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Free radical mediated neurotoxicity induced by cypermethrin in rats

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Abstract
The present study was designed to investigate the neurotoxicity induced by cypermethrin in female and male albino rats. The rats were divided into three group, each group consisting of six rats. Control rats were given olive oil, while other two group of rats were orally intubated with sub-chronic does levels of $1/25^{th}$ and $1/50^{th}$ LD_{50} of CYP dissolved in olive oil respectively daily for four weeks. OS biomarkers viz; catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), gluthione-S-transferase (GST), and lipid peroxidation (LPO) levels were determined in brain. Brain function parameter, acetylcholinestrase (AChE) was also observed alongwith histopathological observations. Activity levels of CAT and SOD decreased while GST, GR, GPx and LPO increased in brain of CYP treated rats. CYP treated female (1.862±0.12 µmoles) and male (1.920±0.06 µmoles) rats at both the doses showed significant inhibition in the ATPase activity in brain as compared to control rats (3.417±0.18 and 3.494±0.11 µmoles) respectively. Cypermethrin treatment decreased the levels of acetylcholinesterase (AChE) in a dose-dependent manner in plasma and brain of female and male rats. Histomorphological studies further revealed number of abnormalities as extensive neuronal damage, marked neuronal degeneration; neurons decreased in number and shrunken with slight vacuolation, pyknotic nuclei, apoptosis nuclei and dilated blood vessels (DV). The results infer that exposure of female and male albino rats to CYP leads to number of pathophysiological conditions in the brain at dose dependent manner.

Keywords: Acetylcholinesterase, ATPase, brain, cypermethrin, oxidative stress, plasma

1. Introduction
Environmental contamination is a global public health issue as the irrational use of pesticides intended for managed agricultural and other domestic practices not only produces adverse biological effects against the target species but also have the potential to affect the health of non-target species [1-3]. Pyrethroid pesticides have emerged as a major class of highly active efficacy and relatively low toxicity compared to organochlorine and organophosphorous pesticides [4].

Cypermethrin-induced neurotoxicity is indeed a matter of concern, as mammals are exposed to it in their day-to-day life [4]. Despite variability in neurotoxicological response due to various factors, such as doses, time and routes of exposure and model organisms, it behaves as a fast-acting neurotoxin in insects [5]. Cypermethrin cause neurotoxicity in mammals and insects through the inhibition of acetyl-cholinesterase (AChE), which leads to the accumulation of acetylcholine [6] and have been found toxic to axonal transport of neurons alongwith neuroinflammation and cognitive impairment [7-9]. Neurotoxicity can also be caused by a long-lasting prolongation of the normal transient increase in sodium permeability of nerve membrane during excitation causing voltage-dependent sodium channels to stay open for much long than normal [10]. These nerve impulses cause the nerves to release the neurotransmitter acetylcholine and stimulate other nerves [11]. Furthermore, CYP-induced neurotoxicity is contributed by its ability to produce free radical generation via changes in the normal antioxidant homeostasis that results in the depletion of antioxidants. CYP readily enters the brain and induces oxidative stress leading to dopaminergic neurotoxicity [12, 13].

Oxidative stress is one mechanism that has been assessed as possible link for pesticide exposures to number of health outcomes observed in epidemiological studies [11]. Pyrethroid toxicity alters the antioxidant enzyme levels due to cell stress with the involvement of free radical intermediates [14, 15]. Pyrethroid induced OS, is also evidenced by increased level of malondialdehyde (MDA) *i.e.* end product of lipid peroxidation (LPO) and by differentially modified endogenous antioxidants like catalase (CAT), glutathione-S-transferase (GST),

superoxide dismutase (SOD) and glutathione peroxidase (GPx), which can lead to development of moderate to severe pathophysiological changes [11, 16]. Several studies demonstrated that it provokes a range of physiological, biochemical and toxicological changes in different experimental animals [10]. Therefore, the present study was undertaken to evaluate the degree of injury induced by cypermethrin in brain of rats following its repeated oral administration. Acetylcholinesterase (AChE) activity was evaluated to relate its changes with oxidative stress and toxicity.

