Original article

Neurological effects of acute exposure to caffeine and organophosphates in mice

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Abstract

Organophosphate (OP) pesticides are commonly known for their neurotoxicity. In the current experiments, two OPs used agriculturally, chlorpyrifos and dimethoate, were separately administered with centrally acting caffeine that is known to affect the pharmacological action of other substances. The aim of this study was to determine whether the combination of OP and caffeine may influence their neurotoxic potential. For this purpose, some neurobehavioral effects of this concomitant exposure were assessed in adult Swiss mice. All substances were given intraperitoneally (i.p.) as single injections. In the passive avoidance task, chlorpyrifos (100 mg/kg) administered together with caffeine (40 mg/kg) significantly impaired acquisition. In the rota-rod test, the addition of caffeine at doses of 20 and 40 mg/kg, induced motor coordination impairment in chlorpyrifos (100 mg/kg)-treated mice. Neurobehavioral impairments were not observed for caffeine, chlorpyrifos and dimethoate (50 mg/kg) given separately as well as for the combination of dimethoate and caffeine. Chlorpyrifos (100 mg/kg) alone and in combination with caffeine (40 mg/kg) significantly reduced acetylcholinesterase (AChE) activity. The current study shows that concomitant exposure to caffeine and chlorpyrifos can cause neurotoxic effects in mice despite the absence of these effects when caffeine and chlorpyrifos are administered alone. However, the possible mechanisms involved need further investigations.

Key words: organophosphates, caffeine, memory, motor coordination, mice

Introduction

Organophosphates (OPs) are widely used pesticides, which main pharmacological effect is associated with the inhibition of the enzyme acetylcholinesterase (AChE). Inhibition of AChE by OPs leads to accumulation of acetylcholine (ACh) at cholinergic synapses in the central and peripheral nervous systems, causing overstimulation of cholinergic receptors of both musca-

rinic and nicotinic type (Costa et al. 2008). Neurotoxic effects of OPs in humans and animals include impairments of motor functions and different forms of learning and memory (Valenzuela-Harrington et al. 2012). It can be hypothesized that the action of OPs in the central nervous system may be affected by other centrally acting substances. According to our knowledge, effects of co-exposure to OPs and caffeine (1,3,7-trimethylxanthine), a nonselective adenosine A_1 and A_{2A} receptor

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antagonist and well known psychoactive substance (Fredholm et al. 1999), on cognitive and motor functions have not been investigated yet. The question of combined exposure to OPs and caffeine is important. Firstly, due to the common use of caffeine worldwide, which is found in many beverages like coffee, tea and soft drinks, as well as products containing cocoa or chocolate, medications and dietary products (Heckman et al. 2010). On the other hand, the wide use of OPs brought about that they are one of the most ubiquitous synthetic chemicals in the environment (Morgan et al. 2014). Secondly, caffeine (similarly to OPs) is an inhibitor of AChE (Fabiani et al. 2018). However, in the case of caffeine, this pharmacological activity is thought to be, at least in part, responsible for its cognition-enhancing properties (Mohamed et al. 2013).

The current study sought to assess the effect of caffeine on two OPs effect i.e. chlorpyrifos (CPF) and dimethoate (DM), which are still in use in agriculture, on acquisition of the passive avoidance task and motor coordination in the rota-rod test. OPs and caffeine have been reported to be active in these tests (e.g. Angelucci et al. 1999, Valenzuela-Harrington et al. 2012, Vatanparast et al. 2013). Because adult rodents are less susceptible to acute toxic effects of OPs than young animals (Won et al. 2001), we used high doses of DM and CPF, up to 50 and 100 mg/kg, respectively. Caffeine was used at doses (20-40 mg/kg) not only active behaviorally in mice but also able to compromise the pharmacological action of other substances (Nehlig et al. 1992, Fredholm et al. 1999). Therefore, it was hypothesized that co-exposure to OPs and caffeine may affect their neurotoxic potential.

