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RESEARCH ARTICLE

Toxic effect of acetamiprid on *Rana ridibunda* sciatic nerve (electrophysiological and histopathological potential)

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ABSTRACT

In this study, the effects of a neonicotinoid insecticide acetamiprid on the sciatic nerve of *Rana ridibunda* were investigated by using electrophysiological and histological methods. A total of 35 preparations of sciatic nerve isolated from 35 frogs (*Nervus ischiadicus*) were used in the experiments. Experiments were designed as four different dose groups ($n = 8$ per group). Acetamiprid solutions of 1 (group 1), 10 (group 2), 100 (group 3), and 1000 µM (group 4) were applied to the nerves in dose groups. In each group, action potentials were recorded before application of acetamiprid which served as control data. The extracellular action potentials were recorded for each group of 30th, 60th, 90th and 120th min of application time. Action potential amplitude and area were measured from recordings. Histological evaluation was performed by transmission electron microscopy.

In electrophysiological examination, all doses in which acetamiprid applied have shown the effect from the 30th min and suppressed the sciatic nerve action potential. Acetamiprid significantly reduced the amplitude at the rate of 78–96% and the area at the rate of 79–98% ($p < 0.05$). In electron microscopic examination, the control nerves were in normal appearance. Disorganization, irregularity, dense ovoid body formation, fragmentation of the myelin sheath, and loss on some axoplasm of the nerves in the dose group have been observed. Our findings showed that acetamiprid can cause neuropathic changes in sciatic nerve at all applied doses. These results indicate that acetamiprid as other insecticides can have harmful effects on non-target organisms.

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Sciatic nerve; action potential; acetamiprid; frog; histopathology

Introduction

Insecticides are chemicals that are widely used worldwide for killing, removing, or controlling of organisms harmful to plants and animals (Schroeder and Flattum 1984). Their type, dose, and residues depending on the residence time at surroundings can lead to a reduction in the reproduction and survival of non-target species (Farooqui 2013). Insecticides are divided into five main groups as chlorinated hydrocarbons, organophosphorus compounds, methyl carbamates, pyrethroids, and neonicotinoid insecticides.

Neonicotinoids is a new group of synthetic insecticides with nitromethylene and nitroimin as well as cyanoimin group (Soloway *et al.* 1979, Matsuda *et al.* 2001). They were act as selective agonists at the nicotinic acetylcholine receptors (nAChRs). Acetamiprid is a neonicotinoid insecticide which is widely used against crop pests. It is commonly used against harmful insects on agricultural products such as cotton, tobacco, potato, tomato, and nuts in many countries. Although acetamiprid is considered safe for use in the environment of human and animals, it can cause headaches, nausea, dizziness, vomiting, and other symptoms after the exposure (Bass *et al.* 2015). The long-term intake of high doses of acetamiprid can lead to breast cancer in adult mouse models and rib malformations in fetal mice (Green *et al.* 2005a,b). Acetamiprid also causes mutagenesis in human peripheral lymphocytes *in vitro*, and has a synergistic mutagenesis effect with alpha-cypermethrin (Kocaman and Topaktaş 2010). Additionally, it affects male reproductive function through inducing oxidative stress in the testes (Zhang 2011).

There are limited studies of neonicotinoid-induced neurotoxicity in the vertebrates and these studies were consisted only of thiamethoxam, imidacloprid, and clothianidin (Kimura-Kuroda *et al.* 2012, Akbas *et al.* 2014). Imidacloprid has been reported to act as an agonist or an antagonist of nAChRs in rat pheochromocytoma (PC12) cells (Nagata *et al.* 1998) and to change the membrane properties of neurons in the mouse cochlear nucleus (Bal *et al.* 2010). Exposure to imidacloprid *in utero* causes decreased sensorimotor performance and increased expression of glial fibrillary acidic protein in the motor cortex and hippocampus of neonatal rats (Abou-Donia *et al.* 2008). Furthermore, it has been reported that the neonicotinoids thiamethoxam and clothianidin induce dopamine release in the rat striatum via nAChRs (Rodrigues *et al.* 2010) and that thiamethoxam alters behavioral and biochemical processes related to the rat cholinergic systems.

