Original Paper

Single Dose Effects of Chlorpyrifos and Lead Combination on Neurobehavioural Aspects in Wistar Rats

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ABSTRACT

Chlorpyrifos, a well known organophosphorus insecticide, and the heavy metal lead, were evaluated for their simultaneous interactive effects on neurobehavioural parameters in Wistar rats after single dose exposure via oral gavaging. The study comprised of functional observation battery and motor activity tests. The study was designed using two different dose levels of chlorpyrifos and lead acetate and grouped into seven groups; control (group 1), chlorpyrifos-5mg/kg (group 2), lead acetate- 100mg/kg (group 3), chlorpyrifos-5mg/kg + lead acetate- 100mg/kg (group 4), chlorpyrifos-50mg/kg (group 5), lead acetate-1000mg/kg (group 6) and chlorpyrifos-50mg/kg + lead acetate-1000mg/kg (group 7). Excitotoxicity and motor activity changes were evident in groups 5 and 7 animals. The animals treated with chlorpyrifos at 50mg/kg exhibited behavioural changes after 2–3 hours of oral gavaging and waned over 2 days. At 50mg/kg chlorpyrifos + 1000mg/kg lead acetate, severe cholinergic signs were noticed approximately 24 hours of exposure and symptoms regressed over 4 days. The incidence and severity of cholinergic behavioural changes were more pronounced in group 7 animals. Chlorpyrifos in the presence of lead delays the cholinergic effects which might be due to its chelating properties with metals and predominant behavioural changes suggest potentiating role of lead on excitotoxicity of chlopyrifos. The present study will be potentially relevant for physicians/scientists to decipher more about variability of action that could arise from accidental poisoning by these agents.

Key Words: Chlorpyrifos, Lead, Neurobehaviour

Introduction

Pesticides and heavy metals as food contaminants are posing a serious threat to human and other life forms, especially over the last 4 or 5 decades due to increased pollution. It is well known that the toxicity of chemicals is influenced by a number of factors. The combination of two or more chemicals at a given time can lead to various interactions, some of which are so complex and obscure that they remain unclear.

Behavioural changes due to toxic effects of chemicals on the nervous system of humans and animals have been known since a long time. Due to the fact that behavioural abnormalities in many cases precede demonstrable morphological changes in the neurological system, behavioural tests serve as more sensitive indicators of many chemicals' neurotoxicity.[1] The heavy metal, lead is a known neurotoxicant, while chlorpyrifos is a highly toxic organophosphorus compound, both inducing behavioural changes in mammals/vertebrates/ insects. It was therefore decided to undertake a study to evaluate the effects of a combination of chlorpyrifos and lead on the neurobehavioural aspects of Wistar rats.

Materials and Methods

Test Items: Chlorpyrifos technical (CPF) of 98.0% purity, and lead acetate (LA) of 99.103 % purity were used for the study.

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Test Species and Husbandry: Healthy Wistar rats, 5-6 weeks old, were obtained from the Breeding Facility. The animals were acclimatized for a period of 5 days. The experimental room temperature was 22±3°C, with 12 hours of artificial lighting and 12 hours of darkness maintained alternately. The relative humidity was maintained at 55–65%. The rats were housed singly in solid-floor polypropylene cages. A sterile rice husk was used as bedding material. The rats were provided with *ad libitum* laboratory rat feed and charcoal-filtered, UV-sterilized water. Individual animals were identified with picric acid marking over the body coat, and coloured cage labels having group details.

Dose Selection: A dose-range finding study was conducted to select the right doses for the main study. Nine male and nine female rats were divided into 3 groups; each group comprising 3 males and 3 females. Chlorpyrifos and lead acetate were given at 150mg/kg and 1000 mg/kg doses respectively to the first two groups of rats, while the third group was treated with a combination of both. All the rats of chlorpyrifos group died on the first day of dosing, while in the combination group, mortality was observed on the second day. Excitotoxicity was observed both in the chlorpyrifos group as well as the combination group before mortality. Based on these results, the main study was conducted using the following doses: control (group 1), chlorpyrifos-5mg/kg (group 2), lead acetate-100mg/kg (group 3), chlorpyrifos-5mg/kg + lead acetate-100mg/kg (group 4), chlorpyrifos-50mg/kg (group 5), lead acetate-1000mg/kg (group 6), and chlorpyrifos-50mg/kg + lead acetate-1000mg/kg (group 7).

