Fish Cholinesterases as Biomarkers of Sublethal Effects of Organophosphorus and Carbamates in Tissues of *Labeo Rohita*

Ghazala,[1] *Shahid Mahboob,*[2,3] *L. Ahmad,*[3] *S. Sultana,*[3] *K. AlGhanim,*[2] *F. Al-Misned,*[2] *and Z. Ahmad*[2]

[1] Department of Environmental Sciences, Government College University, Faisalabad, Pakistan

[2] Department of Zoology College of Science, King Saud University, Riyadh 11451, Saudi Arabia; E-mail: *shahidmahboob60@hotmail.com*

[3] Department of Zoology, Government College University, Faisalabad, Pakistan

Received 21 September 2013; revised 2 November 2013; accepted 24 November 2013

ABSTRACT: Organophosphates and carbamates are major agrochemicals that strongly affect different neuroenzymes and the growth of various fish species. Here, we study the effect of sublethal concentrations of profenofos and carbofuran on the activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) and the associated health risk in fish. *Labeo rohita* fingerlings were exposed to three sublethal concentrations of profenofos and carbofuran. The minimum cholinesterase activities in the brain, gills, muscle, kidney, liver, and blood were after exposure to profenofos (0.06 mg/L). The minimum AChE and BuChE activities in the brain, gills, muscle, kidney, liver, and blood were after exposure to carbofuran (0.28 and 0.198 mg/L). Exposure to both types of pesticides affected the functions of these organs, including metabolism and neurotransmission, to various extents at different exposure concentrations. These findings suggest that they are required to be properly monitored in the environment, to reduce their toxic effects on nontarget organisms © 2014 Wiley Periodicals, Inc. J Biochem Mol Toxicol 28:137–142, 2014; View this article online at wileyonlinelibrary.com. DOI 10.1002/jbt.21545

KEYWORDS: **Profenofos; Carbofuran; Acetylcholinesterase; Butyrylcholinesterase; Inhibition**

INTRODUCTION

Environmental monitoring of pesticides is urgently needed. Contamination by pesticides is an important public health problem, mainly in developing countries. Approximately 0.1% of applied pesticides reaches the target pests, whereas the rest spreads throughout the environment [1]. Pesticides are among the most important classes of insecticides and acaricides in usage and economic impact [2]. Increasing pollution of water resources in Pakistan and the consequential effects on human health as well as the environment are matters of grave concern. The drinking and surface water in densely populated areas is polluted due to various anthropogenic activities [3]. As an agricultural country, pesticide usage and the import of pesticides has greatly increased in Pakistan, from 225,176 tons to 305,938 tons, in the past two decades [4].

Presently, concern over the accumulation and persistence of pesticides in the aquatic ecosystem, which poses a serious threat to biological life, including human beings, [5] is increasing. Fish are directly exposed to these pesticides by absorption through the skin, breathing, and oral intake of pesticide-contaminated water or pesticide-contaminated prey [6]. Among the various biomarkers of pesticide exposure, the family of cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), has widely been used to evaluate the noxious effects of pesticides, i.e., carbamates and organophosphates. The primary and best-known target of organophosphorus and carbamate compounds is a family of enzymes (cholinesterases; ChEs) consisting of AChE, and BChE. The first is synthesized in hematopoiesis, is located in the brain, end plate of the skeletal muscle, and erythrocyte membranes, and mainly regulates neuronal communication by hydrolyzing the ubiquitous neurotransmitter acetylcholine in the synaptic cleft [7]. The second is

Correspondence to: Shahid Mahboob.

synthesized in the liver and is present in plasma, smooth muscle, pancreas, adipocytes, skin, brain, and heart [8]. Aquatic vertebrates (terrestrial, aquatic) and invertebrates indicate pesticide intoxication prior to their death, so they are used as model organisms [9,10]. AChE plays an important role in neurotransmission at cholinergic synapses and neuromuscular junctions by rapidly hydrolyzing acetylcholine to choline and acetate. Other cholinesterases, including BuChE, are crucial for different parts of the immune system. Although its physiological functions are not well defined, BuChE is considered to be one of the core detoxifying enzymes [11,12]. Some studies suggest that BuChE protects AChE against xenobiotics such as pesticides [13].

