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# NEUROTOXIC EFFECT OF DERMALLY-APPLIED CHLORPYRIFOS AND CYPERMETHRIN IN WISTAR RATS

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**Abstract:** The aim of the study was to evaluate the neurotoxic effect of a dermally-applied mixture of chlorpyrifos and cypermethrin in rats based on cognitive function, activity of the blood cholinesterase and brain acetylcholinesterase, as well as histologic brain examination. Nurelle D 550 EC (500 g of chlorpyrifos and 50 g of cypermethrin) was used in the study. The application liquid was in the form of a water solution. The investigation covered eight groups of animals: six experimental groups and two control groups, of 15 rats each. Experimental groups received 5.6 mg/cm$^2$ chlorpyrifos and 0.5 mg/cm$^2$ cypermethrin, or 27.8 mg/cm$^2$ chlorpyrifos and 2.7 mg/cm$^2$ cypermethrin dermally, for one day, one week and four weeks, except for Saturdays and Sundays. The preparations examined were applied to the tail skin of rats. The animals were anaesthetized at the end of exposure period. Plasma cholinesterase and brain acetylcholinesterase activities were determined. The brain for histological examination was perfused with a solution of methanol, formalin and glacial acetic acid, and the sections stained by the Nissel method. The behaviour of the animals was evaluated in the open field test four times: before exposure, and after one, two and four weeks of the experiment. The results of the study showed that chlorpyrifos and cypermethrin applied in a mixture caused an inhibition of cholinesterase and acetylcholinesterase activity and elicited the pycnosis of brain neurocytes.

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**Key words: dermal toxicity, chlorpyrifos, cypermethrin, histopathology, cholinesterase acivity, open field test.**

## INTRODUCTION

Dermal absorption of pesticides is important in occupational poisonings, especially in occupations where means of plant protection are used. Some pesticides may be composed of two or more active substances. The application of pesticides in mixture may result in decreased toxicity of mixture components, additivity or synergistic toxicity.

Nurelle D 550 EC is an insecticide containg two substances, chlorpyrifos and cypermethrin. Chlorpyrifos (0,0-diethyl-0-[3,5,6-trichloro-2-pirydinyl]phosphorotioate) is an organophosphorous insecticide which inhibits acetylcholine decompositon. Therefore it increases the acetylcholine level in the synaptic cleft and stimulates specific receptors [14]. Chlorpyrifos applied dermally in pregnant rats inhibited maternal and foetal brain acetylcholinesterase (AChE) activity within 24 h of dosing (48% and 67% of control activity, respectively) [2]. The neurotoxic effect of chlorpyrifos has been examined extensively [5, 12, 15, 16, 24]. Applied to human skin, as a commercial concentrate or as a reference standard dissolved in ethanol, it was recovered from skin after 24 h. Griffin *et al.* [8] therefore suggest that skin could act as a reservoir and may release chlorpyrifos over a longer period.

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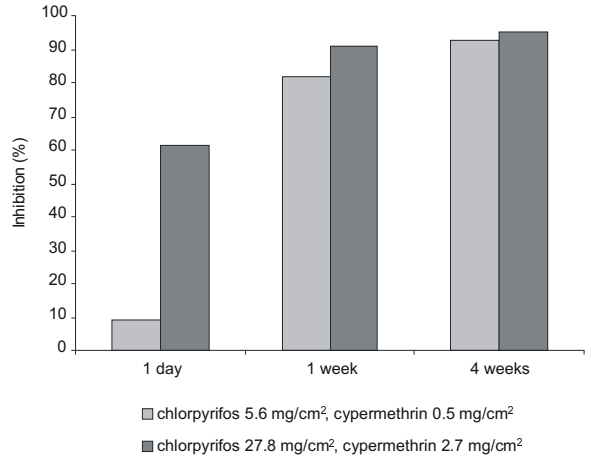


**Figure 1.** Effect of Nurelle D 550 EC applied to tail skin of female rats on plasma cholinesterase (ChE) activity, compared to control group I.

