

## Combined Effects of Chlorpyrifos and Lead on Biochemical Parameters after Repeated Dose 90-Day Dietary Exposure in Wistar Rats

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### ABSTRACT

The presence of organophosphorus pesticides and heavy metals as food contaminants along the food production line is a common finding. To investigate the interactive/combination effects of chlorpyrifos (CPF) and lead acetate (LA) on biochemical parameters, Wistar rats were exposed to both via dietary mode for a period of 90 days.

The study was designed using two different dose levels of CPF and LA, and grouped into seven groups: control - 0 (Group 1), CPF - 1 (Group 2), LA - 50 (Group 3), CPF - 1 + LA - 50 (Group 4), CPF - 10 (group 5), LA - 1000 (Group 6) and CPF - 10 + LA - 500 (Group 7) ppm. The haematology and clinical chemistry parameters were evaluated at the end of weeks 4 and 13 of exposure period, and after 4 weeks of recovery period.

There were no significant changes in haematological parameters, except for a slight anaemic effect in lead-treated animals. Serum biochemistry revealed reductions in serum and RBC cholinesterase enzymes at the end of weeks 4 and 13 in groups 5 and 7. The 90-day exposure followed by a post-treatment free period of 28-days revealed higher inhibition of RBC cholinesterase enzyme in the recovery group of Chlorpyrifos-plus-Lead treated group, when compared with CPF-alone treated group. A similar trend was observed in serum glucose level of animals treated with a combination of CPF and lead after the treatment-free period of 28 days. The cholinesterase activity and serum glucose concentration observed

in group 7 animals were not comparable to group 5 animals after 28 days of recovery period.

The findings suggest long lasting and/or persistence of effects of a combination of chlorpyrifos and lead on glucose homeostasis and cholinesterase activity.

**Key Words:** Chlorpyrifos, Lead, Serum Biochemistry, Cholinesterase

### Introduction

It is well known that organophosphorus (OP) pesticides form chelating complexes with metals. The chances of humans being simultaneously exposed to these two different classes of chemicals are higher in recent times. The usage of effluents for agricultural purposes, leading to contamination of food with heavy metals has been reported by many authors.<sup>1-3</sup> Pesticides are used very often to control various pests. Pesticide residues deposited on the surface of plants have the tendency of penetrating into the plant tissue. As a consequence, both heavy metals and pesticides can enter different trophic levels of food chain due to primary uptake by plants.

Chlorpyrifos, a well-known OP compound, exhibits its toxicity in insects and vertebrates mainly by inhibiting cholinesterase enzyme. Its systemic toxicity through oxidative processes has also been reported.<sup>4</sup> On the other hand, the heavy metal lead, exerts its systemic effects on the haematopoietic system, kidney, brain, gastrointesti-

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nal system, heart and the immune system. The presence of these two chemicals along the food production line is not uncommon. Thus it is desirable to know their interactive effects when simultaneous exposure occurs. Clinical pathological methods can be employed to detect the potential toxic effects on many organ systems. This is also true with regard to many haematological and clinical chemistry methods which are amenable to intraspecies and interspecies extrapolation.<sup>5</sup>

## Materials & Methods

**Test Substance:** Chlorpyrifos (98.0%) and Lead acetate (99.103%), were used for the study.

**Test Species and Husbandry:** Healthy Wistar rats comprising 94 males and an equal number of females of approximately 4–5 weeks age were used. The animals were acclimatized to environmental conditions for a minimum of 5 days. The experimental room temperature was  $22\pm3^{\circ}\text{C}$ . The relative humidity was 55–65%. In the experimental room, 12 hours of artificial fluorescent lighting and 12 hours of darkness were maintained. The experimental room was cleaned and mopped daily with a disinfectant. The animals were housed two/cage/sex in solid floor polypropylene rat cages. Separate hoppers were attached to each cage. The bottom of the cage was layered with clean, sterile rice husk. The animals were provided with ad libitum laboratory rat powder feed and charcoal-filtered UV-sterilized water.

