

Assessment of chlorpyrifos and lead acetate combination on neurobehavioral aspects in Wistar rats after subchronic dietary exposure

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Abstract

Organophosphorus insecticide, chlorpyrifos, and heavy metal, lead, were studied for their interactive effects on neurobehavioral aspects in Wistar rats when exposed for a period of 90 consecutive days through experimental diet. The tests used for the assessment of neurobehavioral changes were include functional observation battery, grip strength measurement, foot splay measurement and motor activity. The study was designed using two different dose levels of chlorpyrifos and lead acetate and grouped into seven groups including concurrent controls. Neurobehavioral observations were performed at the end of 4 and 13 weeks of exposure and after 4 weeks of recovery period. Repeated dietary exposure at a dose level of 10 ppm of chlorpyrifos (i.e., equivalent to 1mg/kg body weight/day) and in a combination of 10 ppm chlorpyrifos plus 500 ppm of lead acetate (i.e., equivalent to 44.0mg/kg body weight/day) to groups of animals revealed mild cholinergic symptoms and decreased rearing counts at the end of week 4. In addition, combination group animals (Chorpyrifos plus Lead) treated at the low dose level (Chlorpyrifos-1ppm and lead-50 ppm) also revealed reduction in the vertical movements. The lack of persistence and/or cumulative effects of these changes after 13 weeks of exposure is due to tolerance induced by the chlorpyrifos. The rearing movements measured in the open field are considered to be more indicative of exploratory behaviour and emotional tendencies than of general motor activity. A decrease in rearing counts of combination group animals (Chorpyrifos plus Lead) treated at the low dose level after week 4 was noticeable, irrespective of sex, suggests that even at low dose levels, the combination of chlorpyrifos and lead produces behavioral changes. However, many higher levels of tests for detection of cognitive functions should also be considered. No other behavioral changes were noticed in the studied behavioral tests.

Keywords: Neurobehavior, Combination, Lead, Chlorpyrifos.

Introduction

Pesticides and heavy metals as food contaminants have raised serious threat to human and other organisms due to pollution boom during the last 4-5 decades. Pesticides in food are predominantly residues from their application on different steps/stages of growth and processing of agricultural products, whereas, heavy metals contaminate food at various stages along the food production line, starting from agricultural lands to food processing. Due to large scale use of these two kinds of chemicals their common existence everywhere throughout food chain is inherent.

Lead has been considered as a well known developmental neurotoxicant. Though animal studies revealed reduced or less action of lead on adult nervous system, severe central nervous system damage (encephalopathic and subencephalopathic symptoms) and peripheral nerve damage and peripheral nerve dysfunction have all been reported in adult human beings

during occupational studies. Cognitive deficits have also been observed in lead workers. The overt clinical signs and brain damage and cognitive deficits depended on the exposure concentration.

Chlorpyrifos (CPF) being an OP compound exerts its toxic action on nervous system by inhibiting cholinesterase enzymes. Repeated subchronic exposure studies conducted for neurotoxicity screening and systemic toxicity studies revealed mild behavioral changes at dose levels of 5 and 15mg/kg/day (Mattson *et al.*, 1996; Yano *et al.*, 2000). No studies have documented their cumulative effects after long term exposure. Tolerance to CPF exposure has been reported by many workers. However, the action of a chemical changes dramatically when it interacts with other chemical(s). Therefore, the present study was undertaken to investigate the interactive effects of a combination of chlorpyrifos and lead acetate on neurobehaviour

in Wistar rats upon oral administration relatively at lower concentrations via diet for a period of 90 consecutive days.

Materials and Methods

Test Substance

Chlorpyrifos technical (98.0% purity) and lead acetate (99.103 % purity), was used for the study.

Test Species and Husbandry

Healthy Wistar rats consisting of 94 males and 94 females of approximately 4-5 weeks old were obtained from the Breeding Facility of Jai Research Foundation. Age of the animals at the time of start of treatment was approximately 5 to 6 weeks. Animals were acclimatized to environmental conditions for a minimum of 5 days. During the acclimatization period, animals were observed for good health. Rats were maintained in an environment controlled room. The experimental room temperature was 22 ± 3 °C. The relative humidity was 55 – 65%. In the experimental room, 12 hours of artificial fluorescent lighting and 12 hours of darkness were maintained. Light hours being 6:00 to 18:00 h. 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 are 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 (Aquaguard water filter system). Individual animal was identified with picric acid marking over the body coat, and colored cage label showing Group N° and sex, Dose and cage N°.

