Symbiosis

www.symbiosisonlinepublishing.com
ISSN Number: 2572-3162

International Journal of Scientific Research in Environmental Science and Toxicology

Research Article

Open Access

Inhibition of Serum Esterases in Juvenile Rats Repeatedly Exposed to Low Levels of Chlorpyrifos

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Received: March 24, 2022; Accepted: May 07, 2022; Published: May 13, 2022

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Abstract

Chlorpyrifos (CPF) is an organophosphorus insecticide that has gained significant attention cue to the reported toxicity associated with developmental exposure. While the canonical mechanism of toxicity of CPF involves the inhibition of brain acetylcholinesterase (AChE), we have reported that exposure of juvenile rats to levels of CPF that do not yield any inhibition of brain AChE results in neurobehavioral alterations at later ages. However, it is unclear what effect exposure to these low levels of CPF has on blood esterase activities which are frequently used not only as biomarkers of exposure but also to set exposure levels in risk assessment. To determine this, male and female rat pups were exposed orally from postnatal day 10 to 16 to either corn oil (vehicle) or 0.5, 0.75, or 1.0 mg/kg CPF. At 12 h after the final exposure, serum cholinesterase (ChE), butyrylcholinesterase (BChE), and carboxylesterase (CES), and red blood cell (RBC) and brain AChE activities were determined. There were no differences between sexes in either the controls or individual treatments for all enzymes. Only the highest dosage of 1.0 mg/kg CPF yielded significant brain AChE inhibition (22-24%) but all dosages significantly inhibited the blood esterases with inhibition being highest with serum CES (65-85%) followed by serum BChE (57-76%), RBC AChE (35-65%), and then serum ChE (16-32%). Our data verify that blood esterases are inhibited at dosages of CPF that alter neurobehavioral performance in the absence of effects on brain AChE activity.

Keywords: Chlorpyrifos (CPF); Organophosphate insecticides; Developmental; Biomarkers.

Introduction

Agriculture is a major industry in the United States, accounting for nearly 11% of the workforce according to the United States Department of Agriculture [1]. Due to the size of this industry, consideration of environmental and public safety factors is essential. Insecticide regulations for agricultural regions have been an important issue due to the toxic effects of different insecticides including the organophosphate (OP) insecticides. One compound of this class, chlorpyrifos (CPF), has garnered more attention than many others. Prior to the year 2000, CPF was heavily used both in households and agriculture but due to its suspected developmental toxicity, voluntary restrictions phased out of household use of CPF. In fact, CPF is represented in a significant portion of the epidemiological studies involving the developmental exposure to OP insecticides. These studies have reported associations between developmental OP exposure and negative effects in children, such as decreased motor skills, decreased cognitive abilities, and increased signs of attention deficit disorder and attention-deficit/hyperactivity disorder (ADHD) [2-8].

Despite the restriction preventing the use of CPF in households, it is still frequently used for agricultural purposes in rural areas [9, 10]. It is also a rather controversial compound. In 2015, the United States Environmental Protection Agency (EPA) proposed to revoke all food residue tolerances for CPF, effectively banning its use [11]. A subsequent EPA-sponsored scientific review of the data did not support the Agency's proposal for revocation [12] and, following an administrative policy change, the Agency decided to allow continued use of CPF [13]. In August 2018, a court order was issued that required EPA to cancel all of the registrations for CPF [14]. Even so, the EPA decided in July 2019 to allow continued use of CPF [15]. There was then a subsequent August 2019 lawsuit by six states to attempt to reverse this decision [16]. In early 2020, Corteva (formerly Dow AgroSciences) announced that it would phase out the manufacture of CPF in the United States [17]. In August 2021, the EPA announced that it would cancel all registered food uses of CPF in the United States [18]. However, non-food uses of CPF in the United States are still allowed including use on golf courses, in turf grass production, on- road medians, in nurseries and greenhouses, for mosquito control, and in other facets. CPF will continue to be used internationally on foods [19] and to combat invading insects [20] because it is cheap and effective. Thus, it will continue to pose a threat to children worldwide.