**Study Design:** Based on the available literature, and considering the objective of the study, two doses were selected. Ten-fold differences were maintained between low- and high-dose groups. The animals were randomly allocated to 7 groups. Each main group comprised 10 male and 10 female rats. The groups were designated as group 1 (G1 – vehicle control), group 2 (G2 – chlorpyrifos 1 ppm), group 3 (G3 – lead acetate 500 ppm), group 4 (G4 – chlorpyrifos 1 ppm + lead acetate 50 ppm), group 5 (G5 – chlorpyrifos 10 ppm), group 6 (G6 – lead acetate 500 ppm), group 7 (G7 – chlorpyrifos 10 ppm + lead acetate 500 ppm).

Additionally, at high dose level, 6 males and 6 females per group were included in the study to investigate persistence or recovery effects, if any, and, grouped as group 8 (G1R – vehicle control recovery), group 9 (G5R – chlorpyrifos 10 ppm), group 10 (G6R – lead acetate 500 ppm), group 11 (G7R – chlorpyrifos 10 ppm + lead acetate 500 ppm).

At the start of treatment, body weight variation among the animals was within the  $\pm 20\%$  of mean body weight range.

**Route of Administration and Experimental Diet Preparation:** The route of administration of the test substances was oral through diet. According to dose groups, required quantities of test substance and feed were weighed using calibrated balance. Each dose group was prepared separately and maintained in the respective container. Chlorpyrifos was dissolved in acetone before premixing with the feed. Lead acetate was ground with a small amount of feed in a mortar vessel and mixed with approximately 10% of untreated feed for 5 minutes to form a premix. The premix was then brought to the appropriate final concentration, i.e., 1, 50, 1+50, 10, 500 and 10 + 500 ppm in diet for group 2 (chlorpyrifos 1 ppm), group 3 (lead acetate 500 ppm), group 4 (chlorpyrifos 1 ppm + lead acetate 50 ppm), group 5 (chlorpyrifos 10 ppm), group 6 (lead acetate 500 ppm) and group 7 (chlorpyrifos 10 ppm + lead acetate 500 ppm) respectively, with untreated feed, and mixed for 15 minutes in a blender. The experimental diet thus prepared was transferred to polyethylene bags and stored in labeled stainless steel containers inside the study room. Based on the results of stability test, experimental diet was prepared on weekly basis. Animals were fed the test substance *ad libitum* for a period of 90 days. Recovery group animals were observed post-treatment for a period of 28 days.

**Method of Analysis of Chlorpyrifos and Lead Acetate in the Test Diet:** The stability and homogeneity of chlorpyrifos in the test diet was analysed using HPLC. The stability study was performed at 0, 4th and 7th day. The test substance was found to be stable for 7 days and homogeneous with the experimental diet.

The homogeneity of lead in the test diet was analyzed using atomic absorption spectrophotometer. Homogeneity tests were done in three random samples. The results indicated that lead was homogeneous with the diet.

**Blood Collection and Observations:** Blood samples were collected by puncturing the orbital sinus plexus with the help of a fine capillary tube under ether anaesthesia at the end of weeks 4, 13 and 17 of experimental period. Animals were fasted overnight before blood sampling.

**Haematology:** 0.5 mL of blood was collected in vials containing EDTA for haematology analysis. One drop of blood was taken on a clean glass slide, spread and stained with Leishman's stain for differential leucocyte count. For determination of clotting time, blood was allowed to flow into a 7.5 cm capillary tube, and the time required for clotting was recorded manually.

**Haematological Parameters and Instrument Used:** Erythrocytes (RBC), haemoglobin (Hb), haematocrit (HCT), MCV, MCH, MCHC, platelets, clotting time, total WBC count and differential leucocyte count were the haematological parameters analysed, using haematology analyzer (Sysmex K-1000).

