CHAPTER-1

COMBINATION EFFECT OF CHLORPYRIFOS AND LEAD ON NEUROBEHAVIORAL ASPECTS BY SINGLE AND NONLETHAL HIGH DOSE LEVELS

Introduction

Hazards and immediate toxicity of chemicals can be assessed at relatively higher doses by a single dose exposure. Acute studies have been widely used for toxic level classification, detection of lethal dose (LD50), to obtain knowledge on accidental exposure of chemicals to skin and eyes and, packaging details i.e., for transport and handling of chemicals. Various regulatory agencies have been asking for different kinds of acute studies based on chemical properties and nature. Not all chemicals show toxicity or clinical signs of toxicity immediate to toxicant administration. Some chemicals produce toxicity several days after the xenobiotic exposure. Depending on the properties of chemicals, effects may be reversible or irreversible and, may be for short duration or long duration. Some organophosphorus chemicals produce delayed neuropathy in human and animals. Organophosphorus induced delayed neuropathy (OPIDN) is a generally progressive, irreversible disorder that causes clinical manifestations appearing days to weeks after exposure.

The accidental exposure to two chemicals (belonging to same class or different classes) at about the same time may produce some toxic effects through interactions like potentiation, synergism etc. In such cases, appropriate studies will be helpful for clinicians or scientists to be aware of the possibility and degree of interaction of two chemicals. In this context, the

present study was undertaken to evaluate the effect of a combination of chlorpyrifos and lead on neurobehavioral aspects in the Wistar rats.

Materials and Methods

Test Substance

Chlorpyrifos (Technical 98.0%) purity was obtained from Enzymes, Pharmaceuticals & Industrial Chemicals Ltd (Factory: 32, Vithoba Industrial Complex, Village Lohop, and Post. Mazgaon, Tal. Khalapur, Dist. Raigad 410 206. Maharashtra). Lead acetate (99.103 % purity) manufactured by s.d. fine CHEM Ltd., was used for the study.

Animals

Healthy Wistar rats (50 males and 50 females) approximately 6 weeks old were obtained from Animal Breeding Facility of Jai Research Foundation. Age of the animals at the time of start of treatment was approximately 6 to 7 weeks. Animals were acclimatized to environmental conditions for a minimum of 5 days. During the acclimatization period, animas were observed for good health.

Environmental Conditions

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 (Suprasol L.C 5%).

Housing, Diet and Water

The animals were housed in groups of two of same sex per cage in solid floor polypropylene rat cages. Each cage was fitted with stainless steel top grill

and having provision for keeping water bottles and pellet feed. The bottom of the cage was layered with clean, sterile rice husk. The animals were provided with ad libitum laboratory rat feed and charcoal filtered, UV sterlised water (Aquaguard water filter system).

Grouping

The animals were randomly allocated to 7 groups. Each group comprised of 5 male and 5 female rats. At the start of treatment, body weight variation among the animals was within the ± 20% of mean body weight range. The groups are as follows.

Group 1 (G1) – Vehicle control
Group 2 (G2) – Chlorpyrifos (low dose)
Group 3 (G3) – Lead acetate (low dose)
Group 4 (G4) – Chlorpyrifos (low dose) + Lead acetate (low dose)
Group 5 (G5) – Chlorpyrifos (high dose)
Group 6 (G6) – Lead acetate (high dose)
Group 7 (G7) – Chlorpyrifos (high dose) + Lead acetate (high dose)

Animal Identification

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°.

Dose Selection

A range finding study was conducted to select doses for the main study. Nine males and nine females were divided into 3 groups; each group comprising of 3 males and 3 females. Chlorpyrifos and lead acetate were given at 150 mg/kg body weight and 1000 mg/kg body weight doses respectively to the first two groups of rats and, the third group of rats was treated with a combination of the first two. All the rats of chlorpyrifos group died on first

day of dosing and in the combination groups, mortality was observed on the second day. No treatment related clinical signs or mortalities were observed in lead acetate alone treated group. Severe cholinergic symptoms were observed both in chlorpyrifos alone and in combination groups before mortality. Based on these results, main study was conducted using the following doses.

Group 1 (G1) – Vehicle control

Group 2 (G2) – Chlorpyrifos – 5 mg/kg b.wt

Group 3 (G3) – Lead acetate – 100 mg/kg b.wt

Group 4 (G4) – Chlorpyrifos - 5 mg/kg b.wt + Lead acetate - 100 mg/kg b.wt

Group 5 (G5) – Chlorpyrifos - 50 mg/kg b.wt

Group 6 (G6) – Lead acetate - 1000 mg/kg b.wt

Group 7 (G7) – Chlorpyrifos – 50 mg/kg b.wt + Lead acetate -1000 mg/kg b.wt

Route and Mode of Administration

The route of administration of test substance was through oral gavage. Chlorpyrifos was suspended in corn oil and lead acetate was dissolved in distilled water. Dose volume administered was 5 mL/kg body weight.

Duration of Treatment

All animals were treated only once during the course of experiment and were observed for a period of 14 days thereafter. The combination group (G4 and G7) animals received chlorpyrifos and lead acetate simultaneously.

Observations

Clinical Signs

Animals were observed for mortality and morbidity twice a day. 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.

Neurobehavioral Observations (Functional Observational Battery)

Functional observational battery was conducted to assess the behavioral and neurological status of each animal. Following observations were made once before commencement of treatment and on 1st, 2nd and 14th days post-treatment.

Functional observational battery are designed by United States Environmental Protection Agency (U.S. EPA) consisting of wide range of neurobehavioral tests. A number of investigators have proposed series of tests that are generally intended to evaluate various aspects of behavioural, neurological, and autonomic status. These batteries consist of series of semiquantitative measurements appropriate for the initial level of behavioural assessment. The tests are, in effect, rating scales concerning the presence or absence (and, in some cases, the relative degree of presence) of certain reflexes. In addition, eating and drinking behaviour and body weight should also be considered in the context of primary behavioural assessment (OPPTS 870.6200, 1996). The following observations were made.

Home Cage Observations

In home cage, animals were observed for posture and for the presence or absence of convulsions.

Posture

The posture of animals was observed in the home cage upon initial approach by the observer and description of the posture was recorded as

- Flattened, limbs may be spread out
- Lying on side
- Curled up often asleep
- Sitting but with head hung down
- Sitting normally, feet tucked in
- Sitting or standing alert, watching
- Rearing
- Vertical jumping
- Writhing
- Circling

Convulsion

In the home cage, animals were also observed for presence or absence of convulsions.

Observations during Removal and Handling

After completing home cage observations, the rat was picked up by the observer and observed for the ease of removal from the home cage, handling reactivity of the animal, eye abnormalities, and autonomic signs such as palpebral closure, lacrimation, salivation and piloerection.

Ease of Removing Rat from Cage

The reactivity of the animal to being removed from its home cage was ranked based on the intensity of its reaction as very easy, easy, moderately difficult, difficult or very difficult.

Handling Reactivity

A subjective measurement of the reaction of the animal while being held by the observer was rated as very easy, easy, moderately easy, freezes or difficult.

Palpebral Closure

The degree of closure of the eyelid during the time when the animal was held by the observer was ranked as eyelids wide open, slightly closed, ptosis or eyelids completely closed.

Lacrimation

The degree of lacrimation was rated and recorded as none, slight or severe.

Eye Examination

Eyes were observed for presence or absence of exophthalmos, opacity, cataract, chemosis, conjunctivitis, and discharge and, other abnormalities, if any.

Piloerection

Piloerection was differentiated from scruffy or ungroomed coat by patting the back of the animal in a rostral to caudal direction. When the animal's hair erected even after patting, it was considered as piloerection. The presence or absence of piloerection was recorded.

Skin Examination

Animal's skin was examined for abnormalities such as rough coat, alopecia and dermatitis.

Salivation

The degree of salivation was rated as none, slight or severe.

Open Field Observations

For open field observations, rats were placed in an open arena with a flat surface covered with clean absorbent paper on it and observed for a period of 3 minutes. Fresh absorbent paper was placed for each animal. During the 3 minute period, following observations were made and recorded.

Gait

The walking pattern of the rats was evaluated by observing movements of the rat in the open field box during the 3 minute test period. The observations were ranked in terms of severity as normal, slightly abnormal or severely abnormal.

Arousal Level

A ranking of the level of unprovoked activity and alterations of the animal in the open field was recorded during the 3 minutes observation period as very low, low, high or very high.

Vocalizations

The actual number of spontaneous or unprovoked vocalizations was recorded.

Rearing

The number of times the rat raises its front paws off the floor is considered rearing. The number of these actions was counted for the 3 minute observation period, and the total number of rearing was recorded.

Respiration

Any apparent alteration in the rate and/or ease of respiration was recorded as normal, dyspnea, abdominal breathing, gasping, snuffles or tachypnea.

Clonic or Tonic Movements

In the open field, each animal was observed for presence or absence of clonic or tonic movements. The observation for clonic movement was recorded as chewing (clonus of the jaws), mild clonic tremors of limbs, repetitive clonic tremors of the whole body or absent. Tonic movements were recorded as tonic contraction of hind limbs, opisthotons – backward, emprosthotonos – forward or absent.

Urination and Defecation

Actual number of urine pools and fecal boluses at the end of the 3 minute observation period was recorded.

Stereotypy

Stereotypy can be defined as the pronounced repetition of specific gestures or movements i.e., the presence of excessive or repetitive behaviors that appear purposeless to the observer.

Bizarre Behavior

Bizarre behavior includes any unusual behavior that will not be normally observed in the test species. Presence or absence of such behavior was recorded.

Sensory Reactivity Measurements

After the 3 minute observation period, while the animal was in the open field box, the following tests were conducted to measure sensory reactivity to different stimuli.

Approach Response

The animal was approached at the nose level with the end of a blunt object which was held approximately 3 cm away from the face of the animal for

approximately 5 seconds to allow time for the animal to respond. The degree of the elicited response was recorded as absent, slow or fast response.

Touch Response

Approaching the animal from the side, the rump of the animal was gently touched with a blunt object. The contact was brief and deliberate but not forceful. The degree of the elicited response was recorded as present or absent.

Tail-pinch Response

The tail was squeezed approximately 2 to 3 cm from the tip using a forceps (always applying the same amount of force for each animal). The degree of elicited response was recorded as normal, slight or absent.

Pupil response

A beam of flash light was brought from a lateral position towards the center of the face of the animal. Constriction of the pupil was observed as a positive response. The degree of elicited response was recorded as normal or absent.

Air Righting Reflex

The animal was held supine, with the hands of the observer under the back and shoulders of the animal for support. The animal was dropped from a height of approximately 30 cm. The ease and uprightness of the landing was recorded as normal or abnormal.

Landing Hind Limb Foot Splay

The feet of each rat were marked with a non-permanent, non-toxic ink just prior to testing. The animal was 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 average of three foot splay values was calculated. A clean recording sheet was used for each animal.

Grip Strength

Grip strength of both the forelimbs and hind limbs were measured using grip strength meter (Columbus Instruments, Ohio, USA) to determine the ability of the animal to grasp and hold on to the mesh platform. The grip strength of each animal was measured for 3 consecutive times; the results were averaged separately for the forelimbs and hind limbs.

Grip Strength Meter (Columbus Instruments, Ohio, USA)

This instrument is used for grip strength measurement of small laboratory rodents. The instrument employs an electronic digital force gauge, which measures the peak force exerted on them by grasping action of the animal. The grip strength meter reads the gauze and displays the results (Grip Strength Meter, Manual).

Forelimb Grip Strength Measurement - Procedure

Grasp the animal close to the base of the tail and allow the animal to hold the pull bar. Once the animal grasps the mesh, slowly pull it away from the pull bar at a rate of approximately one inch/ second. Reading displayed on the meter should be recorded and procedure can be repeated three times to have mean values.

Hind Limb Grip Strength Measurement - Procedure

Grasp the animal around the neck region with one hand and at the base of tail with the other hand so that the position of the animal's head points opposite to the direction of the pull bar. Allow the animal to grasp the pull

bar with its hind limbs and then slowly pull the animal's back towards the force gauze with the same speed as for the forelimb.

Motor Activity

Motor activity of each animal was monitored using an automated animal activity measuring system (Opto-varimex Micro, Columbus Instruments, Ohio, USA) equipped with a computer analyzer. Animals were monitored for three consecutive 10 minute intervals allowing for examination of both exploratory and acclimation activity levels. During this period, total and ambulatory activity of the animal was evaluated. Stereotypic activity was calculated by subtracting ambulatory activity from total activity.

The following motor activity parameters were reported and used for comparisons: Total activity, Ambulatory activity and Stereotypic activity.

