INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
13–371/2014/16–2–406–410
http://www.fspublishers.org

Full Length Article

Cholinesterases: Cholinergic Biomarkers for the Detection of Sublethal Effects of Organophosphorous and Carbamates in *Catla catla*

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Abstract

Cholinergic enzyme inhibition is the most authentic tool to assess the environmental impact of pesticides on aquatic fauna. In current investigation we determined the sublethal toxic effects of carbofuran (carbamate) and profenofos (organophosphate) on acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) of various fish organs (brain, gills, kidneys, flesh, liver and blood). Fingerlings of *Catla catla* were exposed to three sublethal concentrations of profenofos (0.038, 0.019 and 0.012 mg/L) and carbofuran (0.198, 0.099 and 0.066 mg/L) for a period of two months. A highly significant inhibition in both of the cholinergic enzyme activities was observed even at the lowest concentrations of profenofos and carbofuran. Maximum inhibition of AChE activity was determined in the flesh (92.24%), whereas maximum inhibition of BuChE activity was determined in brain (93.10%) when exposed to profenofos. Similarly, carbofuron appeared to most detrimental for kidney (78.09%) when AChE activity was determined and it appeared to be most harmful for the liver (89.19%) when BuChE activity was assessed. Hence, profenofos proved to be the most potent inhibitor of cholinesterases (AChE and BuChE) as compared to cabofuran thus appearing to be more toxic for these aquatic animals.

Keywords: Profenofos; Carbofuran; Biomarkers; AChE; BuChE; Inhibition

Introduction

The increasing pollution of water sources in Pakistan and the consequent effects on human health as well as the environment is an issue of great concern. Drinking and surface water in densely populated areas is polluted due to various anthropogenic activities (Ahmed, 2007). As an agricultural country, pesticide usage and of course import of pesticides has increased to a great extent in Pakistan, from 228,789 metric tons to 310,847 metric tones in the last two decades (Anonymous, 2011). The intensity and amount of pesticide import as well as its expanded use for pest control and improvement of crops in intensive agriculture are posing serious hazards to terrestrial as well as fragile aquatic ecosystems and biota including fish (Mora *et al.*, 1999; Yaqin and Hensen, 2010; Mahboob *et al*., 2011; Ahmad *et al*., 2012; Muhammad *et al*., 2012).

Being strictly aquatic, fish are directly exposed to these pesticides by absorption through skin, breathing in and oral intake of pesticide-contaminated water or pesticide contaminated prey (Mathur and Singh, 2006). Among the various biomarkers of pesticides exposure, the family of cholinesterases [acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)] have widely been used as biomarker to evaluate the noxious effects of pesticides i.e. carbamates and organophosphate. Aquatic vertebrates (terrestrial, aquatic) and invertebrates give the nippy signal of pesticide intoxication long before their death so are taken as model organisms (Rodriguez-Fuentes and Gold-Bouchot, 2000; Torre *et al.,* 2002; Ferreira *et al*., 2010; Adedejio, 2011). AChE plays an important role in neurotransmission at cholinergic synapses and neuromuscular junction by rapid hydrolysis of neurotransmitter acetylcholine to choline and acetate. Other cholinesterases including BuChE are crucial for different parts of the immune system. Though its physiological functions are not well defined, BuChE is considered as one of the core detoxifying enzymes (Shea and Berry, 1984; Soreq and Zakut, 1993; Behra *et al.*, 2002; Assis *et al*., 2010; Gandahi *et al.*, 2013). Some of the investigations hypothesize that BuChE protect AChE against xenobiotics like pesticides (Whitaker, 1986).

Exposure to concentrations of pesticides that are not lethal, may affect fish physiology and behaviour, ultimately demolishing survival, reproduction, metabolic disturbances and growth (Murty, 1986; Kegley *et al*., 1999). The prevelance of most of the 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

To cite this paper: Ghazala, S. Mahboob, S. Sultana, T. Sultana, L. Ahmad and M.R. Asi, 2014. Cholinesterases: cholinergic biomarkers for the detection of sublethal effects of organophosphorous and carbamates in *Catla catla. Int. J. Agric. Biol*., 16: 406–410

(Mahboob *et al.*, 2009; 2011). The organophosphates and carbamates had been found to pose deleterious effects on biota including fish, determined in term of various biomarkers, growth and general health.

The current investigation hypothesized that organophosphates and carbamates are major agrochemicals, sternly affecting different neuro enzymes and ultimately growth of various indigenous fish of Pakistan, commonly cultured and consumed. The objective of this study was to assess the risk levels of pesticides (organophosphates and carbamates) in this popular indigenous fresh water fish.

