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Neurologic dysfunction and genotoxicity induced by low levels of chlorpyrifos

Mariel Muller [a], Leonardo Hess [a], Agostina Tardivo [a], Rafael Lajmanovich [b,c], Andres Attademo [b,c], Gisela Poletta [b,c], Maria Fernanda Simoniello [b], Agustina Yodice [a], Simona Lavarello [a], Dante Chialvo [a,c], Oscar Scremin [a,c,d,*]

[a] PROFISIO, Facultad de Ciencias Medicas, Universidad Nacional de Rosario, Santa Fe 3100, Rosario, Argentina
[b] Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Paraje El Pozo, Santa Fe, Argentina
[c] Consejo Nacional de Investigaciones Cientificas y Tecnicas, Argentina
[d] Greater Los Angeles VA Healthcare System and David Geffen School of Medicine at UCLA, 11301 Wilshire Building, Los Angeles, CA 90073, USA

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ABSTRACT

Chlorpyrifos (CPF) is an organophosphorus cholinesterase inhibitor widely used as an insecticide. Neuro and genotoxicity of this agent were evaluated following daily subcutaneous injections at 0.1, 1 and 10 mg/kg or its vehicle to laboratory rats during one week, at the end of which somatosensory evoked potentials (SEP) and power spectrum of the electroencephalogram (EEGp) were recorded under urethane anesthesia. In another group of conscious animals, auditory startle reflex (ASR) was evaluated followed, after euthanasia, with measurements of plasma B-esterases, and genotoxicity with the alkaline comet assay (ACA) at the same CPF doses. The results indicated a CPF dose related inhibition of B-esterases. Enhanced inhibition of the ASR by a subthreshold pre-pulse was observed at all doses and ACA showed a significant higher DNA damage than vehicle controls in animals exposed to 10 mg/kg CPF. A trend to higher frequencies of EEGp and an increase in amplitude of the first negative wave of the SEP were found at all doses. The first positive wave of the SEP decreased at the CPF dose of 10 mg/kg. In summary, a shift to higher EEG frequencies and alterations of somatosensory and auditory input to the central nervous system were sensitive manifestations of CPF toxicity, associated with depression of B-esterases. The changes in electrical activity of the cerebral cortex and DNA damage observed at doses that do not elicit overt toxicity may be useful in the detection of CPF exposure before clinical signs appear.

1. Introduction

Although pesticides in general and the insecticide chlorpyrifos (O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl), CPF) in particular have improved agricultural productivity, the undesirable effects of their use on the environment and human health are increasingly degrading the sustainability of agriculture (Altieri, 1987). CPF is an organophosphorus (OP) acetylcholinesterase (AChE) inhibitor that affects nervous system functions by enhancing the availability of acetylcholine (ACh) at synaptic sites (Taylor, 1990), although non-cholinergic actions of this and other OP AChE inhibitors have been described (Checler, 1990; Johnson, 1975; Rao et al., 1999).

The widespread use of this insecticide is highlighted by the fact that CPF exposure has been detected in 86–96% of the U.S. population (Hill et al., 1995; Barr et al., 2005). Moreover, CPF metabolites have been found in 100% of urines tested from children in Ohio (Morgan et al., 2011).

Large amounts of CPF are used in agriculture in Argentina, estimated from import records of this insecticide at 6.8 million kilograms per year, and are used on various commodity crops and fruits, grains and vegetables of local consumption (SENASA, 2011). The ecological impact of the intensive use of this OP AChE inhibitor is reflected in persistent toxicity to soil organisms and runoff events into water bodies with invertebrate, anuran and fish kills (Marino and Ronco, 2005; Mugni et al., 2012; Jergentz et al., 2004; Loewy et al., 2011).

* Corresponding author at: Greater Los Angeles VA Healthcare System and David Geffen School of Medicine at UCLA, 11301 Wilshire Building, Los Angeles, CA 90073, USA. Tel.: +1 310 268 3895; fax: +1 310 268 4209.
E-mail address: oscremin@ucla.edu (O. Scremin).

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Once incorporated to the organism, CPF is metabolized to its active form, chlorpyrifos oxon (Ma and Chambers, 1994; Khokhar and Tyndale, 2012) that phosphorylates the active site of AChE rendering it inactive. This leads to disruption of cholinergic neural transmission in the central, autonomic and peripheral nervous systems. Neurotoxic effects of CPF include cortical arousal, disorientation, alterations of the sleep-wakefulness cycle, fasciculations and convulsions. Peripheral autonomic effects include hypersecretion of salivary and lachrymal glands, hypertension, tachycardia, sweating, miosis and bronchorrhea. Respiratory insufficiency (Clegg and van Gemert, 1999; Barron and Woodburn, 1995) and convulsions (Jett, 2012) are observed at high doses.

Long term neurological sequelae of intoxication with OP AChE inhibitors have also been described (Rosenstock et al., 1991; Savage et al., 1988; Steenland et al., 1994).

Activity of B-esterases has been widely used for the evaluation of exposure to pesticides (Wheelock et al., 2008; Manzo et al., 2001). Chlorpyrifos and other organophosphorus (OP) and carbamate AChE inhibitors inhibit the activity of B-esterases following patterns characteristic of every agent (Taylor, 1990).

The electrical activity of the cerebral cortex has often been used for the evaluation of pesticides toxicity. EEG patterns and EEG power spectrum alterations in response to CPF exposure have been reported for a single exposure at doses of 10 and 40 mg/kg (Timofeeva and Gordon, 2002) but there is no information in the literature about effects of this pesticide at lower doses on EEG or at any dose on evoked electrical activity, a technique that has provided sensitive biomonitoring in the case of other neurotoxicants (Nagymajtenyi et al., 1998; Mwanza et al., 2012; Desi and Nagymajtenyi, 1988; Scremin et al., 2011).

