Original Paper

Immunotoxicity Induced by Subacute Acephate Exposure in White Leghorn Cockerels

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ABSTRACT

Acephate (Ace) is an organophosphate foliar spray insecticide of moderate persistence with residual systemic activity of about 10-15 days. This study was designed to evaluate the immunotoxic effect of oral subacute acephate exposure in 125-day old WLH Cockerel chicks for 28 days. The experimental birds were randomly divided into five groups (C1, C2, T1, T2 and T3), each comprising 25 birds. The birds of group C1 were given no treatment and served as control. Group C2 was administered groundnut oil (1ml/kg) and served as control (vehicle). Group T1 was given $ALD_{50/40}$ (21.3 mg/kg), and Group T2 was put on $ALD_{50/30}$ (28.4 mg/kg), while group T3 received $ALD_{50/20}$ (42.6 mg/kg) of acephate suspended in groundnut oil. Experimental birds of all groups were vaccinated with New Castle disease vaccine at day 7.

Blood was collected at two-week intervals for evaluation of humoral immune response. Parameters such as TLC, TP, antibody titre against ND vaccine, DNCB dye test, and histopathology of immune organs were studied to evaluate immunotoxicity.

The results were statistically compared ($p<0.05$) with the control. Acephate produced decreased humoral immune response in terms of New Castle disease vaccine antibody titre, total protein, serum globulin, and serum albumin. Cell mediated immune response was checked with 1-chloro 2, 4 dinitrobenzene dye dermal sensitization test, and it did not reveal any significant differences. Lymphoid organs such as thymus, spleen, bursa, and liver were weighed during necropsy for calculation of organ weight: body weight ratio. After 28 days of acephate exposure, organ:body weight ratios of immune organs were significantly reduced except liver:body weight ratio on 14 days of exposure, which was increased in all treatment groups as compared to control groups. Histopathologically, bursa and spleen showed mild depletion of lymphocytes. To further identify the specific type of cell death as apoptotic or necrotic, DNA ladder assay was performed. DNA fragmentation assay detected ladder pattern (180bp) in DNA from hepatocytes and splenocytes of acephate-treated birds.

It is concluded that acephate is immunotoxic, and exerts its immunotoxicity through induction of apoptosis and alteration of immunological parameters.

Key Words: Acephate, White Leghorn Cockerel, Immunotoxicity, Humoral immunity, Cell-mediated immunity, Apoptosis, Histopathology

Introduction

The lowered immunocompetence in animals and birds due to environmental pollutants may lead to increased susceptibility, occurrence of recurrent infections, epidemics of disease and vaccinal failure. Application of pesticides has become inevitable in agriculture today to aug-

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ment the farming output. However, it is now being realized that the unsystematic and negligent use of pesticides might endanger the disease fighting capabilities of man and other animals, 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 systems. Acephate is one of the top ten organophosphate insecticides sold throughout the world. It is a water-soluble insecticide belonging to the phosphoramidothioate group of organophosphate insecticides, and is considered non-phytotoxic on many crop plants.[1] Since the presence of insecticide residues in agriculture produce is toxic to both animal and human health, the present study was conducted to investigate the effect of repeated (28 day) oral administration of acephate on the immune system of male white leghorn (WLH) cockerel chicks.

Materials and Methods

Experimental Animals

The present study was conducted on one-day old healthy, male White Leghorn (WLH) chicks. The chicks were procured and housed in pens at the Central Poultry Research Station, Anand Agricultural University, Anand. The chicks were provided with ad libitum standard feed and water, as followed by the research station. Standard procedures were adopted to keep the birds free from all stress.

Experimental Design

The birds were randomly divided into five groups (C1, C2, T1, T2, and T3), each containing 25 birds. Apparent LD_{50} of acephate (852 mg/kg) was taken into consideration for calculation of different dose groups.[2] Birds were treated with acephate at dose rates of $LD_{50/40}$, $LD_{50/30}$ and $LD_{50/20}$ in three treatment groups for a period of 28 days starting from day 1 of age. Group C1 was given no treatment and served as control. Group C2 was administered groundnut oil and served as vehicle control. Group T1 was given $1/40^{th}$ of LD_{50} (21.3 mg/kg), group T2 was put on $1/30^{th}$ of LD_{50} (28.4 mg/kg), and group T3 received $1/20^{th}$ of LD_{50} (42.6 mg/kg) of acephate suspended in groundnut oil.

The daily oral administration was continued for 28 days, and the live weight was recorded at weekly intervals.

On the 7^{th} day of experiment, the birds were vaccinated with Newcastle disease Lasota strain vaccine (Indovax) through intra-ocular route. The birds were also vaccinated against Marek's disease (MD) using MD Bivalent vaccine (Merial) 0.2ml at day old age, and IBD using Georgia strain - Indovax, at day 14.

