CHAPTER 4

DISCUSSION

The advent of the twentieth century has made man supreme conquer of nature. His intelligence has intervened into every field of study and agriculture is by no means an exception. No doubt, his inventions and scientific technologies increased the production of agro industry and agroecosystem, but also have rendered the plants more susceptible to disease causing fungus and insects. As a result more numerous fungicides, insecticides etc, has been poured into the market and injudiciously used in the agro ecosystem. For effective control of insect pests use of combination insecticides has increased extensively. However, very little or no data is available on the toxicity potential of these combination insecticides. Moreover, in order to formulate comprehensive safety evaluation strategies it is very essential to have a detailed understanding of the effect(s) of such combination insecticides on non-targeted organisms.

In the present investigation a relatively new combination (cypermethrin 4 % + profenofos 40 %) of insecticides has been studied in detail for its acute toxicity, acute neurotoxicity, sub chronic toxicity and developmental neurotoxicity in rat. A survey of the literature shows that not much of the work has been done on this product; hence, it necessitates studying the toxic effects in depth.

To find out the oral LD_{50} for the insecticide combination, test substance was administered at the dose level of 0 (G1), 325 (G2), 400 (G3) and 460 (G4) mg/kg body weight. Animals from the entire dose groups exhibited test article related clinical signs/symptoms such as tremors, salivation, piloerection, lacrimation, ataxia, and lethargy. In addition, symptoms such as abdominal breathing, nasal discharge, cataract, clonic-convulsion were observed in few animals. Similar findings were noted for profenofos by **Kobel and Gfeller (1983)**. Acute OP poisoning is characterized by the cholinergic syndrome which is due to inhibition of

acetylcholinesterase (AChE) at nerve terminals causing accumulation of the neurotransmitter acetylcholine (ACh). This, in turn, induces over stimulation of nicotinic and muscarinic receptors in the central and peripheral nervous systems and the consequent signs and symptoms **(Lotti, 1991)**. The currently observed symptoms were very well established in organophosphorus poisoning as well as in pyrethroid intoxication. **Taylor (1985)** has explained such symptoms as cholinergic symptoms, which appear due to the effect on muscarinic, nicotinic, and central nervous system receptors.

A total of 30% mortalities in 325g/kg body weight, 60 % mortalities in 400 mg/kg body weight and 100% mortalities in 460 mg/kg body weight dose groups were observed. The calculated LD_{50} for combination insecticide in this study was 374 mg/kg body weight. This value was fairly less than the declared LD_{50} value for technical profenofos (combined for male and female) *i.e.,* 630 mg/kg in rats **(U.S. EPA Document, 1999)**. This suggests that in the presence of cypermethrin, the toxicity of profenofos may have potentiated. This confirms the suggestions of **Ballantyne *et al.* (1995)** that the effects produced in combination may be additive, synergistic, potentiating or antagonistic.

As per EPA classification, the present test article can be categorized under Class-II toxicity category.

Gross lesions *viz.,* mottled liver and congested kidney observed in treated animals could be related to the test article because no such lesions were apparent in control group animals.

In acute neurotoxicity screening battery tests, test substance was administered in a single maximum tolerated dose (MTD) of 25, 75 and 225 mg/kg body weight dose levels for low, mid and high dose groups respectively.

Cholinergic symptoms such as tremors, salivation, writhing, hyperactivity to sound/touch, abnormal gait and ataxia were observed in 75 and 225 mg/kg body weight dose groups immediately after dosing. These signs/symptoms were persisted for 8 hours post dosing. However, all the symptoms were gradually

disappeared after three days post treatment. Observed neurotoxic symptoms could be due to the treatment with the test article. **Righi and Palermo (2003)** reported that rats treated with cyhalothrin, a synthetic pyrethroid revealed signs and symptoms of intoxication that included salivation, tremors, and liquid feces. **Stevens *et al.*, (2001)** stated that organophosphorus insecticides react with acetlycholinesterase at the serine hydroxyl group within the enzyme active site. In this reaction, serine hydroxyl group is phosphorylated, yielding a leaving group. The phosphorylated acetlycholinesterase is inactivated, blocking acetacholine degradation in the synapse. This results in a buildup of this neurotransmitter and central nervous system hyperstimulation. The signs of intoxication include restlessness, hyperexcitability, tremors, convulsions and paralysis.