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 of 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 – 50 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). 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 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 grounded with small amount of feed in 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 – 50 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 polythene 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.

Duration of Treatment

Animals were fed *ad libitum* the test substance incorporated diet for a period of 90 days for 24 hours. Recovery group animals were observed post-treatment for a period of 28 days.

Analysis of Chlorpyrifos Technical 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 percent recovery at 0, 4th and 7th day was 96.5, 94.25 and 89.7 for 1 ppm and 97.05, 93.95 and 89.55 for 10 ppm of chlorpyrifos in diet. The test substance was homogeneous with the experimental diet.

Analysis of Lead Acetate in the Test Diet

The homogeneity of lead acetate in the test diet was analyzed using atomic absorption spectrophotometer. Homogeneity tests were detected in three random samples. The homogeneity results indicated that lead acetate was homogeneous with the feed and, the percent recovery for 500 ppm of lead acetate in experimental diets was 92.4, 92.5 and 92.3% for samples 1, 2 and 3 respectively.

Observations

Clinical Signs: Animals were observed for mortality and morbidity twice a day. Observations were made daily for visible signs and symptoms such as skin and fur changes, eye and mucous membrane changes, respiratory, circulatory, autonomic and central nervous system disturbances as well as, somatomotor activity, behavioral pattern and general changes .

Neurobehavioral Tests (Functional Observational Battery)

Functional observational battery (FOB) was conducted to assess the behavioral and neurological status of each animal. FOB was performed at the end of 4th, 13th and 17th weeks. In addition, observations were made for visible signs and symptoms such as skin and fur changes, eye and mucous membrane changes, respiratory, circulatory changes, autonomic and central nervous system responses, somatomotor activity, behavioural pattern and, general changes, daily.

Neurobehavioral observations were conducted through functional observational battery to assess the behavioral and neurological status of each animal. The methods adopted in the functional observational battery were based on Moser *et al.*, (1988); Moser (1989) and OPPTS 870.6200 (1996). The observations and recording /measurement types are dictionary based and, presented in the Table 1.

Body Weight and Food Consumption

Individual animal was weighed on the day of commencement of treatment and at weekly intervals during the experimental period. The food consumption was calculated on weekly basis.

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 Observations

No mortality was observed during the entire course of experimentation in any of the groups. Treatment related clinical signs such as perennial staining of nose, chromorhinorrhoea, lacrimation and chromodacryorrhoea were observed in group 5 (CPF-10 ppm) and group 7 (CPF-10 ppm + LA-500 ppm) animals of either sex. These treatment related clinical signs were observed predominantly during the early phase of the experimentation i.e., during weeks 2 and 3 as represented in Figures 1A and 1B. No marked differences were observed in clinical signs associated with treatment between group 5 and group 7 animals i.e., between chlorpyrifos alone and in combination with lead acetate.

Neurobehavioral Observations

Activity Measures (Posture, Rearing and Motor Activity)

Vertical movements (rearing) in the open field were slightly reduced but not statistically significant, in groups 4, 5 and 7 of male animals at the end of 4th week. With reference to females, in addition to groups 4, 5 and 7, group 6 females also showed slight reduction in rearing count after exposure to the test substance for a period of 4 weeks. The percent reduction in the mean values of rearing counts at the end of 4 weeks in males was 5.7, 9.0 and 9.0 % in groups 4, 5 and 7 respectively. In females, percent reduction was 12.2, 16.3, 13.2, and 17.3% in 4, 5, 6 and 7 groups of animals respectively. At the end of 13 weeks, mean values of rearing counts for treatment group were comparable to the control group of animals (Table 2).