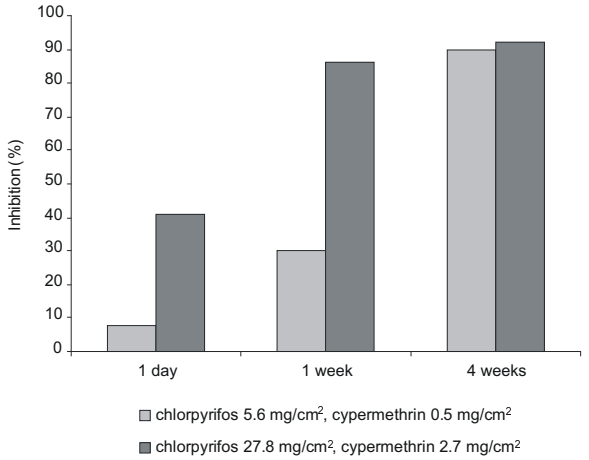


**Figure 2.** Effect of Nurelle D 550 EC applied to tail skin of female rats on plasma cholinesterase (ChE) activity, compared to control group II.

Cypermethrin (R-cyano-3-phenoxybenzyl (1R)-cis-3 (2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) is an active pyrethroid. Pyrethroids are known to stimulate nerves by causing pronounced repetitive acivity. The effect is observed in the central nervous system, sense organs and neuromuscular junctions. Synapses are more sensitive to pyrethroids than nerve fibres. Noncyano pyrethroids, such as permethrin, cause nerve impulse trains of short duration, whereas cyano pyrethroids, such as cypermethrin or deltamethrin, induce long-lasting trains of repetitive nerve impulses. Alpha-cyano pyrethroids induce intense repetitive activity in peripheral nerves that may lead to frequency-dependent depression of the nerve impulse. The major effect of pyrethroids is to delay the closing of the sodium channel, so that a prolonged sodium tail current persists after the membrane repolarization [19, 21, 22].

An interesting example of synergism with relation to the toxic effect of substances administered in mixtures are studies conducted on hens. After intragastric administration of pirydostygmine and subdermal administration of DEET (N,N-diethyl-m-toluamide) or chlorpyrifos, considerably greater neuropathological changes were noted in cases of co-exposure, compared to those observed after administration of each preparation separately [1].

The aim of this study was to evaluate the neurotoxic effect of dermally-applied mixture of chlorpyrifos and cypermethrin based on the blood cholinesterase and brain acetylcholinesterase activity, histologic brain examination, as well as behavioural function (open-field test).

## MATERIALS AND METHODS

Nurelle D 550 EC (500 g of chlorpyrifos and 50 g of cypermethrin, Dow Elanco, USA) was used in the study. The application liquid was in the form of a water solution.

The study was conducted on 3-month-old female Wistar rats, in good condition, with no macroscopic

**Table 1.** Effects of dermally-applied Nurelle D 550 EC on plasma cholinesterase (ChE) activity in rats.

| Examined groups | ChE ( IU/l)[a] $\bar{x} \pm SD$ | | |
|---|---|---|---|
| | 1 day exposure | 1 week exposure | 4 weeks exposure |
| Control group I<br>n = 8 | 1481. 20 ± 155.41 | 1447.88 ± 180.87 | 1482.80 ± 114.94 |
| Control group II<br>n = 10 | 1469.70 ± 180.65 | 902.2 ± 148.24 | 997.00 ± 387.70 |
| Rats exposed to:<br>chlorpyrifos 5.6 mg/cm$^2$<br>cypermethrin 0.5 mg/cm$^2$<br>n = 10 | 1345.75 ± 148.75* | 270.50 ± 25.93** | 101.60 ± 90.00** |
| Rats exposed to:<br>chlorpyrifos 27.8 mg/cm$^2$<br>cypermethrin 2.7 mg/ cm$^2$<br>n = 10 | 869.60 ± 213.34** | 128.80 ± 15.80** | 76.01 ± 8.30** |

*–**: significantly lower compared to control group I; *p < 0.05 , ** p < 0.0001. [a]International Units per litre (1 International Unit = 1 µmol/min).