Materials and Methods

Animals

The experiments were performed on adult male Swiss mice, weighing 27-31 g. They were housed in colony cages in a ventilated room with an ambient temperature of $22\pm2°C$, a relative humidity of $55\pm5\%$, and under a 12-h light/dark cycle. Animals had free access to standard laboratory feed and tap water ad libitum. Experimental groups, consisting of 7-9 animals, were chosen randomly and each mouse was used only once. All experiments were performed between 9.00 a.m. and 4.00 p.m. All experimental procedures were approved by the Second Local Ethics Committee at the University of Life Sciences in Lublin (License no.: 47/2012) and complied with the EU Directive 2010/63/EU for animal experiments.

Chemicals

CPF and DM were purchased from Sigma-Aldrich and caffeine (Coffeinum-natrium benzoicum) from Pharma Cosmetic (Kraków, Poland). Caffeine and DM were dissolved in saline (0.9% NaCl), whereas CPF was suspended in a 1% aqueous solution of Tween 80 (Sigma-Aldrich). All substances were administered intraperitoneally (i.p.) in a volume of 5 ml/kg body weight. Behavioral tests (rota-rod and passive avoidance test) were assessed for the following experimental groups: control (saline), caffeine (40 mg/kg), DM (50 mg/kg), CPF (100 mg/kg), DM (50 mg/kg) + caffeine (40 mg/kg) and CPF (100 mg/kg) + caffeine (40 mg/kg). Due to positive effects found in the CPF + caffeine groups, this combination was also tested using lower doses of caffeine (20 mg/kg) and CPF (50 mg/kg). All experimental groups used in this study have been presented in Table 1 and 2. Caffeine and DM were administered 30 min while CPF was injected 60 min prior to behavioral tests, as well as, before brain sampling for assessment of AChE activity.

Passive avoidance test

A step-through passive avoidance test was used in the current study. On the first day, mice were pretreated with pesticides and/or caffeine before training. During the training trial, pretreated animals were individually placed in an illuminated box (12×20×15 cm), connected to a dark box (24×20×15 cm) equipped with an electric grid floor. A 4×7 cm doorway was located at floor level in the centre of the common wall. Entrance of animals to the dark box was punished by an electric foot shock (0.6 mA for 2 s). Mice that did not enter the dark box within 180 s, were gently helped to enter it. Twenty-four hours later, a retention trial was conducted in which the same animals (without any treatment) were placed again into the illuminated box and the latency (retention time) to enter the dark box was recorded. The trial ended when the mouse entered the dark box or until 180 s had elapsed. Animals avoiding the dark box for 180 s were regarded as remembering the task.

Rota-rod test

Motor coordination was evaluated with the use of rota-rod apparatus (model 47600, Ugo Basile, Varese, Italy). On the first day, mice were trained on a 3-cm diameter rod rotating at a constant speed of 6 rpm. Only animals which were able to remain on the rod for at least 60 s in two consecutive trials (120 s) were further tested. Twenty-four hours later, the animals were pretreated with pesticides and/or caffeine and placed again for 120 s on the rotating rod. The time at which the animals fell

Table 1. Effects of organophosphates and caffeine on acquisition in the passive avoidance task in mice.

Substance (mg/kg)	Retention time (s)
Saline	180 (180, 180)
CAF (40)	180 (180, 180)
DM (50)	180 (180, 180)
DM (50) + CAF (40)	180 (102, 180)
Saline	180 (180, 180)
CAF (40)	180 (120, 180)
CPF (100)	180 (180, 180)
CPF (100) + CAF (40)	45 (12, 98)***
CPF (100) + CAF (20)	130 (40, 180)

Results are shown as median values (in s) along with the 25th and 75th percentiles (in parentheses). The number of mice (n) in groups was 9 except for CPF (100) + CAF (20) group where n=8. CAF – caffeine, DM – dimethoate, CPF – chlorpyrifos. *** $p < 0.001$ vs. control (saline) group (Kruskal-Wallis/Dunn's test).

