Cytotoxicity and Genotoxicity induced by the pesticide Acephate on Cultured Human Peripheral Blood Lymphocytes

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Abstract

Acephate is a broad-spectrum organophosphate insecticide extensively used in India to control pests for agricultural and household purposes. The aim of this investigation was to determine its toxicity profile in-vitro, using lymphocytes from peripheral blood samples of healthy human donors. We found the LC50 of Acephate to be 45M as measured by Trypan blue dye exclusion method. Chromosomal analyses of the metaphase plates of the samples treated with sub-lethal concentrations of Acephate revealed satellite associations, chromatid breaks and gaps indicating its effect on chromosomes. The results were further supported by comet assay, where single strand breaks in DNA were observed as comet tail lengths. The results were statistically significant ($p<0.05$, t-test). Hence, it may be proposed that in-vitro assays like comet assay and chromosomal aberrations test, which indicate genetic damage could be used to study the effect of organophosphorus pesticide poisoning in humans.

INTRODUCTION

Acephate is an organophosphate foliar spray insecticide of moderate persistence used for control of a wide range of biting and sucking insects, especially aphids, including resistant species, in fruit, vegetables, vine, and hop cultivation and in horticulture ([1,2,3,4]). Acephate has contact and systemic action inhibiting the enzyme acetylcholinesterase, which is necessary for the control of nerve transmission ([4,5,6]). Exposure effects of Acephate in humans can include cardiac responses, central nervous system impairment, eye problems, gastrointestinal and respiratory effects ([5,7]). Maximum Residue Limits have been established for acephate's transformation product, methamidophos, at 1.0 mg/kg for lettuce and peppers, and 0.5 mg/kg for tomatoes. Trade names for products containing Acephate include Orthene,Asataf, Pillarthene, Kitron, Aimthane, Ortran, Ortho 12420,Ortril, Chevron RE 12420, and Orthene 755 ([4,7]) .

The chemical details of Acephate are as follows:

Chemical family : Organophosphate Molecular formula : $C_4 H_{10} NO_3 P S$ IUPAC name : O, S-Dimethyl acetylphosphoramidothioate

Many studies have been carried out to evaluate the mutagenic and genotoxic potential of acephate in vitro and in vivo ([1,2,4,6,8]). Acephate showed weak mutagenicity in genotoxic studies using S.typhimurium, Macaca monkey lymphocytes and CD1 mice bone marrow cells employing Ames test, Micronucleus testing and chromosomal aberrations testing ([1,2,4]). These studies concluded that acephate had genotoxic potential in studies in vitro, but studies in vivo gave negative results for gene mutations and conflicting results for chromosomal aberrations ([1,4]). A test in vitro for point mutations (bacterial reverse mutation) gave negative results, as did all tests in vivo for sex-linked recessive lethal mutations, micronuclei in bone marrow, somatic cell mutation in mice and unscheduled DNA synthesis in liver cells ([4]). The evidence indicates that acephate produced positive responses in gene mutation in some in vitro assays with Salmonella, E. coli, and S. cerevisiae. Acephate has been reported to produce mutations in mouse lymphoma cells, sister chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) cells, and mitotic recombination in Saccharomyces. Several in vivo assays for SCEs and cytogenetic endpoints have been negative ([4,8]).

In the present study, an attempt has been made to evaluate the cytotoxic and genotoxic effects of Acephate in human lymphocyte cultures in vitro. The invitro model system as

described by Kaiser Jamil et al., 2004 ([9]), has been used in this investigation.

MATERIALS AND METHODS

PREPARATION OF PESTICIDE SOLUTION

Technical grade Acephate (94%) was obtained from Indian Institute of Chemical Technology, Hyderabad, AP. From this pesticide, a stock solution was prepared in 10ml of 1%DMSO (i.e., by diluting DMSO with water) by weighing 10mg of the test chemical. The concentration of the pesticide Acephate in DMSO was 5mM. From this stock solution, concentrations ranging from 10 to 70 µM were used in the experiments. Pesticide solutions were prepared as described earlier by Wang etal., 2003 ([8]) . Three sets of experiments along with equal numbers of controls were carried out for all the in-vitro assays.