Sensory inputs to the higher levels of the central nervous system are under modulatory control (sensory gating) that can be demonstrated by the phenomenon of pre-pulse inhibition (PPI), in which a sub-threshold sound pulse can inhibit the response to an alerting, high intensity auditory stimulus that follows it (Koch, 1999). This inhibition appears to be mediated by cholinergic muscarinic receptors at the level of the nucleus reticularis pontis caudalis (Jones and Shannon, 2000; Bosch and Schmid, 2006). It is then conceivable that OP AChE inhibitors like CPF might enhance PPI, a phenomenon known to occur with carbamate AChE inhibitors (Clark et al., 2005).

DNA damage and oxidative stress could mechanistically link pesticide exposures with a number of health outcomes observed in epidemiological studies (Muniz et al., 2008). Single cell gel electrophoresis (comet assay) has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies, genotoxicity testing and human biomonitoring. This assay was adapted to measure oxidized purines and pyrimidines by the incubation of the nucleoids with bacterial DNA repair enzymes. Formamidopyrimidine glycosylase (FPG) is used to detect oxidized purines, mostly 8-oxo-7,8-dihydroguanine (8-oxo-G) (Collins et al., 1997). A great variety of oxidized bases have been identified in nuclear DNA but 8-oxo-G is one of the most abundant and readily formed oxidized DNA lesions (Azqueta et al., 2009).

Chlorpyrifos may be administered by cutaneous, oral, intraperitoneal, inhalational, intravenous or subcutaneous routes. The lethal dose 50% (LD50) of this agent depends on the route of administration and animal species. For the subcutaneous (s.c.) route in rats it has been estimated at 147 mg/kg (WHO, 1975). The rationale for the use of subcutaneous administration of CPF in oil in the present experiments is based on the fact that it results in a slow sustained release of the pesticide into the systemic circulation which approximates most human dermal exposures (Ellison et al., 2011). Since exposures to CPF of agricultural workers or the population of urban centers within zones of pesticide drift often consist of repeated, daily exposure, the present work was designed to assess changes in neurological biomarkers of exposure and effect as well as possible genotoxicity following one week of daily s.c. doses of this agent. Doses ranging from approximately 1/1000 to 1/10 of the LD50 were chosen since they are usually devoid of overt toxicity, mimicking a scenario with no alerting clinical signals that may erroneously lead to the assumption of lack of danger.

The biomarkers of exposure (activity of B-esterases) and effects (EEG and comet assay) have been extensively used to assess exposure to pesticides in agricultural workers and at risk bystanders. See reviews by Reigart and Roberts (1999), Seppalainen (1975), and Valverde and Rojas (2009), respectively. Although somatosensory evoked potentials have not been used in humans for this purpose, it is a technique routinely used for the evaluation of patients with many neurological conditions and its implementation is well standardized. Thus the results of this study are readily translatable to the human population.

2. Materials and methods

2.1. Animal care and drug administration

Two batches of animals were used. Experiments including blood enzymatic activity, comet assays and auditory startle were performed in male Sprague-Dawley adult rats and experiments including power spectrum analysis of the electroencephalogram and somatosensory evoked potentials were performed in male Wistar adult rats. The reason for strain selection was based on the fact that Sprague-Dawley rats express more strongly the phenomenon of pre-pulse inhibition than Wistar rats and for this reason they were selected for the study arm including ASR. On the other hand, Wistar rats have been used in recent studies of cholinergic modulation of cortical somatosensory function (Alenda and Nunez, 2004, 2007) and in a previous study of somatosensory evoked potentials with dichlorvos, also an OP cholinesterase inhibitor (Papp et al., 1996). Thus, this strain was selected for the arm with EEG power spectrum and somatosensory evoked potentials to facilitate comparisons with previous work. B-esterase activity measurements and comet assays were carried out in the rats tested for the auditory startle response that were euthanized by decapitation. Those tests were not performed in Wistar rats, used in recording EEG and evoked potentials, in order to avoid the possible confounding effects of prolonged anesthesia with urethane, a known genotoxic agent (Schlatter and Lutz, 1990). The project received Institutional approval from the University of Rosario (Argentina) Medical School and all procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition.

Animals were maintained in an environmentally controlled space, with lights on at 07:00 h and off at 19:00 h and air temperature between 20 and 25 °C. They were housed in polycarbonate cages with sawdust bedding, two animals to a cage, with access to rat chow and water ad libitum. Body mass was recorded daily and they were observed for signs of cholinergic toxicity (salivation, lachrymation, fasciculations, tremors, tail dorsiflexion, convulsions, and drop in body mass or rectal temperature). Chlorpyrifos (Supelco©, CHEM SERVICE, Inc. West Chester, PA) was dissolved in sunflower oil at the concentrations of 0.1, 1, and 10 mg ml^{-1} and administered subcutaneously at doses of 0.1, 1, and 10 mg/kg body mass once daily during 7 days to three groups of animals. One additional group was injected once daily with the same volume (1 ml/kg/body mass) of the CPF vehicle. Number of animals in the first batch for the drug vehicle, 0.1, 1 and 10 mg/kg doses were 10, 5, 9 and 4 and for the second batch 6, 5, 5 and 6 respectively. Body mass and rectal temperature were recorded daily.

