GENERAL CONSIDERATIONS

It is a fact that agriculture remains one of the largest contributors to the world economy. A large amount of money goes into agricultural research. The use of pesticides in agriculture can be recorded back to 2500BC, and even today, the search for the perfect pesticides runs on. With exhaustive studies and numerous tests being carried out on both potential and existing pesticides, there is ample information available in the public domain about the toxicological manifestations of these.

However, although the toxicological data of technical grade pesticides is plentiful, information about formulation pesticides and their toxic effects fails to match the former. Acephate 97% Dry Flowable (DF) is one such formulation pesticide, for which limited information currently exists. While the toxicity of this pesticide has been extensively evaluated by oral dosing (Roberts and Hutson, 1999; Spassova *et al.*, 2000; USEPA, 2012), there remains a dearth of knowledge about its effects if inhaled. Studying the inhalation toxicity becomes crucial since one of the major routes of application of acephate on crops is through aerosol sprays from aircraft and also by workers in fields.

To evaluate the toxicological properties of any pesticide/chemical, it is very much necessary to first evaluate the median lethal concentration (LC_{50}) of the pesticide/chemical. The LC_{50} data provides a basis of dose concentration selection for further long term toxicity testing. In this test, the acute inhalation toxicity of Acephate 97% DF (*Lancer 97 DF)* was evaluated by using Wistar rats of both the sexes as per standard guideline (OECD, 2009). Rats were treated with 5.113 mg Acephate 97% DF/L air for four hours. As per OECD guidelines (2009) if the breathing zone concentration is found to be >5 mg/L air and no mortality is observed, then test is restricted at this single concentration level. In the current study, the LC_{50} was therefore found to be greater than 5.113 mg Acephate 97% DF/L air, since no mortality was observed at this highest recommended concentration.

Although acephate is widely regarded as one of the safest organophosphate pesticides because of its reported relatively safe mammalian metabolic disposition (Roberts and Hutson, 1999), some studies indeed demonstrate toxic effects in mammalian models including humans (Bennett and Morimoto, 1982; Poovala *et al.*, 1998; Datta *et al.*, 2010).

Acephate also tends to get absorbed through lungs rather quickly (Chuckwudebe *et al.*, 1984), and in case of applicators and formulators of this pesticide, this remains a major means of exposure. Therefore, it was deemed essential to study the outcomes of repeated dose inhalation of commercial acephate formulation in rats.

For this study, animals were divided into three treatment groups that were exposed to low, mid and high concentrations of acephate in air. Apart from these, a recovery group had also been studied wherein the rats were exposed to high concentration of acephate for 14 days and were maintained without exposure for an additional 14 days to assess signs of recovery, if any, from the effects of the 14 day treatment period. A respective control group had also been included.

No toxic signs or mortality were observed in treated rats at the end of the treatment period. Statistically significant increase in the body weights 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. Hence it was concluded that this change in body weight was due to initial differences in body weights of the control and high dose recovery groups. Statistically, body weights and percent body weight change were comparable with the respective control groups. No treatment related effects in body weight and percent body weight change were observed in male and female rats during the course of the experiment.

No treatment related change was found in the mean food consumption as well. Evaluation of hematological parameters also did not reveal any treatment related changes. The battery of biochemical parameters studied also remained within the normal range in all the groups of animals.

A statistically significant decrease in serum cholinesterase (ChE) activity was observed in the treated animals (both male and female) from all the groups when compared to that of the controls. However, in the high concentration recovery group, ChE activity was found recovered to the normal range within 14 days from the last day of treatment. This result shows a rescue of the serum ChE activity during the additional 14 days of recovery period, when no Acephate treatment was given to the rats.

Other parameters tested in the serum and also urine did not show significant changes after the 14 day exposure to Acephate. Also, external and visceral examination of terminally sacrificed animals did not reveal any lesions of pathological significance.

Histopathological examination revealed varying degrees of pathological lesions comprised of **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.

All of these microscopic lesions observed in various organs are 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.

Therefore form the repeat dose study it is evident that the test article Acephate 97% DF at the selected dose levels hampered only the serum ChE activity amongst all the parameters studied. However, since the lowest dose selected for the study also reduced the ChE activity to a statistically significant level, it could be construed 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) via inhalation route should be less than 0.238 mg/L air. Further careful studies using lower doses of Acephate 97% DF is needed to arrive at a more realistic NOAEL. What also remains to be elucidated is the mechanism of dephosphorylation or quick replenishment of serum ChE after repeated exposure to the selected organophosphorus formulation.

