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Immunotoxic effects of cypermethrin in mitogen stimulated chicken lymphocytes due to oxidative stress and apoptosis

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Abstract
Today chemical pesticides have become crucial as these are playing vital role in controlling agricultural, industrial, home garden and public health pests globally. However, pesticides are constantly contaminating environment and adversely affecting animal and human health. Cypermethrin is a composite pyrethroid; a broad spectrum, non-cumulative insecticide; and, a fast-acting neurotoxin. Present communication reports cypermethrin induced immunotoxicity, oxidative stress and apoptosis in chicken lymphocyte cell culture system employing lymphocyte proliferation assay, nitric oxide (NO) assay, annexin-V assay (AVA) and DNA fragmentation analysis. There was a marked reduction in B and T cells proliferation due to *in vitro* exposure of cypermethrin. Oxidative stress was increased significantly in cypermethrin treated cells as revealed through NO estimation. AVA revealed significant increase in cells undergoing apoptosis in cypermethrin treated cells. This was further confirmed by DNA agarose gel electrophoresis. Thus, from the findings it could be inferred that low level dose of cypermethrin induces significant immunotoxic effects because of higher oxidative stress and apoptosis in avian lymphocytes.

Keywords: Cypermethrin; immunotoxicity; oxidative stress; apoptosis; chicken lymphocytes

1. Introduction
Cypermethrin (*IUPAC*: (RS)-ý-cyano-3-phenoxybenzyl (1RS)-cis, trans- 3-(2, 2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate; *Molecular formula*: $C_{22}H_{19}Cl_2NO_3$; *Molecular weight*: 416.32) is a composite pyrethroid; a broad spectrum insecticide. It is moderately toxic to mammals. It is active against a wide range of insect pests. Cypermethrin is both a stomach poison and a contact insecticide [1]. Cypermethrin is primarily absorbed from the gastrointestinal tract. It may also be absorbed by inhalation of spray mist and only minimally through the intact skin. Pyrethroids are extremely toxic to aquatic organisms [2]. Though, elimination of cypermethrin is reported to be rapid in most animals and half-life is approximately one day in the most tissues. However, cypermethrin is reported to produce detrimental effects on both invertebrates [3] and vertebrates [4, 5].
The metabolism and elimination of cypermethrin have been extensively studied in rats and mice [6]. Cyanohydrines are formed during metabolism of cypermethrin which are further degraded into cyanides and aldehydes. These substances can stimulate production of reactive oxygen species (ROS) [7]. Enhanced ROS mediate lipid peroxidation that leads to cytotoxicity and genotoxicity in higher vertebrates during the pesticide exposure [8]. There is a clearly established relationship between ROS/ free radicals and apoptosis. Since ROS/ free radical intermediates mediate many immune cell functions and apoptosis, it is likely these two events could arise simultaneously during certain chemical exposures [9]. The toxic effects and NOEL dose of cypermethrin is well documented [10-12]. The pesticide dose selected for present study was NOEL/10^3 which was found to be suitable for the *in vitro* studies in avian lymphocyte cell culture system. Present communication evaluates immunotoxic effects, oxidative stress and apoptosis in chicken lymphocytes due to *in vitro* exposure of low level dose of cypermethrin employing lymphocytes proliferation assay, cytokine assay, annexin V assay, nitric oxide estimation and DNA fragmentation analysis.

2. Materials and Methods

2.1 Chicken lymphocytes

Chicken spleens were collected from healthy birds from local slaughter house, and lymphocytes were isolated under laminar air flow as per standard procedure [13]. Lymphocytes were separated through density gradient centrifugation (Histopaque 1077, Sigma) as per the Method described by Rose and Friedman [14].

2.2 Cell viability assay

Percentage cell viability was determined by 0.1 per cent trypan blue dye exclusion test using haemocytometer [15] and final cell count was adjusted to 10^7 cells/ ml in RPMI-1640 medium and made into one ml aliquots in eppendorf tubes and cells were pelleted by centrifugation at 1400 rpm for 10 min.

2.3 Pesticide treatment

Commercial preparation of cypermethrin was purchased from local market and it's thousand times diluted NOEL (5.0 mg/ kg body weight) dose in RPMI- 1640 medium (Hi – Media, India) was used for the *in vitro* exposure of avian lymphocytes for two hours at 37 ºC. After incubation cells were washed twice and finally suspended in 1 ml of RPMI-1640 medium supplemented with 10% FCS (Sigma, USA).