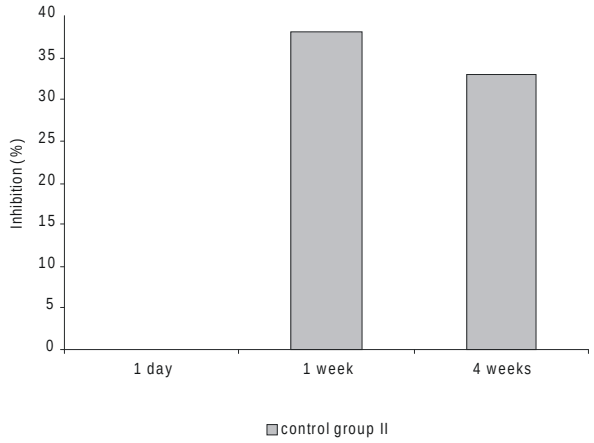


**Figure 3.** Plasma cholinesterase (ChE) activity in control group II, compared to control group I.

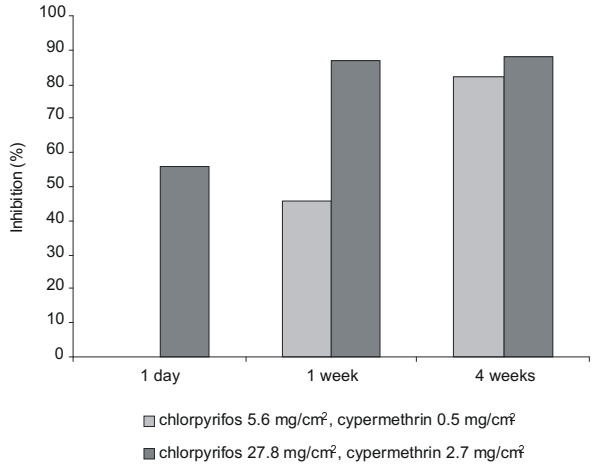


**Figure 4.** Effect of Nurelle D 550 EC applied to tail skin of female rats on brain acetylcholinesterase (AChE) activity, compared to control group I.

changes of the tail skin. The animals were fed with standard feed LSM and water *ad libitum* [9]. The initial body weight of the rats was 170-220 g. The investigation covered eight groups of animals (six experimental and two control) of 15 rats each. The experimental groups received dermally 5.6 mg/cm$^2$ of chlorpyrifos and 0.5 mg/cm$^2$ of cypermethrin, or 27.8 mg/cm$^2$ of chlorpyrifos and 2.7 mg/cm$^2$ of cypermethrin, for one day, daily over one week, or daily over four weeks, except for Saturdays and Sundays. The preparation examined was applied on the tail skin (area 9 cm$^2$ ) of rats with the use of an absorptive fabric FPP-15, and covered with aluminium foil [20]. The time of exposure was four hours daily. The animals of the first control group (I) were not exposed and handled. Rats of the second control group (II) were only immobilized at the same time as animals of the experimental groups.

After one day, one week, and four weeks of the experiment, the animals were anaesthetized. Blood from the heart and brain was taken to evaluate cholinesterase activity. Blood cholinesterase and brain acetylcholinesterase activity was determined by Ellman *et al.* [7] colorimetric method, with the use of propionylotiocholine iodide and 5,5'-dithio-bis(2-nitrobenzoic acid) as cholinesterase stimulator.

The brain for histologic examination was perfused transcardially with a solution of methanol, formalin and glacial acetic acid, embedded in paraffin and cut into sections which were stained by the Nissel method [23].

The behaviour of animals was studied by means of open field test (ambulation, grooming, rearing, object exploration, defecation score) [18], four times: prior to exposure, after one week, two weeks, and four weeks of the experiment. Parallelly, a group of unexposed animals kept in the same condition was tested as a control.

Statistical analysis was performed by the parametric Student's t- test.

**Table 2.** Effects of dermally-applied Nurelle D 550 EC on brain acetylcholinesterase (AChE) activity in rats.

| Examined groups | AChE ( IU/g)[a] $\bar{x} \pm SD$ | | |
|---|---|---|---|
| | 1 day exposure | 1 week exposure | 4 weeks exposure |
| Control group I<br>n = 8 | 6.71 ± 0.32 | 6.66 ± 0.23 | 6.17 ± 0.26 |
| Control group II<br>n = 10 | 6.72 ± 0.33 | 6.27 ± 0.24 | 4.74 ± 0.18 |
| Rats exposed to:<br>chlorpyrifos 5.6 mg/cm$^2$<br>cypermethrin 0.5 mg/cm$^2$<br>n = 10 | 6.77 ± 0.19 | 3.59 ± 0.14** | 1.14 ± 0.25** |
| Rats exposed to:<br>chlorpyrifos 27.8 mg/cm$^2$<br>cypermethrin 2.7 mg/ cm$^2$<br>n = 10 | 2.96 ± 0.22** | 1.54 ± 0.13** | 0.77 ± 0.14** |

**: significantly lower compared to control group I, $p < 0.0001$. [a] International Units per gram (1 International Unit = 1 µmol/min).

