



ORIGINAL ARTICLE

Neurobehavioral Alterations Induced by Cyfluthrin in Male Swiss Albino Mice

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ABSTRACT

β -Cyfluthrin, is a broad-spectrum type II synthetic pyrethroid insecticide. People are exposed to cyfluthrin by consuming contaminated food and from residues persisting after indoor or outdoor applications of cyfluthrin containing products. The aim of this study was to evaluate the neurobehavioral toxicity of cyfluthrin after oral administration to Swiss albino mice. Male weanlings (21 days old) were selected and divided into two groups. Group I received low dose i.e. 14.55 mg/kg (1/20 of LD₅₀) and Group II received high dose i.e. 29.10 mg/kg body weight (1/10 of LD₅₀) upto postnatal day (PND) 60. Control animals received only the vehicle i.e. corn oil, by oral intubation. All the animals were observed daily for food consumption; observations for locomotory, exploratory and emotional behaviors were taken on every alternate day, by using open field arena and hole-board. Learning and memory was assessed with Hebb-William's maze. On PND 60 the animals were sacrificed and brain was dissected out. The neurosomatic index i.e. brain weight to body weight ratio was estimated. Acetylcholinesterase enzyme activity in brain tissue was evaluated spectrophotometrically. Pesticide administration to mice caused cholinergic signs viz. excitability, salivation, tremors and lethargy. Learning and memory in treated animals decreased. A significant decrease in open field activity and exploratory behavior occurred. Emotional behavior was also affected as indicated by increase in fecal boles and frequency of urination, which is a sign of anxiety. Neurosomatic index was lowered by cyfluthrin. Acetylcholinesterase activity in brain was significantly decreased. Exposure to animals to β -cyfluthrin thus elicited significant changes in neurosomatic, neurochemical and neurobehavioral parameters studied.

Keywords: Cyfluthrin, learning, acetylcholinesterase, exploratory behavior, mice.

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INTRODUCTION

Brain is the most important organ in the animal body which is a site of controlling and coordinating with other vital organs and their functions. It has centers for controlling all physiological activities of the body. In both active and steady state of the animal, the brain is always active; however, its functioning can be altered by the effects of any xenobiotics. [1].

Pesticides exert harmful effects on many non-target organisms including humans. Synthetic pyrethroids are commonly used as potent means for pest control and account for 30% of all insecticides used worldwide for agricultural, domestic and veterinary uses [2]. Some studies have reported that pyrethroids affect the behavior of adult mammals [3] by interfering with the nerves and brain functioning. High doses of pyrethroids cause numbness, itching, burning, stinging, tingling, or warmth that could last for a few hours [4]. They are also known to cause dizziness, headache, nausea, muscle twitching, reduced energy, convulsions and loss of consciousness that could last for several days. Cyfluthrin is a relatively new synthetic pyrethroid insecticide which is the active ingredient of many insecticide products. It is used for a wide array of pests in agriculture, in and around the home, and in food handling establishments [5-8]. Its ISO approved common name is 3-(2,2-Dichloro-vinyl)-2,2-dimethyl-cyclo propane-carboxylic acid cyano-(4-fluoro-3-phenoxy-phenyl)-methyl ester, [9]. Like all synthetic pyrethroids, cyfluthrin is a neurotoxicant. It causes hyperexcitation of the nervous system, which leads to convulsions and ultimately death [10]. It induces alterations in nerve membrane, causing abnormal sodium and potassium flow, resulting in repetitive discharges from the neurons, causing convulsions and also blockage of further nerve impulses [11]. Cyfluthrin also affects calcium

concentration in nervous tissue by inhibiting Ca⁺ ATPase involved in calcium transport. This in turn increases the amount of the neurotransmitter acetylcholine released at the junction between nerves [12, 13]. Acetylcholinesterase (AChE) enzyme and the peripheral nervous system play important role in behavioral outputs of the animals and appear to be most vulnerable to synthetic pyrethroids [14].

Behavior of an animal is the output of interaction and coordination of central nervous system (CNS) and peripheral nervous system (PNS). Acetylcholinesterase plays an important role in propagation of nerve impulses to neuromuscular junctions by hydrolyzing the neurotransmitter acetylcholine into choline and acetate. Pyrethroids inhibit this enzyme by affecting its catalytic site, which results in accumulation of acetylcholine in synaptic clefts leading to non-availability of choline for its resynthesis [15, 16].