2.1.1. Recording of brain electrical activity

Before recording brain electrical activity animals were anesthetized with urethane (1.5 g/kg by intraperitoneal route). Two platinum needles were inserted into the left forepaw and connected to a Signal Generator and Stimulus Isolator (Grass Instruments Inc., Models SD5 and SIU7 respectively). The skull surface was exposed through a skin incision and the galea was dissected away. Two stainless steel self-tapping screws were inserted into the skull avoiding brain direct contact at the projections of the forepaw primary somatosensory area on the left (Bregma 0 mm, Left Lateral 4 mm) and right cortex (Bregma 0 mm, Right Lateral 4 mm) (Paxinos and Watson, 1998). The left forepaw was stimulated with 0.05 ms duration and 0.5 Hz frequency pulses, with intensity supramaximal for elicitation of the S1 contralateral evoked response. The electro-cortical activity during the 120 ms that followed the stimulation pulse was amplified with an ISO-4 low noise amplifier (World Precision Instruments, Inc., Sarasota, FL) and digitized at 2 kHz with a MacLab data acquisition system (AD Instruments, Inc., Colorado Springs, CO). The electroencephalogram (EEG) was recorded from the same electrode leads as the evoked potentials. The EEG in early stages of urethane anesthesia often shows a burst suppression pattern that is associated with depressed evoked potentials lacking an early negative wave (Scremin et al., 1973). For that reason, the EEG was continuously monitored to avoid eliciting evoked potentials during periods of burst suppression. Several averaged evoked potentials of 32 consecutive samples were obtained during the course of each experiment.

EEG power spectrum for the EEG frequencies 1–48 Hz was obtained from 6 second segments of recording in the absence of forepaw stimulation using a Cosine-Bell windowing function and 1024 window size with 50% overlap (MacLab, AD-Instruments). EEG power was computed in the following frequency bands: <2 Hz; 2–4 Hz; 4–8 Hz; 8–12 Hz; 12–24 Hz and 24–48 Hz. The data was normalized by dividing power in each frequency band by the average power of all six-frequency bands for each animal (Fractional Power). Fractional power was analyzed by analysis of variance (ANOVA) with factor chlorpyrifos dose 0 (controls injected with CPF vehicle only), 0.1, 1, and 10 mg/kg body mass and compared. If the ANOVA *F*-ratio was statistically significant ($P < 0.05$) significance of the difference of every CPF dose against the vehicle control group was assessed by post hoc Dunnet's multiple comparisons tests.

In order to avoid possible subjectivities associated with selecting wave peaks on every individual evoked potential, a Matlab script was used to obtain ensemble averages of all the average evoked potentials recorded in each animal for every experimental group with their standard deviations, standard errors and 95% confidence intervals. Those grand average tracings are shown in Fig. 5 from which the peak amplitudes and latencies of the following cortical electrical activity stimulus-evoked waves were measured: first positive (P1), first negative (N1) and second positive (P2). Bonferroni adjusted *t*-tests were performed to determine statistical significance ($P < 0.05$) between means of each CPF dose and vehicle controls.

2.2. Cell viability using fluorescent dyes

The same cell suspension used in the comet assay was mixed with fluorescent DNA-binding dyes (100 μg ml^{-1} acridine orange and 100 μg ml^{-1} ethidium bromide was prepared in Ca^{2+} and Mg^{2+} free PBS) and examined by fluorescent microscopy to visualize and count cells with aberrant chromatin organization. The percentages of each of these cellular states in relation to the total cells were obtained (Mercille and Massie, 1994).

2.2.1. Comet assay modified for detection of oxidized bases (FPG)

The standard procedure (Singh et al., 1988) was used with modifications. Briefly 50 μl of fresh blood was added to 950 μl of RPMI 1640 at 37 °C, and subsequently centrifuged at 1000 × *g* for 4 min. Cell pellets (containing approximately 60,000 cells) were mixed with 200 μl of low melting point agarose 1% and two slides were prepared. To lyse the cellular and nuclear membranes of the embedded cells, the key-coded slides were immediately immersed in freshly-prepared ice-cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM trizma base, 1% Triton X-100 and DMSO 10%; pH 10) and left at 4 °C overnight. After lysis, slides were washed and excess liquid dabbed off with tissue, 50 μl of FPG enzyme solution or buffer alone as control was placed on the gel and covered with a cover slip. Slides were put into a moist box (prevents desiccation) and incubated at 37 °C for 30 min (Collins et al., 1997). The slides were then immersed in freshly prepared alkaline electrophoresis solution (300 mM NaOH and 1 mM Na_2EDTA; pH > 13), first for unwinding (20 min) and then for electrophoresis (0.7–1 V cm^{-1}, 300 mA, 20 min at 4 °C). All of the steps were carried out on darkness.

Once electrophoresis was completed, the slides were neutralized and dehydrated with ethanol. Slides were stained with acridine orange at the moment of analysis and one hundred randomly selected comets from each animal were visually classified into five classes according to tail size and intensity (from undamaged, class 0, to maximally damaged, class 4), resulting in a single DNA damage score (damage index, $DI = n1 + 2{\cdot}n2 + 3{\cdot}n3 + 4{\cdot}n4$), where *n*1, *n*2, *n*3 and *n*4 are the number of cells in each class of damage, respectively. Zero category is considered without damage and not included in the final score (Kobayashi et al., 1995).

Statistical analysis was performed using the SPSS 14.0 software package for Windows. Normality was tested by the Kolmogorov–Smirnov test and homogeneity of variances by Levene test. Differences in DI and FPG sites between exposed groups and the CPF vehicle control were tested by one way ANOVA followed by Dunnet's test. A probability < 0.05 was considered statistically significant. The frequency of FPG sites was estimated by subtracting the values obtained without FPG from the values obtained with the enzyme.

2.2.2. B-esterase assays

Plasma acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were determined colorimetrically (Ellman et al., 1961). The reagents included 25 mM Tris–HCl containing 1 mM $CaCl_2$ (pH = 7.6), 2 mM dithio bis 2-nitrobenzoic acid, acetylthiocholine, and butyrylthiocholine iodide (AcSCh and BuSCh, respectively) and plasma. Variation in optical density was measured in duplicate at 410 nm at 25 °C for 1 min using a Jenway 6405 UV–vis spectrophotometer. The activity of plasma AChE and BChE was expressed in μmol or nmol of hydrolyzed substrate per minute per milliliter of plasma using a molar coefficient extinction of 13.6 × 103 M^{-1} cm^{-1} and 14.15 × M^{-1} cm^{-1} (Sanchez-Hernandez et al., 2004).

