CHAPTER 2

REPEATED DOSE INHALATION TOXICITY: 14-DAY STUDY USING ACEPHATE FORMULATION

INTRODUCTION

The development of inhalation toxicology as a distinct discipline can be traced back to well over a century. Fraser and coworkers provided a brief review of this early period and a description of inhalation technology up to 1959 (Fraser *et al.*, 1959). Inhalation toxicology technology has experienced continuous development in the types of materials and designs used in constructing inhalation chambers. Excellent reviews of inhalation technology have been provided by Campbell (1976), MacFarland (1983), Phalen (1984) and Drew (1985).

Phalen (1976) has described five basic types of inhalation toxicology based on the mode of exposure namely whole body, head only, nose only, lung only and partial lung. However, one of the widely accepted modes of inhalation exposure is head/nose only since the advantages of using this method heavily overshadows it's reported drawbacks.

According to Phalen, head only and nose only inhalation exposure systems are similar in terms of their advantages and disadvantages (Phalen, 1976). The advantages include relative efficiency in usage of test material, reduction or elimination of multiple exposure routes, and containment of highly toxic materials. However, he cited labor intensive nature of head/nose only exposure systems and the stress related to the repeated restraint necessary for head/nose-only exposure studies as the major disadvantages. Stress has, until recently, been a major limiting factor in determining the duration of head/nose only exposure studies (Drew, 1985). Laper and Burgess (1981) have reported an increase in the acute inhalation toxicity related to restraint-induced stress. However, Smith *et al.*, (1980) have reported that long-term nose only exposures, up to 6 hr per day, were possible with little or no stress. They measured parameters such as body weight, rectal temperature, clinical pathology, and plasma corticosterone levels to indicate the lack of measurable stress in rats and hamsters

subjected to prolonged head/nose only inhalation exposure. Therefore, in the current study we followed head/nose only inhalation exposure.

Acephate though reported as one the safest organophosphate pesticides because of its reported relatively safe mammalian metabolic disposition (Roberts and Hutson, 1999), moderate to severe toxicity due to acephate was reported from mammalian studies including that in human (Bennett and Morimoto, 1982; Poovala *et al.*, 1998; Datta *et al.*, 2010). Moreover, Chuckwudebe *et al.* (1984) have reported that acephate can quickly get absorbed through lung. Since this is one of the routes through which man (especially the formulators and applicators of the pesticide) accidently contract the compound in question it was thought worth studying the repeated dose inhalation toxicity of commercial acephate formulation (*Lancer 97 DF)* in rats.

OBJECTIVE

The objective of this study was to investigate the sub-acute toxicity and No Observed Adverse Effect Level (NOAEL) of Acephate 97% DF due to repeated inhalation exposure for a period of 14 days (6 hours per day for 5 days a week).

JUSTIFICATION FOR SELECTION OF TEST SYSTEM

The rat was selected as a test system due to several reasons. It is a readily available laboratory rodent species. It has been historically shown to be a suitable model for repeated dose toxicity assessment. The inhalation exposure represents a possible route of exposure to human subjects. The results of the study are believed to be of value in predicting the toxicity of the Acephate 97% DF to human and higher mammals.

INSTRUMENTS AND EQUIPMENT

1. Sartorius Semi Micro Balance, BP 210 D (Range 0.01mg to 210g)
2. Electronic Weighing Scale - SMART (Range 5.0 g to 3.0 kg)
3. Air Compressor, Manufactured by Ingersoll-Rand, Model T 30/2475
4. Infusion Pump, Manufactured by B. Braun, Germany, Model Perfusor Compact
5. Inhalation Chamber, Fabricated by Smit Fabricators, Model Head/Nose Only.
6. Vacuum Pump, Manufactured by Technics Incorporation, Model TID-75-P

