CHAPTER-3

SINGLE DOSE EFFECT OF CHLORPYRIFOS AND LEAD COMBINATION AT CELLULAR LEVEL

Introduction

The nervous system and organs like liver, kidney etc show reserve functional capacity. Due to this, despite animals suffering from structural loss or damages to a portion of these organs no normal functions are manifested. Normal behavior of animals also can be seen after structural loss in the nervous system. The serum urea nitrogen concentration does not increase until approximately 75% of the nephrons are nonfunctional. It is important to study histopathology to correlate the observed changes in behavioral and clinical pathology parameters.

Though single dose exposures to chemicals rarely causes permanent structural changes, some organophosphate chemicals do produce delayed (after a period of time) permanent injury to neuronal tissues after single dose exposure. A mild distal axonopathy was reported some weeks after poisoning incident with chlorpyrifos in a human at a dose of 300-400 mg/kg (Wagner, 1999).

Nephropathy is a characteristic manifestation of lead toxicity. Two types of nephropathy, acute and chronic, have been observed in humans. Acute nephropathy occurs during the early stages of excess exposure, especially in children. The characteristics of early or acute lead-induced nephropathy in humans include nuclear inclusion bodies, mitochondrial changes, and

cytomegaly of the proximal tubular epithelial cells and dysfunction of the proximal tubules. Glomerular effects are either minimal or absent. The acute form is reported in lead-intoxicated children, whose primary exposure is via the oral route, and sometimes in lead workers (Davidson, 1996 and ASTDR, 1999).

Overt encephalopathic symptoms/irreversible severe brain damage can occur after exposure to high concentrations of lead. Overt symptoms of subencephalopathy system (CNS) and peripheral nerve damage are reported to occur at blood lead concentrations ranging from 40-60 µg/dL. Severe lead induced encephalopathy is generally not observed in adults except at extremely high blood lead levels. Reports suggest that acute lead poisoning can cause severe gastrointestinal symptoms and/or signs of encephalopathy in some adults at blood lead levels that range from approximately 50 to >300 µg/dL (Davidson, 1996). Considering systemic toxicity of lead, and to correlate behavioral and biochemical findings with changes at cellular level and, to assess any lead-chlorpyriofos interactive effects, the histopathological studies on below mentioned organs were carried out.

Materials and Methods

Procedure

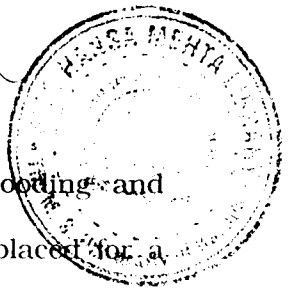
The animals belonging to single dose exposure of chlorpyrifos and lead acetate were sacrificed on day 15. The following organs were collected and preserved in 10% neutral formalin; brain, pituitary, sciatic nerve, spinal cord, (cervical, thoracic and lumbar), eye, muscle, liver, kidney, spleen, adrenal, thyroid and parathyroid and thymus. Histopathological examination was carried out for four males and four females each belonging control and, high dose groups of chlorpyrifos (group 5), lead (group 6) and chlorpyrifos

plus lead (group 7). The dose levels and groups and experimental procedure used are mentioned in the chapter 1.

Tissue Processing

After trimming the tissues, they were filled in capsules. Tissue capsules were washed in running tap water at least for 6 hours. Washed capsules were processed in Automatic Tissue Processor, manufactured by Leica Automated Vacuum Tissue Processor (Model N° ASP 300). Automated Tissue Processor contains dehydration, clearing and wax impregnation units. Tissues were treated in alcohol, xylene and wax in the following conditions.

