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Full Length Research Paper

Investigation of monocrotophos toxic effects on human lymphocytes at cytogenetic level

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The genotoxic and cytotoxic effects of monocrotophos, an organophosphate insecticide, was investigated on human lymphocytes cultured *in vitro*. Utilizing the trypan blue dye exclusion technique assay the IC50 (half maximal inhibitory concentration) of monocrotophos was found to be 16 µM. Based on IC50 value, monocrotophos was found to be highly toxic to lymphocyte culture. Chromosomal aberrations induced by monocrotophos were determined using karyotyping. The analysis revealed that more satellite associations, breaks and gaps were found which were statistically significant ($P < 0.05$) when compared to controls. Comet assay was used to assess the possibility of monocrotophos induction of DNA damage where the increase in comet tail length relates to the extent of DNA single strand breaks. These results indicate that *in vitro* assays could be used as indicators of cyto- and genotoxic effects of the pesticide on humans, and their end points could be used as biomarkers.

Key words: Chromosomal aberrations, cytotoxicity, DNA damage, genotoxicity, monocrotophos.

INTRODUCTION

Monocrotophos is a broad-spectrum organophosphate insecticide and acaricide used widely for agricultural and household purposes and which works systemically and on contact. It is used to control a variety of sucking, chewing and boring insects and spider mites on cotton, sugarcane, peanuts, ornamentals, and tobacco (Kidd et al., 1991). It has been classified as a moderately hazardous pesticide by the WHO (World health organisation, 1986). It is extremely toxic to birds and is used as a bird poison. It is also very poisonous to mammals (Smith et al., 1993). Monocrotophos affects the central nervous system by inhibiting cholinesterase, an enzyme essential for normal nerve impulse transmission. The dose, which kills half of the test animals, the IC50, is 17 - 18 mg/kg for male rats and 20 mg/kg for female rats. The IC50 for dermal exposure is 126 mg/kg for male rats, 112 mg/kg for female rats, and 354 mg/kg for rabbits (Budavari, 1989). Symptoms of monocrotophos poisoning are similar to those of other organophosphate compounds. Its cholinesterase-inhibiting activity causes nervous system effects. Cases of human poisoning are characterized by muscular weakness, blurred vision, profuse perspiration, confusion, vomiting, pain, and small pupils. There is a risk of death due to respiratory failure (Senanayake et al., 1987). No teratogenic effects were found at 2 mg monocrotophos/kg/day in rats, the highest dose tested. Studies show that monocrotophos may be weakly mutagenic (U.S. Environmental Protection Agency, 1985). No significant carcinogenic lesions were observed when rats were exposed to monocrotophos aerosol at concentrations from 97 - 308 mg/m^3 for one hour Monocrotophos is metabolized and excreted rapidly and does not appear to accumulate within the body. In mammals, 60 - 65% is excreted within 24 h, predominantly in the urine (Gallo et al., 1991).

In recent years monocrotophos pesticide toxicity has been extensively investigated on insects and animal models but there are few reports of cytotoxicity and genotoxicity in humans in using *in vitro* models. (Rupa, 1989; Corbett, 1984; Amer and Aly, 1992). Also there are no human data specific to monocrotophos. The aim of the

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present study was to evaluate the effects of monocrotophos at cytotoxic and genotoxic levels *in vitro* in peripheral blood samples of healthy human volunteers. The *in-vitro* model system as described by Rambabu et al. (2005) has been used in this investigation, and the main aim was to identify biomarkers of the pesticide toxicity.

METHODS AND MATERIALS

Preparation of pesticide solution

A stock solution (1%) of monocrotophos pesticide was prepared in DMSO and various aliquots from the stock solutions were used through out the experiments. All experiments were carried out in triplicates and averages recorded. Preparations of reagents and solutions were carried out as per the standard procedures descry-bed by the relevant authors (Cremlyn, 1978).

Collection of blood samples

Fresh blood from healthy non-smoking individuals was collected in heparinized syringes and transferred in the eppendorf tubes and used immediately for the determination of cytotoxicity, chromosomal aberrations and DNA damage. Short-term lymphocyte culture using the whole blood samples was set up following the methods described earlier by Kaiser Jamil et al. (2004). Simplest method of lymphocyte culture is incubation of a small amount of whole blood without previous separation of erythrocytes and granulocytes. This utilizes all available lymphocytes, which are usually 25 - 50% of the leukocyte count that is, 1,800 - 5,000 cells per mm^3 of blood.

