



Neurotoxic effects of combined exposure to caffeine and pyrethroids in mice

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Abstract

Introduction and Objective. Pyrethroids are manufactured insecticides known for their neurotoxicity, which can cause seizures, impairment of cognitive and motor functions. Caffeine (CAF), a nonselective adenosine A₁ and A_{2A} receptor antagonist and commonly used psychoactive substance, may affect these disorders in a dose-dependent manner. The aim of the study was to determine the neurotoxic potential of co-exposure to pyrethroids and CAF in mice.

Materials and method. CAF in combination with deltamethrin (DEL), β -cyfluthrin (CYF) or tefluthrin (TEF) was evaluated against pyrethroid-induced seizures, as well as in the passive avoidance task and rotarod test for the assessment of learning and motor coordination, respectively. All substances were administered acutely by intraperitoneal injections and CAF was used at doses capable of interfering with pharmacological activities of other compounds.

Results. The CD₅₀ value for each pyrethroid (median pyrethroid convulsive dose causing seizures in 50% of mice) was unaffected by CAF (40 mg/kg). Pyrethroids at their 1/5 CD₅₀ did not impair learning, whether administered alone or with CAF. In the rotarod test, CAF (40 mg/kg) potentiated the harmful impact of TEF (2.3 mg/kg) on motor coordination, while having no effect on two other pyrethroids. TEF (2.3 mg/kg) alone and in combination with CAF (40 mg/kg), reduced acetylcholinesterase (AChE) activity in the brain at comparable levels.

Conclusions. This study demonstrates that TEF neurotoxicity may be increased in the presence of CAF co-exposure. The interaction of TEF and CAF with the cholinergic system, resulting in a decrease in AChE activity in the brain, is not responsible for the effect observed in the rotarod.

Key words

pyrethroids, caffeine, seizures, memory, motor coordination, mice

INTRODUCTION

Pyrethroids are widely used as insecticides domestically and in agriculture, as well as in medicine for the treatment of head lice and malaria control in tropical countries [1]. Pyrethroids have a wide range of structures, including those with α -cyano phenoxybenzyl moiety, such as deltamethrin (DEL) and β -cyfluthrin (CYF), and those without, such as tefluthrin (TEF) [2]. These pyrethroids were employed in the current study. The nervous system is thought to be a common target for all pyrethroids, and they have historically been classified as causing a T (tremor), CS (choreoathetosis with salivation) or intermediate syndrome of intoxication based on their biological effects [2]. It is possible that the effects of pyrethroids in the central nervous system can be affected by other centrally-active substances. One of them could be caffeine (CAF; 1,3,7-trimethylxanthine), a nonselective adenosine A₁ and A_{2A} receptor antagonist, and the most widely consumed psychoactive drug worldwide [3]. Coffee and tea are among the most popular beverages throughout the world containing CAF. In addition, CAF can also be synthesized and is added to foods, soft drinks, energy drinks, energy shots, and some medications [4]. The consumption

of moderate amounts of CAF is thought to increase energy availability, alertness, wakefulness, the ability to concentrate and focus attention and to enhance cognitive functioning capabilities and motor performance, as well as decrease fatigue and the sense of effort associated with physical activity [5]. In turn, CAF at high doses, for example, has been shown in experimental studies to produce learning deficits [6, 7] and increase seizure susceptibility in rodents [8]. CAF may also produce seizures *per se* at excessive doses around 400 mg/kg injected intraperitoneally (i.p.) in mice [9]. On the other hand, seizures, learning impairments, and motor coordination impairment have been attributed to the neurotoxic effects of pyrethroids [2, 10, 11].

OBJECTIVE

It was hypothesized that co-exposure to CAF and pyrethroids may affect their neurotoxic potential. Therefore, their combined effects on seizures, learning and motor performance were assessed in the study. The molecular targets of pyrethroids include voltage-gated sodium (the main target of pyrethroid-induced neurotoxicity), chloride, and calcium channels, as well as ligand-gated ion channels (GABA receptors, nicotinic acetylcholine receptors, and glutamate receptors) [12]. Recently, a possible role of acetylcholinesterase (AChE) activity in the biological effects

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of the interaction between CAF and organophosphates has been investigated [13]. Consequently, the brain activity of this enzyme is assessed in the current study, also taking into consideration that both CAF and some pyrethroids, including DEL and CYF, have been reported to inhibit AChE [11, 14, 15]. In our previous study, the effect of CAF on the neurotoxicity of organophosphates was investigated at the highest CAF dose of 40 mg/kg [13]. For comparative reasons, the highest dose of CAF used in this study was also 40 mg/kg.