The present study was undertaken to investigate neurobehavioral alterations in mammalian model i.e. Swiss albino mice by β -cyfluthrin. To assess learning, memory, locomotory, exploratory behaviors and emotionality viz. anxiety and response to stress in animals, a battery of tests is needed. The neurotoxic effects were analyzed by a number of neurobehavioral parameters, neurosomatic index and AChE activity in brain.

MATERIALS AND METHODS

Test compound

Technical grade β -cyfluthrin PESTANAL® (CAS 68359-37-5), obtained from sigma Aldrich Laborchemikalien GmbH D-30918 Seelze, was selected for treatment.

Animals

Swiss albino mice were obtained from the Indian Veterinary Research institute, Bareilly (UP, India), and were housed in air cooled room. The animals were kept in polypropylene cages with bedding of wood savings and covered by steel wire grid. The colony was maintained on standard mice feed obtained from Hindustan Lever Ltd., New Delhi and tap water was provided *ad libitum*. An inbred colony was maintained. In the animal room, temperature was maintained at 28±2°C, relative humidity at approximately 30-40% and a 12 hrs light-12 hrs dark photoperiod. The pregnant females were observed for parturition and day of birth of the offspring was recorded as postnatal day 0 (PND 0).

The experiments were performed according to the guidelines for care and use of animals in scientific research of the Indian National Science Academy (2000), New Delhi and approved by Institutional Ethical committee (1678/GO/a/12/CPCSEA).

Treatment

Male weanlings having body weight 11±2 g were selected for the study at postnatal day 21. They were divided into three groups according to the dose of cyfluthrin administered. The reported oral LD₅₀ of β -cyfluthrin for male mice is 291 mg/kg body weight (U.S. EPA., 1987). One group of 6 animals was given Low dose (LD) of cyfluthrin (1/20 of LD₅₀) i.e. 14.55 mg/kg and another group received High dose (HD) i.e. 29.10 mg/kg of body weight (1/10 of LD₅₀). β -cyfluthrin is lipophilic in nature hence hydrophobic medium i.e. corn oil was used as vehicle.

Pesticide was administered to experimental animals by oral gavage from PND 21 to PND 60 once daily, and control group received vehicle only i.e. corn oil. Animals were observed daily for clinical signs of toxicity and food consumption was noted daily.

During the treatment period behavioral observations were taken on every alternate day. The animals were subjected to the following behavioral tests:

Open field arena (for locomotary parameters) – The open field was constructed from plywood (painted white) and measured 60×60 cm, with 15 cm high walls. On the floor 64 squares, each 7.5×7.5 cm were made using black paint. In such an arena, the overall level of locomotion and time spent in the centre of the arena (which is assumed to be aversive to rodents) are interpreted as measures of exploratory behavior. For the observations each animal was placed in the centre of the arena and the following parameters were noted for 2 minutes each (i) locomotor activity (total number of squares entered by the animal with all four paws) (ii) rearing frequency (number of times the mouse rose onto its hind paws) (iii) grooming frequency i.e. number of times the animal touched or rubbed its snout with its paws [17]. For judging emotional status of animals, the time spent in the centre by the animal was recorded. The frequency of urination and number of fecal boli deposited on the board were counted.

Hole board (for exploratory behavior) - This is used for measuring the response of the animal to an unfamiliar environment. A hole-board i.e. a 60×60 cm wooden box with the floor having holes at random positions was used. The number of times each animal dipped its nose in the holes was counted. The time duration for observations was 2 minutes for each animal [18].

The observations with the open field and hole board were scored using manually operated counter and stop watch and movements were recorded in the camera. The observations were taken at the same time

of the day between 11.00 am to 1.00 pm on every alternate day. The arena was cleaned by alcohol solution before testing each animal to remove the odor left by previous animal.

Hebb-William's maze (For learning) - Hebb-William's maze is used to study the spatial and latent memory in rodents [19]. This maze consists of two ends having rooms with doors; and a zigzag pathway that contains different roads ending blindly, only one leads to correct pathway. Animals were deprived of food for a day and observations were taken on next day. Each animal was placed in one room of the maze and the door was closed. After one minute door was opened and the animal was allowed to explore the maze to reach the other end having food as reward. The time taken to reach the food was recorded and entry into blind ends or alleys was recorded as an error. The maze was cleaned by alcohol solution before testing each animal to remove the odor left by previous animal.