All kinds of motor activity i.e., total activity, ambulatory activity and stereotypic activity of treatment groups, were comparable to the control group of animals at the end of weeks 4, 13 and 17. Posture observation during home cage observation did not reveal any treatment related posture change in either sex. No treatment related changes were noticed in

convulsive domain (tremor, clonic and tonic convulsion), excitability measures (ease of removal and handling reactivity, arousal, vocalization, circling and stereotypic and bizarre behaviors) and autonomic measures

(lacrimation, salivation, pupil response, palpebral closure, pupil response, piloerection, eye examination, skin examination, urination and defecation counts).

Table 1: Summary of neurobehavioral tests and recording types.

Observation	Recorded as
Home cage observations	
a. Posture	Description
b. Convulsion	Present or absent
Handling Observations	
a. Ease of removal from cage	Rank
b. Handling reactivity	Rank
c. Palpebral closure	Rank
d. Lacrimation'	Rank
e. Salivation	Rank
f. Piloerection	Present or absent
g. Eye examination	Description
h. Skin examination	Description
Open field Observations	
a. Gait abnormalities	Rank
b. Mobility score	Rank
c. Arousal level	Rank
d. Vocalization	Actual counts
e. Rearing	Actual counts
f. Respiration	Description
g. Clonic or tonic movement	Description
h. Urination and defecation	Actual counts
i. Stereotype	Description
j. Bizarre behaviour	Description
Sensory Reactivity Measurements	
a. Approach response	Rank
b. Touch response	Rank
c. Click response	Rank
d. Pupil response	Present or absent
e. Air righting reflex	Rank
f. Tail pinch response	Rank
g. Grip strength	Grams force
h. Landing hind limb foot splay	Distance between hind feet(mm)
Motor activity	Total, ambulatory and stereotypic activity

Neuromuscular Measures (grip strength, hind limb foot splay, gait and air righting response) and Sensorimotor Measurements (approach response, touch response, click response and tail flinch response)

The forelimb and hind limb grip strength measurements, hind limb foot splay values performed at the end of weeks 4, 13 and 17 did not reveal any treatment related statistically

significant changes. All the animals belonging to either sex had normal gait during the scheduled behavioral tests. When the animals were dropped from a height of approximately 30cm to measure the air righting reflex, the ease of uprightness of the landing was normal in all the animals. Sensory motor measurements did not reveal any treatment related changes.

Table 2: Rearing Counts in Open Field – Summary by Group.

Rearing count	Experimental Period							
	Male				Female			
	5 th week		13 th week		5 th week		13 th week	
Group N°	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1	11.1	3.4	8.5	2.87	12.3	2.38	11.9	3.64
G2	13.5	1.93	9.5	3.02	11.5	2.67	12.1	1.13
G3	12.9	3.72	9.9	2.36	11.3	2.76	11.1	2.47
G4	10.5	4.78	7.3	1.67	10.8	2.19	13.4	2.92
G5	10.1	2.36	8.3	1.83	10.3	1.83	12.8	1.91
G6	12.6	1.69	8.8	2.43	10.6	2.13	12.3	4.5
G7	10.1	2.7	8.5	2.2	10.1	2.47	13.0	2.27

G1- 0; G2 (CPF) - 1; G3 (LA) - 50; G4 (CPF+LA) - 1+50; G5 (CPF) - 10; G6 (LA) – 500; G7 (CPF+LA) - 10+500 ppm in diet