MATERIALS AND METHOD

Animals. The experiments were carried out on adult male Swiss mice (body weight 25–30 g), purchased from a licensed dealer (J. Kolacz, Warsaw, Poland). Animals were housed under standard laboratory conditions (room temperature $22 \pm 2^\circ\text{C}$, relative humidity $55 \pm 5\%$, 12-h light/dark cycle) in colony cages with free access to food and tap water *ad libitum*. Experimental groups, consisting of 7–8 mice, were made up at random and each mouse was used only once. All experimental procedures were approved by the Second Local Ethics Committee at the University of Life Sciences in Lublin, and complied with the EU Directive 2010/63/EU for animal experiments.

Substances. The following pyrethroids were used: deltamethrin (DEL), β -cyfluthrin (CYF) and tefluthrin (TEF) (purchased from PESTANAL[®] Sigma-Aldrich). Caffeine – CAF (Coffeinum-natrium benzoicum) was purchased from Pharma Cosmetic (Kraków, Poland). Pyrethroids were suspended in a 1% aqueous solution of Tween 80 (Sigma-Aldrich) whereas CAF was dissolved directly in saline (0.9% NaCl). All substances were administered i.p. in a volume of 5 ml/kg body weight as single injections. Pyrethroids were given 60 min and CAF 30 min before the behavioural tests and sampling for assessment of AChE activity.

Seizure activity. The seizure activity of pyrethroids was assessed based on the observations presented by Weiner et al. [2]. According to this report, pyrethroid-induced clonic convulsions were defined as the occurrence of at least one of the following conditions: repetitive movements of mouth/jaw, back twitches, head/body twitches, irregular jerking, convulsions of the whole body. The doses of pyrethroids were: DEL 20–100 mg/kg, CYF 5–50 mg/kg and TEF 11–22 mg/kg. At least 3 groups of mice (consisting of 8 animals per group) were administered with different doses of pyrethroids or in combination with CAF (40 mg/kg). After injection of the substance, animals were placed separately in transparent Plexiglass cages (25×15×10 cm) and observed for up to 3 h for the occurrence of clonic seizures. To estimate the respective CD_{50} value (median pyrethroid convulsive dose causing a seizure response in 50% of mice) for pyrethroid alone or in the combination with CAF, a dose-response relationship curve was calculated based on the percentage of animals having seizures. Pyrethroids doses at their $1/5 CD_{50}$ s were used further in the behavioural tests described below.

Passive avoidance learning. A step-through passive avoidance task was used to assess learning in mice. Animals were pretreated with pesticides and/or CAF on the first day before training. During the training trial, they were placed

individually in an illuminated box (12×20×15 cm) adjacent to a dark box (24×20×15 cm) that was equipped with an electric grid floor connected to a generator. A 4×7 cm doorway was located at floor level in the centre of a common wall. When the mouse entered the dark box, it was punished with an electric foot shock (0.6 mA for 2 s). On the next day, 24 h later, a retention test was conducted in which the same animals (without any treatment) were returned to the lighted box and the latency (retention time) of entering the dark box was recorded. The trial ended when the mouse entered the dark box or after 180 s. Animals avoiding the dark box for 180 s were considered remembering the task.

Rotarod performance. For the assessment of motor coordination in mice, a rotarod apparatus (model 47600, Ugo Basile, Varese, Italy) was used. On the first day, animals were trained on a 3 cm diameter rod rotating at a constant speed of 6 rpm. Animals able to stay on the rod for at least 60 s in 2 consecutive trials (120 s) were subjected to further testing. On the next day, 24 h later, animals were pretreated with pesticides and/or CAF and placed back on the rotating rod for 120 s. The time at which the animals fell off the rotarod was recorded. Mice that did not fall off within the allotted time were given a score of 120 s.