**Clinical Chemistry:** Hitachi 902 was used for the analysis of clinical chemistry parameters except sodium, potassium and chloride. The latter parameters were analysed in Rapid Chem 744. Hitachi 902 is a fully automated instrument. The instrument was calibrated using calibrator for automated systems provided by the manufacturer of reagents. Apart from calibration, control samples, i.e., precinorm and precipath supplied by manufacturer were also checked before analyzing the test samples.

The studied clinical chemistry parameters included glucose, protein, albumin, globulin, cholesterol, blood urea nitrogen (BUN), urea, creatinine, calcium, phosphorus, ALT, AST, ALP, ChE (serum), ChE (RBC), sodium, potassium and chloride. 1.5 mL of blood was collected from each animal in clean centrifuge tubes for serum preparation. Approximately 0.5 mL of blood was used for RBC acetylcholine esterase estimation, as per Ellman et al.<sup>6</sup>

**Evaluation of Data:** The statistical evaluations were performed using validated statistical software. All the parameters characterized by continuous data were subjected to Bartlett's test to meet the homogeneity of variance before conducting Analysis of Variance (ANOVA) and Dunnett's t-test. Where the data did not meet the homogeneity of variance, Student's t-test was performed to calculate significance. The significance was calculated at 5% ( $P \leq 0.05$ ) and 1% ( $P \leq 0.01$ ) level.

## Results

**Haematology:** After 13 weeks of exposure, a significant reduction in Hb content was observed in group 4 males as compared to control males. A slight but not

significant reduction in concentrations of RBC, Hb and HCT were observed in animals of groups 3, 4, 6 and 7 males (**Table 1**). In females, a slight reduction in RBC content, Hb content and HCT level was observed in animals of groups 3, 4, 6 and 7 as compared to control group of animals after 4 weeks of exposure (**Table 2**).

**Clinical Chemistry:** The activities of serum and RBC cholinesterases were significantly decreased in groups 5 and 7 males at the end of 13 weeks of exposure. The RBC cholinesterase activity was reduced by 27.4 and 17.3% respectively in groups 5 and 7 males as compared to control group of males after 4 weeks of exposure. After cessation of treatment for 28 days, 7.9 and 17.1% reduction were observed in RBC cholinesterase activity of group 5 recovery (G5R) and group 7 recovery (G7R) animals, respectively. Females also showed similar results for cholinesterase activity with comparatively increased inhibition after the treatment-free period, particularly in group 7.

The serum glucose concentration was significantly increased in groups 4 and 7 males after 13 weeks of exposure (**Table 3**). Male and female animals belonging to a combination group of chlorpyrifos and lead (90-consecutive days of exposure followed by a treatment-free period of 28-days) (G7R) revealed significant increase in the concentration of serum glucose (**Table 6**).

Other treatment-related changes included significant increase in the concentrations of sodium and chloride content in groups 3, 4, 6 and 7 in both the sexes after 4 weeks of exposure (**Tables 3 & 4**). Variations in the activities of ALT and AST were also noticed in lead-treated animals (**Tables 3, 4 & 6**). Calcium concentration in males of G6 (at week 13) and G7 (at weeks 4 and 13), and females of groups 3 and 6 was significantly increased as compared to respective control animals (**Tables 3, 4 and 5**). The remaining parameters of serum of treated group animals were comparable to control group animals.

## Discussion

**Haematology:** Anaemic effects were observed after 13 weeks of exposure in males, and at the end of 4 weeks in females due to exposure to lead. The mechanism of lead-induced anaemia is well known. The hypochromic and microcytic anaemia that occurs in lead poisoning results from two basic mechanisms: shortened lifespan

and impairment of haeme synthesis. Shortened life span of red blood cells is thought to be due to increased mechanical fragility of the cell membrane. Depressed haeme formation is associated with failure to insert iron into protoporphyrin. The biochemical basis for this effect is not known, but the effect is accompanied by inhibition of sodium and potassium dependent ATPases.<sup>7</sup> Further, low concentrations of lead acetate can change the function of receptors and channels over the plasma membrane of RBC.<sup>8</sup>

Slight anaemic effect due to lead treatment was observed in males at the end of week 13, and after week 4 in females. This sex-related differential response with regard to differential time scale needs to be studied further. No interactive effects such as synergism or potentiation were observed in any of the parameters of haematology.