Study Design: The animals were randomly allocated to 7 groups. Each group comprised 5 male and 5 female rats. At the start of the treatment, body weight variations among the animals were within ±20% of mean body weight range. All animals were treated only once during the course of the experiment, and were observed for a period of 14 days thereafter. The combination group animals (G4&G7) received chlorpyrifos and lead acetate simultaneously. The route of administration of test substance was through oral gavage, and a dose volume of 5 mL/kg was used. Chlorpyrifos was suspended in corn oil, while lead acetate was dissolved in distilled water.

The animals were observed for morbidity and mortality twice a day. Observations were made with regard to visible clinical signs such as skin and fur changes, eye and mucous membrane changes, respiratory and circulatory changes, autonomic and central nervous system responses, somatomotor activity, and behavioural pattern.

Neurobehavioural observations: Neurobehavioural observations were conducted to assess the behavioural and neurological status of each animal. The methods adopted in the neurobehavioural observations were based on Moser (1988)[2], Moser (1989)[3] and OPPTS 870.6200 (1996).[4] Neurobehavioural observations were made once before the commencement of treatment, followed by 1st, 2nd and 14th day post-treatment. The observations and recording/measurement types were dictionary-based.

Recording was done with regard to home cage observations, handling observations, open field observations, sensory reactivity measurements, landing hind limb foot splay, grip strength measurements, and motor activity. Open field observations and sensory reactivity measurements were performed in the open field box.

Motor Activity: Motor activity of each animal was monitored using an automated animal activity measuring system (Columbus Instruments). The animals were monitored for three consecutive 10-minute intervals, allowing for examination of both exploratory and acclimation activity levels. During this period, the total, ambulatory and stereotypic activities were evaluated.

Grip Strength Measurement: Grip strength of both the forelimbs and hindlimbs were measured using grip strength meter (Columbus Instruments) to determine the ability of the animal to grasp and hold on to the mesh platform. The grip strength of each animal was measured three consecutive times; the results were averaged separately for the forelimbs and hindlimbs.

Landing Hind Limb Foot Splay: The feet of each rat were marked with non-permanent ink just prior to testing. The animal was then suspended in a prone position and then dropped from a height of approximately 30 cm on to a recording sheet. This procedure was repeated three times. The distance between two foot prints was measured, and the average of three foot splay values was calculated.

Body Weight and Food Consumption: Individual rats were weighed on the day of initiation of treatment, and subsequently on days 2nd, 8th and 15th of the study period. Weekly food consumption of the animal was calculated.

Evaluation of Data

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

Clinical Signs

Treatment-related clinical signs were observed in rats treated with chlorpyrifos at 50 mg/kg, and rats treated with a combination of chlorpyrifos (CPF-50mg/kg) and lead acetate (LA-1000mg/kg). Tremor, perennial soiling due to urine and faeces, motor in-coordination, decreased muscle tone, cold to touch, chromodacryorrhoea, chromorhinorrhoea, and exophthalmos were observed at differential time scales.

Group 5 (CPF-50mg/kg) animals exhibited symptoms on day 1 and 2, whereas group 7 (CPF-50+LA-1000mg/kg) animals revealed symptoms from day 2 to day 4. The incidence and severity were higher in group 7 rats as compared to group 5 rats. Chromodacryorrhoea, chromorhinorrhoea, and exophthalmos were observed only in females.

Neurobehavioural Observations

Neurobehavioral observations performed before treatment, and on days 1, 2 and 14 revealed significant changes on days 1 and 2 in group 5 and 7 animals.

Activity Measures

Posture and Rearing Count: Observation for posture did not reveal consistent postural changes in either sex. Vertical movements in the open field were reduced in group 5 males by 42.1% on day 1; however, they were not statistically significant. On day 2, during open field observation, group 7 males showed significant reduction in rearing count as compared to control males. In case of females, rearing counts of group 5 on day 1, and group 7 on day 2 were significantly reduced as compared to control females (**Table1**).

Motor Activity

Males: Total, ambulatory and stereotypic activities were reduced in group 5 on day 1, but the decrease was not statistically significant. On day 2, total and ambulatory activity of group 7 males were significantly decreased during the first interval (**Table 2**).

Females: On day 1, significant reduction in motor activity was observed in animals of all three high dose levels (**Table 3**). Total, ambulatory and stereotypic activities of group 7 females during first interval were significantly decreased as compared to control group females on day 2.