There are a few studies investigating the effects of acetamiprid on the nervous system. In one of these studies, Kimura-Kuroda *et al.* (2012) investigated effect of acetamiprid

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on cerebellar neurons from neonatal rats and found that acetamiprid has excitatory effects on mammalian nAChRs. Gasmi *et al.* (2016) demonstrated that the subchronic exposure to acetamiprid-induced neurotoxicity in adult male rats. Sano *et al.* (2016) suggested that *in utero* and lactational acetamiprid exposure interferes with the development of the neural circuits required for executing socio-sexual and anxiety-related behaviors in male mice specifically. In all of these studies, the neurotoxic effect of acetamiprid was investigated in the central nervous system. There is no study in the literature about the effect of acetamiprid on the peripheral nerve of vertebrates and in this regard. Therefore, in this study, it is aimed to investigate the effects of acetamiprid on isolated frog sciatic nerve using electrophysiological and histological techniques.

Material and method

Experimental animals

Acetamiprid ($C_{10}H_{11}ClN_4$; 33,674, Sigma, purity: %100) containing solution was prepared immediately before performing each experiment. Doses used in the present study ranged from 1 to 1000 μM acetamiprid, which was selected according to our preliminary experiments. Thirty-five *Rana ridibunda* individuals weighing 50–60 g were used in the experiments. After decapitation, each frog's sciatic nerve was isolated and placed in Ringer's solutions (NaCl, 115 mM; KCl, 2.47 mM; $CaCl_2$, 1.8 mM; Na_2HPO_4, 2.15 mM). The pH of Ringer's solution was adjust to 7.2 and all experiments were performed at room temperature. Ethical permission was approved by Mersin University Local Ethical Committee for Experimental Animals (HADYEK, Protocol number: 2014/10; Date: March 27 2014) for the animals used in this study. In all transactions with frogs, the criteria for the care and use of laboratory animals indicated in guidelines prepared by National Institutes of Health (NIH) have been applied.

Electrophysiological recordings

Thirty-two isolated sciatic nerves were divided into four groups: Group 1 (1 μM), group 2 (10 μM), group 3 (100 μM), and group 4 (1000 μM acetamiprid) ($n = 8$ per group). Three isolated nerves were used as control for histological evaluation. Electrophysiological recordings were carried out by extracellular recording techniques (Kleinelp 1991). After 15 min stabilization in Ringer's solution, the isolated nerves were placed in a 5 cm × 15 cm plexiglass nerve chamber containing Ag/AgCl electrodes. The distance between the electrodes was 0.5 cm. The nerves were stimulated these electrodes by using supramaximal pulse at the 0.5 ms duration. Compound nerve action potentials (CNAPs) were recorded using BIOPAC MP 100 Acquisition System Version 3.5.7 (Santa Barbara, CA, USA) from each nerve before application of acetamiprid doses and these data were accepted as control. After recordings of control, nerves were treated by acetamiprid and action potentials were recorded 30th, 60th, 90th and 120th minutes of treatment in all groups. Data were transferred to the computers translating to the numerical signals by 16-bit A/D converter for the off-line analysis. The sampling rate was chosen as 20,000 samples. BIOPAC Acknowledge Analysis Software (ACK 100 W) was used to measure CNAP amplitude and area. The dose-amplitude values were used to calculate IC_{50} value at 30th min (the concentration of acetamiprid that inhibits CNAP by 50% under specific conditions (pH, temperature, time, etc.)) by using the GNUPLOT package program (http://www.gnuplot.info).

Histological experiments

After recording of 120th min nerve preparations were used for histological examination, three isolated nerves in which acetamiprid is not applied were used as control ($n = 3$ per group). The standard tissue processing method was applied for electron microscopic examination to the nerves. The sciatic nerve tissue taken for histological examination was cut into 1 mm^3 pieces and was fixed in 2.5% glutaraldehyde solution for 6 h. Tissue processing was applied after nerve tissue washed with phosphate buffer and embedded in epoxy resin blocks. Semi-thin sections with 1 μm thickness were taken from the blocks with the aid of Leica Ultracut 125 UCT ultramicrotome (Leica Microsystems GmbH, Vienna, Austria). Then, 70 nm ultra-thin sections were taken on 300 mesh copper grids. Tissues, after contrasted with uranyl acetate and lead citrate, were evaluated and photographed with the JEOL JEM-1011 transmission electron microscope (JEOL Ltd. Tokyo-Japan) integrated with MegaView III digital camera (Olympus GmbH, Germany). Ultrastructural damage in nerves in dose group was examined by comparing with the control nerves.