The canonical mechanism by which OP insecticides exert their

neurotoxicity has been the inhibition of brain acetylcholinesterase (EC 3.1.1.7; AChE). At higher levels of exposure, inhibition of AChE leads to accumulation of acetylcholine in the cholinergic synapse. This leads to excessive stimulation of the receptors in the autonomic and central nervous systems and at nicotinic receptors on skeletal muscles. This excessive stimulation results in the characteristic signs of OP toxicity. However, environmental exposures do not involve high levels of an OP insecticide, with the exception of accidental exposures or suicide attempts. Real-world scenarios involve low-level exposures that do not yield significant inhibition of brain AChE activity. However, exposure to low levels of OP insecticides cannot be considered safe especially when this exposure occurs during periods of brain development. Usually, changes in neurobehavioral function in adults correlates with higher levels of inhibition of brain AChE [21-23]. In contrast, changes in neurobehavioral function in developing animals have been observed following exposure to OP insecticides at levels that induce only minimal inhibition of brain AChE [24-29]. In our laboratory, we have reported that exposure of juvenile rats to low dosages of CPF that do not inhibit brain AChE results in persistent changes in the proteome of the amygdala with alterations in glutamatergic and GABAergic signalling and disrupts neurobehavioral function [30-32]. These changes were detected once those rats' reached adolescence.

The most frequently used clinical biomarkers for OP exposure entail measuring the inhibition of blood esterases. The three main esterases that are inhibited following exposure to an OP insecticide include: (1) acetylcholinesterase (EC 3.1.1.7, AChE) found suspended in blood and on the erythrocyte membrane; (2) butyrylcholinesterase (EC 3.1.1.8; BChE) found suspended in the blood; and (3) carboxylesterase (EC 3.1.1.1, CES) found suspended in blood. Other enzymes have also been considered for use as biomarkers, such as acyl peptide hydrolase, but it is not inhibited by all OPs and can fluctuate depending on disease state [33]. Many studies suggest that the inhibition of the blood esterases can be used as surrogate markers to track the impact of OP pesticides on exposed individuals [34, 35]. However, few studies have determined what impact exposure to low dosages of OPs has on these surrogate markers of exposure. Using low dosages of CPF that we have previously demonstrated to induce neurobehavioral effects, this project determined the effect that level of treatment had on the biomarkers of exposure.

Materials and Methods

Chemicals

Chlorpyrifos (>99% purity) was a generous gift from DowElanco Chemical Company (Indianapolis, IN). All other chemicals were purchased from Cayman Chemicals (Ann Arbor, MI) or Sigma Chemical Co. (St. Louis, MO).

Animals

Adult male and female Sprague Dawley rats (Hsd:SD; Envigo, Indianapolis, IN) were used for breeding to obtain male and female rat pups. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility in a temperature-controlled environment (22 ± 2EC) with a 12hr dark-light cycle with lights on between 0700 and 1900. Lab Diet rodent chow and tap water were freely available during the experimentation. The day of birth was designated as postnatal day 0 (PND0). All procedures were approved by the Mississippi State University Institutional Animal Care and Use Committee.

Exposure

Beginning on PND10, rats were exposed daily for 7 days to 0.5, 0.75, or 1.0 mg/kg CPF by oral gavage at a volume of 0.5 ml/kg. CPF was dissolved in corn oil and delivered to the back of the throat using a 25-µl tuberculin syringe equipped with a 1-inch 24-gauge straight intubation needle (Popper and Sons, Inc., New Hyde Park, NY). The dosages of CPF were designed to span the range between no inhibition of brain AChE and low inhibition of brain AChE [30, 31, 36-38]. Each day, body weights were recorded and body weight gain was calculated as the difference between the body weights on PND11-16 and the original body weight at the initiation of treatment on PND10.

Tissue Collection

On PND16, male and female rat pups were sacrificed at 12 hrs following the last administration of CPF. Blood was collected and centrifuged to obtain serum which was stored at -80°C until assay. Brains were rapidly removed and dissected to obtain the forebrain (excluding the medulla and cerebellum). The forebrain was frozen on a stainless-steel plate on top of dry ice and maintained at -80°C until assay. In an additional cohort of rats, blood was collected in heparinized tubes and haemoglobin-free erythrocyte ghosts were prepared as described previously [39]. Briefly, heparinized blood was centrifuged (3000g for 10 min) and the plasma was removed, and the volume was measured. The erythrocytes were washed 3 times with 2 volumes of 0.1M phosphate buffer (pH 7.4) and centrifuged (1000g for 10 min) and the supernatant was removed. The packed erythrocytes in the pellet were lysed in 20 volumes of 6.7M hypotonic phosphate buffer (pH 7.4) for 10 min on ice followed by centrifugation at 50,000g for 30 min. The supernatant was removed and the pellet was re-suspended and diluted to the original volume with 0.1M phosphate buffer (pH 7.4). The resuspended erythrocyte ghosts were stored at -80°C until use.