7. Temperature, Humidity, Oxygen and Carbon dioxide Indicator, Manufactured by Chemo Electronic
8. Seven Stage Cascade Impactor and Impactor Control Unit, Manufactured by In. Tox. USA, Model RC-FB.
9. Open Phase Sampler, Manufactured by Bio-tox Instrumentation, New Delhi.
10. AIMS^{TM} Tattoo Machine

SOLVENT AND CHEMICALS

Disinfectant	:	Dettol 2.5%
Sodium Hydroxide Pellet	:	Excella R Grade
Distilled Water	:	Millipore Elix 10 water purification System

ANIMALS

Thirty male and thirty female healthy Wistar rats *(Rattus norvegicus)*, obtained from the Wockhardt Research Centre, Aurangabad, India, were used for the study. Females were nulliparous and non-pregnant. The rats were examined for good health at the time of receipt and acclimatised to the controlled environmental conditions for a period of five days. At the start of the exposure the male rats weighed between 216- 289.2g, while the female rats weighed between 176.1- 221.9g; the rats were between 10 and 11 weeks old. Before the start of the study, rats were randomised and assigned to the required number of groups using in-house developed validated computer software.

ACCLIMATISATION

Rats were allowed to acclimatise to the experimental room conditions for a period of minimum five days prior to randomization. During the acclimatisation period, the rats were observed for abnormal behavior or clinical signs of if any. Rats were also acclimatised to the restrainer tubes for 4 hours for 5 days before of exposure.

ENVIRONMENTAL CONDITIONS

Rats were maintained in an environmentally controlled room. The experimental room temperature and humidity were recorded daily. The temperature range was 20 to 23 °C and the relative humidity level during the experimental period was 65 to 67%. A minimum of 15 air changes per hour was maintained in the experimental room. In the experimental room,

12 hours of artificial fluorescent lighting and 12 hours darkness were maintained, light hours being 0600 hrs to 1800 hrs. The experimental room was cleaned and mopped daily with a disinfectant (Dettol 2.5%).

HUSBANDRY PRACTICES

The rats were housed (2 to 3 rats/cage) in solid floor polypropylene rat cages (size: 410 mm x 282 mm x 180 mm). Each cage was fitted with a stainless steel top grill 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 six tier racks and their positions were rotated weekly. The cages and bedding material were changed on a daily basis.

FOOD AND WATER

The rats were provided with food and water *ad libitum*. The quality of food and water is regularly monitored at Jai Research Foundation. There were no known contaminants in the food and water at levels that would have interfered with the experimental results obtained.

Food: Teklad Certified Global High Fiber Rat/Mice feed manufactured by Harlan, U.S.A.

Water: UV sterilized water filtered through Kent Reverse Osmosis water filtration system.

GROUPING

The rats were randomly allocated to six groups, *viz.*, vehicle control (G I), low concentration (G II), intermediate concentration (G III), high concentration (G IV), control recovery (G V) and intermediate concentration recovery (G VI) using in-house developed validated computer software. Each group comprised of 5 male and 5 female rats.

ANIMAL IDENTIFICATION

After receiving in experimental room rats were marked with permanent ink marker prior to randomization and with unique numbers on the tail using a tattoo machine post randomization. Appropriate cage cards were attached to the cages indicating the experiment number, test chemical name, group number, sex, dose concentration, type of study, cage number and animal number.

DOSE SELECTION

Based on the results of acute toxicity study (Chapter 1), experiments for the repeated dose inhalation in Wistar rats were carried out at below dose concentrations:

Control (G I and G V)	-	0 mg/L air (Exposed to distilled water only)
Low concentration (G II)	-	0.238 mg/L air
Intermediate concentration (G III)	-	0.694 mg/L air
High concentration (G IV and G VI)	-	1.202 mg/L air