Statistical analysis

SPSS 17.0 statistical package program (SPSS, Chicago, IL, USA) was used for statistical evaluation of the data obtained in this study. The checks of normality of continuous measurements are tested with Shapiro Wilk test and were evaluated if the data show normal distribution. As to whether the differences between the groups (one-way ANOVA) were tested by analysis of variance for the data showing normal distribution. On the other hand, Kruskal Wallis test was used for the data not showing normal distribution. Time-dependent changes within the group were investigated by the repeated measures of analysis (repeated measures ANOVA). Statistically significant difference was accepted as $p < 0.05$.

Results

Electrophysiological findings

One recording sample for all groups is given in Figure 1. As seen from the images, the acetamiprid inhibited the action potential at all doses.

Acetamiprid significantly reduced the nerve action potential amplitude compared to the control values ($p < 0.05$). All doses in which acetamiprid applied have shown the effect from the 30th min and inhibited the sciatic nerve action potential (Figure 2). Change of application time did not change this inhibition significantly ($p > 0.05$) (Figure 3(A–D)).

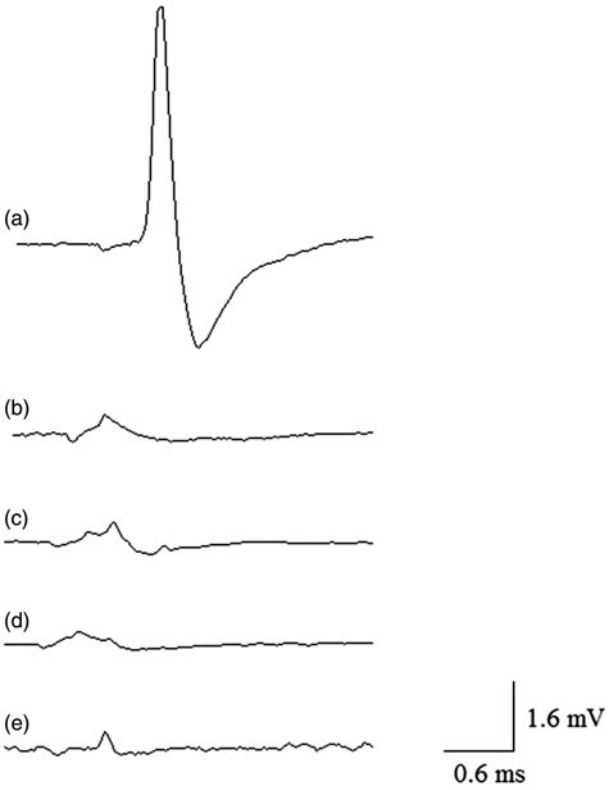

Figure 1. Effects of acetamiprid on CNAP recordings in 30 min (a: Control, b: Group 1, c: Group 2, d: Group 3, e: Group 4).

About 1 μM acetamiprid significantly reduced nerve action potential amplitude at the rate of 78%, 86%, 89%, 85%; 10 μM acetamiprid 88%, 86%, 89%, 85%; 100 μM acetamiprid 90%, 92%, 95%, 96%; 1000 μM acetamiprid 91%, 93%, 94%, 96%, respectively, compared to the control values at the time of 30, 60, 90, and 120 min ($p < 0.05$). When compared the groups with each other, significant differences were not observed on inhibition values ($p > 0.05$).

Acetamiprid administered all dose groups significantly reduced area of action potential compared with the control values (Figure 4). 1 μM acetamiprid significantly reduced nerve action potential area at the rate of 79%, 93%, 93%, 93%; 10 μM acetamiprid 91%, 97%, 94%, 94%; 100 μM acetamiprid 88%, 90%, 98%, 98%; 1000 μM acetamiprid 97%, 97%, 97%, 98%, respectively, compared to the control value at the time of 30, 60, 90 and 120 min compared to the control values ($p < 0.05$). When compared the groups with each other, significant differences were not observed on area values ($p > 0.05$).