2.4 Lymphocyte proliferation assay (LPA)

LPA or B and T cell blastogenesis assay was carried out as per the method described by Rai-el-Balhaa *et al*. [16]. Concanavalin-A (ConA) (Sigma, USA) was used as a T cell mitogen whereas lipopolysaccharide (LPS) (Sigma, USA) as a B cell mitogen at a concentrations of 5 µg/ml, each, in RPMI-1640 medium. Flat bottom 96 well tissue culture plates (Corning, USA) were used for the assay. Each well was seeded with 200µl of lymphocytes suspension and plate was incubated in CO_2 incubator at 37 ^{0}C. After 68 hours post cell seeding in media was removed from each well carefully and 20µl of MTT (5mg/ml stock) in 200µl of media was added to each well and further incubated for 4 hours in dark at 37 ^{0}C in CO_2 incubator. After incubation, dark blue formazan crystals were solubilized with 200µl of DMSO and absorbance was measured at 570 nm using computerized Micro Scan ELISA Reader. Survival rate was calculated from the relative absorbance at 570 nm and expressed as the percentage of control.

2.5 Statistical analysis

Analysis of variance (ANOVA) and student's t-test were used to estimate significant difference between control and treated cells. The values were expressed as mean delta Optical Density ± standard error (mean Δ OD± SE). Student t-test was employed for comparing the mean ODs [17].

2.6 Oxidative Stress Assay

Macrophages were isolated from spleen on the basis of their adherent properties [18]. Cypermethrin treated and control cells were seeded in 24-well culture plates. Cells were then incubated at 37 ºC in 5% CO_2 for 4 hrs to allow the adherence of macrophages. After incubation, cells were washed vigorously four times with DMEM to remove non-adherent cells. These cells were incubated at 37 ºC in CO_2 incubator in presence of LPS (Sigma) at a concentration of 5 µg/ml. Nitric oxide (NO) production by macrophages in the medium was measured by microplate assay method [19]. The standard curve to calculate the NO production was prepared using different dilutions of $NaNO_2$.

2.7 Annexin-V binding assay

Annexin-V binding assay (Immunoperoxidase technique) was carried out using apoptotic cell separation kit (Sigma, USA). Slides were finally examined under light microscope under 40x objective. Cells which exhibited brownish stain around their plasma membrane were identified as apoptotic cells and were counted in percentage.

2.8 DNA fragmentation assay

Genomic DNA from avian lymphocytes was isolated from cypermethrin treated and control cell suspensions. After pesticide treatment, cells were harvested, pellet was washed with phosphate buffer saline pelleted out by centrifugation at 1400 rpm for 10 min. The pellet was then re-suspendeed in 200 µl of suspension buffer (50 mM glucose, 25 mM TrisHCl, 10mM EDTA, and pH 8.0) and mixed by pipetting gently. The cell suspension was treated with 10 µl of 10% SDS solution and mixed by inverting slowly for three to four times and finally 10 µl of proteinase-K [Sigma, USA (20 mg/ml)] was added and incubated for 4-6 hours at 37 ºC. It was observed that the yield of apoptotic DNA was increased when digested with proteinase-K for longer period. To the 220 µl of apoptotic DNA solution, 30 µl of 4 N NaCl solution was added, mixed by inverting slowly for two to three times, and kept in water bath at 60 ºC for 10 min. Equal amount (250 µl) of phenol–chloroform mixture was added, mixed thoroughly either by inverting or vortexing, then centrifuged at 12000 rpm for 10 min. Supernatant was collected into new eppendorf tube. To it 1/6th volume of ice cold isopropanol (Propan–2-ol, Qualizens^R, Mumbai) was added, mixed gently by inverting two to three times, and kept at -20 ºC overnight. After precipitation, DNA was recovered by pelleting for 10 min at 10000 rpm at 4 ºC. Supernatant was decanted and pellet obtained was washed with 70% ethanol. The pellet was then dried and reconstituted in TE buffer (pH 8.0) and treated with RNase for one hour at 37 ºC. The DNA samples were then loaded onto 1% agarose gel and electrophoresed. After complete electrophoresis, gel was observed for fragmentation of genomic DNA under U.V. trans-illuminator and results were stored in gel documentation system [20].

3. Results

3.1 Lymphocyte proliferation assay (LPA)

The *in vitro* exposure of avian lymphocytes to NOEL/10^3 dose of cypermethrin showed significant decrease in B cell blastogenesis in the presence of B cell mitogen LPS with the overall decrease of 56.12% in B cell blastogenesis in cypermethrin treated cells (Table 1 and Fig. 1). Cypermethrin treated avian lymphocytes showed marked decrease of 56.22% in T cell blastogenesis in the presence of mitogen ConA (Table 2 and Fig. 2).