EXPERIMENTAL OUTLINE

Group No.	Group	Concentration (mg/L air)	Number of Rats (Males + Females)			
			Treated	Clinical Pathology		Histo-pathology
				Terminal	Recovery	
G I	Control	0	5 + 5	5 + 5	-	5 + 5
G II	Low concentration	0.238	5 + 5	5 + 5	-	-
G III	Intermediate concentration	0.694	5 + 5	5 + 5	-	-
G IV	High concentration	1.202	5 + 5	5 + 5	-	5 + 5
G V	Control Recovery High	0	5 + 5	-	5 + 5	-
G VI	Concentration Recovery	1.202	5 + 5	-	5 + 5	-

FREQUENCY OF EXPOSURE

The rats were exposed to the Acephate 97% DF aerosols for a period of 6 hour/day for 5 days/week.

ROUTE OF ADMINISTRATION

The route of administration in the present study was inhalation.

DURATION OF TREATMENT

Rats were exposed continuously for 6 hours per day and 5 days/week for a period of 14 days. The rats belonging to G I, II, III and IV were bled for haematology and biochemical parameters and were sacrificed immediately post completion of 14 day exposure. The

recovery group rats were maintained for further 14 days post completion of the 14 day exposure.

EXPERIMENTAL DESIGN

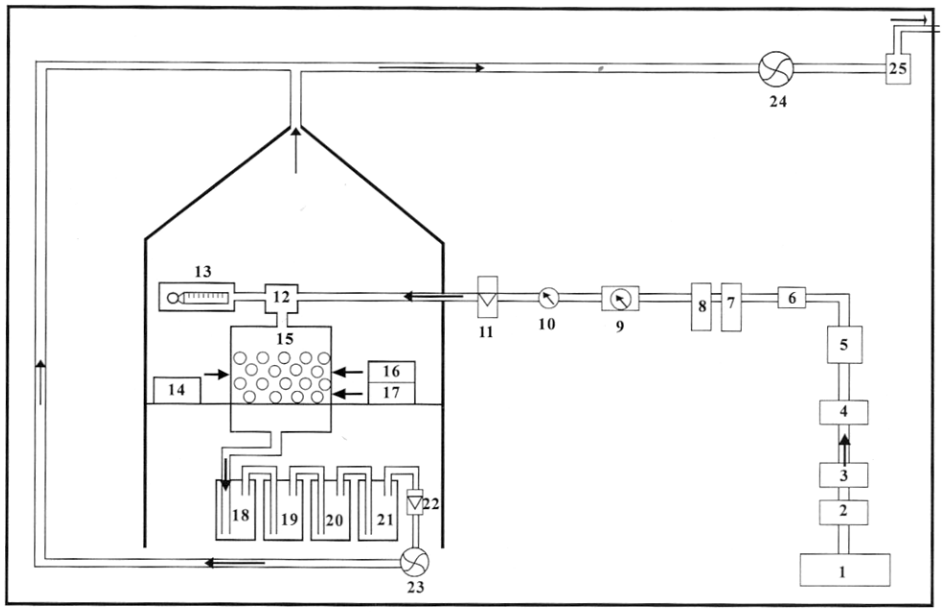
To study the repeated dose inhalation toxicity (14 days) of Acephate in rats, an experiment was carried out using inhalation equipment (head/nose only exposure) designed by Jai Research Foundation and fabricated by Smit Fabricators, Gujarat, India. The dynamic inhalation equipment consisted of an inhalation chamber, inhalation chamber cabinet, air compressor, flow meter, continuous infusion pump, spray atomiser, temperature monitor, humidity monitor, oxygen monitor, carbon dioxide monitor, rat exposure tubes, exhaust system and scrubber.

INHALATION EQUIPMENT

The inhalation equipment (nose only exposure) was designed to sustain a dynamic airflow of 12 to 15 air changes per hour and to ensure adequate oxygen content of 19%. A slightly negative pressure was maintained inside the chamber to prevent leakage of the test substance into the surrounding area.