Histological findings

In the control nerves, almost all of the myelinated and unmyelinated axons were normal. No signs of damage were observed in the myelin sheath and the axoplasm of the control nerves (Figure 5). In this study, minor damage was identified in the nerve fibers in group 1 in which low doses of acetamiprid is applied. Focal losses were observed in some of the myelin sheath, on the other hand rare ovoid bodies were also determined (Figure 6(A)). Myelin ovoid bodies were observed in the nerves of second group which is one of the

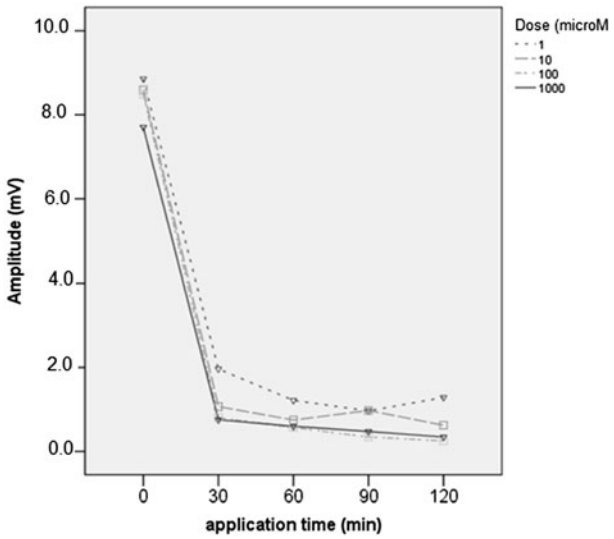

Figure 2. Effects of acetamiprid on amplitude of CNAP. Acetamiprid significantly reduced the nerve action potential amplitude compared to the control values in all application times for all groups ($p < 0.05$).

low dose of acetamiprid. An increase has also been observed in the focal separation of the myelin sheath of the nerves in this group (Figure 6(B)). Focal separations of the myelin sheath in the nerves in group 3 (one of the high dose group) were observed and large vacuolar structures connected to these separations were found. In addition, myelin ovoid bodies were seen in nerve tissue of this group (Figure 6(C)). Severe damages were observed in the nerves of the highest dose group 4 of acetamiprid. Intensive formation of the myelin sheath ovoid body and a fragmentation were present in this group. In addition to these findings, curling and disorganization were observed in some nerve myelin sheath. Axoplasm loss in severely damaged fibers was also determined (Figure 6(D)).

Discussion

In our study, the effects of acetamiprid (a neonicotinoid insecticide) on the frog sciatic nerve were investigated by using electrophysiological and histological methods. It was also observed that this insecticide had neurotoxic effect by causing damage on axonal and myelin.

There is limited published information related to investigating the neonicotinoid insecticides on vertebrate's nervous system. Kimura-Kuroda *et al.* (2012) investigated the acetamiprid (98% pure), imidacloprid (98% pure), and nicotine (99% pure) on primary cell cultures of cerebellar granule cells that were collected from newborn rats and they reported that these insecticides have excitatory effect greater doses than 1 μM on nAChRs. Christen *et al.* (2017) observed that the neurotoxic effects of different insecticides on cerebellar neurons and found that 10 μM of acetamiprid, one of these insecticides, caused neurotoxic effect. In addition to these effects of acetamiprid in the central nervous system, in the present study, it was observed that acetamiprid (100% pure) has neurotoxic effects in peripheral nerves. In our study, all applied doses of acetamiprid showed the effect starting from 30 min and suppressed the compound action potential of