Animal Activity Measuring System (Opto-varimex Micro Animal Activity Measuring System)

Opto-varimex Micro Animal Activity Measuring System is an instrument intended to measure animal activity in multiple cages. The system consists of interchangeable sensor units, transparent acrylic made cages with metal grid and platforms to be placed at the bottom. It is endowed with sensor bracket assembly, computer interface and computer system with "Microsoft Windows 95" operating system. All the sensor units are identical in design and each contains microprocessor, 8 infrared emitters and 8 infrared detectors. When a pair of such sensors is placed facing each other, they produce 16 infrared beams, which intersect an area between them. Sensor pair beam information is received by Opto-varimex Micro program, which collects, processes and saves it for export to other programs like Microsoft Excel for analysis. Up to 64 sensor pairs can be used in a single opto-

varimex micro animal activity measuring system to monitor 64 animal cages with 16 infrared beams. The sensors are connected in a "daisy chain" fashion by interconnecting cords from one sensor to the next (Opto varimex Micro Animal Activity Measuring System Manual).

Individual tests of screening battery were grouped into several domains of neurobiological function.

Functional Domains	Tests
Activity Measures	Posture, Rearing and Motor Activity
Convulsive Domain	Clonic and tonic convulsions, Tremor during home cage and open field.
Excitability Measures	Ease of Removal and Handling Reactivity, Arousal, Vocalisation, Circling and Stereotypic and bizarre behaviors
Autonomic Measures	Lacrimation, Salivation, Pupil response, Palpebral closure, Piloerection, Eye examination, Skin examination. Urination and Defecation counts
Neuromuscular Measures	Landing hind limb foot splay, Grip Strength measurements (forelimb and hind limb), Gait, Air Righting Reflex
Sensorimotor Measurements	Approach Response, Click response, Touch Response, Tail-pinch Response

Other Measures

Body Weight

Individual animal was weighed on the day of commencement of treatment and on 2nd, 8th and 14th days of experimental period.

Food Consumption

The weekly food consumption of the animal was calculated on 8th and 14th days of experimental period using the following formula.

$$\text{Food consumption (g/rat/week)} = \frac{\text{Feed input in the week} - \text{feed left over in the week}}{\text{Number of rats in the cage}}$$

Evaluation of Data

Statistical evaluations were performed using validated statistical software (Developed by jai research Foundation). 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

Single dose exposure to chlorpyrifos and lead acetate separately and in combination was carried out at two different dose levels to investigate their interactive effects on neurobehavioral changes. The results obtained from the study are as follows.

Clinical Observations

No mortalities were observed during the entire course of experimentation in any of the groups. Treatment related clinical signs were observed in group 5 (CPF–50 mg/kg body weight/day) and group 7 (CPF-50 mg/kg body weight + LA-1000 mg/kg body weight/day). Tremor, perennial soiling due to urine and feces, motor in-coordination, decreased muscle tone, cold to touch, chromodacryorrhoea, chromorhinorrhoea, and exophthalmos were observed at differential time scales.

Males

Group 5 animals showed tremor (2), perennial soiling due to urine and feces (3), motor incordination (4), decreased muscle tone (4), and piloerection (3) on day 1 i.e., after approximately 2-3 hours of dosing. On day 2, motor incordination and perennial soiling was persistent in 1 animal belonging to G5, and thereafter symptoms disappeared (Figures 1A – 1E).

Group 7 animals on day 2 showed tremor (4), perennial soiling (4), motor incordination (5), decreased muscle tone (5), piloerection (5) and cold to touch (2). Motor in-cordination in one animal and muscle tone decrement in two animals were persistent till the 3rd day. Perennial soiling due to urine, and feces were present in 3 animals on 3rd day and 1 animal on 4th day. No animals from group 7 exhibited symptoms on first day after dosing. No

treatment related clinical symptoms were observed in groups 2, 3, 4 and 6 (Figures 1A – 1E).

Females

Like males, females also showed similar symptoms. Group 5 animals exhibited tremor (3), perennial soiling (3), motor incordination (4), muscle tone decrease (4), and piloerection (4) on day 1 i.e., after approximately 2-3 hours of dosing. On day 2, tremor (1), perennial soiling (3), motor incordination (2), decreased muscle tone (2), chromodacryorrhoea (1), chromorhinorrhoea (2) and exophthalmos (1) were observed. All animals belonging to group 5 recovered from the exhibited symptoms on day 3 (Figures 1A - 1F).

Group 7 animals on day 2 showed tremor (4), perennial soiling (5), motor incordination (5), muscle tone decrement (5), piloerection (5), chromodacryorrhoea (1), chromorhinorrhoea (5) and exophthalmos (4). On day 3, persistent symptoms were tremor (2), perennial soiling (3), motor incordination (2), muscle tone decrement (5), chromorhinorrhoea (2) exophthalmos (4) and piloerection (3). Perennial soiling (2), muscle tone decrement (2) and exophthalmos (2) were exhibited by two animals and clonic movement of jaws (1) by one animal on day 4. Like males, group 7 females also did not exhibit symptoms on first day after dosing. Additionally, chromodacryorrhoea, chromorhinorrhoea and exophthalmos were observed, as compared to male animals. All animals appeared normal on day 5 onwards. No treatment related clinical symptoms were observed in other treatment groups (Figures 1A - 1F).

Neurobehavioral Observations

Neurobehavioral observations performed before treatment, on day 1, day 2 and day 14 post-treatment revealed treatment related predominant neurobehavioral changes on days 1 and 2 in groups 5 and 7 animals.

Activity Measures (Posture, Rearing and Motor Activity)

Posture

Males

Posture observation did not reveal consistent postural change in either sex of animals. However, three animals belonging to group 5 (chlorpyrifos – 50 mg/kg body weight) on day 1 and 5 animals from group 7 (chlorpyrifos – 50 mg/kg body weight + lead acetate – 1000 mg/kg body weight) on day 2 were observed to be sitting with head hung down. Lack of rearing and loss of alertness was noticed in group 5 and group 7 animals. Though there were some animals across groups before treatment and after treatment for head hung down posture, they did not exhibit tremors or any kind of unusual behaviors (Figure 2).

Females

Animals exhibiting tremors were found to be in head hung down posture (Figure 2).

Rearing

Males

The vertical movements in the open field were reduced in group 5 animals by 42.1% on day 1; however, they were not statistically significant. On day 2 during open field observation, group 7 animals showed significantly reduced rearing count as compared to control group animals (Table 1 and Figure 2).

Females

The rearing counts of animals belonging to group 5 on day 1 and group 7 on day 2 were significantly reduced as compared to control group animals (Table 1 and Figure 2).

Motor Activity

Males

No statistically significant differences were observed in motor activity of treatment group animals as compared to control group animals on day 1. However, reduction was observed in the mean values of motor activity counts of group 5 on day 1. Total, ambulatory and stereotypic activity were reduced by 26.7, 26.4 and 27.1 % (during first interval), 46.1, 48.9 and 38.0 % (during second interval) and 19.7, 19.3 and 19.8 % (during third interval) on day one. On day 2, total and ambulatory activity of group 7 males were significantly decreased during first interval and, non-significantly reduced activity was also observed during second and third intervals (Tables 2A and 2B; Figures 3A and 3B).

Females

Day 1: Total activity and ambulatory activity of animals belonging to group 5, group 6 and group 7 during first interval were significantly decreased as compared to control group animals. During second interval, total and ambulatory activity of groups 5 and 7 animals were significantly decreased. During third interval, total activity of groups 5 and 6 and ambulatory activity of group 5 and stereotypic activity of groups 3, 5, 6 and 7 were all significantly reduced when compared with control group animals. The decreased activity of animals belonging to groups 5, 6 and 7 were treatment related (Tables 2C and Figure 3C).

Day 2: Total, ambulatory and stereotypic activity of group 7 females during first interval were significantly decreased as compared to control group females and, non-significantally reduced activity was also observed during second and third intervals (Table 2D and Figure 3C).

Convulsive Domain (Tremor, Clonic and Tonic convulsion)

Males

Observation for tremor, clonic and tonic convulsion in the home cage revealed tremors in two animals on first day belonging to group 5 and in four belonging to group 7 on day second. These were correlated with head hung down posture of animals and same animals also exhibited tremors during open field observation (Figure 4).

Females

During home cage and open field observations, three animals (60%) on day 1 and one animal on day 2 belonging to group 5 showed tremor. Four animals belonging to group 7 showed tremors on day 2. Clonic movement of jaws (chewing) was observed in one animal from group 7 on day 2 (Figure 4).

Excitability Measures (Ease of Removal and Handling Reactivity, Arousal, Vocalisation, Circling and Stereotypic and bizarre behaviors)

Ease of Removal and Handling Reactivity

Males

Ease of removing rat from the cage was comparatively very easy (3) in group 5 animals on first day of dosing and in group 7 (4) animals on day 2 of dosing. Though there was lack of consistent pattern across the groups, lack of alertness in the cage and higher frequencies on days 1 and 2 were the reason to consider very easy as treatment related changes. After removing

from the cage, reactivity of the animals while being held by the observer in the hand was observed. Two animals belonging to group 5 (on day 1) and three animals belonging to group 7 (on day 2) were freezing in hand. No animals from other groups revealed freezing reactivity (Figure 5).

Females

Four animals from group 5 on day 1 and 5 animals belonging to group 7 on day 2 showed ease of removal as very easy (Figure 5).Three animals from group 5 and four animals from group 7 revealed freezing reactivity in hand respectively on day 1 and 2 (Figure 5).

Arousal

Males

Arousal rate in the open field was low in four animals and very low in one animal of group 5 on day 1. All the animals of group 7 showed very low arousal rate on day 2 (Figure 6).

Females

Arousal rate in the open field was low in three animals and very low in two animals of group 5 on day 1. Like males, all females belonging to group 7 showed very low arousal rate on day 2 (Figure 6).

No animal from either sex revealed vocalizations, stereotypy and bizarre behaviors during the course of experiment.

Autonomic Measures (Lacrimation, Salivation, Pupil response, Palpebral closure, Piloerection, Eye examination, Skin examination. Urination and Defecation counts)

Males

Lacrimation (slight) was observed in 2 animals from group 5 on day 1. Three animals belonging to group 7 showed lacrimation (2-slight 1-severe) on day 2. Salivation was exhibited by two animals (1 -slight, 1-severe) from group 5 on day 1 and three animals (2-slight, 1-severe) belonging to group 7 on day 2. Piloererection was exhibited by 3 animals belonging to group 5 on day 1 and 5 animals belonging to group 7 on day 2 during handling observation. When light stimulus was given to pupil, two animals from group 5 did not respond on day 1. All other animals from remaining groups revealed normal response on day 1. On day 2, one animal each from groups 4 and 6, two animals from group 5 and three animals from group 7 did not respond to light stimulus on day second of sensory reactivity measurements. During handling eye lids were examined for their opening condition. All animals from either sex showed wide opening of eyelids. No treatment related eye abnormalities were observed in male animals treated with test substance. Skin examination of both the sexes did not reveal any treatment related changes. (Figures 7A and 7C).

Females

Three animals (2-slight, 1-severe) belonging to group 5 on day 1 and all animals from group 7 (2-slight, 3-severe) on day 2 revealed lacrimation. Presence of salivation was observed in three animals (2-slight, 1-severe) in group 5 (on day 1) and four animals (3-slight, 1-severe) in group 7 (on day 2). Piloerection was exhibited by 4 animals belonging to group 5 on day 1 and 5 animals belonging to group 7 on day 2 during handling observation.

Pupil response to light stimulus was absent in one animal belonging to group 4 and four animals belonging to group 5 on day 1 of experiment. On day 2, one animal from group 4, two animals from group 6 and three animals each from groups 5 and 7 did not respond to light stimulus. One animal each from groups 5 and 7 exhibited chromodacryorrhoea on day 2 of experimental period. Further, one animal from group 5 and four animal from group 7 showed presence of exophthalmos on day 2 during handling observation (Figures 7B and 7C).

Urination and Defecation Counts

Males

Mean values of defecation counts of group 5 (on day 1) and group 7 animals (on day 2) and urination counts of group 7 (on day 2) animals were significantly decreased as compared to control group animals. The decreased urine and defecation pools in open field might be due to uncontrolled discharge of urine and feces as it was noticed as perennial staining during clinical observation (Tables 3 and 4).

Females

The mean urination count of groups 5 and 7 females (on day 2) and defecation count of group 5 females on days 1 and 2 and group 7 females on day 2 were significantly decreased (Tables 3 and 4).