A severe allergic reaction called Allergic Contact Dermatitis (ACD) is often caused by exposure to certain skin-sensitising chemicals. Acephate 97% DF, although extensively studied for various adverse health effects, has so far not been tested for its skin-sensitizing potential. Since, the pesticide is largely being sprayed by applicators in fields without proper protective gear, it is of great relevance to understand how it affects non-target organisms, coming in direct contact. Of the several conventional animal models available for evaluation of the ACD potential of a chemical including the Buehler Test (Buehler,

1965) and the Guinea Pig Maximisation Test - GPMT (Magnusson and Kligman, 1970), the murine Local Lymph Node Assay (LLNA) developed by Kimbler and coworkers (1986) is largely accepted as a standard protocol to evaluate ACD potential of a chemical. Therefore, the LLNA was used in the current study to evaluate the ACD potential of Acephate 97% DF.

This method (LLNA) is based on the fact that dermal sensitization requires the elicitation of an immune response. This immune response requires proliferation of a lymphocyte subpopulation. The test relies on the detection of increased DNA synthesis via tritiated thymidine incorporation. Sensitization is measured as a function of lymph node cell proliferative response induced in a draining lymph node following repeated topical exposure of the test animal to the test article.

The NIH recommends that only female CBA mice be used for the LLNA, as they reportedly develop a stronger contact dermatitis response when compared to males; however if males are to be used, systematic studies evaluating potential sex differences should be conducted (ICCVAM, 1999). There are, in fact, several advantages to consider with the inclusion of male mice in LLNA testing including a more refined and responsible use of animals. Therefore, to begin to systematically assess the appropriateness of using male mice in the LLNA a comparative guideline study was conducted with individual housing of mice using a possible non-sensitizer and a sensitizer, *viz.*, Acephate 97% DF and α-hexylcinnamaldehyde (HCA) respectively.

Based on the preliminary assay, CBA/J male and female mice were used for test. Acephate was applied topically for three consecutive days (days 0, 1 and 2) at dose levels of 10% (G 2), 25% (G 3) and 50% (G 4) (w/v) Acephate 97% DF in Dimethylformamide (DMF) and 25% (G 5) (v/v) α-Hexylcinnamaldehyde in DMF on the dorsal surface of both ears (25 µL/ear) using a calibrated micropipette. Mice from control group (G 1) were treated in a similar manner with DMF only. No treatment was applied on days 3 and 4. On day 5, all mice were injected intravenously (tail vein) with approximately 20 (±1) µCi of tritiated methyl thymidine. Five hours post-administration, the uptake of ^{3}H-thymidine into the auricular (local) lymph nodes draining the site of chemical application was measured a Liquid Scintillation Analyser in order to assess the proliferative response of the lymph node. The DPM values were measured individually for each mouse. Stimulation Index (SI Value) was calculated.

No clinical signs or local erythema were observed in mice treated with all tested concentrations of Acephate 97% DF and control group. Very slight erythema (barely perceptible) (on days 1 to 4) was observed in HCA treated mice (10/10 mice). The mean body weight of positive control as well as Acephate 97% DF treated mice was comparable to that of the control groups. There was no noteworthy increase observed in ear thickness on days 2 and 5 in all treated groups as well as in vehicle and positive control groups.

A Stimulation Index (SI) of three or more (SI value of treated groups over the control) indicates potential to cause skin sensitisation.

The SI obtained for Acephate 97% DF at all the tested concentrations showed a less than three-fold increase over the control value of male and female mice. Therefore, Acephate 97% DF did not demonstrate dermal sensitisation potential in the local lymph node assay.

The SI of 4.31 for male mice and 5.44 for female mice obtained for the concurrent positive control, HCA, showed greater than a three-fold increase over the control value indicating a positive response in agreement with the historical control (ICCVAM, 2009) for this known weak sensitiser. This confirmed the reliability of this test procedure.

Moreover, Stimulation indices (SI) for the three Acephate 97% DF treatment groups were comparable for female and male mice. SI for the HCA treated animals was also comparable for both the sexes. Also, the SI values observed for both male and female mice of positive control were within the range of lab historical data for female mice. These data provide initial support for the use of male mice in the LLNA and maybe used for further studies after further validation.

The current inhalation toxicity study of Acephate 97% DF revealed that the selected organophosphorous compound is relatively safe for mammals if contracted accidently via nasal route. However, even though the routine haematology and histopathology did not reveal any test compound induced changes, the serum cholinesterase activity showed marked decline in the animals from all the treated groups. The phosphorylation of cholinesterase by organophosphates is well documented and hence the above observation is not a surprising one. However, the basis of expeditious recovery shown by the animal in terms of ChE activity remains to be elucidated.