REPRODUCTION AND NEUROTOXICOLOGICAL INVESTIGATION OF A COMBINATION INSECTICIDE IN RATS

[CONCISE SUMMARY]

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CONCISE SUMMARY

Pesticides, used for combating the pests including insects, weeds, fungii, rodents and other form of human life including virus, bacteria and other micro-organisms have made a phenomenal contribution worldwide towards the production and preservation of food, fibre and cash crops, as also the eradication of diseases and maintenance of public health **(Dhaliwal *et al.*, 1999)**.

The importance of continued development in the pesticides is more so in a country like India which is predominantly agricultural with about 80% of the population depending upon agriculture and living in rural areas. Indian population is expected to cross the 100 mark by 2000 AD, with the corresponding requirement of food grains exceeding 220 million tonnes. As against this, the availability of land per capita is expected to be 0.14 hectares. As such, the critical role of pesticides in preventing crop losses, making available food at cheap rates and improvement in quality of food can not be overemphasized **((Kathpal and Kumari, 1998))**.

Besides ensuring the food supplies, pesticides have also to make as substantial contribution in increasing the production of cash crops like cotton, tea, coffee, tobacco etc. which in addition to meeting the domestic requirement are so important for export earnings.

Equally important is the contribution the pesticides have to make in preventing the diseases and maintaining health of the increasing population. According to WHO 2.5 lakh to 5 lakh children die annually due to Malaria. It is imperative that this trend be arrested.

In spite of severe environmental pressures the world market for pesticides has grown phenomenally from just US$ 26 billion in 1990. Western Europe and USA are the world leaders with shares of about 30.2% and 22.7%. There is a boom in the

global pesticide market and new insecticides, herbicides and fungicides and their formulations are being introduced with greater level of activity, however, with conscious efforts for minimizing the hazards to the human beings and the environment.

Indian pesticide industry has also made a remarkable progress having achieved the status of second longest basic pesticide manufacturer in Asia after Japan. Further encouraging developments in the recent years include almost total self reliance with imports constituting less than 5% of total consumption of about 70,000 tonnes annum, indigenous development of several new products and processes, and penetration into overseas markets with exports already having touched a level of about Rs.150 crores. However, there is still a long way to go considering that the per capita consumption of pesticides in India is just 475gm per hectare as compared to 11800gm per hectare in Japan and 3000gm per hectare in USA **(Kannan, 1997)**.

There has been a considerable concern and debate on the possible adverse effects of pesticides on life and environment due to toxicity, residues, carcinogenicity, contamination of soil, water and air etc. Despite the restrictions and regulations on pesticide use, India accounts for one third of the total poisoning cases in the world **(Dhaliwal *et al.*, 1999)**.

In response to this concern, the development in pesticide is directed towards discovering new products with lower toxicity and ecological impact, as well as reduced dosages and employing processes which are less hazardous. An interesting example would be the decreasing oral LD-50 toxicity in the successive generations of insecticides DDT (Organochlorine) - 118 mg/g; Fenitrothion (organophosphate) - 570 mg/g; Permethrin (pyrethriod) - 1500 mg/kg; chlorfruazuron (insecticide growth regulator) - 8500 mg/g **(Oehme, 1987)**.

In agriculture it is becoming a common practice to use combination of pesticides in order to combat resistance to weeds and insects. One of the combinations that have become most popular in agriculture is a combination of pyrethroids and organophosphates. Considerable body of literature has been found on toxicity related studies of organophosphorus and synthetic pyrethroid insecticides

independently but very limited information is available on combination of these classes of insecticides. Particularly, there is a paucity of data regarding neurotoxicity and postnatal development aspects in rats. Hence, the present investigation has been envisaged to determine the toxic effects of combination insecticide (Profenofos 40% + Cypermethrin 4% EC) on nervous system, general health, reproduction and post natal development in Wistar rats (*Rattus norvegicus*) when exposed over a period of time.

The route of exposure used in the present investigation was oral because it is the most common route of exposure encountered in toxicological literature and it is a route by which a large number of substances gain entrance into the animal's body.

In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. It provides information on health hazards likely to arise from a short-term exposure by the oral route. Data from an acute study may serve as a basis for classification and labeling. It is an initial step in establishing a dosage regimen in sub-chronic and other studies and may provide initial information on the mode of toxic action of a substance. Acute toxicity studies will thus identify highly toxic chemicals and provide information on the possible hazards, which could occur where humans are, exposed. Oral LD_{50} evaluated for the present test compound, following a single acute exposure, was 374 mg/kg body weight. From the available literature LD_{50} value of profenofos technical and cypermethrin technical reported are 630 mg/kg **(U.S. EPA Document, 1999)** and 250 mg/kg body weight **(Tomlin, 2000)**, respectively. The obtained data showed that the toxicity of the combination insecticide is much less than the reported LD_{50} value of profenofos. This suggests that in the presence of cypermethrin, the toxicity of profenofos may have potentiated.

