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RESEARCH ARTICLE

Determination of the effects on learning and memory performance and related gene expressions of clothianidin in rat models

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Abstract Clothianidin (CLO) is one of the pesticides used to protect against insects, and its potential toxic effects on cognitive functions are not clearly known. This study aims to evaluate the possible effects of dose-dependent CLO on learning and memory in infant and adult male rats and the expression of related genes in the hippocampus. Doses of 2, 8 and 24 mg/kg of CLO were administered to newborn infant and adult albino Winstar rats in the form of gavage and dissolved in vehicle matter. Their cognitive and learning functions were evaluated by the Morris water maze and probe tests. Expression levels of N-methyl D-aspartate 1 (GRIN1), muscuranic receptor M1, synoptophysin (SYP) and growth-associated protein 43 (GAP-43) of tissues isolated from the hippocampus were determined using the real-time PCR method. In the Morris water maze test, no change ($p > 0.05$) was exhibited in the adult and infant rats after CLO was applied, although there was a significant difference ($p < 0.05$) in performance between infants and the control group after 24 mg/kg was applied in the probe test. Also, expression levels GRIN1, M1, SYP, GAP-43 did not change when compared to the control ($p > 0.05$). Our study shows that exposure to high doses of CLO causes deterioration of cognitive functions in infant rats.

Keywords Clothianidin · Cognitive function · N-methyl D-aspartate 1 · Muscarinic receptor M1 · Synoptophysin · Growth-associated protein 43

Introduction

Pesticides are often used to remove or kill pests in various organisms in different fields. Insecticides are a subgroup of pesticides and are commonly used in agriculture to protect against harmful insects. In addition to their effects as insecticides, pesticides can cause adverse effects to other organisms due to their toxic properties. Today, due to an

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increase in the prohibition of organophosphorus compounds and methylcarbamats, neonicotinoid insecticides has been used more often (Tomizawa and Casida 2003). That insecticide category includes CLO [(E)-1-(2-chloro-1,3-thiazol-5-yl methyl)-3-methyl-2-nitroguanidine], which has a broad application in the neonicotinoid insecticide spectrum (Tomizawa and Casida 2005) and is effective on postsynaptic nicotinic acetylcholine receptors. However, it shows its effect on target organisms rather than mammals at much lower concentrations (Matsuda et al. 2001). Its toxic effects on non-target organisms and on the ecosystem of pesticides are a topic of interest worldwide (Pimentel et al. 1998).

As a nicotinic acetylcholine agonist, CLO is shown to be effective in the case of many harmful insects and is used to protect organisms against them (Courjaret and Lapied 2001). In insects, CLO has a distinct pharmacological effect on nAChRs and can induce cell death through apoptosis and necrosis (Tomizawa and Casida 2005). Neonicotinoids play a role as agonists in postsynaptic insect nAChRs due to their high affinity (Tomizawa and Casida 2003). Since the use of pesticides has become widespread, it is important to investigate the potential toxic risk to non-target organisms. Mammalian nAChRs are heteromeric complexes that exist in α ve β subunits (Romanelli and Gualtieri 2003), and the primary binding area for neonicotinoids are α4β2 receptors in brain (Tomizawa and Casida 2005).

Nicotinic acetylcholine receptors have been identified in neuromuscular synapses, and the latest studies reveal that these receptors are present not only in nerve cells, but also in adipocytes, macrophages, lymphocytes, keratinocytes, hepatic, dendritic, lung, and intestinal epithelial cells (Gahring and Rogers 2006; Abou-Donia et al. 2008). Moreover, it has been reported that CLO has also caused DNA damage (Feng et al. 2005). Some studies have shown the cytogenetic, genotoxic, and neurotoxic effects of CLO application, in addition to its adverse effects on offspring development during pregnancy (Abou-Donia et al. 2008).

CLO, which can enter the nutritional chain easily, is reported being safer for people compared to other insecticides. The reason for this is that CLO is metabolized to 2-chloro-1,3-thiazole-5-carboxylic acid and then conjugated to glucoronic acid in mammalian cells (Ford and Casida 2006). It can also undergo different metabolic reactions (Casida 2011). Moreover, made on mammals effects are limited, and additionally the studies generally use rats (Faro et al. 2012; Oliveira et al. 2010).

The levels of neurodevelopmental protein in the brain region can play an important role in the development of cognitive functions. Such proteins include GRIN1, M1, SYP, and GAP-43, and the determination of mRNA levels of the mentioned proteins in presence of CLO may be important to observe changes in memory and learning patterns during neurodevelopment. According to our literature survey, research shows that the effects of CLO on nervous system and cognitive functions of mammalian are limited. This aim of this study was to determine the effect of CLO on memory and learning in infant and adult rats, in addition to the expression levels of GRIN1, M1, SYP, and GAP-43. We believe that this study will reveal more information on the possible effects of neonicotinoid insecticidess on the mammalian.

