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Assessment of DNA Damage in Agricultural Workers Exposed to Mixture of Pesticides in Assam (India)

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ABSTRACT

In agricultural fields, pesticides are used to protect the crops, but they pose a potential hazard to the farmers and the environment. The aim of the present study is to investigate the relationship between the occupational exposure of different kinds of pesticides and DNA damage of an individual due to directly spraying the pesticides in the agricultural field. Blood samples of 70 exposed workers (after a day long spraying) and 70 control subjects belonging to Kolgochia Area of Barpeta district of Assam, were evaluated using the comet assay. Significant differences were found in the DNA damage among freshly exposed workers and controls group. The two groups had similar mean ages and smoking habits. Mean comet tail length was used to measure the DNA damage. Length of duration of pesticide exposure on the workers had significantly large tail length (62.75±21.97, 63.48±20.15, P=0.05). There was a significant increase in the comet parameters, viz., mean comet length and frequency of cells showing migration in exposed workers as compared to the control (20.73± 20.23 vs. 63.48±20.15, P=0.054641 and 31,07±23.81 vs. 62.75±21.97 P=0.043). Confiding factors during pesticide exposure, such as age, smoking, drinking and dietary habit were expected to modulate the damage. The evidence of genetic hazard related to exposure, resulting from the exposure of pesticides, is the matter of concern. There is a need for educational programs in agriculture to reduce the use of such chemicals.

INTRODUCTION

The use of pesticides in agriculture has been increasing steadily. At present there are more than 1000 chemicals classified as pesticides. These pesticides are used to control pest and to increase food production. Large amount of these chemicals is released into the environment daily and many of them affect non target species and they may represent a potential hazard to human health.

As most occupational and environmental exposure is the mixture of pesticides, the genotoxic potential evaluated on single compound cannot be evaluated in humans. So, genotoxicological assessment in human population is a useful tool to estimate the genetic risk from the exposure of pesticide mixture.

Genotoxic effects in the population exposed to pesticides have been worked out (Antonucci & Colus 2000) as well as negative findings are determined (Carbonell et al. 1990, Gomez Arroyo et al. 1992, Hoyos et al. 1996).

The pesticides induce the breaking of DNA (Islas et al. 2005) and thus the affect the DNA replication and its ability to carry information (Velazquez et al. 1986). DNA damage together with cellular response can establish instability through multiple pathways (Gontijo et al. 2001), and can be considered as a strategy for risk assessment, and it is a reliable biomarker.

Single cell gel electrophoresis (SCGE) or comet assay has been used increasingly in human biomonitoring studies. This assay is a rapid sensitive tool to demonstrate damaging effect 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 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 population (Garaj-Vrhovac & Zeljezic 2000, 2001, Zeljezic & Garaj-Vrhovac 2001).

Hence, in the current study a group of pesticide spraying workers from some major agricultural spots of Barpeta district, Assam was evaluated for DNA damage in the peripheral lymphocytes using the comet assay.

MATERIALS AND METHODS

Population study: The study involved 140 subjects divided into two groups. The first group consists of 70 workers employed for spraying the crop in the agricultural field of

Barpeta district of Assam. The average duration of their employment in pesticide production was 8 years, ranging from 3 to 13 years. During spraying operation all the subjects were exposed to a complex mixture of pesticides mainly-malathion, phosphomedon, carbamates, carboryl, methyl-parathion and chlordane heptachlor. The control group (70 subjects) was selected from the general population with no history of occupational exposure to pesticides or any environmental agents. The selection criteria for exposed and control personals was based on a questionnaire.

All the subjects were asked to complete a face to face questionnaire which included standard demographic data (age, gender, etc.) as well as medical occupational questionnaire (hours per day working, year of exposure, use of protective measures, the environment at the time of spraying, wind direction, temperature etc.). The exposed group was identified and selected by a medical doctor. Only those subjects, who worked for at least 3 years in pesticides spraying operation, were considered eligible. It was ensured that pesticide exposed and control workers did not markedly differ from each other, except occupational exposure, and the exposed and control subjects had not been taking any medication, nor they had smoking habit. Those who smoked for a year were considered as smoker in both control and exposed group. The blood samples were collected during the month of September to October. Table 1 shows the main characteristics of the both groups. The study was approved by the local ethical committee. Before the collection of blood sample, all the subjects had to sign on the consent form.