Immunological Parameters

After 14 and 28 days of oral administration, the birds were weighed and blood samples were collected before final culling of birds, for estimating total leukocyte count (TLC).[3] Blood in glass tubes were kept under refrigeration, to separate serum which was utilized for estimation of antibody titer against ND by ELISA (using ELISA kit from Symbiotics Corporation, USA), serum total protein, and albumin and globulin levels. DNCB dye test for monitoring cell mediated immunity was done before 48 hours of culling of birds after primary sensitization at day 13. Skin over the left and right lateral abdomen of each bird was defeathered, aseptically cleaned and 2" x 2" area was marked with India ink. Primary sensitization was carried out by intradermal injection of 0.1 ml 0.5% DNCB dye solution in acetone on day 13 on left side, while on the right, acetone 0.1 ml was injected. Skin thickness was measured with Vernier callipers at 0, 24, and 48 hours after challenge. The sensitized birds were challenged after 14 days, i.e., on day 27 with 0.1 ml of 0.05 % DNCB solution in acetone on left side lateral abdominal skin, while the right side was injected with acetone. Reaction was assessed by measuring skin thickness at 0, 24, and 48 hours after challenge. The birds were sacrificed after taking body weight, and the weights of liver, spleen, thymus, and bursa were taken at necropsy for calculation of organ: body weight ratio. Organ: body weight ratio was calculated to know the effect of acephate on over all growth, as well as growth of various organs. O: BW ratio was calculated by dividing organ weight with body weight (g).

O: BW = Organ weight (g)/ Body weight (g) X 100

Histopathology

The organs (liver, kidney, spleen, thymus, and bursa) were then collected in 10% formalin for histopathological examination. Sections were cut at 6-8 microns thickness, and were stained with haematoxylin and eosin (H & E) stain.[4]

Molecular Detection of Apoptosis by DNA Ladder Essay

DNA fragmentation assay was carried out for treated

and control tissues of liver and spleen. The liver and spleen tissue homogenates were used to detect apoptosis. Extraction of genomic DNA from tissues was done as per standard procedure. Briefly, tissues were minced and incubated overnight at 65ºC in lysis buffer containing 25mM Tris HCl, pH 8.0, 10 mM EDTA, 150 µg Proteinase K /500 µl, 10% SDS to make 2% solution, and 30 µg RNAse/100 µl DNA. Each sample was extracted with a mixture of Phenol:Chloroform:Isoamyl alcohol (25:24:1) once, and the DNA precipitated with chilled isopropyl alcohol. The DNA was pelleted by centrifuge at 10000 rpm/25ºC/10min. The DNA was resuspended in Tris EDTA pH 7.4 and quantified using UV Spectrophotometer at 260 and 280 nm. The DNA having ratio of 1.8 was considered as being of acceptable purity. The concentration of DNA was estimated using the following formula:

Concentration of DNA ng/µl = OD 260 ? 50 ? dilution factor

The extracted tissue DNA samples were electrophoresced and compared for their DNA laddering pattern in 0.8% (w/v) agarose gel containing ethidium bromide (0.5µg/ml) in 0.5X TBE buffer using horizontal submarine electrophoresis apparatus.

To confirm the tissue DNA extraction and ladder pattern in DNA, 5µl of extracted genomic DNA from each treatment and control group was mixed with one µl of 6X gel loading buffer and electrophoresced at constant 80 V for 30 min in 0.5X TBE buffer. The electrophoresced DNA was documented by using gel documentation system: Syn Gene (Gene Genius Bio Imaging System, UK).

TBE 5 (5X)

Tris base	54g
Boric acid	27.5g
0.5 M EDTA (pH 8.3)	20 ml
Deionized water up to	1000 ml

Agarose gel loading buffer (6X)

Bromophenol blue	0.25% (w/v)
Xylene cynol FF	0.25% (w/v)
Ficoll	15% (w/v) (Type 400; Pharmacia)

Dissolved in appropriate volume of Deionized water

Ethidium Bromide	1% used @ 5 µl/100 ml agarose gel solution.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare the effects of treatment of various doses of acephate on different biochemical, haematological and immunological variables in control and treated birds. The results were presented as mean ± Standard deviation (SD). Statistical analyses were performed using EXCEL software to determine the one-way analysis of variance, and for assessment of statistical significance (ANOVA, student's t-test). Differences between means were considered significant for $p<0.05$.