Since the animals recovered from cholinergic symptoms, mean body weight of animals from treatment groups were unaffected during the experiment.

Test article caused significant reduction in serum cholinesterase level in treated males at 25, 75 and 225 mg/kg body weight dose levels and also in females treated at 75 and 225 mg/kg body weight dose levels as compared to control group. The observed reduction in cholinesterase was consistent and also dose dependent and considered to be due to the organophosphorus entity of the test article *i.e.*, profenofos. Observations of **McDaniel *et al.*, (2004)** support our observations as they reported significant reduction in cholinesterase due to profenofos treatment in rats. The Organophosphate (OP) compounds act primarily by phosphorylation of the acetlycholinesterase enzyme (AChE) at nerve endings (**Stevens *et al.*, 2001**). The enzyme is critical to normal control of nerve impulse transmission from nerve fibers to smooth and skeletal muscle cells, glandular cells, and autonomic ganglia, as well as within the central nervous system (CNS). Some critical proportion of the tissue enzyme mass must be inactivated by phosphorylation before symptoms and signs of poisoning become manifest **(Du Bois, 1971)**.

Carey (1999) stated that all OP anticholinesterases potentially have a mechanism of toxicity in common *i.e.*, the phosphorylation of AChE causing accumulation of ACh as explained in the earlier paragraph.

Neurobehavioural observations performed on rats at peak time of effect *i.e.,* at 4 hours post dosing, revealed various symptoms. In home cage, males treated at 225 mg/kg body weight dose level and females treated at 75 and 225 mg/kg body weight dose level exhibited symptoms *viz.,* abnormal postures, vertical jumping, writhing and tonic-clonic convulsions. Adverse behavioral effects are now a recognized outcome of exposure to many industrial and environmental chemicals **(Weiss, 1988).**

During handling observations, ease of removal and handling reactivity was found very difficult in most of the males and females treated at 225 mg/kg body weight dose level. In addition, most of the females from 225 mg/kg body weight dose group showed lacrimation. All the males and females from 225 mg/kg body weight dose group revealed piloerection. Salivation was noticed in one or two females from 75 and 225 mg/kg body weight dose group. These symptoms are characteristic of the stimulation of parasympathetic nervous system (muscarinic effects), such as salivation, nasal discharge, lacrimation and nicotinic effects which included tremors, and piloerection. Such symptoms are reported to be a characteristic feature in rats administered with both phosphorothioates and phosphorodithioates **(DuBois, 1971).**

In the open field, all the males and females from 225 mg/kg body weight dose group showed slight to severe abnormal gait and slight to totally impaired mobility/movement. Similarly, majority of rats from treated groups revealed low or very low arousal and also revealed tonic/clonic movements. Severity of symptoms was more in high followed by mid and low dose group.

Sensory Reactivity Measurements: Approach response was slight or energetic in almost all the animals from control and test article treated groups, whereas, majority of animals from 75 and 225 mg/kg body weight revealed exaggerated response to touch and click stimulus. No constriction of pupil was observed in animals from 225 mg/kg body weight dose group. All the males from high dose group landed on side in air righting reflex test, whereas, 2 out of 5 females of the same dose group landed on side and rest of the females landed on back. Majority of males and females from 225 mg/kg body weight dose group did not respond to tail pinch and few responded feebly to tail pinch.

Both males and females treated at 225 mg/kg body weight dose levels revealed reduced hind limb grip strength at peak time of effect on the day 1, however, on day 7 and 14 hind limb grip strength was comparable to control group. Also, hind limb foot splay for test article treated animals was considerably increased.