The exposure concentrations of pesticides that are not lethal to fish may affect their physiology and behavior, ultimately decreasing survival, reproduction, metabolic disturbances, and growth [14,15]. The prevalence of most carbamates (carbofuran, carbosulfan, carbaryl) and organophosphates (triazophos, chlorpyrifos, profenofos, endosulfan, methamidophos, diazinon, parathion methyl, and malathion) has been studied in aquatic biota, water, and sediments in Punjab, Pakistan [16]. Profenofos and carbofuran have a relatively short half-life as triazophos (30–250 days) and profenofos (0.33–62 days) in water. Biomarkers are the best parameters for determining their effects when they are no longer present in the water, but their effects on cholinesterase are irreversible; the effects are permanent and may not be present in water. The present study suggests that organophosphates and carbamates are major agrochemicals that strongly affect different neuroenzymes and the growth of various indigenous fish species of Pakistan that are commonly cultured and consumed. Therefore, the primary objective of the study was to assess the effect of sublethal concentrations of profenofos and carbofuran on the activity of AChE and BuChE and the associated health of *Labeo rohita*.

MATERIALS AND METHODS

Live specimens of *L. rohita* fingerlings (L = 90 ± 6 mm, W = 30.00 ± 2.00 g) were transported from the Fish Seed Hatchery, Faisalabad, Pakistan, and were acclimatized in glass aquaria (70 L). Fish were fed with commercial feed at 3% of body weight per day during the acclimatization period (15 days). Water parameters were analyzed and maintained on a daily basis. Technical-grade profenofos (98%) and carbofuran (90%) were obtained from Ali Akbar Enterprises, Lahore, Pakistan.

PESTICIDE EXPOSURE TESTS

Sublethal toxicity tests were performed after determination of acute toxicity. Three sublethal concentrations of all pesticides were prepared in a suitable solvent (profenofos in acetone (Merck, Darmstadt, Germany), carbofuran in ethanol (Merck)) as 1/5th, 1/10th, and 1/15th fraction of the median lethal concentration (LC_{50}) (predetermined). Fish were exposed to these lower concentrations of pesticides in triplicate with 20 fish at each concentration for a period of 60 days. The fish were fed daily with commercial diet at the rate of 3% of their body weight in two fractions at an interval of 8 h. The aqueous solution was renewed every 4 days to maintain a continuous supply of pesticides to the fish. The fish were removed from each aquarium at the end of the experiment and anesthetized with MS-222 (Finquel®). They were dissected to remove the brain, gills, liver, kidney, and muscle samples, which were quickly removed, frozen in liquid nitrogen, and stored at –20°C. AChE and BuChE activity levels were determined according to the procedure described by Ellman et al. [17] and Kuster [18], with certain modifications. Total soluble proteins were determined by the Bradford [19] standard method to assess enzymatic activity/g of protein. Differences among treatments were tested using ANOVA followed by the Tukey HSD test.

RESULTS

Characteristics of Water

The quality of water remained constant. Aquaria were continuously aerated, so that the dissolved oxygen level was maintained at 5.00–5.50 mg/L. The electrical conductivity, pH, and water temperature ranged from 2.23–2.47 mS, 7.5–8.5, and 25–27°C, respectively.

Median Lethal Concentration of Profenofos and Carbofuran

A dose-dependent increase and time-dependent decrease were observed in the mortality rate. As the exposure time increased from 24 to 96 h, the median concentration was reduced. There was a significant difference ($P < 0.05$) among LC_{50} values obtained at different exposure times. At 96 h, LC_{50} for profenofos was 0.31 mg/L (0.26–0.38; Figure 1). The LC_{50} value for carbofuran in *L. rohita* at 96 h was 1.39 mg/L (0.99–2.01; Figure 2).

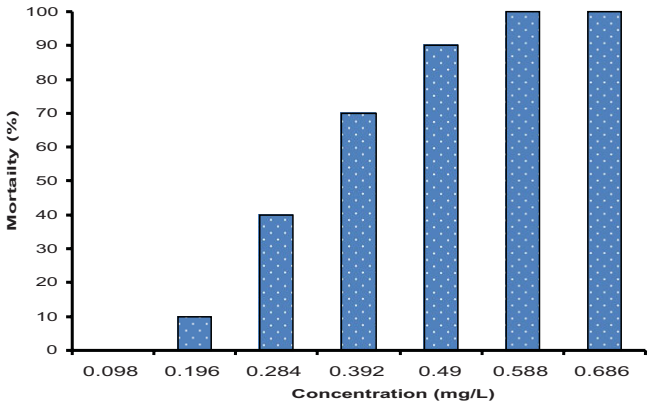

FIGURE 1. Effect of different concentrations (mg/ L) of profenofos on the mortality (% age) in *L. rohita* at 96 h of exposure.

