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Research Article
Effects of Sublethal Doses of Thiacloprid, a Neonicotinoid Insecticide, on Learning and Memory Performance of Mice

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Abstract

Background and Objective: Thiacloprid (THI), a neonicotinoid insecticide, currently one of the most preferred insecticides worldwide. Although they are claimed to be less hazardous on mammals, late studies revealed the harmful effects of this kind of insecticides. However, there are few studies examining the effect of THI on learning and memory performance in the literature. This study was conducted to investigate the effects of sublethal doses of THI on learning and memory functions and to determine the effect of the protocol on biochemical parameters. **Materials and Methods:** In this outcome, 50, 100 and 200 mg kg^{-1} THI were administered by oral gavage for 3 weeks in mice (n:7). At the end of this process, a novel object recognition (NOR) and passive avoidance (PA) tests were conducted to measure learning and memory functions. Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) levels were measured biochemically. **Results:** In the NOR test, reductions in the discrimination index values were observed with THI applications. The step-through latencies of the mice to enter the dark compartment in the retention trial of the PA test was reduced similarly in THI applied groups. The biochemical investigations revealed that BDNF and GPx levels in the brain tissue were significantly reduced in all groups compared to the control group, while a significant reduction in NGF levels was observed only in 200 mg kg^{-1} applied group. There was no significant difference in SOD and CAT levels between test groups. **Conclusion:** These results indicated that sublethal, chronic THI application degenerates the learning and memory functions with affecting BDNF, NGF and GPx levels.

Key words: Thiacloprid, neonicotinoid insecticide, learning-memory performance, novel object recognition test, brain-derived neurotrophic factor (BDNF), glutathione peroxidase (GPx)

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Thiacloprid (THI) is a synthetic insecticide and a member of neonicotinoid (NEO) drugs. Other than THI, thiamethoxam, imidacloprid, clothianidin, flupyradifurone, dinotefuran and nitenpyram are the molecules classified in this class of drug[1,2]. The NEO class drugs are currently one of the most preferred insecticides worldwide to protect the seeds and plants in the early stages against harmful insects as well as in forestry, seafood cultivation and urban pest management[1-3].

THI is a partial agonist on the nicotinic acetylcholine receptors (nAChR) of insects. THI binds to acetylcholine receptors, but the acetylcholinesterase enzyme, which breaks down acetylcholine, cannot break THI down. The drug initially increases cholinergic transmission, but in the later phases, it blocks the nAChR and prevents acetylcholine binding on these receptors. Eventually, the development of paralysis results in the death of insects[4-6].

It is stated that NEO class drugs bind weakly on mammal nAChR and they have low penetration rates through the blood-brain barrier; thus, these drugs are considered safe in mammals[7]. With these unique characteristics, NEO class drugs are favoured instead of organophosphates and carbamates, which are traditional insecticides[8]. Nonetheless, there are an increasing number of studies suggesting their harmful effects on humans. Several *in vivo* and *in vitro* studies stated NEO class drugs could be hazardous in humans in sublethal doses[9-12].

There are not many studies that presented the effects of THI on memory processes, there is a need to understand its effect on this outcome. In the present study, sublethal doses of THI, a neonicotinoid insecticide, were administered for 3 weeks and the effect of this regimen on learning and memory functions was investigated in mice. In light of the results of this experiment, continuous exposure to sublethal doses of THI will be recognized and its safety and side effects on memory will be understood.

MATERIALS AND METHODS

Animals: The study was conducted at Dicle University Health Sciences Application and Research Centre between September and November 2019. Twenty-eight male BALB/c mice (7-8 weeks old, 30-40 g) obtained from an animal colony facility were used in this experiment. One week before the tests, the mice were transferred to the laboratory and kept under standard laboratory conditions; 12 hours dark/light cycle (light onset at 08:00 pm), 23±2°C with 60% humidity. The mice were free to access the food pellets and tap water [13]. All the procedures concerning the animals were conducted with the approval granted from Dicle University Animal Experiments Ethics Committee and following the animal care guidelines designated by National Institutes of Health.

Experimental groups and drug administration: The mice were divided into 4 groups (n:7). Group 1; the control group was given 0.1 mL distilled water for 21 days by oral gavage. Group 2 (THI-50), group 3 (THI-100) and group 4 (THI-200) were given 50, 100 and 200 mg kg^{-1}/day THI (ZELOS OD 240 g L^{-1}), respectively dissolved in distilled water and administered by oral gavage[14]. The learning and memory tests were conducted in the 19th, 20th and 21st days of the procedure. After the completion of the behavioral examinations, the mice were sacrificed under ether anesthesia by cervical decapitation. The prefrontal cortex and hippocampal tissues were taken and stored at -80°C until biochemical analysis.