Plasma carboxylesterase (CbE) activity, determined using α-NA as substrate, was measured following the method of Gomori (1953) adapted by Bunyan et al. (1969). The enzyme assay was carried out with 25 mM Tris–HCl, 1 mM $CaCl_2$ (pH = 7.6) and plasma at 25 °C. The reaction was initiated by adding a-naphthyl acetate (1.04 mg ml^{-1} in acetone) as substrate, and stopped after 10 min by adding 2.5% sodium dodecyl sulphate and 0.1% of Fast Red ITR in 2.5% Triton X-100 in deionizer water (prepared right before use). The samples were left in darkness for 30 min to develop, and the absorbance of the naphthol–Fast Red ITR complex was read at 530 nm. Plasma CbE (α-NA) activity was expressed as μmol of substrate hydrolyzed per minute per milliliter of plasma using a molar extinction coefficient of 33.225 × 103 M^{-1} cm^{-1}.

Table 1
Body mass and rectal temperature recorded before drug or vehicle injection in all experimental groups in which enzymatic activity, comet assay and auditory startle were studied.

Day	Vehicle	CPF 0.1 mg/kg	CPF 1 mg/kg	CPF 10 mg/kg
Body mass (g)				
1	454.2 ± 10.9	410.4 ± 14.5	418.0 ± 13.6	445.8 ± 17.7
2	438.9 ± 10.2	419.0 ± 15.8	411.2 ± 14.3	440.3 ± 18.1
3	440.4 ± 10.9	424.8 ± 16.8	419.8 ± 13.9	448.8 ± 19.1
4	440.3 ± 7.5	408.6 ± 16.4	405.4 ± 13.7	435.8 ± 17.3
5	423.0 ± 10.0	415.0 ± 16.4	411.6 ± 13.9	426.3 ± 18.3
6	437.7 ± 11.2	416.4 ± 16.3	414.4 ± 13.3	431.0 ± 11.0
7	433.0 ± 10.4	411.4 ± 15.8	417.2 ± 11.6	437.3 ± 17.4
Rectal temperature (°C)				
2	37.3 ± 0.1	37.0 ± 0.1	37.1 ± 0.1	36.6 ± 0.1*
3	37.3 ± 0.1	37.0 ± 0.1	37.1 ± 0.1	37.3 ± 0.1
4	37.2 ± 0.1	36.9 ± 0.1	37.1 ± 0.1	37.1 ± 0.1
5	36.9 ± 0.2	36.0 ± 1.0	36.8 ± 0.1	36.7 ± 0.1
6	37.3 ± 0.1	37.0 ± 0.2	37.3 ± 0.1	37.2 ± 0.3
7	37.5 ± 0.1	37.1 ± 0.1	37.2 ± 0.1	37.7 ± 0.1

Means of body mass and rectal temperature were compared between the three CPF dose groups and the vehicle group by T-tests with Bonferroni correction at each day. Mean of rectal temperature for the 10 mg/kg group was significantly lower than the vehicle group on day 2 only. No temperature records were collected on day 1 for a technical problem.
* Significantly different from the Vehicle Group ($P < 0.05$ by t-tests with Bonferroni correction).

2.2.3. Auditory startle response (ASR)

Animals were placed in a loosely fitted plastic tube, 10 cm in diameter with adjustable length between 10 and 16 cm. A three dimensional accelerometer (DE-ACCM-3D, DimensionEngineering.com) was fitted to the floor of the tube. The whole assembly was located in a dark, sound-attenuating chamber provided with a loudspeaker. The accelerometer was interfaced to the computer with a DAQ Card TM-6062E (National Instruments). A script written in MATLAB®2007 was used to generate the sound pulses and to analyze the accelerometer output. Prior to testing, animals were placed in the apparatus for 5 min and presented with constant white noise (70 dB) that continued throughout the testing phase. Following the acclimation period, animals were exposed to three types of stimuli: an acoustic startle pulse alone (PA; 40 ms, 118 dB), a pre-pulse alone (PPA; 20 ms, 80 dB), and pre-pulse preceding a startle pulse (PPP; 100 ms inter-stimulus interval). The output of the three accelerometer axes were added together and integrated over 100 milliseconds following the startle pulse. All responses above background were included in the analysis. Animals were presented with a pseudorandom sequence of 60 trials with a variable inter-trial-interval (mean = 15 s). Each subject received 20 PA, 20 PP, and 20 PPP trials in the same pseudorandom order.

Recording of EEG, and somatosensory evoked potentials as well as the auditory startle response followed by euthanasia and collection of blood samples were done one day after the last CPF or vehicle injection on day 7.

3. Results

Data on body mass and rectal temperature of the animals in which enzymatic activity, comet assay and auditory startle response were studied indicated lack of significant changes over time between treatment CPF dose groups and vehicle controls, except for rectal temperature on day 2 in the CPF 10 mg/kg group (Table 1). No differences in means of those variables between CPF dose groups and vehicle controls were found in animals in which EEG power spectrum and somatosensory evoked potentials were studied (Table 2).

Results from assays of enzymatic activity indicated dose-dependent decreases in AChE and BChE. In contrast, CbE was only decreased at the highest dose of CPF (Fig. 1).

Results from the comet assay demonstrated a significantly higher DNA damage in animals exposed to 10 mg/kg compared to vehicle control group ($P = 0.031$ by ANOVA followed by Dunnet's tests) but no differences were observed with the other exposed groups ($P > 0.05$). No differences were found in FPG sites between any CPF exposed group and the vehicle control group ($P > 0.05$) (Fig. 2 and Table 3). Cell viability in blood samples, determined as recommended by Singh (2000), was greater than 95%.