3.2 Oxidative stress assay

As illustrated in the Table 3 and Fig.3 cypermethrin treated cells exhibited 174.63% more NO concentration as compared to the control.

3.3 Annexin-V binding assay (Immunoperoxidase technique)

Cypermethrin treated cells which were fixed on slides exhibited a typical brownish stain on their surface upon incubation with Annexin-V biotin while untreated control cells did not display brownish colour on their surface. The cells treated with cypermethrin displayed 8.25 ± 1.02%. Annexin-V positive cells as compared to the control value of 3.25 ± 0.65% (Table 4 and Fig. 4).

3.4 DNA fragmentation assay

DNA analysis of pesticide exposed cells exhibited typical 'ladder pattern' which is characteristic biochemical alteration of cells undergoing apoptosis (lane b, Fig. 5), while in the control untreated cells intact DNA was observed as observed by presence of a single band (lane a, Fig. 5). Apoptosis is characterized by the activation of endogenous endonucleases, particularly the caspase-3 activated DNase (CAD), with subsequent cleavage of nuclear DNA into internucleosomal fragments of roughly 180 base pairs (bp) and multiples thereof (360, 540 bp, etc.). The apoptotic DNA fragmentation is being used as a marker of apoptosis and for identification of apoptotic cells via the DNA laddering assay [21].

Table 1: *In vitro* effects of cypermethrin on B cell blastogenesis in chicken lymphocytes

S. No.	Treatments	Mean Δ O.D. ± S.E.**	Percentage change
1	Control	0.417 0.001	–
2	Cypermethrin	0.183 ± 0.003	-56.12
CD at 1% = 0.027 CD at 5% = 0.019			

Significant at $p < 0.01$

Table 2: *In vitro* effects of cypermethrin on T cell blastogenesis in chicken lymphocytes

S. No.	Treatments	Mean Δ O.D. ± S.E.**	Percentage change
1	Control	0.466 ± 0.018	–
2	Cypermethrin	0.204 ± 0.016	-56.22
CD at 1% = 0.070 CD at 5% = 0.050			

Significant at $p < 0.01$

Table 3: *In vitro* effects of cypermethrin on NO concentration (µM/ml) in mononuclear cells

S. No.	Treatments	Mean Conc. ± S.E.**	Percentage change
1	Control	74.68 ± 0.841	–
2	Cypermethrin	205.09 ± 1.475	+174.63
CD at 1% = 6.283 CD at 5% = 6.484			

Significant at $p < 0.01$

Table 4: Evaluation of apoptosis by *in vitro* exposure to NOEL/10^3 dose of cypermethrin (Annexin-V binding assay)

