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Research Article

Subchronic exposure to Chlorpyrifos, Carbofuran, and Cypermethrin increase sciatic nerve damage and degeneration in adolescent rats

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ABSTRACT

Several types of pesticides that are often used are chlorpyrifos (organophosphate), carbofuran (carbamate), and cypermethrin (pyrethroid). Pesticides can kill pests but also can cause toxic effects on humans when exposed. One of the adverse effects of pesticide exposure is a disturbance in the peripheral nervous system. Age is an essential factor in peripheral nerve damage. Nerve injury is one of the most common injuries in children and adolescents and is estimated at 10% to 15% of all exceptional unit cases. However, the outcome of peripheral nerve injury is known better in subjects who sustain the injury at a younger age. This study aims to determine the subchronic exposure effect of chlorpyrifos, carbofuran, and cypermethrin to sciatic nerve histopathology in juvenile rats. This study used 30 male Wistar rats (*Rattus norvegicus*) which were divided into 5 groups, normal group (N), control group (K), chlorpyrifos group (P1), carbofuran group (P2) and cypermethrin group (P3). Subcutaneous injection of pesticides was carried out for 21 days. Rats were sacrificed using the cervical dislocation method, and the right sciatic nerve was taken for histopathological observation. Assessment of the degree of ischiatic nerve histopathological damage is using the Jensen et al., (2018) method. Data were analyzed using the Kruskal-Wallis test and continued with the Mann-Whitney test. This study showed a significantly increased degree of histopathological damage in the chlorpyrifos group, carbofuran group, and cypermethrin group compared to the control group and the normal group ($p<0.05$). It can be concluded that exposure to chlorpyrifos, carbofuran, and cypermethrin pesticides increased damage and degeneration of the ischiadicus nerve in juvenile rats.

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INTRODUCTION

Pesticides are substances that are often used in the agroindustry to kill pests that have the potential to damage crops. Pesticides can be classified based on their target and chemical composition. Based on their chemical composition, pesticides are divided into several groups, including organophosphates, carbamates, and pyrethroids (Kaur et al., 2019). Several types of pesticides that are often used are chlorpyrifos (organophosphate group), carbofuran (carbamate group), and cypermethrin (pyrethroid group). The use of chlorpyrifos is quite high, reaching 60% in the United States and 53% in China (Foong et al., 2020). Chlorpyrifos is also often used by farmers in Indonesia (Sulaeman et al., 2016). In addition to chlorpyrifos, carbofuran is also often used, especially because organochlorines are prohibited, so carbofuran is an alternative (Indraningsih, 2008). For the pyrethroid group, cypermethrin is often used, such as in Botswana, Africa, where more than 50% of farmers use the pesticide cypermethrin (Sharma et al., 2019).

Pesticides can kill pests but also can cause toxic effects on humans when exposed. Pesticides can enter the human body through various ways, such as inhalation, dermal contact, and ingestion. Dietary exposures are also the main source of nonoccupational exposure to chlorpyrifos (Kadem Majeed et al., 2014;(Eaton et al., 2008). Weakness of limbs, muscle tremors, ataxia, convulsions, coma, and even death have been reported as a result of pesticide exposure in humans. Cypermethrin is known to have a toxic dose of 100-1000 mg/KgBW and a potentially lethal dose of 1-10 g/KgWB Exposure to these cypermethrin doses causes symptoms of toxicity and may lead to death (Das & Parajuli, 2007; Yadav, 2018). A study showed that exposure to chlorpyrifos 135 mg/KgBW had a Case Fatality rate (CFR) of 7.6% in 1376 patients, and carbofuran 8 mg/KgBW had a Case Fatality rate (CFR) of 1% in 479 patients (Dawson et al., 2010)(Dawson et al., 2010).