Acetylcholinesterase (AChE) activity. AChE activity was determined using a modification of Ellman's colorimetric method, as described earlier [13]. Mouse brains removed from skulls after decapitation were placed in a freezer at -80°C . The next day, the brains were cleaned and washed out with cooled phosphate buffer (pH 7.8), dried and weighed. The fresh, unfrozen tissue was homogenized with ice-cold 0.05 M sodium phosphate buffer (pH 7.8) at a ratio of 1 g of tissue in 5 ml of buffer. Then an aliquot of 50 μl of the homogenate was added to 20 ml buffer containing 5.5 dithiobis-2-nitrobenzoic acid-DTNB (10 mg/100 ml), and 4 ml of the sample was used to determine AChE activity. The assay was started by adding 50 μl of 20 mM propionylthiocholine iodide (PTC) to the samples. PTC was hydrolyzed by AChE to form thiocholine, reacting with DTNB to yield yellow 5-thio-2-nitrobenzoate. The samples were then centrifugated at $1850 \times g$ for 5 min. Changes in absorbance, directly proportional to AChE activity, were measured with a microplate reader (Bio-Tek ELx800) at a wavelength of 412 nm.

Statistical analysis. CD_{50} values with their 95% confidence limits were calculated by the computer log-probit analysis according to Litchfield and Wilcoxon [16]. The seizure activity of pyrethroids and CAF was analyzed using the log-probit method. Results from the passive avoidance task and the rotarod test were evaluated with Kruskal-Wallis non-parametric ANOVA, followed by Dunn's multiple comparisons test. One-way ANOVA and *post-hoc* Dunnett's test was used to compare AChE activity among experimental groups. Group differences were considered statistically significant at $p < 0.05$.

RESULTS

Seizure activity. CD_{50} values for DEL (70.2 mg/kg), CYF (22.3 mg/kg) and TEF (11.6 mg/kg) were not significantly affected by CAF treatment at the dose of 40 mg/kg i.p. in mice (Fig. 1).

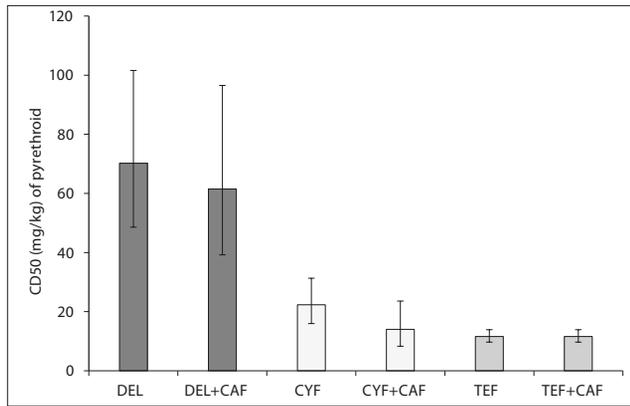


Figure 1. Effect of caffeine on seizure activity of pyrethroids in mice. Results are shown as median convulsive doses (CD_{50} s in mg/kg) with 95% confidence limits. Caffeine was administered at the dose of 40 mg/kg. 24 mice were used to determine each CD_{50} value. CAF – caffeine, DEL – deltamethrin, CYF – β -cyfluthrin, TEF – tefluthrin. $p > 0.05$ vs. respective pyrethroid group (according to Litchfield and Wilcoxon [16])

Behavioural tests. Pyrethroids alone, used at $1/5 CD_{50}$ doses, DEL (14.0 mg/kg), CYF (4.5 mg/kg) and TEF (2.3 mg/kg), did not impair learning in the passive avoidance task. Also, co-administration of pyrethroids with CAF (40 mg/kg) did not cause retention deficits compared to the control group (Fig. 2).

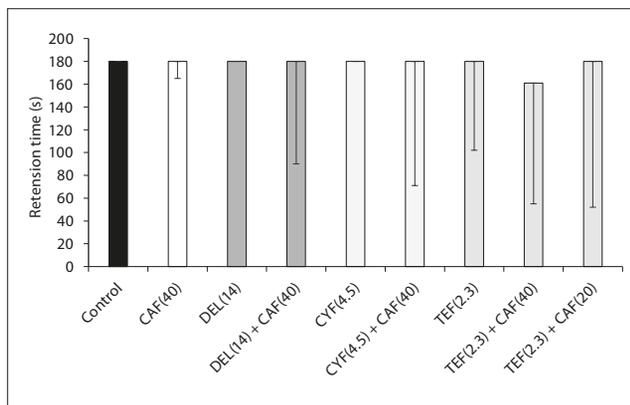


Figure 2. Effect of caffeine and pyrethroids on learning in the passive avoidance test in mice. Data are expressed as median values (in seconds – s) along with the 25th and 75th percentiles. The number of mice (n) in individual groups was 8. CAF – caffeine, DEL – deltamethrin, CYF – β -cyfluthrin, TEF – tefluthrin. $p > 0.05$ vs. control group (Kruskal-Wallis/Dunn's test)