The above neurobehavioural symptoms in treated animals were primarily due to organophosphorous poisoning **(WHO, 1986; Brar *et al.*, 2000a)**. The mode of action of the organophosphate poisoning is inhibition of the acetylcholinesterase **(Casida, 1963; O'Brien, 1967)** Phosphorylation of acetlycholinesterase enzyme allows accumulation of acetylcholine (ACh) in the PNS at cholinergic neuroeffector junctions (muscarinic effects), skeletal nerve-muscle junctions, and autonomic ganglia (nicotinic effects), as well as in the CNS. At cholinergic nerve junctions with smooth muscle and gland cells, high acetylcholine (ACh) concentration causes muscle contraction and secretion, respectively. At skeletal muscle junctions, excess ACh may be excitatory (cause muscle twitching), but may also weaken or paralyze the cell by depolarizing end plate. In the CNS, high ACh concentrations cause sensory and behavioural disturbances, incoordination, depressed motor function, and respiratory depression. Increased pulmonary secretions coupled with respiratory failure are the usual causes of death from organophosphate poisoning. Recovery depends ultimately on generation of new enzyme in all critical tissues **(Taylor, 1985)**.

Observed neurobehavioural symptoms in 75 and 225 mg/kg body weight dose groups revealed very clear evidence of dose dependent severity of the effect of the test article. Effects were most evident on the day of exposure and disappeared over the next few days. No signs of treatment were apparent after 7 days of exposure. Histopathology showed no evidence of treatment-related changes in either the nervous system or muscle

Further, motor activity (both total and ambulatory activities) count for 225 mg/kg body weight dose group was significantly reduced. Similar observations were reported by **Mattsson *et al.*, (1996)** for chlorpyrifos an OP insecticide.

The acute effects of test article over-exposure were found regressed over a period of time. Functional observational battery tests performed on day 7 and 14 in treatment groups did not reveal any abnormal behaviour. Which clearly indicates the recovery from the test article induced effects.

However, no histopathological lesions that were related to treatment with test article were noticed in any of the treatment groups. Similar observations were reported for chlorpyrifos, an OP insecticide by **Mattsson *et al.*, (1996)** in a single dose neurotoxicity screening study in rats. Hence it is logical to conclude that acute dosage of the test article does not evoke structural alterations. Further, to study the effect of test article when administered in repeated sub lethal doses for a minimum period of 90 days, further experiments were designed.

During 90 day of treatment period, few clinical symptoms *viz.*, nasal discharge, soft stool and snuffles were observed in few animals. These symptoms were found transient and sporadic; moreover, these symptoms were noticed in animals' irrespective sex and dose group. Hence, these symptoms were considered as incidental and not related to treatment.

Observed variations in mean body weight and food consumption were inconsistent, transitory and were not observed in high dose group (38 mg/kg body weight). Thus, test article did not affect general health of the animals during the experiment.

Test article did not affect any of the hematology parameters in males treated at various dose levels. Whereas, in females, RBC's were slightly increased in 6 mg/kg body weight dose group and decreased in 15 mg/kg body weight dose group, whereas, no such variations were observed in 38 mg/kg body weight dose group. Therefore, observed variations were considered to be due to random biological variation and incidental. Observed variations in hemoglobin, HCT, MCV, MCH, MCHC and platelets count in test article treated groups were inconsistent with respect to increased dose and were well within the normal reference range, hence, they were considered as incidental and not related to treatment. Moreover, hematology values from treatment recovery group were comparable to control

recovery group. Test article did not alter differential leucocytes counts in both males and females treated at 6, 15 and 38 mg/kg body weight dose levels

Clinical chemistry estimations performed at the end of 90 days treatment period revealed significant increase in serum glucose level in females treated at 15 and 38 mg/kg body weight dose level, whereas, in males no such variations were apparent.

