezic and Garaj-Vrhovac, 2001, 2002). Pesticide production in India is a year-round activity. Pesticide industry workers log 8 h/day, 6 days/week. These workers are constantly exposed to variety of pesticides. Reports on genetic damage in populations exposed to pesticides from India are scanty (Rita et al., 1987; Rupa et al., 1988, 1989a,b,c; 1991; Padmavathi et al., 2000).

The aim of this study was to risk characterization of pesticide exposure in workers employed in a pesticide manufacturing unit. The pesticides being produced were organophosphates, carbamates and pyrethroids. Hence, in the current study a group of pesticide production workers from Hyderabad, India, were evaluated for DNA damage in blood leukocytes using the Comet assay.

Materials and methods

Study population

The study involved 108 subjects divided into two groups. The first group consisted of 54 workers employed in a pesticide manufacturing unit located in Hyderabad, India. The average duration of their employment in pesticide production was 8.57 years (range 3–13 years). During production all subjects were simultaneously exposed to a complex mixture of pesticides (acephate, chlorpyriphos, phorate, fenvalerate, cypermethrin, monocrotophos, dimethoate and carbendazim and a wide range of formulations). The control group (54 subjects) was selected from the general population with no history of occupational exposure to pesticides or any particular environmental agent.

The selection criteria for study persons was based on questionnaire. All subjects were asked to complete a face-to-face questionnaire which included standard demographic data (age, gender, etc.) as well as medical (exposure to X-rays, vaccinations, medication, etc.), lifestyle (smoking, coffee, alcohol, diet, etc.) and occupational questions (hours per day working, years of exposure, use of protective measures, etc.). The exposed group was identified and selected by a medical doctor from an initial 160 workforce. Only those subjects who had worked for at least 3 years in the pesticide production unit were considered eligible. It was ensured that the pesticide-exposed workers and the controls did not markedly differ from each other except for occupational exposure. It was also assured that the exposed subjects and the controls had not been taking any medicines nor had they been exposed to any kind of radiation for 12 months before blood sampling. The subjects who smoked >5 cigarettes/day at least for 1 year were considered smokers, in both the study and control groups. The samples were collected in winter between October and December 2001. Table I shows the main characteristics of both groups. The study was approved by the local ethical committee. Informed consent was obtained from each individual prior to the beginning of the study.

DNA damage analysis using the Comet assay

For the study of DNA damage, blood samples were collected from all the study and control group subjects. A total of 40 µl of blood was taken from a finger prick into a heparinized glass capillary for the Comet assay, which was carried out according to Singh et al. (1988), with slight modifications. To avoid possible bias, the blood samples were coded. The samples were transported on ice to the laboratory and were processed within 2 h. Cell viability determined by trypan blue exclusion technique (Pool Zobel et al., 1994) ranged from 92 to 96% (data not shown).

Slides were prepared in duplicate per subject. Fully frosted microscope slides were covered with 140 µl of 0.75% normal melting point agarose (40–42°C). After application of a coverslip the slides were allowed to gel at 4°C for 10 min. An aliquot of 20 µl of whole blood was then added to 0.5% of 110 µl of melting agarose (37°C). After carefully removing the coverslips a second layer of 110 µl of sample mixture was pipetted onto the pre-coated slides and allowed to solidify at 4°C for 10 min, with the coverslips in place. The coverslips were removed and a third layer of 110 µl of low melting agarose was pipetted onto the slides and allowed to gel at 4°C for 10 min. The slides (without coverslips) were immersed in freshly prepared, cold lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris–HCl, pH 10, 1% sodium N-lauroyl sarcosine, 1% Triton X–100 and 10% DMSO, with the DMSO added just before use) and refrigerated overnight. Slides were then placed in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 13) for 20 min to allow unwinding of the DNA. Electrophoresis was conducted for 25 min at 25 V (0.66 V/cm) adjusted to 300 mA by raising or lowering the buffer level in the tank. Slides were then drained, placed on a tray and washed slowly with three changes for 5 min each of neutralization buffer (0.4 M Tris–HCl, pH 7.5). DNA was precipitated and slides were dehydrated in absolute methanol for 10 min and were left at room temperature to dry. The whole procedure was carried out in dim light to minimize artefactual DNA damage. Slides were stained with 50 µl of ethidium bromide (20 µg/ml) and viewed under a fluorescence microscope. Analysis was performed using a 400x objective with a Leica optiphase microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. Slides were randomized and coded to blind the scorer. All slides were scored by one person, to avoid inter-scorer variability.

A total of 50 individual cells were screened per subject (25 cells from each...
effects of smoking and occupational pesticide exposure on DNA mean comet tail length in workers (Table III). Table IV reveals a significant effect of occupational exposure on mean comet tail length in smoking and non-smoking exposed workers when compared with the controls (P = 0.001). Note that age and gender are not taken into account in Table IV. The results of ANCOVA when age was included as a covariate are summarized in Table V. The effects of smoking and occupational pesticide exposure on DNA damage were quite significant (P < 0.05). Age and gender showed no significant effect on mean comet tail length.