Materials and Methods

Live specimens of fingerlings of *Catla catla* (L=90±6 mm, W= 30.00±2.00 g) were transported from Fish seed hatchery, Satiana road Faisalabad Pakistan and acclimatized in glass aquaria (70 L) at the Department of Wildlife and Fisheries, GC University, Faisalabad. Fish were fed commercial feed @ 3% body weight during acclimatization period (15 days). Water parameters were analyzed and maintained on a daily basis. Pesticide technical grades of Profenofos 98% and Carbofuran 90% were obtained from Ali Akbar Enterprises, Lahore, Pakistan.

Pesticides Exposure Tests

Sublethal toxicity tests were performed after determination of acute toxicity. Three sublethal concentrations of all pesticides were prepared in suitable solvents [profenofos in acetone (Merck, Germany)], carbofuran in ethanol (Merck, Germany)} as $1/5^{th}$, $1/10^{th}$ and $1/15^{th}$ part of LC_{50} (predetermined). Fish were exposed to these concentrations of pesticides in triplicates with 20 fish in each replicate for a period of 60 days. The fish were fed daily with commercial diet at the rate of 3% of live body weight in two fractions with the time interval of 8 h. The aqueous solution was renewed after every four days to maintain a continuous supply of pesticides to fish. The fish were taken out from each aquarium at the end of the experiment and anaesthetized with MS-222 (Finquel®). The fish were dissected to quickly remove brain, gills, liver, kidney and meat samples, which were frozen in liquid nitrogen and stored at -20°C. AChE and BuChE activities were determined following the protocols of Ellman *et al.* (1961) and Kuster (2005), with certain modifications. Total soluble proteins were determined as described by Bradford (1976) to assess the activity of enzymes/g of protein.

Statistical Analysis

A completely randomized design (CRD) with three replications per treatment was used for statistical analysis. Differences among treatment means were ascertained using analysis of variance (ANOVA) followed by the Tukey HSD test using computer software, Minitab package (Version 15).

Results

The current studies evaluated the effects of profenofos and carbofuran (commonly used organophosphate and carbamate insectcides) on cholinesterase activity in various organs of *Catla catla* viz., brain, gills, liver, kidney, flesh and blood under the sublethal exposure for a period of two months. Results of current investigation showed a variation in cholinesterases activities in different organs. Though mortality was not observed during the experiment, however, the animals showed signs of intoxication including lethargy, erratic movement and reduced response for feed at the third or fourth week of exposure till the end of the experiment. Suffocation was also observed for the first few hours of toxic media addition followed by acclimatization.

In the current investigation, cholinesterases (AChE and BuChE) activities were observed in the brain upon exposure to both the pesticides. The brain AChE activity inhibition ranged from 59% to 86% under various exposure concentrations of profenofos (0.012, 0.019 and 0.038 mg/L) as compared to the activity in the control group. BuChE activity was also reduced against various concentrations of profenofos. In *Catla catla*, the activity of BuChE was recorded to ~1.77 µmoL/min/g protein in the brain, under the medium and the highest exposure concentration of profenofos, whereas, significantly different activity (~12.78 µmoL/min/g Protein) was observed in the least concentration of profenofos ($1/15^{th}$ of LC_{50}) (Table 1). Carbofuran also caused a severe inhibition of cholinesterases (AChE and BuChE) in brain of fish from 30 to 75% over control group upon exposure to carbofuran levels with a highly significant difference ($P<0.01$) (Table 2).

Closely related to fish flesh, AChE activities were also found to be reduced in gills as 79.63% and 66.48% under the exposure of profenofos (0.038 mg/L) and carbofuran (0.198 mg/L), respectively. Under the exposure of profenofos (0.038 mg/L), BuChE activity was reduced to 1.00 µmoL/min/g protein in gill which is quietly less than the control group activity (7.71 µmoL/min/g protein), but this inhibition was significant as compared to the group exposed to carbofuran maximum concentration where activity was found to be doubled with 73.95% inhibition (Tables 1, 2). In sublethal toxicity, the AChE and BuChE activities in blood was significantly reduced after the two months of exposure and varied significantly from control group in case of profenofos as well as carbofuran (Table 2).

The activity of BuChE was reduced to 2.50 µmoL/min/g protein as compared to control group (19.80 µmoL/min/g protein) in the flesh when exposed to the high exposure concentration of profenofos. Carbofuran inhibitory effect on flesh AChE ranged from 32 to 70% under various exposure concentrations with highly significant difference among exposure concentrations and from control group.

