MONOCROTOPHOS INDUCED IMMUNOTOXIC EFFECT ON AVIAN LYMPHOCYTES THROUGH APOPTOSIS

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Introduction

Application of pesticides has become inevitable in the modern agriculture to augment the farming output. However it is now being realized that the unsystematic and lackadaisical use of pesticides might endanger the disease fighting potentialities of animals, man including poultry. Organophosphates (OPs) that replaced the use of organochlorines (OCs) are known for their pesticidal potency. There are reports confirming their toxic effects on immune, endocrine, and nervous system (Warren *et al*, 1999). Monocrotophos is a dimethoxy OP compound which is widely used to control pests of various crops. Since the presence of insecticide residue in agriculture produce is toxic to both animal and human health, the present investigation was aimed at elucidating the immunotoxic effect of commercial preparation of monocrotophos on avian lymphocytes exposed to NOEL/10^3 dose (1mg/kg, Charles, 1979).

Materials and methods

Avian lymphocytes were cultured in RPMI-1640 with Hepes buffer and 10% fetal calf serum (FCS). Lymphocyte cell culture was exposed to NOEL/10^3 dose of monocrotophos (Kadett-36) for 60 and 120 min respectively. Lymphocyte blastogenesis assay was carried out by the method of Rai-el Balhaa *et al*, (1987) with slight modifications (Chauhan, 1998) using LPS and Con A as B and T cell mitogens respectively. The reduction of the MTT dye (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) to formazon was used as an indicator of cell proliferation (Altman, 1976; Mosmann, 1983). The optical density was determined at 570nm. The results were reported as mean delta optical density (OD). Annexin-V binding assay was performed by the method of Chauhan and Tripathi (2002). Smears of cells quenched in 10% H_2O_2 were stained with annexin-V-biotin and avidin-peroxidase conjugate. Diaminobenzedine tetrahydrochloride (DAB) was used as substrate. Slides were examined under 40X objective for cells exhibiting brown colour on their surface. DNA fragmentation assay for treated and control cells was carried out using lysis buffer containing 20 μg proteinase K/ml, 25mM tris HCl, pH 8.0, 10mM EDTA, 10% SDS and 10 μg RNase A/ml and phenol-chloroform, precipitated using ethanol then subjected to agarose gel

electrophoresis. Flow cytometry was performed by the method of Nicolette *et al*, 1991. Cells showing increased SSC and decreased FSC on dot plots were identified as apoptotic cells. Electron microscopy (EM) was performed by the method of Malorni *et al*, 1998.

Statistical Analyses

Student's *t*-test was used for finding the significant difference between the treated and control cell populations. The values were expressed as mean delta optical density ± standard error (mean delta OD ±S.E).

Results

Mean delta OD of LPS and Con A stimulated lymphocyte culture in control and that of treated has been shown in Table-1. A significant (P≤0.05) reduction in mean delta OD was observed in monocrotophos treated cells when compared to control. The reduction in mean delta OD was higher at 120 min of exposure when compared at 60 min exposure time. Fixed cells on the slide, upon incubation with annexin-V-biotin displayed brown colour on their surface when compared to control (Fig.1). DNA fragmentation assay revealed ladder pattern of DNA of exposed cells when compared to control (Fig.2). Treated cells following flow cytometric analyses showed increased SSC and decreased FSC when compared to control (Fig.3). SEM showed smoothening of cell surface, formation of membrane blebs, while TEM of exposed cells revealed condensation of chromatin and its margination towards nuclear envelope, formation of apoptotic bodies and their engulfment by neighboring cells (Fig.4), which are the typical structural features of apoptotic cells.

Discussion

The present investigation was carried out to evaluate immunotoxic effects of NOEL/10^3 dose of monocrotophos, an OP insecticide in avian lymphocytes. Immunotoxicity was determined by lymphocyte blastogenesis, annexin-V binding, DNA fragmentation assay and flow cytometry. While electron microscopy was done for studying ultra structural alterations in exposed cells.

Lymphocyte blastogenesis assay revealed decrease in mean delta OD in presence of LPS and Con A in exposed cells when compared to the control. This indicated the depression of both HI and CMI response by monocrotophos. The MTT dye method has been widely used as marker for cell proliferation studies (Rai-el-Bahlaa, 1985; Dennizot and Lang, 1986; Charmichael *et al*, 1987). Depression of humoral response had earlier been recorded with multiple doses of insecticides (Banerjee *et al*, 1998; Tryphonas *et al*, 2000). The dose

dependent suppressive effect of trichlorfen and dichlorvos on lymphocyte proliferation and phagocytic ability of macrophages was earlier reported by Dunier *et al* (1991) while Khurana and Chauhan (2003) documented the immunosuppressive effect of monocrotophos in sheep.