*Clinical Chemistry:* Clinical chemistry estimations performed at the end of weeks 4 and 13 revealed treatment-related changes in electrolytes content, glucose concentration, calcium concentration, and serum and RBC cholinesterase enzyme levels. After 4 weeks of exposure, electrolytes (sodium and chloride) content in lead-treated animals were significantly increased. Whereas, at the end of the treatment period, sodium and chloride contents of treated groups were comparable to control groups (**Tables 3 & 4**). This increase might be due to minor variations in the functional capacity of nephrons due to the action of lead on proximal tubules of kidney. Reserve functional capacity of kidney or adaptation to chemical insult might be the reason for comparable levels of electrolytes at the end of the treatment period. Kidney has remarkable ability to compensate for loss of renal function. There are a number of cellular and molecular responses to a nephrotoxic insult which can ameliorate or prevent cell death. Two of the most notable

**Table 1** RBC, Hb and HCT – Group Mean Values (Sex: Male; Period: 13<sup>th</sup> Week)

Parameter/Group		G1	G2	G3	G4	G5	G6	G7
RBC (10 <sup>6</sup> /microlitre)	Mean	8.6	8.5	8.2	8.1	8.4	8.1	8.1
	SD	0.20	0.54	0.27	0.40	0.48	0.39	0.20
Hb (g/dL)	Mean	16.3	16.1	15.8	15.6	16.3	15.7	15.8
	SD	0.71	1.31	0.40	0.46	0.76	0.58	0.59
HCT (%)	Mean	45.5	45.4	43.9	43.0	44.4	43.9	43.2
	SD	2.19	3.73	1.41	1.89	2.08	1.77	1.16

**Table 2** RBC, Hb and HCT – Group Mean Values (Sex: Female; Period: 4<sup>th</sup> Week)

Parameter/Group		G1	G2	G3	G4	G5	G6	G7
RBC (10 <sup>6</sup> /microlitre)	Mean	6.5	6.3	6.0	5.9	6.4	6.0	6.1
	SD	0.45	0.54	0.59	0.52	0.40	0.78	0.45
Hb (g/dL)	Mean	14.1	14.2	13.7	13.2	14.2	13.7	13.9
	SD	0.91	1.40	0.77	1.02	0.66	1.61	1.05
HCT (%)	Mean	38.4	37.3	35.5	35.5	36.9	35.8	35.5
	SD	1.93	3.11	1.86	1.67	1.71	1.50	1.66

**Table 3** RBC, Hb and HCT – Group Mean Values (Sex: Male; Period: 4<sup>th</sup> Week)

Parameter/Group		G1	G2	G3	G4	G5	G6	G7
Glucose (mg/dL)	Mean	105.2	105.3	98.9	114.5	115.3	109.4	117.3
	SD	32.40	13.01	21.69	21.12	19.99	18.88	22.70
Calcium (mg/dL)	Mean	11.1	11.4	11.0	11.0	11.1	10.7	10.4**
	SD	0.47	0.25	0.41	0.34	0.40	0.26	0.38
Phosphorus (mg/dL)	Mean	9.8	10.2	10.1	10.1	10.2	10.4	10.3
	SD	0.49	0.33	0.90	0.53	0.71	0.33	0.52
Sodium (mEq/L)	Mean	153.9	156.3	161.8**	162.7**	157.9	161.1**	161.4**
	SD	2.31	1.45	2.33	2.08	2.29	0.88	0.45
Chloride (mEq/L)	Mean	114.3	115.1	117.7*	118.9**	115.4	118.4**	117.8*
	SD	1.38	1.39	1.99	1.31	1.58	1.61	1.68
ALT (IU/L)	Mean	48.5	47.1	51.3	47.4	45.7	39.5	38.5*
	SD	9.63	5.33	3.32	4.09	6.04	5.31	7.28
* = significant at 5% level ( $p \leq 0.05$ ); **= significant at 1% level ( $p \leq 0.01$ )								