Table 2. Effects of organophosphates and caffeine on motor coordination in the rota-rod test in mice.

Substance (mg/kg)	Impairment of motor coordination (s)
Saline	120 (120, 120)
CAF (40)	120 (120, 120)
DM (50)	120 (120, 120)
DM (50) + CAF (40)	120 (115, 120)
Saline	120 (120, 120)
CAF (40)	120 (120, 120)
CPF (100)	47.5 (9, 120)
CPF (100) + CAF (40)	8.5 (4, 33)**
CPF (100) + CAF (20)	20 (11, 23)*
CPF (50) + CAF (40)	120 (120, 120)

Data are shown as median values (in s) along with the 25th and 75th percentiles (in parentheses). The number of mice (n) in groups was 8 except for CPF (100) + CAF (20) group where n=7. CAF – caffeine, DM – dimethoate, CPF – chlorpyrifos. ** $p < 0.01$, * $p < 0.05$ vs. control (saline) group (Kruskal-Wallis/Dunn's test).

off the rota-rod was recorded. Mice that did not fall off in the time allotted received a score of 120 s.

Measurement of acetylcholinesterase (AChE) activity

The pretreated animals were decapitated at times scheduled for the behavioral tests. Brains of mice were removed from skulls and placed into the deep freeze at -80°C. 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 the use of ice-cold 0.05 M sodium phosphate buffer (pH 7.8) in a proportion of 1 g of tissue in 5 ml of buffer. Next, a 50 µl aliquot of homogenate was added to 20 ml buffer containing 5.5 dithiobis-2-nitro-benzoic acid-DTNB (10 mg/100 ml) and 4 ml of the sample was used for determination of AChE activity using a modification of Ellman's colorimetric method (Ellman et al. 1961). 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 the yellow 5-thio-2-nitrobenzoate. Subsequently, the samples were centrifugated at $1850 \times g$ for 5 min. Changes in absorbance, being directly proportional to AChE activity, were measured using a microplate reader (Bio-Tek ELx800) at a wavelength of 412 nm.

Statistical analysis

Statistical analysis of data from the passive avoidance and the rota-rod test was performed with Kruskal-Wallis non-parametric ANOVA followed by Dunn's multiple comparisons test. AChE activity was analyzed with one-way ANOVA and post hoc Dunnett's test. Group differences were considered statistically significant at $p < 0.05$.
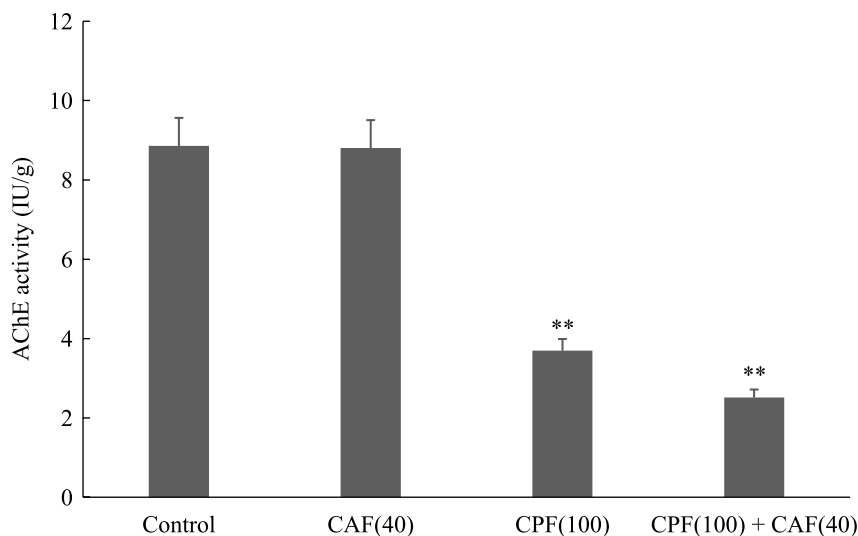
Fig. 1. Effects of chlorpyrifos (CPF 100 mg/kg) and caffeine (CAF 40 mg/kg) on brain acetylcholinesterase (AChE) activity in mice. Results are presented as mean ± SEM of eight determinations and expressed in IU/g (International Units per gram) of wet brain tissue. **p<0.01 vs. control (saline) group (ANOVA/Dunnett's test).