Annexin-V binding assay using immunoperoxidase technique revealed translocated phosphatidylserine in the treated cells. Translocation of phosphatidylserine in cells undergoing apoptosis is one of the hallmarks of that process. During the stages of apoptosis, due to structural alterations in the plasma membrane, phosphatidylserine is translocated onto outer surface serving as a biomarker of cells undergoing apoptosis (Fadok *et al*, 1992). Therefore the detection of phosphatidylserine in exposed cells indicated monocrotophos induced apoptosis in avian lymphocytes. There are many reports of demonstration of phosphatidylserine using flurochrome coupled to annexin-V and then subjecting for fluorescent activated cell sorting (FACs) [Knoopman *et al*., 1994; Vermes *et al*.1995].

DNA fragmentation assay detected ladder pattern of DNA in treated cells. DNA fragmentation is one of the distinct features of apoptosis (Cohen and Duke 1984; Arends *et al*., 1990; Compton 1992; Schwartzman and Cidlowski, 1993; Nagata, 2000), thus the appearance of DNA ladder confirmed the apoptotic death induced by monocrotophos in avian lymphocytes.

Both TEM and SEM showed prominent ultrastructural changes in the cells exposed to monocrotophos. TEM demonstrated the condensation of chromatin, its margination towards nuclear periphery, and formation of apoptotic bodies while SEM revealed cells with loss of microvilli on their surface and formation of membrane blebs, which are the distinct features of apoptosis, further strengthening the evidence of monocroptophos induced immunotoxicity in avian lymphocytes.

Thus based on these findings it can be concluded that monocrotophos is immunotoxic and induction of apoptosis is one of the way by which it executes immunotoxicity.

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Time interval (min)	B cell blastogenesis	T cell blastogenesis
Control	0.254±0.066	0.256±0.015
60	0.185±0.082*	0.172±0.003*
120	0.155±0.019*	0.144±0.005*

* Significant at $P \leq 0.05$

Table 1 *In-Vitro* effect of Monocrotophos on B and T-lymphocyte blastogenesis using LPS and Con A respectively. (Mean delta OD ±S.E.)

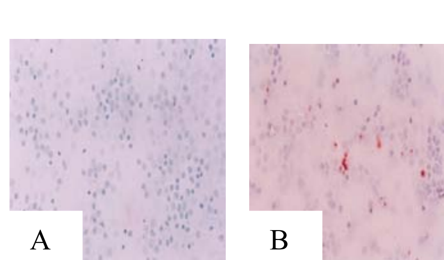


Fig 1. Photomicrographs of avian lymphocytes by immunoperoxidase technique using Annexin-V **A)** Normal lymphocytes (x400) and **B)** Lymphocyte showing exposed phosphatidylserine on it surface (x400).

1 2 3

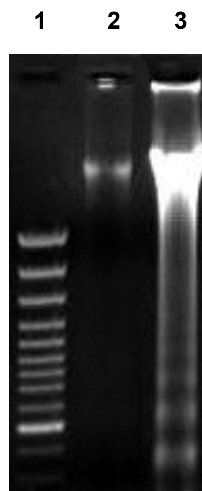


Fig 2. DNA analysis of avian lymphocytes exposed to NOEL/10^3 dose of monocrotophos with control upon 1 % agarose gel electrophoresis. Lane 1: Marker; Lane2: control; Lane 3: DNA ladder upon exposure to monocrotophos.

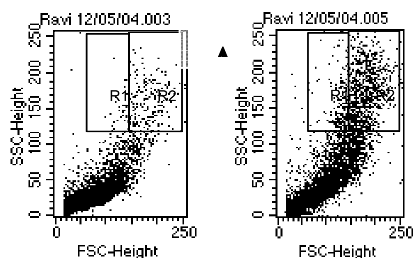
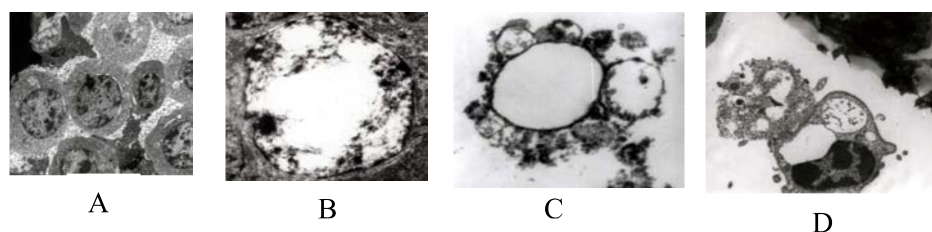


Fig 3. Dot plots from flow cytometric analysis of avian lymphocytes **A:** Control population **B:** After monocrotophos treatment for 8 h. Region (R1) represents apoptotic cells and R2 represents non-apoptotic cells.



A B C D

Fig 4.Transmission electron microscopic photographs showing apoptosis in avian lymphocytes induced by monocrotophos: - **A)** Normal lymphocyte (x11000), **B)** Margination of chromatin (x15000), **C)** Formation of apoptotic bodies (x11000), and **D)** Phagocytosis of apoptotic bodies (x11000)

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