CHAPTER 2

MATERIAL AND METHODS

The details regarding the materials, experimental procedures and techniques followed during current investigation are described in this chapter under the following heads.

Test Substance:

The details of the test substance provided in the label are:

Product Name	:	**Polytrine C** 44 EC
Ingredients	:	Cypermethrin 4% + Profenofos 40% EC
Chemical name (IUPAC)	:	
1) Cypermethrin	:	(RS)-∝-cyano-3-phonoxybenzyl(1RS)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
2) Profenofos	:	(RS)-O-4-bromo-2-chlorophenyl O-ethyl S-propyl phosphorothioate
CAS No.	:	
1) Cypermethrin	:	52315-07-8
2) Profenofos	:	41198-08-7
Physical Appearance	:	Colorless liquid
Storage Conditions	:	Stored in original conditions as supplied by the manufacturer at room temperature
Manufactured by	:	Novertis India Limited, 14, J. Tata Road, Mumbai-400020
Date of Manufacture	:	July 2001
Date of Expiry	:	June 2003

EXPERIMENTAL PROCEDURE

Animal Husbandry

Healthy, young rats (*Rattus norvegicus*) of Wistar strain obtained from Breeding Facility, Jai Research Foundation, Valvada-396 108, Dist. Valsad, Gujarat, India were used for the experiments in the current investigation. Females were nulliparous and non-pregnant. All the animals were examined for good health at the time of receipt.

Rats were maintained in a controlled environment. The experimental room temperature and humidity were between 19 - 25 °C and 50 - 70 % respectively. In the experimental room 12 hours of artificial fluorescent lighting and 12 hours darkness was a maintained, light hour being 6.00 to 18.00. The experimental room was cleaned and mopped with disinfectant (Suprasol LC 5%) daily.

The animals were housed in solid floor polypropylene rat cages (size: 410 x 282 x 180 mm). Animals were housed individually. Each cage was fitted with a stainless steel top grill having provision for keeping food pellet and a polypropylene water bottle with stainless steel drinking nozzle. The bottom of the cage was layered with clean, sterile rice husk. The cages were kept on a 5 tier rack and their positions were rotated weekly.

The animals were provided *ad libitum* laboratory rat pellet food (Amrut Brand, manufactured by Pranav Agro Industries Ltd., Pune, India) and drinking water (filtered through Aquagaurd water filter system). Fresh food was supplied at least once a week and water bottles were refilled daily.

Rats were randomly allocated to different groups based on body weight stratification method using in-house developed validated computer software. At the commencement of treatment, the body weight variation among the animals was within 20 % of the mean body weight for each sex.

Individual animals in the experiment was identified with picric acid marking on the head region representing group number and on body coat representing animal

number and colored cage label showing study No, Group No & Sex, Study, Cage No. and Animal No.

Protocols for the present experiments were approved by IAEC (Institutional Animal Ethics Committee) according to CPCSEA, India (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Route and Mode of Test Substance Administration

The route of administration was oral by gavage. Animals were dosed once daily by gavage using a metal cannula attached to a Borosilicate hard glass syringe, which was graduated up to 5 ml. The rats from control group were administered distilled water (vehicle) only. Required quantity of test substance was weighed and dissolved in distilled water to attain the desired concentrations. Fresh solutions of test substance in distilled water were prepared just before dosing. The dose volume administered was calculated on the basis of a constant factor of 10 ml/kg body weight.

2.1 Phase I: Acute Oral Toxicity of Combination Insecticide in Rats

As an initial step to study the toxicity of Polytrin C, an acute oral toxicity (LD_{50}) study was conducted to determine the median lethal dose (LD_{50}) of the test substance following single oral dose to Wistar rats of either sex.

A dose range finding study was conducted to establish dose levels for main LD_{50} study. Five groups of Wistar rats each comprising 2 males and 2 females were dosed combination insecticide at dose levels of 0, 50, 100, 200 and 400 mg/kg body weight.