2. Materials and Methods

2.1 Chemicals

All the chemicals were purchased from SDFCL (SD Fine-Chem Ltd) and SRL (Sissco Research Laboratories Pvt. Ltd). All chemicals used were of analytical grade. Cypermethrin (Rallis India Limited, Secunderabad, Hyderabad) having 25% Emulsifiable Concentrate (EC) was used in the present study.

2.2 Animals

The female and male albino rats of 8–10 weeks age and weighing 100– 150 g were procured from the Department of Livestock Production and Management, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. Rats were housed in polypropylene cages with paddy husk bedding in laboratory, where the humidity (50 ± 5%), temperature (25 ± 2˚ C) and a normal photoperiod of 12–12 h light–dark cycle were environmentally controlled. Rats were provided the standard rat feed and water ad libitum. All methods and procedures of animal handling used in research were in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and experiment conducted in the present study was duly approved by Institutional Animal Ethics Committee (IAEC), GADVASU, Ludhiana vide letter no. 212-237 dated 11.09.2015.

2.3 Experimental design

This two year study was carried out in Punjab Agricultural University. Female and male albino rats were acclimatized for 10 days in laboratory conditions and were divided into three groups each consisting of six rats. The first group of rats serving as control were given only olive oil and to the remaining two groups, cypermethrin was given at a dose level of $1/25^{th}$ and $1/50^{th}$ of LD_{50} by oral intubation for 30 days.

2.4 Sample preparation

After 30 days of treatment, the rats were fasted overnight and were anaesthetized before dissection. After dissection, blood samples from rats were collected directly from heart in heparinised vials and centrifuged at 2300 r.p.m. for 15 minutes. Supernatant was obtained as plasma to estimate acetylcholinestrase activity. Brain was excised immediately and cleared off the adhering tissue. 0.5 g of brain was homogenised in 0.1 M PBS (pH 7.4), centrifuged and supernatant was obtained. Brain supernatant were used for the biochemical parameters which were assayed by standard methods viz., acetylcholinestrase activity by Voss and Sachsse [17], total proteins by Lowry *et al*. [18], CAT (catalase) by Aebi [19], SOD (Superoxide Dismutase) by Marklund and Marklund [20]. GST (glutathione-Stransferase) by Habig *et al*. [21], GR (glutathione reductase) by Carlberg and Mannervik [22], GPx (glutathione peroxidase) by Hafeman *et al*. [23], LPO (Lipid peroxidation) by Stocks and Dormandy [24] and ATPase activity by the method of Dhanya *et al*, [25].

2.5 Histological studies

Brain tissue was placed in alcoholic Bouin's fixative for 24 hours and then dehydrated in graded series of alcohols, cleared in benzene and embedded in paraffin wax (melting point 58-60°C). The 5µm thick sections were cut using microtome and stained with haematoxylin and eosin and slides were observed under OLYMPUS CH20i microscope and photographed.

2.6. Statistical analysis

The experimental results are expressed as mean ± standard error of the mean (SEM) for n=6. Statistical analysis of data was performed by using CPCS1 and one-way ANOVA was done to check any significance and the criterion for statistical significance was set at $P < 0.05$.

3. Results

3.1 Body weight and organ weight

No statistically significant change in body weight was observed in all the experimental group rats, and reduced growth rate was observed in cypermethrin treated rats as compared to control at P<0.05. Weight of brain was comparable in all the experimental group rats in comparison to control rats (Table 1).

Table 1: Effect of cypermethrin treatment on weight (g/100g b.w.) and growth rate of treated female and male wistar rats

	Female			Male		
	Control	**$1/25^{th}$ of LD_{50}**	**$1/50^{th}$ of LD_{50}**	**Control**	**$1/25^{th}$ of LD_{50}**	**$1/50^{th}$ of LD_{50}**
Initial b.w.	160.00±12.25	158.00±8.85	155.00±6.19	111.25±16.63	112.00±5.77	104.50±11.09
Final b.w.	188.75±12.77	179.00±10.95	176.51±9.18	135.50±21.68	131.75±8.78	125.50±5.07
Growth rate	0.69±0.19	0.49±0.08*	0.47±0.08*	0.93±0.11	0.77±0.10*	0.86±0.03*
Brain	1.60±0.05	1.40±0.11	1.46±0.07	1.24±0.13	1.01±0.09	1.19±0.02