S. No.	Treatments	Mean percent ± S.E.**
1	Control	3.25 ± 0.65
2	Cypermethrin	8.25 ± 1.02

Significant at $p < 0.01$

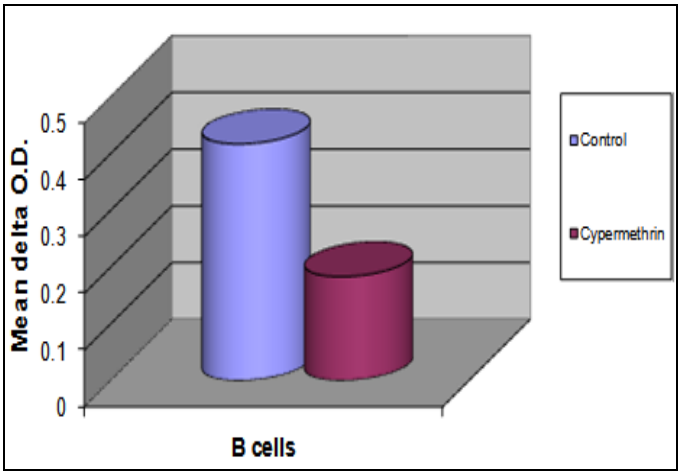

Fig 1: Effect of Cypermethrin on B Blastogenesis in Avian Lymphoy

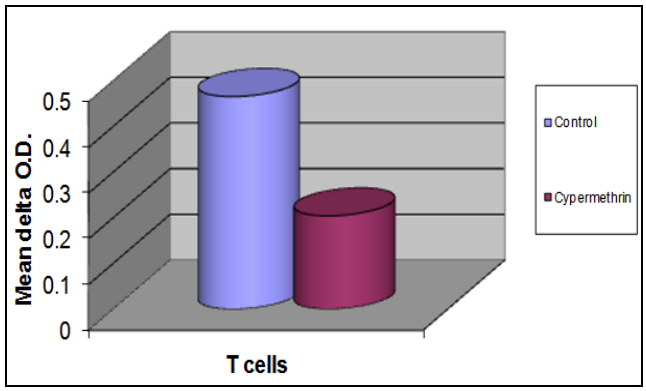

Fig 2: Effect of Cypermethrin on T cell Blastogenesis in Avian Lymphoy

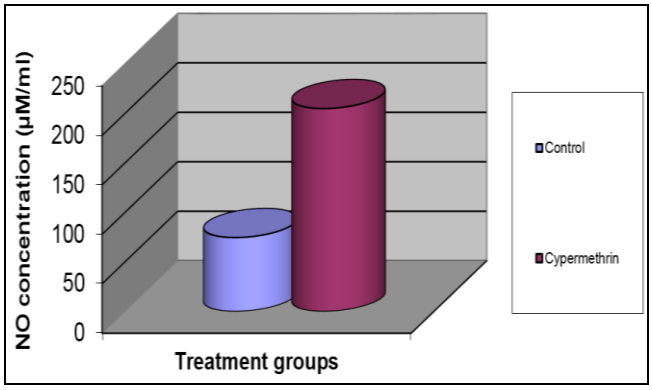

Fig 3: Effect of cypermethrin on nitric oxide (No) Concentration in mononuclear Cells

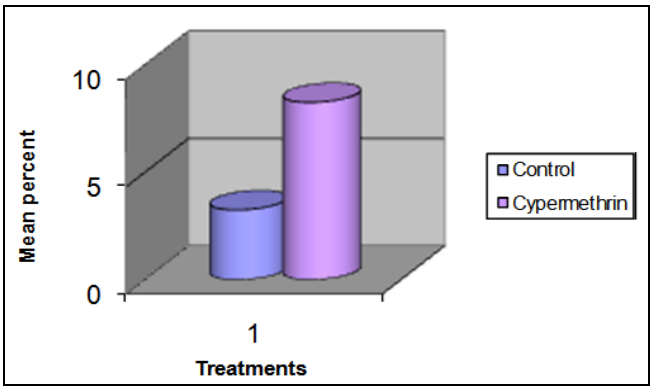

Fig 4: Detection of Apoptosis Due to *In Vitro* Exposure of Cypermethrin in Avian Lymphocytes by Immunope Rox Idase Technique

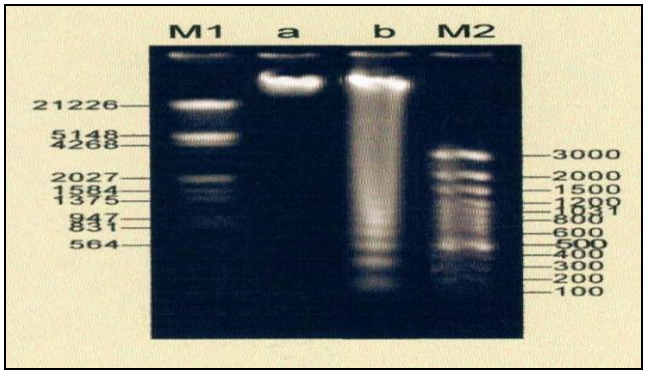

Fig 5: DNA fragmentation analysis of genomic DNA isolated from avian lymphocytes after *in vitro* exposure of cypermethrin
M1 λ *Hind* III/ *EcoR* I double digest
M2 100 bp DNA ladder marker
A Control cells
B Cypermethrin treated cells

4. Discussion and conclusions

The acute toxicity of many pesticides used is well known and poisoning cases often documented. In contrast, much less is known about longer-term impacts on different systems of the human/ animal body including the nervous, hormone, reproductive and immune systems. Rehman *et al.* [22] systematically reviewed on pyrethroid toxicity with special reference to deltamethrin.