The dose volume was calculated based on a constant factor of 10 ml/kg body weight. The distilled water was used as a vehicle.

Dose Group (mg/kg b. wt)	No. of Rats Dosed		Mortality		Symptoms Observed
	Male	Female	Male	Female	
I (0)	2	2	0	0	No symptoms
II (50)	2	2	0	0	No symptoms
III (100)	2	2	0	0	Males found lethargic immediately after dosing
IV (200)	2	2	0	0	Revealed abnormal gait, ataxia, salivation, hyperesthesia
V (400)	2	2	2	0	Revealed abnormal gait, ataxia, salivation, hyperesthesia, tremors

Based on results of dose range finding study, following doses were selected for main LD_{50} study:

G1 - 0 mg/kg body weight

G2 - 325 mg/kg body weight

G3 - 400 mg/kg body weight

G4 - 460 mg/kg body weight

A total of 20 male and 20 female rats were obtained from the Animal Breeding Facility of Jai Research Foundation, Valvada-396 108, Gujarat, India and acclimatized to the experimental room conditions for 5 days. Animals were randomly distributed to four group *viz.*, G1, G2, G3 and G4 by body weight stratification. Each group comprised 5 male and 5 female rats. All the animals were deprived of food for 12 h before dosing. The rats were dosed at the dose level of 0 (G1), 325 (G2), 400 (G3) and 460 (G4) mg/kg b. wt. Distilled water was used as vehicle. Dose volume was calculated based on a constant factor 10 ml/kg body weight.

All animals were observed at hourly interval during first 3 hours post dosing and once a day thereafter for visible clinical signs and mortality. All the animals were weighed on the day of dosing and on day 7 and 14 post dosing. On day 15 all the surviving animals were sacrificed and subjected to gross necropsy.

LD_{50} was calculated by Probit analysis using Finny's method **(Gad and Weil, 1994; Campbell, 1974).**

2.2 Phase II: Acute Neurotoxicity Screening Battery Study of Combination Insecticide in Rats

Neurotoxicity is considered to be an adverse effect on either the structure and/or function(s) of the nervous system (either reversible or irreversible) following acute or chronic exposure to chemicals **(Moser, 1996)**.

Organophosphorous compounds are very well established to cause neurotoxicity to organisms through inhibition of acetyl cholinesterase and/or neurotoxic esterase (NTE) **(De Bleecker, 1993).** Whereas, pyrethroids are also known as neuropoisons acting on the axons in the peripheral and central nervous system by interacting with sodium channels in mammals **(Bloomquist, 1993)**.

Extensive database is available regarding the neurotoxic, developmental and reproductive toxic potential of many organophosphorous and synthetic pyrethroids individually, whereas, very little or no information is available about combination of organophosphorous and synthetic pyrethroids. Before the assessment sub-chronic neurotoxicity, it is necessary to screen the neurotoxic potential of combination insecticide following sub-lethal acute doses. Hence, neurotoxicity screening battery study was undertaken.

Neurotoxicity screening battery study consists of a functional observational battery (FOB), motor activity and neuropathology. Functional Observational Battery (FOB) is a type of neurological examination in which a wide range of neurological functions is assessed including measures of sensory, motor and autonomic functions. The functional observational battery consists of non-invasive procedures designed to detect gross functional deficits in rats **(Moser, 1996)**.

The neuropathological techniques are designed to provide data to detect and characterize histopathological changes in central nervous system and peripheral nervous system. Functional observational battery tests are made during following times:

- In home cage
- While hand held
- In open field
- During manipulative tests

Range Finding Study to Determine Bench Mark Dose (BMD):

A range finding study was carried out to determine the Bench Mark Dose (BMD), which is defined by EPA as the highest dose non-lethal dose, which shows some neurotoxic or other toxic effects. Time of peak effect was also determined in the range finding study.