In the analysis of the rotarod data, the Kruskal-Wallis test revealed a significant overall group effect ($H = 42.131$, $p < 0.0001$). Further analysis with Dunn's test showed impaired motor coordination in TEF (2.3 mg/kg) group ($p < 0.05$) and TEF (2.3 mg/kg) + CAF (40 mg/kg) group ($p < 0.01$), compared to the control group. The combinations of TEF and CAF, when one of these substances was at doses lower than 2.3 mg/kg (TEF) and 40 mg/kg (CAF), did not impair motor coordination in mice. Co-exposure to CAF (40 mg/kg) and other 2 pyrethroids, DEL (14.0 mg/kg) or CYF (4.5 mg/kg), did not affect the performance of mice in the rotarod (Fig. 3).

AChE activity. Analysis of data with one-way ANOVA revealed a significant overall group effect [$F(3, 28) = 9.349$; $p = 0.0002$]. Further analysis with Dunnett's test showed that TEF (2.3 mg/kg) alone and the combination of TEF

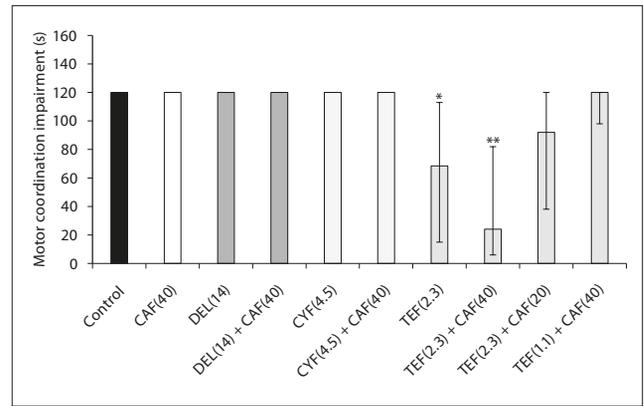


Figure 3. Effect of caffeine and pyrethroids on motor coordination in the rotarod test in mice. Data are shown as median values (in seconds – s) along with the 25th and 75th percentiles. The number of mice (n) in individual groups was 8 except for TEF (2.3 mg/kg) + CAF (40 mg/kg) group where $n = 7$. CAF – caffeine, DEL – deltamethrin, CYF – β -cyfluthrin, TEF – tefluthrin. ** $p < 0.01$, * $p < 0.05$ vs. control group (Kruskal-Wallis/Dunn's test)

(2.3 mg/kg) with CAF (40 mg/kg) significantly reduced AChE activity in mouse brains, $p < 0.01$ for each group (Tab. 1).

Table 1. Effects of caffeine and tefluthrin on brain acetylcholinesterase (AChE) activity in mice

Substance (mg/kg)	AChE activity (IU/g)
Control (saline)	8.855 \pm 0.330
Caffeine (40)	8.803 \pm 0.439
Tefluthrin (2.3)	7.093 \pm 0.383**
Tefluthrin (2.3) + caffeine (40)	6.821 \pm 0.238**

Results are shown as mean \pm SEM of eight determinations and expressed in IU/g of wet brain tissue. The number of mice (n) in individual groups was 8. ** $p < 0.01$ vs. control group (ANOVA/Dunnett's test)

DISCUSSION

The present study showed that CAF (at the subconvulsive dose of 40 mg/kg i.p.), which is a psychoactive substance able to attenuate the anticonvulsant action of antiepileptic drugs in mice when used at similar doses [9], did not affect the seizure activity induced by tested pyrethroids, DEL, CYF and TEF. On the other hand, it is the first study to show that acute CAF can increase the neurotoxic effects of pyrethroids, as observed with TEF in the rotarod test.

The pronounced impairment of motor coordination in the rotarod test caused by simultaneous exposure to CAF and TEF requires further investigations. Both DEL and CYF have previously been reported to impair rotarod performance in animals [17, 18], therefore the observed impairment cannot be attributed to a specific effect of TEF on the rotarod. Since TEF belongs to a group of pyrethroids (Type I pyrethroids) other than DEL and CYF (Type II pyrethroids), it should be taken into account in an attempt to explain this phenomenon. Experimental studies showed some of the differences in the mechanisms of toxicity between Type I and Type II pyrethroids [19]. Despite common biological targets for all pyrethroids, including particularly voltage-gated sodium, calcium, and chloride ion channels, Type I pyrethroids, have quantitatively and qualitatively different effects than Type II pyrethroids at all levels of organization. These include the level of the mentioned ion channels, the macroscopic levels

