Cytogenetic analysis of Pakistani individuals occupationally exposed to pesticides in a pesticide production industry

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Although several cytogenetic biomonitoring studies on workers exposed to pesticides have been reported, there is only limited information on this topic from developing countries where pesticides have been widely used over the years. People in developing countries are at higher risk from exposure, due to poor working conditions and a lack of awareness of the potential hazards during manufacturing and application of the pesticides. The present study has assessed the genotoxic effects of pesticides on workers involved in the pesticide manufacturing industry. Subjects in the exposed group (29) were drawn from workers at a pesticide production plant in district Multan (Pakistan). The control group (unexposed) composed of 35 individuals from the same area but was not involved in pesticide production. Liver enzymes, serum cholinesterase (SChE), microrunon assay and some haematological parameters were used as biomarkers in this study. A statistically significant ($P < 0.001$) increase in levels of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase was detected in exposed workers with respect to the control group. There was a significant ($P < 0.001$) decrease in the level of SChE in the exposed group. Exposed individuals exhibited cytogenetic damage with increased frequencies ($P < 0.001$) of binucleated cells with micronuclei and total number of micronuclei in binucleated lymphocytes in comparison with subjects of the control group. A decrease ($P < 0.001$) in cytokinesis block proliferation index similarly demonstrates a genotoxic effect due to pesticide exposure. The results indicate that the pesticide industry workers have experienced significant genotoxic exposure. This study highlights the risk to workers in the pesticide manufacturing industries of developing countries such as Pakistan and the need for implementation of suitable safety measures to prevent/limit exposure to harmful toxins.

Introduction

Pesticides constitute a heterogeneous category of chemicals specifically designed for the control of pests. Their application is still the most effective and accepted means for the protection of plants from pests, and has significantly contributed to the enhanced agricultural productivity and crop yield (1). In recent years the use of pesticides in agriculture has been increasing steadily. At present there are more than 1000 chemicals classified as pesticides (2). A total of about 890 active ingredients are registered as pesticides in USA and currently marketed in some 20 700 pesticide products (3). Because large amounts of these chemicals are released into the environment daily and many of them affect non-target organisms, they may represent potential hazard to human health. Many of these compounds because of their environmental persistence will linger in our environment for many years to come (1).

Pesticides are released most frequently into the environment because of their widespread use in public health and agriculture. Despite the beneficial effects associated with the use of pesticides, many of these chemicals may pose potential hazards to man and environment. The use of these agricultural chemicals without necessary protection may lead to alterations in the genetic material and the possible development of some types of tumors.

Occupational exposure to pesticides has been associated with several neoplastic diseases. In particular a significant increase was found in the incidence of soft tissue sarcoma (4), Hodgkin’s lymphoma (5), multiple myeloma (5,6), bladder and pancreatic cancer (7), leukaemia and non-Hodgkin lymphoma (8,9), reproductive problem (10) and more recently the incidence of Parkinson disease (11,12). Although pesticides act selectively against certain organisms without adversely affecting others, absolute selectivity is difficult to achieve.

Organophosphate pesticides are cholinesterase inhibitors and are extensively used in agriculture and community health programmes. Cholinesterase inhibition is an indirect indicator used to monitor organophosphates exposure (13). They can also cause hepatotoxicity (14). Studies on organochloride insecticides in animals and humans have detected hepatotoxicity at high and low doses, which might be linked to hepatic enzyme induction (15).

Biomonitoring studies using somatic cells have been extensively conducted to evaluate the possible genotoxic risk of a defined exposure and some indicators, such as chromosomal aberrations, have been shown to be a relevant biomarker for further cancer incidence (16). In addition, the use of appropriate biomarkers in these biomonitoring studies can provide useful tools to elucidate the mechanisms of action of the exposure. A certain number of field studies have been carried out, obtaining an association between the occupational exposure to pesticides and the presence of chromosomal aberrations as a factor which increase the cancer risk (17–19). In biomonitoring studies the use of micronucleus assay in peripheral lymphocytes is increasing as a useful technique to screen chromosomal aberrations and to detect clastogenic and aneuploidogenic agents (20,21).

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

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exposed workers from different countries (22). There are only a few reports of health effects due to chronic occupational exposure to pesticides in developing countries. People in developing countries are at higher risk for chronic exposure to these chemicals because of poor working conditions and an unawareness of the potential hazards in manufacturing and application of these insecticides (23).