TRYPAN BLUE DYE EXCLUSION ASSAY

Fresh blood was collected from clinically healthy non-smoking individuals in heparinized tubes. Lymphocytes were separated under sterile conditions using Ficoll-density gradient centrifugation ([10]). The cell count was determined using Neubauer's chamber and the final volume of lymphocytes was adjusted to achieve a cell density of 2 x 106 cells/ml and used for toxicity evaluation studies. Aliquots of the lymphocytes were distributed in sterile Eppendorf tubes and then treated with varying concentrations of Acephate ranging from 10 to 70µM. After two hrs of incubation, the viability of the treated and untreated samples was determined using 0.4% Trypan blue solution ([11]).

CHROMOSOMAL ABERRATIONS ASSAY

Blood samples were collected in heparinized tubes for initiation. Lymphocytes were stimulated to divide using phytohemagglutinin in an atmosphere of 5% CO_2. After 48 hrs of division, sub-lethal concentrations (1-7 µM) of the pesticide were added into the tubes and incubated further. At the 70th hour, the cultures were harvested after addition of colchicine ([12]). Slides stained with 4% Geimsa solution were then screened for chromosomal aberrations.

SINGLE CELL GEL ELECTROPHORESIS (COMET ASSAY)

The DNA damaging effects caused by the pesticide Acephate were observed using comet assay i.e., Single Cell Gel Electrophoresis (SCGE) with slight modifications ([13,14]). Experiments were set up by incubating lymphocytes for two hours with concentrations of Acephate ranging from 1.0 to 7.0 µM. Positive controls consisted of cells treated separately with Cyclophosphamide at 1 µM ([15]). Negative controls were set up by incubating lymphocytes with the solvent DMSO at a final concentration of 1%. 10 l of the 100 l aliquots of the lymphocytes treated as above along with untreated samples were mixed with 120 l of 0.5% low melting agarose and layered on the surface on glass slides previously coated with 140 l of 1% normal melting agarose. After the application of coverslips, the slides were allowed to gel at 4oC for 20 mins. After carefully removing the coverslips a second layer of 0.5% low melting agarose was pipetted onto the slides and allowed to gel for a further 20mins at 4oC. The slides were immersed in freshly prepared cold lysing solution and refrigerated overnight followed by alkali treatment, electrophoresis and neutralization. The dried slides were then stained using silver nitrate solution after appropriate fixing ([16]). The whole procedure was carried out in dim light to minimize artefactual DNA damage ([14,17]). Analysis was performed at a magnification of 100x using a light microscope after coding the slides ([13,14,17]). A total of at least 100 cells were screened per slide.

STATISTICAL ANALYSIS

All the data were subjected to statistical analyses using t-test. The results of cell viability, chromosomal aberrations , SCGE were tabulated after analysis and the data presented in tables and figures. Probit regression analyses (Statistical software package) was applied to determine the concentrations required to reduce the cell viability by 50% ([18]). Probit regression analyses transforms cell viability expressed as percentage, into standardized normally distributed values (probits) and produces a linearised model of the relationship between cell viability and the concentrations of the test substances (expressed as decimal logarithms) ([18,19,20])

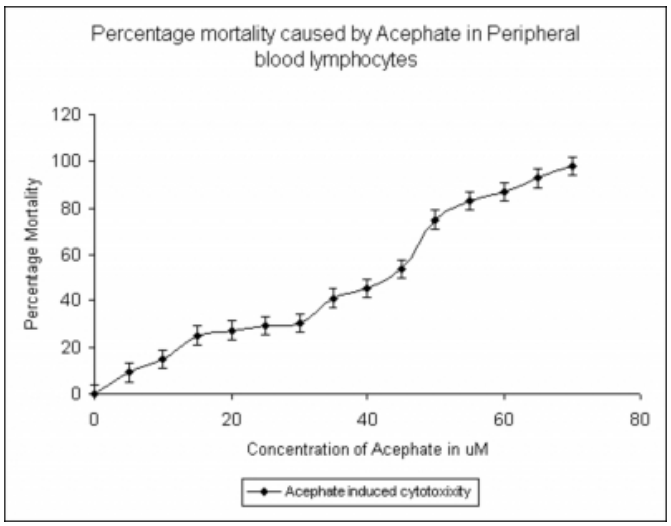
RESULTS

The cytotoxic effects of the Acephate 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 1. From this data, the LC50 values were calculated using probit analyses, and it was found that Acephate at a concentration of 45µM gave 50% cytotoxicity when incubated with lymphocytes as described

earlier.