of organization within electrically-conductive tissue (nerve-muscle, spinal cord, hippocampus and cortical neurons), as well as the level of the whole animal, where toxicologically relevant responses and lack of such effects can be observed [20]. Based on current results it is rather impossible to assess whether the observed impairment in the rotarod is a pyrethroid group-related effect, or just specific for CAF-TEF interaction in this test. Studies with CAF and other representatives of pyrethroids, both belonging to Type I and Type II groups, could help clarify this issue. However, this study shows that some pyrethroids, in addition to previous findings on organophosphates [13], are pesticides whose toxicity may also increase after acute CAF treatment.

In the current study, CAF potentiated the toxic effect of TEF on rotarod performance, which is a sensitive test of motor function [21]. The rotarod with a rotating rod at constant speed was used, which is applied to assess motor coordination and balance in mice [22]. Recently, mechanisms related to neurotransmitter systems that can contribute to motor skill learning impairments in rodents assessed by some behavioural tests, including the accelerating rotarod test, have been presented [23]. It is likely that these systems may be also involved in motor coordination impairment evaluated in the constant-speed rotarod. These neuromodulator and neurotransmitter systems include dopaminergic, cholinergic, endocannabinoid, GABAergic and glutamatergic systems [23]. Reports are showing that pyrethroids may affect some of these systems; for example, allethrin (Type I pyrethroid), cyhalothrin (Type II) and DEL (Type II) following systemic (i.p.) administration, were investigated for their effects on glutamatergic and GABAergic neurons in the rat hippocampus [24].

Since pyrethroids act on channels such as voltage-gated sodium channels (VGSCs) and voltage-gated calcium channels (VGCCs), this effect could lead to alterations in neurotransmission which may be involved in pyrethroid neurotoxicity [24, 25]. The current study shows for the first time that TEF is an AChE inhibitor in rodents. The inhibiting effect on AChE activity by other pyrethroids has been reported previously [12]. Gestational and lactational exposure to bifenthrin (Type I) and CYF (Type II) in rats caused a significant decrease in the activity of AChE in the cerebellum, corpus striatum and hippocampus, as well as impairment of motor coordination in the constant-speed rotarod in pups on postnatal day 21 [26]. In a study by Syed et al. [18], AChE activity in different regions of the brain and motor coordination in the rotarod was reduced by chronic CYF administration in adult rats. The decreased level of AChE activity has been also correlated with disturbed locomotor activity and exploratory behaviour in adolescent Swiss mice administered with CYF [11]. Moreover, it has been demonstrated that DEL intoxication reduced AChE activity in mice [14]. The mechanism by which TEF impaired motor coordination in the rotarod test needs to be elicited. However, it is known that the reduction of AChE activity induced by organophosphorus pesticides leads to accumulation of acetylcholine (ACh) in the synaptic cleft, which results in over-stimulation of nicotinic and muscarinic ACh receptors, impeded neurotransmission and the occurrence of many symptoms including muscle weakness and muscle fasciculations [27]. The same disturbances could occur after TEF intoxication and affect mice performance in the rotarod. CAF, as an inhibitor of AChE [15], might further decrease its

activity when co-administered with TEF but this mechanism does not seem to be responsible for CAF-induced potentiation of TEF effect in the rotarod. Exposure to the TEF group and TEF + CAF group reduced the activity of AChE in the brain by 20% and 24%, respectively, compared to the control group, and there was no statistical difference between these groups; therefore, other mechanisms are probably involved. Since CAF is an antagonist of A_1 and A_{2A} adenosine receptors [28], consideration should be given to the possible role of adenosine receptor blockade in potentiating the effect of CAF on TEF-induced motor coordination impairment. It would be interesting to conduct a study with selective A_1 and A_{2A} adenosine receptor antagonists and agonists on the motor coordination of animals treated with TEF in the rotarod test.

CONCLUSIONS

Based on the results in the current study, it is suggested that the acute neurotoxicity of TEF may be higher in the case of CAF co-exposure. This effect is task-dependent as it was only observed in the rotarod, and not in the passive avoidance test. Additional tests should be carried out to confirm this effect on other motor functions, while on the other hand, other memory tests should be performed to rule out this effect on memory systems. Although CAF as well as TEF, as shown in this study, are AChE inhibitors, the interference of TEF and CAF with the cholinergic system leading to a reduction in AChE activity in the brain is not responsible for the phenomenon in the rotarod.

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