DESCRIPTION OF INHALATION CHAMBER (Figure 2.1: Schematic layout of exposure system)

The dynamic inhalation chamber had 4 main parts namely inlet unit, two exposure chambers and an outlet unit. Each part was 30 cm in height and 30 cm in internal diameter. The total capacity of the chamber was 84.80 litres. The inlet unit (upper) was made up of a glass cylinder with facility for the attachment of a spray atomizer. The exposure chambers (middle) were made up of stainless steel with 20 portholes in each chamber, to accommodate 40 rat exposure tubes. Transparent rat exposure tubes were made up of polyacrylic material and provided with orifices to eliminate excreta and urine. The transparent rat exposure tubes were accommodated in the portholes of the inhalation chamber. The outlet unit (lower) was made up of stainless steel with an outlet provision connected to a vacuum pump. The outgoing air from the chamber was passed through the impingers containing 1.0% sodium hydroxide solution, and moisture traps containing silica gel (Figure 2.2).



Key: 1 = Air compressor, 2 = Air filter -1, 3 = Air filter -2, 4 = Air filter -3, 5 = Moisture separator, 6 = High flow filter, 7 = Charcoal filter, 8 = Silica gel filter, 9 = Air filter with regulator, 10 = Pressure gauge, 11 = Flow meter, 12 = Spray ato miser, 13 = Infusion pump, 14 = Manometer, 15 = Inhalation chamber, 16 = Particle size & gravimetric concentration analysis, 17 = Temperature, humidity and oxygen concentration, 18 = 1% NaOH, 19 = 1% NaOH, 20 = Moisture trap, 21 = Moisture trap, 22 = Flow meter, 23 = Vacuum pump, 24 = Blower, 25 = Scrubber