Main aim of the present study was to assess the genotoxic effects of pesticides in workers occupationally exposed to these chemicals at a pesticide production plant in Pakistan.

Materials and methods

Population

A total of 64 subjects (29 exposed and 35 unexposed) from Multan district of the Punjab Province of Pakistan were included in this study. The exposed group (29 men) consisted of workers from a pesticide production industry. This industry was involved in the manufacturing of a wide range of pesticides, specifically organophosphates and pyrethroids. All members of this group were regularly exposed to complex mixtures of pesticides. All participants signed a written consent and answered a standard questionnaire covering standard demographic questions (age, gender, etc.), as well as the questions pertaining to medical history (genetic disorders, vaccination, medication, etc.), smoking habit and occupational exposures (working hours/day, years of exposure, etc.). For smoking habit, individuals were divided into three groups, i.e. smokers, ex-smokers and non-smokers. Individuals who had left smoking in last 1–5 years were categorized as ex-smokers and those who never smoked or had left this habit in last more than 5 years were placed in non-smokers category. Unexposed group was composed of 35 men from same area but were not involved in pesticide production.

Sample collection

Ten millilitres of blood sample per individual was collected using sterile disposable syringes. Five millilitres of the collected blood was transferred to sterile heparin vacutainers to prevent from clotting and to use for DNA studies. Remaining 5 ml was left in the same disposable syringe and was allowed to clot to obtain serum for enzyme analysis. Samples were subjected to haematological studies immediately after collection.

Haematological study

Freshly collected blood samples were analysed for haematological assay using an automatic analyser Sysmex-KX21, Japan. Different tested haematological parameters were as follows: haemoglobin (Hb), the measure of the total Hb g/dl of blood, haematocrit (HCT), the measurement of the percentage of red blood cells (RBCs) in whole blood and total RBC count, total number of RBC per µl of blood. Moreover, cell indices, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and MCH concentration (MCHC), were also calculated from the data obtained. MCH gives the average weight of Hb in single RBC. MCV reflects the size of RBCs by expressing the volume occupied by a single RBC. MCHC measures the average concentration of Hb in RBCs.

Enzyme analysis

Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and serum cholinesterase (SChE) were checked in RBCs.

Lymphocyte cultures and micronuclei analysis

Blood samples from each subject were coded and processed within 24 h. 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 1% l-glutamine (all obtained from Gibco, UK). Lymphocytes were stimulated by 1% phytohaemagglutinin (PHA; Gibco) and incubated for 72 h at 37°C. Two cultures per subject were established. A cytochalasin B (CyB-B) solution was prepared in dimethylsulphoxide at a final concentration of 6 µg/ml (24) and added to the culture after 44 h incubation to arrest cytokinesis. After 72 h of incubation, cultures were harvested by centrifugation at 800 r.p.m. for 8 min. In order to eliminate RBCs and to preserve lymphocyte cytoplasm cells were treated with a hypotonic solution (2–3 min in 0.075 M KCl at 4°C). Cells were centrifuged 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, Germany) in phosphate buffer (pH 6.8) for 10 min and then scored. Two slides for each sample were prepared.

To determine the frequency of binucleated cells with micronuclei (BNNM) and the total number of micronuclei in binucleated lymphocytes (MNL), a total of 1000 binucleated cells (500 per replicate) with well-preserved cytoplasm were scored per subject on coded slides. This number of the cells usually scored in most of the laboratories (25). In addition, 500 lymphocytes were scored to determine the percentage of cells with 1–4 nuclei and the cytokinesis block proliferation index (CBPI) was calculated according to Surralles et al. (26) by using the following formula:

$$\text{CBPI} = \frac{\text{MI} + 2\text{MII} + 3(\text{MIII} + \text{MIV})}{\text{Total number of cells}}$$

To minimize the variability, the same researcher carried out all the microscopic analysis.

Statistical analysis

The results were statistically analysed using Mann–Whitney U-test.

Results

Table I shows the main characteristics of the population studied. There was no difference in the age of the two groups (Mann–Whitney U-test, P > 0.05). Age in control group ranged from 29 to 42 years (mean 35.20 ± 3.52) whereas the age in exposed group was in the range of 27–39 years (mean 34.17 ± 2.96). Workers exposed to pesticides had an exposure period of 3–18 years (13.48 ± 3.84). Regarding smoking habit, the population studied was divided into three subgroups, i.e. smokers, ex-smokers and non-smokers. While interpreting the results smoking habit was kept in consideration to describe any possible interference of tobacco. About half of the individuals in control group were non-smokers (51%), whereas in exposed group they were 38%. Exposed group had more number of individuals in smokers’ category (52%), which were 29% in control group. Higher number of individuals suffered from allergy and dermatitis, i.e. 59 and 41%, respectively, in exposed group as compared to control group where they were 26 and 14%, respectively. Problem of conceiving was relatively higher in exposed individuals than the control.