ANOVA of the ASR PA response (high intensity sound pulse alone) data showed statistical significance for the factors "treatment" (Vehicle, CPF 0.1, 1 and 10 mg/kg) ($P < 0.0001$) and "trials" (20 consecutive, pseudorandom events) ($P = 0.006$) but no interaction between treatment and trial. Dunnet's post hoc multiple contrasts indicated a lower response than vehicle controls ($P < 0.05$) only for the CPF 10 mg/kg dose (Fig. 3). ANOVA of the ASR PPP response (high intensity sound pulse preceded by a sub-threshold sound pulse) showed significance for the factor treatment ($P = 0.0015$) but no significance for the factor trial ($P = 0.07$) or the interaction between them. Dunnet's post hoc multiple contrasts indicated a lower response (greater inhibition of the PA) in all experimental groups (Fig. 3).

The EEG power spectra showed two opposite trends for frequency ranges up to 4 Hz (a decrease as a function of CPF dose)

Table 2
Body mass and rectal temperature recorded daily before drug or vehicle injection in all experimental groups in which EEG power spectrum and somatosensory evoked potentials were studied.

Day	Vehicle	CPF 0.1 mg/kg	CPF 1 mg/kg	CPF 10 mg/kg
Body mass (g)				
1	312.9 ± 3.1	296.0 ± 6.5	310.6 ± 5.3	314.5 ± 6.2
2	335.4 ± 13.6	306.5 ± 12.7	323.7 ± 10.3	333.0 ± 16.1
3	345.6 ± 11.7	311.3 ± 12.5	327.6 ± 13.4	332.7 ± 15.7
4	341.6 ± 10.9	310.8 ± 11.3	344.0 ± 22.5	327.3 ± 13.8
5	346.7 ± 10.3	317.5 ± 12.0	330.6 ± 10.0	334.7 ± 13.1
6	346.6 ± 11.4	313.7 ± 13.2	329.7 ± 10.8	333.2 ± 0.5
7	344.4 ± 11.6	318.7 ± 12.7	336.9 ± 10.9	336.2 ± 13.0
Rectal temperature (°C)				
1	37.2 ± 0.2	37.2 ± 0.1	37.1 ± 0.1	37.0 ± 0.1
2	37.1 ± 0.2	37.1 ± 0.1	37.7 ± 0.1	36.8 ± 0.2
3	36.7 ± 0.2	37.1 ± 0.1	37.2 ± 0.1	37.0 ± 0.2
4	36.5 ± 0.3	36.9 ± 0.2	37.1 ± 0.2	37.3 ± 0.3
5	36.9 ± 0.3	37.2 ± 0.3	37.4 ± 0.1	37.0 ± 0.3
6	36.7 ± 0.2	37.0 ± 0.1	37.2 ± 0.2	36.9 ± 0.1
7	37.0 ± 0.1	37.6 ± 0.2	37.3 ± 0.1	37.0 ± 0.1

Means of body mass and rectal temperature were compared between the three CPF dose groups and the vehicle group by t-tests with Bonferroni correction at each day. No significant differences were found.

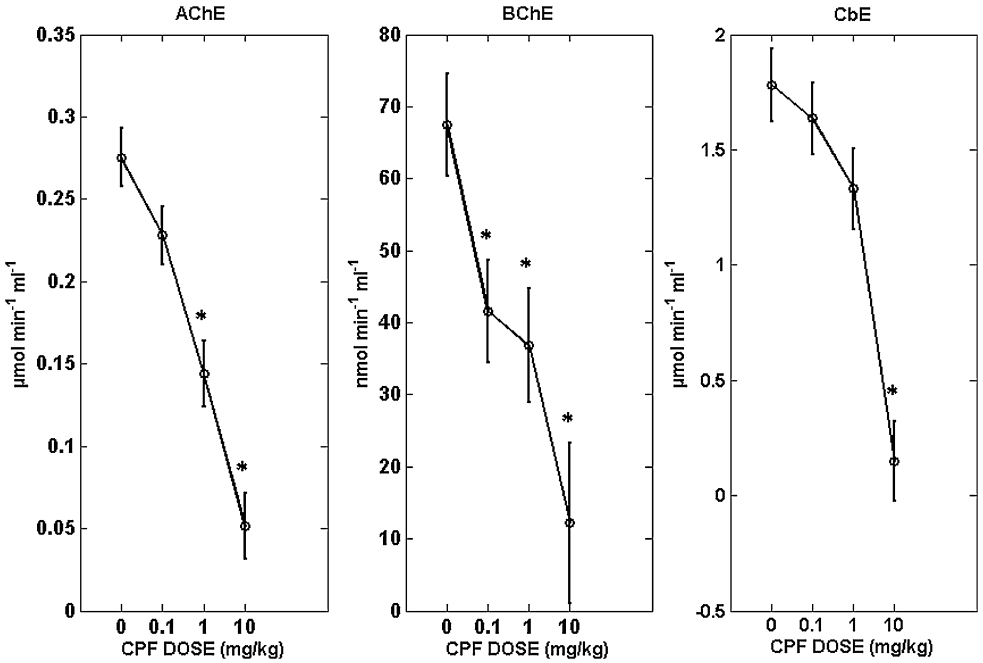

Fig. 1. Means and standard errors of activity (left to right), of AChE, BChE and CbE. * Significantly different from the vehicle group by ANOVA and multiple comparisons with Dunnet's test. Number of cases for the drug vehicle, 0.1, 1 and 10 mg/kg CPF doses were 10, 5, 9 and 4, respectively.

and for frequency ranges above 4 Hz (an increase as a function of CPF dose) with power spectra for the bands 4–8 Hz, 12–24 Hz and 24–48 Hz becoming significantly higher than the corresponding bands of vehicle controls at the dose of 10 mg/kg (Fig. 4).