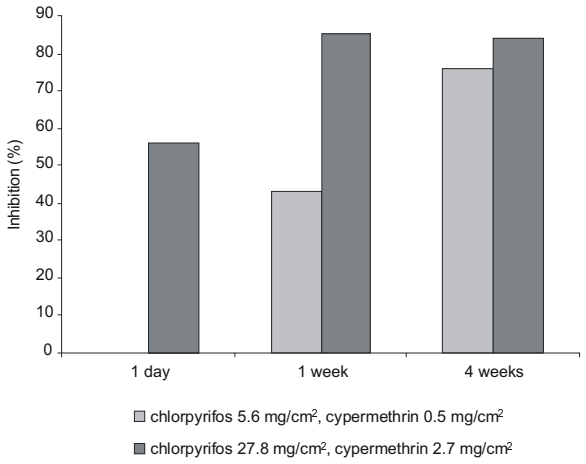


**Figure 5.** Effect of Nurelle D 550 EC applied to tail skin of female rats on brain acetylcholinesterase (AChE) activity, compared to control group II.

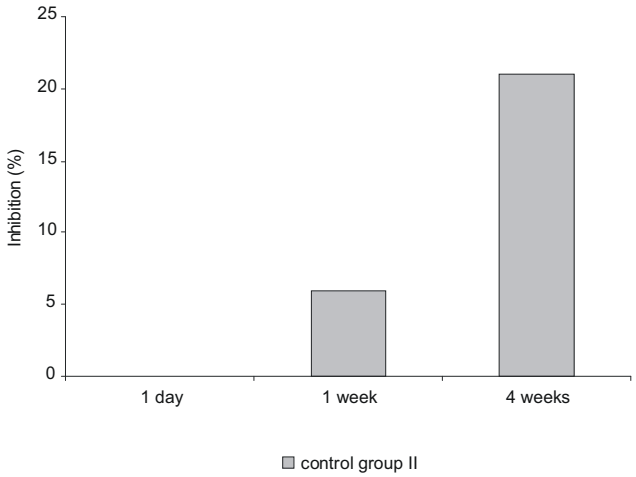


**Figure 6.** Brain acetylcholinesterase (AChE) activity in control group II, compared to control group I.

## RESULTS

**Biochemical studies**. Cholinesterase (ChE) activity in plasma of experimental rats which received the lower dermal dose of the mixture (5.6 mg/cm$^2$ chlorpyrifos and 0.5 mg/cm$^2$ cypermethrin) decreased considerably, compared to both control groups ($p < 0.0001$) after one week and four weeks of exposure. A single dose slightly decreased ChE activity, compared to the control groups ($p < 0.05$). After administration of a higher dose of the mixture, ChE activity was markedly inhibited in all experimental groups exposed to dermal absorption for one day, one week, and four weeks ($p < 0.0001$), compared to both control groups (Tab. 1). In these groups, directly after exposure lasting one day, one week or four weeks, the plasma ChE activity decreased by 61%, 91% and 95%, compared to control group I, and by 41%, 86% and 92%, compared to control group II (Fig. 1 and Fig. 2). In animals of control group II, which were only immobilized for one week or four weeks, plasma ChE activity decreased by 38% and 33% respectively, compared to the control group I (Fig. 3).