Figure 2.1: Schematic layout of exposure system

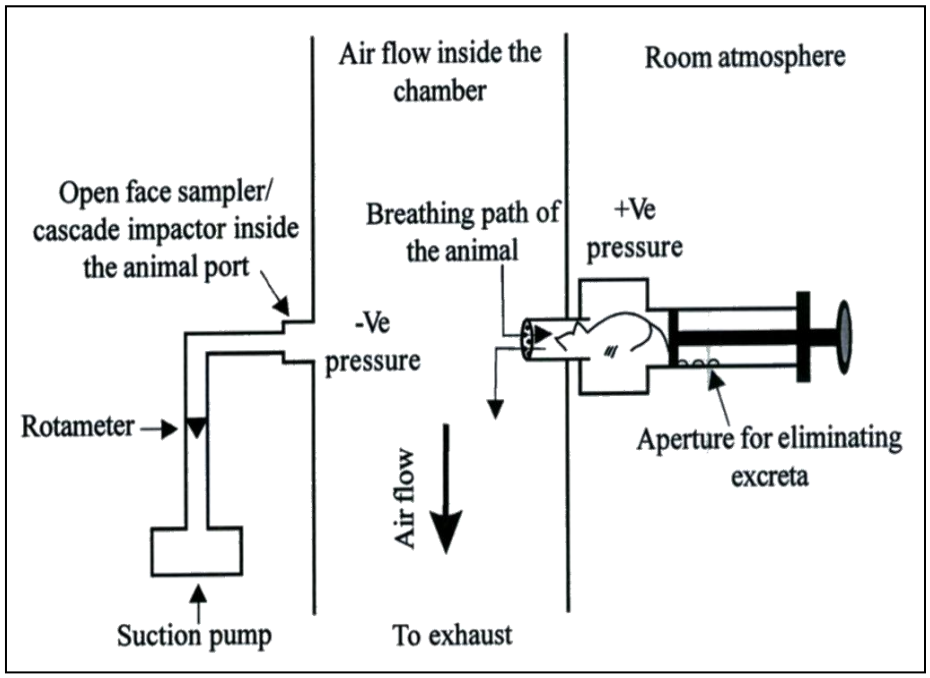


Figure 2.2: Schematic layout of exposure tube and sampling port

DESCRIPTION OF INHALATION CHAMBER CABINETS

The inhalation unit was housed in an inhalation chamber cabinet. The cabinet was elevated at 75 cm above the ground level. The dimension of the cabinet was 150 cm x 150 cm x 120 cm in length, breadth and height, respectively. The cabinet was fitted with a conical dome at the top, which was connected to the cabinet exhaust. Each cabinet had a panel attached at the side to mount the air filters, air regulators and the flow meters. The oxygen monitor and the temperature and humidity indicator were mounted on the front panel of the cabinet. A pressure manometer was also fitted on the front wall of the cabinet to monitor the negative pressure inside the chamber. The probe from the manometer was fixed inside the inhalation exposure chamber. The cabinet had glass window opening at the front and rear of the cabinet for clear view of the chamber.

DESCRIPTION OF EXHAUST SYSTEM

Exhaust of inhalation exposure chamber

The air coming from the chamber was passed through the 1% sodium hydroxide solution kept in the filtration flasks and then through the moisture traps. This was connected to individual vacuum pumps before entering into the cabinet exhaust line.

Cabinet exhaust

The cabinet was connected to an individual suction pump and the pump was connected to a booster blower. The air coming out of the exhaust system finally entered inside the scrubber.

Scrubber

A scrubber was used to neutralize the toxic aerosols coming out of the exhaust from the inhalation chamber. It was a cylindrical structure having a height of 305 cm and a diameter of 30.5 cm. The total volume of the scrubber was 222.725 litres. The scrubber was packed with Fiber Glass Reinforced Plastic (FRP) material. The gas that entered inside the scrubber moved upwards while the sprinkler sprinkled caustic lye solution from the top to neutralize the gas. The sprinkler was connected to ½ HP motor pump that drew caustic lye solution from a storage tank having a capacity of 500 litres.

PROCEDURE

For application in the fields, Acephate 97% DF is first diluted with water and sprayed on crops. Hence Acephate 97% DF was diluted with distilled water at 1:1 ratio to achieve the stock solution concentration at 500 mg/mL. Prepared stock solution was loaded into an infusion syringe, positioned on the continuous infusion syringe pump (manufactured by B. Braun Melsungen AG, Germany). The prepared solution of Acephate 97% DF was infused into the spray automizer, where an aerosol was formed and distributed into the inhalation chamber.

Rats from G II, G III and G IV were exposed to different nominal concentrations by changing the infusion rate of prepared solution of Acephate 97% DF and by keeping the air inflow and outflow constant for each group exposure. The infusion rates were 5, 10 and 15 mL/h for G II, III and IV, respectively and the infusion rate was 15 mL/h for G VI (recovery high dose group).

The above procedure was followed on all the exposure days to prepare test substance solution. The control and the control recovery group rats were exposed to air only. The nominal concentrations 2.083, 4.167 and 6.250 mg/L air, were determined for G II (low concentration), G III (intermediate concentration) and G IV (high concentration), respectively. The air inflow and outflow rate was maintained at 20 and 25 litres per minute respectively to maintain a negative pressure inside the chamber, and this was monitored throughout the exposure period.

The chamber temperature and the relative humidity recorded using the thermo-hygrometer was 20 to 22 °C and 31 to 91%, respectively. The oxygen concentration inside the chamber was 19.2 to 20.7%. The carbon dioxide concentration inside the chamber was 0.03 to 0.41%. Temperature, relative humidity, carbon dioxide and oxygen were measured with Temperature, Humidity, Oxygen and Carbon dioxide Indicator, Manufactured by Chemo Electronic.

Each rat was accommodated individually in transparent perspex exposure tube with adjustable unit. The exposure tubes were fitted to the portholes of the inhalation chamber. The adjustable unit of the exposure tube was set so that the rats breathe Acephate 97% DF aerosols through the window panel of the exposure tube. Observations during the 6 hour inhalation exposure were made through the transparent perspex exposure tubes.