Neuromuscular Measures (Grip Strength, Hind Limb Foot Splay, Gait, Air Righting Reflex)

Grip Strength

Males

Forelimb and hind limb grip strength of group 5 animals on day 1 was reduced by 7.0 and 29.7 % respectively as compared to control group

animals. However, the reduction was not statistically significant. On day 2, forelimb and hind limb grip strength of group 7 animals were significantly decreased as compared to control group animals (Table 5 and Figure 8).

Females

Hind limb grip strength of group 5 females was significantly decreased as compared to control group animals. Forelimb grip strength was reduced by 25.2% and was not statistically significant on day 1. Forelimb and hind limb grip strengths of group 7 animals were significantly reduced on day 2 as compared to control group animals (Table 5 and Figure 8).

Hind Limb Foot Splay

Males

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

Females

Mean hind limb foot splay values of treatment group animals were comparable to control group animals on day 1. Like males, hind limb foot splay measurement was also not performed for group 7 females on day 2.

Gait

Males

Slight abnormal gait and severe abnormal gait changes were observed in three and one animals respectively belonging to group 5 on day 1. One animal which had severe abnormal gait on day 1 showed slight abnormal gait on day 2. All five animals of group 7 showed gait changes (4-slightly

abnormal, 1-severely abnormal) on day 2. Gait changes were observed as short step walking pattern to toe walking type (Figure 9).

Females

All the animals of group 5 showed gait changes (4-slightly abnormal, 1-severely abnormal) on day 1 and on day 2, one animal had slight gait problem. Animals belonging to group 7 exhibited gait changes (1-slightly abnormal, 4-severely abnormal) during open field observations on day 2. Gait changes were observed as short step walking pattern (slightly abnormal animals) to toe walking type (severely abnormal animals) (Figure 9).

Air Righting Reflex

Males

When the animals were dropped from the height of approximately 30 cm, the ease of uprightness of the landing was slightly abnormal for one animal belonging to group 5 on day 1. All other animals from remaining groups showed normal landing posture. In group 7, the ease of uprightness of the landing upon dropping was slightly abnormal for four animals and air righting reflex was not performed for one animal due to severe cholinergic symptoms on day second of sensory reactivity measurements (Figure 9).

Females

On day 1 the ease of uprightness of the landing was slightly abnormal for four animals and severely abnormal for one animal belonging to group 5. No other animals showed abnormal landing posture. On day 2, two animals from group 5 and four animals from group 7 landed slightly abnormally. Like in male, air righting reflex was not performed for one female on day 2 (Figure 9).

Sensorimotor Measurements (Approach response, Touch response, Click response and Tail flinch response)

Males

On first day there was not much difference among the treatment groups when approach response was carried out for sensory reactivity measurements. On day 2, one animal from group 7 responded slowly and the rest 4 animals did not respond at all. The absent response in group 7 animals was due to the treatment. Presence of touch response was shown by all animals from all groups on day 1. On day 2, four animals from group 7 did not respond to touch by a blunt object. There was no clear difference between groups to sound stimuli in all occasions of sensory reactivity measurements. No treatment related changes were observed on day 1 in any of the groups when tail tip was squeezed with forceps. On day 2, four animals belonging to group 7 showed slight reaction to tail flinch stimuli (Figure 10).

Females

Like males, females also did not show clear cut difference in approach response on day 1. In group 7, one animal responded slowly and 4 animals did not respond on day second of sensory reactivity measurements. One animal from group 5 did not respond to touch on day 1. All the 5 animals belonging to group 7 did not respond to touch by blunt object on day 2 of sensory reactivity measurements. On day 2, all five animals from group 7 reacted slightly to forceps stimuli. Like in males, in females as well, there was no clear difference between groups to sound stimuli in all the occasions of sensory reactivity measurements (Figure 10).

In summary, neurobehavioral tests performed using functional observation batteries revealed predominant cholinergic symptoms only in group 5 (on day 1) and group 7 (on day 2) animals. On day 2, animals belonging to group 7 revealed higher degree of treatment related behavioral changes as compared to the effects exhibited by group 5 animals on day 1 of treatment. Group 6 (lead acetate at 1000 mg/kg dose level) animals apart from a decrease in motor activity, did not reveal any consistent changes with reference to other parameters. Remaining treatment groups of animals i.e., group 2, group 3 and group 4, did not reveal consistent treatment related changes in any of the studied parameters.

Other Measures

Body Weight

Males

On day 2, the mean body weight of group 7 animals was significantly reduced as compared to control group animals. Group 5 animals also revealed 10.9 % reduction in mean body weight as compared to control group animals; however, the decrease was not statistically significant. No statistically significant difference was observed in mean body weight of treatment group animals as compared to control group animals on days 8 and 14. However, mean body weight of group 7 males was reduced by 11.5 % on day 8 and by 10.8 % on day 14 as compared to control group males (Table 6 and Figure 11A).

Females

Like males, group 7 females also showed significant reduction in mean body weight on day 2 as compared to control group females. The non-significant reduction observed in mean body weight of group 5 females was 9.8 % as

compared to control group females. Slight reduction in mean body weight was observed in group 7 females on days 8 (7.7 %) and 15 (6.6 %) as compared to control group females (Table 6 and Figure 11B).

Food Consumption

Males

Group 7 males showed slight reduction in mean food consumption during weeks 1 and 2 as compared to control group males. The percent reduction was 17.7 % and 12.2 % respectively on weeks 1 and 2. The reduction was not statistically significant (Table 7 and **Figure 12**).

Females

Group 7 females showed non-significant slight reduction (9.7 %) in mean food consumption during week 1. During week 2 food consumption of treatment group animals were comparable to control group animals (Table 7 and **Figure 12**).

The reduction in mean food consumption was correlated with decrease in mean body weights in either sex of animals.

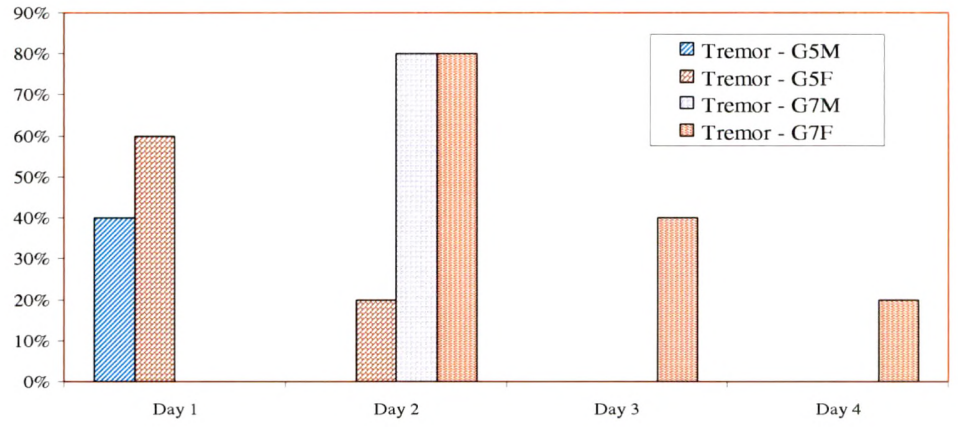

Figure 1A. Percent of group 5 (CPF-50 mg/kg b.wt) and group 7 animals (CPF– 50 mg/kg b.wt + LA - 1000 mg/kg b.wt) showing clinical sign -**Tremor**

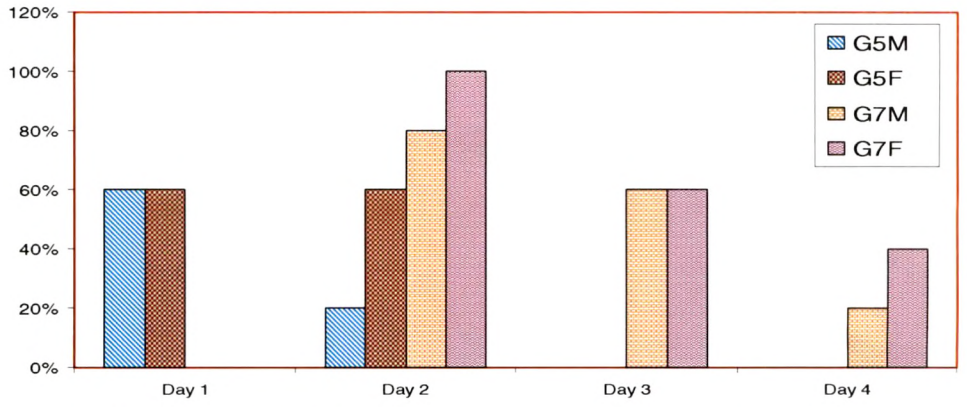

Figure 1B. Percent of G5 (CPF-50 mg/kg b.wt) and G7 animals (CPF– 50 mg/kg b.wt + LA - 1000 mg/kg b.wt) showing clinical sign - **Perennial soiling**

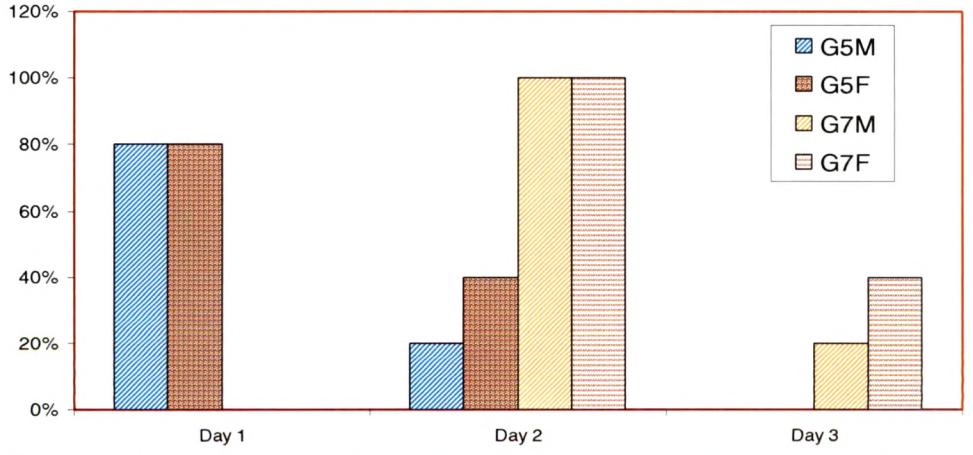

Figure 1C. Percent of group 5 (CPF-50 mg/kg b.wt) and group 7 animals (CPF– 50 mg/kg b.wt + LA - 1000 mg/kg b.wt) showing clinical sign - **Motor Incordination**

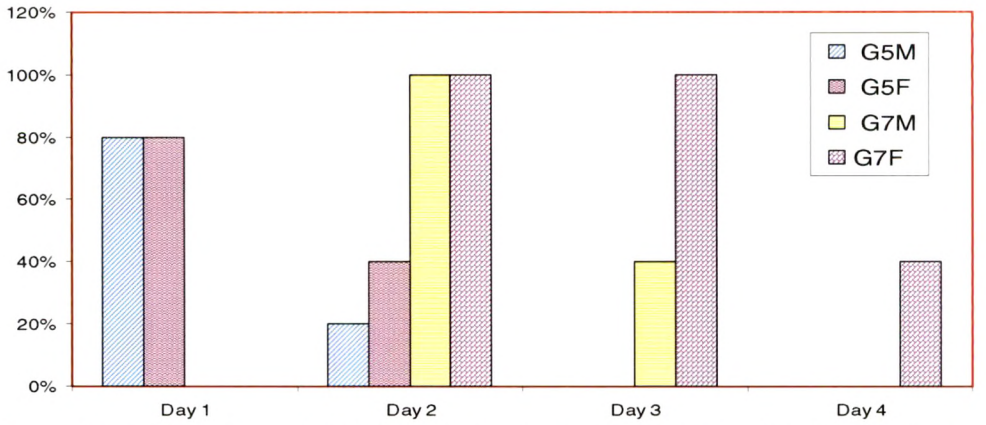

Figure 1D. Percent of group 5 (CPF-50 mg/kg b.wt) and group 7 animals (CPF– 50 mg/kg b.wt + LA - 1000 mg/kg b.wt) showing clinical sign - **Muscle Tone Decrement**
Key : Day 1 = 3-4 hours after treatment ; Day 2 = 24 hours after treatment