Table 1: Comparison of means (±S.E) for acetylcholinesterase and butyrylcholinesterase activity (µmol/min/g protein), of control group and three exposure concentrations (mg/L) of profenofos in different organs of *Catla catla*

Enzymes	Treatments (mg/L)	Brain	Gills	Flesh	Kidney	Liver	Blood
Acetylcholinesterase	Control	34.55±0.029a	14.24±0.139a	13.80±0.173a	36.39±0.052a	121.99±0.006a	22.40±0.058a
	0.038	4.87±0.041d	2.90±0.520d	1.07±0.040d	4.19±0.110d	31.98±0.058d	2.98±0.012d
	0.019	6.88±0.049c	5.56±0.323c	2.09±0.052c	11.70±0.404c	43.87±0.040c	9.34±0.023c
	0.012	14.09±0.055b	9.80±0.462b	5.13±0.075b	19.74±0.023b	85.65±0.029b	16.71±0.006b
Butyrylcholinesterase	Control	25.67±0.040a	7.71±0.077a	19.03±0.017a	29.05±0.029a	158.55±0.029a	52.00±0.577a
	0.038	1.77±0.043d	1.00±0.058d	2.50±0.289d	5.79±0.058d	15.79±0.055d	5.89±0.052d
	0.019	1.77±0.043d	2.56±0.035c	8.71±0.058c	8.52±0.012c	39.87±0.040c	13.00±0.289c
	0.012	12.78±0.043b	5.89±0.052b	12.90±0.520b	19.34±0.196b	98.00±0.289b	29.80±0.115b

Table 2: Comparison of means (±S.E) for acetylcholinesterase and butyrylcholinesterase activity (µmol/min/g protein), of control group and three exposure concentrations (mg/L=ppm) of carbofuran in different organs of *Catla catla*

Enzymes	Treatments (mg/L)	Brain	Gills	Flesh	Kidney	Liver	Blood
Acetylcholinesterase	Control	34.55 ±0.029a	14.25±0.144a	13.80±0.462a	36.39±0.052a	121.99±0.006a	22.40±0.058a
	0.198	8.60±0.346c	4.78±0.046d	4.19±0.110d	7.98±0.046d	49.99±0.046d	6.78±0.046d
	0.099	8.77±0.463c	8.67±0.040c	6.78±0.046c	14.70±0.115c	64.78±0.012c	13.24±0.139c
	0.066	24.05±0.029b	11.45±0.260b	9.45±0.260b	23.47±0.040b	98.59±0.088b	17.15±0.087b
Butyrylcholinesterase	Control	25.69±0.059a	7.68±0.046a	19.03±0.017a	29.05±0.577a	158.55±0.029a	52.00±0.577a
	0.198	7.13±0.088c	2.00±0.289d	5.50±0.115d	7.90±0.520d	17.19±0.052d	18.89±0.029d
	0.099	7.59±0.335c	3.60±0.346c	9.22±0.127c	15.42±0.058c	68.70±0.115c	23.65±0.029c
	0.066	17.80±0.462b	5.90±0.520b	15.90±0.231b	23.40±0.231b	107.00±0.577b	37.64±0.003b

Values sharing same letters are non-significantly different (P>0.05) within a column

The maximum flesh BuChE activity was observed at 15.90 µmoL/min/g protein when fish were exposed to 0.066 mg/L carbofuran but it was highly significantly less than control group (Tables 1, 2).

In addition to the brain, gills, flesh and blood, AChE activity was also observed to be reduced in other metabolizing organs i.e., liver and kidney. Maximum inhibition was observed in kidneys (88%) and liver (74%) at the highest concentration of profenofos. Less inhibition in both the organs was observed, when fish were exposed to various concentrations of carbofuran (Tables 2, 3). BuChE activity also followed the same trend with maximum inhibition upto 90% in liver caused by exposure of profenofos followed by carbofuran exposure (0.198 mg/L) as 89.15%. A significant difference between maximum inhibition caused by profenofos and carbofuran, was observed in the kidney (Tables 1, 2). As far as the effects of both of the pesticide are concerned, we found that the highest concentrations of profenofos and carbofuran had affected the cholinesterase level maximum in all of the organs.