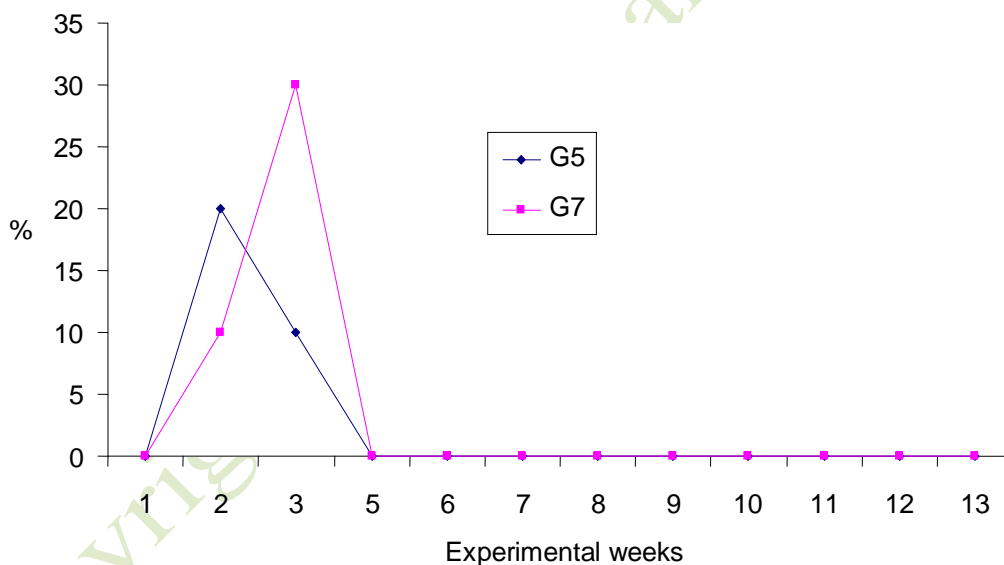


Figure 1A. Per cent of group 5 (chlorpyrifos -1 ppm) and group 7 animals (chlorpyrifos – 1 ppm plus lead acetate – 500 ppm) showing clinical signs – Lacrimation – Main group males

Other Measures

Body Weight and Food Consumption

No statistically significant differences were observed in weekly mean body weights of treatment group animals as compared to respective control groups throughout the exposure period. However, close observation of mean body weight data revealed slight reduction in mean body weight of group 7 males from week 2 to week 13 as compared to control group

males. The percent decrease was 3.8, 2.4, 4.9, 5.6, 5.5, 7.1, 8.6, 6.9, 7.0, 6.2 and 5.1 respectively in weeks 2 – 13, as compared to control group animals. Weekly mean body weights of treatment group females were comparable to control group females. No significant differences were observed in mean food consumption of treatment group animals as compared to control group animals.

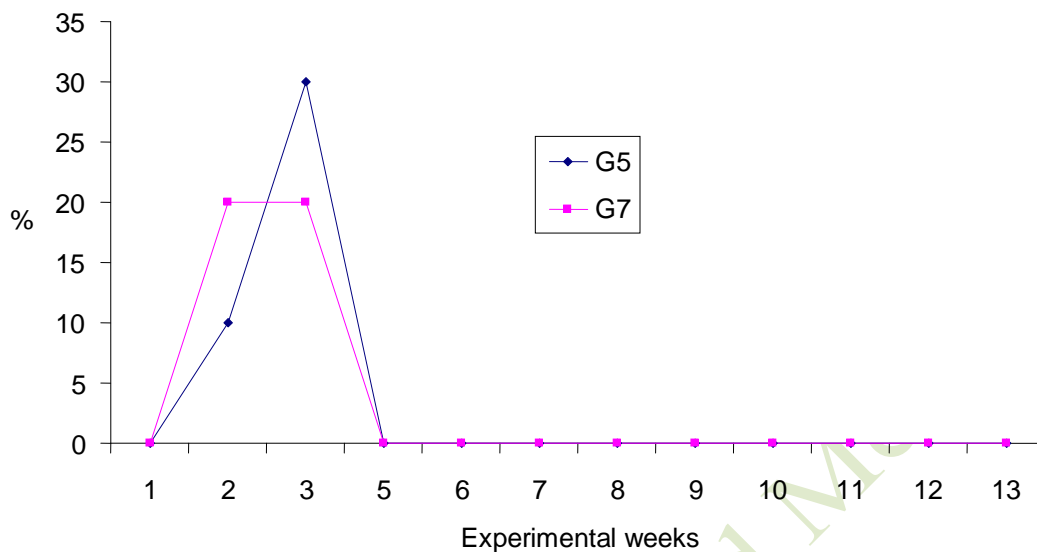


Figure 1B: Per cent of group 5 (chlorpyrifos -1 ppm) and group 7 animals (chlorpyrifos – 1 ppm plus lead acetate – 500 ppm) showing clinical signs – Lacrimation – Main group females

