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TOXIC NUCLEAR EFFECTS OF THE ORGANOPHOSPHORUS INSECTICIDE DICHLORVOS (DDVP) IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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Organophosphorus insecticides are used worldwide in the control of agricultural, household and veterinary pests. Dichlorvos (2,2-dichlorovinyl dimethyl phosphate) is a commonly used organophosphorus insecticide. In this study, in order to elucidate the toxic nuclear effects of dichlorvos, freshly isolated human peripheral blood lymphocytes were incubated with 5, 10, 20, 40, 80 and 100 µg/mL of dichlorvos. According to the results, dichlorvos induced micronuclei, decreased the mitotic and replication indexes. It is a genotoxic product causing chromosomal damage (an increase in micronucleus) and cell death (decrease in mitotic and replication indexes).

Keywords: Dichlorvos – toxicity – micronucleus – mitotic index – replication index

INTRODUCTION

Organophosphorus (OP) insecticides are used worldwide in the control of agricultural, household and veterinary pests. OP compounds exert acute toxic effects which are mainly due to the suppression of neuronal acetylcholinesterase activity [22]. The widespread uses of OP insecticides indicate the extensive availability and potential for accidental and intentional human exposure [5].

Dichlorvos (DDVP) is an OP insecticide that has been in use for more than 40 years. It has been evaluated in a wide range of toxicology assays including bioassays for cytotoxicity, genotoxicity, carcinogenity and mutagenity [1]. The oral LD_{50} for DDVP is 61–175 mg/kg in mice, 100–1090 mg/kg in dogs, 15 mg/kg in chickens, 25–80 mg/kg in rats, 157 mg/kg in pigs, and 11–12.5 mg/kg in rabbits [9, 11]. DDVP primarily affects the nervous system through cholinesterase inhibition, i.e. the

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blockage of an enzyme required for proper nerve functioning. In addition, the EPA has classified it as toxicity class I (highly toxic), because it may cause cancer and there is only a small margin of safety for other effects. DDVP has been classified as a possible human carcinogen because it caused tumors in rats and mice in some studies [6–7]. The cytotoxicity evaluations have included a wide range of test systems and endpoints, including assays both *in vivo* and *in vitro*. There is general agreement that DDVP is cytotoxic *in vitro* [1].

The micronucleus (MN), mitotic index (MI) and replication index (RI) analysis methods are cytogenetic tests used both *in vivo* and *in vitro*. Micronuclei are formed as a result of chromosomal non-disjunctions after genotoxic damage and are a very sensitive index of genetic damage. The MI assay is used to characterize proliferating cells and identify compounds that inhibit or induce mitotic progression. RI measures cell division kinetics by counting the percent of cells containing 1, 2, 3 or more nuclei per individual [29].

In this study, the *in vitro* toxic nuclear effects of DDVP using as OP insecticide were investigated in human peripheral lymphocytes.

MATERIALS AND METHODS

Chemicals

Dichlorvos (2,2-dichlorovinyl dimethyl phosphate-DDVP) (Agrofarm), a yellow liquid, is formulated for the use as an insecticide. Some of DDVP doses (5, 10, 20 and 40 μg/mL) used in our study was prepared as described earlier by Dean [3]. Two high doses (80 and 100 μg/mL) were added. Peripheral blood (PB) karyotyping medium (Biological Industries), Colcemid (Sigma) and Giemsa staining (Merck) were used in peripheral blood culture. PB karyotyping medium is based on RPMI-1640 basal medium supplemented with L-glutamine, foetal bovine serum, antibiotics (gentamycin) and phytohemagglutinin (PHA-M).

Sample preparation for MN, MI and RI assay

After getting approval from Local Ethic Committee, heparinized blood samples (0.4 mL), obtained from healthy donors, were placed in sterile culture tubes containing 5 ml of PB karyotyping medium. The culture tubes were incubated at 37 °C for 72 h. After a culture period of 24 h, DDVP concentrations (5, 10, 20, 40, 80 and 100 μg/mL) were added to each culture tubes. After a culture period of 70 h, 0.1 mL colcemid solution (10 μg/mL) was added to each culture tubes. After incubation for 72 h, cells were harvested by centrifugation, by giving hypotonic treatment (0.075 M KCl) and they were fixed in fresh fixative solution (methanol : acetic acid, 3 : 1). This fixation step was repeated for three times. Slides were air-dried and stained with 5% Giemsa staining for 6.5 min.

Staining and examination of micronucleus

The slides were immersed in 5N HCl for 20 min at room temperature. After washing with tap water, the slides were kept in Schiff solution (250 mg basic fuchsin is melted in boiling water and cooled to 50 °C. After 250 mg sodium-bisulphate was added, the color should change to red wine color) for 90 min in the dark. The slides were treated with sodium bisulphite for 2 min thrice. The slides were washed with tap water thrice, 30 min each time, stained for 40 min in 5% fast green solution (250 mg fast green is weighted and completed to 50 mL with 95% ethanol) and washed with tap water to remove extra stain.