Study design: The alkaline single cell gel electrophoresis assay was carried out according to technique given by Singh et al. (1988), with slight modification to the original technique. To avoid possible bias, blood samples were coded. To study the DNA damage, blood samples were collected from exposed and controlled groups. A total of 140 blood samples were taken from finger trip in a heparin zed glass capillary for the comet assay. Samples from the both groups were transported on ice to the laboratory and processed within two hours. Cells viability was determined by tryptophan blue exclusion technique. Slides were prepared in duplicate for each person. Fully frosted microscopic slides were covered with 140 µL of 0.75% normal melting agarose (40°-42°C). After application of a cover slip, slides were allowed to gel at 4°C for 10 minutes. An aliquot of 20 µL of whole blood was then added to 0.5% of 110 µL of low melting point agarose (37°C). After carefully removing the cover slips, a second layer of 110 µL of sample mixture was pipette onto the pre-coated slides and allowed to solidify at 4°C for 10 minutes, with the coverslip in place. Again the coverslip was removed and a third layer of 110 µL of low melting point agarose (LMPA) was pipette onto the slides and allowed to gel at 4°C for 10 min. The slides (without cover slips) were immersed in freshly prepared, cold lysing solution (2.5 M NaCl, 100 mM Na, EDTA, 10 mM Tris-HCL, pH 10, 1% sodium N-lauroyl sarcosinate, 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 minutes 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 minutes each of neutralization buffer (0.4 M Tris HCL, pH 7.5). DNA was precipitated and slides were dehydrated in absolute methanol for 10 minutes 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 (20µ/mL) and viewed under a fluorescence microscope. Analysis was performed using a 400x objective with a Leica opt phase 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 was screened per subject (25 cells from each slide). Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet. The length of DNA migration in the comet tail, which is an estimate of DNA damage was measured using an ocular micrometer. Quantification of the DNA damage for each cell was calculated as: comet tail length (µm) = (maximum total length)-(head diameter).

Statistical analysis: Mean and standard deviation (means) were calculated for each parameter studied. The statistical analysis of differences in DNA damage, as measured by the comet assay, was carried out using *t*-test. The selection of *t*-test was made after finding variables to lie in the normal distribution curve. χ^2-test and multifactor analysis of variance (ANOVA) were used to check the significant differences. Correlation between different variables was determined by Spearman rank correlation test. The critical level for rejection of the null hypothesis was considered to be a p-value of 5%. All analysis was performed with the SPSS 10.0 version software packages.

RESULTS

The effect of occupational exposure to pesticides on the levels of DNA damage in leukocytes of pesticide produc-

tion workers and control subjects was assessed by the comet assay. Table 1 represents the distribution of subjects with respect to sex, age, smoking and years of exposure. The mean age of the subjects in the control group and in the group exposed to pesticides was almost similar (26.26±7.47, 28.93±9.49). The gender distribution and smoking habit was similar (Table 1). The mean comet tail lengths in leukocytes of exposed workers and the control subjects using the comet assay are summarized in Table 2 and Table 3.

The exposed workers had a significantly greater mean DNA tail length than the controls (P=0.001). The smokers had a slightly greater mean tail length than the non-smokers (P<0.024). Although those who were older, had a longer mean tail length than those who were younger and men had a longer mean tail length than women, while the differences were not significant in pesticide-exposed workers. A slight difference was observed between workers with <10 and those with ≥ 10 years exposure (69.48±20.15, 62.75±21.97, p=.0321) (Table 2). Age and gender showed significant effects on DNA in control persons only (Table 3). Table 4 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 4. The results of ANOVA, when age was included as a covariate, are summarized in Table 5. The effects of occupational pesticide exposure on DNA damage were quite significant (P<0.05).