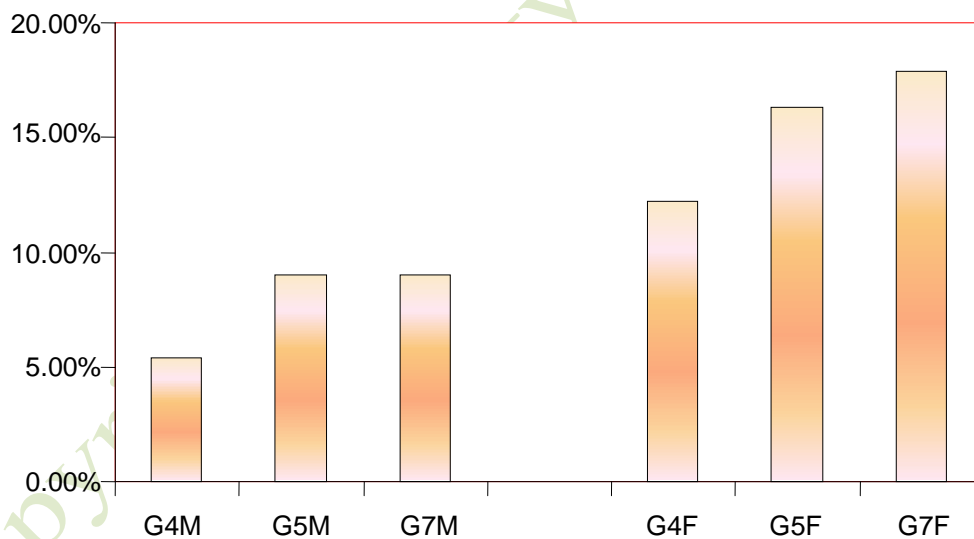


Figure 2: Percent decrease of mean rearing counts in open field on weeks 5 and 13 in animals belonging to G4 (chlorpyrifos – 1 ppm + lead acetate 50 ppm), G5 (chlorpyrifos – 10 ppm), G6 (lead acetate – 500 ppm) and G7 (chlorpyrifos – 10 ppm + lead acetate 500 ppm) as compared to control group animals.

Discussion

Dietary exposure to chlorpyrifos and lead acetate combination (10 ppm chlorpyrifos i.e., equivalent to 1mg/kg body weight/day and 500 ppm acetate i.e., equivalent to 44.0mg/kg body

weight/day) in Wistar rats for a period of 13 weeks, did not reveal serious alterations in the studied neurobehavioral aspects. Clinical signs such as perennial staining of nose, chromorhinorrhoea, lacrimation and

chromodacryorrhoea were observed in some of the animals belonging to groups 5 and 7. Though incidences of clinical signs observable in groups 5 and 7 were less, no animal from control group revealed any of these signs and, these cholinergic signs were also evident in single dose study. Hence, these clinical signs can be considered treatment related.

After 4 weeks of exposure, the rearing counts (vertical movements) in the open field were slightly reduced in either sex of groups 4, 5 and 7 (Table 2 and Figure 2). Mattsson *et al.*, (1996) observed a decrease in motor activity at 15 mg/kg/body weight/day of chlorpyrifos at week 4. When compared with motor activity, the rearing movements measured in the open field could be of more indicative of exploratory behaviour and emotional tendencies (Meyer, 1998). This observation also indicates that vertical movement measurements in open field are more sensitive to gauge the effects of chemicals on motor activity and emotional tendencies. Locomotor activity of an animal is dependent not only on the animal's motor system but also on the sensory and motivational factors (Meyer, 1998).

The occurrence of cholinergic clinical signs was more during weeks 2 and 3 as compared to remaining weeks of exposure. The rearing counts of treatment group animals were comparable to control group animals after 13 weeks of exposure. The reduction of vertical movements at week 4 and higher number of clinical signs before week 4 indicate lack of persistence or cumulative effects. This is indicative of tolerance to repeated exposure of chlorpyrifos. Many authors have reported tolerance to chlorpyrifos after repeated exposure [Bushnell *et al.*, 1993; Bushnell *et al.*, 1994; Pope *et al.*, 1992; Mattsson *et al.*, 1996]. Tolerance to organophosphate cholinesterase inhibitors indicates that functional recovery accompanies neurochemical compensations for the inhibited enzyme. It is evident from several studies that a cholinesterase inhibiting organophosphate pesticide like chlorpyrifos induces tolerance to repeated exposure. Tolerance i.e., reduction in effects with continued treatment, in case of OP treatment, is generally characterized by decrease in the symptoms of cholinergic over stimulation (such as lacrimation, salivation and hypothermia) and, down regulation of muscarinic and N-methyl-D-aspartate (NMDA) receptors in various regions of the brain.