Trypan blue dye exclusion assay for cytotoxicity

The *in vitro* cytotoxic effect of the monocrotophos pesticide was estimated by the Trypan blue dye exclusion technique. Trypan blue penetrates dead cells through damaged membrane, staining the nucleus. Cells were counted using a Neubauer's chamber (Haemocytometer) and the number of viable (opaque) and dead cells were scored by direct observation under the bright field microscope.

Probit analysis and calculations for lethal concentrations

The cytotoxicity of monocrotophos was determined at various concentrations against lymphocytes killed. The log concentrations and percent kill data obtained as above were subjected to probit analysis by using the probit model program of Reddy et al. (1992) and half maximal inhibitory concentration (IC50) for test compound was calculated.

Chromosomal analysis

The protocol for chromosomal aberration analysis was essentially as described earlier (Moorhead et al., 1960). Chromosome preparations were screened after adding colchicine to arrest the cells in metaphase stage (after 72 h of initiation of cultures). These were fixed in methanol and acetic acid (3:1) and plates were flame dried and stained with 4% giemsa before viewing under microscope and recording the images in the Medi-image software program.

Experiments were carried out to standardize the procedures to detect chromosomal aberrations with proper controls. Normal and treated blood samples were processed as described above and well-spread metaphase plates were analyzed for the chromosome aberration frequency. Attempt was made to screen at least 100 cells per each concentration every time.

Initiation for the culturing of lymphocytes was done in duplicates under sterile conditions. 2 units of PHA or Lectin were added to each media vial (Kolodny and Hirschhorn, 1964; Coulson and Chalmers, 1964). Then 15 µl of freshly collected whole blood was added to each vial and the vials were kept for incubation at 37°C for 72 h. At the end of 48th h of incubation, various concentrations of monocrotophos pesticide solution was added to the tubes and incubated further for another 24 h (IAEA 1983). The tubes were shaken every morning until processed.

The experiments were carried out using various aliquots from the stock solution of the monocrotophos. The concentrations used for the chromosomal aberration experiment were sub-lethal or low doses of the IC50 in varying concentrations. The lymphocytes were incubated with the monocrotophos pesticide for 24 h.

Statistical analysis

Statistical analyses of results were performed using the simple analysis of variance (ANOVA) and the standard deviation (SD) was applied to evaluate chromosomal aberration frequencies between treated and untreated sample.

DNA damage studies

The DNA damage studies were carried out using comet assay, that is, Single Cell Gel Electrophoresis (SCGE) as described by Singh et al. (1988) with slight modifications. Pre cleaned slides were layered with 140 µl of 1% regular agarose to promote even and firm attachment of subsequent layers. Second layer includes 110 µl of 0.5% low melting agarose along with the 20 µl of sample material (that is, monocrotophos treated blood). The final layer comprised of 110 µl of low melting agarose alone. After solidification of agarose, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA and 300 mM NaoH, 1% sodium sarcocinate, pH adjusted to 10, 10% DMSO and 1% Triton X 100 added fresh) and stored overnight at 4°C. The slides were removed from lysing solution and were placed on a horizontal gel electrophoretic unit. The unit was filled with freshly made alkaline buffer (1 mM Na_2 EDTA and 300 mM NaoH, pH > 13). The slides remained submerged in the buffer for 20 min. Electrophoresis was carried out in the same buffer for 25 min at 25 v and 30 mA. DNA fragments in each cell migrate at a rate inversely proportional to the size of the fragments. Slides were then washed gently 2 - 3 times, at intervals of 5 min each with 0.4 M Tris at pH 7.5 (neutral buffer). After final wash the neutral buffer was drained and each slide was stained with 60 ml of silver nitrate covered with a micro glass coverslips and sealed. The slides were viewed under a microscope, which has a CCD camera attachment and connected to a computer with Medi-Image software containing frame grabber, viewer, and saving in a library and finally printable version was obtained.