Glucose in body fluid is important in the diagnosis and management of glycemic level, adrenal dysfunction and various stress conditions. Hyperglycemia is reported to occur due to chronic liver disease **(Ellefson and Caraway, 1976).** Dose dependent increase in glucose level of females indicates the correlation of test article treatment. Increase in the serum glucose level was reported by **Mandal *et al.*, (2000)** in cypermethrin treated goats. Increase in the blood glucose level was also observed in cypermethrin treated rats by **Lock and Berry (1981). Shakoori *et al.*, (1988)** reported increased serum glucose level in rats following cypermethrin treatment for six months in a feeding study. It is presumed that the hyperglycemia induced may be due to elevated gycogenolysis **(Ayub and Gupta, 1997).**

In males, treated at 38 mg/kg body weight dose level ALT activity was significantly increased as compared to controls. Females treated at 38 mg/kg body weight dose level also revealed similar trend of increase in ALT activity, but the variation was statistically insignificant. ALT is found in most of the tissues but liver is by far richest in ALT. The increased ALT values are also reported in a study conducted on rats due to organophosphate and are indicative of hepatic and renal injury. Incidence of liver damage under toxic exposure elevates the level of ALT **(Lynch *et al.*, 1964; Hanke and Piotrowski, 1980).** Increases in ALT and bilirubin level is commonly found in liver disease, also action of certain drugs and toxic substances on liver causes most marked increase in the levels of ALT **(Varley *et al.*, 1980).**

Further, total bilirubin values were significantly increased in males treated at 38 mg/kg body weight dose level as compared to control group. This could be attributed to hepatic dysfunction/injury. Serum total bilirubin is increased in hepatocellular damage (infectious hepatitis, and other toxic hepatopathy), intra- and

extra hepatic biliary tract obstruction, intravascular and extra-vascular hemolysis **(Friedman, 1980).**

Creatinine was significantly increased in both males and females of 38 mg/kg body weight dose group; however, in females variation was statistically insignificant. Creatinine is a non-protein waste product of creatine phosphate metabolism by skeletal muscle tissue. Creatinine production is continuous and is proportional to muscle mass. Creatinine is freely filtered and therefore the serum creatinine level depends on the Glomerular Filtration Rate (GFR). Renal dysfunction diminishes the ability to filter creatinine and the serum creatinine rises **(Relman and Levinsky, 1971)**. Increased serum creatinine levels are seen in impaired renal function **(Levey *et al.*, 1988)**.

Increased total protein and albumin values were observed in males as well as females treated at 6, 15 and 38 mg/kg dose level. A clear dose dependent increase in the levels of total protein and albumin were evident and indicate the relevance of test article treatment. Increase in total protein in serum reflects increases in albumin, globulin, or both. Generally, significantly increased total protein is seen in volume contraction, venous stasis, or in hypergammaglobulinemia **(Tietz, 1983).** Increased serum proteins were reported by **Shakoori *et al.*, (1988)** in six months feeding study of cypermethrin in rats.

The current study revealed significantly reduced cholinesterase in males and females compared to control group animals. A steady reduction with increase in dose level was apparent. It is considered that reduced cholinesterase level was due to test article administration. It has been well established that organophosphorus insecticides cause acetlycholinesterase inhibition **(Stevens and Breckenridge, 2001)**. Similar reduction in cholinesterase enzyme was evident in acute neurotoxicity battery study also.

Clinical chemistry estimations performed at the end of 28 days recovery period revealed elevated levels of glucose. This indicates that to recover completely from observed alteration it may require more time than 28 days.

Functional observational battery tests performed during 13th week of treatment period did not reveal any treatment related neurobehavioural, functional or motor abnormalities. Forelimb and hindlimb grip strength and hindlimb foot splay in animals of both the sexes treated with test article were not affected. However, slightly increased motor activity was observed in 15 and 38 mg/kg body weight dose group animals.

Absolute and relative weight of organs from the animals kept for studying reversal or delayed occurrence of any toxicity did not reveal any variation as compared to that of control group.

Exposure to chemical substances can cause adverse effects on the male and female reproductive systems. Reproductive toxicity may be expressed as alterations in sexual behavior, decreases in fertility, or loss of the fetus during pregnancy. A reproductive toxicant may interfere with the sexual functioning or reproductive ability of exposed individuals from puberty throughout adulthood **(Schardein, 1999)**. Toxicants that target the female reproductive system can cause a wide variety of adverse effects *viz.*, changes in sexual behavior, onset of puberty, cyclicity, fertility, gestation period, pregnancy outcome and lactation **(Freeman, 1994)**.