Results

The results of immunological study of oral subacute acephate exposure to day old WLH Cockerel chicks on TLC (total leukocyte count), protein metabolism, and organ: body weight ratios are displayed in **Tables 1** to **3**. *Gross Lesions* - The liver was pale in colour and was friable. It was greasy and easy to cut, and appeared fatty. At 14 days of acephate treatment, the size of liver was found to be enlarged in all the treatment groups as compared to those of control groups. Similarly, at 28 days of treatment also, there was enlargement of liver in T1 and T2 groups, whereas a marked reduction in size of liver was observed in T3 group. Further, areas of haemorrhages, necrosis, and congestion were noted in the treatment groups, but the severity varied from mild to intense on the basis of the acephate dose administered. Consistency of spleen was normal in all the groups, but congestion was observed in birds given acephate. Size of spleen was reduced corresponding to the toxic dose of acephate. No observable changes were found in the bursa at necropsy. There was hypotrophy of individual thymic lobes in the thymus. Size and consistency of kidneys were normal in all the groups, but marked congestion was observed in the treatment groups at necropsy.

Histopathological Findings - The changes were mainly noticed in the group T3. Microscopic alterations were observed in almost all the collected organs of acephate treated birds except in the thymus. Sinusoidal congestion and parenchymatous degeneration of hepatic cells and fibrosis in hepatic portal areas were observed in liver. Spleen showed congestion and severe lymphoid depletion. There was severe atrophy of bursal follicles and fibrosis in the interfollicular areas in the bursa of

birds sacrificed on 29th day. However, no changes could be observed in the thymus.

Discussion

As the immune system is one of the most sensitive targets of pesticides owing to continuous proliferation and differentiation, and as the study was conducted on day-old WLH cockerels, these chicks were more susceptible to toxicity on the immune system, as the lymphoid organs were in the growing phase.

Different non-functional (serum total protein, serum total albumin, serum total globulin and organ: body weight ratio), and functional (ELISA and DNCB) immunological parameters were studied after 14 and 28 days of oral subacute acephate exposure in these chicks.

TLC was done to see the effect of acephate on leukopoietic organs. ND antibody titres were measured to see the effect of acephate on development of humoral immunity, while DTH was evaluated by DNCB dye test to monitor development of cell-mediated immunity. Organ: body wt. ratio of immune organs was calculated after taking the body weight of immune organs at necropsy. The collected organs, namely liver, spleen, bursa, thymus, and kidney were also used for observing histopathological changes in these organs.

TLC - The administration of acephate did not generate any significant change in the Total Leukocyte Count in WLH cockerels after 14 and 28 days of oral administration of acephate, but at both 14 and 28 days of exposure, there was a non-significant reduction in TLC in the birds of treatment group, whereas no effect was observed on birds of control group (**Table 1**).

Toxin exposure causes a specific immunologic imbalance unseen by other causative agents, including a general decrease in cell-mediated immunity (CMI), and an increase in humoral immune response.[5,6]

In the present study, antibody titres against ND vaccine were measured by enzyme linked immunosorbent assay (ELISA) to monitor humoral immune response, and DNCB dye test was done to monitor cell-mediated immunity in the birds, previously vaccinated at 7 days of age with Lassota strain ND vaccine. The results indicated no significant change in antibody titres against ND vaccine at 14 days exposure, except an increased antibody titre in the group T1 (21.3 mg/kg body weight). However, there was significant reduction in antibody titres against ND vaccine at 28 days exposure. Similarly, a reduction of antibody titres was observed in rats in the 6-7 weeks of an immunization procedure that commenced either one day before, or one day after an oral dose of half of the LD50 of parathion.[7] Most organophosphorous pesticides elicit autoimmune reactions and suppress the production of antibodies against vaccines.[8]

Elevation of humoral immunity often results in production of antibodies to various tissues,[9] and is also seen in exposure to chlorinated pesticides.[10]

Rishi and Garg (1993) observed a stimulatory effect on total antibody titre at low doses of malathion in chicks; however, immunosuppression was observed at higher dose level.[11]

Various groups of pesticides have been found to cause an immunosuppressive effect in birds. Suppression of humoral immune response was observed by chlorpyriphos,[12] quinalphos,[13] lindane,[14] and alphamethrin[15] in chickens.

Table 1 Effect of Daily Oral Administration of Acephate on Total Leukocyte Count (no./cmm) in WLH Birds

Groups	TLC (MEAN±S.E.)	
	14 day	28 day
Group C1 (Control)	15750.00[a]± 946.485	15678.33[a]± 959.408
Group C2 (Vehicle Control)	15854.50[a]±1087.170	15833.33[a]± 1301.708
Group T1 (LD50/40)	15733.33[a]±437.163	15399.50[a]± 524.811
Group T2 (LD50/30)	15483.33[a]±416.667	15278.33[a]± 640.096
Group T3 (LD50/20)	15350.00[a]±963.933	15066.67[a]± 517.472

Values indicate Mean ± S.E. (figures in brackets indicate 'n')
Means having different superscripts at a particular period of treatment differ significantly (P<0.05)

In general, low level of organophosphorous pesticide exposure appears to have a stimulatory effect, while higher level of exposure depresses immune functions.[16] The exact mechanism by which immunosupression occurs is in many cases not clear.