DISCUSSION

The investigation was conducted to evaluate the genetic damage in the workers employed in the pesticides spraying operation in the agricultural field, utilizing the comet assay. The result shows that occupational exposure of the mixture of pesticides induced a significant increase in the level of DNA damage in the Kolgochia area of Assam.

Assessment of the level of DNA damage in 70 farmers of Kolgochia area of Barpeta district was compared with the 70 control subjects. The investigations on genotoxic potential of workers handling pesticides using comet assay are few. The assay was used to quantify the level of DNA damage in mononuclear leucocytes, conducted in agricultural areas exposure to pesticides. The French farmers who were occupationally exposed to a number of pesticides, showed a significantly high amount of genetic damage (Lebailly et al. 1998a). In another study by the same authors, the comet assay was used to assess DNA damage in the farmers. The result showed an increase in the genetic DNA damage level after one day spraying with a pesticide mixture (Lebailly et al. 1998b).

Evaluation of genetic DNA damage of workers involved in the production of a variety of pesticides using the comet assay revealed an increase in DNA damage in the peripheral blood lymphocytes (Garaj-Vrhovac & Zeljezic 2000). Similar study of DNA damage in Croatian workers occupationally exposed to a complex mixture of pesticides showed an increase in the values of the comet tail length parameter (Garaj-Vrhovac & Zeljezic 2001). There are few studies in which no significant increase in DNA damage in exposed workers in comparison to control was found, which could be due to the difference in working conditions like use of varied quality of protective equipments and variable duration of exposure etc. In another study it was found that lack of protective measures by the workers increased the amount of DNA damage or genotoxic damage. Since DNA damage is an important step in the events leading carcinogenic pesticides exposed to cancer, comet assay in the peripheral lymphocytes is an important potential risk evaluation in the monitoring of cancer. There are many other studies performed on the agricultural workers exposed to a variety of pesticides which examined genotoxicity in different ways (Nehez et al. 1988, Rupa et al. 1989, Munnia et al. 1999). However, some studies showed negative result also (Carbonell et al. 1990, Gomez-Arroyo et al. 2000).

Since the potential genotoxic effect may be due to the cumulative effect of all or some of the pesticides, it is not possible to attribute the damage to any particular agent. The result of such type of study on the subjects occupationally exposed to pesticides using a variety of genotoxic assays, suggest that the mixture of pesticides in long term occupational exposure affected the DNA of somatic cells. The detected DNA damage is 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 crosslink's. Occupational exposure to xenobiotics may result in their covalent binding to DNA, which may lead to chromosome alterations and could be an initial event for 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 their mechanisms such as oxidative stress or inflammatory processes (Lebailly et al. 1998b). Again, increased xenotoxicity is found in individuals occupationally exposed to pesticides in related to cancer risk and genetic illness. Agricultural chemicals may induce individual genetic variability in the enzymes, which metabolize xenobiotics, when there is no effect of detoxification; the metabolic sub-products accumulate, contributing to the turmorigenic process

Table 1: Demographic characteristic of study population with respect to age, sex, smoking and year of exposure.

Parameter	Exposed subjects (n= 70)	Control subjects (n= 70)	t, p and χ^2 value
Age (mean±SD)	28.93± 9.49	26.26±7.47	t=1.0164 p=0.3184
Year of exposure(Mean±SD)	9.4±3.29		
Gender			
Male	42(60%)	47(67%)	χ^2=0.7711
Female	28(40%)	23(33%)	p=.0.379879
Smoking			
Smoker	56(80%)	51(73%)	χ^2=0.9912
Non-smoker	14(20%)	19(27%)	p=0.319444

Table 2: Mean comet tail length in pesticides workers (exposed) according to smoking, exposure, age and gender.