**Table 4** Serum Chemistry – Group Mean Values (Sex: Male; Period: 13<sup>th</sup> Week)

Parameter/Group		G1	G2	G3	G4	G5	G6	G7
Glucose (mg/dL)	Mean	88.5	101.5	99.1	111.6*	104.4	112.2	116.0**
	SD	9.71	7.58	13.93	13.84	18.13	18.48	15.16
Calcium (mg/dL)	Mean	10.0	10.0	9.7	9.9	9.8	9.5**	9.6**
	SD	0.30	0.32	0.35	0.16	0.32	0.18	0.34
Chloride (mEq/L)	Mean	109.8	109.6	108.7	109.6	109.2	108.3	107.5
	SD	3.43	3.95	1.59	2.17	2.11	3.86	3.99
ALT (IU/L)	Mean	47.9	42.9	56.6	46.0	51.8	41.5	44.9
	SD	6.12	7.73	13.23	7.64	3.91	7.70	10.99
AST (IU/L)	Mean	220.2	194.2	196.0	195.1	199.1	174.6*	172.1*
	SD	27.15	30.63	24.67	24.55	21.03	42.86	28.31
* = significant at 5% level ( $p \leq 0.05$ ); **= significant at 1% level ( $p \leq 0.01$ )								

**Table 5** Serum Chemistry – Group Mean Values (Sex: Female; Period: 4<sup>th</sup> Week)

Parameter/Group		G1	G2	G3	G4	G5	G6	G7
Glucose (mg/dL)	Mean	100.1	103.4	113.4	103.5	95.7	98.8	102.1
	SD	20.54	22.93	19.99	19.35	23.63	11.65	14.28
Calcium (mg/dL)	Mean	11.6	11.3	10.4**	11.5	11.1	10.6**	11.4
	SD	0.38	0.42	0.46	0.59	0.43	0.62	0.18
Phosphorus (mg/dL)	Mean	9.2	9.2	8.8	9.0	8.9	9.4	9.2
	SD	0.46	0.26	0.67	0.76	0.53	0.64	0.48
Sodium (mEq/L)	Mean	145.0	146.8	149.8**	149.5**	147.1	148.5**	152.6**
	SD	1.73	1.79	2.32	2.40	2.35	1.47	1.57
Chloride (mEq/L)	Mean	106.9	108.3	109.2	108.5	106.5	108.3	110.7*
	SD	1.57	2.07	2.45	1.95	2.63	1.42	1.45
ALP (IU/L)	Mean	227.2	238.0	200.2	201.4	216.4	235.8	240.0
	SD	71.20	48.53	101.61	37.27	54.36	39.24	72.19
* = significant at 5% level ( $p \leq 0.05$ ); **= significant at 1% level ( $p \leq 0.01$ )								

**Table 6** Serum Chemistry – Group Mean Values (Period: 17<sup>th</sup> Week)

Parameter/Group	Male					Female			
		G1R	G5R	G6R	G7R	G1R	G5R	G6R	G7R
Glucose (mg/dL)	Mean	107.4	111.6	120.3	127.3*	110.0	114.7	116.0	145.0**
	SD	10.45	8.50	11.26	7.72	4.79	5.17	5.98	8.52
ALT (IU/L)	Mean	76.3	67.2	66.1	64.9*	57.0	60.8	50.7	60.0
	SD	10.54	14.21	13.23	6.10	5.37	15.04	9.79	12.69
AST (IU/L)	Mean	212.7	221.4	167.5*	134.9**	154.1	139.4	131.7	148.8
	SD	22.94	16.02	20.49	19.09	19.35	13.35	13.44	29.93
ALP (IU/L)	Mean	220.2	139.1	180.5	168.4	130.9	132.8	108.5	141.2
	SD	69.03	34.97	15.16	57.34	27.0	55.98	23.33	49.31
* = significant at 5% level (p ≤ 0.05); **= significant at 1% level (p ≤ 0.01)									