Materials and methods

In the study of effects of CLO on juvenile rat models, 16 pregnant albino Winstar female rats were used. The animals were divided into four groups.

Animal groups

1. The control group (n = 4): The same amount of vehicle ingredient (water) was given by gavage to six newborn male pups and to all other groups on a daily basis from the seventh day. Buoyancy was tested until 97 days after cultivation and the animals then decapitated under anesthesia.
2. CLO-2 group (n = 4): A 2 mg/kg live-weight dose of CLO dissolved in vehicle ingredient (water) was given by gavage to six newborn male pups every day from the seventh day. Buoyancy was tested until 97 days after cultivation and the animals then decapitated under anesthesia.
3. CLO-8 group (n = 4): An 8 mg/kg live-weight dose of CLO dissolved in vehicle ingredient (water) was given by gavage to six newborn male pups from the seventh day. Buoyancy was tested until 97 days after cultivation and the animals then decapitated under anesthesia.
4. CLO-24 group (n = 4): A 24 mg/kg live-weight dose of CLO dissolved in vehicle ingredient (water) was given by gavage to six newborn male pups from the seventh day. Buoyancy was tested until 97 days after cultivation and the animals then decapitated under anesthesia.

In the study of male rat models and CLO, 24 (8–9 week-old) albino Wistar rats were used. The animals were divided into four groups.

1. The control group (n = 6): To the animals in the control group, the vehicle ingredient (corn oil) for a period of 1 month and the amount of the same period of time was given by gavage.
2. CLO-2 group (n = 6): CLO dissolved in a vehicle ingredient (corn oil or DMSO) in a 2 mg/kg live-

weight dose was given by gavage every day for 3 months. The buoyancy was then tested and the animals decapitated under anesthesia.

3. CLO-8 group (n = 6): CLO dissolved in a vehicle ingredient (corn oil or DMSO) in an 8 mg/kg live-weight dose was given by gavage every day for 3 months. Buoyancy was then tested and the animals decapitated under anesthesia.
4. CLO-24 group (n = 6): CLO dissolved in a vehicle ingredient (corn oil or DMSO) in a 24 mg/kg live weight dose was given by gavage every day for 3 months. Buoyancy was then tested and the animals decapitated under anesthesia.

Morris water maze learning performance

The Morris water maze procedure has been described in previous research (Waller et al. 1960). In the case of our study, a circular water tank (120 cm in diameter and 50 cm in height) was filled to a depth of 30 cm with water at 25 ± 1 °C and the tank was virtually divided into four equal quadrants, labeled N–S–E–W. The water was made opaque by the addition of semi-skimmed milk.

An escape platform was hidden 1.5 cm below the surface of the water in a fixed location in one of the four quadrants of the pool. The platform remained in the same quadrant during the entire experiment. The rats were required to find the platform using only the distal spatial cues available in the testing room. The cues remained constant during testing. The rats were trained to locate the platform and escape onto it in four trials per day for five consecutive days. A different starting position was used in each trial (in a quadrant not containing the platform). During a given trial, the rat was introduced into the pool at one of four pseudo randomly chosen start points. The animal was allowed 60 s to find the platform. After climbing onto the platform, the animal remained there for 30 s before the commencement of the next trial. There was a 30-second intertrial interval. If the rat did not find the platform after 60 s, it was placed on it and allowed to remain there for the same amount of time. The time taken to reach the platform (latency in seconds) was measured.

Probe trial

24 hours after the last training trial, a probe test was conducted in which each rat received a 60-second free swim in the pool with the platform removed. The time spent in the target quadrant indicated the degree of memory consolidation that had taken place after learning. For these trials, the percentage of time spent in the target quadrant was recorded. The percentage was calculated as a measure of spatial memory. To test visual acuity and motor functions, the rats were tested in the water maze with a visible platform in a new location after the last day of training. For the visual test, the hidden platform was replaced by a visible platform located in the opposite quadrant. The animals were tested and the latency times taken to reach the platform were recorded for each trial. All the rats were then killed by decapitation without anesthesia. The brain of each animal was rapidly removed, and the hippocampus and cortex were dissected on ice for the biochemical studies. Samples were used fresh or kept at −80 °C until the measurements were performed.