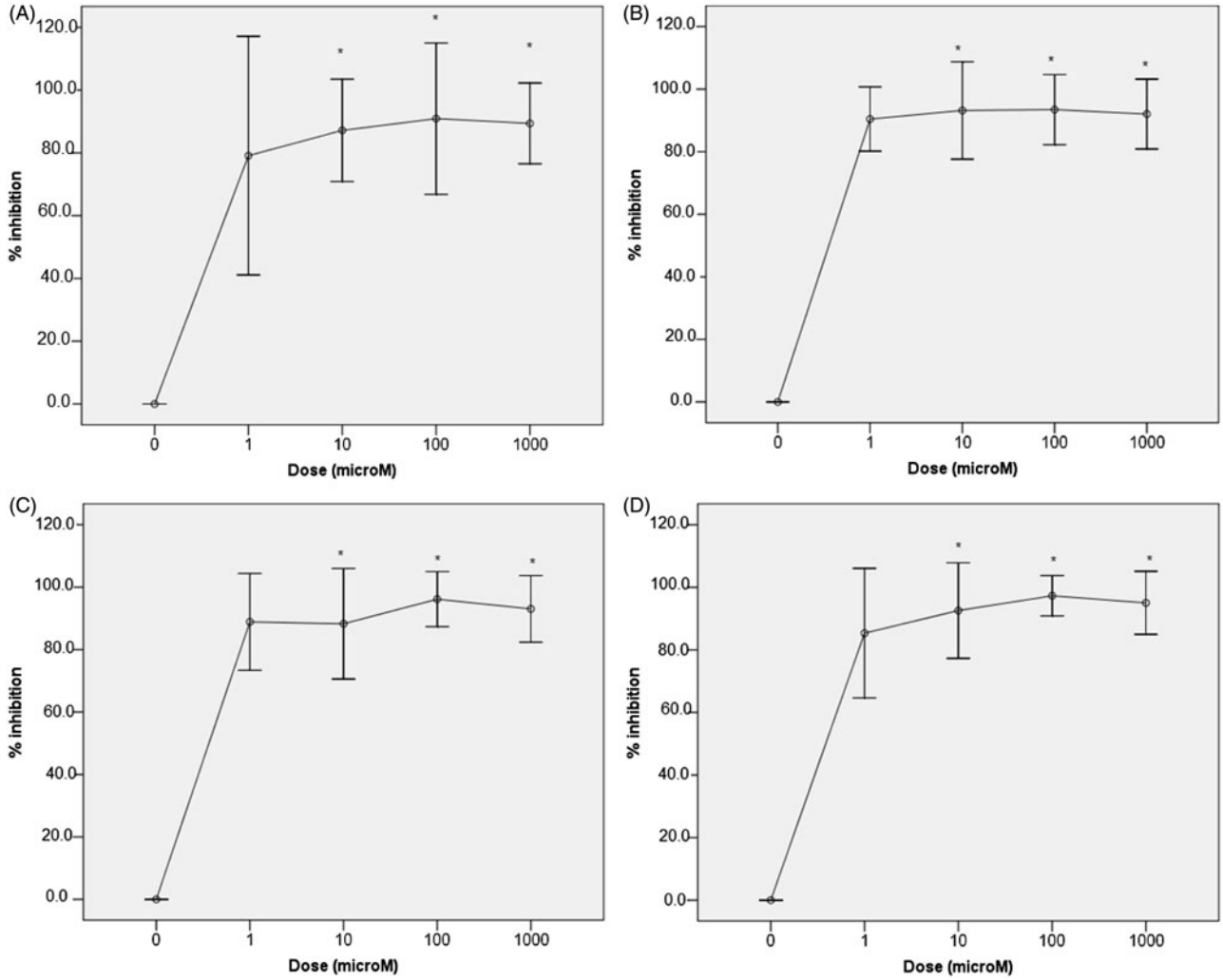

Figure 3. Dose–response relationship of the acetamiprid. (A) 30 min after application, (B) 60 min after application, (C) 90 min after application, (D) 120 min after application. *When compared to the control value (0 min), $p < 0.05$.

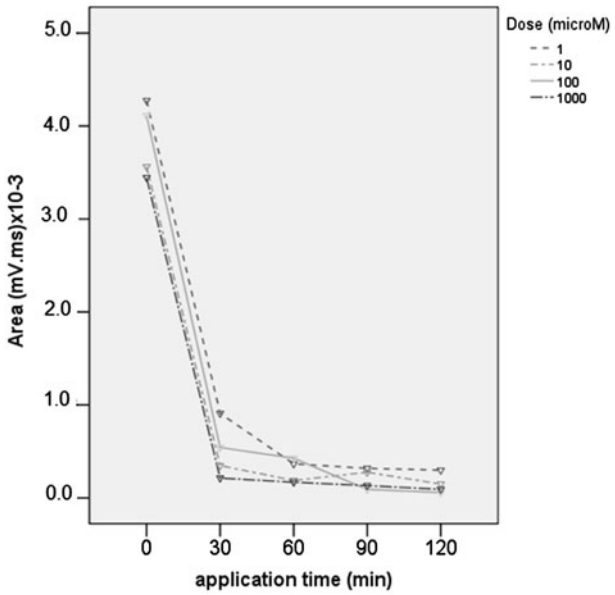

Figure 4. Effects of acetamiprid on area of CNAP. Acetamiprid significantly reduced area of the nerve action potential compared to the control values in all application times for all groups ($p < 0.05$).