### Discussion

The aim of this study was to evaluate the genotoxic damage in workers employed in pesticide production in India. The investigation was conducted utilizing the Comet assay. The results of this study indicate that occupational exposure to a mixture of pesticides induces a significant increase in the level of DNA damage. In vivo studies that have investigated the genotoxic potential of workers handling pesticides using the Comet assay are few. The assay was used to quantify the level of DNA damage in mononuclear leukocytes of French farmers who were occupationally exposed to a number of pesticides. The findings showed that genetic damage was significantly high (Lebailly et al., 1998a). In another study by the same authors, the Comet assay was used to assess DNA damage in the farmers. An increase in DNA damage levels was observed after 1 day spraying with pesticide mixtures (Lebailly et al., 1998b). Evaluation of workers involved in the production of a variety of pesticides using the Comet assay revealed an increase in DNA damage in peripheral blood lymphocytes (Garaj-Vrhovac and Zeljezic, 2000). Similarly, a study of possible genetic damage in Croatian workers occupationally exposed to a complex mixture of pesticides showed an increase in the values of the Comet assay parameters (Garaj-Vrhovac and Zeljezic, 2001). Our results are consistent with the above reports. Other studies performed on populations exposed to a variety of pesticides, which examined genotoxicity with different assays, support our findings (Yoder et al., 1973; Dolut et al., 1985; Paldy et al., 1987; Rita et al., 1987; Nehez et al., 1988; Rupa et al., 1989c; 1991; De Ferrari et al., 1991; Kourakis et al., 1992; Bolognesi et al., 1993; Carbonell et al., 1993; Lander and Ronne, 1995; Falck et al., 1999; Munnia et al., 1999; Antonucci and Colus, 2000; Gomez-Arroyo et al., 2000; Shaham et al., 2001). However, results obtained by some researchers using various cytogenetic assays with populations exposed to pesticides elucidated negative results (Crossen et al., 1993; Carbonell et al., 1990; Gomez-Arroyo et al., 1992; Hoyos et al., 1996; Scarpato et al., 1996; Davies et al., 1998; Venegas et al., 1998; D’Arce and Colus, 2000; Lucero et al., 2000; Pastor et al., 2001a,b; Zeljezic and Garaj-Vrhovac, 2001).

Since the clastogenicity may be due to cumulative effects of all or some of the pesticides, it is not possible to attribute damage to any particular agent. The results of this study, as well as the results of research performed on subjects occupationally exposed to pesticides using a variety of genotoxic assays, suggest that mixtures of pesticides in long-term occupational exposure could act as clastogens on the DNA of somatic cells. The detected DNA damage could be due to cytotoxic and/or genotoxic effects. The genetic damage...
demonstrated in the current study and evaluated as an increase in comet tail length could possibly originate from DNA single-strand breaks, repair of DNA double-strand breaks, DNA adduct formation or DNA–DNA and DNA–protein crosslinks. Occupational exposure to xenobiotics may result in their covalent binding to DNA, which may lead to chromosome alterations and could be an initial event in the process of chemical carcinogenesis (Fairbairn et al., 1995; Shah et al., 1997). Exposure to known genotoxic compounds could induce DNA damage not only directly but also through other mechanisms, such as oxidative stress or inflammatory processes (Lebailly et al., 1998b). It is well known that increased genotoxicity in individuals occupationally exposed to pesticides is related to cancer risk and genetic illness. The individual genetic variability in the enzymes which metabolize agricultural chemicals may also be involved in this process. When they are not efficient in detoxification, the metabolic subproducts accumulate, contributing to the tumorigenic process (Hodgson et al., 1991).

The genetic damage seen in the current investigation may be due to insufficient protective measures taken by the exposed workers. Similarly, positive genotoxicity has been reported in studies where the pesticide-exposed workers had used very few protective measures (Gomez-Arroyo et al., 2000; Padmavathi et al., 2000). In contrast, some cytogenetic studies of pesticide sprayers showed that using proper safety procedures significantly reduced the genetic risk (Venegas et al., 1998; Lucero et al., 2000). Therefore, using more or fewer safety measures is also a factor that may explain the positive findings of this study. However, no data on environmental measurements in the work area are available.

This study has demonstrated significantly greater DNA migration in smokers of the control group as well as in the pesticide industry workers. Likewise, a significantly higher level of DNA damage was found in smokers after a period of pesticide production with the Comet assay (Zeljezic and Garaj-Vrhovac, 2001). A significantly increased comet tail length was observed in pesticide manufacturing workers with >10 years exposure. A similar increase in genotoxic effects in workers exposed to pesticides with increase in duration of exposure has been reported (Carbonell et al., 1993; Padmavathi et al., 2000). Our investigation has revealed an insignificant effect of age on DNA damage in the study subjects. These results are in agreement with those of Lebailly et al. (1998a).

Our study did not show a significant difference between genders. Similarly, a significant difference in gender was not detected in a study of occupational exposure to pesticides (Zeljezic and Garaj-Vrhovac, 2001).

In the present investigation the blood samples were collected in a single month (October–December) for the study of genetic damage in pesticide industry workers. However, in some studies variations in genetic markers were evaluated at different times in pesticide exposed workers and contrasting results were observed (Carbonell et al., 1995; Laurent et al., 1996). Biomonitoring studies of populations exposed to pesticides are rather specific because different populations have different lifestyles, nutritional habits, climatic and environmental conditions and are exposed to different mixtures of pesticides. This could explain why some studies have found an increase in genetic damage in populations exposed to pesticides whilst in other studies the results were negative. Although the exposure level can be considered significant and information is available on the genotoxicity of several of the pesticides used, there is a lack of information concerning genotoxic effects of complex mixtures. Another explanation for the genotoxic damage observed could be the lack of protective measures taken by the workers. Therefore, there is a need to educate those who work with pesticides about the potential hazards of occupational exposure and the importance of using protective measures. Since DNA damage is an important step in events leading from carcinogen exposure to cancer, our study represents an important contribution to the correct evaluation of the potential health risks associated with agrochemical exposure.

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