Step 1	Reagent	Time Duration (hours)
1	70 % Alcohol	1
2	80 % Alcohol	1
3	90 % Alcohol (Bottle I)	1
4	90 % Alcohol (Bottle II)	1
5	100 % Alcohol (Bottle I)	1
6	100 % Alcohol (Bottle II)	1
7	Xylene (Bottle I)	1
8	Xylene (Bottle II)	1/2
9	Xylene (Bottle III)	1/2
10	Wax (Bottle I)	1
11	Wax (Bottle II)	1
12	Wax (Bottle III)	3



Decalcification of bony tissues

Sternum, femur and spinal cord should be decalcified. Gooding and Stewart's fluid was used as decalcifying fluid. Tissues were placed for a minimum of 4 days in decalcifying fluid.

Embedding

TBS 88 Paraffin Embedding System Dispenser Unit and TBS 88 Paraffin Embedding System Cool Unit (Model- Medite/TBS 88) were used for embedding of tissues. During embedding, temperature of the paraffin tank, working plate and cooling plate was between 63-67 °C, 58-63 °C and -5 to 0 °C respectively. The heated working plate was used for providing optimal warming of tissue capsules. Tissues were placed in proper orientation in paraffin block by using cold point provided in the working plate. Disposable plastic block holders were used for moulding purpose and tissue identification labels were placed above the mould. Wax Embedding moulds were kept on the cooling plate (-5 to 0 °C) for wax solidification.

Section Cutting

Semi Automatic Rotary Microtome (Model - Leica RM 2145) was used for getting sections of tissues. Initially the blocks were trimmed (thickness of 15 – 20 μ) and then the gauge of the microtome was set at 3-5 μ to obtain thin sections. The tissue sections were transferred to warm water bath (temperature- 48 – 55 °C) to make them free of folds. Clean slides applied with egg albumin were inserted at 45° angle to transfer sections in to the slides. After labeling the slides with lead pencil they were placed in slide warming table (48 to 55 °C) for 2 -3 minutes. Once the wax got melted out slides were placed in staining rack.

Staining and Mounting

Hematoxylin-eosin staining was performed as per the following routine;

Step N°	Reagent	Time Duration (minutes)	Step N°	Reagent	Time Duration (minutes)
1	Xylene (I)	2	11	Ammonia Water	3 – 4 dips
2	Xylene (II)	2	12	Water	1
3	100 % Alcohol	1	13	95 % Alcohol	1
4	90 % Alcohol	1	14	Eosin	1
5	80 % Alcohol	1	15	70 % Alcohol	1
6	70 % Alcohol	1	16	80 % Alcohol	1
7	Water	2	17	90 % Alcohol	1
8	Hematoxylene	5	18	100 % Alcohol	1
9	Water	2	19	Xylene	2
10	Acid Alcohol	1 dip	20	Slides were mounted using DPX	

RESULTS

The microscopic examination of organs such as brain, pituitary, sciatic nerve, spinal cord, (cervical, thoracic and lumbar), eye, muscle, liver, kidney, spleen, adrenal, thyroid and parathyroid and thymus were carried out to decipher the changes at cellular level after single dose exposure chlorpyrifos and lead acetate or a combination of two.

Certain spontaneous/incidental pathological findings have been made in different organs: liver (periportal MNC infiltration, hepatocellular vaculation/fatty chage, extramedullary hematopiosis and congestion) kidney (cyst, cystic tubular dilation, congestion, MNC infiltration focus/ interstitial nephritis), spleen (lymphoid depletion/atrophy, congestion, extramedullary hematopoisis) brain (ependymal cell hyperplasia - focus), pituitary (Rache's cleft with secretion) adrenal (accessory cortical nodule/ accessory adrenal tissue, cortical tissue hypertrophy) and, thyroid (Ultimobronchial cyst). The number of animals exhibiting particular lesions group wise are represented in the summary Table 1.

In thymus, starry sky appearance was shown by four animals (50% male + 50% female) belonging to group 7 and 3 animals belonging to group 6 (25% male + 50% female. Lymphoid depletion was exhibited by two animals (50% male) belonging to group 6; and one in group 7 (Table 1; Figure 1; Plate I). The changes observed in thymus are considered to be treatment related.