Novel object recognition test: An open field test apparatus (40×40×20 cm) was used to conduct the novel object recognition (NOR) test. The experiment was carried out in a room illuminated continuously with a bulb about 100 lux beyond the centre of the apparatus. This test consists of habituation, training and retention trials. Habituation trial, the mice were put in the centre of the apparatus with no object placed and allowed to habituate for 5 min. Training trial, 30 min after the habituation trial, the same mice were put in the apparatus with 2 similar objects placed 10 cm above the sidewall in a symmetrical position and were allowed to explore for 5 min. Retention trial, 1 h after the training trial, the mice were put in the apparatus while one of the objects was replaced with a novel object and they were left to explore freely for 5 min. The test was video recorded and the time spent exploring the novel (N) and familiar (F) objects were determined using Ethovision XT 11 (Noldus Inf. Tech. Netherlands). The mice directing its nose towards the objects or touching it with the nose was considered as the exploration behavior. The mice with a normal recognition memory were expected to spend more time exploring the novel object[15]. A discrimination index (DI) was used to determine recognition memory performance. The DI was identified with the time spent exploring the novel object divided by the total exploration time of both objects multiplied by 100 (DI = N/(N+F)x100). A higher DI was regarded as greater memory retention[16].

Passive avoidance test: The passive avoidance test apparatus (MAY-PA 1014-M, Ankara Turkey) consisted of 2 compartments (11×12×20 cm) connected with an automatic door. One compartment was enlightened (2000 lux) and had white-colored walls while the other compartment was dark and had black-colored walls with a grid floor. The test was carried out in 2 days. On the 1st day (acquisition trial), the mice were put in the enlightened room and waited for 30 sec, then the door connecting the 2 compartments was opened. The step-through latency to enter the dark compartment was calculated and noted. After the entrance of the mice to the dark compartment, the door was shut and an electric shock was given (0.75 mA for 1 sec). The mice were left in the room for 15 sec and were put in their home cage afterward. Twenty-four hours after the acquisition trial, a retention trial was performed. In this test, the mice were placed in the light compartment, the door was opened after 30 sec once again and the step-through latencies were calculated. If the mice did not enter the dark compartment for 300 sec, the test ended afterward. The step-through latencies of the mice in the retention trial were considered as the index of emotional memory. After each trial, the apparatus was cleaned with 20% alcohol and then with tap water to eliminate the olfactory cues not to affect the behavior of the next mice[17].

Biochemical analysis: Brain tissues were homogenized in 9 (w/v) volumes of ice-cold phosphate-buffered saline (PBS, 0.01M, pH: 7.4). The homogenates were centrifuged for 5 min (5000 g) and the supernatants were collected for biochemical analysis. Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glutathione peroxidase (GPx), superoxide dismutase (SOD) levels were analyzed using enzyme-linked immunosorbent assay (ELISA) in the brain tissue samples using an auto ELISA plate analyzer (Robonik readwell touch, Thane, India)[18]. Catalase (CAT) levels were analyzed spectrophotometrically (UV-1205 Shimadzu, 405 nm) using a commercially kit (Elabscience, Wuhan, China)[19]. The BDNF, NGF, GPx and SOD levels were expressed as nanogram g^{-1} tissue and CAT levels were expressed as U g^{-1} tissue.

Statistical analysis: The data were analyzed using SPSS (Chicago, USA) and displayed as the mean values±standard deviations. A one way ANOVA followed by *post hoc* Tukey test was used and the p-values below 0.05 were considered as significant.

RESULTS

Novel object recognition test: The NOR test results (Fig. 1) revealed that while the interest of the mice to the familiar object did not alter between the THI applied groups ($p>0.05$), it was reduced exploring the novel object in the THI applied groups compared to the control group ($p<0.001$). The discrimination index values were significantly reduced in the 50, 100 and 200 mg kg^{-1} THI applied groups when compared the control group ($p<0.05$).