Figure 1

Figure 1 : Cytotoxicity caused by Acephate on lymphocytes



p<0.01 (t-test)

Metaphases suitable for cytogenetic analyses were screened for the frequency of chromosomal aberrations. All experiments for chromosomal aberrations were carried out in triplicates, and for each set 100 metaphases were screened. The chromosomal aberration frequency was found to be dose-dependent, with increasing gaps , breaks and satellite associations as compared to untreated cells (Table 1).

Figure 2

Table 1: The frequency of chromosomal aberrations in lymphocyte cultures treated with sub-lethal concentrations of Acephate

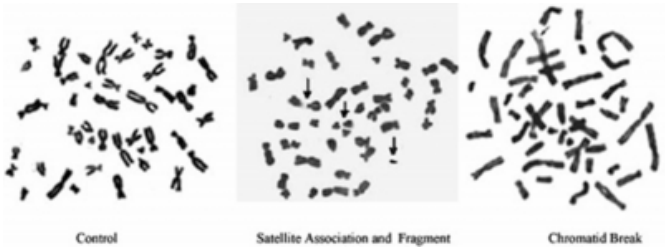
S no.	Concentration of Acephate in µM	No. of cells scored	Breaks	Gaps	Satellite associations	Fragments	Aneuploid cells
1	Control	100	Nil	1±0.1	2±0.140	Nil	Nil
2	1.0µM	100	2±0.140	1±0.1	3±0.171	1±0.1	1±0.1
3	2.0µM	100	2±0.140	2±0.140	3±0.171	1±0.1	1±0.1
4	3.0µM	100	3±0.171	4±0.196	6±0.238	2±0.140	2±0.140
5	4.0µM	100	4±0.196	5±0.219	6±0.238	1±0.1	3±0.171
6	5.0µM	100	5±0.219	5±0.219	7±0.256	2±0.140	3±0.171
7	6.0µM	100	6±0.238	7±0.256	7±0.256	3±0.171	5±0.219
8	7.0µM	100	7±0.256	9±0.287	8±0.272	2±0.140	4±0.196

p< 0.05 (t-test)

Metaphase plates with aberrations are presented in Fig 2. It was found that, satellite associations (8%), breaks (7 %) and gaps (9 %) increased with the increase in concentration of Acephate. The satellite associations between acrocentric chromosomes (D-D, D-G and G-G) were clearly demonstrated.

Figure 3

Figure 2: Metaphase plates showing chromosomal aberrations



The length of DNA migration in the comet tail, which is an estimate of the DNA damage for each cell was calculated as comet tail length after visualizing through a light microscope. The results of comet assay for various concentrations of the pesticide Acephate along with positive controls and untreated samples are tabulated in Table 2.

Figure 4

Table 2: Comet assay –Assessment of DNA damage.