responses are metallothionein induction and heat shock response. Heat shock proteins are believed to play an important housekeeping role in the maintenance of normal protein structure and/or the degradation of damaged proteins, and thereby provide a defense mechanism against toxicity and/or facilitate recovery and repair. Metallothionein is a low molecular weight, cysteine-rich, metal-binding protein that has a high affinity for heavy metals. The binding capacity of metallothionein to heavy metals renders heavy metals biologically inactive and thereby reduces toxicity. Metallothionein production can be induced by nontoxic concentrations of metals.<sup>9</sup>

In males, calcium concentration was decreased in group 7 animals at weeks 4 and 13, and in group 6 animals after 13 weeks of exposure (**Tables 3 & 5**). In females, the calcium concentration was decreased in group 6 animals at the end of week 4 and 13, and in group 3 animals at the end of week 4 (**Table 5**). Sex-specific decrease in calcium levels is inferable as seen by the decrease in males of group 7, but with no such effect in group 7 females. There are studies which indicate that lead interacts with calcium content at various levels, and at moderate exposure levels, results in decreased serum calcium content. Disorders of intestinal calcium absorption, transport, distribution, deposition and excretion can be caused due to direct action of lead on cell membranes, and on hormones like parathormone, osteocalcine, calcitonine, and 1,25-dihydroxy vitamin D; factors which could mediate hypocalcaemic status.<sup>10,11</sup>

Groups 6 and 7 revealed reduction in activities of ALT and AST. Decreased levels of these enzymes at dietary concentrations of 100 and 1000 ppm of lead have been reported by Davidson.<sup>12</sup> Decreased AST/ALT activity generally observed in toxicology studies may indicate decreased hepatocellular production or release, or an effect on the coenzyme pyridoxal 5-phosphate.<sup>13</sup>

The male animals of groups 4 and 7 after 13 weeks of exposure, and male and female animals of recovery group 7 (G7R) at the end of recovery period, revealed significant increase in glucose concentration as compared to respective control group animals (**Tables 3, 4 & 6**). Increased kidney gluconeogenic enzymes and adenylate cyclase-cyclic AMP system have been demonstrated by Stevenson et al<sup>14</sup> after chronic exposure to lead in rats. They also observed suppressed insulinogenic index (the ratio of serum IRI to blood glucose concentration) in lead-

treated animals. This enhancement of gluconeogenesis and decreased insulin action might be the cause for lead-induced hyperglycaemia observed in this study.

Reduction in serum and RBC cholinesterase activities were evident at the end of week 4 and 13. The percentage reduction of cholinesterase activity reveals that the inhibition of both serum and RBC cholinesterase activities was slightly higher in group 5 animals as compared to group 7 at the end of week 4. After week 13, the percent inhibitions were comparable or slightly higher in group 7 animals. At the end of the recovery period, serum and RBC cholinesterase activities of group 7 recovery females (G7R) were decreased by 3.9% and 34.50% respectively against 0% (serum) and 14.7% (RBC) in group 5 recovery females (G5R). In the case of males, 7.9 and 17.1% reduction were observed in RBC cholinesterase activity of group 5 recovery (G5R) and group 7 recovery (G7R) animals respectively.

Persistence of hyperglycaemia, and incomplete reversal to normal activities of cholinesterases in group 7 recovery animals after cessation of test substances for a period of 28 days suggest long-lasting effects of exposure to lead and chlorpyrifos combination. The chelating complex formation properties of lead and chlorpyrifos could delay the biotransformation processes and hence, availability to the body for long time.<sup>15</sup> The persistent increase in serum glucose even after the withdrawal of test substances is also an indication of metabolic and other disorders. However, additional studies pertaining to the cellular mechanisms involved, and the exact mechanism associated with glucose imbalance need to be carried out to support these findings.

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