Passive avoidance test: Step-through latencies to enter the dark compartment of the mice were similar between test groups in the acquisition trial of the PA test ($p>0.05$, Fig. 2). In the retention trial, the control group's step-through latency was 286±26.2 sec and it was 201.4±65.9, 183.4±91.7 and 106.8±68.2 sec in the THI-50, THI-100 and THI-200 groups, respectively. The THI applied groups step-through latencies to enter the dark compartment were lower than the control group, yet only THI-100 ($p<0.05$) and THI-200 ($p<0.001$) groups were found statistically significant compared to the control group (Fig. 2).

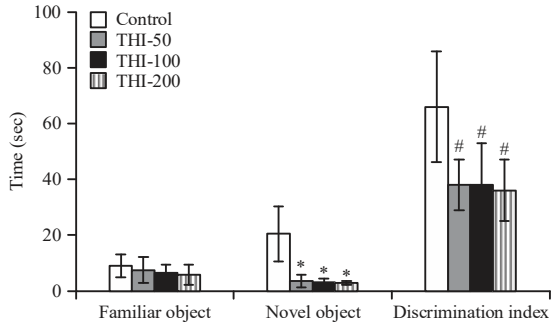

Fig. 1: Novel object recognition test results of the mice
THI: Thiacloprid, *p<0.001, #p<0.05 vs. control

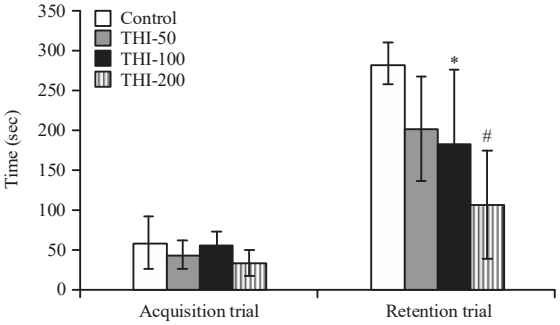

Fig. 2: Step-through latencies to enter the dark compartment of the mice in the acquisition and retention trial of the passive avoidance test
THI: Thiacloprid, *p<0.05, #p<0.001 vs. control