The average waveforms of somatosensory evoked potentials and their 95% confidence intervals are shown in Fig. 5 for all experimental groups. The averages and SE of peak latencies and amplitudes of the three waves analyzed are present in Table 4. The first positive wave (P1) decreased significantly in amplitude only at the CPF dose of 10 mg/kg while the first negative wave (N1) amplitude was significantly different from the vehicle controls at all CPF doses. No statistically significant differences with regard to vehicle controls were found for the second positive wave (P2) at any of the CPF doses. The peak latencies of the three waves were not different from vehicle controls for any of the CPF doses.

4. Discussion

The rationale for the use of subcutaneous administration of CPF in oil in the present experiments is based on the fact that it results in a slow sustained release of the pesticide into the systemic circulation which approximates most human dermal exposures (Ellison et al., 2011).

The inhibition of AChE observed in the present experiments at the end of the 7th day of treatment was dose-related and reached 20% of control at the daily CPF dose of 10 mg/kg. This is a greater inhibition than previously reported for a single administration of 10 mg/kg CPF by the same route to rats (48%) (Terry et al., 2003) a fact most likely related to a cumulative effect of repeated CPF administration. In fact, a recent study by Ellison et al. (2011) have shown that a pharmacodynamic model based on parameters

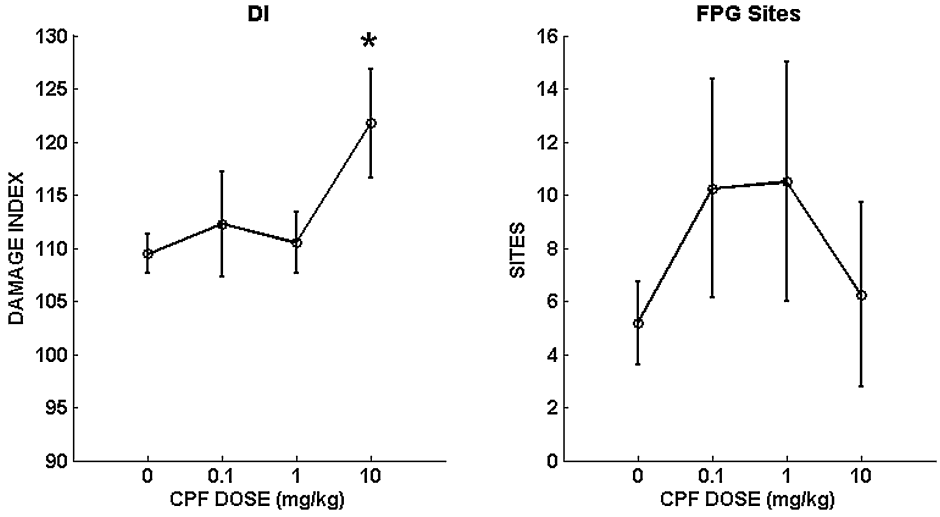

Fig. 2. Means and standard errors of the results of comet assays. A significant increase in damage index (DI) over the vehicle controls was found at the CPF dose of 10 mg/kg. * Significantly different from the Vehicle group by ANOVA and multiple comparisons with Dunnet's test. Number of cases for the drug vehicle, 0.1, 1 and 10 mg/kg CPF doses were 10, 5, 9 and 4, respectively.

Table 3
Damage Index (DI) and FPG sites in the comet assay in CPF exposed groups and the CPF vehicle control.

Treatment	DI (mean ± SE)	FPG sites (mean ± SE)
Vehicle	109.45 ± 1.82	5.18 ± 1.55
0.1 mg/kg	112.25 ± 4.91	10.25 ± 4.13
1 mg/kg	110.50 ± 2.84	10.50 ± 4.51
10 mg/kg	121.75 ± 5.11*	6.25 ± 3.47

* Significantly different from the vehicle group by one way ANOVA followed by Dunnet's test ($P < 0.05$).

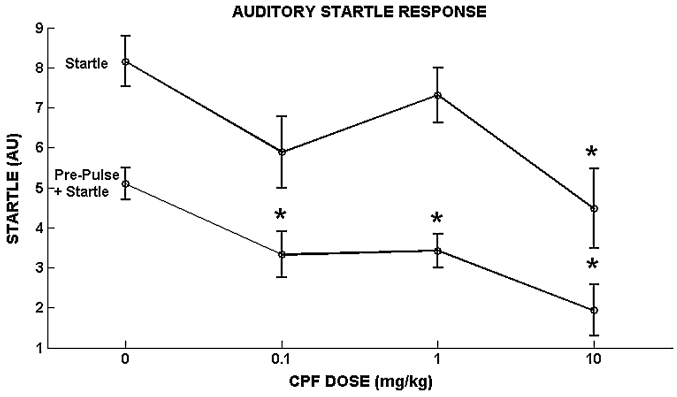

Fig. 3. Means and standard errors of the auditory startle response to a high intensity sound pulse (top) and the attenuation of the same stimulus by a preceding subthreshold sound pulse (bottom). * Significantly different from the vehicle group by ANOVA and multiple comparisons with Dunnet's test. Number of cases for the drug vehicle, 0.1, 1 and 10 mg/kg CPF doses were 10, 5, 9 and 4, respectively.

obtained from single doses can predict a final AChE inhibition level after repeated daily doses close to the one reported in our study (Ellison et al., 2011). A similar pattern and comparable levels of AChE inhibition have been reported in cotton agricultural workers with 9–10 days exposure to CPF supporting the validity of this experimental model (Farahat et al., 2011).