On PND 60, the animals were weighed and sacrificed by cervical dislocation; brain was removed and weighed. Neurosomatic index was calculated and acetylcholinesterase activity in brain was evaluated:

Neurosomatic index - Brain/body weight ratio was calculated. Comparative increase in body weight on PND 60 - PND 21 was calculated.

Acetylcholinesterase - The activity of the acetylcholinesterase (AChE) enzyme in the brain tissue was measured spectro-photometrically using the method given by Ellman's *et al.*, (1961). The enzyme activity is measured by following the increase intensity of yellow color produced by the action of thiocholine when it reacts with dithiobisnitrobenzoate ion (DTNB). The intensity was measured at 412 nm by using a sensitive colorimeter. AChE activity in moles of substrate hydrolyzed/minute/gm of tissue calculated.

Statistical analysis - The behavioral and biochemical estimations data were statistically analyzed by analysis of variance (ANOVA) using a statistical software SPSS 10.0. The data is expressed as Mean \pm Standard error. One way ANOVA Multi comparisons Post Hoc test followed by Tukey Honest Significant difference (HSD) was used for comparison between treated and control group and within groups. The level of significance $p < 0.05$ and $p < 0.01$ were set as significant and highly significant respectively.

RESULTS

Signs of toxicity were observed viz. burrowing behavior, scratching, blinking of eyes, lacrimation, excessive salivation, excitation, aggressiveness, tremors, lethargy and decreased food intake after pesticide administration. However, intensity of symptoms was mild to severe in different dose groups.

Neurosomatic Index

The body weight of animals significantly decreased ($p < 0.01$), in both treatment groups as compared to controls. Brain weight was not significantly affected (Table 1). However, neurosomatic index i.e. brain/body weight ratio was affected significantly in Group II (0.016 ± 0.001) as compared to control (0.013 ± 0.001). The low dose did not affect this ratio as shown in Fig 1, Table 1. Increase in body weight from PND 21 to PND 60 was significantly affected ($p < 0.01$) by treatment (Table 1).

Spontaneous behavior in open field

Fig. 2 shows the activity of animals in the open field. The various locomotory parameters were studied which showed that activity of control animals increased during the time period whereas it decreased in treatment groups.

Number of floor squares crossed by animals in 2 minutes decreased in dose dependent manner at the level of $p < 0.01$. It was noticed throughout the period that the animals preferred to stay at the periphery (Fig 3) Exposure to cyfluthrin significantly decreased the *rearing frequency* (Group I, 9.16 ± 0.27 ; Group II, 7.14 ± 0.37) as compared to control (11.42 ± 0.36). Likewise *grooming frequency* was also significantly reduced by cyfluthrin treatment ($p < 0.01$) as seen in Fig. 2.

Exploratory behavior of the animals

Exploration was measured by hole-board and is represented in Fig. 3. There was a significant decrease in mean nose dip counts as compared to control animals (9.37 ± 0.06 in Group I; 8.01 ± 0.22 in Group II vs control, 11.26 ± 0.17).

Emotional behavior or anxiety

Emotional status of the animals judged by measuring the frequency of urination and counting the fecal boles deposited on the floor which increased significantly (Fig. 4) in both treatment groups in dose dependant manner as compared to control (Table 2). These parameters showed anxiety in treated groups.

Learning and memory

Learning ability of mice was judged by Hebb- William maze. The time taken by animals to reach the food as reward significantly ($p < 0.01$) increased in the high dose group (46.85 ± 3.26) as compared to control group (29.92 ± 1.50), but was not significant in low dose group. However, the errors in reaching the goal significantly increased in both treatment groups as shown in the Fig.5 and Table 5.

Achetylcholinesterase activity

Acetylcholinesterase activity was calculated as moles of substrate hydrolyzed i.e. break down of acetyl choline per minute per gram of brain tissue. In this study AChE activity in brain tissue decreased significantly in both the treated groups (Table 2). Decrease in this activity was also dose dependant as seen in the Fig. 6.