Enzymes Assays

Forebrains were homogenized in cold 0.05M Tris-HCl buffer (pH 7.4 at 37°C) in a glass mortar using a Wheaton motorized tissue grinder and a Teflon pestle. Prior to the brain AChE assay, the homogenate was diluted in cold 0.05M Tris-HCl buffer (pH 7.4 at 37°C) to a final tissue concentration of 1.0 mg/ml. For serum CES, serum was diluted with the same buffer to obtain a final tissue concentration of 2.5 µl/ml. For serum ChE and BChE, the final concentration was 15 µl/ml. For ghost erythrocyte (RBC)

AChE, the final concentration was 50 µl/ml. The activity of serum ChE and BChE was measured spectrophotometrically using a modification [40] of Ellman et al. [41] with acetylthiocholine as the substrate for AChE and butyryl thiocholine as the substrate for BChE (1 mM final concentration) and 5,5'-dithiobis (nitrobenzoic acid) as the chromogen. The activity of serum CES was measured spectrophotometrically using 4-nitrophenyl valerate as the substrate (0.5 mM final concentration) and monitoring 4-nitrophenol, one of the hydrolysis products, as previously described [42]. Protein concentrations were quantified with the Folin phenol reagent using bovine serum albumin as a standard [43]. Specific activities were calculated as n moles product produced min-1 mg protein-1. Since BChE can hydrolyse acetylthiocholine, serum ChE activity is defined as a mixture of acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) activities as we did not utilize specific inhibitors in the assay.

Statistical Analysis

Statistical analysis was performed using SAS statistical package (SAS Institute Inc., Cary, NC). The sphericity of the body weight gain data was initially tested by analysis of variance (ANOVA) using the general linear model with a repeated measures paradigm and was found to violate the assumption of sphericity. Therefore, subsequent analysis by ANOVA using the Mixed procedure [44] was conducted with a repeat measure's paradigm with a Huynh-Feldt covariance structure [45]. Enzyme activities were also analysed by ANOVA using the Mixed procedure [44]. All analyses determined significant differences in sex, treatment, and sex × treatment interactions. Mean separation was performed by least-square means. The criterion for significance was set at $p < 0.05$.