**Table 3.** Effect of dermally-applied Nurelle D 550 EC on the rats spontaneous activity measured in open field test.

| Examined groups | | Control group n = 8 $\bar{x} \pm$ SEM | Rats exposed to: chlorpyrifos 5.6 mg/cm$^2$ cypermethrin 0.5 mg/cm$^2$ n = 10 $\bar{x} \pm$ SEM | Rats exposed to: chlorpyrifos 27.8 mg/cm$^2$ cypermethrin 2.7 mg/ cm$^2$ n = 10 $\bar{x} \pm$ SEM |
|---|---|---|---|---|
| Prior to exposure | A | 100.75 ± 11.09 | 92.20 ± 8.13 | 99.60 ± 5.16 |
| | B | 2.00 ± 0.57 | 2.10 ± 0.43 | 2.50 ± 0.37 |
| | C | 18.20 ± 3.12 | 20.90 ± 1.43 | 19.10 ± 1.67 |
| | D | 6.25 ± 2.13 | 6.40 ± 1.09 | 6.30 ± 0.76 |
| | E | 2.40 ± 0.87 | 2.40 ± 0.61 | 3.30 ± 0.57 |
| After 1 week exposure | A | 63.25 ± 8.71 | 59.50 ± 8.71 | 51.00 ± 6.58 |
| | B | 2.00 ± 0.91 | 2.00 ± 0.29 | 1.90 ± 0.34 |
| | C | 6.00 ± 2.54 | 8.90 ± 1.64 | 8.00 ± 1.27 |
| | D | 1.00 ± 0.70 | 2.20 ± 0.44 | 1.90 ± 0.48 |
| | E | 3.20 ± 1.39 | 4.60 ± 1.03 | 3.40 ± 0.76 |
| After 2 weeks exposure | A | 91.70 ± 14.41 | 99.20 ± 10.66 | 64.70 ± 11.06 |
| | B | 1.40 ± 0.45 | 2.00 ± 0.36 | 1.10 ± 0.38 |
| | C | 9.60 ± 1.80 | 12.30 ± 1.31 | 8.20 ± 1.77 |
| | D | 5.80 ± 1.25 | 7.10 ± 1.26 | 4.50 ± 1.23 |
| | E | 1.40 ± 0.93 | 0.80 ± 0.59 | 0.20 ± 1.13 |
| After 4 weeks exposure | A | 58.40 ± 12.95 | 79.90 ± 11.42 | 43.80 ± 9.76 |
| | B | 1.20 ± 0.29 | 1.90 ± 0.35 | 1.10 ± 0.31 |
| | C | 4.70 ± 1.20 | 7.00 ± 1.77 | 4.70 ± 1.28 |
| | D | 1.90 ± 0.95 | 3.30 ± 0.93 | 2.40 ± 0.82 |
| | E | 1.90 ± 0.86 | 1.60 ± 1.15 | 0.30 ± 0.30 |

A -ambulation; B - grooming; C - rearing; D -object exploration; E - defecation

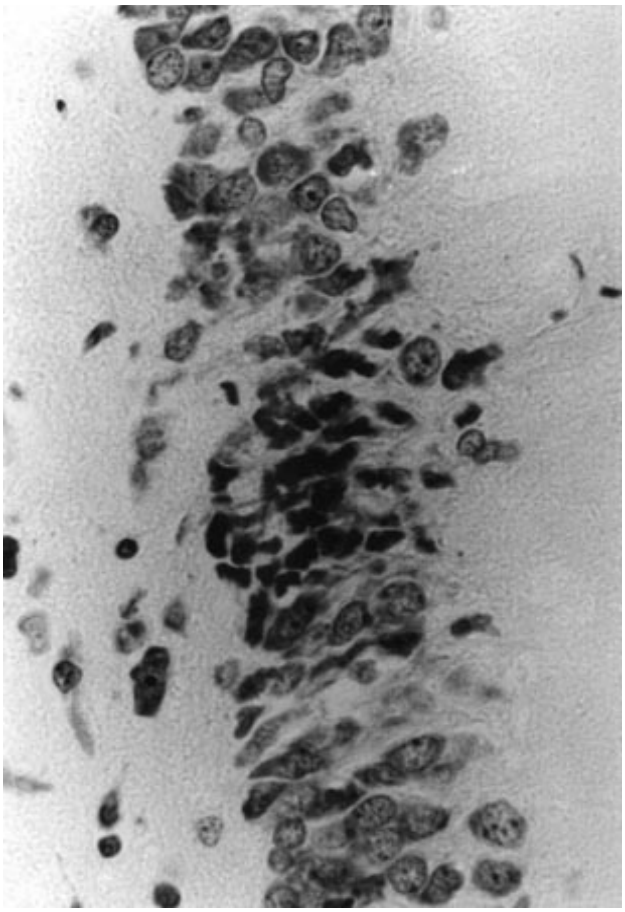

**Figure 7.** Brain of rat exposed to dermal absorption of 27.8 mg/cm$^2$ chlorpyrifos and 2.7 mg/cm$^2$ cypermethrin. Focal pycnosis of the cytoplasm in the cells of stratum granulosum areae dentatae. Stained according to the Nissel method. H+E, × 160.