Table 1: Effect of cyfluthrin on Neurosomatic Index of male Swiss albino mice.

Dose	Control Group (received Corn oil)	Low dose group (14.55 mg/kg of cyfluthrin)	High dose group (29.10 mg/kg of cyfluthrin)
Weight			
Body weight (g)	27.83±0.30	25.16±0.30**	22.16±0.30**
Brain weight (g)	0.31±0.019	0.34±0.002	0.36±0.017
Brain/Body weight	0.0134±0.0007	0.0135±0.00013	0.0163±0.0007**
Body weight increase (PND 60 - PND 21)	16.33±0.33	13.66±0.42**	10.50±0.42**

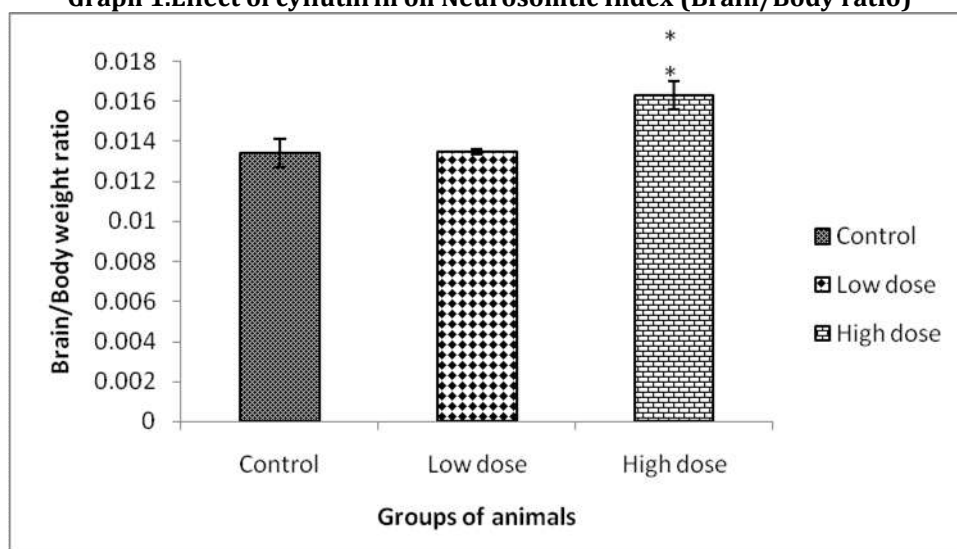
*P<0.05, **P<0.01

Table 2: Effect of cyfluthrin on Behavioral parameters of male Swiss albino mice.

Behavioral Tests	Dose	Control Group (received Corn oil)	Low dose group (14.55 mg/kg of cyfluthrin)	High dose group (29.10 mg/kg of cyfluthrin)
Open field studies	Squares crossed/2min.	70.96±3.2	54.41±2.09**	43.55±1.64**
	Rearing	11.42±0.36	9.16±0.27**	7.14±0.37**
	Grooming	3.22±0.1	1.89±0.65**	1.55±0.09**
Hole - board	No. of nose dips/2 min.	11.26±0.17	9.37±0.06**	8.01±0.22**
	Time spent in centre	2.53±0.12	2.01±0.12**	0.9±0.02**
Emotional behavior	Urination	1.1±0.11	1.93±0.13**	3.1±0.21**
	Defecation	3.71±0.22	5.64±0.41**	8.32±0.11**
Learning	Time taken to find the goal (sec.)	29.92±1.5	36.53±2.41	46.85±3.26**
	Errors	7.07±0.34	9.93±0.58*	13.08±1.11**
Biochemical Parameter of brain	AchE activity (moles/hydrolyzed/ min./g of tissue.	20.14±0.68	8.58±0.49**	4.65±0.45**