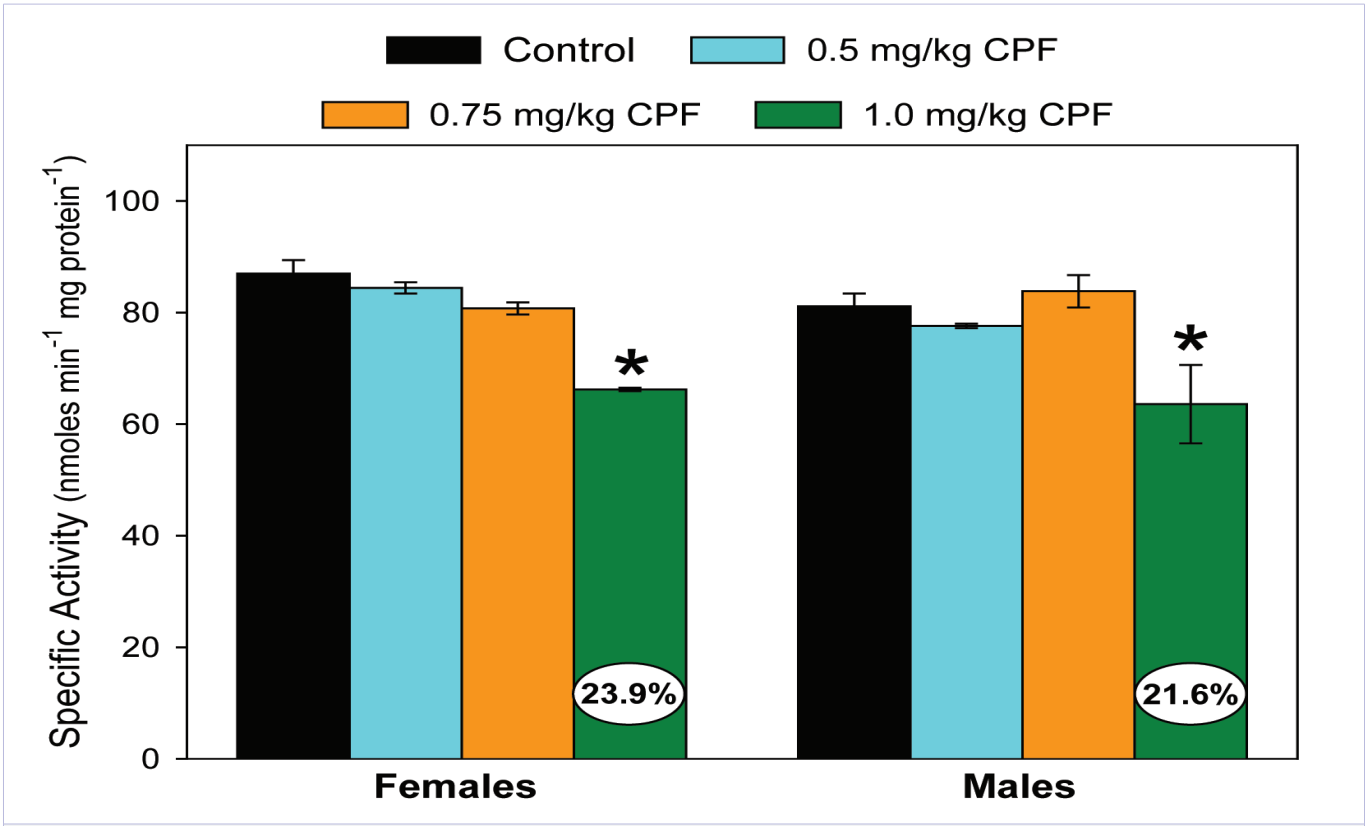

Figure 1: The specific activity of forebrain acetylcholinesterase (AChE) of rat pups following daily oral exposure from postnatal day 10 through 16 to either corn oil (control) or 0.5, 0.75, or 1.0 mg/kg chlorpyrifos (CPF) and determined 12 h after the last administration. Values are expressed as nmole product min−1 mg protein−1 ± SE (n = 4–5). Percent inhibition for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Statistical differences from control values are indicated by asterisks above the corresponding bar (* = $p \leq 0.05$).

Results

Following repeated developmental exposure to CPF, there were no signs of overt toxicity or cholinergic hyperstimulation with any dosage. There was no significant effect of treatment or sex on growth body weight gain. There was a significant overall effect of day ($p < 0.0001$) for all treatment groups indicating significant growth occurred over the treatment period. This lack of effect on body weight gain by the dosages of CPF used in this study is in agreement with our previous observations [30, 31, 38, 46].

For all enzyme biomarkers of exposure, there was a significant effect of treatment but no significant effect of sex or any significant sex × treatment interaction for all enzymes. As presented in Figure 1, a significant decrease in brain AChE specific activity was only observed at the highest dosage of CPF tested (1.0 mg/kg)

and the level of inhibition was similar between sexes. This pattern of inhibition is similar to what we observed in our previous studies. For serum CES specific activity (Figure 2), serum BChE specific activity (Figure 3), serum AChE specific activity (Figure 4), and ghost RBC ChE activity (Figure 5), there was a statistically significant decrease in activity for all three dosages of CPF. The level of inhibition was similar between sexes for all biomarker enzymes.