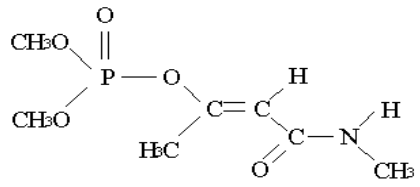

Figure 1. Structure the organophosphorus pesti-cide, monocrotophos. Molecular formula: $C_7H_{14}NO_5P$. Molecular weight: 223.1. IUPAC name: Dimethyl (E) 1-methyl-2- (methylcarba-moyl) vinylphosphate.

Table 1. Frequency of chromosomal aberrations in untreated (control) and treated (monocrotophos) lymphocytes.

Monocrotophos Concentrations in µM (24 h)	Number of metaphases with breaks	Number of metaphases with gaps	Number of metaphases with Satellite associations	Number of metaphases with Aneuploide cells	Percent cells with aberrations
Control	Nil	Nil	2	Nil	2
1.6	4	6	10	4	24
2.6	11	8	16	6	41
3.6	11	14	16	6	47
4.6	14	15	16	7	52
5.6	17	19	17	8	61

All experiments for chromosomal aberrations were carried out in triplicates, and for each set 100 metaphases were screened.

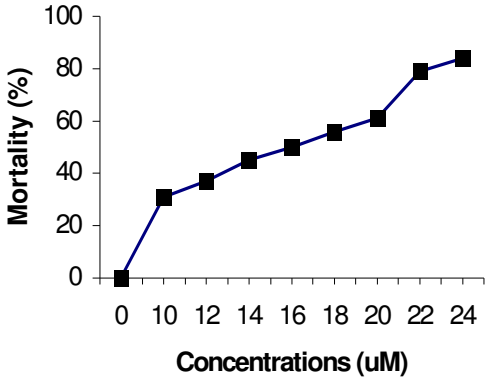

Figure 2. Concentration versus mortality of monocrotophos pesticide. The IC50 value of the monocrotophos obtained from the trypan blue viability test is 16 µM.

Table 2. Comet tail length (µm) in lymphocytes treated with monocrotophos.

Monocrotophos Concentrations (µM)	Average tail length (µm; mean ± SD)
Control	07.158 ± 0.765
1.6	51.194 ± 1.779
2.6	76.437 ± 1.181
3.6	88.719 ± 1.923
4.6	103.63 ± 1.836
5.6	131.95 ± 2.253

For each set 100 cells were screened.

RESULTS

Cytotoxicity

The cytotoxic effects of the monochrotaphos pesticide were determined by the loss of membrane integrity by trypan blue dye exclusion method. Our experiments indicated a clear dose dependent cytotoxic effect of the pesticide on lymphocytes. The percent viability of the cells decreased with increase in the concentration of the pesticide. The results are represented in Figure 2. From this data, the IC50 values were calculated using probit analyses, and it was found that monochrotaphos at a concentration of 16 mM gave 50% mortality when incubated with lymphocytes.

Chromosomal aberration frequencies

Chromosomal aberrations in the form of chromatid breaks, gaps and satellite associations were observed at sub lethal concentrations (that is, 1/10 IC50). Results obtained from the chromosomal analysis data of metaphase plates are presented in the Table I and Figures 3 - 6. It was found that the number of breaks, gaps and satellite associations increased as the concentration of the pesticide increased.

Comet assay

The DNA damaging effects induced by the monocrotophos pesticide was studied *in vitro* using Alkaline Single Cell Gel Electrophoresis (comet assay). The results of comet assay for various concentrations of the pesticide monocrotophos are tabulated in Table 2.

Figure 3. Untreated metaphase cells showing normal chromosomes with out any aberrations (control).

Figure 4. Monocrotophos treated metaphase cells showing satellite association between 'D' groups of chromosomes that is, D-D satellite associations and having 45 chromosomes (aneuploidy).

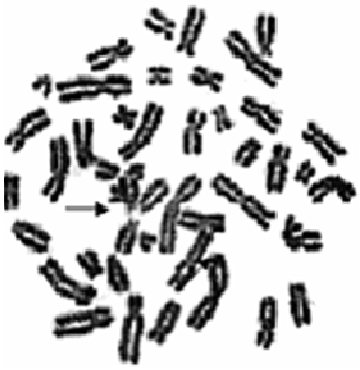

Figure 5. Monocrotophos treated metaphase cells showing 'gap'on chromosome of 'A' group and satellite association between only 'D' group of chromosomes i.e. D-D-D satellite associations and having 45 chromosomes (aneuploidy)

The morphology of normal cells and treated cells visualized as comets is shown in Figure 7 and 8. It is

Figure 6. Monocrotophos treated metaphase cells showing break on chromosome of 'C' group and satellite association between 'D' and 'G' groups of chromosomes i.e. D-G satellite associations and having 45 chromosomes (aneuploidy).