Three rats per sex per dose were given combination insecticide dissolved in distilled water at dose levels of 28, 42, 63, 95, 142, 213 and 320 mg/kg b. wt. Mortalities occurred at 320 mg/kg body weight. Time of maximal effects of clinical signs was about 4 - 5 hr after dosing. Based on the results of this study, doses of 0, 25, 75 and 225 mg/kg b. wt. were chosen for the single dose neurotoxicity study.

Main Study:

Five rats per sex per dose were stratified by weight and then randomly assigned to a dose group using a computer program. The numerous observations in a short time period made it necessary to stagger- start the study over 2 days. Therefore, males and females were treated on two separate days. Five rats per sex per dose group were used for the FOB and motor activity assays pre exposure, on day of treatment (day 1), and on days 7 and 14. After 24 hr of treatment, serum cholinesterase was assayed for all the animals. At the end of the study neuropathology of central and peripheral nervous tissues (perfusion fixed) was conducted on all the rats.

Animals and Husbandry:

Male and female Wistar rats of 5 week old were obtained from Breeding Facility of Jai Research Foundation, Valvada-396 108, Guajarat, India. Animals were allowed to acclimatize to the experimental conditions for a period of 5

days. The animals were housed individually in polypropylene rat cages. A 12 h light/dark cycle was maintained in the room, and food and water were available *ad libitum*.

In-life Observations:

All the animals were observed for overt toxicity, morbidity, and mortality at least twice a day. Body weights were taken on days 1, 7 and 14. FOB and motor activity testing was performed as per the methods described by **Moser *et al.*, (1988)** and the intervals were day -1(Pre exposure), on the day of dosing (day1) at the time of peak clinical effect (approximately 4-5 h), and then on days 7 and 14 of the study period.

Functional observational battery was conducted during following times:

- In home cage
- While hand held
- In open field
- During manipulative tests

Home Cage Observations

In home cage, the rats were observed for posture and presence or absence convulsions if any.

Posture

The posture of the animal was observed in 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
- Rearing

- Vertical jumping
- Writhing (to twist, roll or turn about) or
- Circling

Convulsions

In the home cage, animals were also observed for presence or absence of convulsions such as clonic or tonic movements.

Observations during Handling

Followed by home cage observations, the animals were picked up and observed for the ease of removal from the home cage, handling reactivity of the animal, skin abnormalities, eye abnormalities, and autonomic signs such as palpebral closure, lacrimation, salivation and piloerection.

Ease of Removal from Home Cage

The reactivity of the animal 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, freezes, or difficult.

Palpebral Closure

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

Lacrimation

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

Eye Examination

Eyes were examined for presence or absence of exophthalmos, microphthalmos, opacity, cataract, chemosis, conjunctivitis, 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 was considered as piloerection. Presence or absence of piloerection was recorded.

Skin Examination

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

Salivation

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

Open Field Observation

For open field observations, rats placed (one at a time) in an open arena (size: 495x 495 x 203 mm), a flat surface placed with a clean absorbent paper and observed for 3 minutes. Absorbent paper was replaced for each animal. During the 3 minutes period the following observations were made and recorded:

Gait

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

Mobility Score

A measure of ability of the animal to locomote despite of gait abnormalities was recorded. The ranking of the degree of impairment of locomotion was recorded as normal, slightly impaired or totally impaired.

Arousal Level

A ranking of the level of unprovoked activity and alertness in the open field was recorded during the 3 minutes observation period. Observations on arousal were ranked as very high, low, high or very high.

Vocalization

The actual numbers of spontaneous or unprovoked vocalization if any were recorded.

Rearing

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

Respiration

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

Clonic or Tonic Movements

In the open field each animal was observed for the presence of absence of clonic or tonic movements. The observations for clonic movements were recorded as chewing (clonus of the jaws), mild clonic tremors of limbs, and repetitive clonic tremors of the whole body or absent. Tonic movements were recorded as tonic (contraction of hind limbs), opistotonos-backward, emprosthtonos-forward or absent.

Urination and Defecation

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

Stereotype

Stereotype can be defined as pronounced repetition of the specific gestures or movements i.e., the presence of excessive or repetitive behaviour that appears to the observer. The observations were recorded as absent, circling or excessive grooming.