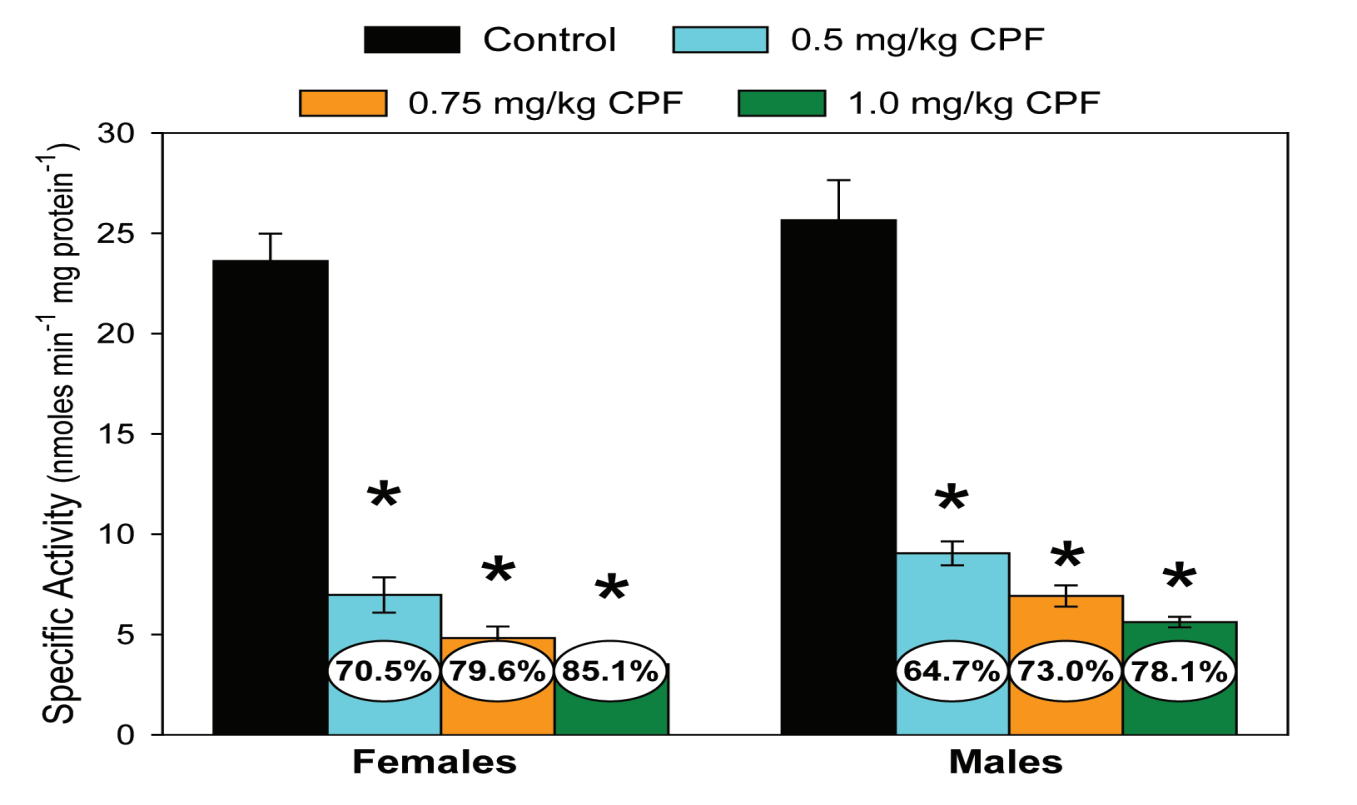

Figure 2: The specific activity of serum carboxylesterase (CES) of rat pups following daily oral exposure from postnatal day 10 through 16 to either corn oil (control) or 0.5, 0.75, or 1.0 mg/kg chlorpyrifos (CPF) and determined 12 h after the last administration. Values are expressed as nmole product min−1 mg protein−1 ± SE (n = 4–7). Percent inhibition for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Statistical differences from control values are indicated by asterisks above the corresponding bar (* = $p \leq 0.05$).

Discussion

It is commonly recognized that the inhibition of blood esterases is the most sensitive marker for exposure to OP compounds. There have been conflicting reports over the years as to which enzyme provides the best endpoint to use for risk assessment. For example, in a laboratory study related to developmental exposures, Chen et al. [47] reported that RBC AChE inhibition is a more sensitive indicator of CPF exposure than is neurological tissue. Therefore, it is an appropriate surrogate for exposure and can be used to set margins of safety for both adults and infants. However, Thetkathuek et al. [48] reported that plasma ChE may be the best indicator for exposure assessment for farmworkers. In contrast, Ramírez-Santana et al. [49] reported that plasma BChE was a better predictor for reduced neurobehavioral functioning in farmworkers. Nonetheless, RBC AChE inhibition serves as the key health measure that the EPA uses as the basis for setting its point of departure for developing a tolerance for OP insecticides including CPF [50, 51]. In addition, health agencies from other countries have utilized RBC AChE inhibition as the basis for determining the safety of CPF and other OPs [52, 53].

The most significant impact of low-level CPF exposure on blood enzyme activity occurred with serum CES activity. This was not surprising given that exposure of juvenile rats to higher levels of CPF (using the same exposure paradigm as was used in this study) yields greater inhibition of serum CES activity than of serum ChE activity [54]. In addition, the level of inhibition following 0.5 mg/kg observed in the present study is consistent with the level of inhibition observed in our previous study [38]. During in vivo OP exposures, the inhibition of CES serves as a bio scavenger that stoichiometrically binds to and inactivates the active metabolite of OP insecticides. This functions to reduce the number of OP molecules available to reach the brain and inhibit AChE [55, 56]. However, while most mammalian laboratory species contain CES activity as a component of their blood, humans do not [57]. This limits the usefulness for utilization of serum CES in OP toxicity assessment for humans but it can be used as marker for exposure in many animal species.