One of the adverse effects of pesticide exposure is a disturbance in the peripheral nervous system. Chlorpyrifos, carbofuran, and cypermethrin have been shown to cause toxic effects in humans, especially on peripheral nerves. Some of these clinical symptoms in peripheral nerves are numbness, paresthesias, muscle weakness, especially in distal leg muscles, and urinary disturbances (Eaton et al., 2008; Mishra et al., 2020; Yilmaz et al., 2008; Grisold & Carozzi, 2021). In addition to clinical symptoms, the toxicity of pesticides on peripheral nerves can also be assessed from the histopathological features of peripheral nerves. Several studies conducted on rats exposed to chlorpyrifos, carbofuran, and cypermethrin found features of axon degeneration in peripheral nerves. The histopathological picture is vacuolation, digestion chamber, axon swelling, and perineurial edema (Rogers-Cotrone et al., 2010; Sundukov, 2006; Kadem Majeed et al., 2014).

The sciatic nerve is the largest peripheral nerve in the body and has both sensory and motor components. The sciatic nerve has an important role in the body's motor because it is responsible for innervating most of the muscles in the body's lower extremities (Elder, 2007). Pesticides have been known to cause damage to the sciatic nerve (Bostan, 2017). The high use of pesticides increases the risk of sciatic nerve injury in workers in the agroindustry sector due to exposure to pesticides, especially workers who are teenagers. Based on data from the Central Statistics Agency (BPS), the majority of youth aged 16-30 years in rural areas work in agriculture, with a percentage of 39.41%. The data also shows that the highest percentage of rural youth who work in agriculture is in the 16-18 year age group, with a percentage of 32.70%.

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Age is an essential factor in peripheral nerve damage. Nerve injury is one of the most common injuries in children and adolescents and is estimated at 10% to 15% of all cases in specialized units (Costales et al., 2019). However, the clinical outcome of peripheral nerve injury is known to be better in subjects who experienced the damage at a younger age (Chemnitz et al., 2013). Therefore, this study aimed to determine subchronic exposure effect of chlorpyrifos, carbofuran, and cypermethrin to sciatic nerve histopathology in juvenile rats.

METHODS

Male Wistar rats (*Rattus norvegicus*) aged 30 to 60 days in healthy condition characterized by active movement and good appetite were used in this study. Rats were obtained from the Laboratory of Animal Physiology of UIN Maulana Malik Ibrahim Malang, East Java. Rats were placed in separate cages with each cage containing 2 rats. Rats were fed with pellets and fed with mineral water.

The pesticides chlorpyrifos, carbofuran, and cypermethrin are trademarks of Sigma-Aldrich. The pesticides were then made into stock solutions by dissolving them in dimethyl sulfoxide (DMSO) and sterile distilled water. Chlorpyrifos 20 mg/Kg, carbofuran 0.2 mg/Kg, and cypermethrin 20 mg/Kg were each dissolved in DMSO. The mixture was then dissolved in sterile distilled water until homogeneous. The chlorpyrifos stock solution was stored at low temperature while the carbofuran and cypermethrin stock solutions were stored at room temperature.

In this study, rats were divided into 5 groups, namely groups N, K, P1, P2, and P3. The normal group (N) did not get treatment. The control group was given a subcutaneous injection of 5% DMSO solution. Treatment group 1 (P1) was given a subcutaneous injection of 20 mg/Kg of chlorpyrifos solution. Treatment group 2 (P2) was given a subcutaneous injection of 0.2 mg/Kg of carbofuran. Treatment group 3 (P3) was given a subcutaneous injection of cypermethrin 20 mg/Kg. The administration of DMSO solution and pesticide injection was once a day and was carried out for 21 days. At the end of the study, the rats were sacrificed using the cervical dislocation method. The rats were then dissected, and the right sciatic nerve was taken for histopathological preparations and observation. This research has received ethical approval from the Ethics Commission of the Faculty of Medicine, University of Jember, number 1.605/H25.1.11/KE/2022.

The sciatic nerve was taken in the right part with a length of 1 cm. Histopathological preparations of the sciatic nerve using longitudinal sections and Hematoxylin-Eosin (H&E) staining. Histopathology was observed by double blinding using an Olympus microscope with a magnification of 400x. Assess the degree of histopathological damage to the sciatic nerve using the Jensen et al., (2018) method. Data were analyzed using the Kruskal-Wallis test and continued with the Mann-Whitney test, with a significance level of 95% (α=0.05), with a p-value of <0.05 considered significant.