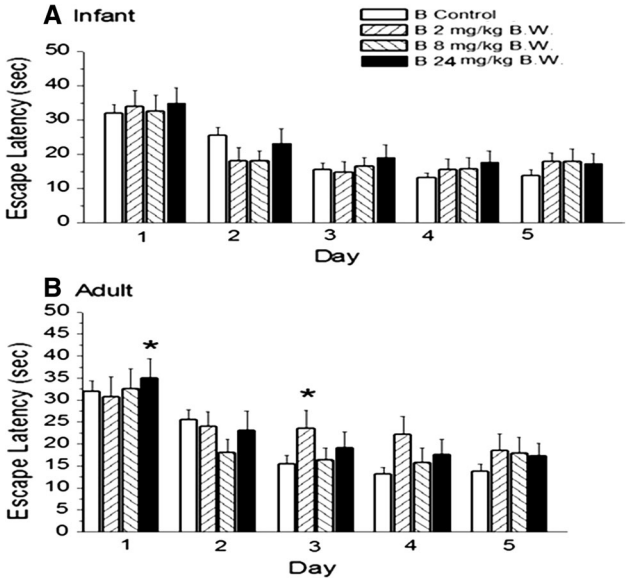

Fig. 1 Spatial learning of infant and adult rats in Morris water maze test. There was no relationship between infant rats who received different doses and the control. On the first day of the treatment, the adult rats with the 8 mg/kg CLO dose showed latencies when compared to the control, and on the third day, the adult rats with the CLO 2 mg/kg dose showed higher latencies when compared to the control

Determination of gene expression

RNA was obtained by using a RNA isolation kit of brain tissue (hippocampus) and, the gene expressions GRIN1, M1, SYP and GAP-43 were studied by using Real Time PCR method.

Statistical analysis

Statistical analysis was performed with SPSS 15.0 for Windows (SPSS Inc.). The one sample Kolmogorov–Smirnov test was used to determine whether the data was distributed normally. Groups were compared using the One way ANOVA. The results were reported as mean ± SD. p value < 0.05 was considered statistically significant.

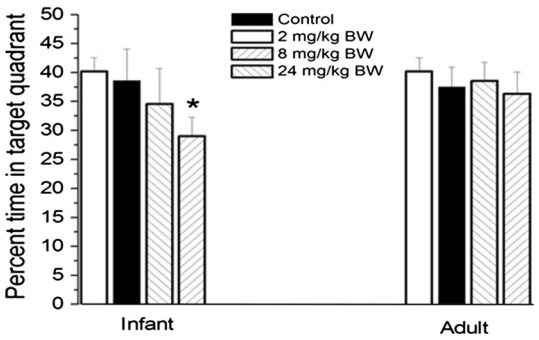

Fig. 2 Mean percentage time spent in the target quadrant of groups. There was a significant difference in performance between the infants who were treated with a 24 mg/kg dose and the control rats ($p < 0.05$), There was no relationship between adult rats who received different doses and the control

Results

In this study, different doses of CLO were applied to newborn infant and male rats. After the application, the rats were tested by means of learning exercises, and the expression levels of the gene of interest were determined and evaluated statistically.

Morris water maze test

Learning and memory behaviors were investigated using a platform test and a spatial version of the Morris water maze test. The results are shown in Fig. 1.

Figure 1 displays how long it took the adult and young animals on average (mean latencies) to find the submerged platform over the 5 days of testing in the Morris water maze. As can be seen from the results, the adult and infant rats who received a dose of CLO did not exhibit any changes with regard to cognitive function and memory. On the first day of the treatment, the adult rats with the 8 mg/kg CLO dose showed higher latencies when compared to the control, and on the 3rd day, the adult rats with the CLO 2 mg/kg dose showed higher latencies when compared to the control. The mean latencies for finding the platform during the visible platform trials of CLO-treated adults were not significantly different to those of the infant rats. The spatial learning ability of CLO-treated adult and infant rats were tested by using statistical analysis, and there was no difference between CLO-treated adults and young control rats ($p > 0.05$).

Probe trial

As shown in Fig. 2, although there was a significant difference in performance between the infants who were treated with a 24 mg/kg dose and the control rats ($p < 0.05$). There was no relationship between adult rats who received different doses and the control. As can be seen from the probe trial results for the adult groups, there was a non-significant difference when compared to the control.

The expressions of GRIN1, SYP, GAP-43 and M1 were determined in the hippocampus tissues of adult, infant, and control groups. In the case of all doses applied to the adult and infant groups, the mRNA expression levels of GRIN1, SYP, GAP-43 and M1 did not change when compared to the control ($p > 0.05$). Also, the expression levels were not significant (Table 1).

Discussion

The insecticides used in ecological and agricultural practices are used for pest control; however, the residues of these chemicals enter the nutritional chain, directly or indirectly, and may have negative effects on many living organisms, including humans. Calderón-Segura et al. (2012) reported that neonicodinoid insecticides administered in a concentratation-dependent manner, for example, clothianidin (CLO), thiacloprid, and imidacloprid, especially in high doses, cause damage to DNA on human peripheral blood cells. Moreover, it has been reported that pesticides have a negative impact on the neurological system (Gahring and Rogers 2006; Muñoz-Quezada et al. 2013).