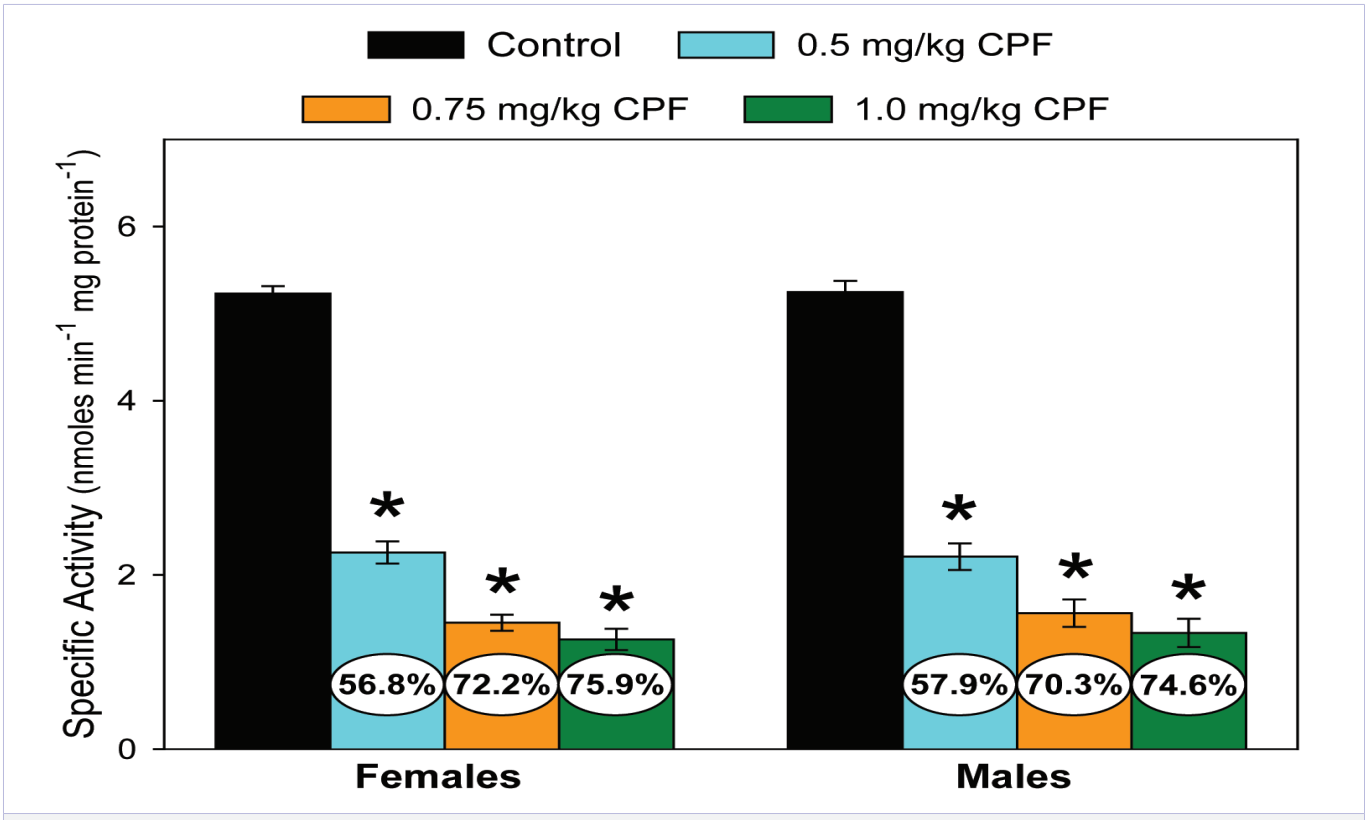

Figure 3: The specific activity of serum butyrylcholinesterase (BChE) of rat pups following daily oral exposure from postnatal day 10 through 16 to either corn oil (control) or 0.5, 0.75, or 1.0 mg/kg chlorpyrifos (CPF) and determined 12 h after the last administration. Values are expressed as nmole product min−1 mg protein−1 ± SE (n = 4–7). Percent inhibition for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Statistical differences from control values are indicated by asterisks above the corresponding bar (* = $p \leq 0.05$).

The second most significant impact of low-level CPF exposure on the blood enzyme activity occurred with serum BChE activity. In in vitro studies, BChE is much more sensitive to inhibition than AChE [58]. Much like CES, BChE also serves as a bio scavenger that stoichiometrically binds and inactivates the active metabolites of OP insecticides during in vivo OP exposures [59]. Because of its higher sensitivity to inhibition by OPs, BChE is a useful laboratory marker of OP exposure [60, 61], but it is not useful as a predictor of toxicological outcomes because it is not involved in neurotransmission [62]. In addition, the usefulness of BChE as a biomarker suffers from the increased individual variability in activity levels especially between sexes [63, 64]. Since it is produced in the liver, blood activity levels of BChE can fluctuate depending on the disease state of the liver [65]. As it is not involved in neurotransmission, experts have suggested that it should not be used to establish reference doses in the process of the risk assessment of OP insecticides [66].