RESULTS

The data is the degree of nerve damage based on the criteria of Jensen et al., (2018). The degree of damage to the sciatic nerve can be seen in Table 1. Based on the data on the average degree of damage to the sciatic nerve in each group, it is known that the average degree of damage starts from the highest to the lowest degree, P2 (carbofuran), P1 (chlorpyrifos), P3 (cypermethrin), K (control group) & N (normal group). The highest was in the P2 group (carbofuran) and the lowest was in the normal group. The higher means the damage is

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Table 1. The sciatic nerve damage degree

Group	Degree of Damage (Average ± SD)
N (Normal)	2.45 ± 0.274
K (Control)	2.50 ± 0.586
P1 (Chlorpyrifos)	3.85 ± 0.821
P2 (Carbofuran)	4.00 ± 0.433
P3 (Cypermethrin)	3.65 ± 0.822

Table 2. The Kruskal Wallis test result

	Degree of Damage
Kruskal-Wallis H	16.358
df	4
Asymp. Sig.	.003

Table 3. The post hoc Mann-Whitney test result

	N	K	P1	P2	P3
N		0.588	0.008*	0.007*	0.011*
K	0.588		0.015*	0.008*	0.035*
P1	0.008*	0.015*		0.448	0.592
P2	0.007*	0.008*	0.448		0.340
P3	0.011*	0.035	0.592	0.340	

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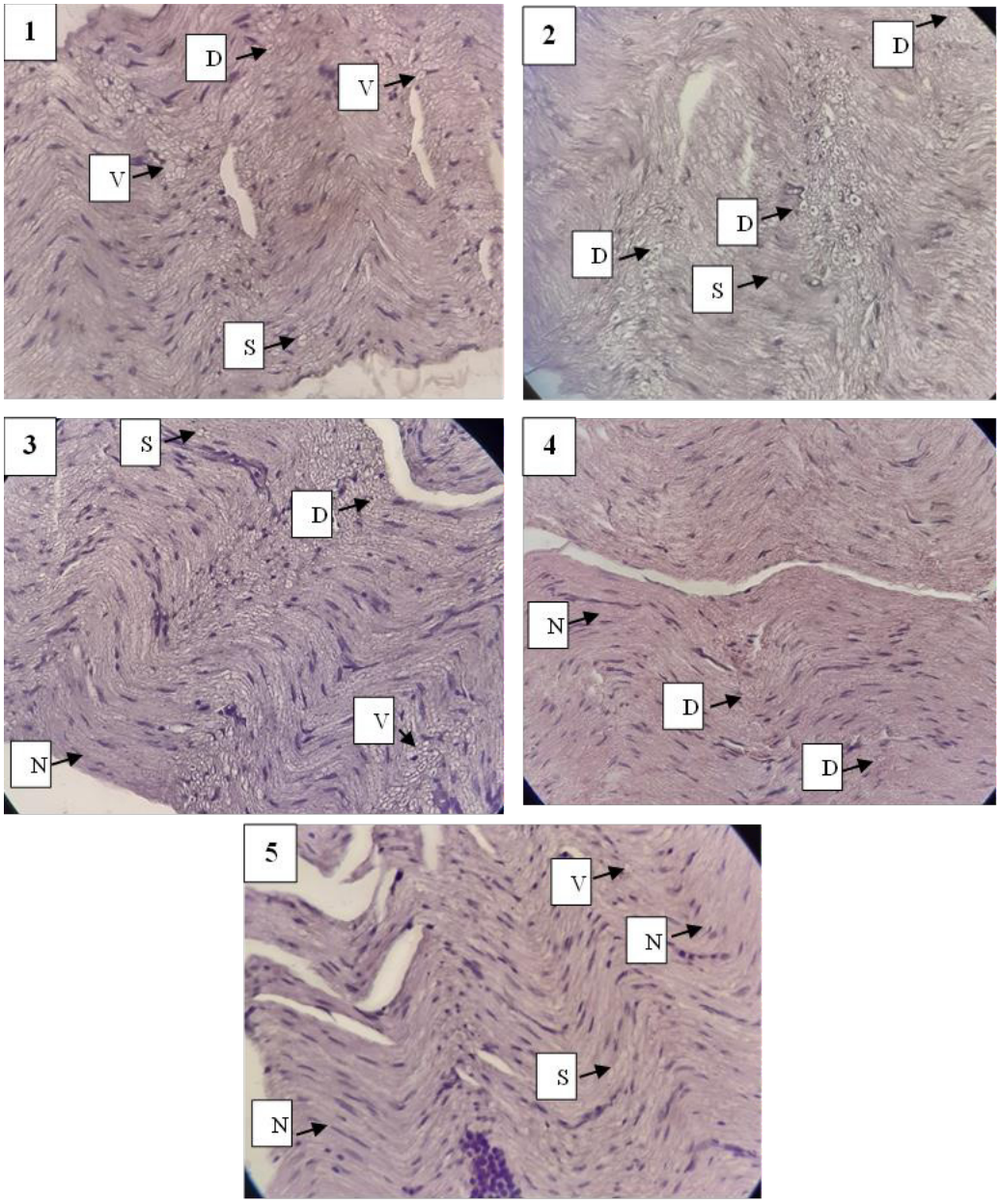