Table 1

Dose: G1- 0; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) – 50 +1000 mg/kg body weight/day

Organ/Lesion	Group 1		Group 5		Group 6		Group 7	
	M N = 4	F N = 4	M N = 4	F N = 4	M N = 4	F N = 4	M N =4	F N = 4
Liver								
1) Periportal MNC infiltration	1	1	1	1	0	1	0	1
2) Hepatocellular vaculation/fatty chage	0	0	1	1	1	1	1	1
3) Extramedullary hematopiosis	0	0	1	0	0	0	0	0
4) Congestion	0	1	0	0	0	0	0	0
Kidney								
1) Cystic tubular dilation	0	0	1	1	0	1	1	1
2) MNC infiltration focus/ interstitial nephritis	0	0	0	0	0	1	0	1
3) Cyst	0	0	0	0	0	1	0	0
4) Congestion	0	0	0	0	0	0	1	0
Spleen								
1) Lymphoid depletion/atrophy	0	0	1	1	0	1	0	1
2) Extramedullary hematopiosis	0	0	0	0	1	0	1	0
3) Congestion	0	0	0	1	1	1	1	0
Brain								
1) Ependymal cell hyperplasia - focus	0	1	0	0	0	0	0	1
Pituitary								
1) Rache's cleft with secretion	1	1	1	0	0	0	1	0
Adrenal								
1) Accessory cortical nodule/ accessory adrenal tissue	0	0	0	0	0	1	1	0
2) cortical tissue hypertrophy	0	0	0	1	0	0	1	1
Thyroid								
1) Ultimobronchial cyst	0	0	1	0	0	0	1	0

Table 1 (continued)

Dose: G1- 0; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) – 50 +1000 mg/kg body weight/day

Organ/Lesion	Group 1		Group 5		Group 6		Group 7	
	M N = 4	F N = 4	M N = 4	F N = 4	M N = 4	F N = 4	M N =4	F N = 4
Thymus								
1) Congestion	1	0	0	1	0	0	0	0
2) Starry sky appearance	0	0	0	0	1	2	2	2
3) Lymphoid depletion	0	0	0	0	2	0	1	0

N = Number of animals examined histopathologically

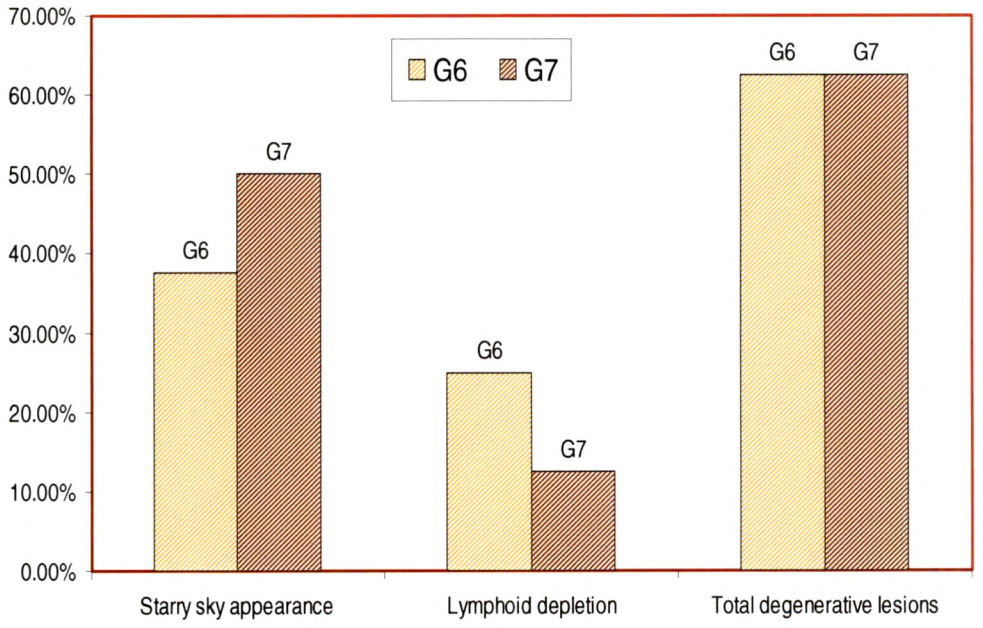


Figure 1. Degenerative Changes of Thymus

Plate I

Figures A (H & E 4X) and **B (H & E 10X)**: Thymus from animal belonging to control group showing normal structure of cortex and medulla with normal population of cells.