In this study there was no significant difference in increase in dermal thickness, i.e., DTH response to DNCB dye between control and treatment groups on day 14 and day 28 on left and right sides after 24 hrs and 48 hrs of sensitization. The comparison of increase in thickness of left side (injected with dye) and right side (injected with vehicle) showed that there was more increase in thickness on left side as compared to that of the right. This result suggests that acephate may be non-toxic with regard to cell-mediated immune response at dose rates administered in this study. Similarly, quinalphos in mice and monocrotophos in goats did not cause suppression of CMI as tested by DNFB dye test.[17] Lack of statistically significant effect of aldicarb on immune system was observed when mice received water containing aldicarb for 34 days, while chlorpyriphos,[12] butachlor,[18] fenvalerate,[19] quinalphos,[14] cypermethrin,[20] and alphamethrin,[15] cause suppression of CMI in chickens. Suppression of CMI in lambs was observed on exposure to monocrotophos,[21] fenvalerate,[20] and carbofuran.[22] Chahal, (1995) studied the toxicological effect of fenvalerate in buffalo calves, and reported suppression of cell-mediated immunity on dermal spray of fenvalerate.[23]

Koller et al., (1976) observed no significant alteration of primary and secondary immune response in mice by feeding leptophos up to concentration of 500 ppm for 12 weeks.[24] Similarly, triphenyl phosphate (TPP), a potential feed contaminant in the diet did not produce significant effect on immune response.[25]

In the present study, after exposure to acephate, the serum total proteins, serum total albumin, and serum total globulin were found significantly decreased (**Table 2**), as compared to control, indicating significant damage to vital organs and interference with protein metabolism. Similarly, decreased level of serum proteins has been noted in white leghorn birds with subacute oral administration of acephate for 28 days in other studies.[26,27] A decrease in serum total proteins and albumin has also been observed due to exposure of different insecticides in birds,[28] whereas no effect on serum albumin was observed due to cypermethrin in chicken.[29] Serum globulins were found to be decreased due to feeding of endosulfan and quinalphos in chicks,[30] and lindane and monocrotophos in lambs.[21]

The fall in total serum protein could be due to the stressogenic effect of acephate, or general toxic action that also leads to decrease in weight gain in the insecticide-treated birds. Reduction in total serum protein has also been reported in cockerels following prolonged feeding of fenvalerate-medicated ration at the rate of 4000 ppm.[31] Serum globulin level was decreased in all treated groups following feeding of broiler chicks with 20-ppm fenvalerate, 2 ppm monocrotophos, and 2 ppm endosulfan.[13]

In the present study, organ: body wt. ratios of immune organs were calculated after taking the body weight of immune organs at necropsy. The weight and gross morphology of lymphoid organs were the first parameters

Table 2 Effect of Daily Oral Administration of Acephate on Serum Protein, Albumin & Globulin in WLH Birds

Groups	**Serum Total Protein** (MEAN±S.E.)		**Serum Albumin** (MEAN ±S.E.)		**Serum Globulin** (MEAN ±S.E.)	
	14 day	**28 day**	**14day**	**28 day**	**14day**	**28 day**
Group C1 (Control)	$2.83^{b,c}$±0.21	3.13^{b}± 0.32	$1.39^{b,c}$±0.10	$1.15^{b,c}$±0.09	1.45^{a}±0.21	1.98^{a}±0.33
Group C2 (Vehicle Control)	3.06^{c}±0.13	3.15^{b}±0.40	1.46^{c}±0.02	1.18^{c}±0.06	1.61^{a}±0.13	1.97^{a}±0.43
Group T1 (LD50/40)	$2.48^{a,b}$±0.20	2.21^{a}±0.15	$1.20^{a,b}$±0.08	0.98^{a}±0.05	1.28^{a}±0.24	1.24^{a}±010
Group T2 (LD50/30)	2.17^{a}±0.19	2.25^{a}±0.12	$1.19^{a,b}$±0.09	0.94^{a}±0.06	0.98^{a}±0.23	1.38^{a}±0.13
Group T3 (LD50/20)	2.08^{a}±0.18	2.06^{a}±0.11	1.13^{a}±0.02	0.91^{a}±0.05	0.94^{a}±0.19	1.16^{a}±0.14