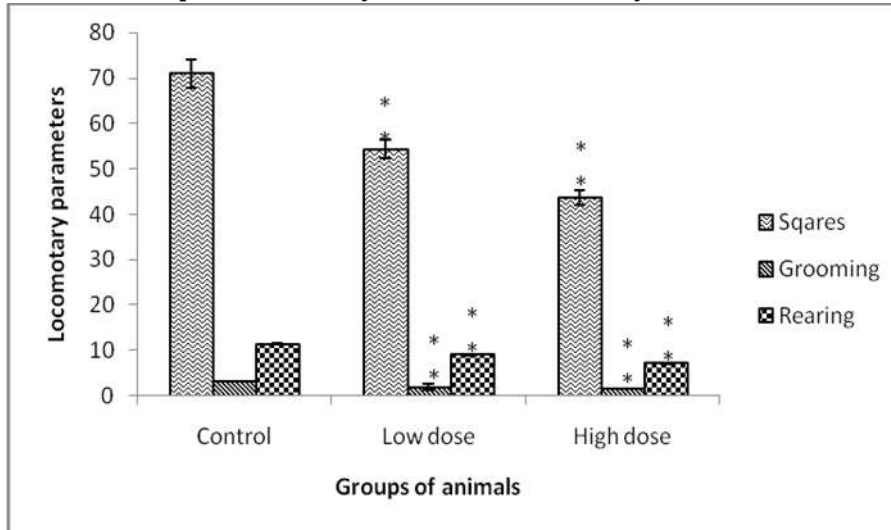
*P<0.05, **P<0.01

Graph 1. Effect of cyfluthrin on Neurosomatic Index (Brain/Body ratio)



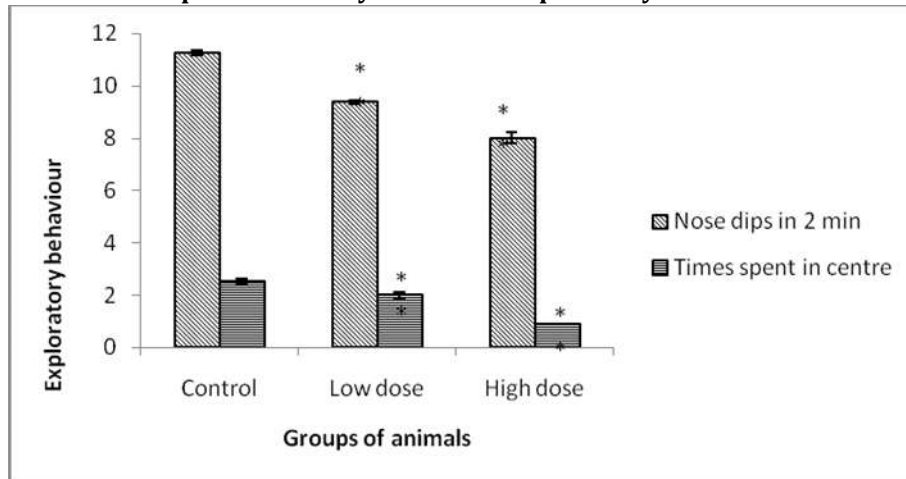
Level of significance * P<0.05, **P<0.01

Graph 2. Effect of cyfluthrin on locomotory behaviour



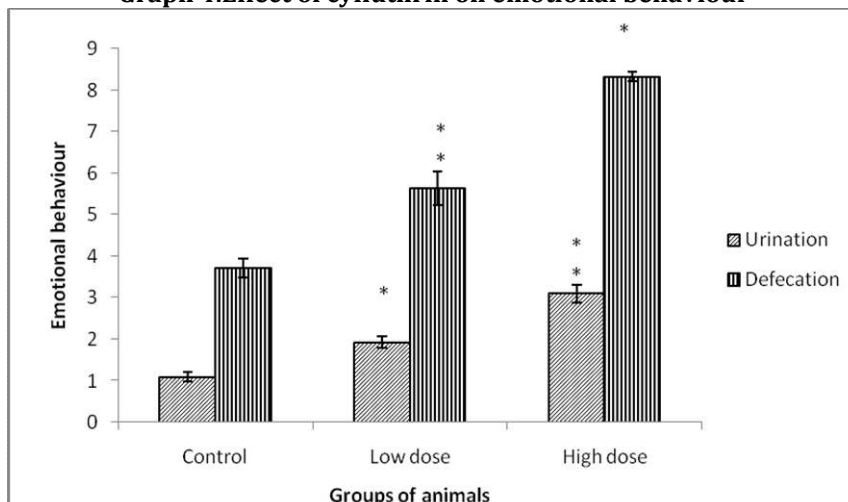
Level of significance * P<0.05, **P<0.01