Bizarre Behaviour

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

Sensory Motor Measurements

After the 3 minutes observation period, while the animal was in the open field box, the following tests were conducted as per the procedures described by **Moser (1996)**. The animal was allowed to move freely in the open field box for these tests but may have been positioned in the box by the observer in order to administer stimulus.

After removal from the open field box, pupil response to light was observed. The animal was then tested for air righting reflex and hind limb foot splay.

Approach Response

The animal was approached at nose level with the end of a blunt object, which was held approximately 3 cm from the face of the animal for approximately 4 seconds to allow time for the animal to respond. The degree of the elicited response was recorded as no reaction, slight reaction, energetic or exaggerated.

Touch Response

Approaching the animal from the side the ramp of the animal was touched with a blunt object. The contact was brief (approximately 1 to 2 seconds) and deliberate but not forceful. The degree of the elicited response was recorded as no reaction, slight reaction, energetic or exaggerated.

Click Response

A clicker was positioned approximately 5 cm above the animal with care taken not to have the clicker in the animal's field of vision. The clicker was held in the palm of the hand to ensure consistency of the sound from test to test. The degree of the elicited response of the animal to the click sound was recorded as no reaction, slight reaction, energetic or exaggerated.

Tail-Pinch Response

The tail was squeezed approximately 2 - 3 cm from the tip using forceps. The degree of the elicited response was recorded as no reaction, slight reaction, flinch or exaggerated.

Pupil Response

The beam of a pocket-sized flashlight was brought from a lateral position medially 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, slight response or no constriction.

Air Righting Reflex

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

Landing Hind Limb Foot Splay

Landing foot splay was evaluated according to a procedure similar to that published by **Edwards and Parker (1997)**. The tarsal joint of each hind foot of each rat was marked with non-permanent 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 for each animal. The distance between two foot prints was measured and average of the three of the three foot splay values was calculated. A clean recording sheet was used for each animal.

Grip Performance

Hind limb and forelimb grip performance were tested using a grip strength meter (Columbus Instruments, Ohio, USA) as per the procedure described by **Mattson *et al.* (1986)**. The grip strength each animal was measured for 3 consecutive times; the results were averaged separately for the forelimbs and hind limbs.

Motor Activity:

The motor activity was measured using an automated animal activity measuring system (Columbus Instruments, Ohio, USA) equipped with a computer analyzer. Each animal was individually and no entry into the test room was allowed during the testing period. Rats were monitored for 3 consecutive ten minutes intervals allowing for examination of both exploratory and acclimatization activity levels. During this period total and

ambulatory activity of the rat was evaluated. Streotypic activity was calculated by subtracting ambulatory activity from total activity.

Cholinesterase Estimation:

Blood samples were collected from all animals of control and treatment groups after 24 of dosing. Serum was separated and cholinesterase activity was estimated (Hitachi-902).

Neuropathology:

At the end of the study period i.e. on 15th day all the rats were perfused *in-situ* with 10 % neutral formalin. Rats were examined for gross pathological alterations. Brain, spinal cord, sciatic nerve and skeletal muscle were collected and evaluated for histopathology.

2.3 Phase III: Repeated Dose 90-Days Neurotoxicity Study of Combination Insecticide in Rats

After obtaining initial information on neurotoxic propensity of the test material following single oral dose, a repeated oral dose 90-days neurotoxicity study was conducted. The study was conducting with reference to OECD guideline number 408.

Animals and Animal Husbandry

For this study a total of 60 male and 100 female Wistar rats were obtained from the Animal Breeding Facility of Jai Research Foundation, Valvada-396 108, Gujarat, India and acclimatized to the experimental conditions for a period of 5 days. Age of the animals at the start of treatment was 5-6 weeks. Stringent animal husbandry practices were followed.