The control group rats were subjected to "sham" treatment and exposed to distilled water only. Treatment and treatment recovery groups were exposed continuously for 6 hours after an equilibration period.

GRAVIMETRIC CONCENTRATION ANALYSIS

The concentration of Acephate 97% DF aerosol was determined gravimetrically as discussed in Chapter 1.

OBSERVATIONS

Clinical Signs

Rats were observed for morbidity and mortality twice a day. All visible signs and symptoms such as skin and fur changes, eye and mucous membrane changes, respiratory, circulatory, autonomic and central nervous system, somatomotor activity, behavioural pattern and general changes were recorded once a day.

Body Weight

Individual rat was weighed on the day of commencement of treatment and at weekly intervals thereafter.

Food Consumption

The daily food consumption of the rats was calculated throughout the experimental period in repeated dose experiment. Food consumption was calculated using the following formula:

$$\text{Food Consumption (g/rat/day)} = \frac{\text{Total feed input in a day (g)} - \text{Total feed left over in a day (g)}}{2 \text{ or } 3^{*}}$$

*as 2 to 3 rats housed in a single cage

Clinical Pathology Observations

Blood samples were collected from all the surviving animals at terminal sacrifice under light anesthesia (isoflurane) by puncturing the orbital plexus. The animals were fasted overnight (water allowed) prior to blood collection and approximately 0.5 mL of blood was collected in vials containing 4% K_2EDTA for haematological analysis. Approximately 2.0 mL of blood was allowed to clot at room temperature for about 30 minutes and then centrifuged at 2500-3000 rpm to collect serum, which was analysed for various clinical

chemistry parameters.

Following haematological parameters were analyzed from whole blood using a calibrated Automated Hematology Analyzer *MEK-6450K* (Nihon Kohden, Japan)

- Total leukocyte count (WBC) [$x10^3/\mu L$]
- Erythrocyte count (RBC) [$x10^6/\mu L$]
- Haemoglobin (HGB) [g/dL]
- Haematocrit (HCT) [%]
- Mean corpuscular volume (MCV) [fL]
- Mean corpuscular haemoglobin (MCH) [pg]
- Mean corpuscular haemoglobin concentration (MCHC) [g/dL]
- Platelets count (PLT) [$x10^3/\mu L$]

Following clinical chemistry parameters from seum were estimated using a computer controlled Automatic Analyzer *Biotecnica BT 2000 plus* (Chema Diagnostica, Italy)

- Alanine aminotransferase (ALT) [IU/L]
- Albumin [g/dL]
- Aspartate aminotransferase (AST) [IU/L]
- Alkaline phosphatase (ALP) [IU/L]
- Blood urea nitrogen (BUN) [mg/dL]*
- Creatinine [mg/dL]
- Creatinine kinase (CK) [IU/L]
- Cholinesterase [IU/L]
- Glucose [mg/dL]
- Inorganic Phosphorus [mg/dL]
- Total bilirubin [µmol/L]
- Total protein [g/dL]
- Total Cholesterol [mg/dL]
- Urea [mg/dL]
- Globulin [g/dL]
- Albumin: Globulin [ratio]

*relative parameter (calculated by instrument on the basis of value obtained for urea).

Urine samples were collected after overnight fasting from the surviving animals using rat metabolic cages in graduated collecting tubes and following urine biochemical parameters were analyzed using Siemens Urinalysis Analyzer CLINITEK- Status®

Parameter	Sample Type	Instrument
Specific gravity	Urine	CLINITEK- Status®
pH		
Glucose		
Ketone [mg/dL]		
Blood		
Bilirubin		
Urobilinogen [E.U./dL]		

Pathology

At scheduled sacrifice date, all the surviving animals belonging to different groups were euthanised by intraperitoneal injection of sodium thiopentone(150 mg/kg body weight). Terminally sacrificed rats were examined externally in unopened condition. Immediately the animals were exsanguinated by opening of the carcasses and examined for the topography of different organs.