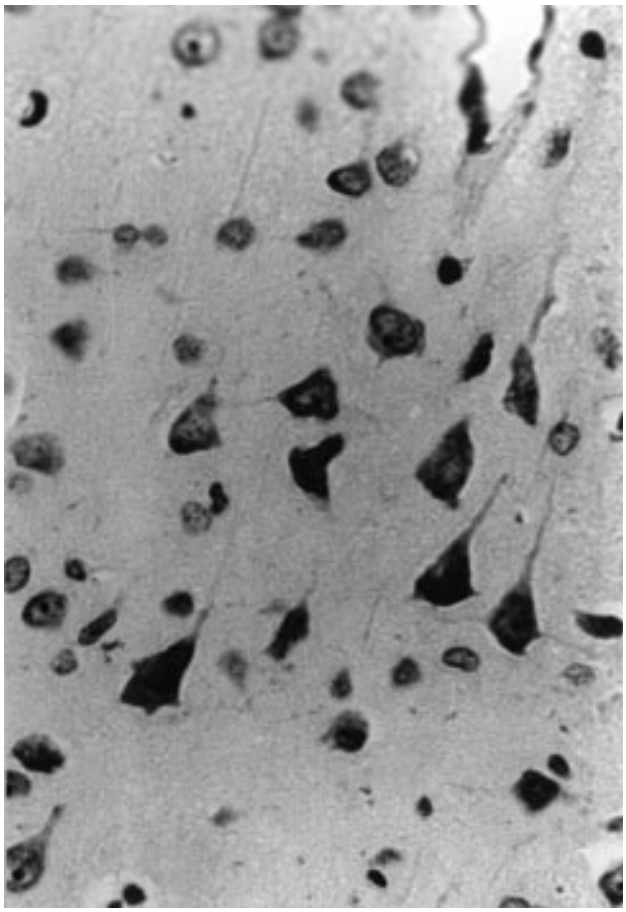

**Figure 8.** Brain of rat exposed to dermal absorption of 27.8 mg/cm$^2$ chlorpyrifos and 2.7 mg/cm$^2$ cypermethrin. Pycnosis of the cytoplasm in single cells of layer pyramidale of cortex cerebri. Stained according to the Nissel method. H+E, × 160.

Single exposure to a low dose of the mixture did not affect brain acetylcholinesterase (AChE) activity, howewer, the exposure lasting one and four weeks resulted in a marked decrease of enzyme activity as compared to the both control groups ($p < 0.0001$) (Tab. 2). A one day, one week and four weeks administration of a higher dose of the mixture evoked a statistically significant decrease of AChE activity. AChE actvity inhibition was: 56%, 87% and 88% respectively, compared to control group I (Fig. 4) and 56%, 85% and 84%, compared to control group II (Fig. 5). In animals of control group II, brain AChE activity decreased after exposure lasting one week and four weeks as compared to the control group I by 6% and 21%, respectively (Fig. 6).

**Histologic examinations**. Changes in the brain were noted in the animals which were administered a higher dose of chlorpyrifos and cypermethrin on the tail skin after one week and four weeks. These changes were manifested by pycnosis of neurocytes in various areas of the brain. Most often, the changes were clear in the cells of the stratum granulosum in areae dentatae (Fig. 7) and stratum pyramidale hippocampi (CA 1). Pycnosis of the cytoplasm in single cells of layer pyramidale of the cortex cerebri (Fig. 8), area pyriformis of the cortex cerebri, striatum, Purkinje cells (Fig. 9), and nucleus medialis in the cerebellum was observed.

**Open field test**. No significant differences in the number of ambulation, grooming, rearing, object exploration, and defecation scores were found between the experimental and control groups (Tab. 3).

## DISCUSSION

Subcutaneous administration of a single dose of 279 mg/kg of chlorpyrifos resulted in the inhibition of cholinesterase activity in plasma and erythrocytes, as well as high acetylcholinesterase inhibition in the whole brain (over 90%) which persisted for up to seven days after administration of the preparation [15].