Graph 3. Effect of cyfluthrin on exploratory behaviour

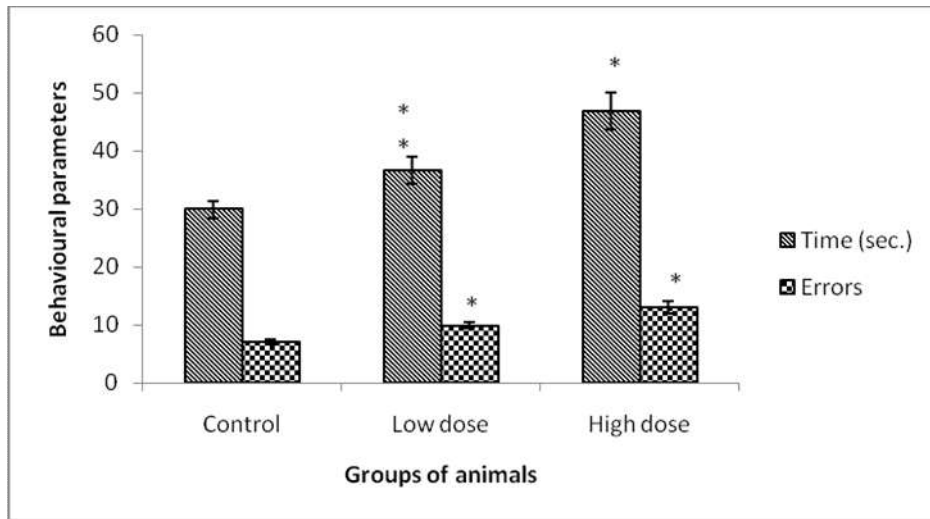


Level of significance * P<0.05, **P<0.01

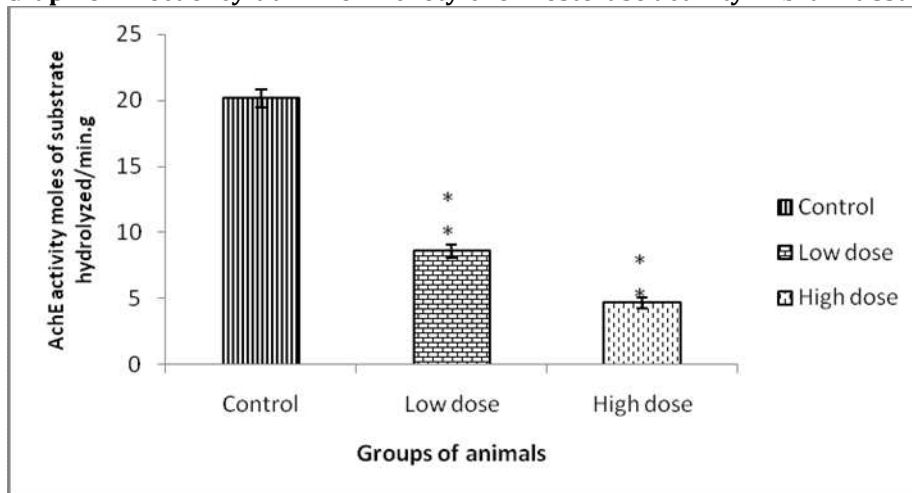
Graph 4. Effect of cyfluthrin on emotional behaviour



Level of significance * P<0.05, **P<0.01

Graph 5. Effect of cyfluthrin on learning and memory

Level of significance * $P < 0.05$, ** $P < 0.01$

Graph 6. Effect of cyfuthrin on Achetylcholinesterase activity in brain tissue

Level of significance * $P < 0.05$, ** $P < 0.01$

DISCUSSION

Cyfluthrin induces salivation, incoordination, muscle trembling, jerky movements, behavioral changes and convulsions. It was chosen for the study due to its widespread use in India [20]. It is sold under the brand name Baygon and Solfac [21]. It is common household insecticide used to control houseflies, cockroaches and mosquitoes [22].

After administration of cyfluthrin, animals showed behavioral changes like shivering, scratching, burrowing behavior (dip their head on to saw dust), salivation, lacrimation, some of which were for short duration and some were permanent. This may be due to reversible and non reversible damage on nervous system. Similar signs were observed by Sheets, [23] with pyrethroids. They also reported hypothermia, repetitive chewing and pawing movements, gait incoordination, flattened posture and decreased activity [24]. Pyrethroids are reported to reduce previously learned behavior and decreased memory [25, 26].