Grouping

At the end of the acclimatization period animals were randomly allocated to six groups by using validated computer software. First four groups (*i.e.*, G1, G2, G3 and G4) comprised 10 males and 20 females each and last two groups (G5 and G6) comprised 10 animals per sex per group. At the start of commencement of treatment, the body weight variation among the animals was within (±) 20 % of the mean for each sex.

Route and Mode of Administration

All the animals were administered combination insecticide (Polytrin C) dissolved in distilled water by oral gavage at the dose levels of 6, 15 and 38 mg/kg body weight/day for low (G2), mid (G3) and high (G4) dose groups, respectively for 90 consecutive days. Control group (G1) animals were given vehicle (distilled water) only.

Experimental Outline

Group No.	Dose (mg/kg b. wt./day)	No. of Animals		Clinical Pathology at 13th Week		Clinical Pathology at the End of Recovery Period	
		Male	Female	Male	Female	Male	Female
G1 (Control)	0	10	20	10	10	-	-
G2 (low dose)	6	10	20	10	10	-	-
G3 (Mid dose)	15	10	20	10	10	-	-
G4 (High dose)	38	10	20	10	10	-	-
G5 (Control Recovery)	0	10	10	-	-	10	10
G6 (High dose Recovery)	38	10	10	-	-	10	10

All the animals in the experiment were dosed for a period of 90 consecutive days; after completion of 90 days of treatment clinical pathology estimations were performed on blood samples collected from 10 animals of either sex in main groups *i.e.,* G1 to G4. Animals in the recovery groups were observed for period of 28 without administration of test substance for recovery or delayed occurrence of clinical signs, if any. Further, clinical pathology estimations were performed on 29th day of recovery period. All the animals in the recovery group subjected to necropsy at the end of recovery period and specified organs were collected and histopathological examinations were performed.

OBSERVATIONS

Clinical Signs:

All the animals in the experiment were observed daily for visible signs and symptoms such as skin and fur changes, eye and mucous membrane changes, respiratory, circulatory, autonomic and central nervous system, somatomotor activity, behaviour pattern and general changes.

Ophthalmoscopy

A complete ophthalmologic examination was performed on each rat using an ophthalmoscope once before commencement of treatment and at the end of 90 days treatment.

Neurobehavioural Observations (NBO):

Neurobehavioural observations were performed to assess the neurobehavioural and neurological status of each animal once a week during 90 days of treatment period. The details of the observations were explained in the section 2.2.

Functional Observational Battery (FOB) Tests:

During 12^{th} week of experiment, functional observational battery tests (FOB) including neurobehaviuoral observations, sensory motor measurements, hind limb foot splay, grip strength and motor activity measurements were performed on 5 male and 5 female rats from each group. The detailed method followed for performing FOB was as explained in phase-II.

Body Weight:

Individual body weight was recorded for all animals on the day of commencement of exposure and at weekly intervals thereafter.

Food Consumption:

During the experiment, feed input and leftover was recorded once a week. Food consumption was calculated on a weekly basis.

Clinical Pathology:

Clinical pathology (haematology and clinical chemistry) estimations were made on blood samples of 10 male and 10 female rats from main groups *i.e.*, G1, G2, G3 and G4 on 91st and 92nd day of experiment respectively. Clinical pathology estimations for recovery group animals done at the end of 28 day recovery period.

Animals were fasted overnight (water allowed) and blood samples were collected by orbital sinus plexus puncture using a fine capillary tube under light ether anesthesia **(Riley, 1960)**.

For determination of clotting time, blood allowed flowing into a 7.5 cm capillary tube and the time required for clotting was recorded manually. Approximately 1 ml blood was collected in vials containing EDTA for hematology analysis.

One drop of blood was taken on a clean glass slide, spread and stained with Leishman's stain for differential leucocytes count. The differential leucocytes count was performed manually by counting a total of 100 leucocytes for each animal **(Jain, 1986)**.

Two to three ml of blood was collected from each animal in clean centrifuge tubes for serum separation. The blood was allowed to clot at room temperature and serum was separated by centrifugation at low speed, Separated serum was used for clinical chemistry analysis.