Simultaneously gross lesions were recorded as per relevant standard operating procedures. Organs like testes, epididymis, ovaries and eyes were fixed in modified Davidson's fixative. The remaining organs, showing gross abnormality were fixed in 10% neutral buffered formalin. The fixed tissues and gross lesions were processed as per routine procedures to have Hematoxylin-Eosin stained sections. The prepared slides were examined under microscope by a pathologist to note histopathological lesions in different organs. Amongst the control and high dose groups, all collected organs and gross lesions observed from remaining groups and target organs in all the groups were subjected to histopathological examination.

Evaluation of results

All the parameters characterised by continuous data such as body weight, percent change in body weight, feed consumption, urine, haematological and clinical chemistry 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.

Significance was calculated at 1% as well as 5% level and indicated in the summary tables as follows:

↑ - Significantly higher than control ($p \leq 0.05$)
↓ - Significantly lower than control ($p \leq 0.05$)
↑↑ - Significantly higher than control ($p \leq 0.01$)
↓↓ - Significantly lower than control ($p \leq 0.01$)
*↑ - Significantly higher than control recovery ($p \leq 0.05$)
*↓ - Significantly lower than control recovery ($p \leq 0.05$)
*↑↑ - Significantly higher than control recovery ($p \leq 0.01$)
*↓↓ - Significantly lower than control recovery ($p \leq 0.01$)

RESULTS AND DISCUSSION

Concentration Details of Acephate 97 % DF Technical in the Inhalation Chamber

The mean breathing zone concentrations inside the inhalation chamber during the exposure period were 0.238, 0.694, 1.202 mg/L air for low, intermediate and high concentrations respectively (6-hour mean measured value by gravimetric analysis) of Acephate 97% DF. The Mass Median Aerodynamic Diameter (MMAD) of Acephate 97% DF aerosols was found to be 2.09 to 2.76 µm with a geometric standard deviation (GSD) of 2.15 to 2.44 (Tables 2.1 to 2.3, Figures 2.3 to 2.5, Appendix 2.1 and 2.2).

The chamber aerosol concentration of Acephate 97% DF was determined from the entire mass collected on the glass fibre filter x 0.5 (dilution factor).

Mortalities

No mortality was observed in the rats exposed to Acephate 97% DF at the exposed concentrations of 0.273 and 0.727 mg/L air (Table 2.4, Appendix 2.3).

Clinical Observations

No toxic signs were observed in any of the rats exposed to Acephate 97% DF at the dose concentrations of 0.273 and 0.727 mg/L air as well as in the control group rats (Table 2.5; Appendix 2.4 and 2.5).

Body Weight

A statistically significant increase in the body weight was observed in female rats from high dose concentration recovery group on days 7, 14 and 21 but percent body weight change

was statistically comparable to the control recovery group (Table 2.6). Hence, it was concluded that this significant change in body weight was due to initial difference in body weight of the control and high dose recovery group. Statistically, body weights and percent body weight change was comparable with the respective control groups (Table 2.7, Figures 2.6 to 2.13, Appendix 2.6, 2.7)

No treatment related effect in body weight and per cent body weight change was observed in male and female rats during the course of the experiment.

Food Consumption

Statistically, no significant increase or decrease in the food consumption was observed in male and female rats from all the treated groups in comparison to the control group (Table 2.8).

Haematology

Statistically, no significant changes in the haematological parameters were observed in male and female rats from all the treated groups in comparison to those of the control group (Table 2.9, 2.10).

Clinical Chemistry

In males and females, a significant decrease in ChE (in G II, G III and G IV) was observed when compared to the control group (GI). This decreased in ChE was due to the treatment. But in recovery high concentration group, ChE was found to be insignificantly different from the recovery control group (Table2.11, 2.12). Therefore, the ChE level in serum is being restored during the recovery period i.e. 7 days (Figures 2.14 to 2.17).