In this study, a decrease in body weight was observed, which is correlated with the decrease in food intake in dose dependent manner. Other pyrethroids also have been reported to decrease body weight [27, 28]. The reduction in weight gain may be due to effect of pesticide on gastrointestinal tract, resulting in decreased appetite and absorption of nutrients from gut [29]. This may also be due to direct cytotoxic effect of the pesticide on somatic cells or indirectly through the central nervous system which controls the food and water intake and regulates the endocrine function [30].

Quantitative measurements of motor activity are the interaction between the test subject and the testing apparatus; motor function is considered behavioral domain which may be impaired by pyrethroids.

Spontaneous behavior is movement of the animal in the absence of any intervention which influences the willingness of the animal to move during the test period. Motor activity is the summation of movements of the animals whether it is horizontal or vertical. Locomotor activity refers to ambulation, i.e. a change in the animal's position in enclosed device (in this study it was open arena) by walking, circling, running and rearing [31], whereas, non locomotor activity refers to behaviors such as scratching, pawing, grooming and sniffing [6]. Nicotinic acetylcholine receptors are the mediator of fast excitatory neurotransmission at vertebrate neuromuscular junctions and in central nervous system [32]. Pyrethroids are found to inhibit these receptors which lead to blockage of neurotransmission which may be a cause of uncoordination between muscles results into uncoordinated or impaired movement of organs [33].

In the open field test, there was a significant decrease in total activity as indicated by the decreased number of squares crossed by the animals [17] and rearing frequency (animal standing on its hind limbs) also decreased by the treatment. Similar results were reported due to treatment of rats with bifenthrin; permethrin, cypermethrin, cyhalothrin, bifenthrin, cyhalothrin and permethrin [34-36]. Grooming frequency was also decreased by the pesticide (Table 2). This may probably be due to decreased muscle movements or change in animal's mood abnormally by the cyfluthrin [17].

Exploration or head dipping on a hole-board is frequently used as an indicator of exploratory tendencies in rodent studies [37]. Drugs with diverse pharmacological properties cause alteration in head dipping suggesting that many neurotransmitter systems are involved in the expression of exploratory behavior [38]. It has been shown that changes in head-dipping behavior by cyfluthrin may reflect the anxiogenic state of animals [39]. Cyfluthrin treated mice showed decreased exploration as indicated by less number of nose dips in treated mice compared to control animals. Animals also showed anxiety viz. increased frequency of urination and defecation and decreased tendency to explore the arena. Mice preferred to stay at periphery of arena which is a sign of neophobia [40, 41].

Acetylcholinesterase is known to have many non classical functions. During early development AChE expression is tightly correlated with neuronal outgrowth and cell survival [42].

Laboratory studies have demonstrated that AChE activity is modulated by several conditions that result in progressive and regressive neuronal and behavioral plasticity. Chronic deprenyl administration-induced increase in dendritic arborisation in the primate brain is associated with an increased AChE activity in the hippocampus and cortex [43]. Intracranial self-stimulation treatment has been shown to result in increased dendritic arborisation, enhance learning in operant conditioning tasks, and reverse chronic restraint stress-induced behavioral deficits. This enhancement of cognitive function is associated with an increase in AChE activity [44, 45]. Cyfluthrin is known to block the catalytic site of AChE [1]. Administration of substances like inorganic arsenic, metanil yellow and 2, 4-dichloro phenoxy acetic acid causes behavioral dysfunction in an operant conditioning task. This has been found to be coupled with decreased AChE activity [46, 47, 48]. Several studies demonstrate that chronic stress leads to cognitive dysfunction and results in disorders like depression, anxiety and impairment of learning and memory [45, 49]. Hence decreased activity of AChE activity in brain in this study may be correlated with the decreased learning and memory of the animals, as indicated by increase in number of errors in the maze learning experiment. There is substantial clinical evidences that muscarinic receptor blockage by drugs results in disruptions of behavior, working (short term) memory, reference memory, attention, decisional processes, movement and strategy selection and altered sensory processing [50]. Hence it can be concluded that cyfluthrin is a neurotoxicant at the tested dose levels.

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