The literature survey of pesticides revealed that they are harmful to man and environment **(FAO and WHO working groups, 2000; White et al., 2000; Kartikeyan et al., 2001)**, but the data available are only for the technical grade products or their formulations **(Rechardson and Chambers, 2002)**. The combination of two pesticides may have additive, synergistic, potentiative or antagonistic effect **(Ballantyne et al., 1995)**.

Further, the lack of toxicity data concerning to neurotoxic propensity of insecticide combination (Profenofos 40% + Cypermethrin 4% EC) calls for a detailed classical neurotoxicity screening battery tests following a single oral dose.

The insecticide combination was dosed to several groups of experimental animals, one dose being used per group. The animals were observed under carefully standardized conditions with sufficient frequency to ensure the detection and quantification of behavioral and/or neurologic abnormalities, if present. Various functions that could be affected by neurotoxicants were assessed during each observation period. The evaluation of behavioral toxicity is a new kind of challenge to pesticide toxicology because of the enormous structural and chemical heterogeneity of the nervous system and the even greater complexity of behavior **(Weiss, 1988)**. In addition, serum cholinesterase (ChE) was estimated after 24 hours of dosing. Measurements of motor activity of individual animals are made in an automated device. At the end of 14 days of observation period, animals were perfused and tissue samples from the nervous system were prepared for microscopic examination.

Majority of animals revealed symptoms such as tremors, salivation, writhing, hyperactivity to sound/touch, abnormal gait immediately after dosing. Similar symptoms were documented for chlorpyriphos and cypermethrin by **Hardman *et al.*, (1996)**.

During handling observations, ease of removal and handling reactivity was found very difficult in most of the animals treated at higher doses. In addition, lacrimation, piloerection and salivation were noticed in few animals. Severity of symptoms was prominent with the increased dose.

In the open field, all the animals treated at higher doses of test article showed slight to severely abnormal gait and slight to totally impaired mobility. Further, arousal was low or very low arousal and also revealed tonic/clonic movements. Severity of symptoms was more mid and high dose group animals reflecting clear evidence of dose dependent pattern. Such observations are primarily due to organophophorus poisoning **(WHO, 1986)**, the mod of action being the inhibition of the acetyl

cholinesterase **(Casida, 1963; O'Brien, 1967)**, and the signs and symptoms were typically cholinergic in nature.

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 test article treated groups' revealed exaggerated response to touch and click stimulus. No constriction of pupil was observed in majority of animals from high dose group. Majority of animals from high dose group landed on side or on back showing abnormal landing pattern in air righting reflex test. Also, majority of males and females from test article treated group did not respond to tail pinch and few responded slightly to tail pinch.

Hind limb grip strength and motor activity of test article treated group was reduced and hind limb foot splay was increased at peak time of effect on the day 1, however, on day 7 and 14 hind limb grip strength was comparable to control group. Similar observations were reported by **Mattsson *et al.*, (1996)** for chlorpyrifos another OP insecticide.

In the present study, a significant reduction in serum cholinesterase activity was noticed at 24th hour post dosing in test article treated group animals. Anticholinesterase agents act by inhibiting the activity of the cholinesterase enzyme **(Taylor, 1996)** further is an important marker to assess the exposure of an individual to chemicals, especially those which are neurotoxicants and cholinesterase inhibitots. It has been reported that profenofos is stereo specifically converted to a more potent inhibitor of acetyl cholinesterase by mouse liver microsomal mixed-function oxidase system **Wings, *et al.*, (1983).**

Observed neurobehavioural symptoms in mid and high 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 by 7 days of post exposure. Recovery from neurological symptoms by 7th day post dosing indicates the complete elimination of test article as it was evidenced by the observations of **Ifflander *et al.*, (1974),** where he dosed four male and three female rats a single dose of ring labeled-^{14}C profenofos

at 4.8 mg/kg b. wt. Within 6 days essentially the entire radioactive dose was eliminated in the urine (81.8 % in males and 96.4% in females) and faeces (15.7% in males and 2.5 % in females). Most of the urinary and faecal excretions occurred within the first 24 hours of dosing.

Mean body weight of animals from treatment groups were unaffected on day 7 and 14 of experiment. The reason could be fast recovery from the cholinergic symptoms.

Histopathology showed no evidence of treatment-related changes in either the nervous system or muscle. The possible reason could be quick elimination of test article from the body through urine and faeces, further, unavailability of sufficient quantity of chemical required to cause tissue damage. Similar observations were reported for chlorpyrifos, an OP insecticide by **Mattsson *et al.*, (1996)** in a single dose neurotoxicity screening study in rats; hence, 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.