Table II shows the mean values ± SD for different haematological parameters. Except MCH and MCHC all other values differed significantly in exposed and unexposed individuals at different probability levels in Mann–Whitney U-test. For adult males, reference value for Hb ranges from 13.5 to 17.5 g/dl. The mean ± SD of Hb in exposed group was 13.79 ± 0.81. In control group this value was 14.33 ± 0.56. These Hb values differed significantly in two groups at a probability level

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Exposed</th>
<th>Unexposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>29</td>
<td>35</td>
</tr>
<tr>
<td>Years of pesticides exposure</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Smokers</td>
<td>15 (52)</td>
<td>10 (29)</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>3 (10)</td>
<td>7 (20)</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>11 (38)</td>
<td>18 (51)</td>
</tr>
<tr>
<td>Suffer from allergy</td>
<td>17 (59)</td>
<td>9 (26)</td>
</tr>
<tr>
<td>Suffer from dermatitis</td>
<td>12 (41)</td>
<td>5 (14)</td>
</tr>
<tr>
<td>Have difficulty in conceiving</td>
<td>4 (14)</td>
<td>3 (9)</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>35</td>
</tr>
</tbody>
</table>
Table II. Haematology of group studied (mean ± SD)

<table>
<thead>
<tr>
<th>Haematology</th>
<th>Exposed</th>
<th>Unexposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dl)</td>
<td>13.79 ± 0.81ab</td>
<td>14.33 ± 0.56</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>41.93 ± 1.44b</td>
<td>42.54 ± 1.48</td>
</tr>
<tr>
<td>RBC (million/μl)</td>
<td>4.94 ± 0.54c</td>
<td>5.35 ± 0.25</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>28.24 ± 3.45d</td>
<td>26.86 ± 1.73</td>
</tr>
<tr>
<td>MCV (μm³)</td>
<td>85.76 ± 8.67b</td>
<td>79.73 ± 4.97</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>32.91 ± 2.09d</td>
<td>33.74 ± 1.92</td>
</tr>
</tbody>
</table>

The two samples are significantly different at probability level.

*aP < 0.01.

*bP < 0.05.

*cNo significance difference in two samples (P ≥ 0.05).

Table III. Enzyme analysis of studied groups

<table>
<thead>
<tr>
<th>Enzyme (IU/l)</th>
<th>Exposed</th>
<th>Unexposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>55.40 ± 9.86a</td>
<td>36.97 ± 7.63</td>
</tr>
<tr>
<td>AST</td>
<td>61.10 ± 17.26c</td>
<td>42.26 ± 11.09</td>
</tr>
<tr>
<td>ALP</td>
<td>29.08 ± 30.57a</td>
<td>246.52 ± 29.55</td>
</tr>
<tr>
<td>SChE</td>
<td>8174.59 ± 2437.41a</td>
<td>11 715.79 ± 1608.76</td>
</tr>
</tbody>
</table>

The difference between the two samples is significantly high (P < 0.001).

P < 0.01. Standard reference range of HCT in adult males is 40–54%. Level of HCT also differed in two groups at a probability level P < 0.05. The RBC count was significantly higher (P < 0.001) in control as compared to exposed group. Normal reference range for total RBC count is 4.2–5.6 million/μl. Among cell indices, there was no difference in MCH and MCHC between exposed and control groups and the value of these two indices were within the normal reference range (MCH = 27–31 pg, MCHC = 32–36 g/dl). MCV was also within the reference range in both groups (MCV = 80–100 μm³); however, it was significantly lower (P < 0.05) in the exposed than the control group.