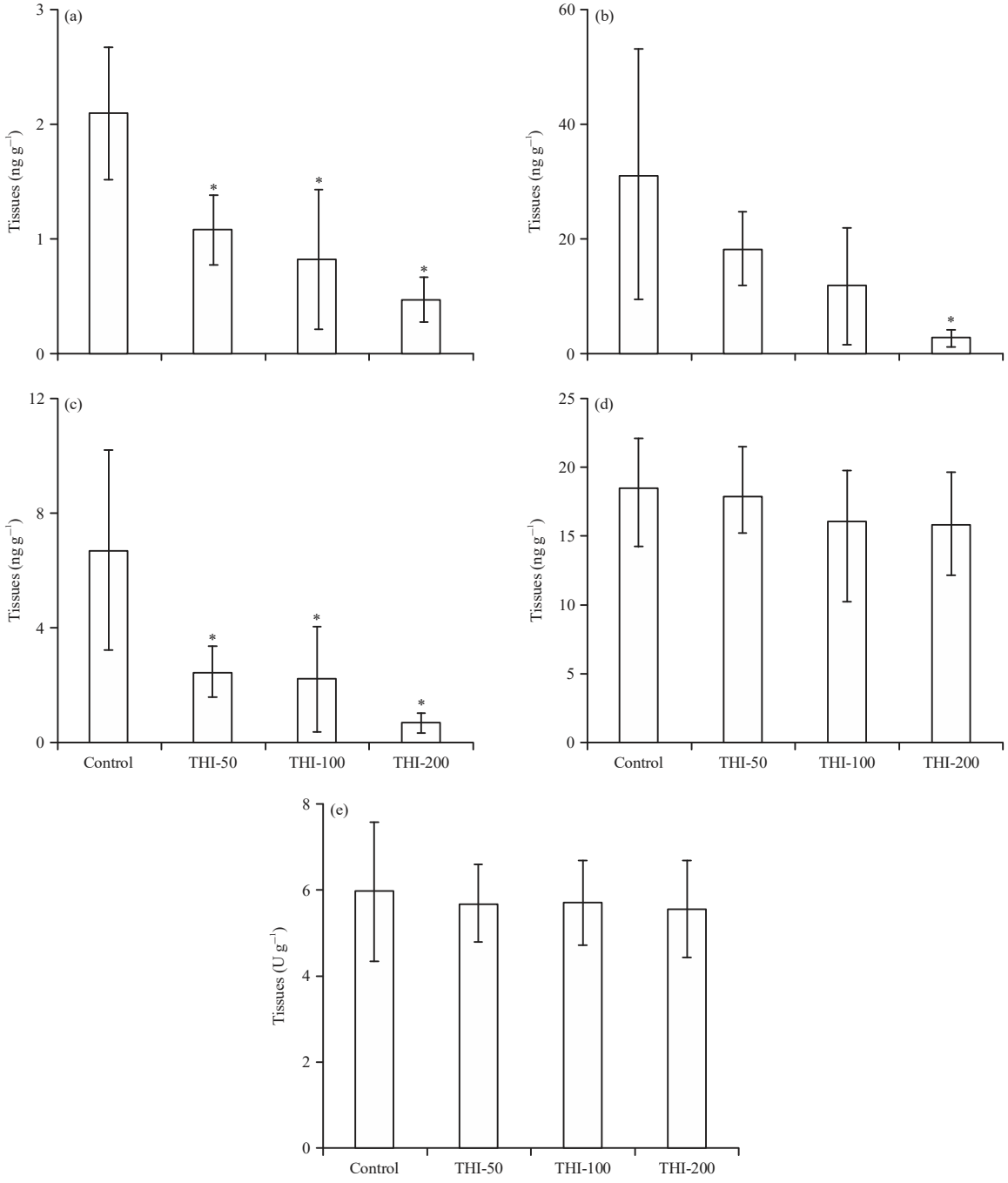

Fig. 3(a-e): Brain tissue (a) BDNF, (b) NGF, (c) GPx, (d) SOD and (e) CAT levels of the mice

THI: Thiacloprid, BDNF: Brain-derived neurotrophic factor, NGF: Nerve growth factor, GPx: Glutathione peroxidase, SOD: Superoxide dismutase, CAT: Catalase, *p<0.01 vs. control

BDNF, NGF, SOD, GPx and CAT levels: The BDNF levels of the control, THI-50, THI-100 and THI-200 were 2.10±0.58, 1.08±0.30, 0.83±0.61, 0.47±0.19, respectively (Fig. 3a). The results of THI applied groups were significantly lower than the control group ($p<0.01$). The NGF levels were not statistically significant in the THI-50 (18.4±6.47) and THI-100 (11.8±10.4) groups ($p>0.05$) while THI-200 (2.71±1.63) group had lower values ($p<0.01$, Fig. 3b) compared to the control group.

The GPx levels were 6.72±3.50 in the control group, 2.48±0.88 in the THI-50 group, 2.24±1.86 in the THI-100 group and 0.70±0.37 in the THI-200 group (Fig. 3c). The GPx levels were found significantly lower in the THI applied groups compared to the control group ($p<0.01$). The SOD and CAT levels did not differ between test groups ($p>0.05$). The SOD (Control: 18.5±4.16, THI-50: 18.0±2.64, THI-100: 16.2±5.89, THI-200: 16.0±3.68) and CAT (Control: 6.01±1.61, THI-50: 5.73±0.89, THI-100: 5.74±0.89, THI-200: 5.61±1.14) levels were displayed in Fig. 3d and e, respectively.

DISCUSSION

The NEO class drugs are classified as systemic insecticides and are widely being used in plant protection. Nicotinic acetylcholine receptors are the target of NEO class drugs. Initially, they stimulate the nAChR, yet they block cholinergic transmission later on by competing with acetylcholine, which results in paralysis and death[4]. The target of these molecules has vital functions of humans primarily in learning, memory and behavior[12,20]. The NEO class drugs are considered relatively safe in humans as a general conclusion due to their low affinity on the nAChR and low penetration rate towards the central nervous system[21]. Because of these advantages of the NEO class drugs, they are preferred instead of highly toxic organophosphate and carbamate type insecticides[7,8].

The studies revealed that the NEO class insecticides do not only stay on the surface of the plants, but they also reach the flowers, pollen and penetrate their fruits[1,22]. Furthermore, the waste products of these drugs were found in the soil and water[20,23]. According to the Total Diet Study reported by the Food and Drug Administration in 2012, NEO class drugs were the most common pesticide found in the food of neonates and newly walking children[24]. As a consequence, a number of studies were conducted to reveal their effects on mammals. These studies have shown their genotoxic, cytotoxic, carcinogenic effects and their potential to make an endocrine disturbance other than their effects on the central nervous system[25-27].