Values indicate Mean ± S.E. (figures in brackets indicate 'n')
Means having different superscripts at a particular period of treatment differ significantly (P<0.05)

studied in assessing toxicity, as the response to injury is often expressed as changes in tissue or organ weight, size, colour, and gross appearance. These observations were combined with leukocyte counts, studies of differentiation, and the results of histopathological evaluations of lymphoid organs and tissues. After 14 days of acephate treatment, the liver to body weight ratio increased in all treatment groups compared to control groups. However, the ratio at 28 days of acephate exposure increased in T1 and T2 groups, whereas a significant reduction was recorded in T3 group (**Table 3**). A similar increase in liver: body wt. ratio has been observed due to malathion treatment in mice,[32] and PCB in mice.[33] This trend of increase and decrease in various treatment groups could be substantiated on the basis of the principle that there will be acute inflammatory reactions which lead to increase of weight and size of organ critically, followed by reduction in both, particularly in group T3, which might be due to the toxic effect of acephate during its metabolism in the liver as evidenced by histopathological changes in the hepatic parenchyma. The exact route of exposure also affects the organ weight at necropsy. In one study, significant depression in liver weight was found at all oral exposure levels of carbaryl, while dermal exposure revealed no significant change.[34]

In the present study, no significant change in spleen: body weight ratio was noted on 14 days of acephate exposure, however there was significant decrease in spleen: body wt. ratio on 28 days of acephate exposure (**Table 3**). Similar reduction in spleen: body wt. ratio has been observed following cypermethrin,[29] carbaryl,[35] carbofuran,[36] and chlorpyriphos[12] exposures in broiler chickens. A decrease in spleen: body weight ratio at the highest dose suggests that acephate might be immunotoxic at higher doses. Decrease in spleen: body wt. ratio was supported by histopathological observations indicating depletion of lymphocytes in individual splenic follicles.

There was significant reduction in bursa: body weight ratio observed after 14 and 28 days of acephate exposure (**Table 3**). Similar results have been observed by various earlier workers following exposure to cypermethrin,[29] carbaryl,[35] carbofuran,[36] and chlorpyriphos[12] in broiler chickens. Rupture of bursal follicles and reduction in follicular density are suggestive of decreased bursa weight due to acephate toxicity on lymphoid organs. Decrease in bursa: body weight ratio was supported by gross and histopathological observations.

A dose dependent reduction in thymus: body weight ratio was also observed in this study. However, there was more significant reduction in thymus: body weight ratio in T3 group as compared to control groups (**Table 3**). A similar decrease in thymus: body weight ratio has been observed due to exposure to PCB,[33] and carbofuran[36] in broiler chickens. The significant reduction in thymus: body wt. ratio might be due to the immunotoxic effects of subacute acephate exposure at higher dose levels. Since in the case of cockerels, the postnatal growth of thymus occurs up to 8th week, only negligible histopathological changes could be observed, which can be substantiated on the basis of compensatory mechanism of growing tissues. The decrease in thymus: body wt. ratio was also supported by gross pathological observations, which showed hypotrophy of individual thymic lobes, but the histopathological examintaion did not show any decrease in lymphocyte populations.

Table 3 Effect of Daily Oral Administration of Acephate on Organ:Body wt Ratio of Immune Organs in WLH Birds

Groups	Liver: Body wt ratio		Spleen: Body wt ratio		Bursa: Body wt ratio		Thymus: Body wt ratio	
	14-day	28-day	14-day	28-day	14-day	28-day	14-day	28-day
Group C1 (Control)	3.03^{a}±0.09	3.13^{b}±0.14	$0.13^{a,b}$±0.01	0.21^{b}±0.01	0.63^{b}±0.02	0.57^{b}±0.06	$0.25^{a,b}$±0.01	0.28^{b}±0.03
Group C2 (Vehicle Control)	3.03^{a}±0.10	3.06^{b}±0.14	$0.14^{a,b}$±0.01	0.21^{b}±0.01	0.64^{b}±0.05	0.66^{b}±0.05	0.29^{b}±0.03	0.29^{b}±0.04
Group T1 (LD50/40)	3.32^{a}±0.11	4.55^{c}±0.28	0.19^{b}±0.05	0.16^{a}±0.02	0.58^{b}±0.07	0.54^{b}±0.06	$0.23^{a,b}$±0.03	$0.21^{a,b}$±0.06
Group T2 (LD50/30)	3.26^{a}±0.08	4.31^{c}±0.27	$0.14^{a,b}$±0.01	0.15^{a}±0.01	0.57^{b}±0.01	0.30^{a}±0.03	0.21^{a}±0.01	$0.20^{a,b}$±0.04
Group T3 (LD50/20)	3.31^{a}±0.10	2.04^{a}±0.28	0.10^{a}±0.01	0.15^{a}±0.02	0.43^{a}±0.04	0.27^{a}±0.05	0.20^{a}±0.02	0.13^{a}±0.01

