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Neurobehavioral and biochemical changes in *Nauphoeta cinerea* following dietary exposure to chlorpyrifos

Isaac A. Adedara [a,b,c], Denis B. Rosemberg [a], Diego de Souza [a], Ebenezer O. Farombi [c], Michael Aschner [d], Diogo O. Souza [b], Joao B.T. Rocha [a,*]

[a] Departamento de Bioquímica e Biologia Molecular, CCNE, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[b] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600-Anexo, 90035-003 Porto Alegre, RS, Brazil
[c] Drug Metabolism and Toxicology Research Laboratories, Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria
[d] Department of Molecular Pharmacology; Albert Einstein College of Medicine Forchheimer 209; 1300 Morris Park Avenue, Bronx, NY 10461, USA

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ABSTRACT

The present study aimed to increase our understanding about the mode of toxic action of organophosphate pesticides in insects by evaluating the biochemical and neurobehavioral characteristics in *Nauphoeta cinerea* exposed to chlorpyrifos (CPF)-contaminated diet. The insects were exposed for 35 consecutive days to CPF at 0.078, 0.15625, 0.3125 and 0.625 μg/g feed. Locomotor behavior was assessed for a 10-min trial in a novel arena and subsequently, biochemical analyses were carried out using the cockroaches' heads. In comparison to control, CPF-exposed cockroaches showed significant decreases in the total distance traveled, body rotation, turn angle and meandering, along with significant increase in the number of falls, time and episodes of immobility. The marked decrease in the exploratory profiles of CPF-exposed cockroaches was confirmed by track plots, whereas occupancy plot analyses showed a progressive dispersion at 0.15625 μg/g feed group. Moreover, the heads of CPF-exposed cockroaches showed marked decrease in acetylcholinesterase activity and antioxidant status with concomitant significant elevation in dichlorofluorescein oxidation and lipid peroxidation levels in CPF-treated cockroaches. Gas Chromatography–Mass Spectrometry analyses revealed bioaccumulation of CPF in cockroaches exposed to concentrations above 0.078 μg/g feed. The findings from this investigation showed *N. cinerea* as a value model organism for the risk assessment of environmental organophosphate contamination in insects.

1. Introduction

Chlorpyrifos (CPF) is a broad spectrum organophosphate pesticide widely used throughout the world in agriculture and non-agriculture applications. CPF is also applied on non-food sites including golf course turf, sod farms, wood products, greenhouses and industrial sites [1,2]. Exposure to CPF occurs by inhaling the chemical secondary to wind-drifts. Dietary intake of CPF is an important source of exposure for the general population [3]. Adverse effects of CPF in humans include neurological effects, developmental and autoimmune disorders. Prenatal exposure to CPF increases the risk of mental and motor developmental delay, with increased incidence of pervasive developmental neurotoxicity, including attention deficit/hyperactivity disorder [4]. CPF elicits its noxious effects via impairments of axonal transport, increased generation of reactive oxygen species and induction of intracellular oxidative stress, following both acute and chronic exposures in experimental animals [5–9]. CPF oxon, the toxic metabolite of the CPF has been implicated in the neurodevelopmental toxicity both in humans and rodents [10].

The biophysical principles of the nervous system function in insects and mammals are analogous, because they possess similar neurotransmitters, although their distribution varies [11,12]. The distribution of acetylcholinesterase (AChE) in insects indicates that the head contained most of the AChE activity [13–15]. Literature indicated that the enzyme isoforms are mainly found associated with nerve cells in the brain and thoracicoabdominal region [15–18]. Moreover, the structure of insect AChE is similar to those of vertebrates [19]. However, the subtle ultrastructural differences between the enzymes from insects and vertebrates have been related to the diverse biochemical behavior of AChE in mammals and insects [14,15,20]. Recently, cockroaches have been shown to be a useful non-vertebrate model for assessing the effects of electric field and mercury [21–23]. Cockroaches have several inherent advantages in various bioassays over more conventional test animals, such as rodents. They are smaller, simpler to house and maintain, easier to breed in large numbers because of their high reproductive potential [24]. Furthermore, the use of cockroaches in research may contribute to the understanding of unknown toxicological mechanisms involved

* Corresponding author.
E-mail addresses: jbtrocha@gmail.com, jbtrocha@yahoo.com.br (J.B.T. Rocha).

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in the behavioral changes in other insects explored economically, such as honey bees.

In basic neuroscience research, the novel environment test is an evolving behavioral tool that is used to assess novelty-associated behavioral stress responses [25]; however, there are only few studies investigating the behavioral response to novelty in insects [23,26,27]. To evaluate the behavioral and biochemical endpoints using a non-vertebrate species, CPF-induced neurobehavioral model in nymphs of lobster cockroach *Nauphoeta cinerea* was performed in a novel environment paradigm. Herein we report, for the first time, behavioral tests in a cockroach model, which assessed exploratory profiles and locomotor endpoints following subchronic environmental contaminant exposure. In addition, determination of biochemical assays, including acetylcholinesterase activity, antioxidant and oxidative stress parameters were performed in the brains of CPF-exposed cockroaches.