An increase in total bilirubin was observed inG III female rats. But this value observed was in the range in normal total bilirubin level (Table2.11, 2.12). Hence, although the change was statistically significant, it was biologically insignificant.

Urine Analysis

Statistically no significant change in the pH and specific gravity was observed in urine of male and female rats from all the treated groups in comparison to the control group (Table2.13, 2.14).

Pathological Findings

Gross Pathology Findings (Table 2.15, Appendix 2.8)

External

External examination of terminally sacrificed animals did not reveal any lesions of pathological significance.

Internal

Visceral examination of terminally sacrificed animals did not reveal any lesions of pathological significance.

Microscopic Observations (Table 2.15, Appendix 2.8)

Histopathological observation of preserved tissues revealed various pathological lesions, described as follows.

Liver: Mononuclear cell infiltration, hepatocytes degeneration, oval cell proliferation; **Kidneys:** Urothelial hyperplasia, cortical basophilic tubules, mononuclear cell infiltration, tubular and pelvis dilation

Lungs: mononuclear cell infiltration, alveolar foamy macrophages, alveolar haemorrhages, hyperplasia of bronchiolar epithelium

Heart: myocardial degeneration and cardiomyopathy

Larynx: Hyperplasia of respiratory epithelium, infiltration of inflammatory cells

Nasal cavity: Inflammation and degeneration of respiratory and olfactory epithelium; **Trachea:** Mononuclear cell infiltration.

Representative photographs are of these lesions are presented in Figures 2.18 to 2.23.

All of these microscopic lesions were observed in various organs at lower rate of occurrence. Further these lesions were mostly minimal to mild in nature, non specific and insignificant. Hence, these lesions were considered to be spontaneous or incidental in nature encountered in rats of this age kept under laboratory conditions.

It is apparent from the above results that the Acephate 97% DF at the selected doses did not induce any significant toxic manifestation in the hemetological and biochemical front when challenged through the inhalation route. Moreover, no apparent structural abnormalities were evident in the histological preparations of the target organs studied. However, at the end of 14 day repeated inhalation treatment statistically significant decrease in the serum cholinesterase level was observed in animals from all the treatment groups. It is well documented that organophosphorus compounds hamper the cholinesterase activity by irreversibly phosphorylating the active site of the enzyme (Poovala, *et al.*, 1998; Nigg and Knaak, 2000; Harrington, 2002; CDC, 2009; USEPA, 2012; Kumar and Upadhay, 2013). Therefore, it is prudent to presume that a similar phosphorylayion by the Acephate 95%DF could be the reason for the observed reduction in the activity of serum cholinesterase level in the treated animals.

However, surprisingly the animals in the high dose recovery group revived the ChE activity appreciably in 14 days post treatment and the values for the cholinesterase activity were found statistically comparable ($p > 0.05$) with the recovery control group at the terminal sacrifice. Nevertheless, with the current body of evidence it is not possible to explain the mechanism behind the rapid recovery in the ChE activity following repeated head/nose only inhalation exposure to acephate formulation. Further, mechanistic study aimed at understanding the dephosphorylation of phosphorylated ChE and /or the regulation of de novo synthesis of the enzyme needs to be envisaged to unearth the reasons for the rapid recovery of ChE activity following acephate intoxication. Notwithstanding the above concern, based on the current results, it could concluded that the "No Observed Adverse Effect Level (NOAEL)" of Acephate 97% DF in Wistar rats exposed over a period of 14 days (6 h per day for 5 days a week) should be less than 0.238 mg/L air since that was the lowest concentration tested during the present study and that the animals from group also showed significant reduction in serum ChE. Therefore, additional follow up studies using still lower doses of Acephate 97% DF need to be conducted to deduce an absolute NOAEL value that would not alter the serum ChE.