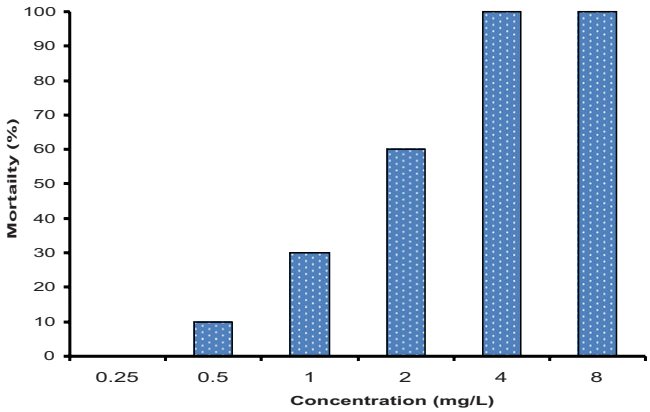

FIGURE 2. Effect of different concentrations of (mg/L) of carbofuran on the mortality (%) in *L. rohita* at 96 h of exposure.

Cholinesterase Activity

In this study, cholinesterase activities were determined in the brain after exposure to both pesticides, and the inhibition of brain AChE ranged from 1.04 to 14.87 μmol/min/g protein at various exposure concentrations of profenofos compared to the control group. BuChE activity was also reduced after exposure to various concentrations of profenofos. In *L. rohita*, the minimum and maximum activity levels of BuChE in the brain were 8.38 $\pm$ 0.046 and 28.78 $\pm$ 0.04, respectively, after exposure to different concentrations of profenofos, whereas significantly different activity was observed for AChE (14.87 $\pm$ 0.04 μmol/min/g protein; Table 1). The sensitivity of fish to pesticide exposure, especially organophosphate, was dependent on the level of brain AChE activity. Carbofuran also significantly inhibited cholinesterases (AChE and BuChE) in fish brains by 66%–86.8% compared to the control group after exposure to different concentrations of carbofuran ($P < 0.01$; Tables 2 and 3). The activity of BuChE in the gills was reduced to 2.50 $\pm$ 0.11 μmol/min/g protein compared to the control group (7.28 $\pm$ 0.16 μmol/min/g protein) after exposure to 0.06 mg/L profenofos. The carbofuran inhibitory effect on muscle AChE ranged from 37% to 77% after exposure concentrations; significant differences were observed between different treatments and between the treatments and the control group. The maximum muscle BChE activity was 14.25 $\pm$ 0.14 μmol/min/g protein after the fish were exposed to 0.09 mg/L carbofuran, but this value was significantly less than that for the control group (Table 3). The AChE activities were also reduced in the gills by 97.05% and 76.83% after exposure to profenofos (0.06 mg/L) and carbofuran (0.28 mg/L), respectively. The BuChE activity was reduced to 2.50 $\pm$ 0.29 μmol/min/g protein after profenofos exposure (0.06 mg/L), which was less than that of the control group (7.28 $\pm$ 0.16 μmol/min/g protein), but this inhibition was doubled at 65.71% inhibition in gills with 0.28 mg/L of carbofuran (Tables 2 and 3).

In addition to the brain, gills, muscle, and blood, AChE activity was also reduced in other metabolizing organs, i.e., liver and kidney. Maximum inhibition was observed in the kidneys (94.3%) and in the liver

TABLE 1. Comparison of Means ($\pm$ SE) for AChE and BuChE Activity (μmol/min/g protein), of the Control Group and Three Exposure Concentrations (mg/L = ppm) of Profenofos in Different organs of *L. rohita*