Table III shows the results of serum enzymes analysis. Enzymes are used as very important biomarkers in biomonitoring studies. SChE was found to be significantly lower (P < 0.001) in exposed group (8174.60 ± 2437.40) as compared with control group (11 715.80 ± 1608.76). Baseline value for SChE in the population of studied area was recorded as 12 819.32 ± 324.72. Baseline value was calculated by detecting the SChE level in 150 individuals from different localities of the same region. From the results of SChE values it was observed that among exposed individuals 75.86% had values below the normal baseline while among control individuals 20% were below this line. The normal reference range of ALT is 46 IU/l. The value of ALT was significantly higher (P < 0.001) in exposed individuals (55.40 ± 9.86) as compared with control group (36.97 ± 7.63). Considerable number (20.68%) of individuals of exposed group had ALT above the reference range as compared to control group (5.71%). The AST values in control and exposed groups differed significantly (P < 0.001). Exposed group had higher values for AST (61.11 ± 17.25) as compared with control group (42.27 ± 11.09). Large number of exposed individuals (72.41%) had AST outside the reference range when compared with control (25.71%). Normal upper threshold value for AST is 46 IU/l. Analysis of the ALP showed that most of the exposed workers had higher ALP value (290.86 ± 30.58) as compared with unexposed group (246.53 ± 29.56). Significant number (65.51%) of exposed individuals had AST values outside the reference range than unexposed individuals (14.28%). Normal upper limit for ALP is 280 IU/l. Values of ALP also differed significantly (P < 0.001) between exposed and unexposed groups. Figure 1 represents the percentage of individuals with abnormal values for the tested serum enzymes.

A summary of the mean data of cytogenetic variables studied and the CBPI is given in Table IV. The mean ± SD for BNMN, MNL and CBPI for control group were 6.11 ± 1.95, 9.03 ± 2.46 and 1.71 ± 0.26, respectively. The corresponding values for the exposed group were 12.62 ± 1.47, 20.41 ± 4.63 and 1.26 ± 0.21, respectively. Values for both BNMN and MNL were significantly higher (P < 0.001) in exposed group than the unexposed group, while CBPI of exposed group was significantly lower (P < 0.001) than the unexposed group. To highlight possible effects of smoking, values for cytogenetic analysis were also expressed in separate subgroups of smokers, ex-smokers and non-smokers. In general, smokers and non-smokers of exposed group presented significantly higher
A linear correlation between duration of exposure to pesticides and BNMN and MNL values was observed (Table V). The exposed group had an exposure period ranging from 3 to 18 years (mean 13.48 ± 3.84). Maximum values were observed in the group of individuals with more than 15 years of exposure. Proliferation index was minimum in the individuals with maximum exposure time.

Discussion

The main objective of this study was to detect that whether the exposure to pesticides mixture in a pesticide manufacturing industry induces increase in the levels of cytogenetic damage. The study was carried out simultaneously in exposed and a control group, both from the same area with similar individual characteristics. In this study, a battery of molecular biomarkers was used to evaluate a complete range of damage to the exposed individuals.

As a preliminary study towards cytogenetic damage, haematology was evaluated for both the groups. A significant difference was observed for the studied parameters in these groups.

In this study, experimental group was exposed to a variety of chemical substances of organophosphate and pyrethroid nature. Liver and nervous system enzymes were used as important biomarkers for detection of hepatotoxic and neurotoxic nature of these pesticides. Four enzymes, three hepatic (ALT, AST and ALP) and one nervous system (SChE), were evaluated. All the evaluated variables differed significantly in both the groups at a probability level \( P < 0.001 \). Change in ALT, AST and ALP may indicate the liver damage and disruption of liver function. Our findings indicate that liver damage may occur in the workers exposed either to high levels of toxic substances or to low doses of these substances for a longer period or in the workers not complying with rules for using personal protection equipments. These results are also supported by some other studies conducted on the same classes of chemicals. An increase in ALP confirms the presence of hepatic damage (27). A significant rise in AST occurs after chronic exposure to pesticides (28). It was found that administration of Fenthion (an organophosphate) results in the \textit{in vitro} and \textit{in vivo} increase in serum enzymes including ALT, AST and ALP (29). SChE is an enzyme responsible for the degradation of neurotransmitter, acetylcholine, which if persists in the synapse can impair the normal functioning of nervous system. SChE level differed significantly in exposed and control group.