Figure 1. The Histopathological picture of the sciatic nerve in the Hematoxylin-Eosin (H&E) staining, 400x magnification; 1. chlorpyrifos administration 20mg/Kg; 2. Carbofuran administration 0.2 mg/Kg; 3. Cypermethrin administration 20 mg/Kg; 4. Control; 5. Normal. (V: vacuolation, D: Digestion chamber, S: swelling of axons and myelin, N: normal axons).

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more severe, and the lower means the damage is lighter.

In the normal and control groups, the histopathological assessment results showed an average degree of slight damage or grade 2. Histopathological appearance at this grade showed that more than 7 axons to less than 25% of axons looked abnormal or degenerated. In the treatment group that was exposed to pesticides, namely groups P1 (chlorpyrifos), P2 (carbofuran), and P3 (cypermethrin) had the same average degree of damage, marked or grade 4. Histopathological appearance at this stage shows that 50-80% of axons appear abnormal or degenerate. Nerve degeneration is indicated by vacuolation and digestion chamber images on axons. The histopathological appearance of the sciatic nerve can be seen in Fig 1.

The data were analyzed using the Kruskal-Wallis non-parametric test. The results of the Kruskal-Wallis test can be seen in Table 2. Based on the results of the Kruskall-Wallis test, the result is 0.003 ($P < 0.05$). These results indicate differences in the degree of damage between groups in the study. The data then continued with the post hoc Mann-Whitney test. The Mann-Whitney test table can be seen in Table 3.

The result showed that the normal group did not show a significant difference in the degree of damage when compared to the control group. The normal group and the control group showed significant differences in the degree of damage when compared to groups P1 (chlorpyrifos), P2 (carbofuran), and P3 (cypermethrin). There was no significant difference in the degree of damage between the P1 (chlorpyrifos), P2 (carbofuran) and P3 (cypermethrin).

DISCUSSION

Pesticides can increase nerve damage and degeneration through increased production of Reactive Oxygen Species (ROS), resulting in increased oxidative stress on nerves. Increased ROS and oxidative stress can increase nerve damage and degeneration (Fukui, 2016). Pesticides also induce neuronal microtubule damage, interfering with the distribution of the Nicotinamide mononucleotide adenylyl transferase enzyme (Nmnat), which protects neurons from degeneration (Li & Collins, 2019). In this study, adolescent rats were exposed to three pesticides: chlorpyrifos, carbofuran, and cypermethrin. This study showed increased damage and degeneration of the sciatic nerve in juvenile rats exposed to chlorpyrifos, carbofuran, and cypermethrin.