Parameter	No. of subjects N=70	Comet tail length (Mean±SD)(µm)	t and p value
Smoking			
Smokers	56	64.98±21.51	t=5.2543
Non-smokers	14	55.02±17.91	p=0.001
Year of exposure			
<10	23	63.48± 20.15	t=2.3223
≥10	47	62.75±21.97	p=0.0321
Age			
<35	56	57.05±17.03	t=2.8189
≥35	14	86.73±18.24	p=0.0100
Gender			
Male	42(60%)	69.10±21.22	t=2.6956
Female	28(40%)	53.83±17.49	p=0.0159

Table 3: Mean comet tail length in control persons according to smoking, age and gender.

Parameters	No. of subjects	Comet tail length (Mean±SD) (µm)	t and p value
Smoking			
Smokers	51	31.09±29.03	t=3.2593
Non-smokers	19	6.5±1.29	p=0.0039
Age			
<35	60	28.91±28.67	t=3.3731
≥35	10	5.5± 0.70	p=0.0027
Gender			
Male	47	33.2±29.70	t=3.1224
Female	23	7.2±1.92	p=0.0059

Table 4: Effect of pesticides and smoking on DNA mean comet tail length.

Parameters	No.of subjects (Non-smoker)	Comet tail length (Mean±SD)(µm)	No.of subjects (Smokers)	Comet tail length (Mean±SD)(µm)	t-test smokers versus non-smokers
Controls	19	6.5±1.29	51	31.09±29.03	t=3.2593 p=0.0039
Pesticide Exposed	14	55.02±17.91	56	64.98±21.51	t=5.2543 p=0.001
t-test exposed versus control	t=2.2297 p=0.0673		t=3.4918 p=0.0021		

Table 5: Comet parameters in 70 freshly exposed cases showing DNA damage in relation to duration of exposure, smoking, drinking and dietary habits.

Parameters	No. of Subjects (%)	Frequency of cells showing migration Mean±SD	Anova P=value	Mean tail length(Mean±SD)	Anova P=value
Duration (Years)					
≤10	23(33.33%)	20.73±20.23	F=0.51793	63.48±20.15	F=21.44
>10	47(67.14%)	31.07±23.81	P=0.54641 Not significant at <0.05	62.75±21.97	P=0.0436 Significant at <0.05
Age(Years)					
≤30	47(67.14%)	26.20±14.20	F=0.23529	53.36±15.86	F=2.98166
>30	23(33.33%)	32.12±19.12	P=0.6755 Not significant at <0.05	82.25±15.21	P=0.226355 Not significant at <0.05
Smokers	56(80%)	26.00±20.12	F=0.05009	64.98±21.51	F=1.30136
Non-smokers	14(20%)	33.54±25.05	P=0.82924 Not significant at <0.05	55.02±17.91	P=0.372156 Not significant at <0.05
Alcoholics	47(67.14%)	31.45±21.6	F=0.08492	64.14±22.31	F=4.92568
Non- alcoholics	23(33.33%)	32.60±12.60	P=0.79818 Not significant at <0.05	60.7±19.04	P=0.156662 Not significant at <0.05
Vegetarian	28(40%)	27.21±21.40	F=1.46701	67.86±24.14	F=12.06154
Non-vegetarian	42(60%)	26.20±20.04	P=0.349513 Not significant at <0.05	59.74±18.77	P=0.073842 Not significant at <0.05
With protective measure	23(32.85%)	30.09±30.7	F=0.59172	69.71±9.17	F=4.97633
Without protective measure	47(67.14%)	21.40±10.2	P=0.522182 Not significant at <0.05	59.63±24.26	P=0.155419 Not significant at <0.05

(Hodgson et al. 1991).

No significant increase in DNA damage with some factors like age, smoking, drinking, dietary habit were found in our results. Similarly, no significant relationship was found between the DNA damage in the workers and smoking habit or drinking habit.

It can be concluded that pesticides did cause DNA damage along with the duration of exposure. The confounding factor including age, smoking and diet were expected to modulate the genotoxic effect of xenobiotic, but not significantly in the absence of any positive correlation between these factors. The comet parameter suggested that the DNA damage was probably caused by carcinogenic pesticides which were sprayed in the agricultural fields by the farmers.

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