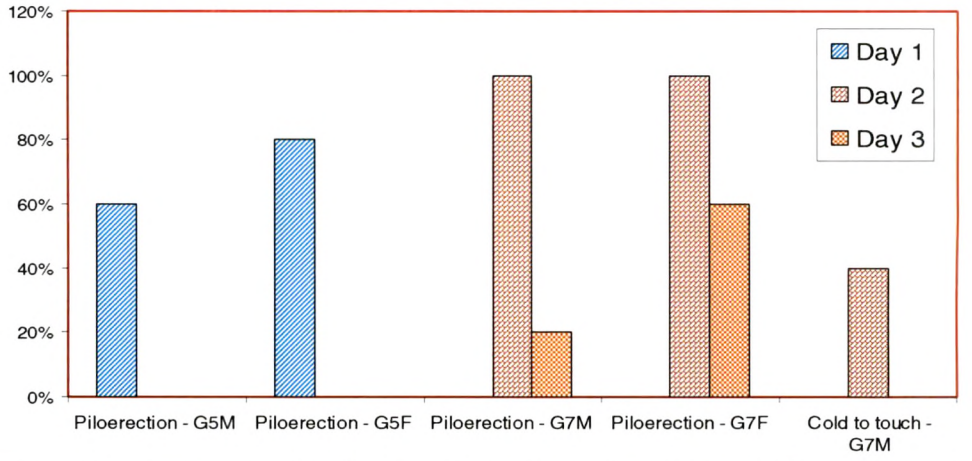

Figure 1E. Percent of G5 (CPF-50 mg/kg b.wt) and G7 animals (CPF– 50 mg/kg b.wt+ LA - 1000 mg/kg b.wt) showing clinical sign– **Piloerection and Cold to touch**

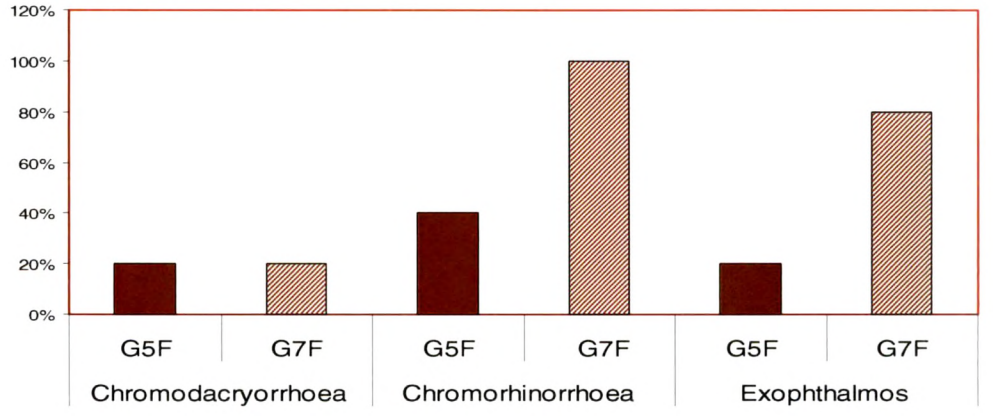

Figure 1F. Percent of G5 (CPF-50 mg/kg b.wt) and G7 animals (CPF– 50 mg/kg b.wt + LA - 1000 mg/kg b.wt) showing clinical sign – **Eye abnormalities on day 2.**

Table 1

Rearing Count - Group Mean Values

Open Field Observations	Experimental Period							
Parameter : Rearing count	Male							
Group N°	PE		1 Day		2 Day		14 Day	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1 (N=5)	9.2	4.60	7.6	3.51	6.00	4.58	11.6	6.66
G2 (N=5)	12.8	2.77	11.6	4.45	9.00	2.0	8.8	5.07
G3 (N=5)	13.4	3.21	7.8	2.49	9.2	3.27	5.2	5.07
G4 (N=5)	12.6	5.18	8.4	2.7	7.4	3.58	7.6	4.22
G5 (N=5)	13.8	4.44	4.4	3.05	8.6	6.73	8.8	4.6
G6 (N=5)	14.0	5.24	7.8	3.83	6.8	4.32	6.2	4.02
G7 (N=5)	15.2	3.11	7.0	2.00	2.2*	2.00	9.2	5.40

Open Field Observations	Experimental Period							
Parameter : Rearing count	Female							
Group N°	PE		1 Day		2 Day		14 Day	
	Mean	SD	Mean	SD		Mean	SD	Mean
G1 (N=5)	10.2	2.39	7.8	3.03	7.6	2.97	10.8	6.53
G2 (N=5)	13.2	4.66	10.2	2.39	6.6	3.21	12.0	3.24
G3 (N=5)	17.2	1.10	12.4	1.34	8.0	3.81	7.6	4.88
G4 (N=5)	11.6	3.65	7.4	0.89	9.2	2.49	3.4	5.46
G5 (N=5)	11.2	3.49	3.4*	2.30	4.4	3.05	9.6	4.62
G6 (N=5)	14.6	2.79	6.0	3.32	7.8	4.97	8.2	3.77
G7 (N=5)	12.2	5.17	6.2	2.17	0.00**	0.00	8.0	4.95

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

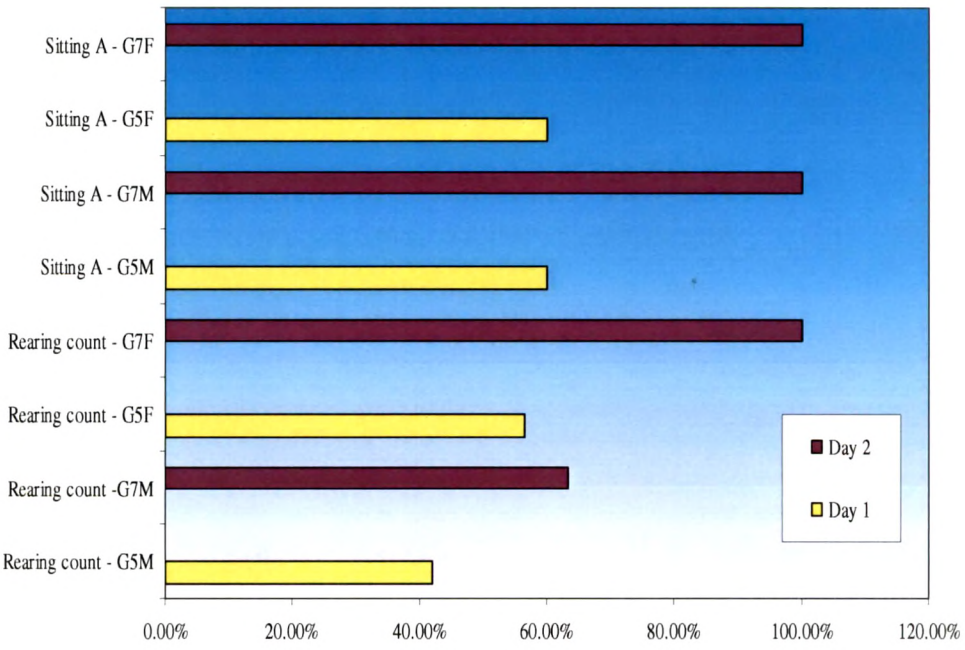

Figure 2. Percent of animals showing effects of CPF - 50mg/kg b.wt (G5) and CPF -50 mg/kg b.wt + LA - 1000 mg/kg b.wt (G7) on posture; Sitting A = Sitting but with head hung down in home cage and percent decrease of mean rearing counts in open field on days 1 and 2.

Table 2A

Motor Activity - Group Mean Values

Sex : Male **Period : 1st Day**

Group N°		Time Interval and Parameter								
		0-10 minutes			11-20 minutes			21-30 minutes		
		Total	Ambu-latory	Stereo-typic	Total	Ambu-latory	Stereo-typic	Total	Ambu-latory	Stereo-typic
G1	**Mean**	20.08	17.68	9.37	17.48	15.18	8.5	13.35	11.16	7.18
(N=5)	**SD**	5.16	5.22	1.63	7.11	7.02	2.26	3.83	3.97	1.20
G2	**Mean**	24.25	21.64	10.72	18.50	16.55	8.22	14.31	11.98	7.44
(N=5)	**SD**	3.69	4.07	1.93	4.68	4.66	1.20	7.69	7.72	2.63
G3	**Mean**	20.35	17.97	9.48	17.11	14.75	8.5	16.36	14.02	8.25
(N=5)	**SD**	5.68	5.30	2.43	7.58	7.31	2.84	7.09	6.72	2.97
G4	**Mean**	22.48	19.66	10.81	16.73	14.62	8.00	13.67	11.66	6.92
(N=5)	**SD**	6.64	6.43	2.39	6.77	6.46	2.61	6.98	6.71	2.71
G5	**Mean**	14.71	13.02	6.83	9.42	7.76	5.27	10.72	9.01	5.76
(N=5)	**SD**	4.73	4.28	2.12	4.60	4.2	2.08	4.80	4.12	2.60
G6	**Mean**	19.34	17.56	7.93	17.03	14.62	8.44	13.74	11.35	7.53
(N=5)	**SD**	9.49	8.56	4.54	7.42	7.48	2.28	6.66	6.42	2.71
G7	**Mean**	16.28	14.34	7.69	14.19	11.64	8.04	8.57	6.11	5.95
(N=5)	**SD**	5.29	4.76	2.37	4.80	4.54	1.95	2.39	1.95	1.68

Key : Day 1 = 3-4 hours after treatment ; Day 2 = 24 hours after treatment

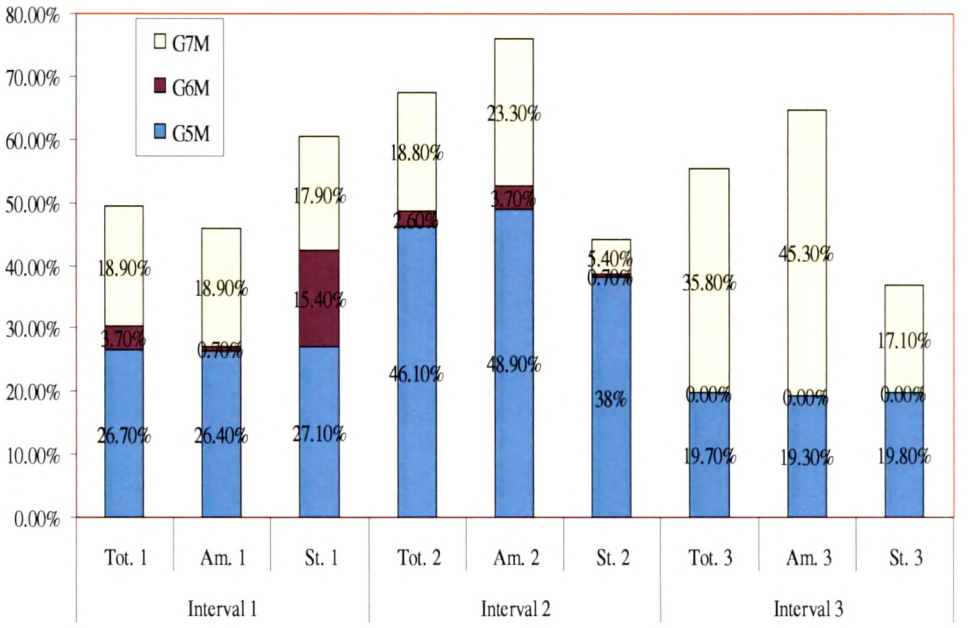

Figure 3A. Percent decrease of motor activity of G5M (CPF -50 mg/kg b.wt), G6M (LA -1000 mg/kg b.wt) and G7M (CPF - 50 mg/kg b.wt + LA-1000 mg/kg b.wt) on day 1. Tot.- Total activity ; Am.- Ambulatory activity ; St.- Stereotypic activity. **Key :** Day 1 = 3-4 hours after treatment ; Day 2 = 24 hours after treatment

Table 2B

Motor Activity - Group Mean Values

Sex : Male **Period : 2nd Day**

Group N°		Time Interval and Parameter								
		0-10 minutes			11-20 minutes			21-30 minutes		
		Total	Ambu-latory	Stereo-typic	Total	Ambu-latory	Stereo-typic	Total	Ambu-latory	Stereo -typic
G1 (N=5)	Mean	23.90	21.62	9.76	21.91	19.4	10.04	17.64	14.29	8.77
	SD	3.27	3.62	1.21	2.91	2.44	1.88	4.92	4.59	1.41
G2 (N=5)	Mean	23.01	20.77	9.84	14.67	12.61	7.21	10.29	8.5	5.69
	SD	7.8	7.28	3.07	5.57	5.92	1.16	5.22	4.8	2.39
G3 (N=5)	Mean	19.09	16.85	8.98	13.12	11.12	6.72	9.39	6.09	6.68
	SD	2.47	2.14	1.24	7.12	6.92	2.60	3.97	4.34	2.23
G4 (N=5)	Mean	21.15	19.05	9.17	17.64	14.53	9.0	13.75	11.13	7.74
	SD	4.17	3.63	2.15	6.98	8.18	2.36	5.58	5.78	2.1
G5 (N=5)	Mean	20.95	19.0	8.83	15.62	13.83	7.1	13.66	12.07	6.36
	SD	6.27	5.79	2.42	9.12	8.48	3.74	7.94	7.18	3.5
G6 (N=5)	Mean	21.93	19.93	9.08	12.85	11.16	6.26	11.84	9.29	6.83
	SD	3.31	3.33	1.16	6.68	6.36	2.42	3.83	4.72	1.11
G7 (N=5)	Mean	13.54 *	11.68*	6.67	12.30	10.17	6.8	11.59	9.05	6.91
	SD	4.36	4.43	1.53	3.5	3.57	1.33	2.78	3.4	1.41