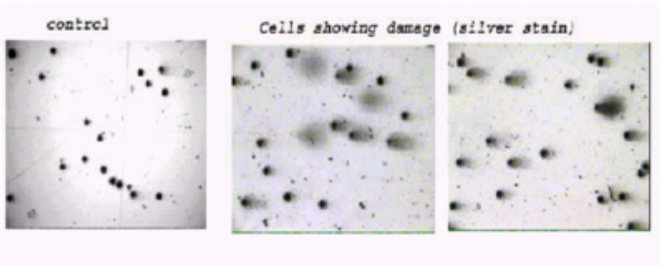
S no.	Concentrations of Acephate used (µM)	No. of cells scored	Average tail length in µm (mean ± SD)
1	Control (untreated)	100	0.11±0.03
2	1.0µM	100	0.16±0.106
3	2.0µM	100	0.21±0.140
4	3.0µM	100	0.32±0.153
5	4.0µM	100	0.35±0.156
6	5.0µM	100	0.54±0.178
7	6.0µM	100	0.72±0.199
8	7.0µM	100	0.95±0.299
9	8.0µM	100	Necrosis
10	9.0µM	100	Necrosis
11	10.0µM	100	Necrosis
12	1.0µM (Cyclophosphamide) (Positive Control)	100	0.905±0.190
13	Cells treated DMSO (1%) (Negative Control)	100	0.12±0.01

p<0.01 (t-test) ; * length ± standard error

The morphology of normal cells and treated cells visualized as comets is shown in Fig 3.

Figure 5

Figure 3: Control cells and cells showing DNA damage as comet tail length



It is evident from these results that the comet tail length increased from 0.11µm to 0.95µm with the increase in the concentration of Acephate from 0 to 7µM. This is indicative of the single strand DNA breaks in these treatments.

DISCUSSION

Organophosphorus pesticides along with the carbamate and organochlorine pesticides are widely used by farmers in India and abroad in agriculture because of their high efficiency towards the target organisms ([21,22]). Extensive use of these pesticides in crop protection and for household purposes has resulted in their wide spread distribution in the environment causing part of the world wide environmental contamination ([22]). Besides their great contribution to the animal and human prevention of vectors of diseases, their use also creates many problems because of their toxicity to non-target organisms, persistence and combined effects with other agro-biochemicals and environmental factors ([4,21,22]) .

The toxicity profile of Acephate on peripheral blood of healthy humans under in vitro test conditions was investigated. In recent years this pesticide toxicity has been extensively investigated on experimental models but there are very few reports of cytotoxicity and genotoxicity in humans in the in-vitro models. Acephate was found to show conflicting results in a variety of invitro tests in bacteria, yeasts and mammalian cell systems when evaluated for its genotoxic potential ([4]). However, our studies on cultured human peripheral blood lymphocytes and other studies showed positive results.

Cytotoxicity data indicate that after two hours of exposure to the pesticide, almost 100% cells became non-viable at 70µM concentration. Cytogenetic analysis for chromosomal aberrations like breaks, satellite associations, gaps, etc., can be used to monitor pesticide- induced genotoxic effects on chromosomes under invitro and invivo conditions ([23,24,25,26,27]) have reported similar findings with certain pesticides. Genotoxicity assessment of Acephate at the tested concentrations revealed that this pesticide could cause significant chromosomal aberrations in cultured peripheral blood lymphocytes of humans' invitro. The percentage if chromosomal aberrations increased with the increase in the concentrations of the pesticide. The results were found to be statistically significant ($p<0.05$, t-test). Chromosomal analysis using human lymphocytes is therefore a sensitive assay of genotoxic evaluation.

DNA damage studies by single cell gel electrophoresis technique showed that as the concentration of the pesticide increases (0 to 7µM) DNA damage also increased (0.11±0.03µm to 0.95±0.29µm) respectively. Cells with damaged DNA display increased migration of DNA fragments from the nucleus generating a comet shape while undamaged cells have an intact nucleus ([28,29,30]) .At further higher concentrations tested (8-10µM), there was a predominance of necrotic cells. This assay can be used as a rapid and sensitive tool to demonstrate the damaging effects of chemicals on DNA at the individual cell level. Similar studies have also been reported. ([28,30,31,32,33,34]).

The results of in-vitro experiments may give some insight into the different mechanisms involved in cellular and genetic toxicology. At the same time they lay foundations for further experiments at the molecular level as well as invivo conditions. These invitro methods are less expensive than the in vivo methods, give rapid results and conform to the worldwide reduction in animal testing. This study is therefore significant as the literature on the cytotoxic and genotoxic potential of this pesticide on cultured human lymphocytes was scarce. Moreover, these results may be used to study the effects caused by Acephate exposure under field conditions.

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