In a study comparing the effects of exposure to several OP insecticides on blood enzyme activity, serum ChE was not inhibited by all OPs tested while RBC AChE activity was suppressed by every OP tested [67]. Similar to the inhibition of BChE, the inhibition of RBC AChE is not considered a direct adverse effect. However, RBC AChE is similar kinetically to both brain and skeletal muscle AChE [68] and, thus, can be considered the critical effect". The critical effect has been defined as the first adverse effect or its known precursor that occurs as the dose rate increases [51, 69].

In our study, the effect of CPF exposure on RBC AChE activity was less than the effect on serum CES and BChE but was greater than the effect observed on serum ChE. Significant effects were observed in RBC AChE activity at exposure levels that did not alter brain AChE activity. Previously, Zheng et al. [70] orally exposed rat pups daily from PND7-PND21 to increasing dosages of CPF (0.15-15 mg/kg) and reported that the No Observed Effect Level (NOEL) for brain, plasma, and RBC AChE was 0.75 mg/kg. While our data agrees with these findings, we observed significant inhibition of serum ChE and RBC AChE activity following exposure to a lower dosage of CPF. In a different study, Marty et al. [71] orally exposed rat pups daily from PND11-21 to increasing dosages of CPF (0.05-3.5 mg/kg) and reported that the lowest dosage to affect brain AChE activity was 1.0 mg/kg and the lowest dosage to affect RBC AChE activity and plasma ChE activity was 0.5 mg/kg. While the age range of exposure differs between our studies, our data agree with the findings of Marty et al. concerning the inhibition of brain AChE, RBC AChE, and blood ChE following low-level repeated exposure of rat pups to CPF.

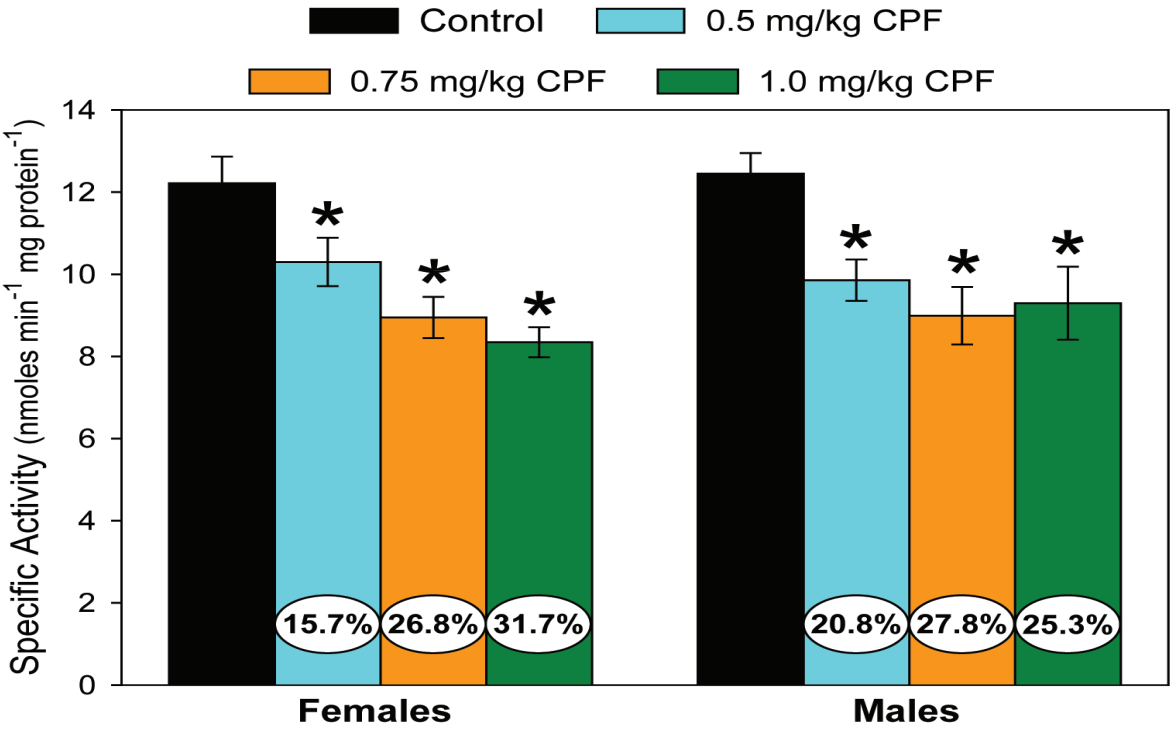

Figure 4: The specific activity of serum cholinesterase (ChE) of rat pups following daily oral exposure from postnatal day 10 through 16 to either corn oil (control) or 0.5, 0.75, or 1.0 mg/kg chlorpyrifos (CPF) and determined 12 h after the last administration. Values are expressed as nmole product min−1 mg protein−1 ± SE (n = 4–7). Percent inhibition for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Statistical differences from control values are indicated by asterisks above the corresponding bar (* = p ≤ 0.05).