In the present study there was a decrease in B and T cell blastogenesis in cypermethrin treated cells as compared to control cells. This was in good agreement with several other workers. Some *in vivo* studies have described effect of cypermethrin on the immune functions, and both inhibition and stimulation of the immune system have been reported [23, 24]. There was marked reduction in thymus weight in type II pyrethroid, deltamethrin treated mice or rats [25, 26]. Similarly, significant lymphocyte depletion was observed in the thymus, spleen and lymph nodes of cypermethrin-treated rats [27]. Several studies reported immunosuppressive effects on humoral and cell-mediated immune responses in different species like adult mice, rats, and goats due to high doses of cypermethrin, supercypermethrin forte and deltamethrin [25, 27, 29-34]. Studies have found permethrin, a synthetic pyrethroid insecticide to be toxic to the immune system. Permethrin inhibited the mitogentic response of murine splenic lymphocytes to concanavalin-A and lipopolysaccharide [28]. Topical exposure to permethrin was found to cause reduction of macrophage function and antibody production in the spleen, indicating that exposure may produce systemic immune effects [35]. Ambwani *et al.* [36] reported immunotoxic effect due to allethrin exposure in chicken lymphocytes.

Diel *et al.* [37] for the first time demonstrated the immunotoxicologic properties of the synthetic pyrethroids S-bioallethrin in *in vitro* approach with human lymphocytes and basophils. Diel *et al.* [38] examined *in vitro* effects of the synthetic pyrethroids S-bioallethrin alone and in combination with the common synergist piperonyl-butoxide (PBO) on human blood lymphocytes and basophils in atopic individuals and non-atopic control subjects. S-bioallethrin and PBO also caused inhibition of lymphocyte proliferation (MTT-test) after a 72-hr culture period in a concentration dependent manner. In contrast to the MTT-measurements the combined agents are more effective in inhibiting interleukin-4 (IL-4) and interferon-γ (IFN-γ) production. Chauhan and Agrawal [39] studied immunopathological effects of alphamethrin, a synthetic pyrethroid, in six cross bred male bovine calves. The results showed that the blastogenic activity of T and B lymphocyte was reduced by 48% and 40%, respectively in comparison to the controls. The immunosuppressive effects were observed due to alphamethrin.

Present study showed enhanced oxidative stress through NO estimation in cypermethrin treated cells. Raina *et al.* [40] reported enhanced oxidative stress and lipid peroxidation due to dermal exposure of cypermethrin in rats. There is a clearly established relationship between ROS/ free radicals and apoptosis. Since ROS/ free radical intermediates mediate many immune cell functions and apoptosis has been established in immune cell populations, it is likely these two events could arise simultaneously during certain chemical exposures. In particular, two different studies reported induced apoptosis in rat and murine thymocytes and concluded an association between the onset of apoptosis and the increase in ROS [9, 41]. Wang et al. [42] showed that β-cypermethin and 3-PBA have immunotoxic effects on macrophages due to enhanced ROS production. In summary, there is sufficient evidence for relationship between ROS generation, immune cell regulation and induction of apoptotic processes.

There was an increase in cells undergoing apoptosis when treated with cypermethrin which is in accordance with several other reports [43]. Annexin-V binding assay is one of the most widely accepted and standardized technique which takes advantage of the changes in the membrane phospholipids that occur early in apoptotic cells [44-48]. cypermethrin treated cells showed typical DNA ladder pattern on agarose gel electrophoresis. It is also well established that pesticides cause genotoxicity and DNA damage weather *in vivo* or *in vitro* in majority of animal species [20, 49-54]. Undeğer and Başaran [55] evaluated genotoxic effects in freshly isolated human peripheral lymphocytes due to *in vitro* exposure of various concentrations of dimethoate and methyl parathion from the organophosphate class, propoxur and pirimicarb from carbamates, and cypermethrin and permethrin from pyrethroids through comet assay. The cells were incubated with various concentrations of the test substances for 0.5 hr at 37 ^{0}C and significant increase in DNA damage in human lymphocytes was observed at certain dose levels. Even 30 min exposure of these substances could induce genotoxic effects in peripheral lymphocytes.

Analyzing the outcome of the present studies it can be concluded that *in vitro* exposure of low level dose of cypermethrin caused immunotoxicity, oxidative stress and apoptosis in avian lymphocytes. The *in vitro* system was found to be a versatile and scientifically convenient system for such immunotoxicity studies and Annexin-V binding assay and DNA fragmentation analysis are proficient techniques to study and analyze pesticide induced apoptosis. It can be further emphasized that though Synthetic pyrethroids are increasingly used as insecticides and marketed as having relatively low human toxicity, there long term exposure and environmental accumulation may pose health problems in poultry, animals and humans. *In vitro* lymphocytes cell culture system can prove helpful in preliminary screening of low level pesticide exposure and thereby reduce experimental animal testing.

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