For this study a total of 60 male and 100 female Wistar rats were obtained and randomly distributed to four groups (*i.e.*, G1, G2, G3 and G4) comprised 10 males and 20 females each. In addition 10 male and 10 female rats were assigned for recovery group. 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.

No treatment related clinical sign or change in body weight and food consumption was noticed. Hematological parameters estimated at the end of 90 days of dose administration did not reveal any change which is attributable to test article. Whereas, clinical chemistry estimations revealed increased levels of glucose, ALT, total bilirubin, creatinine, total protein and albumin and significantly reduced serum cholinesterase. Chronic exposure to organophosphorus pesticides is associated with decreased acetylcholinesterase activity and hepatic dysfunction **(Gomes *et al.*, 1999)**. 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).** 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).** 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)**. Generally, significantly increased total protein is seen in volume contraction, venous stasis, or in hypergammaglobulinemia **(Tietz, 1983).** It has been well established that organophosphorus insecticides cause acetlycholinesterase inhibition **(Stevens and Breckenridge, 2001)**.

Clinical chemistry estimations performed at the end of 28 days recovery period revealed increased glucose, total bilirubin and albumin and reduced AST values in high dose recovery group when compared to control recovery group. Even after withdrawal of treatment for 28 days, level of total bilirubin and albumin were found increased. This indicates that to recover from observed alteration it may require more time than 28 days.

In the present study, functional observational battery tests performed during 13th week of treatment did not reveal any functional abnormality in test article treated group. The possible reason could be due to the sub lethal doses used in the experiment.

Exposure to chemical substances can cause adverse effects on the male and female reproductive systems. Toxicants that target the female reproductive system can cause a wide variety of adverse effects.

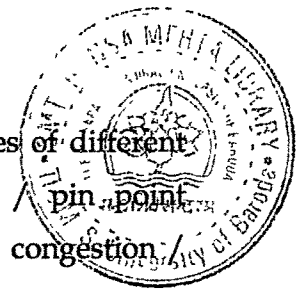
In order to study the effect of insecticide combination on reproductive end points such as mating behavior, organ weight, pathological changes on reproductive and other organs, male fertility index, female fertility index, parturition index, live birth index, pups survival index, litter weight and litter size, males and females treated for 90 days were allowed to cohabitate at 1:2 ratio. Treatment was continued during cohabitation, gestation, lactation through weaning.

In the present investigation, test article did not exert any adverse effect on estrous cycle and gestation period of females. **Chen *et al.*, (2002)** tested various organophosphates including fenvalerate for estrogenic potential and reported that they showed no estrogenic potential. Further, body weights and food consumption of test article treated females were remained unaffected during gestation and lactation periods. These observations are in line with the observations of **IBTL, (1978)** in a three generation rat study with profenofos.

Administration of the test article at the doses used in this study had no effect on: mating behavior, mean gestation length, gestation index, lactation index, live birth index and mean litter weight. Similar observations were reported in a two generation reproduction study of profenofos in rat by **US EPA (1999),** where, administration of profenofos at 0.36, 7.3, and 29 mg/kg body weight/day dose level did not affect mating behavior, mean gestation length and number of litters with live pups.

However, in the present study reduced survival index and mean litter size (both male and female pups) was observed, further, increased mortality of pups during lactation period at 38 mg/kg body weight dose group. This 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.

Absolute and relative weights of organs in parental animals did not show any changes. Whereas, in pups of test article treated groups, absolute, relative and relative brain weights of liver, kidney and spleen were significantly increased. Absolute and relative brain weights of test article treated groups were comparable to control group.



The gross pathology of the parental animals revealed varying degrees of different lesions in **liver** (hyperemic /congestion /consolidation /mottling / pin point hemorrhages / petechial hemorrhages), **spleen** (diffused white foci / congestion / atrophy / enlarged), and **kidneys** (pallor / congestion).

Histopathology of organs from parental animals revealed lesions in **liver** (congestion/minimal fatty changes centribular hypertrophy/multi focal degeneration / necrotic foci), **kidney** (cortical tubular dilatation with flattened epithelium/ congestion / multifocal regenerating tubules), **nerve** (demyelination / vacuolation/ axonal degeneration), **adrenals** (congestion /vacoulation), **brain** (inflammatory cell infiltration / gliosis), **ovary** (atretic follicle), **lymph nodes** (depleted germinal center / focal cyst), **pituitary** (congestion / focal increased acidophils), **spleen** (Congestion/lymphoid hyperplasia) and **thymus** (atrophy / 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.

Present study shows varying degrees of different gross lesions in kidneys, liver, brain, nerve and ovary in parental animals, which were more prominent in the test article treated groups and hence they may be considered as related to the administration of insecticide combination. 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.

Microscopic examination of different organs from test article treated group pups showed lesions in brain (mltiple 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).

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.