Key : N= Number of observations; * = significant at 5% level ($p \leq 0.05$)

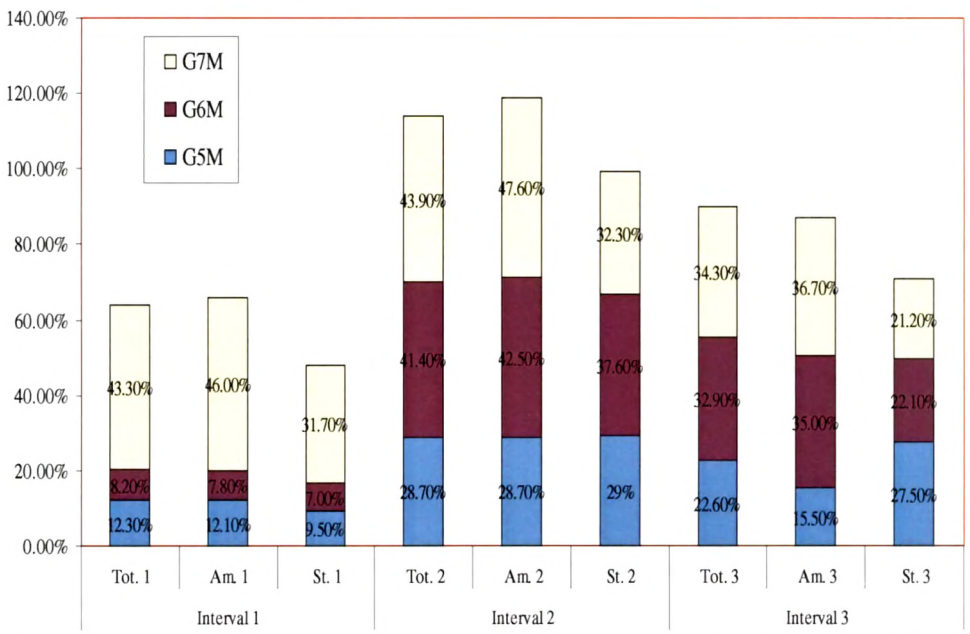

Figure 3B. Percent decrease of motor activity of G5M (CPF -50mg/kg b.wt), G6M (LA -1000 mg/kg b.wt) and G7M (CPF -50 mg/kg b.wt + LA -1000mg/kg b.wt) on day 2. Tot.- Total activity ; Am.- Ambulatory activity ; St.- Stereotypic activity.
Key : Day 1 = 3-4 hours after treatment ; Day 2 = 24 hours after treatment

Table 2C

Motor Activity - Group Mean Values

Sex : Female **Period : 1st Day**

Group N°		Time Interval and Parameter								
		0-10 minutes			11-20 minutes			21-30 minutes		
		Total	Ambu-latory	Stereo-typic	Total	Ambu-latory	Stereo-typic	Total	Ambu-latory	Stereo-typic
G1	Mean	26.06	23.72	10.76	20.76	18.51	9.36	15.66	13.67	7.62
(N=5)	SD	2.69	2.54	1.12	3.88	3.76	1.42	1.33	1.41	0.37
G2	Mean	27.03	24.54	11.32	22.33	20.03	9.80	17.28	14.73	8.74
(N=5)	SD	1.47	1.36	0.8	1.35	1.63	1.13	4.05	4.67	1.04
G3	Mean	24.31	21.80	10.62	19.05	16.55	9.03	11.35	9.51	5.94*
(N=5)	SD	8.63	8.38	2.77	7.03	7.53	1.42	9.49	9.01	3.55
G4	Mean	26.16	23.65	11.10	22.11	19.95	9.39	20.15	18.04	8.86
(N=5)	SD	7.73	7.54	2.27	10.73	10.27	3.65	8.34	8.03	2.75
G5	Mean	18.96**	17.05**	7.97	11.51*	9.59*	6.12	8.48*	6.93*	4.83**
(N=5)	SD	8.45	8.46	2.51	4.59	4.84	1.31	2.57	2.54	0.95
G6	Mean	17.64*	15.17**	8.59	13.74	11.12	7.83	9.75*	7.88	5.16**
(N=5)	SD	6.44	6.97	1.42	6.08	6.08	2.21	8.07	8.10	2.73
G7	Mean	20.06**	18.07**	8.64	12.18*	10.12*	6.43	10.66	9.38	4.98**
(N=5)	SD	8.34	7.99	2.79	7.26	7.09	2.79	4.21	4.08	1.44

Table 2D

Motor Activity - Group Mean Values

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

Group N°		Time Interval and Parameter								
		0-10 minutes			11-20 minutes			21-30 minutes		
		Total	Ambu-latory	Stereo-typic	Total	Ambu-latory	Stereo-typic	Total	Ambu-latory	Stereo-typic
G1	Mean	23.19	20.79	10.23	17.78	15.8	8.05	12.02	10.36	5.99
(N=5)	SD	1.49	1.18	1.26	3.48	3.2	1.98	6.53	6.12	2.57
G2	Mean	25.05	22.53	10.87	21.78	19.41	9.82	16.22	13.82	8.33
(N=5)	SD	2.53	2.8	0.92	5.23	5.05	1.76	4.66	4.86	1.17
G3	Mean	22.81	19.9	10.84	12.10	10.3	6.15	19.36	7.93	4.75
(N=5)	SD	5.82	6.45	1.02	7.2	6.68	3.23	9.71	8.72	4.59
G4	Mean	29.39	26.95	11.70	23.02	20.56	10.18	21.29	19.33	8.92
(N=5)	SD	3.81	3.83	0.79	7.44	7.54	1.75	4.4	4.17	1.51
G5	Mean	23.8	21.83	9.45	18.61	16.86	7.83	15.18	13.23	7.39
(N=5)	SD	5.14	4.85	1.9	2.91	2.45	1.82	3.12	2.96	1.44
G6	Mean	23.45	21.02	10.39	20.9	18.21	10.21	19.04	16.73	9.07
(N=5)	SD	4.39	3.99	1.93	2.46	2.48	1.06	3.52	3.46	1.12
G7	Mean	14.76*	12.74*	7.33*	10.3	8.26	6.01	10.79	8.78	6.14
(N=5)	SD	4.64	4.46	1.92	3.63	3.72	1.30	4.41	4.44	1.38

Key : N= Number of observations ; * = significant at 5% level ($p \leq 0.05$); ** = significant at 1% level ($p \leq 0.01$) Day 1 = 3-4 hours after treatment; Day 2 = 24 hours after treatment

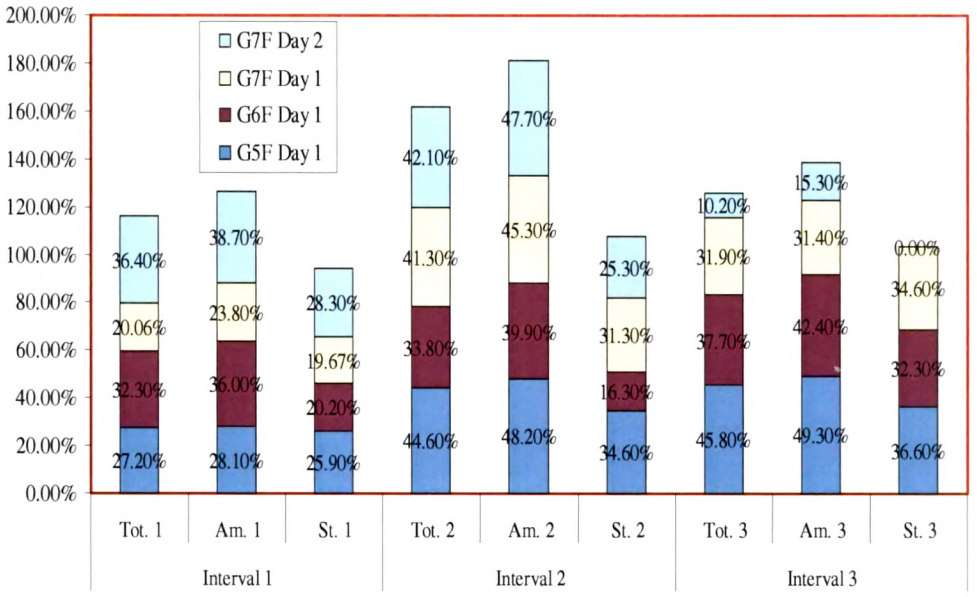

Figure 3C. Percent decrease of **motor activity** of G5F (CPF -50 mg/kg b.wt) and G6F (LA -1000 mg/kg b.wt) on day 1 and G7F (CPF -50 mg/kg b.wt + LA -1000 mg/kg b.wt) on days 1 and 2. Tot.- Total activity ; Am.- Ambulatory activity ; St.- Stereotypic activity.

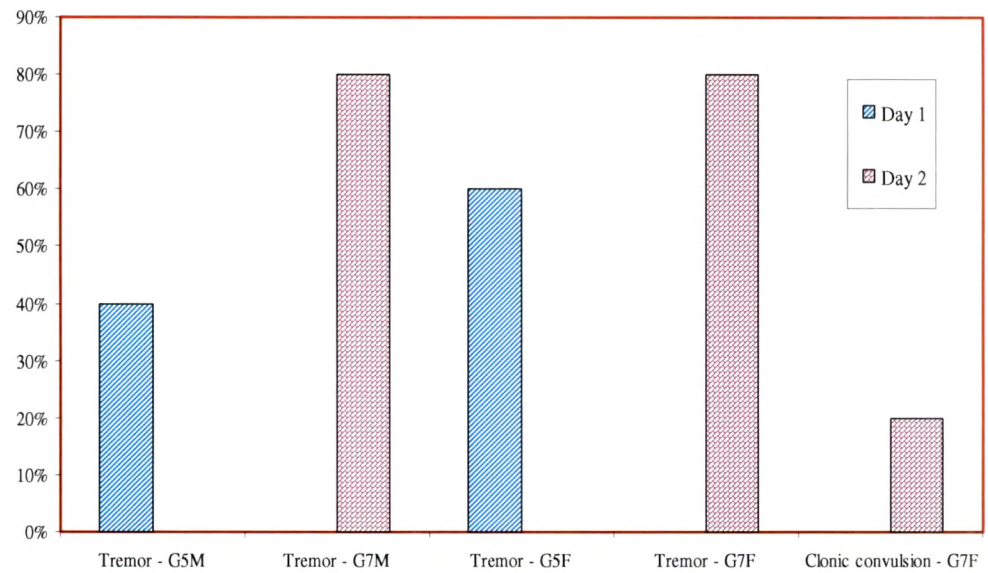

Figure 4. Percent of animals showing effects of CPF - 50 mg/kg b.wt (G5, day 1) and CPF - 50 mg/kg b.wt + LA - 1000 mg/kg b.wt (G7, day 2) on **Convulsive Domain** (tremor and clonic convulsion**).**
Key : Day 1 = 3-4 hours after treatment ; Day 2 = 24 hours after treatment

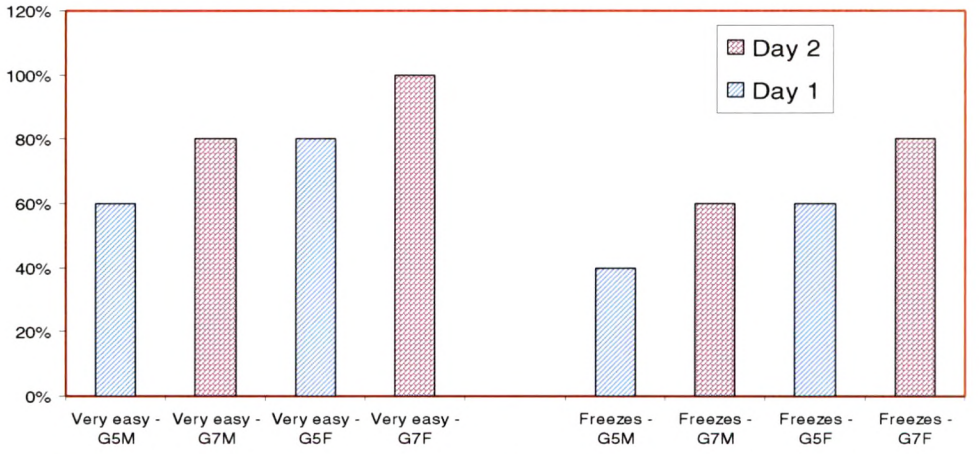

Figure 5. Percent of animals showing effects of CPF - 50mg/kg b.wt (G5, day 1) and CPF -50 mg/kg b.wt + LA -1000 mg/kg b.wt .(G7,day 2) on **ease of removal and handling reactivity.**