Results

Behavioral tests

Caffeine (40 mg/kg, i.p.) as well as tested pesticides, CPF (100 mg/kg, i.p.) and DM (50 mg/kg, i.p.), administered separately did not impair acquisition of the passive avoidance task in mice. CPF (100 mg/kg) co-administered with caffeine (40 mg/kg) induced retention deficits as compared to the control group (p<0.001). The combination of CPF with caffeine at the lower dose of 20 mg/kg did not show this effect (Table 1).

Caffeine (40 mg/kg) and DM (50 mg/kg) given alone did not impair motor coordination in the rota-rod whereas CPF (100 mg/kg) showed a strong tendency towards this impairment (statistically not significant). The addition of caffeine at the dose of 40 mg/kg to CPF (100 mg/kg) resulted in clear-cut motor coordination impairment (p<0.01). Caffeine at the lower dose of 20 mg/kg combined with CPF (100 mg/kg) also impaired motor coordination (p<0.05) in the rota-rod test. However, the addition of caffeine (40 mg/kg) to the lower dose of CPF (50 mg/kg) did not induce motor incoordination (Table 2). Caffeine (40 mg/kg) combined with DM (50 mg/kg) did not affect either acquisition or motor coordination (Table 1 and 2).

AChE activity

CPF (100 mg/kg) alone (p<0.01) as well as the combination of CPF (100 mg/kg) with caffeine (40 mg/kg) (p<0.01) significantly reduced AChE activity in mouse brains. Caffeine (40 mg/kg) alone did not significantly affect AChE activity (Fig. 1). The activity of AChE was not determined after DM and caffeine treatment due to negative results in the behavioral tests.

Discussion

The current study showed that the combined exposure to caffeine and CPF caused neurotoxic effects in adult mice. The phenomenon was observed in the passive avoidance task and the rota-rod test. It was accompanied by a reduction in AChE activity in CPF + caffeine group and in mice subjected to CPF solely which, in turn, did not exhibit neurological deficits.

Decreased ACE activity and the lack of effects in the passive avoidance task after CPF alone administration need some discussion. In general, the effect of CPF on passive avoidance behavior seems to depend on the time of exposure and the age of animals (Ricceri et al. 2003, Vatanparast et al. 2013). In one study, subcutaneous administration of CPF at a single dose of 60 mg/kg did not cause retention deficits in adult rats but decreased AChE activity in different brain regions i.e. the cortex, hippocampus, basal forebrain and caudate putamen (Ehrich et al. 2004). AChE activities in these brain regions were around 33%, 45%, 43%, and 39% of control, respectively (Ehrich et al. 2004). Similarly, in our study, adult mice acutely treated with CPF at the dose of 100 mg/kg i.p. did not show impairment of acquisition of the passive avoidance task but a significant decrease in AChE activity was noted i.e. 42% of control as detected in the whole brain. It would suggest that although AChE inhibition is a reliable endpoint for acute CPF toxicity in adult animals (Dementi 1999), it does not participate in passive avoidance impairment.