The slides were randomized and scored by a single observer. About 500 cells were examined at 600 check magnification from each slide and when MN cells were located they were examined under 1000 magnification. The criteria suggested by Scarpato [24] for recognizing micronuclei were followed. Dead or degenerating cells were excluded from evaluation. Nuclear blebbing (MN-like structure connected with the main nucleus with a bridge) were not considered. Only micronuclei equal to or smaller than one-fifth of the main nucleus were considered. Multimicronucleated cells were also scored but not included in the evaluation of MN frequency.

Examination of MI and RI

MI was calculated as the proportion of metaphase for 1000 cells for each donor and concentration. A total of 500 cells were scored for the determination of the RI. RI was calculated by using the following formula: $RI = (1 \times M1 + 2 \times M2 + 3 \times M3)/500$, where M1, M2 and M3 are the cells containing 1, 2, 3 or more nuclei, respectively [29].

Statistical analysis

The computer software program SPSS 10.0 was used to analyze the data. The statistical significance of the effects of DDVP on MN, MI and RI was tested by repeated measures of analysis of variance (ANOVA) and differences between groups were determined by the least significant differences (LSD) test with $p < 0.05$ and $p < 0.01$ considered significant.

RESULTS

Six different concentrations (5, 10, 20, 40, 80 and 100 μg/mL) and three different parameters (MN, MI, RI) were evaluated to test the nuclear effects of DDVP on human peripheral lymphocytes *in vitro*.

The results of MN test are given in Table 1. Increasing doses of DDVP induced a statistically significant increase in MN formation compared with the control ($p < 0.01$, $p < 0.05$). These increases were dose-dependent. According to control, a significant increase was observed in the 5 μg/mL concentration range (4.10 ± 0.29) for MN. MN rate after addition of 5 μg/mL DDVP (4.10 ± 0.29) was approximately 3.5 times than in the control (1.25 ± 0.61) (Table 1). There are not differences among female and male donors ($p > 0.05$).

The effects of DDVP on MI and RI are summarized in Tables 2 and 3. The results showed that increasing doses of DDVP decreased MI and RI. According to the control group, MI rates of three DDVP doses (40, 80 and 100 μg/mL) were found as statistically significant ($p < 0.01$, $p < 0.05$). RI rates of four DDVP doses (20, 40, 80 and 100 μg/mL) were different from the control group ($p < 0.01$, $p < 0.05$) in Tables 2 and 3, respectively. According to the control, a significant decrease was observed in the

Table 1
Micronucleus (%) in human lymphocyte cultures exposed to DDVP

DDVP (μg/mL)	Female	Male	Mean MN ± SD
Control	1.05 ± 0.35	1.45 ± 0.91	1.25 ± 0.61
5	4.30 ± 0.14**	3.90 ± 0.28**	4.10 ± 0.29**
10	4.15 ± 0.07**	3.90 ± 0.28**	4.02 ± 0.22**
20	4.15 ± 0.35**	3.95 ± 0.35**	4.05 ± 0.31**
40	6.00 ± 0.14**	5.60 ± 0.28**	5.80 ± 0.29**
80	5.70 ± 0.70**	5.45 ± 0.07**	5.57 ± 0.43**
100	7.75 ± 0.49**	7.70 ± 0.70**	7.72 ± 0.49**

* Significantly different from control ($p < 0.05$)
** Significantly different from control ($p < 0.01$ and $p < 0.05$)
MN: Micronucleus
SD: Standard deviation

Table 2
Mitotic index (%) in human lymphocyte cultures exposed to DDVP

DDVP (μg/mL)	Female	Male	Mean MI ± SD
Control	4.59 ± 0.89	3.95 ± 0.45	4.27 ± 0.68
5	4.43 ± 0.54	3.72 ± 0.13	4.08 ± 0.52
10	4.33 ± 0.70	3.89 ± 0.17	4.11 ± 0.48
20	4.05 ± 1.31	2.95 ± 0.09**	3.50 ± 0.99
40	2.33 ± 0.29*	2.28 ± 0.07**	2.30 ± 0.17**
80	1.80 ± 0.09**	1.59 ± 0.07**	1.69 ± 0.14**
100	1.07 ± 0.06**	1.02 ± 0.08**	1.04 ± 0.06**

* Significantly different from control ($p < 0.05$)
** Significantly different from control ($p < 0.01$ and $p < 0.05$)
MI: Mitotic index
SD: Standard deviation

Table 3
Replication index in human lymphocyte cultures exposed to DDVP

DDVP (μg/mL)	Female	Male	Mean RI ± SD
Control	1.809 ± 0.114	1.823 ± 0.084	1.816 ± 0.082
5	1.732 ± 0.183	1.776 ± 0.027	1.754 ± 0.110
10	1.737 ± 0.196	1.708 ± 0.008*	1.722 ± 0.114
20	1.541 ± 0.027	1.501 ± 0.018**	1.521 ± 0.030**
40	1.433 ± 0.019*	1.505 ± 0.010**	1.469 ± 0.043**
80	1.241 ± 0.065**	1.233 ± 0.047**	1.237 ± 0.047**
100	1.098 ± 0.022**	1.108 ± 0.024**	1.103 ± 0.020**

* Significantly different from control ($p < 0.05$)
** Significantly different from control ($p < 0.01$ and $p < 0.05$)
RI: Replication index
$RI = (1 \times M1 + 2 \times M2 + 3 \times M3)/500$
M1: The number of cells in first metaphase
M2: The number of cells in second metaphase
M3: The number of cells in third or more metaphase
SD: Standard deviation

40 μg/mL concentration range (2.30 ± 0.17) for MI (Table 2) and in the 20 μg/mL concentration range (1.521 ± 0.030) for RI (Table 3). These decreases were dose-dependent ($p < 0.01$, $p < 0.05$). There are not differences among female and male donors ($p > 0.05$).