Enzyme	*Treatment (mg/L)*	*Brain*	*Gills*	*Flesh*	*Kidney*	*Liver*	*Blood*
AChE	Control	40.44 $\pm$ 0.02 A	20.36 $\pm$ 0.03A	18.09 $\pm$ 0.05A	24.39 $\pm$ 0.05A	98.06 $\pm$ 0.03A	26.52 $\pm$ 0.01A
	0.06	1.04 $\pm$ 0.02D	0.6 $\pm$ 0.03D	1.89 $\pm$ 0.05D	1.35 $\pm$ 0.20D	4.24 $\pm$ 0.02D	1.65 $\pm$ 0.03D
	0.03	7.99 $\pm$ 0.05C	3.24 $\pm$ 0.14C	2.64 $\pm$ 0.02C	6.87 $\pm$ 0.04C	23.59 $\pm$ 0.10C	10.50 $\pm$ 0.17C
	0.02	14.87 $\pm$ 0.04B	6.02 $\pm$ 0.13B	7.34 $\pm$ 0.20B	8.34 $\pm$ 0.20B	45.78 $\pm$ 0.01B	13.78 $\pm$ 0.02B
BuChE	Control	40.43 $\pm$ 0.05A	7.28 $\pm$ 0.16A	19.03 $\pm$ 0.02A	40.18 $\pm$ 0.10A	167.00 $\pm$ 0.29A	49.08 $\pm$ 0.05A
	0.06	8.38 $\pm$ 0.05D	2.50 $\pm$ 0.29C	1.89 $\pm$ 0.05D	4.37 $\pm$ 0.04D	16.94 $\pm$ 0.03D	8.65 $\pm$ 0.02D
	0.03	25.87 $\pm$ 0.04C	4.38 $\pm$ 0.05B	4.73 $\pm$ 0.02C	10.67 $\pm$ 0.04C	32.62 $\pm$ 0.01C	14.87 $\pm$ 0.02C
	0.02	28.78 $\pm$ 0.05B	4.78 $\pm$ 0.04B	12.45 $\pm$ 0.26B	12.92 $\pm$ 0.07B	94.37 $\pm$ 0.04B	23.67 $\pm$ 0.01B

S.E. = standard error.
Means with different letters for each fish in a column are highly significantly different ($P < 0.01$).

TABLE 2. Analysis of Variance for Esterase Activities (μmol/min/g of protein), of the Control Group and Three Exposure Concentrations (mg/L = ppm) of Profenofos and Carbofuran in Different Organs of *L. rohita*

		Mean of Squares											
Enzyme		*AChE*						*BuChE*					
SOV (Cont. × Conc.)	*DF*	*Brain*	*Gills*	*Flesh*	*Kidney*	*Liver*	*Blood*	*Brain*	*Gills*	*Flesh*	*kidney*	*Liver*	*Blood*
Profenofos	3	886.35**	241.15**	167.26**	294.22**	4918.26**	318.42**	526.4^{NS}	11.61^{Ns}	180.25**	753.54**	13976.3**	948.07**
Carbofuran	3	759.61**	159.97**	114.12**	238.87**	3466.22**	254.98**	357.69**	13.45**	120.95**	581.31**	13439.06**	955.37**

Abbreviations: SOV. = source of variation; DF = degree of freedom; Conc. = three exposure concentrations (1/5th, 1/10th, and 1/15th of LC_{50}); Contr. = Control group.
** = highly significant ($P < 0.01$).

TABLE 3. Comparison of Means (±SE) for AChE and BuChE Activity (μmol/min/g protein), of the Control Group and Three Exposure Concentrations (mg/L = ppm) of Carbofuran in Different Organs of *L. rohita*

Enzyme	*Treatment (mg/L)*	*Brain*	*Gills*	*Flesh*	*Kidney*	*Liver*	*Blood*
AChE	Control	40.43 ± 0.02A	20.36 ± 0.21A	18.09 ± 0.05A	24.39 ± 0.05A	98.06 ± 0.03A	26.52 ± 0.01A
	0.28	4.67 ± 0.04D	4.56 ± 0.03D	4.19 ± 0.11D	3.51 ± 0.11D	19.24 ± 0.07D	4.78 ± 0.01D
	0.14	9.18 ± 0.05C	5.24 ± 0.14C	6.40 ± 0.23C	8.67 ± 0.06C	35.66 ± 0.03C	12.56 ± 0.02C
	0.09	17.00 ± 0.06B	10.76 ± 0.03B	11.40 ± 0.23B	13.94 ± 0.03B	47.81 ± 0.06B	18.67 ± 0.02B
BuChE	Control	40.43 ± 0.25A	7.29 ± 0.17A	19.04 ± 0.02A	40.18 ± 0.10A	167.00 ± 0.58A	49.08 ± 0.05A
	0.28	13.70 ± 0.40C	2.50 ± 0.11D	3.89 ± 0.05D	7.37 ± 0.04D	19.63 ± 0.06D	6.78 ± 0.02D
	0.14	26.88 ± 0.05B	3.80 ± 0.11C	13.56 ± 0.03C	16.01 ± 0.01C	36.21 ± 0.12C	18.57 ± 0.03C
	0.09	27.67 ± 0.04B	5.79 ± 0.05B	14.25 ± 0.14B	19.22 ± 0.07B	97.55 ± 0.03B	26.57 ± 0.01B

S.E. = standard error.
Means with different letters for each fish in a column are highly significantly different ($P < 0.01$).