In the present investigation, test article did not exert any adverse effect on estrous cycle and gestation period of females treated for more than 90 days. Further, body weights and food consumption of test article treated females were remained unaffected during gestation and lactation periods.

Administration of test article did not affect male and female fertility indices as well as mating index of test article treated group animals, as these indices were comparable between treatment and control groups.

Gestation index, mean duration of gestation and lactation indices for test article treated group animals were comparable to that of control animals.

In the present investigation, lactation index was significantly reduced and survival index for pups (both male and female pups) was also reduced on days 0, 4, 7 and 14 of lactation in 38 mg/kg body weight dose group. Further, mean litter size (both

male and female pups) in 38 mg/kg body weight dose group was significantly reduced on lactation days 0, 4, 7, 14 and 21. Increased mortality of both male and female pups was noticed during lactation period 0-4 at 38 mg/kg body weight dose group. These changes could be attributed to the effect of the insecticide combination. Results of our study emphasize this aspect. The toxin may cause genetic or chromosomal damage to the germ cell, which may result in fetal death, structural abnormalities or functional abnormalities or stillbirth. The same is also applicable in the female reproductive system. On the other hand if the toxins interfere with the process of development, then it profoundly causes detrimental effect in mother and fetus, **Ratcliffe, *et al.*, (1995); Chapin, *et al.*, (1985); Gray, *et al.*, (1992)** imply that more remarkable demonstration of reproductive toxicity is decrease in pup number. However, mean litter weight of pups *i.e.,* male or female or combination of both sex were comparable to control group.

No variations in the organ weights of parent males and females treated with the insecticide combination were established. However, terminal observations (at weaning) of pups revealed increased absolute weight of liver in 38 mg/kg dose group male pups and 15 mg/kg and 38 mg/kg body weight dose group female pups. Even relative weights (organ to body weight ratio) as well as relative brain weights of both male and female pups at 38 mg/kg body weight were significantly increased. **WHO working group** reported increased liver weights in long-term treatment studies due to cypermethrin toxicity.

The liver has many metabolic activities and is therefore, affected by very large number of xenobiotics. However, the liver has also an immense capacity for self repair, so reverses rapidly on cessation of treatment **(Hinton and Grasso, 1995)**.

Histopathology of liver from pups belong to 38 mg/kg body weight dose group did not reveal any consistent or test article related lesion.

Absolute, relative and weight of spleen in 38 mg/kg body weight dose group male pups were significantly increased. However, no corresponding histopathological lesions were observed in the spleen.

Relative weight of kidneys in 38 mg/kg body weight dose group pups of both sex were significantly increased. The functional integrity of the mammalian kidney is vital to total body homeostasis, as the kidney plays a principal role in the excretion of metabolic waste and in the regulation of extra cellular volume, electrolyte composition, acid-base balance and endocrine function.

Toxic insult to the kidney could disrupt many of its homeostatic function and could have profound effect on the total body metabolism **(Goldstein and Schnellman, 1996)**. Toxicity of cypermethrin to kidney weights **(WHO, 1992)** has been reported. In the present study, microscopic examinations of kidneys of pups revealed tubular dilatation with flattened epithelium and cystic tubules and could be attributed to treatment.

Present study shows varying degrees of different gross lesions in liver, kidney, nerve, brain, ovary and thymus in parental animals, which were more prominent in the test article treated groups. The histopathology of theses organs revealed marked changes in test article treated group animals when compared to control. Observed variation in clinical pathology parameters could be well correlated the histopathological changes. Liver has long been recognized as a target organ for xenobiotic - induced toxicity due to its central role in metabolic disposition **(Carfagna *et al.*, 2001)**. The histopathology of liver showed minimal fatty changes, minimal fatty changes and dilatation of tubules. **Enan et al., (1987)** reported that profenofos caused adverse effects on liver function. Malathion induced severe chronic inflammations, degeneration of hepatocytes, cell vacuolations and lymphocytic infiltrations **(El-Elaimy *et al.*, 1995)**. Kidney from test article treated group showed cortical tubular dilatation with flattened epithelium, congestion and multifocal regenerating tubules. **Perger, *et al.*, (1994)** reported that rats treated with pyrethroids in sub acute study revealed hypertrophy in the liver and kidneys. Histopathological studies of kidneys revealed moderate degree, degenerative changes and presence of eosinophilic proteineous material in lumen of renal tubules and aggregation of mononuclear cells due to the exposure to certain organophosphorus insecticides **(Kaur *et al.*, 2000)**.