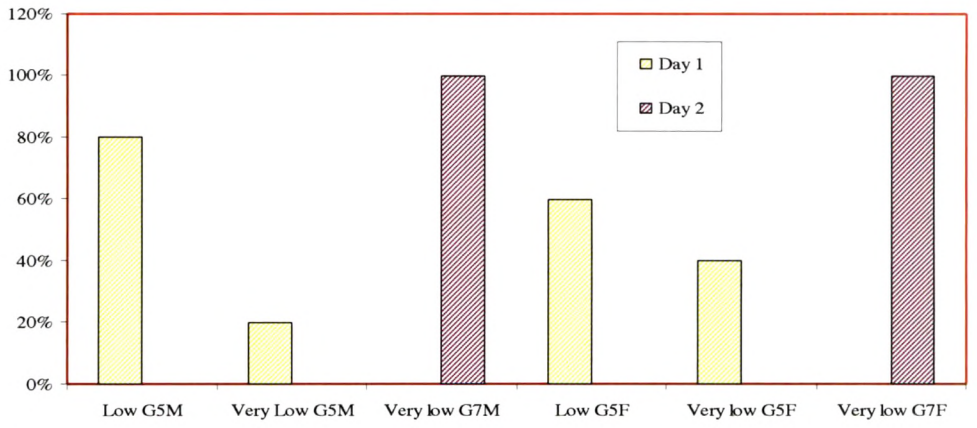

Figure 6. Percent of animals showing effects of CPF - 50 mg/kg b.wt (G5, day 1) and chlorpyrifos - 50 mg/kg b.wt + LA - 1000 mg/kg b.wt (G7) on **arousal level**.

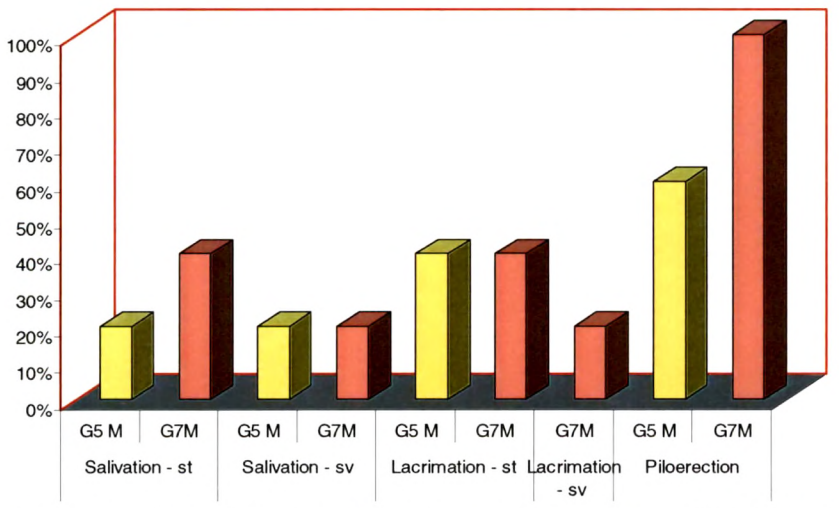

Figure 7A. Percent of animals showing effects on **autonomic measures** at CPF -50 mg/kg b.wt (G5M, Day 1) and CPF - 50 mg/kg b.wt + LA - 1000 mg/kg b.wt (G7M, Day 2).**Key** : st = slight ; sv = severe

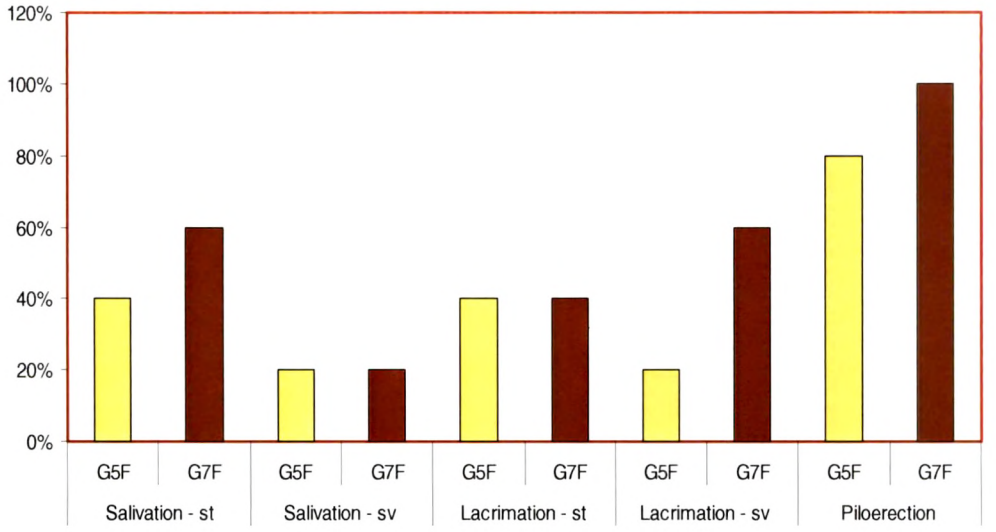

Figure 7B. Percent of animals showing effects on **autonomic measures** at CPF -50mg/kg b.wt (G5F, Day 1) and CPF - 50mg/kg b.wt + LA - 1000mg/kg b.wt (G7F, Day 2).

Key : st = slight ; sv = severe

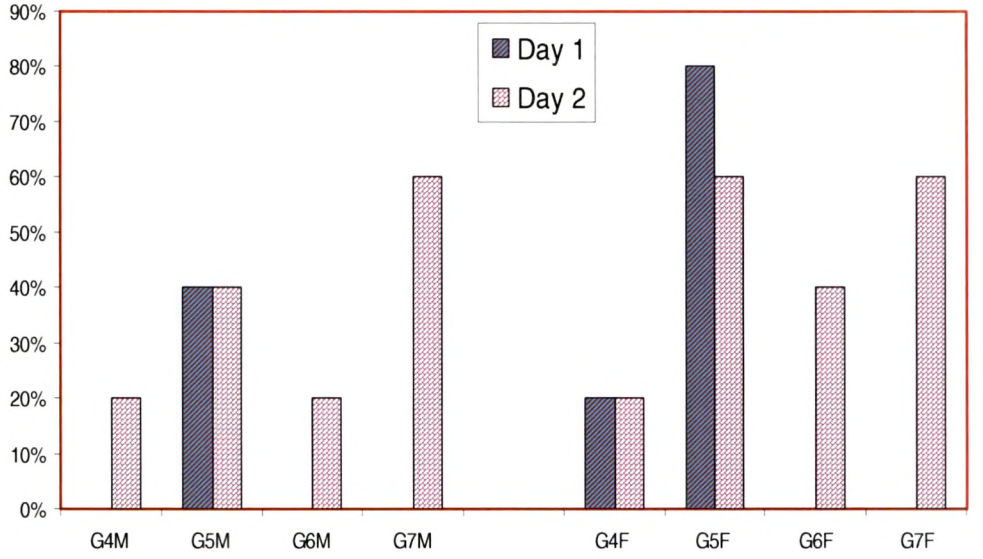

Figure 7C. Percent of animals showing effects on **pupil response** (absent response) in males and females of G4 (CPF- 5mg/kg b.wt + LA - 100 mg/kg b.wt), G5 (CPF -50 mg/kg b.wt) and CPF - 50 mg/kg b.wt + LA - 1000 mg/kg b.wt (G7) on days 1 and 2.

Key : Day 1 = 3-4 hours after treatment ; Day 2 = 24 hours after treatment

Table 3

Urination Count - Group Mean Values

Dose: G1- 0; G2 (CPF) - 5; G3 (LA) - 100; G4 (CPF+LA) - 5+100; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) - 50+1000 mg/kg body weight/day

Open Field Observations			Male					
Parameter : Urination count			Experimental Period					
Group N°	PE		Day 1		Day 2		Day 14	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1 (N=5)	2.6	1.9	2.0	1.4	2.4	1.5	3.6	2.1
G2 (N=5)	5.2	3.2	3.4	2.8	1.4	0.5	1.0	1.2
G3 (N=5)	3.0	1.2	4.2	2.0	2.4	1.1	1.4	2.0
G4 (N=5)	3.2	2.7	2.0	1.6	4	2.4	3.0	2.4
G5 (N=5)	3.8	2.0	1.2	1.0	1.8	1.7	5.0	4.0
G6 (N=5)	2.8	2.3	3.2	4.1	1.2	0.8	3.2	2.6
G7 (N=5)	4.0	3.7	0.8	1.3	0.0**	0.00	5.0	2.1

Open Field Observations			Female					
Parameter : Urination count			Experimental Period					
Group N°	PE		Day 1		Day 2		Day 14	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1 (N=5)	3.0	2.1	0.6	0.9	2.0	1.6	3.2	3.5
G2 (N=5)	4.8	6.2	2.2	3.3	3.4	4.5	1.6	1.1
G3 (N=5)	2.4	2.6	2.0	1.4	2.0	2.0	2.8	2.7
G4 (N=5)	2.6	2.4	1.8	2.5	2.6	3.8	1.6	0.8
G5 (N=5)	6.2	3.3	1.6	1.8	0.0**	0.0	1.0	1.2
G6 (N=5)	1.6	0.9	2.6	3.1	3.0	2.1	2.8	2.0
G7 (N=5)	3.4	1.9	2.2	2.5	0.8*	0.9	2.4	2.8

PE = Pre-exposure, N = Number of animals

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

Key : Day 1 = 3-4 hours after treatment ; Day 2 = 24 hours after treatment

Table 4

Defecation Count - Group Mean Values

Dose: G1- 0; G2 (CPF) - 5; G3 (LA) - 100; G4 (CPF+LA) - 5+100; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) - 50+1000 mg/kg body weight/day

Open Field Observations	Experimental Period							
Parameter : Defecation count	Male							
Group N°	PE		Day 1		Day 2		Day 14	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1 (N=5)	3.8	3.4	2.8	1.6	3.8	3.0	4.2	3.2
G2 (N=5)	3.4	2.3	1.2	1.3	4.8	3.0	4.0	2.5
G3 (N=5)	3.6	1.3	3.6	2.7	2.0	1.4	5.8	2.1
G4 (N=5)	3.0	1.6	3.0	1.5	4.0	3.5	3.6	3.0
G5 (N=5)	3.8	2.0	0.0**	0.0	2.4	1.8	5.2	1.3
G6 (N=5)	1.8	2.1	3.0	2.5	2.8	3.0	4.4	2.9
G7 (N=5)	5.4	1.1	2.0	2.3	0.0**	0.0	3.0	0.7

Open Field Observations	Experimental Period							
Parameter : Defecation count	Female							
Group N°	PE		Day 1		Day 2		Day 14	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1 (N=5)	1.4	1.3	3.2	2.4	4.6	2.7	3.0	2.2
G2 (N=5)	1.6	2.0	1.4	0.9	2.8	2.6	0.8	1.0
G3 (N=5)	4.6	2.4	1.2	0.4	3.4	2.3	3.2	0.4
G4 (N=5)	0.6	0.9	1.8	1.3	1.8	2.6	1.6	2.3
G5 (N=5)	6.2	3.3	0.0**	0.0	0.0**	0.0	3.2	2.3
G6 (N=5)	1.4	0.9	1.4	0.9	1.8	1.3	1.0	1.4
G7 (N=5)	1.8	2.0	1.2	0.4	0.8*	1.2	0.8	1.8

PE = Pre-exposure, N = Number of animals

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

Key : Day 1 = 3-4 hours after treatment ; Day 2 = 24 hours after treatment

Table 5

Grip Strength (g) - Group Mean Values

Dose: G1- 0; G2 (CPF) - 5; G3 (LA) - 100; G4 (CPF+LA) - 5+100; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) - 50+1000 mg/kg body weight/day