Table 1 Rearing Count - Group Mean Values: Females

Group No.	Experimental Period							
	PE		1 Day		2 Day		14 Day	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1	10.2	2.39	7.8	3.03	7.6	2.97	10.8	6.53
G2	13.2	4.66	10.2	2.39	6.6	3.21	12.0	3.24
G3	17.2	1.10	12.4	1.34	8.0	3.81	7.6	4.88
G4	11.6	3.65	7.4	0.89	9.2	2.49	3.4	5.46
G5	11.2	3.49	3.4*	2.30	4.4	3.05	9.6	4.62
G6	14.6	2.79	6.0	3.32	7.8	4.97	8.2	3.77
G7	12.2	5.17	6.2	2.17	0.00**	0.00	8.0	4.95

* = significant at 5% level ($p \leq 0.05$); ** = significant at 1% level ($p \leq 0.01$)

Table 2 Motor Activity - Group Mean Values

Sex: Female **Period : 2nd Day**

		First Interval		
Group No.		Total	Ambulatory	Stereotypic
G1	Mean	23.90	21.62	9.76
	SD	3.27	3.62	1.21
G2	Mean	23.01	20.77	9.84
	SD	7.8	7.28	3.07
G3	Mean	19.09	16.85	8.98
	SD	2.47	2.14	1.24
G4	Mean	21.15	19.05	9.17
	SD	4.17	3.63	2.15
G5	Mean	20.95	19.0	8.83
	SD	6.27	5.79	2.42
G6	Mean	21.93	19.93	9.08
	SD	3.31	3.33	1.16
G7	Mean	13.54*	11.68*	6.67
	SD	4.36	4.43	1.53

* = significant at 5% level ($p \leq 0.05$)

Table 3 Motor Activity - Group Mean Values

Sex: Female **Period : 2nd Day**

		First Interval		
Group No.		Total	Ambulatory	Stereotypic
G1	Mean	26.06	23.72	10.76
	SD	2.69	2.54	1.12
G2	Mean	27.03	24.54	11.32
	SD	1.47	1.36	0.8
G3	Mean	24.31	21.80	10.62
	SD	8.63	8.38	2.77
G4	Mean	26.16	23.65	11.10
	SD	7.73	7.54	2.27
G5	Mean	18.96**	17.05**	7.97
	SD	8.45	8.46	2.51
G6	Mean	17.64*	15.17**	8.59
	SD	6.44	6.97	1.42
G7	Mean	20.06**	18.07**	8.64
	SD	8.34	7.99	2.79

* = Significant at 5% level ($p \leq 0.05$); ** = significant at 1% level ($p \leq 0.01$)

Convulsive Domain

Observation for tremor, clonic and tonic convulsions in the home cage revealed tremors in 40% (4/10) of animals belonging to group 5 on day 1. In group 7, tremor and clonic convulsions were exhibited by 40% and 70% (7/10) animals respectively, on day 2.

Excitability Parameters

Ease of removing rat from the cage was comparatively easy (7/10) in group 5 animals on first day of dosing, and in group 7 animals (9/10) on day 2. After removing from the cage, reactivity of the animals while being held by the observer in the hand was observed. Five rats of group 5 (day 1), and 7 rats of group 7 (day 2) were freezing in hand. Low and very low arousal rates were exhibited by the animals of groups 5 and 7.

Autonomic Parameters

Autonomic symptoms such as lacrimation, salivation, and piloerection were exhibited by the animals of groups 5 and 7 (**Fig 1**). Abnormal pupil response was noticed when light stimulus was directed towards pupil.

Urination and Defaecation Counts: Mean values of defaecation counts of males of groups 5 (day 1) and 7 (day 2), and urination counts of group 7 (day 2) males were significantly decreased as compared to control group males. The mean urination count of groups 5 and 7 females (on day 2), and defaecation count of group 5 females on days 1 and 2, and group 7 females on day 2 were significantly decreased. Uncontrolled discharge of urine and faeces was noticed as perennial staining during clinical observation.

Neuromuscular Measures

Grip Strength: Forelimb and hindlimb grip strength of group 5 males on day 1 was reduced (statistically insignificant) by 7.0 and 29.7 % respectively, as compared to control group males. On day 2, forelimb and hindlimb grip strength of group 7 males were significantly decreased as compared to control males (**Table 4**). In females, hindlimb grip strength of group 5 on day 1, and forelimb and hindlimb grip strengths of group 7 on day 2, were significantly reduced.

Hind Limb Foot Splay: No statistically significant differences were observed in the mean hind limb foot splay values of treatment group animals of either sex as compared to respective control group animals on day 1. On day 2, group 7 animals did not land properly on to the sheets due to severe cholinergic symptoms, and hence hindlimb foot splay measurement was not performed for the group 7 animals.