In the normal and control groups, the histopathological assessment results showed an average degree of slight damage or degree 2. In the normal and control groups, histopathological features were found indicating axon degeneration, namely digestion chamber, vacuolation, and axon swelling, although there were slight. The degenerate axon picture can also be found in normal nerves because the axon degeneration process also occurs in normal neurodevelopment. This is in accordance with other studies that the process of axon degeneration can also occur in normal development. Axon degeneration in normal development aims to eliminate unwanted or inappropriate axon branching in its region (Saxena & Caroni, 2007; Luo & O'Leary, 2005).

The treatment group that was exposed to pesticides, namely groups P1 (chlorpyrifos), P2 (carbofuran), and P3 (cypermethrin) had the same average degree of damage, marked or grade 4. The picture of vacuolation, digestion chambers, and axonal swelling on

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the axons showed nerve degeneration. The results of the comparison between the treatment group and the normal group, and the control group showed a significant difference in the degree of damage. The treatment group had a higher degree of injury when compared to the normal group and the control group. These results indicate that the administration of three types of pesticides in the treatment group both increased damage and degeneration of the nerves. Nerve degeneration is shown by vacuolation and digestion chamber images on axons. In the treatment group, there was an increase in the picture of the digestive chamber and vacuolation of the nerves when compared to the normal group and the control group. This is in accordance with several studies conducted by Rogers-Cotrone et al., (2010), Sundukov, (2006), Kadem Majeed et al., (2014) that the three types of pesticides increase nerve damage and degeneration. The increase in axon damage and degeneration in this treatment group may occur because pesticides can increase the production of Reactive Oxygen Species (ROS) and induce microtubule damage resulting in increased nerve damage and degeneration (Karami-mohajeri & Abdollahi, 2011; Gupta et al., 2007). Research conducted on adult rats exposed to organophosphate pesticides showed an increase in vacuolation of the lumbar region of the nerves (Rogers-Cotrone et al., 2010). Another study conducted using adult rats exposed to chlorpyrifos and carbaryl either alone or in combination showed a pathological effect on the sciatic nerve which was observed through histopathological and biochemical observations (Sundukov, 2006). Another study conducted on adult rats with exposure to cypermethrin increased neurodegeneration. The increase in nerve degeneration is indicated by an increase in the vacuolation of the number and size of the spinal and sciatic nerves (Kadem Majeed et al., 2014).

Increased ROS, decreased antioxidants, and microtubule damage can be caused by excessive excitation of nerves due to pesticides (Karami-mohajeri & Abdollahi, 2011; Gupta et al., 2007). The three pesticides in this study, namely chlorpyrifos, carbofuran, and cypermethrin, work by inhibiting the action of acetylcholinesterase (AChE) and modulating sodium and calcium channels and then increasing excitation in the nerves (Eaton et al., 2008; Saquib et al., 2021; Kadem Majeed et al., 2014). Increased excitation of nerves leads to dysfunction of mitochondria. Neurons cannot control ROS production, so ROS production is excessive and results in increased oxidative stress and neurodegeneration (Fukui, 2016; Karami-mohajeri & Abdollahi, 2011). In addition, excess intracellular calcium due to the effects of pesticide exposure induces phosphorylation of Collapsin Response Mediator Protein (CRMP-2) and an increase in HDCA5, which results in microtubule damage. Microtubule damage to neurons disrupts the distribution of Nicotinamide mononucleotide adenylyl transferase enzyme (Nmnat) which protects neurons from damage and degeneration (Li & Collins, 2019). Sub-chronic exposure to chlorpyrifos, carbofuran, and cypermethrin in this study showed an increase in damage and degeneration of the sciatic nerve in adolescent rats. The limitation of this study is that no clinical assessment was carried out to determine the effect of pesticide exposure on the clinical condition of the lower extremities of subjects.

CONCLUSION

Based on this study, it can be concluded that subchronic exposure to chlorpyrifos, carbofuran, and cypermethrin increase damage and degeneration of the sciatic nerve in adolescent rats.

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