In this study, the effects of sublethal doses of thiacloprid, a NEO class insecticide, on learning and memory functions were investigated. To this end, 3 different doses of THI were administered for 21 consecutive days and a NOR and a PA test were conducted. These tests are widely and successfully practiced tests to measure learning and memory functions in experimental studies[15,28]. The NOR test is preferred to measure hippocampal-dependent short-term visual memory functions[15], while the PA test is practiced in the assessment of amygdala and hippocampus-dependent instrumental learning and contextual fear conditioning[29]. The PA test is a commonly used test to measure emotional memory in rodents[30]. A conducted study stated that THI acts as a significant risk factor for honey bees by interrupting learning and memory functions[31]. In another study, different doses of THI were given to rats via food pellets and memory functions were evaluated using a passive avoidance test. It is reported that female rats had increased latencies in the retention trial of the test with 40.8 mg kg^{-1} THI application[32]. The present study was conducted to investigate the effect of 50, 100 and 200 mg kg^{-1} THI applications on learning and memory functions. In the retention trial of the PA test, although there were reductions in step-through latencies of the THI applied groups, only 100 and 200 mg kg^{-1} THI applications were significantly reduced the latencies compared to the control group. The results of the conducted NOR test have revealed that all three doses of THI produced a decrease in the interest of the mice towards the novel object and reduced the discrimination index. These results indicated that sublethal applications of THI could produce visual and emotional memory disruptions.

Furthermore, BDNF and NGF levels were measured with biochemical methods in the tissue samples obtained from the sacrificed mice. BDNF and NGF are essential growth factors that are responsible for the regeneration of the brain and maintaining their health. Both growth factors are produced in the cortex and hippocampus of the brain[33]. The NGF plays vital roles mainly in the development of cholinergic neurons, while BDNF has essential roles in dopaminergic, serotonergic, cortical and hippocampal neuron development in addition to its effects on cholinergic neurons. In the literature, there is no study that examined the effects of THI and other NEO class insecticides on BDNF and NGF levels. Reductions in these parameters were stated to be related to several neuropsychiatric and neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease and depression[34]. Developments of neurotoxic effects of organophosphates, which are another group of insecticides, are reported to be closely related to decreased levels of BDNF and NGF[35]. In the present study, lower BDNF levels were observed in all 3 doses of THI applied groups. Similarly, NGF levels were found to be lower in the 200 mg kg^{-1} THI applied groups compared to the control group. The results of the behavioral and biochemical tests revealed that sublethal dose application of THI causes learning and memory function deficits with affecting BDNF and NGF levels.

It also should be clarified if the memory function deficits caused by THI application were related to oxidative damage. To this end, GPx, SOD and CAT levels were measured, which are known as first-line antioxidant defense systems. The mentioned enzymes are responsible respectively in the breakdown of hydrogen peroxides, dismutation of superoxide radicals and converting hydroperoxides to harmless molecules (H_2O_2/alcohol and O_2)[36]. This defensive system also includes transferrin, which has roles in metal ion binding and ceruloplasmin, that chelates iron or sequesters copper, consequently preventing the free radical production. Superoxide and singlet oxygen formed by various reactions in the cells are converted to hydrogen peroxide (H_2O_2) and molecular oxygen. The enzyme prevents the accumulation of H_2O_2 is CAT, which converts H_2O_2 to water and molecular oxygen and protects the cells from oxidative damage[37,38]. However, the mitochondria of the cells do not contain the CAT enzyme. In the mitochondria, instead of the CAT enzyme, the selenium-dependent GPx enzyme undertakes the reduction responsibility[39,40] of H_2O_2. A study reported reduced antioxidant enzyme levels in plasma and tissue after THI applications in rats[14]. In this study, the GPx levels were significantly reduced in the THI applied groups compared to the control group. Differing from the literature, the same reduction was not observed in the SOD and CAT levels. These results indicate that THI application does oxidative damage in the brain, particularly in mitochondria.

CONCLUSION

With the results concerning the present study, it was concluded that 3 weeks of THI administration increases mitochondrial oxidation, reduces BDNF and NGF levels and deteriorates learning and memory functions. When considered that human is continuously exposed to insecticides in low doses, enlightening the long-term effects of these compounds on public health is crucial, especially in a generation with increased rates of neurodegenerative and neuropsychiatric disorders.

SIGNIFICANCE STATEMENT

The present study discovered the detrimental effects of thiacloprid on learning and memory functions. The study will help future researchers to uncover the critical areas of insecticides, degenerated memory functions and neurotrophic factors that many researchers have not been able to explore to date.

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