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Study of Cypermethrin Cytogenesis effects on Human Lymphocytes Using In-Vitro Techniques

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ABSTRACT: The Cytogenetic effects of Cypermethrin a synthetic pyrithroid insecticide was investigated on human lymphocytes cultured in-vitro. Utilizing the trypan blue dye exclusion technique assay the LC50 of cypermethrin was found to be 36 uM. Based on LC50 value, cypermethrin was found to be low toxic to lymphocyte culture. Cypermethrin showed an increase in the frequency of chromosomal aberrations and found to be significant. Karyotype analysis revealed more satellite associations and chromosomal breaks in cypermethrin treated samples. Low-doses of the pesticide also induced single-strand breaks in the DNA as assessed by comet assay. The pesticide caused increase in the comet tail length with increase in pesticide concentration, implicating genotoxicity in somatic cells. It is concluded that In vitro assays could give important information of the mechanism of toxicity at low dosages and impact on genetic material of human origin. @JASEM

Pyrethriods are synthetic analogues of pyrethrins, the active substances in the flowers of Chrysanthemum, *Cineraria folium*. Pyrethroids can be classified into two large groups. Type I pyrethroids do not contain a cyano group in their molecules and include allethrin, tetramethrin, permethrin, and phenothrin. Type II pyrethroids contain a cyano group at the _ carbon position and include newer compounds, such as deltamethrin, cyphenothrin, cypermethrin, and fenvalerate. The two types of pyrethroids cause somewhat different symptoms of mammalian poisoning. Poisoning with type I pyrethroids is characterized by hyper excitation, ataxia, convulsions, and eventual paralysis; poisoning with type II pyrethroids, by hypersensitivity, choreoathetosis, tremors, and paralysis. Despite differences in the symptoms, both types of pyrethroids have the same major target site; the sodium channel of nerve membrane, i.e., the channel directly responsible for generating action potentials (Biologic basis of Neuro toxicity., 1992).

Cypermethrin has become one of the most important insecticides in wide scale use. It has wide uses in cotton, cereals, vegetables and fruit, for food storage, in public health and in animal husbandry. Cypermethrin, an analogue of pyrethrins is classified by the World Health Organization (WHO) as moderately hazardous (class II). It interacts with the sodium channels in nerve cells through which sodium enters the cell in order to transmit a nerve signal. These channels can remain open for up to seconds compared to the normal period of a few milliseconds, after a signal has been transmitted (Clark et al., 1989). Cypermethrin also interferes with other receptors in the nervous system. The effect is that of long lasting trains of repetitive impulses in sense organ (Abbassy et al., 1983).

Since cypermethrin is highly toxic to fish and bees, humans are indirectly affected as humans consume fish in their food and also honey which is made by the bees. Thus humans can be exposed to cypermethrin toxicity directly through spraying or contact methods or indirectly through consuming the pesticide-contaminated products like fish and honey. Hence there is a need for evaluating the toxicity at low doses, which can be studied easily using in vitro methods. The aim of the present investigation is to evaluate the cyto and genotoxicity of cypermethrin at low doses. The In vitro model system has been used instead of the animal model studies. The oral LD50 for cypermethrin in rats is 250 mg/kg (in corn oil) or 4,123 mg/kg (in water) (Meister., 1992). There is little human evidence that pyrethrins or pyrethroids cause health problems due to exposures. However, several animal studies have given us reasons for concern, specially the widespread use of these chemicals.

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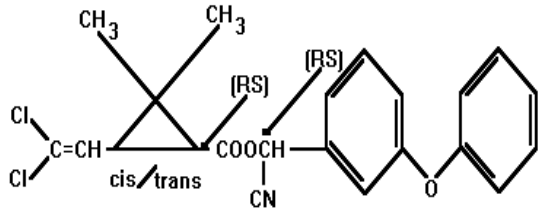
The aim of the present study was to evaluate the effects of cypermethrin at cyto toxic and genotoxic levels in-vitro in peripheral blood samples of healthy human volunteers. The invitro model system as described by "Rambabu et al (2005), has been used in this investigation, and the main aim was to identify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

METHODS AND MATERIALS

The Pyrethroid pesticide used in this study was cypermethrin having the following structure:



Cypermethrin (8 isomers)

Molecular formula: $C_{22}H_{19}Cl_2NO_3$
Molecular weight: 416.32

IUPAC: (RS)-ý-cyano-3-phenoxybenzyl (1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate

Stock solution (1%) of cypermethrin 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 described by the relevant authors (Cremlyn., 1978.).
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 i.e. 1,800-5,000 cells per mm^3 of blood.

The in-vitro cytotoxic effect of the cypermethrin 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.

The cytotoxicity of cypermethrin 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 lethal concentration $_{50}$ (LC_{50}) for test compound was calculated. 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 hrs 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 analysed 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 15µl of freshly collected whole blood was added to each vial and the vials were kept for incubation at 37^0C for 72 hrs. At the end of 48th hr of incubation, various concentrations of cypermethrin pesticide solution was added to the tubes and incubated further for another 24hrs (IAEA., 1983). The tubes were shaken every morning until processed. The experiments were carried out using various aliquots from the stock solution of the cypermethrin. The concentrations used for the chromosomal aberration experiment were sub-lethal or low doses of the LD_{50} in varying concentrations. The lymphocytes were incubated with the cypermethrin pesticide for 24 hours.