Interestingly, in spite of the pronounced AChE inhibition observed, no overt toxicity was present in our animals with the exception of loose stools in the rats that received 10 mg/kg daily. Paucity of toxic signs is probably related to the development of tolerance to the effects of CPF as observed with other OP AChE inhibitors (Chippendale et al., 1972; Sumerford et al., 1953; Lopez-Crespo et al., 2007). The lack of overt toxicity in our experiments was also in line with a lack of a decrease in body mass even at the highest dose, in agreement with a previous study also in rats (Terry et al., 2003) in which a decrease in body mass was only observed at daily CPF doses of 25 mg/kg and higher. Inhibition of AChE is most likely the main mechanism underlying the presently observed changes in cortical electrical activity and ASR. However, inhibition of other B-esterases, in particular BChE, may also play a role. Evidence has been recently adduced pointing to a role of BChE in cholinergic transmission, including the preservation of cholinergic function in mice that lack AChE but with normal levels of BChE that substitutes for it (Li et al., 2000; Mesulam et al., 2002). In this regard, the parallelism between BuChE inhibition (Fig. 1) and the ASR response (Fig. 3) reported here is striking although by no means supportive of a mechanistic relationship between both variables.

The comet assay has applications in areas of biomedical and environmental health science such as biomonitoring of animal and human populations for environmental and occupational exposure to genotoxic agents. The assessment of DNA damage and the relationship with oxidative stress through an early modification of the assay allow the quantitative detection of damaged bases, most commonly, oxidized bases (Dusinska and Collins, 2008). In this work, we obtained statistically significant differences in DNA damage (DI) in those rats exposed to 10 mg/kg CPF, but no differences in FPG sites were observed, indicating the absence of oxidized purines. This finding is a new manifestation of the genotoxic potential of CPF described with other methods (Patnaik and Tripathy, 1992; Amer and Fahmy, 1982).

It has long been known that cholinergic mechanisms modulate brain rhythms and evoked electrical activity. Systemic administration of muscarinic cholinergic agonists induces cortical arousal (Yamamoto and Domino, 1967; Domino et al., 1967).

Pharmacological cholinergic blockade or depletion of cortical AChE by cholinergic immunotoxin treatment increase slow-wave power and decrease high-frequency power (Buzsaki and Eidelberg, 1983; Riekkinen et al., 1990; Vanderwolf, 1992; Holschneider et al., 1999), whereas cholinergic agonists result in a reversal of this phenomenon (Vanderwolf, 1992). Furthermore, AChE inhibitors such as physostigmine enhance the synchronization of theta

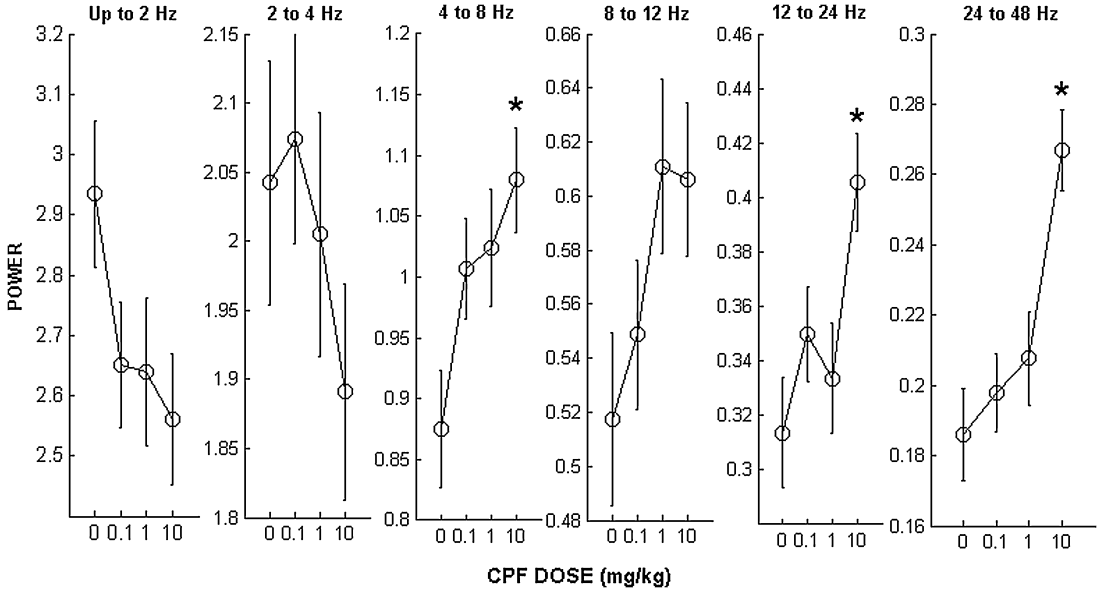

Fig. 4. EEG mean fractional power (circles) and standard error (bars) for the six frequency bands studied. * Significantly different from the Vehicle group by ANOVA and multiple comparisons with Dunnet's test. Number of cases for the drug vehicle, 0.1, 1 and 10 mg/kg CPF doses were 6, 5, 5 and 6, respectively.