Mean ± SE values of 6 animals in each group.
*Significant difference at p<0.05 as compared to control

3.2 Biochemical observations

The present investigation of brain revealed that total protein content was comparable in all experimental female and male group rats at P<0.05. CAT and SOD activity levels were reduced in CYP treated female and male rats. GST, GPx and GR levels increased non-significantly in CYP treated female rats while the increase was found to be significant in treated male rats. LPO in terms of MDA levels were significantly high in CYP treated female and male rats as compared to control rats at P<0.05 (Table 2). CYP treated rats at both the doses showed significant inhibition in the ATPase activity in brain as compared to control rats. The brain of CYP treated female and male rats showed significant inhibition in the activity of AChE at higher dose as compared to control group rats at P<0.05 (Table 2). AChE inhibition was found to be non-significant in plasma of all the CYP treated rats (Fig. 1).

Table 2: Effect of cypermethrin on brain acetylcholinesterase (AChE), ATPase and antioxidant activity of female and male rats.

	Female			Male		
Parameters	**Control**	**1/25th of LD_{50}**	**1/50th of LD_{50}**	**Control**	**1/25th of LD_{50}**	**1/50th of LD_{50}**
Total soluble protein	4.176±0.07	3.677±0.09	4.091±0.11	5.756±0.49	4.764±0.99	5.004±1.02
CAT	17.132±1.01	15.652±1.10	16.185±0.65	16.716±2.23	12.395±1.29	12.699±1.231
SOD	4.921±0.40	4.504±0.83	4.512±1.22	4.627±0.99	3.239±0.59	3.701±0.99
GST	0.064±0.016	0.076±0.021	0.067±0.012	0.064±0.013	0.083±0.009*	0.0785±0.009
GPx	0.416±0.044	0.484±0.073	0.457±0.034	0.561±0.051	0.803±0.078*	0.605±0.031
GR	0.006±0.000	0.008±0.001	0.007±0.001	0.007±0.000	0.009±0.001	0.008±0.000
LPO	3.081±0.244	6.442±0.152*	5.762±0.286*	3.465±0.226	6.790±0.647*	5.123±0.444*
ATPase	3.417±0.18	1.862±0.12*	2.630±0.20*	3.494±0.11	1.920±0.06*	2.361±0.28*
AChE	696.90±34.94	504.55±30.19	617.05±28.18	707.74±29.69	495.36±25.69*	653.21±20.96

Mean ± SE values of 6 animals in each group.
*Significant difference at $p<0.05$ as compared to control

Units: AChE (U/L), ATPase (µmoles Pi h-1mg-1 protein), Protein (mg/g wet wt. of tissue), CAT (µmole of H_2O_2 decomposed/min/mg protein), SOD (U/mg protein), GST (µmoles of GSH-CDNB conjugate formed/ min/mg protein), GR (µmoles of NADPH oxidized/ min/mg protein), GPx (U/mg protein), LPO (nM MDA/100 mg tissue).

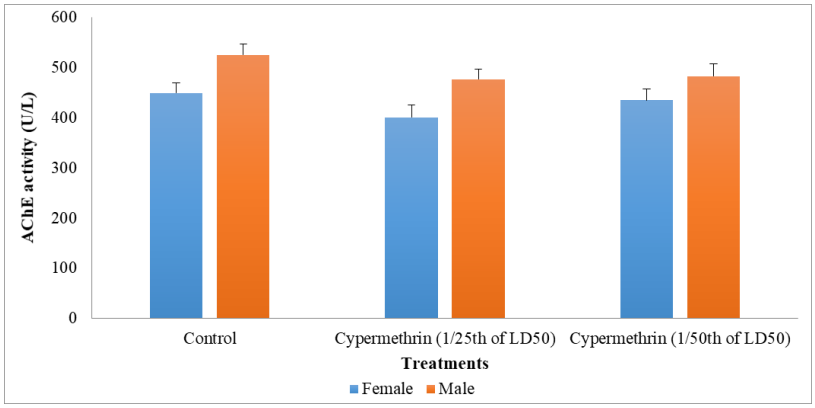

Fig 1: Effect of cypermethrin on acetylcholinesterase (AChE) in plasma of treated female and male rats

3.3 Histological observations

Histopathological examination of the brain sections in the control female and male albino rats showed normal neuronal structure i.e. neurons with the surrounding supporting cells, normal nuclei which showed dispersed chromatin, prominent nucleoli, cytoplasm and microglial cells (Fig 2. A&D).