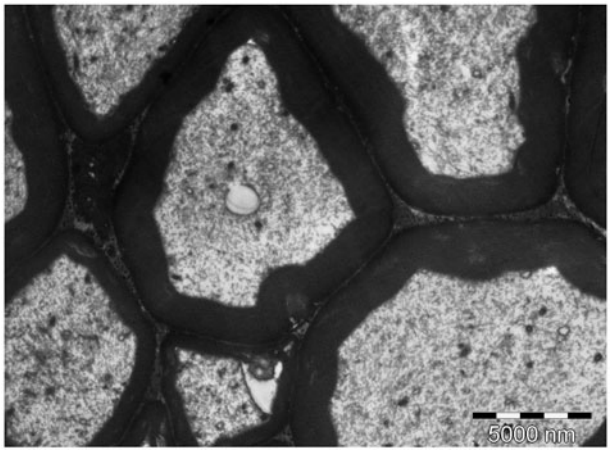

Figure 5. Electron microscopic view of control nerve. Normal sciatic nerve cross section ultra-structure was observed in control nerve (×5000).

sciatic nerve as dramatically. This suppression was observed at all doses and application times. Change of application time and dose did not affect this suppression as significantly. This strong suppression maybe related to its high acute toxicity.

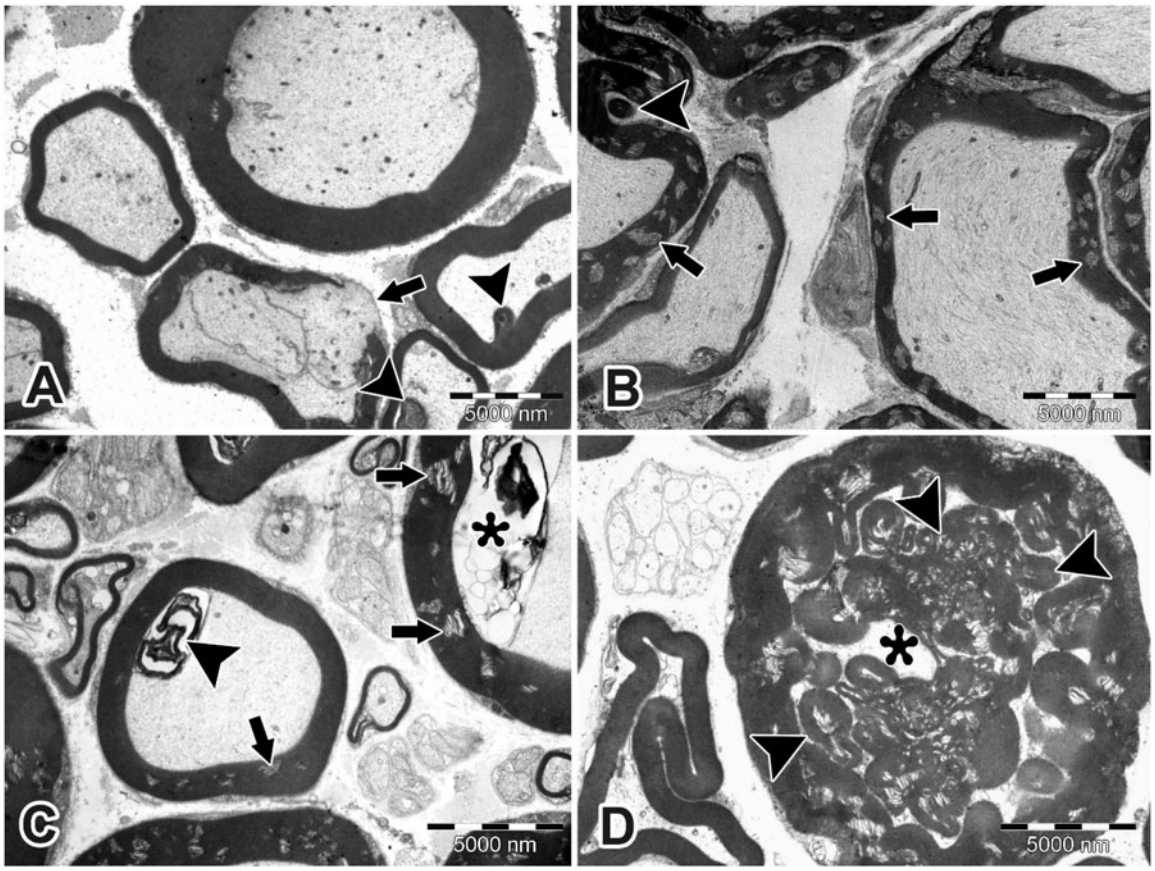

Figure 6. Electron microscopic view of dose groups (×5000). Myelin ovoid bodies (arrowheads) in (A), (B), and (C). Focal separation of myelin lamellae (arrows) in (B) and (C). Loss of myelin sheath (arrow) in (A). Vacuolar structure (asterisk) in (C). Squeezed axoplasm (asterisk), fragmented myelin sheath and ovoid bodies (arrowhead) in (D).

We calculated the IC_{50} value of acetamiprid, which is the indicator of toxicity, as 0.51 μM at 30 min for frog sciatic nerve. This value was found as 0.7 μM for mammalian neuronal nAChRs (α_4/β_2) in rats (Sheets *et al.* 2016).

Nerve signals are transmitted by action potentials, which are rapid changes in cell membrane potential from the resting or depolarized state. Measurements of action potential parameters provide information about membrane signal transmission. We measured amplitude and area values from compound action potential recordings and a decrease on both values was observed. Our electrophysiological findings indicate axonal neuropathy as defined by Aminoff (1998). According to our knowledge, there is no study investigating the effect of acetamiprid on CNAPs of vertebrate peripheral nerves. In addition, Akbas *et al.* (2014) investigated the effects of imidacloprid, a neonicotinoid insecticide, on the action potential of the frog sciatic nerve and found that different doses of imidacloprid (1, 10 and 100 μM) significantly reduced the action potential amplitude and area compared with those of control nerves. Our electrophysiological results are consistent with the results reported by Akbas *et al.* (2014) for imidacloprid.