Clinical Pathology Parameters studied are given below:

Hematology

Parameter	Sample Type	Instrument Used
White blood corpuscles (WBC)	Whole blood	Sysmex K 1000
Red blood corpuscles (RBC)	Whole blood	Sysmex K 1000
Hemoglobin (HGB)	Whole blood	Sysmex K 1000
Haematocrit (HCT)	Whole blood	Sysmex K 1000
Mean corpuscular volume (MCV)	Whole blood	Sysmex K 1000
Mean corpuscular hemoglobin (MCH)	Whole blood	Sysmex K 1000
Mean corpuscular hemoglobin concentration (MCHC)	Whole blood	Sysmex K 1000
Platelet count (PLT)	Whole blood	Sysmex K 1000
Clotting time	Whole blood	Manual
Differential leucocytes count (DLC)	Whole blood	Light Microscope

Clinical Chemistry

Parameter	Sample Type	Instrument Used
Albumin (ALB)	Serum	Hitachi - 902
Aspartate aminotransferase (AST)	Serum	Hitachi - 902
Alanine aminotransferase (ALT)	Serum	Hitachi - 902
Gamma glutamyl transferase (GGT)	Serum	Hitachi - 902
Alkaline phopshatase (ALP)	Serum	Hitachi - 902
Cholinestearse (ChE)	Serum	Hitachi - 902
Creatinine	Serum	Hitachi - 902
Total Bilirubin	Serum	Hitachi - 902
Total Protein	Serum	Hitachi - 902
Urea and Blood Urea Nitrogen (BUN)	Serum	Hitachi - 902
Calcium (Ca)	Serum	Hitachi - 902
Phosphorus (P)	Serum	Hitachi - 902
Chloride (Cl)	Serum	ERBACHEM -5 PLUS
Sodium (Na)	Serum	Flame photometer
Potassium (K)	Serum	Flame photometer
Glucose (Glu)	Serum	Hitachi - 902
Cholesterol (Chol)	Serum	Hitachi - 902

Pathology

All the main group animals were allowed to cohabitate at 1:2 ratio following blood collection on 91st day. Whereas, recovery group animals were kept under observation for 28 days without any treatment and on 29th day all animals were euthanased by carbon dioxide (CO_2) asphyxiation and subjected to a complete necropsy subjected to gross necropsy. The thoracic, abdominal and cranial cavities were then cut opened and thorough examination of the organs was carried out to detect changes or abnormalities, if any. Absolute weights of adrenals, gonads, epididymides, kidneys, brain, heart, spleen, liver, lungs and thymus were recorded immediately after dissection and relative weights were calculated later.

2.4 Phase IV: Developmental Neurotoxicity Study of Combination Insecticide in Rats

Following 91 days of treatment males and females from respective treatment and control groups were kept for cohabitation in the ratio of 1:2 (male: females). Everyday early in the morning vaginal smear from each female was observed for the presence of sperm and stages of estrous cycle as per the method specified by **Hafez (1970)**. The mated females were kept individually and allowed to litter.

Observations during Gestation

The day on which sperm was observed in the smear was considered as 0 day of gestation. Females with sperm positive vaginal smears were weighed on days 0, 7, 14 and 20th day of gestation. Food consumption for each dam was monitored on similar days as body weight. If any male was found unable to impregnate females in the cage during one week, another proven male was used to cohabitate with the females. A maximum of 3 males were used to cohabitate with a female during 3 weeks. If female is unable conceive even after changing 3 males, that female was sacrificed.

Lactation/Litter Observations

The day of delivery of pups was considered as 0 day of lactation. Dams and its pups were weighed individually on days 0, 4, 7, 14 and 21 and food consumption was monitored at similar intervals as body weight. In case of mortality in any litter/litters, sex and body weight of the individual pups/pups were recorded. Dams, which were not littered after 25 days of gestation, were subjected to necropsy.

On 20th day of lactation one male and one female pup from each dam were randomly selected and subjected to complete functional observational battery (FOB) tests. On day 21 of lactation all the dams and pups were euthanized by CO_2 asphyxiation and subjected to complete gross pathology.