In other studies it was observed that a subdermal administration of the above-mentioned high dose of chloropyrifos resulted in a considerable inhibition of cholinesterase activity in the cerebral cortex and corpus striatum in adult rats, after two, four and six weeks (by

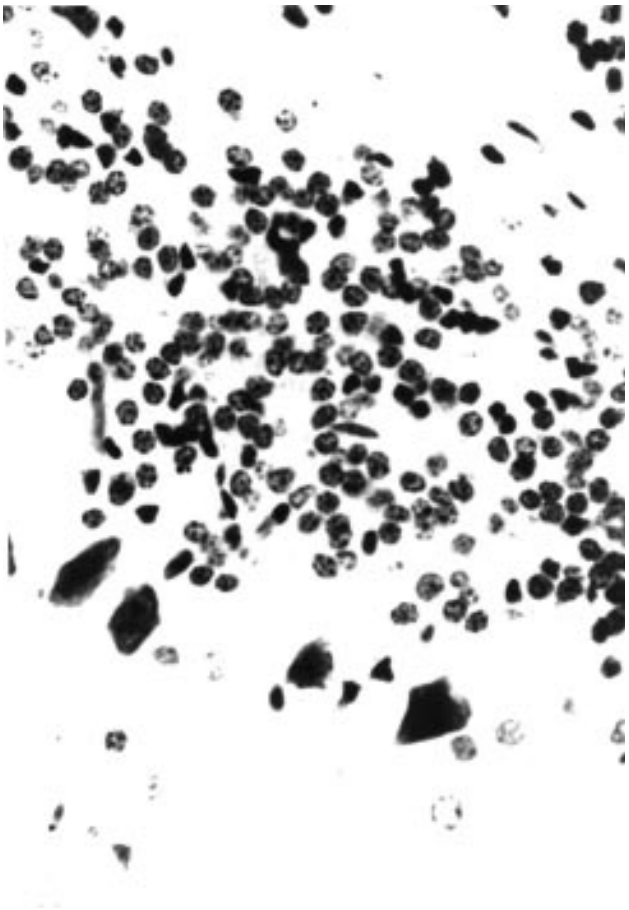

**Figure 9.** Brain of rat exposed to dermal absorption of 27.8 mg/cm$^2$ chlorpyrifos and 2.7 mg/cm$^2$ cypermethrin. Pycnosis of the cytoplasm of the Purkinie cells in cerebellum. Stained according to the Nissel method. H+E, × 160.

94–96%, 82–83%, and 58–60%, respectively), and a decrease in the motor activity two days after administration of the preparation [16].

Subcutaneous administration of single and multiple doses of chlorpyrifos caused cholinesterase inhibition in blood and brain, a decrease in the number of muscarinic receptors, and inhibition of motor functions [3]. Repeated subcutaneous injections of chlorpyrifos (40 mg/kg for four days) caused an extensive inhibition of brain cholinesterase activity in the cortex, hippocampus and striatum among adult rats, four and 14 days after the last injection (by 92–98% and 71–78%, respectively) [4].

Cypermethrin administered intragastrically in doses of 100 mg/kg and 80 mg/kg for a period of six, 12 and 18 days did not cause any significant changes in cholinesterase activity in the blood, plasma, brain or liver [10]. However, Rao and Rao [17] reported that after intragastric administration of permethrin, the inhibition of acetylcholinesterase activity was observed in all areas of the brain: cortex cerebri, cerebellum, striatum, hippocampus, and hypothalamus at four, eight, and 12 h after administration, while cypermethrin led to an increase in the activity of investigated enzyme.

Our study showed a significant decrease in cholinesterase activity in the plasma of experimental rats administered with a mixture of chlorpyrifos and cypermethrin dermally, in a lower dose (5.6 mg/cm$^2$ of chlorpyrifos and 0.5 mg/cm$^2$ of cypermethrin), compared to both control groups ($p < 0.0001$), after one and four weeks exposure. A single dose (4 h) slightly decreased ChE activity, compared to the control groups ($p < 0.05$). After administration of a higher dose of the mixture (27.8 mg/cm$^2$ of chlorpyrifos, and 2.7 mg/cm$^2$ of cypermethrin) a distinct inhibition of ChE activity was noted in all experimental groups ($p < 0.0001$).

No changes were found in the brain AChE activity after four-hour dermal exposure to a lower dose of the mixture; an extreme decrease was observed, however, after one and four weeks lasting administration of this dose, compared to both control groups ($p < 0.0001$). A single, weekly and monthly administration of a higher dose resulted in a statistically significant decrease in the activity of the enzyme examined (in both weekly and monthly experiments the inhibition of AChE was < 90%).