Discussion

Cholenergic biomarkers are generally divided into two main classes: A and BuChE in most of the organisms and are extensively used as a diagnostic tool for ecotoxicological assessment of pesticides as well as xenobiotics in aquatic environment (Torre *et al.*, 2002). These enzymes (cholinestserases) are induced on exposure to toxic substances e.g., organophosphates, carbamates, PAHs, halogenated aromatics and certain types of dioxins (Stegeman and Lech, 1991; Boer *et al.,* 1993). Inhibition of cholinesterase has been studied in several systems and organs with a focus on brain tissues (Fernandez-Vega *et al.,* 2002; Pena-Llopis *et al.,* 2003). Sensitivity of fish to pesticide exposure, especially organophosphate is dependent on the level of brain AChE activity (Murphy *et al.,* 1968).

Cholinesterases are useful indicators of pollution because of their high sensitivity and presenting the first detectable signs of sublethal stress response in organisms (Stegeman *et al.,* 1992; Chambers and Boone, 2002). In current investigation, there was inhibition of cholinesterases in all organs including brain under exposure of both the pesticides. Inhibition of cholinesterase in either nervous system or flesh has been acknowledged as the adverse effect on the organisms because enzyme activity of the target tissues is known to contribute in the neurotransmission (Padilla, 1995). Most of the observations of AChE inhibition in fish have been emphasized in the brain only as the intoxication of the brain contributes a lot towards behavioral changes (Jaffery and Keizer, 1995). Although catfish brain seemed to be the most sensitive to the exposure of aldicarb, the fish with 90% inhibition of AChE was alive with moderate symptoms of intoxication (Everett *et al.,* 2000), our findings have shown less than 90% inhibition in brain under any of the exposed concentrations of both pesticides, whereas more than 90% inhibition was observed as 92% in flesh when fish exposed to the maximum concentration (0.038 mg/L) of profenofos, but fish were still alive during the whole period of exposure. Toni *et al.* (2010) also reported decrease in flesh ChE activities in fish. The flesh cholinesterases represent the largest pool of cholinesterase in the body, it is also important to control the

muscular function; the loss of muscular control can have many problems for fish including loss of swimming control and blockage of the opercular movement. This may result in reduced oxygenation of the blood and consequently lead to hypoxia induced death (Zinkl *et al.*, 1987). This may also attribute towards the changes in behavior of fish when exposed to pesticides. Significant inhibition of cholinesterases was also observed in gills, liver and kidneys in the current investigation. Hence, activity reduction in gills can be attributed to suffocation and reduced respiratory activity which may be a problem in fish as well (Chambers and Carr, 1995).

Heath *et al*. (1993) studied the effect of carbofuran on newly hatched striped bass larvae and decrease in ChE activity was observed at high concentration of carbofuran. In this study, a decrease in ChEs was observed in all organs of fish. More than 50% inhibition of cholinesterase (AChE and BuChE) activities was observed in all the organs of *Catla catla* under the highest exposure concentration (0.038 mg/L), medium exposure concentration (0.019 mg/L) of profenofos and the highest exposure concentration of carbofuran (0.198 mg/L), whereas variation of inhibition was observed under other exposure concentrations of both the pesticides. Poisoning of cholinesterases by pesticides in fish to this extent has been considered as a good indicator of intoxication (Coppage and Matthews, 1974; Westlake *et al.*, 1981).

Overall comparison of cholinesterase inhibitory action of both the pesticides (different in chemical nature) in various organs presented that profenofos (organophophate) has caused more inhibition of cholinesterases as compared to carbofuran (carbamte). Once organophosphorous pesticides enter the body of an organism, most of them are transformed into metabolites, which in many cases are more toxic compounds than the parent compounds or induced directly the target enzymes or organs (Belden and Lydy, 2000). Consequently, the effects of most of the pesticides on AChE activity are considered as an irreversible action since the time of re-synthesis of the enzyme is naturally longer than the duration of dissociation of the organophosphorous-complex, whereas BuChE are inhibited to protect AChE from toxic actions of pesticides (Whitaker, 1986; Gaglani and Bocquene, 2000). Inhibition in the activity of both AChE and BuChE may contribute towards same transformation.

In conclusion, both the pesticides had deleterious effects but profenofos was more toxic as compared to carbofuran in the context of inhibition of AChE and BuChE activities in all of the tested organs and tissues. Hence, these pesticides should be properly monitored in the environment, in order to curtail their venomous effects on living organisms.

Acknowledgements

This study was funded by Higher Education Commission (HEC), Pakistan. The authors are thankful to Dr. Tayyaba Shaheen, Assistant Deputy Director, Fish seed hatchery, Faisalabad for providing fish seeds and Mr. Akram-UL-Haq, Regional Director, the Ali Akbar group of Industries, Pakistan for providing technical grades of pesticides.

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(Received 20 March 2013; Accepted 23 September 2013)