DISCUSSION AND CONCLUSIONS

DDVP is a widely used pesticide with high potential for human exposure. A number of pesticides are acutely clastogenic to the cell, because they affect to the cell divisions. DDVP has been examined in our study in a range of cell types for *in vitro* toxic effects (Table 4).

In living creatures, which are exposed to a mutagen factor, the probability of formation of mitotic and meiotic defects is increased and the rate of MN could increase due to this increase [19]. DDVP increased MN (Table 1). Alcohol consumption, smoking and viral infections increase MN rates in peripheral blood lymphocytes [25]. In this study peripheral blood samples were obtained from people who did neither smoke nor drink alcohol and had no viral infection.

MI and RI are used as indicators of adequate cell proliferation biomarkers. MI measures the proportion of cells in the M-phase of the cell cycle and its inhibition could be considered as cellular death or delay in the cell proliferation kinetics [21]. According to Tables 2 and 3, control and the lowest MI values are 4.27 ± 0.68 and 1.04 ± 0.06, respectively, and control and the lowest RI values are 1.816 ± 0.082 and 1.103 ± 0.020, respectively. The present results also show that RI values were significantly lower in DDVP-treated cells than in controls. This may be attributed to sev-

Table 4
Some cytotoxicity studies of DDVP

Cytotoxicity	Test(s)	Cells	References
+	MN	Chinese hamster lung cells	[11]
+	MN	Chinese hamster ovary cells	[12]
+	MN	Human lymphoblastoid cells	[13–14]
+	MI	Chinese hamster V79 cells	[15]
+	CA*	Human lymphocytes cells	[9]

*CA – Chromosomal aberrations

eral factors, one of which could be the fact that many cells, during the time remained in the circulation (at G0), suffered DNA damage and did not survive the first cell cycle in the culture to express MN, since they would have entered a process of necrosis or apoptosis before this event. Another factor to be considered could be that mitotic delay, by permitting the repair of toxic nuclear lesions, might also modify the frequency of cells that undergo mitosis at a given time in culture, thus changing the frequency of cells with two nuclei or more at harvest.

A negative correlation was observed between MN induction and cell proliferation; namely the higher the MN frequency were detected in exposed individuals, the lower the values of nuclear division progression were expressed as RI. This may mean that cells with greater chromosomal damage may die before cell division or they may be less capable of entering this phase [23]. OP insecticides are also capable of inducing programmed cell death (apoptosis) by multifunctional pathways. Namely they both may act as genotoxic agents and may affect other biochemical pathways. In human peripheral blood lymphocytes under *in vitro* conditions, there is no report whatsoever concerning apoptosis and necrosis caused by DDVP. But it is reported that DDVP showed stronger apoptosis-inducing ability than chlorpyrifos at lower doses in human natural killer (NK) cells [14]. DDVP may affect on general cell viability. Remington et al. [20] noted that 2 μM DDVP had no effect on cell viability at 24 h, however at 20–60 μM there was a loss of 20% viability and 100 μM caused a 40% reduction in cell viability in TK6 lymphoblastoid cells. It is reported that DDVP used at the highest concentration (0.452 mM) did not affect the viability of human NK-92CI cell line [15].

The exact mechanism of the toxic effects of DDVP is not known. OP insecticides containing the P=S bond (known as thion) are converted to P = O (known as oxon) by a system of enzymes called microsomal mixed-function oxidases (MFO) in which the enzyme cytochrome P-450 plays a major role [18]. The oxons are highly toxic compounds, which account for the profound toxic effects of OP insecticides [27].

OP compounds as DDVP show alkylating properties [1, 10] and the methyl esters have a higher alkylating potential than the ethyl esters [10]. Alkylating agents are known to cause DNA damage [8]. Most cytotoxic and genotoxic carcinogens are electrophillic by themselves or activated to electrophillic intermediates that bind to

critical macromolecules [2]. The mutagenic activity of DDVP may be due to the existence of electrophillic sites in the parent molecule or its metabolic intermediates, which are capable of binding to nucleophillic sites in DNA. In DDVP, there are two potential electrophillic sites (the alkyl group(s) and the phosphoryl group) [12]. OP compounds are reported to have the ability to bind to DNA [28] and cause mutations [26].

We conclude that DDVP could be considered as a MN inducer agent, mainly at toxic doses. Finally, the particular results found in the present study indicate that the current usage of DDVP can have potential effects and risks on human and environmental health. On the other hand, further toxicity studies that measure other types of DNA damage, both *in vitro* and *in vivo*, are still necessary.

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