This low level of cholinesterase is typically due to the exposure to organophosphate pesticides which cause irreversible inhibition of the enzyme and making it unable to degrade acetylcholine. This fact has also been reported by other workers (28,30–32). The decrease in SChE level was found higher in workers with longer exposure time than workers with shorter exposures. Pesticide production in Pakistan is a year-round activity. Pesticide industry workers log in 8 h/day, 6 days/week. During working hours, the workers are constantly exposed to a variety of chemicals of pesticide/insecticide nature (J.A. Bhalli, personal communication). Within the same industry, workers employed in different sections were having different levels of SChE depending upon the chances of chemical exposure. Selected individuals were involved in three different sections of the industry. Out of total 29 individuals selected for this study as experimental group, 16 were employed in formulation and mixing section, 7 in packing section, 2 were inspectors of these sections and 4 were employed in administrative section. Those workers, who were in direct contact with pesticides in formulation and mixing, packing and inspection sections, had a significantly lower mean SChE level as compared to the indirectly exposed individuals who were in administrative jobs. These results are confirmed by some other similar studies (33). To evaluate the chromosomal damage, cells of peripheral blood lymphocytes were chosen which are the most common cell targets used for human biomonitoring purposes. These cells have been classically used for detecting genotoxic effects in a large number of studies as they are considered to be adequate for detecting general exposure (22). In addition, these cells are in non-proliferative stage (\( G_0 \)) and have a long half-life, which is about 3 years (34,35).

Results obtained from this study indicate that there was a significant increase in the BNMN and MNL values of exposed group as compared to control. Other workers have also confirmed the induction of cytogenetic damage after exposure to pesticides (36–39). However, in contrast, some studies have shown no apparent change in BNMN and MNL after exposure to pesticides, indicating lack of chromosomal damage related to pesticide exposure (40–44). While considering this difference of results in different studies it must be kept in consideration that in various biomonitoring studies populations have varied response to the pesticide exposure. It may be due to the facts that in different areas variable groups of pesticides are used depending upon the environmental factors and the type of crops. In addition, working conditions are generally different, protective measures taken differently and in some places not at all. The environmental conditions can also influence the absorption kinetics of chemicals by the human body. But above all and the most important factor is the studied population. Studies where no significant difference between exposed and control individuals is reported are on pesticides sprayers.
and field workers, whereas those which show difference in these two groups are on both field workers and pesticides manufacturers. Majority of the studies on pesticide production workers are in accordance with the present findings (37,45–48). Furthermore, the actual pesticide exposure is highly influenced by the protective measures used by the pesticide production workers (22). During the working hours, individuals of exposed group in this study were not using appropriate protective measures (J.A. Bhalli, personal communication).

While interpreting the results for BNMN and MNL, some other confounding factors like smoking habit and exposure time were also analysed. The smoking has an additive effect on the frequency of BNMN and MNL, which is reflected by the higher values of BNMN and MNL in lymphocytes of smokers than the non-smokers. In our study we observed that smokers had higher MNL and BNMN values than ex-smokers and non-smokers of the same group either exposed or unexposed. These findings are in accordance with some previously published studies (49). However, smokers and non-smokers of exposed group presented significantly higher ($P < 0.001$) MNL and BNMN values than their corresponding control subgroup. This increase in cytogenetic damage may be attributed to the pesticide exposure. The linear increase in BNMN and MNL values was also recorded with increase in pesticide exposure time. These results are in accordance with the findings of other workers (36). The difference of BNMN and MNL in ex-smokers of the two groups (exposed and control) was marginally significant ($P < 0.05$). This may be due to the very small sample size of ex-smokers in exposed and control groups, i.e. 3 and 6, respectively. There was a decrease in CBPI value of exposed group as compared to the control. This reduction in the proliferation index of lymphocytes of the group exposed to pesticides suggested that the studied individuals were exposed to chemicals with cytotoxic properties, which affected the cell proliferation kinetics (50,51). A decrease in mitotic index of pesticides exposed individuals is also reported by other workers (34). There was a highly significant difference ($P < 0.001$) in CBPI values for smokers and non-smokers within and between the exposed and control groups. A significant difference was also observed in relation to pesticides exposure. There was a decrease in CBPI with the increase in exposure time. This decrease in CBPI is also confirmed by other studies (44).

In summary, this work shows that there is an increase in ALT, AST and ALP enzymes associated with liver and a decrease in SChE in individuals working in a pesticide manufacturing industry. An increase in BNMN and MNL frequencies indicate the cytogenetic damage in industrial workers. Finally, a decrease in CBPI values in exposed workers again indicates the genotoxic effect of pesticide exposure. Our data highlight the importance of primary preventive measure to avoid pesticide exposure and health surveillance, with particular attention paid to the liver function.

An important outcome of our study is that the management of the pesticide plant where we conducted this work has started implementing the safety measures to prevent/limit pesticide exposure to their workers.

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References


Genotoxic effects of pesticides


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