Brain of the treated animals showed lesions such as demyelination, vacuolation and axonal degeneration. Poisoning by organophosphorus compounds can result in an acute but usually manageable medical crisis and may damage the CNS and the PNS, as well as cardiac and skeletal muscle tissue **(Millard and Broomfield, 1995)**. In ovary atretic follicle were noticed, further thymus revealed atrophy and epithelisation.

Histopathological changes observed in kidneys and nerve in males treated at 38 mg/kg body weight dose group and brain, kidneys, liver, ovary and thymus in females treated at 38 mg/kg dose group were more prominent.

Gross and histopathology examination of F_1 pups from test article treated groups revealed various lesions in brain (multiple pale acellular areas, gliosis, demyelination), liver (cystic tubules, extramedullary hematopoisis, sinusoidal dilation, vacoulation and congestion, sinusoidal dilation), thymus (atrophy characterized by epithiolisation), kidney (tubular degeneration with flattened epithelium) and spleen (extramedullary hematopoisis, megalokaryocytosis). Pathological changes in the cortex of thymus, liver, adrenal glands, lungs and skin were observed in rabbits repeatedly fed high doses of cypermethrin **(U.S. EPA, 1989)**.

Studies by **Jadwiga *et al.*, (1999)** showed that administration of the mixture of chlorpyrifos and cypermethrin for 28 days induced histological changes in the liver and kidneys.

An over all incident of pathomorphological changes in various organs of either sex belonging to different treated groups, the dose related changes indicate the toxic response in the organs of both males and females The consistence and severity of pathological changes in different organs of male and female animals appear to be more prominent in 38 mg/kg body weight dose group.

In conclusion the present experiments revealed that the insecticide combination showed definite signs of potentiation of toxicity. Moreover, at a single sub lethal oral doses of 75 and 225 mg/kg body weight caused cholinesterase inhibition which in

turn induces cholinergic symptoms and an array of functional/neurobehavioural abnormalities in both male and female rats However, effects were reversed to normalcy after short period.

Subsequent subchronic study using various dose levels did not affect any of the hematology parameters whereas, clinical chemical parameters showed definite dose dependent alterations in glucose, ALT, total protein, total bilirubin, creatinine, and albumin. These biochemical responses, in relation to the test article, exhibited a sex related variation. However, the physiological reasons behind this sex related variation in response to test article needs to be further elucidated.

Nevertheless, as expected of any organophosphorus compound a dose related decline in serum cholinesterase level was observed in treated groups. This impairment in cholinesterase activity was however reversible and normalcy was achieved within 28 days.

No treatment related effects were seen in estrous cycle, duration of gestation period, and body weight and food consumption during gestation and lactation, and fertility indices however, survival index of pups was significantly reduced and mortality of pups was significantly increased in 38 mg/kg body weight dose group. Further, the absolute and relative weights of liver, spleen and kidneys were significantly increased in 38 mg/kg body weight dose group pups. Moreover, the test substance seemed to adversely affect many of the vital organs in a dose dependent fashion as evident by the pronounced structural alterations observed in tissues like liver, kidney, spleen and nervous tissue.

Finally, it is apparent from the above findings that the test article at the given dose is a potent neutotoxicant and elicited reversible neurobehavioural alterations. The toxic manifestations are also evident at the biochemical and structural front. However the compound had marginal reproductive toxicity and no adverse influence were noticed in haemogram.