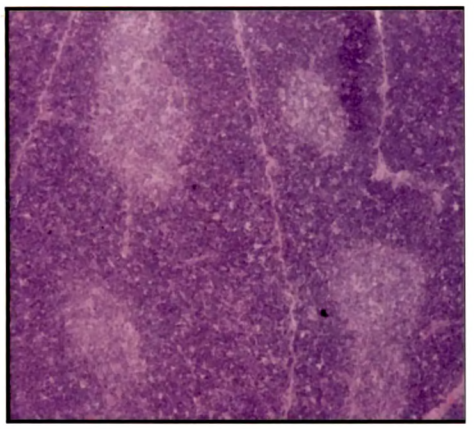
Figure C: High magnification of thymus from animal belonging to control group showing normal lymphoid population in cortex **(H & E 40X)**.

Figure D: Thymus from animal belonging to lead treated group (1000 mg/kg) showing starry sky appearance **(H & E 10X)**.

Figure E: High magnification of thymus from animal belonging to lead treated group (1000 mg/kg) showing lymphoid depletion in cortex well compared with Figure C **(H & E 40X)**.

PLATE I

A



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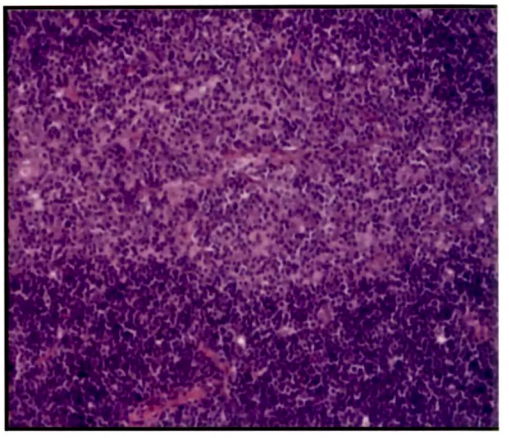
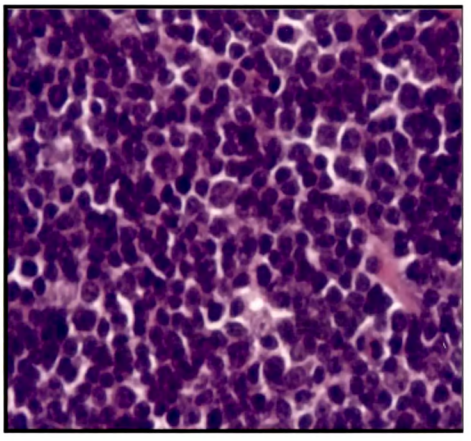
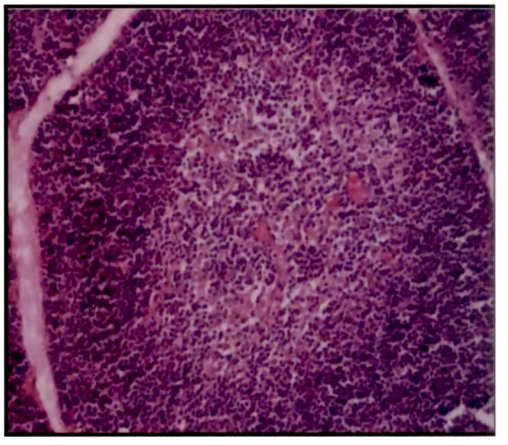