Organ Weights

For parents absolute weights of adrenals, ovaries, uterus, kidneys, brain, heart, spleen, liver, lung and thymus were recorded immediately after dissection from all the dams. Relative weights were calculated later.

One male and one female pup from each dam were selected and organs *viz.*, brain, kidneys, spleen, liver and thymus were collected.

Organs were dissected out, cleared off from the adhering fat and blotted free blood as per the technique specified by **Paine (1995)**.

Pathology

All animals were euthanased by overdose of carbon dioxide (CO_2) asphyxiation and subjected to a complete necropsy subjected to gross necropsy.

Histopathology

The method described by **Godkar (1994)**, which is a standard technique of haemotoxylin and eosin (H & E) staining was followed for histology. All the vital organs including reproductive organs were collected from all the animals and fixed in 10 % neutral buffered formalin at room temperature for 18 h, after which tissues were transferred to fresh formaldehyde (10%). The tissues were then dehydrated by passing through descending grades of isopropyl alcohol series, cleared in xylem, embedded in paraffin wax (58 to 60°C melting point) and transverse sections cut at 4 - 5 micron in a rotary microtome (Leica). These sections were stained in hemotoxylin and eosin (H & E) stain, dehydrated, cleared in xylene and mounted in DPX for microscopy and photography. Brain from all the high dose group and control group was evaluated microscopically.

Evaluation of Data

Parameters listed below were calculated using the formulae as described by **Clegg *et al.*, (2001).**

$$\text{Fertility index} = \frac{\text{N° of Cohabitated females becoming pregnant}}{\text{N° of non-pregnant couples cohabitated}} \times 100$$

Note: Because both sexes are exposed to test article, distinction between sexes often is not possible.

$$\text{Mating Index} = \frac{\text{N° of males or females mating}}{\text{N° of males or females cohabitated}} \times 100$$

Note: Mating is used to indicate that evidence of copulation (vaginal plug or sperm in vaginal smear) was obtained.

$$\text{Gestation index} = \frac{\text{N° of females delivering live young}}{\text{N° of females with evidence of pregnancy}} \times 100$$

$$\text{Weaning index/ Lactation Index} = \frac{\text{N° of live offspring at day 21}}{\text{N° of offspring delivered}} \times 100$$

$$\text{Live birth index} = \frac{\text{N° of live offspring}}{\text{Total N° of pups born}} \times 100$$

$$\text{Sex Ratio} = \frac{\text{N° of male offspring}}{\text{N° of female offspring}} \times 100$$

$$\text{4 Day Survival index} = \frac{\text{N° of live offspring at lactation day 4}}{\text{N° of live offspring delivered}} \times 100$$

$$\text{7 Day Survival index} = \frac{\text{N° of live offspring at lactation day 7}}{\text{N° of live offspring delivered}} \times 100$$

$$\text{14 Day Survival index} = \frac{\text{N° of live offspring at lactation day 14}}{\text{N° of live offspring delivered}} \times 100$$

$$\text{21 Day Survival index} = \frac{\text{N° of live offspring at lactation day 21}}{\text{N° of live offspring delivered}} \times 100$$

Duration gestation (days) = Date of littering - Date of day of "0" gestation

Statistical Analysis

Raw data were processed and analyzed to give group means and standard error with significance, wherever, the difference occurs between the controls and treated groups using validated in-house developed statistical software. All the parameters characterized by continuous data such as body weight, food consumption, grip strength, motor activity, organ weight, hematology and clinical chemistry data were subjected to relevant statistical method (*viz.*, Bartlett's test, ANOVA, Dunnett's test or Student's t-test) using GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego California USA **(Motulsky, 1999)**.

Significance was calculated at 5 % level and indicated in the summary tables as follows:

*↑ = Significantly higher than the control ($P \leq 0.05$)

*↓ = Significantly lower than the control ($P \leq 0.05$)