One of the main findings of the study is that the addition of caffeine to CPF group caused retention deficits in the passive avoidance test, which were associa-

ted with further reduction in AChE activity. In the passive avoidance task, caffeine differentially affects the different stages of memory processing in a dose-dependent manner (Angelucci et al. 1999). On the other hand, a number of studies including the present one showed that caffeine (20-46.2 mg/kg) did not affect acquisition of the passive avoidance task (Chrościńska-Krawczyk et al. 2009, Sanday et al. 2013). Because the inhibitory effect of caffeine on the acquisition is dose-dependent (Angelucci et al. 1999), it can explain the lack of this effect in the current study. However, caffeine (40 mg/kg) when combined with CPF (100 mg/kg) impaired acquisition and further inhibited AChE activity in the brain (28% of control). As an inhibitor of AChE (Fabiani et al. 2018), caffeine could further decrease its activity when co-administered with CPF but this mechanism does not seem to be responsible for the acquisition impairment in the light of other reports. Brain AChE levels could be less than 20% of control without behavioral/clinical detriments after exposure to OP compounds (Ehrich et al. 2004). Additionally, AChE inhibition by caffeine is thought to be partly responsible for its cognition-enhancing activity (Mohamed et al. 2013). Furthermore, in the current study, acquisition impairment has not been observed in mice treated with caffeine and DM at the dose (50 mg/kg) which inhibits AChE (Valenzuela-Harrington et al. 2012), which may also suggest the lack of involvement of AChE inhibition in the phenomenon. Accumulating evidence has confirmed that both CPF and caffeine have numerous targets of action related to cognitive processes other than the cholinergic system. Caffeine is an antagonist of both A_1 and A_2 adenosine receptors with additional action on serotonin-, catecholamine- and glutamate-mediated neurotransmission (Nehlig et al. 1992). Therefore, additional biochemical studies are needed to better understand possible CPF-caffeine interactions at the molecular level that may help to elucidate the behavioral effects in the passive avoidance task.

The second important behavioral finding in this study was the pronounced impairment of motor coordination in the rota-rod test caused by acute co-exposure to CPF (100 mg/kg) and caffeine (20 and 40 mg/kg) in mice. It is not known whether the plausible reason is due to interference of CPF and caffeine with the cholinergic system leading to the enhanced reduction in AChE activity in the brain. Exposure to a single dose (50 mg/kg) of methyl parathion, another OP compound, resulted in significant inhibition of blood cholinesterase activity and was associated with a severe depression of motor coordination in the rota-rod test in adult rats (Zhu et al. 2001). Further, quercetin (an antioxidant) supplementation to CPF-treated rats resulted in a decrease in ACh levels and an increase in AChE activity,

associated with improvement of CPF animals performance in the rota-rod test (Fereidounni and Dhawan 2018). As caffeine is the well-known antagonist of adenosine A_1 and A_{2A} receptors, a possible role of adenosine receptor blockade should be also considered in the potentiating effect of caffeine on CPF-induced motor incoordination. It would be of interest to carry out a study with selective adenosine A_1 and A_{2A} receptor antagonists on motor coordination of CPF-treated animals in the rota-rod test.

Although neither CPF nor caffeine administered individually had an effect on acquisition and motor coordination in mice, based on the current results, it may be suggested that CPF is more toxic in the presence of caffeine than vice versa. CPF appears to have a greater neurotoxic potential than caffeine because the combined use of caffeine and CPF at the doses of 40 and 50 mg/kg, respectively, did not influence the rot-rod testing, but the effect in this test was seen when caffeine was administered at the same dose and CPF at the higher dose. Moreover, the combination of DM and caffeine at the dose of 40 mg/kg did not cause any behavioral abnormalities. Additionally, administration of CPF alone showed a strong tendency towards impairment of motor coordination (statistically not significant) (Table 2). It may also be suggested that the potentiation effect of caffeine on CPF toxicity is more pronounced in terms of motor coordination, as caffeine at a lower dose of 20 mg/kg impaired motor coordination but not acquisition in CPF-treated mice (Table 1 and 2).

The obtained results indicate that the combined exposure to CPF and caffeine may induce toxic effects in mice, even if the individual administration of these compounds does not cause such effects. The underlying mechanisms of this potential toxic effect need further studies. Possible participation of the reduced activity of AChE in the observed neurobehavioral effects seems unclear.

Acknowledgements

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