Bushnell *et al.*, (1994) reported tolerance after repeated injection of chlorpyrifos in rats. Tremor and behavioral changes were observed in rats when blood cholinesterase was inhibited by 60% to 90% after 5 weeks on weekly injections up to a dose of 60mg/kg body weight (0, 15, 30 or 60 mg/kg s.c.). They observed reduced blood cholinesterase inhibition (50% - 75% of control) and absence of behavioral effects, when CPF injection was given every other week. Restart of weekly injections for 10 weeks reduced blood cholinesterase by 75% to 90%. Tremor did not recur while; behavioral changes like motor slowing and working memory impairment persisted throughout the dosing period in all treated groups. They also observed pharmacological evidence for tolerance to the muscarinic effects of CPF (i.e., CPF-treated rats were supersensitive to scopolamine and subsensitive to pilocarpine).

The density of various cholinergic receptors in different parts of brain markedly affects brain cholinergic signaling and subsequent metabolic events and pathological consequences. Repeated exposure to organophosphorus (OP) insecticides both in vivo and in vitro results in a decrease of muscarinic acetylcholine receptors (MRs) in the central nervous system, which is one of the characteristic features of tolerance. The in-vivo effects of disulfoton (OP) exposure on the mRNA levels of the three muscarinic acetylcholine receptor (mAChRs) for subtypes (M1, M2, and M3) were studied by Yagle and Costa (1996) in the brain tissue and, in peripheral mononuclear cells which express the m3 subtype. Sprague Dawley rats were exposed to doses of 2mg/kg/day of disulfoton for 14 consecutive days, and the messenger ribonucleic acid (mRNA) levels of muscarinic receptor subtypes m1, m2 and m3 were assayed immediately after the cessation of exposure as well as, after a withdrawal period of 28-days. There was a significant decrease in the levels of muscarinic receptor subtypes in different brain regions immediately after exposure (m1 mRNA levels in hippocampus (23%); m2 subtype mRNA in both hippocampus (24%) and medulla (19%) and m3 mRNA levels in cortex (10%)). After the recovery period, no variation in reduction in m1 or m3 mRNA levels was observed in any of the brain regions examined while the m2 subtype mRNA levels (in the hippocampus) remained decreased by 29%. This result indicates that m2 muscarinic

receptors in the hippocampus may be more susceptible to OP induced alterations.

The role of NMDA receptor during OP intoxication has been studied using binding agents such as [³H] MK-801. The NMDA receptor is one which is dependent on excitatory amino acids. NMDA receptor down regulation is a kind of tolerance mechanism to OP induced toxicity via excess excitatory amino acids. During NMDA receptor activation, permeability to Na⁺, Ca²⁺, K⁺, and Mg²⁺ increases and the resultant overload of Na⁺ and Ca²⁺ can prove detrimental to cells. Such down regulation of NMDA receptors has been demonstrated by Zhou *et al.* (2005). They studied the effect of an acute exposure to an OP compound, dichlorvos, on NMDA receptor density and, the protective efficacy of memantine on dichlorvos toxicity. Dichlorvos induced significant decrease in NMDA receptor density in rat brain.

Conclusion

No specific interactions could be noticed in the present study based on neurobehavioral changes through functional observational battery. However, a decrease in rearing counts of group 4 (i.e., chlorpyrifos 0.1mg plus lead acetate 4.5 mg/kg body weight/day) animals after week 4 was noticeable. The reduction in rearing counts after the 4th week, irrespective of sex, suggesting that even at low dose levels, the combination of chlorpyrifos and lead produces behavioral changes in the nervous system. Though vertical movements in open field are more relevant to know the effects of chemicals on motor activity and emotional tendencies, many higher levels of tests for detection of cognitive functions should also be considered.

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