Values indicate Mean ± S.E. (figures in brackets indicate 'n')
Means having different superscripts at a particular period of treatment differ significantly (P?0.05)

Histopathological studies were made to evaluate the extent of immunotoxicity caused by acephate at the administered doses from organs such as liver, spleen, thymus, and bursa of Fabricius. Microscopic changes were observed in almost all the organs collected. Severity of microscopic lesions in different organs and different groups of birds given acephate was in accordance with the dose given. Severity of lesions was more in the group given the highest dose of acephate (Group T3: 42.6 mg/kg body weight). The nature of histopathological lesions observed in different organs in various dose groups are listed below.

Liver changes comprised mild to severe congestion, haemorrhages, parenchymatous degeneration of hepatic cells, and fibrosis in hepatic portal areas. These lesions may be attributed to decomposition and metabolism of acephate to methamidophos in liver. Liver being the organ of biotransformation of majority of the compounds, most prominent lesions such as congestion, vacuolar degeneration, accumulation of fat in centrilobular area, focal to extensive necrosis, hyperplasia of kupffer cells and dilation of sinusoids could be observed. Similar nature of lesions was also reported by earlier workers.[35,37-40]

In the present study, no significant gross changes were observed in immune organs such as bursa and thymus, while spleen showed minor lesions in the form of paleness and reduction in size in some of the birds of T3 group. Histopathological examination of bursa of Fabricius showed mild to moderate depletion of lymphocytes, and mild interfollicular fibroblastic proliferation. Spleen showed severe depletion of lymphocytes, while thymus did not reveal any histopathological lesions. The lesions were principally observed in the highest treatment groups, and indicated that higher doses may produce significant signs of toxicity in spleen and thymus.

Similar depletion of lymphocytes in focal areas in bursa of Fabricius and spleen have been observed by Khurana et al (1996a) in broiler chicks.[29] Cha et al (2000) observed depletion of spleen lymphocytes in the periarteriolar lymphoid sheath and marginal zone in white pulp.[41] Fibroblastic proliferation in the bursa of Fabricius was observed by feeding butachlor to birds for 6 months.[42] However, no changes could be traced in the thymus. Some workers failed to demonstrate any histopathological changes following feeding of famphur[43] and aldicarb[44] in rats. It could be due to low doses, or short duration of exposure.

The immune system plays a central role in the maintenance of an organism's health; the interaction of xenobiotics with various components of the immune system has become an area of profound interest. Suppression of immune responses by certain pesticides has been suggested to be the basis of increased allergy, hypersensitivity and malignancy. Therefore, there is an urgent need to obtain more information regarding the manner in which pesticides cause immunotoxicity.

In this study, to further identify the specific type of cell death as apoptotic or necrotic, DNA ladder assay was performed. DNA fragmentation assay detected ladder pattern (180bp) in DNA from hepatocytes and splenocytes of the birds in the treatment groups as compared to control group. Thus, the appearance of DNA ladder confirmed the apoptotic death induced by acephate in avian hepatocytes and splenocytes. DNA fragmentation is one of the well known features of apoptosis. The DNA Ladder assay is based upon the principle that during apoptosis, cellular nuclear DNA is non-randomly cleaved into 180-200 base pair units. When run on an agarose gel, this DNA ladder can be detected, and is an indicator of apoptotic cells. DNA fragmentation has been suggested to be one of the first irreversible events to trigger mature immune cell apoptosis.

Thus based on these findings, it can be concluded that acephate is immunotoxic, and induction of apoptosis is one of the ways by which it executes immunotoxicity. The consistency in results from all experiments strengthens the claim that this pesticide induces apoptosis and causes immunotoxicity. There is no assay that can conclusively detect and measure immunosupression and apoptosis or necrosis in cells. Therefore, it is important to examine multiple parameters, as done in this study, before drawing conclusions, particularly since apoptosis is a very rapid event and it is also likely that both modes of cell death occur simultaneously.[45]

The results indicated that acephate was found to be immumnotoxic at the dose level tested. As immune system is one of the most sensitive targets of pesticides owing to continuous proliferation and differentiation, acephate could be considered as immunotoxic at this exposure level as it was found to be immunotoxic.