An oral administration of chlorpyrifos in doses of 10, 30, 60 or 100 mg/kg produced the following results: the lowest dose caused merely ChE inhibition, and evoked no behavioural changes; the administration of higher doses led to an increase in ChE inhibition and changes in animals behaviour. A partial reversibility of the behavioural effects was noted 24 hours after the experiment, with a slight reversibility of brain ChE activity (in frontal cortex, hippocampus, striatum, hypothalamus, cerebellum, pons medulla) [14]. After an oral administration of chlorpyrifos to seven-day-old rats (15 mg/kg), 21-day-old rats (47 mg/kg) and 90-day-old rats (136 mg/kg) plasma ChE activity decreased only in adult animals; however, no symptoms of functional toxicity were observed [24].

According to Das *et al.* [6], acute and chronic immobilization stress suppresses brain AChE activity in adult mice.

In our study, brain acetylcholinesterase and plasma cholinesterase activity in animals solely immobilised for four hours (control group II), did not change after a single exposure; however, after exposure lasting one or four weeks, inhibition of brain acetylcholinesterase and plasma cholinesterase activity was noted. These results suggest that the repeated prolonged immobilization may cause such stress, which leads to suppression of acetylcholinesterase in the brain and cholinesterase in plasma.

Histologic examinations of the brain showed that the dermal application of alpha-cypermethrin in doses of 50 mg/kg and 250 mg/kg for four weeks resulted in the accumulation of cytoplasm of Purkinje cells in the cerebellum, a focal accumulation of the neurocytes cytoplasm of stratum granulosum, as well as of single neurocytes of the CA 3 hippocampus layer, and of neurocytes in the hypothalamus and the cortex cerebrum [11].

Histologic changes observed in the brain after the administration of the mixture of chlorpyrifos and cypermethrin occurred primarily following the administration of a higher dose of the pesticide, after one and four weeks of the experiment. These changes were manifested as the accumulation of neurocytes cytoplasm in various areas of the brain, and were clear in the neurocytes of stratum granulosum areae dentatae and stratum pyramidale hippocampi (CA 1). Pycnosis of single neurocytes was noted in the area pyriformis of the cortex cerebri, and striatum. Pycnosis of the Purkinje cells or nucleus medialis neurocytes in the cerebellum was also observed.

The dermal application of alpha-cypermethrin in doses of 50 and 250 mg/kg did not lead to changes in the behaviour of the animals in the open field test after two weeks of the experiment. After four weeks, only grooming was increased in rats treated with alpha-cypermethrin in a dose of 250 mg/kg ($p < 0.01$). A slight decrease was noted with respect to the remaining parameters, such as the number of ambulations, rearing, and object exploration. However, the differences were not statistically significant (unpublished data).

In the present study, no significant differences in the open field test were found between the experimental and control groups.

In order to determine whether the organophosphorus insecticide monocrotofos enhanced the neurotoxic effect of cypermethrin, oral doses of monocrotofos (11 g/l), cypermethrin (25 g/l), or a mixture of these two preparations were administered for seven subsequent days. The symptoms of poisoning and neurotoxic effects were related to the exposure to organophosphorus insecticide as a component of the mixture, and no evidence on the enhancement of neurotoxic effect was found [13].

The results of the present study showed that the mixture of chlorpyrifos and cypermethrin exerts a strong cholinergic effect, whereas no significant influence of this pesticide on the behaviour of animals in open field test was noted. Similar results in the open field test were obtained after dermal exposure to alpha-cypermethrin (unpublished data).

It is known that organophosphorous preparations and pyrethroids exert a neurotoxic effect which, in the case of organophosphorous compounds, is manifested by a strong cholinesterase inhibition and leads to the impairment of brain conductivity. Cypermethrin belongs to pyrethroids which contain within their structure an alphacyanide group. These compounds affect the central and peripheral nervous systems, and lead to decreased motor activity.

Due to the fact that organophosphorous compounds and pyrethroids are characterized by a similar effect on the CNS, it may be expected that the symptoms would intensify after the application of the mixture of two preparations which cause a decrease in the motor activity of animals. However, no such interaction was observed in our study performed by the open field test. These results suggest that it is necessary to conduct a more comprehensive analysis with the use of behavioural tests.

The results of our study showed that chloropyrifos and cypermethrin administered in a mixture strongly inhibited cholinesterase activity in plasma and acetylcholinesterase activity in brain, and had a toxic effect on the CNS. However, the effect of the mixture of pesticides on the CNS was not stronger than that caused by alpha-cypermethrin alone. High cholinesterase and acetylcholinesterase inhibition was associated with the effect of chlorpyrifos. Cypermethrin, as reported by other authors, does not cause an inhibition of the activity of the enzymes examined [10].

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