Male								
Period	1st day				2nd Day			
Group	Forelimb		Hind limb		Forelimb		Hind limb	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1 (N=5)	699.2	68.2	367.6	65.2	591.6	80.7	374.6	64.9
G2 (N=5)	678.0	66.9	383.8	67.7	666.6	113.8	408.6	56.9
G3 (N=5)	686.0	67.1	367.4	68.7	630.0	75.4	400.0	48.2
G4 (N=5)	671.2	38.2	341.0	66.3	667.6	115.4	359.2	64.0
G5 (N=5)	650.0	77.5	258.4	11.5	583.2	45.8	301.8	15.1
G6 (N=5)	714.0	47.3	352.0	62.6	665.4	79.8	383.6	56.5
G7 (N=5)	721.2	40.9	365.0	83.3	453.8*	66.7	172.6**	16.5

Female								
Period	1st day				2nd day			
Group	Forelimb		Hind limb		Forelimb		Hind limb	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
G1 (N=5)	649.2	40.1	445.4	114.8	686.4	114.6	399.0	126.5
G2 (N=5)	661.0	113.4	385.0	97.5	686.2	105.8	352.4	104.6
G3 (N=5)	644.8	61.9	389.6	57.9	673.6	68.3	384.8	39.4
G4 (N=5)	593.6	70.4	337.2	64.2	635.6	117.7	341.0	44.1
G5 (N=5)	485.6	41.3	289.0*	30.0	598.0	148.9	266.0	62.0
G6 (N=5)	671.6	53.3	372.2	64.9	664.0	89.7	327.6	54.3
G7 (N=5)	663.6	55.1	333.0	60.5	437.8*	23.9	186.8**	13.5

Key : N= Number of animals; *= significant at 5% level ($p \leq 0.05$);
** =significant at 1% level ($p \leq 0.01$)

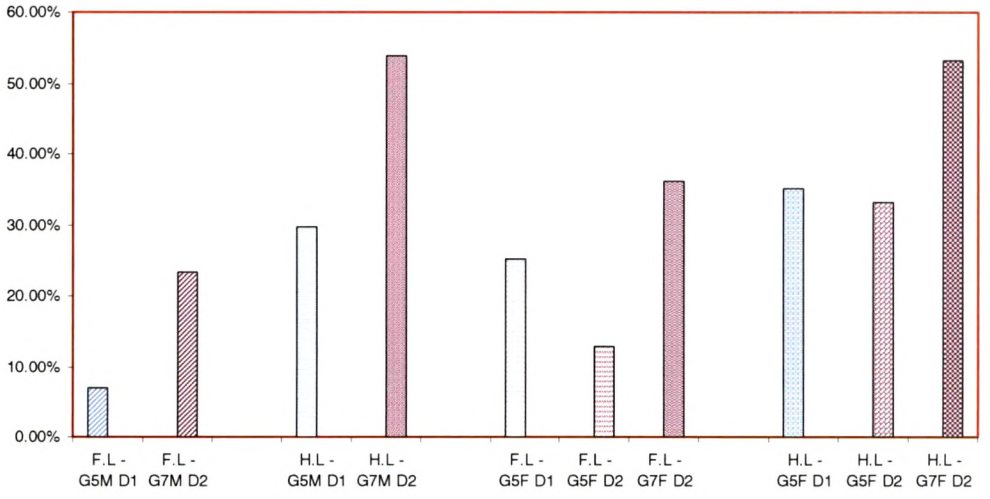

Figure 8. Percent decrease of **grip strength** of animals treated with chlorpyrifos - 50 mg/kg b.wt. (G5M) and chlorpyrifos - 50 mg/kg b.wt. plus lead acetate - 1000 mg/kg b.wt. (G7M).

FL- forelimb; HL- hind limb, D1- Day 1, D2- Day 2

Key : Day 1 = 3-4 hours after treatment ; Day 2 = 24 hours after treatment

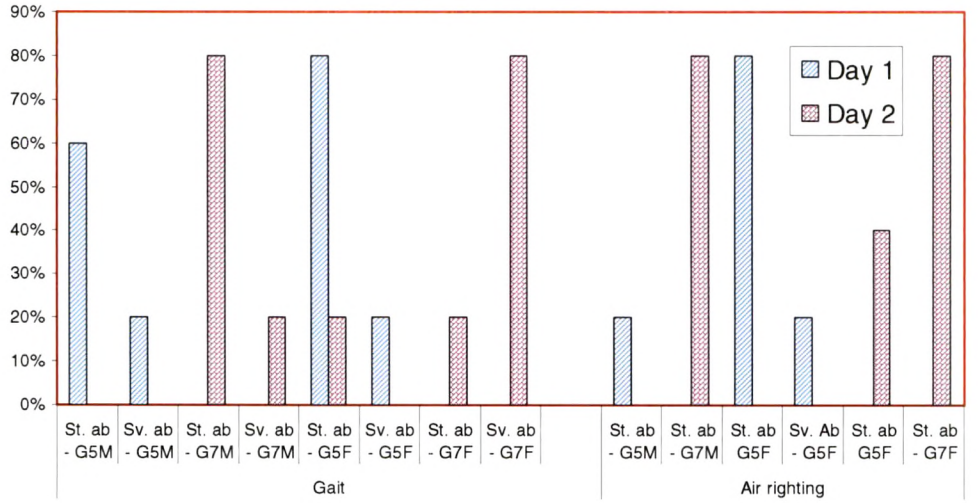

Figure 9. Percent of animals showing effects of CPF - 50mg/kg b.wt. (G5) and CPF -50 mg/kg b.wt. + LA -1000 mg/kg b.wt.(G7)on **gait and air righting reflex.**
St. ab = slightly abnormal; Sv. ab = severely abnormal

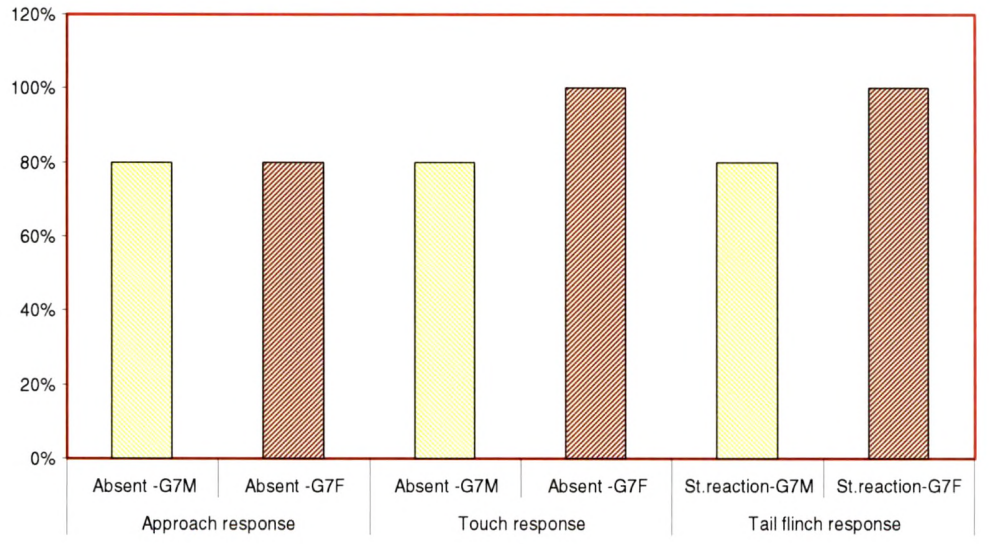

Figure 10. Percent of animals showing effects of CPF -50 mg/kg b.wt. + LA -1000 mg/kg b.wt. (G7, day 2) on **sensory motor domain.** St.- slight reaction
Key : Day 1 = 3-4 hours after treatment ; Day 2 = 24 hours after treatment

Table 6

Body Weight – Group Mean Values

Dose: G1- 0; G2 (CPF) - 5; G3 (LA) - 100; G4 (CPF+LA) - 5+100; G5 (CPF) - 50; G6 (LA) - 1000; G7 (CPF+LA) - 50+1000 mg/kg body weight/day

Sex : Male

Period/Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
@	Mean	122.4	122.2	125.2	123.6	121.8	124.0	123.0
	SD	9.29	10.71	11.69	8.26	9.63	9.51	8.19
2nd Day	Mean	133.4	131.0	130.0	132.8	118.8	129.6	108.0**
	SD	11.59	10.42	10.70	10.23	13.83	7.44	4.18
8th Day	Mean	166.4	163.6	171.0	170.2	154.8	168.2	147.2
	SD	14.59	16.24	9.27	15.19	26.17	10.13	3.42
14th Day	Mean	197.6	199.8	193.80	214.1	206.0	199.2	176.2
	SD	19.98	21.41	10.51	17.92	28.51	8.41	9.68

Sex : Female

Period/Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
@	Mean	115.4	116.4	116.0	118.0	116.0	111.2	115.6
	SD	5.64	9.45	11.11	9.08	7.62	8.87	10.01
2nd Day	Mean	122.2	127.8	122.8	127.2	110.2	117.6	103.0 *
	SD	8.64	8.84	14.75	10.16	12.89	10.74	5.92
8th Day	Mean	142.0	143.6	141.6	145.8	142.2	143.2	131.0
	SD	13.13	14.05	14.69	9.12	15.39	15.77	12.53
14th Day	Mean	158.6	157.2	159.2	160.6	157.8	157.6	148.2
	SD	13.05	15.27	13.26	8.32	15.88	14.79	12.81

Key : N= Number of animals; @ = Day of commencement of treatment

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

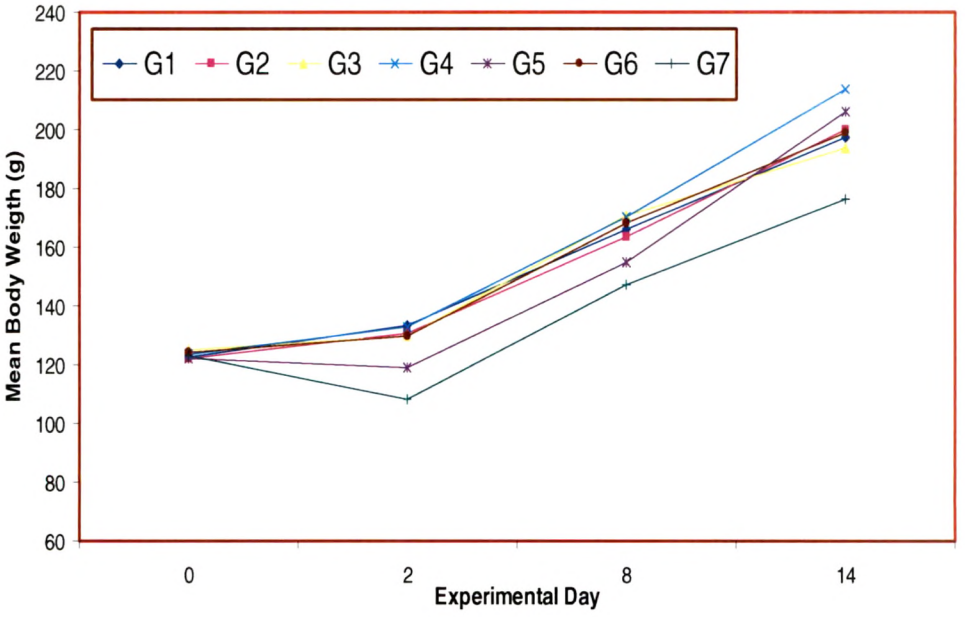

Figure 11 A. Growth Curve (Group Mean Body Weight) – **Males.** Key: 0 = experiment commencement day, 2nd Day = 24 hours after treatment.

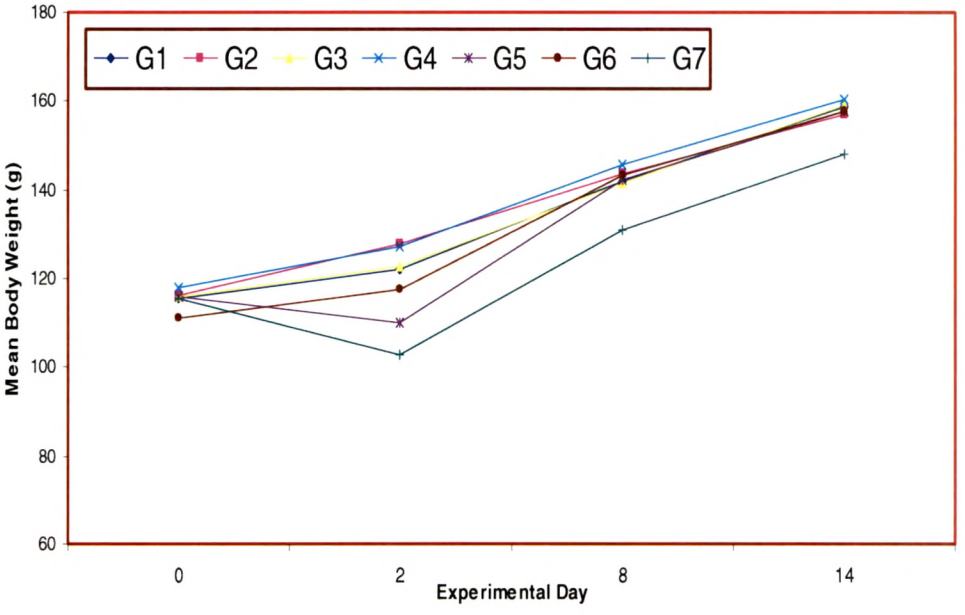

Figure 11 B. Mean Growth Curve (Group Mean Body Weight) – **Females.** Key: 0 = experiment commencement day, 2nd Day = 24 hours after treatment.