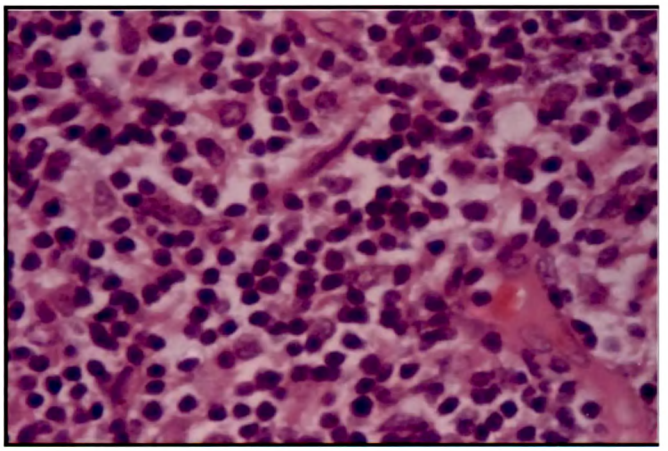
C



D



E



DISCUSSION

The microscopic examination of various organs after acute exposure to chlorpyrifos and lead acetate or their combination via oral gavage in both sexes of Wistar rats did not reveal any treatment related changes either in central nervous system or in peripheral nervous system. The few lesions observed in different systemic organs are considered spontaneous/incidental findings and hence, not related to treatment.

Groups 6 and 7 animals showed degenerative lesions (starry sky appearance and lymphoid depletion) in thymus due to lead treatment (Table 1; Figure 1; Plate I). Necrosis of the cortical lymphocytes may occur in treated rats primarily as a direct effect of the chemical or secondary due to debilitation and stress. Early changes consist of isolated cell debris in the cortex, resulting in the classical "starry sky" appearance of the cortex. Atrophy of thymus is a common age related change in the rat (Stefanski *et al.*, 1990). However, the animals used in the present study are young and not more than 8-9 weeks old at the time of sacrifice. Hence, degenerative lesions observed in thymus of young animals belonging to groups 6 and 7 are considered to be treatment related.

ATSDR (1999) had reported significant decrease in both splenic and thymic weights in lead-exposed mice administered with 2.6 mg lead/kg/day as lead nitrate by gavage once as compared to the controls. Significant decrease in total leukocyte count with no significant change in differential leukocyte count were reported in the lead-exposed animals. However, the present study has not revealed any significant changes in total or differential leucocyte counts and this may be related to the difference in exposure

concentration of lead and species/strain difference. No interactive effects were noticed due to lead and chlorpyrifos on thymus. The observed degenerative changes of thymus were considered to be immunosuppressive effects primarily caused due to lead. Many short term repeated dose studies have revealed lead as immunotoxicant, primarily affecting cellular immunity.

Overall, the present study suggests that single dose of chlorpyrifos or lead or even a combination of the two does not have any significant pathological manifestations in any of the organs studied except for thymus which is primarily and solely attributed to lead intoxication.

SUMMARY

Organophosphorus insecticide, chlorpyrifos and heavy metal, lead have been evaluated for their interactive pathological effects in Wistar rats. The histopathology of nervous system, and some systemic organs were studied after 14 days of single dose exposure. The study was conducted using two different dose levels of chlorpyrifos and lead acetate and grouped into seven groups; control (Group 1), chlorpyrifos-5 mg/kg (Group 2), lead acetate- 100 mg/kg (Group 3), chlorpyrifos-5 mg/kg + lead acetate- 100 mg/kg (Group 4), chlorpyrifos-50 mg/kg (group 5), lead acetate-1000 mg/kg (Group 6) and chlorpyrifos-50 mg/kg + lead acetate-1000 mg/kg (Group 7). No treatment related changes were noticed in the studied nervous system regions (brain, spinal cord, eyes and sciatic nerve). Histopathology of systemic organs reported degenerative lesions (starry sky appearance / lymphoid depletion) in thymus of animals belonging to groups 6 and 7. However, no interactive effects were observed between chlorpyrifos and lead acetate. The observed degenerative changes of thymus are considered to be the manifestation of immunosuppressive effects of lead.