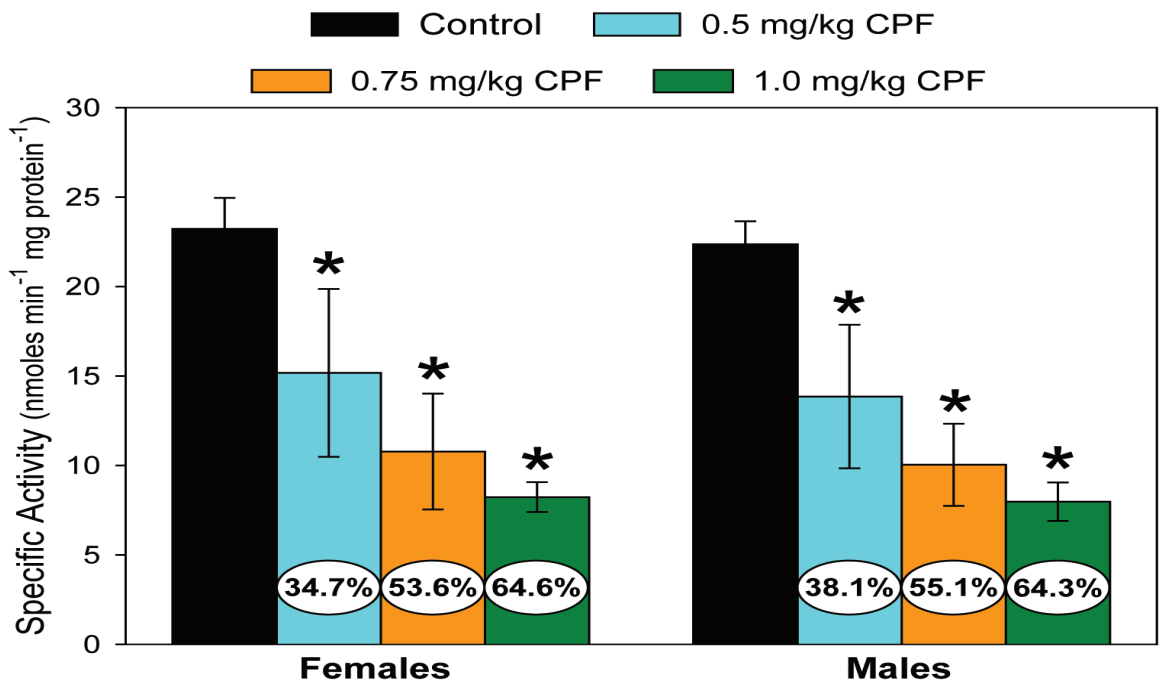

Figure 5: The specific activity of red blood cell (RBC) acetylcholinesterase (AChE) of rat pups following daily oral exposure from postnatal day 10 through 16 to either corn oil (control) or 0.5, 0.75, or 1.0 mg/kg chlorpyrifos (CPF) and determined 12 h after the last administration. Values are expressed as nmole product min−1 mg protein−1 ± SE (n = 4–7). Percent inhibition for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Statistical differences from control values are indicated by asterisks above the corresponding bar (* = p ≤ 0.05).

We have previously reported that oral juvenile exposure to low dosages of CPF that do not result in the inhibition of brain AChE can result in altered neurobehavioral performance at later ages [30, 31]. We report here that these oral dosages of CPF significantly inhibit blood esterase activities including RBC AChE. The inhibition of RBC AChE (greater than 10%) is the level of inhibition commonly used by regulatory agencies in risk assessment when setting the initial point of departure during the development of safety tolerance for OP insecticides [72]. Our current data demonstrate that the inhibition of RBC AChE is a sensitive marker for dosages of CPF that will result in neurobehavioral alterations. However, additional studies are needed to determine if these neurobehavioral alterations will be present following exposure to an OP insecticide that does not significantly alter RBC AChE.

Acknowledgments

The authors acknowledge the statistical expertise of Dr. Robert Wills. Research was supported by the Mississippi Agricultural and Forestry Experiment Station (MAFES), the College of Veterinary Medicine, Mississippi State University, and the National Institutes of Health (R15ES023162).

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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