Table 7

Food Consumption (g/rat/week) – Group Mean Values

Dose: G1- 0; G2 (CPF) - 5; G3 (LA) - 100; G4 (CPF+LA) - 5+100; G5 (CPF) - 50; G6 (LA) – 1000; G7 (CPF+LA) - 50+1000 mg/kg body weight/day

Sex : Male

Period/Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
1st Week	Mean	135.7	132.0	142.3	136.0	131.0	132.7	111.7
	SD	8.08	14.42	5.51	11.53	19.97	5.51	17.90
2nd Week	Mean	119.0	117.5	128.0	124.0	117.5	123.0	104.5
	SD	2.83	3.54	5.66	16.97	16.26	1.41	6.36

Sex : Female

Period/Group		G1 (N=5)	G2 (N=5)	G3 (N=5)	G4 (N=5)	G5 (N=5)	G6 (N=5)	G7 (N=5)
1st Week	Mean	96.7	95.0	97.0	114.3	106.3	112.7	87.3
	SD	17.90	17.32	14.93	15.95	11.02	8.02	3.06
2nd Week	Mean	90.0	90.5	94.0	90.5	91.5	94.0	90.5
	SD	2.83	0.71	7.07	3.54	3.54	4.24	2.12

Key : N= Number of animals

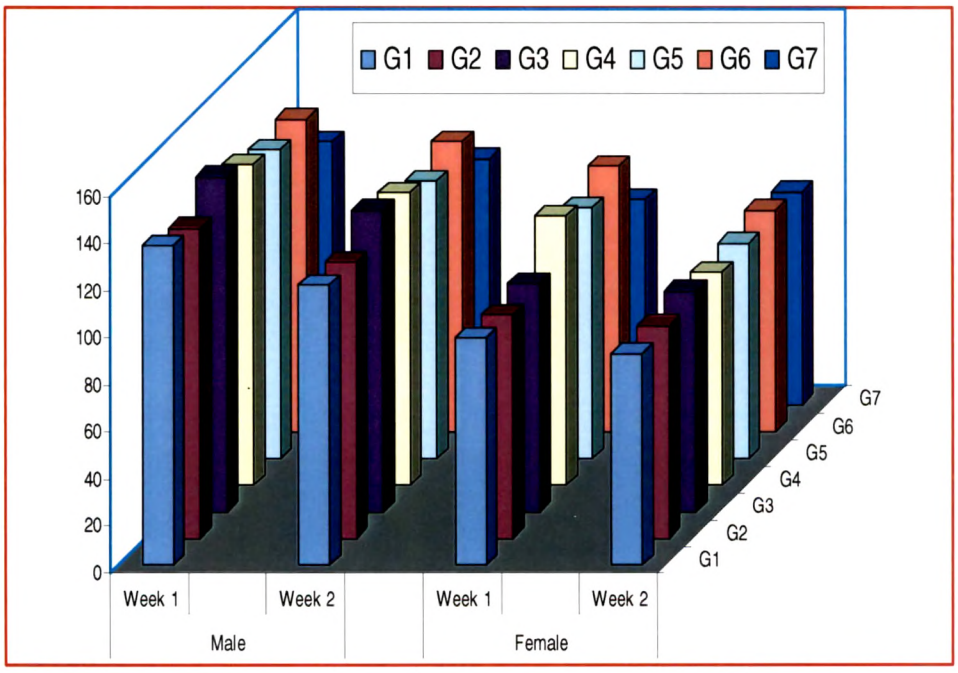

Figure 12. Weekly mean food consumption (g/rat/week) – group mean comparison

DISCUSSION

Neurobehavioral studies performed to investigate interactive effects of chlorpyrifos and lead acetate combination after single dose exposure via oral gavaging in Wistar rats have shown cholinergic over stimulation predominantly on day 1 in group 5 and on day 2 in group 7 animals. Animals treated with either dose of lead acetate revealed no predominant neurobehavioral effects except for a decrease in motor activity on day 1 in the higher dose group. The behavioral effects due to cholinergic over stimulation were more severe in group 7 (chlorpyrifos -50 mg/kg body weight + lead acetate-1000 mg/kg body weight) animals on day 2, compared to effects in group 5 (chlorpyrifos -50 mg/kg body weight) animals on day 1. The overall results indicate that acute toxicity of chlorpyrifos is more hazardous when combined with lead acetate rather than per se and too strangely on day 2, as against its immediate effects 2-3 hours post-dosing.

The clinical signs exhibited by groups 5 and 7 animals are essentially due to the inhibition of cholinesterase by chlorpyrifos. The clinical signs associated with cholinergic toxicity viz., tremor, motor in-coordination, gait changes, perennial soiling, lacrimation, salivation and piloerection were commonly observed in both sexes of groups 5 and 7 animals (Figures 1A - 1F). In group 5, clinical signs were predominant on day 1 and waned over the next 2 days. However, in group 7, the clinical signs were predominant on day 2 and some animals, particularly females, showed persistent symptoms up to fourth day. The severity and incidences of symptoms were also high in group 7 as compared to group 5. Lammers and Kulig (1997) have reported the time of peak effects for acute studies with chlorpyrifos to be 2 hours. The group 5 animals exhibited predominant symptoms 2 to 3 hours post dosing and, the

time of peak effects observed in group 5 animals is comparable to the finding of Lammers and Kulig (1997).

During clinical signs/behavioral observations, two males belonging to group 7 exhibited significant reduction in temperature to be felt too cold to touch (Figure 1D). To evaluate the behavioral and autonomic effects of long-term ChE inhibition, Gordon (1994) monitored core temperature, heart rate and motor activity in Long-Evans rats by using radiotelemetry transmitters. Subcutaneous injection of a sublethal dose of chlorpyrifos at 280 mg/kg following a single injection through peanut oil led to a significant reduction in core temperature during the first night after treatment. In the present study, though temperature measurement was not done, a reduction in temperature could be observed in two animals by hand touch during clinical observations. As the hand held observation was performed once a day, detection of drastic reduction in temperature might have been missed/unnoticed if that would have occurred at a different time interval. Piloerection was observed in most of the group 5 and group 7 animals (Figure 1D) which is an indication of hypothermic response. However, extreme reduction in temperature felt in two of the animals could also be due to severe cholinesterase inhibition. And some of the cholinergic symptoms especially, tremor and gait changes were severe in some animals. Gordon and Fogelson (1993) have shown wide ranging variation in individual motor activity and colonic temperature responses when the inhibition in ChE activity exceeded threshold levels. This may be indicative of marked genetic variability with regard to ChE inhibition. Chromodacryorrhoea, chromorhinorrhoea and exophthalmos (Figure 1F) were observed only in females and the severity and incidences of symptoms were also more in

females as compared to males. Obviously a sex related differential response need to be studied further.

Neurobehavioral Observations

Neurobehavioral observations performed in home cage, during handling and during open field, showed behavioral abnormalities related to cholinergic over stimulation. Like clinical signs, predominant behavioral changes were also observed in group 5 and group 7 animals on days 1 and 2 respectively.

On day 1, no statistically significant differences were observed in motor activity of treatment group males as compared to control group males. However, reduction in motor activity was observed in group 5 animals (Table 2A and Figure 3A). Motor activity analysis of females indicated reduction in activity levels in groups 5, 6 and 7 animals compared to control group animals on day 1(Table 2C and Figure 3C). At first interval, motor activity of male and female animals belonging to group 7 was significantly decreased on day 2. Rearing, the vertical movements in open field was also decreased in group 5 females on day 1 and, on day 2 in group 7 females (Table 1 and Figure 2). The measurement of locomotor functions, clearly indicate that the effects are more prominent in females. Measurements for muscular strength have also given results which can be correlated with motor functions.

With regard to sex specificity, the effects were comparatively more evident in females as compared to males. Some of the cholinergic over stimulation signs i.e., chromodacryorrhoea, chromorhinorrhoea and exophthalmos were observed only in females. Though the rates of both activation and detoxification of chlorpyrifos by hepatic microsomes have been shown to be greater in males than in females, the need for evaluating other processes like hepatic and extra hepatic activation, binding and detoxification of CPF

and CPF-oxon associated with detoxifying routes have also been suggested (Mattsson *et al.*, 1996). Differences in the ratio of activation to detoxification are related with chemical species, gender and, age dependent sensitivity to organophosphates (Timchalk, 2001). It is well known that females are more susceptible to chlorpyrifos than males though the exact basis for the same is not clear.

The cholinergic effects observed in group 5 animals are very much comparable to the effects observed by Mattsson *et al.* (1996) at 100mg/kg body weight dose level of chlorpyrifos in 9 weeks old F344 rats. This difference in the response to dosage could be attributed to the difference in the age of the animals. Behavioral and cholinergic inhibition can be seen in immature rodents at doses five fold lower than doses that are required for similar effects in adults (Claudio *et al.*, 2000). Young animals have minimal activity of the detoxification enzymes compared to adult animals. The age dependent sensitivity of young animals is associated with a decreased carboxylesterase (CaE) – mediated hydrolysis and CYP450 mediated dearylation capacity in young animals relative to adult animals (Timchalk, 2001).

Disappearance of clinical signs by day 5 and normal findings of FOB tests on day 14 reveal that the effects are reversible. Group 7 animals did not land properly onto sheets during hind limb foot splay measurements on day 2. Though there was severe muscular dysfunction in group 7 animals on day 2, remarkably, there was complete recovery by day 14.

The severity and incidences of effects are greater in group 7 (chlorpyrifos -50 mg/kg body weight + lead acetate -1000 mg/kg body weight) animals as compared to the effects in group 5 (chlorpyrifos -50 mg/kg body weight)

animals on day 2. The exact mechanism involved in the more pronounced and delayed manifestation of effects on day 2 is not known. The organophosphorus chemicals form chelating complexes with metals (Pesticide Manual, 1997). The formation of a chelating complex by chlorpyrifos with metals might be involved in delayed absorption, activation and detoxification processes. The toxicity and inhibition of cholinesterase by chlorpyrifos depend mainly on its major metabolite chlorpyrifos oxon. The local activation of CPF to CPF oxon may also be involved. Most activation to oxon occurs in liver; the oxon is also effectively detoxified in the liver and some amount of oxon also enters into the general circulation. In addition, binding of CPF to some plasma proteins limits the clearance of the parent CPF in the liver so that there are chances for some bound CPF to enter into the general circulation and extrahepatic activation, responsible for the clinical syndrome. With regard to lead, initially, lead is distributed in the plasma and soft tissues and, under steady state conditions 99% of the lead in blood is found in 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 and thereby severe cholinergic toxicity.

SUMMARY

Chlorpyrifos, a well known organophosphorus insecticide and heavy metal lead, was evaluated for their simultaneous interactive effects on neurobehavioral parameters in Wistar rats after single dose exposure via oral gavaging. The study was comprised of functional observational 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-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). Clinical signs and behavioral abnormalities associated with cholinergic over stimulation, and motor activity changes were evident in group 5 and group 7 animals. The animals treated with chlorpyrifos at 50 mg/kg exhibited behavioral changes after 2-3 hours of oral gavaging and waned over 2 days. At 50 mg/kg chlorpyrifos + 1000 mg/kg lead acetate (Group 7) severe cholinergic signs were noticed after approximately 24 hours of exposure i.e., on second day and symptoms regressed over 4-5 days. Females were more affected than males. The incidences and severity of cholinergic behavioral changes were more pronounced in group 7 animals. In group 6 i.e., lead acetate at 1000 mg/kg body weight, apart from motor activity decrease no other behavioral changes were prominent. The occurrence predominant behavioral abnormalities at 50 mg/kg chlorpyrifos + 1000 mg/kg lead acetate (Group 7) on day 2 of exposure suggest delay in biotransformation of chlorpyrifos due to presence of lead. The severe cholinergic signs in group 7 animals as compared to group 5 animals revealed higher rate of extrahepatic activation of chlorpyrifos to chlorpyrifos oxon. Chlorpyrifos in presence of lead delays the

cholinergic effects which might be due to its chelating properties with metals and predominant behavioral changes suggest higher scope for local activation.