2. Materials and methods

2.1. Chemicals

Technical grade chlorpyrifos (CPF) from Milenia Agrociências S.A., Paraná, Brazil was used for this study. Thiobarbituric acid (TBA), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), acetylthiocholine iodide, 5′,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Cockroach husbandry and experimental protocol

Nymphs of lobster cockroach *N. cinerea* were obtained from the Laboratório de Bioquímica Toxicologica, Universidade Federal de Santa Maria, Brazil. They were reared in plastic boxes at standard conditions of controlled temperature 25 ± 1 °C, 60–80% relative humidity, and subjected to natural 12:12-h light-to-dark photoperiod cycle. The insects had free access to water and standard cockroach food [23]. CPF was added to the dry food as an ethanol solution, and left till the ethanol was completely evaporated. Control food was treated with an equivalent volume of ethanol. Following ethanol evaporation, the food was stored at −20 °C. Experimental cockroaches were assigned to five groups consisting of 30 nymph cockroaches per group. The control group received standard food only, whereas the remaining four groups were fed with food containing CPF at 0.078, 0.15625, 0.3125 and 0.625 μg/g feed for 35 consecutive days. The exposure time and concentrations were chosen based on preliminary range-finding experiments conducted to determine concentrations that would result in the survival of cockroaches long enough for the manifestation of neurobehavioral changes. The nymphs of *N. cinerea* were used in this study because the developing organism is commonly more susceptible to toxic insult than the adult.

2.3. Behavior in a novel environment

Behavior of cockroaches in a novel environment was measured following CPF exposure period. The cockroaches were randomly selected and placed in a white polystyrene box (15 cm in width × 15 cm in length × 7 cm in height) with a glass covering its top. The behavior was filmed during a 10-min trial using a webcam (DNE webcam, Porto Alegre, Brazil) mounted above the novel apparatus and connected to a laptop for recording the videos. To ensure the same experimental conditions, all experiments were performed during the same time-period each day (from 10:00 a.m. to 4:00 p.m.). The behavioral parameters were automatically measured at a rate of 30 frames per second using an appropriate video-tracking software (ANY-maze, Stoelting CO, USA). Care was taken when transferring the cockroaches from home containers to the novel arena to avoid handling stress. All the cockroaches were handled and tested using standardized procedures (similar manipulation, time period in a day and illumination) during the investigation.

2.3.1. Locomotor parameters

The locomotor activity of the cockroaches was determined by behavioral endpoints including the total distance traveled, immobility, body rotation, turn angle (which represents the changes in direction of the center point of the animal) and meandering (absolute turn angle divided by the total distance traveled).

2.3.2. Vertical exploration

The vertical behavior of cockroaches in the new environment indicates its tendency to climb the walls of the apparatus. Endpoints of vertical activity included the time spent and number of entries into the periphery (wall of the container) area during the 10-min trial.

2.3.3. Exploratory profile

Analysis of the exploratory profile of the cockroaches was performed by representative track and occupancy plots in order to represent the overall activity in both horizontal and vertical regions. Analogous to other models, cockroaches tend to establish a home-base formation during novel environment trial, which is defined as a place in the arena for which the experimental animal shows a preference across time, both in terms of occupancy and as a starting and ending point of exploratory tours [28]. The potential home-base formation of cockroaches was assessed using behavioral data (basically transitions and time spent per section) and was confirmed by both track and occupancy plots.

2.4. Sample preparation for biochemical assays

Following the exposure period, cockroaches from control and CPF-treated groups were anesthetized on ice and weighed. Subsequently, the heads were carefully removed, weighed, homogenized in ice-cold 0.1 M phosphate buffer, pH 7.4 in ratio of 1:40 (mg head: μL buffer) and centrifuged at 6000 × *g* for 10 min at 4 °C to obtain the supernatant which was used for the biochemical estimations. The protein contents of head homogenates were determined by the Lowry method [29].

2.4.1. Determination of acetylcholinesterase activity

Acetylcholinesterase is considered an index for assessing the function of nervous system and its activity is commonly used to diagnose neuro-degenerative diseases and defects [23,30]. The determination of AChE activity was carried out according to the method of Ellman et al. [30]. The system consisted of 135 μL of distilled water, 20 μL of 100 mM potassium phosphate buffer (pH 7.4), 20 μL of 10 mM DTNB, 5 μL of sample, and 20 μL of 8 mM acetylthiocholine as substrate. The degradation of acetylthiocholine iodide was measured for 5 min (30 s intervals) at 412 nm using a SpectraMax plate reader (Molecular Devices, CA, USA) and the results were expressed as μmol/min/mg protein.

2.4.2. Total thiol determination

Total thiol level is a well-known indirect oxidative stress endpoint for determining the oxidative changes in sulfhydryl groups of proteins and peptides in biological samples. Total thiol content was determined according to the method previously described by Ellman [31]. The reaction mixture consisted of 170 μL of 0.1 M potassium phosphate buffer (pH 7.4), 20 μL of sample, and 10 μL of 10 mM DTNB. Following 30 min incubation at ambient temperature, the absorbance was measured at 412 nm. A standard curve was plotted for each measurement using reduced glutathione (GSH) as a standard and the results expressed as mmol/mg protein.