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REFERENCES

1. Worthing, CR. The Pesticide Manual: A World Compendium. 8th edn, 1987. The British Crop Protection Council, UK.
2. Willcox H, Coffey T. Environmental Impact of Acephate (Orthene®) on Forest Insect and Disease Management. 1977. Department of Agriculture Forest Service. Northeastern Area State and Private Forestry, Upper Darby. p.8.
3. Jain NC. Schalm's Veterinary Hematology, 4th edn, 1986. Lea and Febiger, 600 Washington Square, Philadelphia, USA.
4. Luna LG. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd edn, 1968. McGraw Hill Book Co., New York, USA.
5. Colosio C, Barcellini W, Maroni M. Immunomodulatory effects of occupational exposure to mancozeb. Arch Environ Health 1996; 51: 445-451.
6. Casale GP, Scott DM, Anderson JR, et al. A preliminary study of immunologic and hematologic profiles of peripheral blood from Nebraska farmers who apply pesticides to their fields. Clin Toxicol 1998; 36: 183-194.
7. Dandliker WB, Hicks AN, Levinson SA, Stewart K, Brawn RJ. Effects of Pesticides on the Immune Response. 1979. La Jolla, California, Scripps Clinic and Research Foundation (epa 600/1-79-039).
8. Shtenberg AI, Khovaeva LA, Zavarzina MV. Effect of chlorophos and methyl nitrophos on the immune reactions of the organism against the background of protein-deficient nutrition. Vopr Pitan 1974; 8: 35-42.
9. Thrasher JD, Madison R, Broughton A. Immunologic abnormalities in humans exposed to chlorpyrifos: Preliminary observations. Arch Environ Health 1993; 48: 89-93.
10. Queiroz MLS, Bincoletto C, Perlingeiro RCR, Quadros MR, Souza CA. Immunoglobulin levels in workers exposed to hexachlorobenzene. Hum Exp Toxicol 1998; 17: 172-175.
11. Rishi S, Garg BD. Effect of malathion on immune response to T-dependent and T-independent antigens in chicken. Indian J Poult Sci 1993; 28: 208-216.
12. Malik G, Gera S, Dahiya JP, Kadian SK, Agarwal VK. Biochemical and immunological alterations induced by chlorpyriphos intoxication in broiler chickens. Indian J Animal Sci 2005; 75: 668-671.
13. Garg UK, Pal AK, Jha GJ, Jadhao SB. Haemato-biochemical and immuno-pathophysiological effects of chronic toxicity with synthetic pyrethroid, organophosphate and chlorinated pesticides in broiler chicks. Int Immunopharmacol 2004; 4: 1709-1722.
14. Garg S, Singh BP, Chauhan RS. Immunosuppressive effect of lindane on humoral immune response in poultry. In: Proceedings of the XXI World Poultry Congress, Montreal, Canada. 2000; p227-228.
15. Singh SP, Sharma LD, Chauhan RS. Immunotoxic effect of acute toxic effect of alphamethrin in chicks. In: Proc. Nat. Symp. Adv. Vet. Path. Post Independence Era, and XIV Ann. Conf. IAV, Izzatnagar; 1997. p157-159.
16. Galloway T, Handy R. Immunotoxicity of Organophosphorous Pesticides. Ecotoxicol 2003; 12: 345-363.
17. Jha GJ, Mahto LM, Tamang RK, Gupta MK, Chauhan HV. Evaluation of cell mediated immunity during chronic organophosphate intoxication in mice and goats. Acta Vet Hung 1990; 38: 55-60.
18. Kumar R, Singhal LK, Singh BP, Chauhan RS. Effect of butachlor on cell-mediated immunity in chicken. J Immunol Immunopathol 2002; 4: 84-87.
19. Singhal LK, Singh BP, Chauhan RS. Fenvalerate induced stress and its effect on cell-mediated immunity ion chickens. Int Symp End Tech Sec: Anim Welf Consider 2001.
20. Khurana R, Chauhan RS. Immunopathological effects of fenvalarate on cell mediated immune response in sheep. J Immunol Immunopathol 2000; 2: 56-59.
21. Khurana R, Chauhan RS. Immunopathological effects of monocrotophos on humoral immune response in sheep. J Immunol Immunopathol 2003; 5: 54-56.
22. Khurana R, Mahipal SK, Chauhan RS. Effect of carbofuran on cell mediated immunity in sheep. Indian J Toxicol 1998; 5: 1-6.
23. Chahal GS. Toxicological study of fenvalerate in buffalo calves and poultry with special reference to immunosupression. MVSc Thesis, 1995. GB Pant Univ Agri Technol., Pantnagar, India.
24. Koller LD, Exon JH, Roan JG. Immunological surveillance and toxicity in mice exposed to organophosphate pesticide, leptophos. Environ Res 1976; 12: 238-242.
25. Hinton DM, Jessop JJ, Arnold A, Albert RH, Hines FA. Evaluation of immunotoxicity in a subchronic feeding study of triphenyl phosphate. Toxicol Health 1987; 3: 71-89.
26. Purohit TJ. Subacute oral toxicity study of acephate in synthetic white leghorn birds. MVSc Thesis, 2005. Anand Agricultural University, Anand.