The female and male rats treated with CYP (1/25th of LD_{50} and 1/50th of LD_{50}) showed extensive neuronal damage, marked neuronal degeneration; neurons decreased in number and had indistinct boundaries. Neurons appear smaller and shrunken with slight vacuolation. Pyknotic nuclei, apoptosis nuclei and dilated blood vessels (DV) are some of the significant histologic changes observed in the normal tissues, clearly indicating that CYP is a biological toxin in the animal. The effects were more severe for the higher dose of CYP as compared to the lower dose (Fig. 2- B, C, E & F)

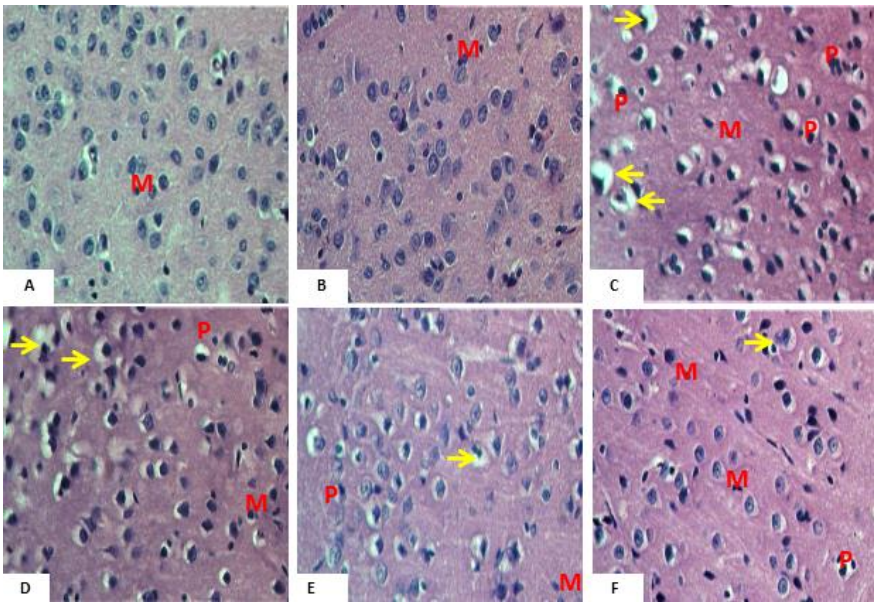

Fig 4: Effect of CYP induced toxicity (Magnification: 400x, Stain: Eosin and hematoxylin) Arrows showing neuron swelling and vacuolation;, P: Pyknotic degenerating neurons; M: microglial neurons. A&D: Brain sections of control female and male rats respectively. B&E: Female and male rats treated with 1/50th of LD_{50} of CYP. C&F: Female and male rats treated with 1/25th of LD_{50} of CYP

4. Discussion

Brain is considered highly vulnerable to oxidative stress than any other organ of the body as it consumes high amount of oxygen, contains high amount of polyunsaturated fatty acids (PUFA) and low level of antioxidant enzymes [26]. Although considered to be safe, there are some recent reports on neurotoxicity of cypermethrin in animal models. Presence of PUFA in brain and lipophilic nature of cypermethrin makes the brain most prone target [11]. In this study decrease in weight of brain was observed in rats exposed to cypermethrin which can be due to its accumulation that may cause brain damage [11]. CYP at daily oral dose of 5 mg/kg b.w. significantly decreased the relative weight of brain in female and male rats. Non- significant decrease was also observed in relative brain weight of rats exposed to 3.83 mg/kg b.w. CYP for 14 days [11]. However, Sayim *et al* [10] observed no significant change in brain weights of rats treated with CYP (5 mg/kg b.w.). The present investigation showed slightly decreased growth rate with CYP treatment. The present results are in accordance with Ratnasooriya *et al.* [27], Hussain *et al.* [28] and Adjrah *et al.* [29], which suggested that CYP intoxication leads to a decreased body weight and growth rate in animals.