Gait: Abnormal gait changes were observed in animals treated with chlorpyrifos at 50 mg/kg (4/10) on day 1. Chlorpyrifos in combination with lead (group 7) revealed increase in the incidence (8/10) and severity on day 2 of experimentation. Gait changes were observed as short-step walking pattern to toe -walking type.

Air Righting Reflex: The change in the air righting reflex was prominent in animals (6/10) treated with combination of chlorpyrifos (50 mg/kg) and lead (1000 mg/kg) on day 2.

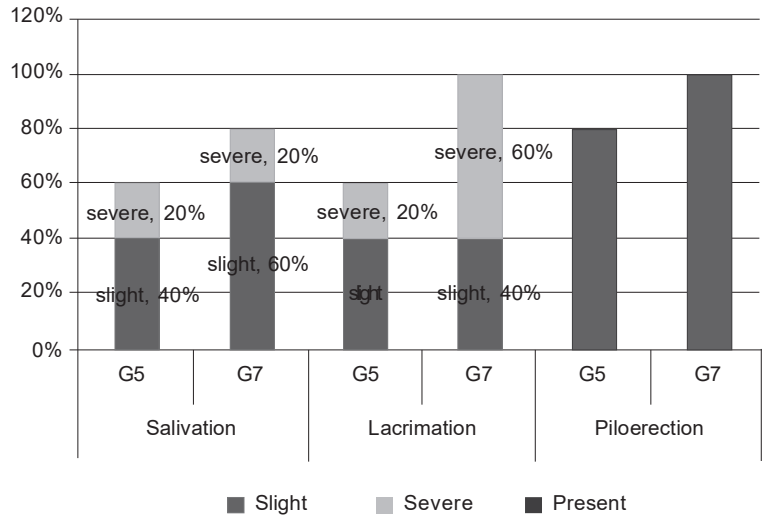

Fig. 1 Changes in autonomic nervous system - females

Table 4 Grip Strength (g) - Group Mean Values: Males

Period	1st day				2nd Day			
Group	Forelimb		Hind limb		Forelimb		Hind limb	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1	699.2	68.2	367.6	65.2	591.6	80.7	374.6	64.9
G2	678.0	66.9	383.8	67.7	666.6	113.8	408.6	56.9
G3	686.0	67.1	367.4	68.7	630.0	75.4	400.0	48.2
G4	671.2	38.2	341.0	66.3	667.6	115.4	359.2	64.0
G5	650.0	77.5	258.4	11.5	583.2	45.8	301.8	15.1
G6	714.0	47.3	352.0	62.6	665.4	79.8	383.6	56.5
G7	721.2	40.9	365.0	83.3	453.8*	66.7	172.6**	16.5

* = significant at 5% level ($p \leq 0.05$); ** = significant at 1% level ($p \leq 0.01$)

Sensorimotor Measurements: On the first day, there was not much difference in sensory reactivity measurements among the treatment groups. On day 2, approach response, touch response, and tail flinch response of group 7 animals were disturbed by the treatment (**Fig 2**).

Discussion

Neurobehavioural studies performed to investigate interactive effects of chlorpyrifos and lead combination after single dose exposure in Wistar rats showed cholinergic over-stimulation in groups 5 and 7 rats. The behavioural effects due to cholinergic over-stimulation were more severe in group 7 (CPF-50 mg/kg + LA-1000 mg/kg) animals on day 2, as compared to the effects in group 5 (CPF-50 mg/kg) animals on day 1. The overall results indicate that acute toxicity of chlorpyrifos is more hazardous when combined with lead, rather than when administered alone, and is more pronounced on day 2, and not immediately.