(95.6%) after exposure to the highest concentration of profenofos (Table 1). Less inhibition in both organs was observed when the fish were exposed to various concentrations of carbofuran, as mentioned in Tables 3 and 3. BuChE activity also followed a similar trend, with maximum inhibitions in the liver of 89.8% after exposure to profenofos (0.06 mg/L) and 88.2% after carbofuran exposure (0.0.28 mg/L). A significant difference between the maximum inhibitions caused by profenofos and carbofuran was observed in the kidney (Tables 1 and 3). In sublethal toxicity, the AChE and BuChE activities in blood were significantly reduced after 2 months of exposure and differed significantly from the control group after both profenofos and carbofuran exposure (Table 2 and 3). More than 50% inhibition of cholinesterase (AChE and BuChE) activities was observed in all organs of *L. rohita* after the highest (0.06 mg/l) and medium (0.03 mg/l) exposure concentrations of profenofos and the highest exposure concentration of carbofuran (0.298 mg/l), whereas variable inhibition was observed at other exposure concentrations of both pesticides. We found that the highest concentrations of profenofos and carbofuran affected the maximum cholinesterase level in all tissues. The overall comparison demonstrates that profenofos inhibited the more cholinesterase activity in all studied tissues in *L. rohita*, although both pesticides are chemically different.

DISCUSSION

Although mortality was not observed during the experiment, the animals showed signs of intoxication, including lethargy, erratic movement, and reduced response for feeding, from the third or fourth week of exposure until the end of the experiment. Suffocation was also observed after the first few hours of toxic medium addition, followed by acclimatization. Cholinesterases are biomarkers that have widely been used in various organisms. These enzymes are induced when the organisms are exposed to toxic substances, i.e., organophosphates, carbamates, PAHs (Polycyclic aromatic hydrocarbons), halogenated aromatics and certain types of dioxins [20,21]. Inhibition of cholinesterase has been studied in several systems and organs with a focus on brain tissues [22, 23]. Murphy et al. [24] reported that fish possessing a higher enzyme activity exhibit more enzyme inhibition after pesticide exposure. Our results agree with these findings. Biomarkers are useful indicators of pollution because of their high sensitivity and presentation of the first detectable signs of sublethal stress response in organisms [20, 25]. Inhibition of cholinesterase in either the nervous system or muscle has an adverse effect on organisms because the enzymatic activity in the target tissues contributes to neurotransmission [26]. AChE inhibition in fish has only been emphasized in the brain because intoxication

of the brain affects behavior [27]. Although the catfish brain is the most sensitive to aldicarb exposure, these fish with 90% AChE inhibition were alive with moderate symptoms of intoxication [28]. Our findings show less than 90% inhibition in the brain after exposure to any tested concentration of either pesticide. In contrast, maximum inhibition was observed in the muscle when the fish were exposed to the maximum concentration (0.06 mg/L) of profenofos; however, the fish remained alive during the whole period of exposure. Toni et al. [29] also reported a decrease in muscle ChE activities in fish. The muscle cholinesterases represent the largest pool of cholinesterases in the body. It is also important to control muscular function; the loss of muscular control causes many problems for fish, including the loss of swimming control and blockage of opercular movement, which may result in reduced oxygenation of the blood and hypoxia-induced death [30]. This effect may also contribute to the changes in fish behavior after exposure to pesticides. The reduction in cholinesterase activity in the gills may contribute to suffocation and reduced respiratory activity, as demonstrated by Chamber and Carr [31], who reported that the primary cause of AChE inhibition–induced death in mammals is generally related to respiratory failure [31], which may also be a problem for fish. Heath et al. [32] studied the effect of carbofuran on newly hatched striped bass larvae, and a decrease in ChE activity was observed at high concentrations of carbofuran. Our findings agree with these previous results [23, 32]. The poisoning of cholinesterases by pesticides in fish is considered to be a good indicator of intoxication [33]. The current findings are in line with Dembele et al. [34].

CONCLUSIONS

We believe that profenofos is highly toxic compared to carbofuran in the context of AChE and BuChE inhibition in all tested organs and tissues: brain, blood, gills, muscle, kidneys, and liver. Exposure to both types of pesticides affected the functions of these organs, including metabolism and neurotransmission, to various extents at different exposure concentrations. Because these pesticides are highly toxic, they are required to be properly monitored in the environment, so that their toxic effects on nontarget organisms, as well as human beings, can be reduced.

ACKNOWLEDGMENTS

The authors would like to their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. RGP-VPP-341.

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