In order to ascertain whether the electrophysiological properties were associated with corresponding alterations in morphological signs of sciatic nerve, histopathological examinations were also carried out. Disorganization, irregularity, dense ovoid body formation, and fragmentation of the myelin sheaths of the nerves in the dose group were observed. Loss of some axoplasms of the nerves was also observed on which acetamiprid applied. The structural damage on the sciatic nerves exposed to high dose groups is relatively more noticeable than to those of the nerves exposed to low dose groups. This strong damage in high doses maybe related to the oxidative stress of acetamiprid. It was previously reported that high doses of acetamiprid caused oxidative stress (Çamlıca *et al.* 2017). Oxidative stress can cause imbalance between free radical production and antioxidant activity and lead to cellular damage (Yu *et al.* 2008). There is a few study to investigate the histopathological effects of neonicotinoid insecticides on against non-target organisms. Signh *et al.* (2015) applied the neonicotinoid insecticide imidacloprid to 5, 10 and 20 μg chicken eggs, and they examined the histopathological effects on embryo cerebellum. They found that imidacloprid generated toxic and important degenerative effects on glial cells, neurons, and neuropils compared with the control group. Goyal *et al.* (2010) examined the histopathological effects of neonicotinoid insecticide thiacloprid on liver, kidney, heart, lung, intestine, brain, and ovary of *Gallus domesticus* by applying the insecticide orally 10 mg/kg/day for 28 days. They observed hepatocyte degeneration and congestion in liver, epithelial cell loss in kidney, tubular cell degeneration, hemorrhage and light blockage in lungs, glial cells in brain, neuronal degeneration around the satellite cells, myocardial hemorrhage in heart. These results support our findings about the neurotoxic effect of acetamiprid.

Conclusions

The effects of different doses of acetamiprid, a neonicotinoids insecticide, on isolated sciatic nerve were investigated for the first time using electrophysiological and histological techniques. Our findings showed that acetamiprid can cause neuropathic changes in sciatic nerve even at low doses. These results indicate that acetamiprid as other insecticides can have harmful effects on non-target organisms. Further studies should be performed in this area and new strategies should be developed for the use of insecticides.

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Disclosure statement

The authors report no declarations of interest.

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