Pesticide accumulation in tissue is associated with induction of oxidative stress and production of ROS [30]. In the present study CAT level was decreased, which may be due to the excessive production of superoxides anions after cypermethrin exposure [11]. Similar observation for CAT has been reported in brain tissue of *Rattus rattus* collected from Bathinda district which is known to be pesticide contaminated region [31]. Significant reduction in SOD activity was observed in brain of rats exposed to cypermethrin in the present study. Reduction in SOD level may be due to excessive ROS. The excessive ROS may attack the thiol group of cysteine residues and PUFAs of biological membranes leading to cell damage [32]. Reduction in SOD shall further increase ROS which in turn inactivated CAT. Ali [33] also illustrated that a daily administration of LTC (lambda-cyhalothrin) at two doses for 4 weeks significantly decreased the activity of CAT and SOD in a dose dependant as compared with that of the control rats. The depression of antioxidant enzyme activities reflects failure of the antioxidant defense mechanisms to overcome the ROS induced by pesticide exposure that leads to the accumulation of free radicals which facilitate the enhancement of LPO, which furthur increases the oxidative damage to the brain tissue [34].

Glutathione-S-transferase (GST) and Glutathione reductase (GR) is thought to be the fundamental antioxidant enzymes. GST catalyzes the conjugation of GSH via a sulfhydryl group to electrophilic centers on a wide variety of substrates. This activity helps in detoxification of breakdown products of pesticides [11]. Cypermethrin exposure enhances GST, GPx and GR levels in rat brain in this study, the enzyme which plays a primary role in minimizing oxidative damage. The changes in these oxidative stress biomarkers have been reported to be an indicator of tissue's ability to cope with oxidative stress. ROS has also been known to decrease the detoxification system produced by GST [35].

Malondialdehyde (MDA) is an end product in lipid peroxidation including phospholipids in the cell membrane and enhanced levels of MDA is an biomarker of oxidative stress. The increase in LPO levels suggest an inappropriate antioxidant defense which could be due to both increase in pesticide-induced ROS formation and SOD inhibition [11].

Pesticides are known to alter the activities of Na^+/K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase [36, 37]. The inhibition of ATPase activities may be a causative factor of neuronal/cellular dysfunction, due to alteration in cationic transport across the membrane and disturbance in uptake as well as release of certain neurotransmitters. Decrease in ATPase activities by CYP exposure in the present study is, likely to alter the ionic flux and thus disturb the normal neuronal functions [38].

AChE is an enzyme that is essential for the normal functioning of the central and peripheral nervous system [39]. Moderate to severe sign of neurobehavioral alterations and AChE inhibition were observed in cypermethrin treated rats. Decrease in the AChE activity in rat brain was also observed on B-cyfluthrin and chlorpyrifos and deltamethrin administration. Cypermethrin treated rats showed cholinergic activity such as salivation, muscles twisting followed by tremors, progressive stretching of hind limbs and immediate thirst for water [11]. The severity of symptoms, thus, correlates with the extent of AChE inhibition and depicts the duration-dependent neurotoxicity of cypermethrin. AChE could be inhibited due to oxidative damage as well as the occupation of its active sites by insecticides [40].

It has also been established that CYP intoxification produced significant histopathological changes in the brain of rats [41].The increase in the CYP dose (250 mg/kg b.w.), for 28 days caused slight histopathological alterations in the brain tissue. These changes manifested due to pyknosis of neuro cells in various areas of brain and in the cells of stratum granulosum and stratum pyramidale hippocampi [42]. The rats treated with CYP showed dilated blood vessels, cerebral edema and necrosis with hemorrhage due to 50mM CYP and carbendazim intoxification at 12 and 24hr [44].

5. Conclusion

The present study results indicated marked changes in plasma and brain in response to cypermethrin, which could be due to its toxic effects primarily by the generation of ROS, causing damage to the various membrane components of the cell. This study suggests the usefulness of these biomarker for providing an integrative measurement of the overall neurotoxic and toxicity risk arising from the burden of cypermethrin in rats.

6. Acknowledgments

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7. Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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