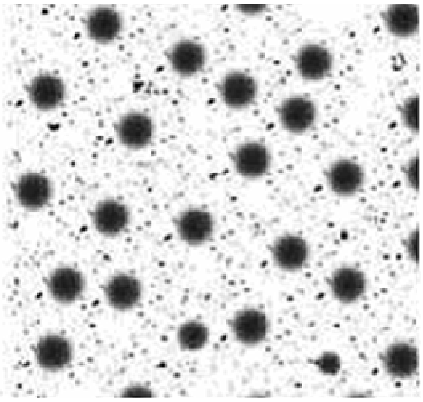

Figure 7. Unexposed cells showing no damage in comet assay (control).

evident from these results that the comet tail length increased from 51.194 µm to 131.95 µm with the increase in the concentration of monocrotophos from 1.6 mM to 5.6 mM. This is indicative of the single strand DNA breaks in these treatments.

DISCUSSION

The toxicity profile of monochrotaphos on peripheral blood of healthy humans under *in vitro* test conditions was investigated. We determined the toxic and subtoxic doses at which the pesticide could induce genotoxicity in human lymphocytes. The damage, which this pesticide incurs on DNA, may be irreversible; however it remains to the established. We have determined the IC50 values of monocrotophos pesticide using peripheral blood lymphocytes *in vitro* in which as low as 16 µM gave 50% kill of the cells. The present investigation on using *in vitro* assays, however, seems to be very useful short-term assays, which could be used for a wide number of chemicals for quick screening methods. The data was statistically analyzed and was found to be significant, and to-

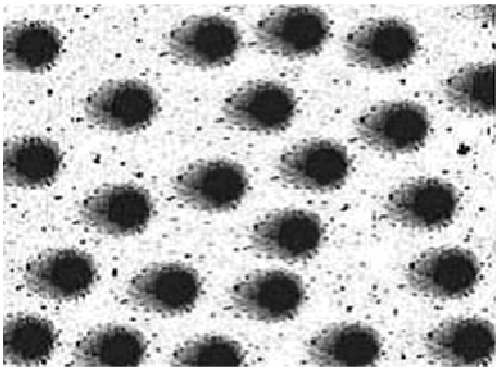

Figure 8. Monocrotophos exposed cells showing DNA damage (as comet tails).

together with other related assays like chromosomal aberration frequency assay and comet assay, it was obvious that at sublethal dosages, the pesticide could induce aberrations in the chromosomes and also cause single strand DNA breaks (1/10 of IC50 dosage).

The genotoxicity assay (comet assay) of monocrotophos indicated dose-dependent increase in tail-lengths of the comets. Banu et al. (2001) and Hartmann et al. (1988) have reported similar results in the mice model. We could measure the tail lengths of the comets by using medi-image software program and it was seen that low doses of the monocrotophos pesticide could induce single strand breaks in DNA. Genotoxicity assessment revealed that monocrotophos at the tested concentrations caused significant chromosomal aberrations in peripheral blood lymphocytes of humans as compared to controls. Analysis revealed more satellite associations, gaps and breaks in treated samples. Our study on the frequency of chromosomal aberrations suggests that conditions like aneuploidy could also occur in humans. The frequency of the occurrence of satellite associations was significantly more compared to controls. The cells showing satellite associations also showed aneuploidy. It is seen from our results that under the influence of monocrotophos, both gaps and breaks were found at certain concentrations.

It has been suggested by many authors that gaps are indicative of toxic phenomena from genetic point of view, and these aberrations could be useful sensitive biomarkers of monocrotophos-induced genetic damage. The advantage of these *in vitro* assays is that the minimum dosage can be estimated, which can be useful in determining the effects on humans with reference to cytotoxicity and genotoxicity. It is therefore concluded that pesticide users and manufacturers should be aware of the hazards of the xenobiotics in general use.

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