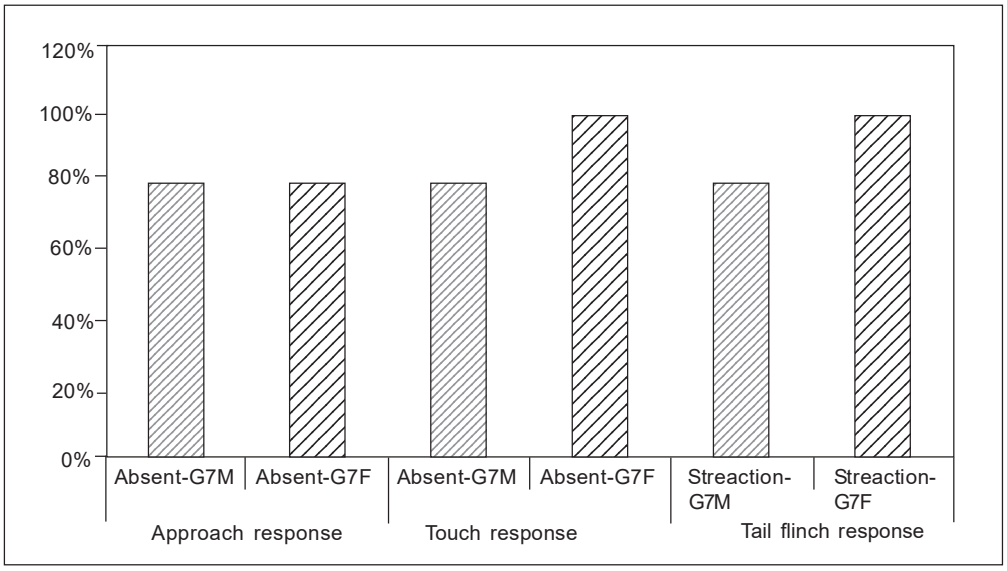

Fig. 2 Changes in autonomic nervous system - females

Clinical Signs

Cholinergic symptoms associated with neuromotor and autonomic systems were observed in both sexes of groups 5 and 7 animals. In group 5, clinical signs were predominant on day 1, and waned over 2 days. Whereas in group 7, the clinical signs were predominant on day 2, and some animals showed persistent symptoms up to fourth day. The severity and incidence of symptoms were also high in group 7 as compared to group 5. The reported time of peak effects for acute studies with chlorpyrifos is 2 hours.[5]

Significant reduction in body temperature in a few animals of group 7, as well as piloerection, an indication of hypothermic response, was observed in most of the animals belonging to groups 5 and 7. The extreme reduction in temperature in Long-Evans rats by using radiotelemetry transmitters has also been reported by Gordon, 1994.[6] Some of the cholinergic symptoms, especially gait and air-righting reflex changes, and sensory reactivity changes were prominent in some animals of group 7. The wide ranging variations in individual motor activity and colonic temperature responses when the inhibition in ChE activity exceeded threshold levels have been reported earlier.[7] This may be due to genetic variability with regard to ChE inhibition.

Neurobehavioural Observations

Neurobehavioural observations performed in home cage, during handling as well as in open field, showed behavioural abnormalities related to cholinergic overstimulation. Just as in the case of clinical signs, predominant behavioural changes were observed in groups 5 and 7 animals, on days 1 and 2 respectively.

Measurements of locomotor functions and muscular strength clearly indicate that the effects are more prominent in females as compared to males. Some of the cholinergic over-stimulation signs i.e., chromodacryorrhoea, chromorhinorrhoea and exophthalmos were observed only in females. Gender-specific processes such as hepatic and extra-hepatic activation, binding and detoxification of CPF and CPF-oxon need to be further studied in-depth.[8] Differences in the ratio of activation (hepatic and extra-hepatic) to detoxification (CPF and CPF oxon) are related to gender.[9,10]

The severity and incidence of behavioural changes were greater in group 7 (CPF-50 mg/kg + LA-1000 mg/kg) animals on day 2, as compared to the effects of group 5 (CPF-50 mg/kg) animals on day 1. The exact mechanism involved in the more pronounced and delayed manifestations of effects on day 2 is not clear. It is well known that organophosphorus compounds form chelating complexes with metals.[11] The formation of a chelating complex by chlorpyrifos with metals might be responsible for delayed absorption, activation, and detoxification processes. The extent of toxicity and inhibition of cholinesterase by chlorpyrifos depends mainly on its major metabolite chlorpyrifos oxon, and local availability of CPF and CPF oxon. The availability of oxon at the site of activation particularly depends on escape from general biotransformation of liver, and binding of CPF to some plasma proteins. With regard to lead, initially it is distributed in the plasma and soft tissues, and subsequently under steady-state conditions, 99% of the lead in blood finds its way into the erythrocytes. Under these circumstances, CPF might get additional scope to form chelating complex with lead, and thus CPF might bypass general liver activation and detoxification processes, providing additional scope for local activation of CPF.

The severity and persistence of cholinergic excitotoxicity for longer duration observed in chlorpyrifos and lead-treated animals suggest increased cholinergic toxicity with chlorpyrifos in the presence of lead. This gives validity for the potentiating role of lead on excitotoxicity of chlopyrifos. Hence, exposure to combination of chlorpyrifos and lead simultaneously is more dangerous than to an exposure of either alone. As chlorpyrifos and lead are extensively used in and around the home environment, there is likelihood of children being exposed to them both inadvertently. Hence, this study on comparatively young animals will be potentially more relevant for physicians/scientists to decipher more about variability/ mechanism of action that could arise from accidental poisoning by these agents.

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