27. Patel N. Toxicological studies of short term exposure of bantamized white leghorn birds to acephate. MVSc Thesis, 2005. Anand Agricultural University, Anand.
28. Bhushan B. Study on immunotoxicological effects of malathion and carbaryl in chickens. MVSc Thesis, 1993. CCS Haryana Agri Univ., Hisar, India.
29. Khurana R, Chauhan RS, Mahipal SK. Immunopathology of chronic cypermethrin toxicity in poultry. In: Proceedings of XIII Annual Conference of IAVR and National Syposium on Impact of Environmental Pollution on Health and Production of Livestock, Poultry and Wild Life. 1996a. Pantnagar, India. p71.
30. Khurana R, Chauhan RS, Mahipal SK, Rishi S. Effect of endosulphan on serum biochemical attributes in chickens. Haryana Vet 1996b; 90-94.
31. Singh G, Sharma LD, Singh SP, Ahmed AH. Haematobiochemical profiles in cockerels following prolong feeding of fenvalerate. Indian J Toxicol 2001; 8: 141-145.
32. Bannerjee BD, Pasha ST, Hussain QZ, Koner BC, Ray AA. Comparative evaluation of immunotoxicity of malathion after subchronic exposure in experimental animals**.** Indian J Exp Biol 1998; 36: 273-282.
33. Koszutsky J, Skrobanek P. Prolonged exposure of broiler chicks to PCB: Clinical and biochemical changes. Vet Med 1994; 39: 397-407.
34. Ladis GS, Smith C, Heaps K, Loveless SE. Evaluation of the humoral immune response of CD rats following a two week exposure to the pesticide carbaryl by the oral, dermal or inhalational routes. J Toxico Environ Health 1994; 42: 143-156.
35. Varshneya C, Singh T, Sharma LD, Bahga H, Garg SK. Immunotoxic responses of cypermethrin, a synthetic pyrethroid insecticide in rats. Indian J Physiol Pharmacol 1992; 36: 123-126.
36. Nasser AM, Zanaty K. Adverse effect of immunity and performance of broiler chicken dipped in some pesticide. Vet Med J 1994; 30: 242-245.
37. Gupta PK Chandra SV. Toxicity of endosulfan after repeated oral administration to rats. Bull Environ Contam Toxicol 1977; 18: 378-384.
38. Lee WG, Whang IW, Sohn TJ. Ultrastructural study on the mechanism of hepatotoxicity of hexachlorochyclohexane and the protective effect of glutathione. Kyungpook Univ Med J 1980; 21: 78-84.
39. Spicer EJF, Phillips LM, Jefferson MD, Blair M. Lifetime oral carcinogenicity study in mice with Orthene.(R) 1982. Technical Report No. 415-006 from International Research and Development Corp., Mattawan, Michigan, USA. Submitted to WHO by Tomen Agro Inc., San Francisco, California, USA.
40. Majumdar S, Chakraborty AK, Mandal TK, Bhattacharya A, Basak DK. Subacute toxicity of fenvalerate in broiler chicks: Concentration, cytotoxicity and biochemical profiles. Indian J Exp Biol 1994; 32: 752-756.
41. Cha SW, Gu HK, Lee KP, Lee MH, Han SS, Jeong TC. Immunotoxicity of ethyl carbamate in female mice: Role of esterase and cytochrome P-450. Toxicol Lett 2000; 115: 173-181.
42. Singh AK, Singh DD, Singhal LK, Chauhan RS, Singh GK. Age related changes in lymphoid organs in poultry due to butachlor. In: Proceedings of XVI Annual Conference of Indian Association of Veterinary Anatomists. Pantnagar, India. 2001. p89.
43. Black WD, Valli VE, Claxton MJ, Marcean-Day ML. The effects of subchronic feeding of the organophosphate famphur to rats. Toxicol Appl Pharmacol 1979; 50: 167-170.
44. Thomas P, Ratajczak HV, Demetral D, Hagen K, Baron R. Aldicarb immunotoxicity: Functional analysis of cell mediated immunity and quantitation of lymphocyte sub-populations. Fundam Appl Toxicol 1990; 15: 221-230.
45. Corcoran GB, Fix L, Jones DP, Moslen MT, Nicotera P, Oberhammer FA, Buttyan R. Apoptosis: Molecular control point in toxicity. Toxicol Appl Pharm 1994; 128: 169-181.