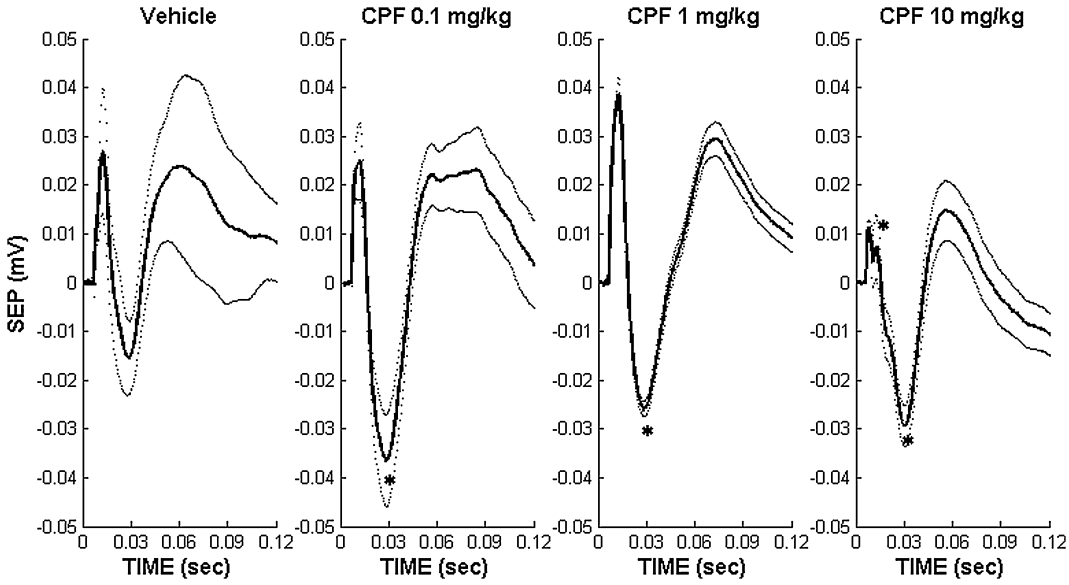

Fig. 5. Average somatosensory evoked potentials (SEPs for the controls injected with the CPF vehicle (dose = 0) and the three treatment groups (CPF doses = 0.1, 1 and 10 mg/kg)). CPF was administered by s.c. route daily during seven days. The SEPs are shown with continuous lines and their 95% confidence limits with dotted lines. Statistically significant differences (Bonferroni adjusted *t*-tests, $p < 0.05$) in the amplitude of SEP peaks are indicated by (*). Number of cases for the drug vehicle, 0.1, 1 and 10 mg/kg CPF doses were 6, 5, 5 and 6, respectively.

activity, whereas cholinergic antagonists or excitotoxic lesions of the basal forebrain diminish it (Dickson et al., 1994; Leung et al., 1994; Holschneider et al., 1997). CPF has been shown to alter the frequency power spectrum in conscious rats (Timofeeva and Gordon, 2001).

The short latency somatosensory cortical evoked response (first positive and first negative waves in the present recordings) show considerable analogy between rats and primates (Allison and Hume, 1981). The positive short latency wave represents depolarization of pyramidal neurons in layer IV and deep layer III of the cerebral cortex, driven by the primary thalamic input after amplification by local interneuron's networks while the following negative deflection corresponds to the apical dendritic depolarization of the same pyramidal neurons (Eccles, 1951; Chang, 1959; Jellema et al., 2004). Although the balance between the two waves is related to the extent of depolarization of pyramidal cells, an additional contribution to a surface negativity is known to occur by depolarization of cells in layers II and III driven by transcallosal and association fibers inputs (Chang, 1953).

The increase in the amplitude of the short latency negative wave (N1) at all CPF doses and the absence of a conspicuous positive wave preceding it induced by the highest dose of CPF argues for a facilitation of the spread of activation towards the superficial layers of the cerebral cortex under the influence of this AChE inhibitor. Since spontaneous cortical arousal is associated with a predominantly negative SEP (Scremin et al., 1973) and the power of higher frequencies reported here increases progressively with CPF dose, it is possible that the primary effect of CPF could be an action on the brainstem mechanisms driving the cortical rhythms rather than a local action at the cortical levels. Moreover, muscarinic cholinergic agonists locally applied to the brain stem induce EEG arousal (Kinney et al., 1998) but when applied locally to the cortex they inhibit the SEP N1 wave (Malcolm et al., 1967) (Scremin et al., 1973). Thus it appears that at low levels of cholinergic pharmacological stimulation the brain stem arousal effect predominates. As for the mechanism mediating the increased negativity of the SEP associated to cortical arousal, it has been postulated that the unspecific thalamo-cortical afferent system anatomically described by Lorente de No (1949) might impinge on apical dendrites of pyramidal cells to lower their threshold for depolarization facilitating the displacement of the current sink towards the superficial cortical layers (Chang, 1959). Discharges from neurons in cortical layers II–III probably also contribute to the surface negativity of the SEP.

Generalized convulsions are a manifestation of CPF severe toxicity in animals (Jett, 2012) and humans (Clegg and van Gemert, 1999). Low doses of CPF that do not induce seizures are nevertheless pro-convulsant based on lowering of the threshold for amygdala kindling in immature rats (Wurpel et al., 1993). We did not observe seizures in the present experiments, but the acceleration of EEG rhythms and the progressive increase in magnitude of the negative wave of the SEP indicate activation of the cerebral cortex with accelerated invasion of the entire thickness of the cortical structures following the trigger stimulus, an expression of enhanced neuronal excitability which may herald the appearance of paroxysmal activity leading to seizures at higher doses or longer times of exposure.

In summary, low CPF doses ranging from 1/1000 to 1/10 of the LD50 repeated daily for one week, model typical exposures of agricultural workers and the population of urban centers within zones of pesticide drift. This pattern induced significant changes in enzymatic biomarkers of exposure, DNA damage and neurological effects. Further exploration of other biomarkers and their long-term persistence are justified to evaluate the full magnitude of the impact on human health of this pattern of CPF exposure.

Table 4
Peak latencies and amplitudes of somatosensory evoked potentials.

Wave	Vehicle	CPF 0.1 mg/kg	CPF 1 mg/kg	CPF 10 mg/kg
Latency (msec)				
P1	12.8 ± 0.5	11.8 ± 0.7	11.9 ± 0.5	12.6 ± 0.3
N1	32.0 ± 2.0	35.2 ± 3.5	36.9 ± 2.9	33.3 ± 2.2
P2	71.6 ± 8.1	70.9 ± 5.8	76.1 ± 3.0	57.1 ± 1.9
Amplitude (μV)				
P1	29.0 ± 6.1	24.8 ± 3.9	38.7 ± 1.6	7.4 ± 3.3*
N1	−13.5 ± 3.7	−36.5 ± 4.3*	−25.7 ± 0.7*	−29.2 ± 2.1*
P2	26.4 ± 7.7	21.9 ± 3.5	29.3 ± 1.7	14.7 ± 3.0

* Significantly different from the vehicle group ($P < 0.05$ by *t*-tests with Bonferroni correction).

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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