The DNA damage studies were carried out using comet assay, i.e. Single Cell Gel Electrophoresis (SCGE). Single Cell Gel Electrophoresis (SCGE), commonly known as comet assay was carried out as

per the procedure described by "Singh et al (1988)" with slight modifications as described below. Pre cleaned slides were layered with 140µl of 1% regular agarose to promote even and firm attachment of subsequent layers. Second layer includes 110µl of 0.5% low melting agarose along with the 20µl of sample material (i.e. cypermethrin 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 (2.5M NaCl, 100 mM Na_2 EDTA 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^o 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 (1mM 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 25v 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. The LD50 value of the cypermethrin obtained from the Trypan blue viability test is 36µM.

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 sat associations as compared to untreated (control).

RESULTS AND DISCUSSION

The cytotoxic effects of the cypermethrin 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 LD-50 values were calculated using probit analyses, and it was found that cypermethrin at a concentration of 36 mM gave 50% mortality when incubated with lymphocytes as described earlier.

Chromosomal aberrations in the form of chromatid breaks, gaps and satellite associations were observed at sub lethal concentrations (i.e. 1/10 LC50). Results obtained from the chromosomal analysis data of metaphase plates are presented in the Table-I. It was found that the number of breaks, gaps and Satellite associations increased as the concentration of the pesticide increased.

Table I. Showing the frequency of chromosomal aberrations in untreated (control) and treated (cypermethrin) lymphocytes.

Cypermethrin Concentrations in µM (24 hrs)	Number of metaphases scored	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	100	Nil	Nil	Nil	Nil	0
3.6	100	Nil	Nil	1	Nil	1
4.6	100	Nil	2	3	1	6
5.6	100	Nil	6	4	3	13
6.6	100	1	7	8	5	21
7.6	100	3	10	12	7	32

The DNA damaging effects induced by the cypermethrin has been studied in-vitro using Alkaline Single Cell Gel Electrophoresis (comet assay). The results of comet assay for various concentrations of the cypermethrin are tabulated in Table II. It is evident from these results that the comet tail length increased from 12.28 µm to 44.00 µm with the increase in the concentration of cypermethrin from 3.6 mM to 7.6 mM. This is indicative of the single strand DNA breaks in these treatments.

In-vitro studies on human blood samples can give information of the toxicological effects of the cypermethrin in human blood samples. So far there are few reports of the impact of cypermethrin on lymphocyte cultures of humans (Amer., 1993). Low-doses of the cypermethrin pesticide could induce single strand breaks in DNA. Animal model studies have also indicated that when administered to pregnant and nursing rats, cypermethrin may lead to genotoxicity and also neurotoxicity by functional delay in the brain maturation of the pups. The

toxicity to young rats is higher, may be because the pathway for degrading cypermethrin is not fully developed in young rats (Cypermethrin Environmental health criteria., 1989). The present study on the frequency of chromosomal aberrations suggests that conditions like aneuploidy could occur in humans. However, these changes may induce certain disease conditions at the individual level, which may not be inherited by the progeny. It is also possible that these aberrations could be rectified by DNA repair mechanisms, indicating recovery from the diseased conditions.

Table II. Means+SD Comet tail length (μm) in lymphocytes treated with cypermethrin pesticide.

Cypermethrin Concentrations in μM	No. Of cells scored	Average tail length in μm (mean ± SD)
Control	100	1.155 ± 0.754
3.6	100	12.28 ± 1.154
4.6	100	16.71 ± 1.052
5.6	100	25.97 ± 1.131
6.6	100	32.58 ± 1.155
7.6	100	44.00 ± 1.788

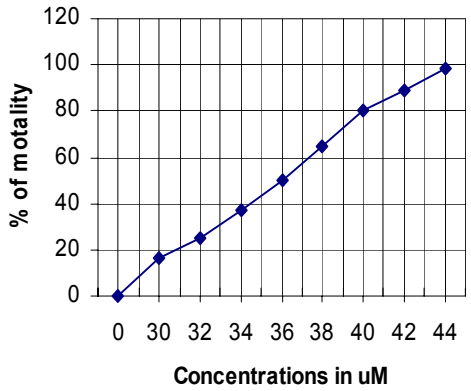


Fig 1. Concentration vs. mortality of cypermethrin pesticide

Comparative biochemical analysis on cypermethrin is not available except for one report on the occurrence of acceptable daily intake (ADI) in rodent juveniles (Ferah et al., 2005), which has not been confirmed so far. The relatively rapid degradation of cypermethrin means that it is not generally found as residue in food. The present investigations on using In vitro assays are, however, seems to be very useful short-term assays, which could be used for a wide number of chemicals for quick screening methods directly related to human toxicology. Medical diagnosis of cypermethrin toxicity indicates that mild poisoning is short-lasting and in case of occupational over-exposure medical intervention are essential (Ellenhorn and Barceloux., 1988). It is reported that 5% sodium bicarbonate lavage can be used if the patient has ingested the pesticide. In severe cases, the medical practice includes phenobarbital or diphenylhydantion or their mixture may be given (Ghousia ., 2005). Cypermethrin has been reported to be highly toxic to aquatic organisms and fish as well as to bees (Davis., 1993; Sarkar at al., 2005). Hence, awareness serves as a vital role for pesticide users in order to overcome the hazards caused by the xenobiotics in gener

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