Some neonicotinoid pesticides are known to present effects on behavioral and biochemical changes in rats (Vural 2005). However, the studies on CLO effects to memory and learning are limited. In this study, different doses of CLO were given to adults and infant rats, and after the application periods, the effects of CLO on learning and memory performance and on the mRNA expressions of related genes in adult and infants were tested with Morris water maze, probe trial test, and PCR. The Morris water maze and probe trial tests were used to evaluate learning and memory in rats. These tests are well validated for spatial learning and memory. The technique of escape from water to motivate learning has long been used in studies of this type (Baydas et al. 2007; Tuzcu and Baydas 2006). In the Morris water maze test, adults and infants displayed similar cognitive performance compared to the control. According to the results, there was no change in the infants compared to the control in the learning experiments. The performance of the adults that were given CLO was sometimes affected, but the results were inconsistent. In the light of these results, CLO exposure appeared not to affect learning and memory performance. In the probe trial (consolidation of memory) test, the infant groups given a high dose of CLO were adversely affected compared to the control group (Fig. 2).

Table 1 Expression levels of the genes

Adult	GRIN1	SYP	GAP43	M1	*p*
Control	0.156 ± 0.06	1.262 ± 0.70	0.153 ± 0.05	0.088 ± 0.07	0.550
2 mg/kg	0.215 ± 0.03	0.960 ± 0.55	0.175 ± 0.03	0.090 ± 0.02	0.110
8 mg/kg	0.137 ± 0.05	0.570 ± 0.69	0.138 ± 0.04	0.025 ± 0.01	0.600
24 mg/kg	0.187 ± 0.05	1.367 ± 0.94	0.180 ± 0.06	0.060 ± 0.01	0.849
Infant					
Control	0.207 ± 0.06	0.989 ± 0.51	0.161 ± 0.07	0.102 ± 0.08	0.306
2 mg/kg	0.215 ± 0.03	0.960 ± 0.55	0.182 ± 0.03	0.097 ± 0.04	0.896
8 mg/kg	0.137 ± 0.05	0.570 ± 0.69	0.145 ± 0.03	0.031 ± 0.03	0.177
24 mg/kg	0.187 ± 0.05	1.367 ± 0.94	0.166 ± 0.05	0.071 ± 0.02	0.733

GRIN1, N-methyl D-aspartate 1; *M1*, muscuranic receptor; *SYP*, synoptophysin; *GAP-43*, growth-associated protein 43. The results were reported as mean ± SD. P value < 0.05 was considered statistically significant

The expression levels of GRIN1, M1, SYP and GAP-43 in the hippocampus were determined using the real-time PCR method. No difference was observed between the expression levels of genes obtained from the hippocampus tissue of either adult or infant rats, and the results were not statistically significant. The absence of differences in the levels of gene expression between the control and the treatment groups could be explained based on the fact that CLO had no effect on the expression of GRIN1, M1, SYP and GAP-43. As far as our literature survey, there is no report on the expression levels in these genes after treatment with neonicotinoid insecticide such as CLO, and we could say that this study is the first report for the expression of those genes.

Neonicotinoid insecticides are highly effective pesticides, and show selective effects on insects, while their toxicities with respect to mammals are low (Fishel 2005; Matsuda et al. 2001). In insects, these agents show their activities on nicotinic acetylcholine receptors (nAChRs), whereas the nAChRs receptors are composed of many subunits in mammals (Romanelli and Gualtieri 2003). The binding of CLO in mammals shows more selectivity compared to insects. Also, CLO is metabolized to 2-chloro-1,3-thiazole-5-carboxylic acid and then is conjugated to glucoronic acid (Ford and Casida 2006). Thus, it does not join with the nAChRs receptors. Therefore, CLO might not exhibit any effect on learning and memory of rats, especially in adults. It should be emphasized that a high dose of CLO could be shown as the reason for the decrease in memory performance observed, especially in infant rats.

Conclusion

Consequently, in light of the above findings, different doses of CLO applied to adult rats do not have an impact on learning and memory or on the related genes in general. However, high doses of CLO seem to decrease learning and memory performance in infant rats especially. We believe this study is important as it is the first to show the effects of CLO on learning and memory and the related gene expression in rats. In addition to these effects, future studies should focus on neurodegenerative and biochemical effects in both adult rats under the effects of CLO. Moreover, mRNA amounts and protein levels should be determined using a Western blot analysis of different genes relating to learning and memory. Also, the expression of genes such as UGTs that play a role in the detoxification of toxic substances should be determined in parallel with studies relating to neonicotinoid insecticides.

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