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Inhibition of Plasma Cholinesterase Activity in Alligator Gar (*Atractosteus spatula*) Following Chronic Exposure to Diazinon

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Abstract

Diazinon is an organophosphate pesticide that has been used in agriculture and veterinary medicine for many years. The extensive use of this chemical has led to its accumulation in aquatic environments and this can result in negative effects on fish health. The most frequent biomarker for exposure to OP pesticides is the inhibition of cholinesterase (ChE) but most studies have focused on the effects of short term exposure to high levels of OP pesticides and on the effects on various organs that require sacrifice of the fish. The objective of this study was to determine the effects of chronic low levels of diazinon on the biomarker plasma ChE in alligator gar (*Atractosteus spatula*) which are large enough to capture, draw blood, and release. Alligator gar were exposed daily either to the ethanol vehicle or to a sub-lethal concentration of diazinon (0.01 mg/L or 0.1 mg/L) for 30 days. Plasma was collected and the diazinon significantly reduced plasma ChE activity in a dose-dependent manner with 62% and 72% in the low and high concentrations, respectively. The results suggest that ChE activity in gar plasma can be used as a reliable non-terminal biomarker for monitoring diazinon contamination in aquatic environments.

Keywords: Alligator gar; Diazinon; Cholinesterase

Introduction

Diazinon (*O*,*O*-Diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl) is a neurotoxic agent from the organophosphate family which has been commonly used as a pesticide in agriculture and veterinary medicine [1]. The extensive use and potential misuse of organophosphates such as diazinon creates the potential for them to reach aquatic habitats where they can have long lasting effects on non-target organisms including crabs, fish, and other inhabitants [2,3]. In fact, diazinon can be detected in the environment for up to 4 months following application, and because of the extensive use, it can keep building up in the aquatic environment [4].

All organophosphate pesticides are known to inhibit the action of acetylcholinesterase which hydrolyzes the neurotransmitter acetylcholine (ACh). However, two types of cholinesterases (ChE), acetylcholinesterase and butyrylcholinesterase, are found in brain, muscles, liver, and blood and both are frequently used as indicators for exposure to organophosphate pesticides in both fish and other animals [5-8].

To inhibit either ChE in fish, as well as other species, diazinon must be metabolized by cytochrome P-450 to diazoxon in the liver [9]. Following activation, diazoxon binds to AChE which blocks its function [10]. As a result, ACh accumulates in cholinergic synapses leading to hypercholinergic activity of the effected nerve [11]. The clinical observations resulting from this hyperactivity in exposed fish are well documented, especially in chronic exposure, and include erratic swimming, decreased feeding, convulsions, muscle weakness, and increased respiratory rate [12,13].

Organophosphate exposure can have adverse effects on many body organs in fish [14-17] . Some of this damage has been suggested to be caused by oxidative stress that is induced during hepatic metabolism which generates reactive oxygen species (ROS) in aquatic organisms [18]. Following production, these ROS can react with vital molecules such as proteins, lipids, nucleic acid, and DNA and damage them [19]. However, many studies reporting the induction of ROS following diazinon exposure were observed following exposure to commercial preparations, which contain other chemical components, rather than technical grade diazinon [20-22].

Alligator gar (*Atractosteus spatula*) are found in large rivers, backwaters, lakes, bottomland swamps, and even brackish water estuaries in North and Central America and Cuba. Gar populations have declined over the past fifty years as a result of habitat loss, overfishing, and possibly exposure to pollutants [23]. Water run-off from crops and pastures carry pesticide residues into alligator gar habitat, exposing these fish to agricultural pollutants. However, little is known about the effects of agricultural pollutants on alligator gar and very few studies have investigated the short-term effects of exposure to pollutants in gar [24-26]. It is possible that exposure to pesticides, such as diazinon, could be a contributing factor in the decline in gar populations.

A recent biomarker study reported that a 96 h exposure of 0.304 mg/L diazinon resulted in decreased activities of acetylcholinesterase, butyrylcholinesterase, carboxylesterase, and glutathione s-transferase, but increased activities of superoxide dismutase and alkaline and acid phosphatases in the liver of alligator gar [26]. However, in natural freshwater, diazinon is only moderately adsorbed by sediment and is soluble in the water column [27]. It has a half-life of up to 14 days and even greater persistence in brackish water suggesting that exposure of gar to diazinon in natural environments would continue beyond the 96 h commonly used in laboratory tests [28].

While Gonzalez et al. [26] demonstrated that hepatic enzyme activities in gar can be used as biomarkers, the hepatic sampling requires sacrifice of the animal. The purpose of this study was to establish a non-terminal method to determine if alligator gar have been exposed to environmental pollutants. Alligator gar are large enough to capture, draw blood, and release. Blood enzymes can be analyzed in the laboratory. Therefore, this study utilized gar blood cholinesterase (ChE) as a biomarker and measured changes in ChE activity following chronic exposure to two different concentrations of diazinon.

Materials and Methods

Chemicals and experimental animals

Diazinon (99% purity) was purchased through Chem Service Inc (West Chester, PA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Diazinon was dissolved in 3 ml ethanol to concentrations of 1.167 mg/ml and 11.667 mg/ml prior to use.

Thirty-two alligator gar (average weight of 773.9 gm and average length of 53.85 cm) were obtained from the Private John Allen National Fish Hatchery in Tupelo, MS. Fish were acclimated for 10 days prior to exposure. Fish were held under natural light cycle and fed daily with pelleted feed (Rangen, Inc.) throughout the experiment.

Stainless steel tanks were used to avoid any reaction between the chemical and tank material. Tanks held 350 liters of well water, and an air stone was in each tank at all times during each experiment. Water in each tank was exchanged every day to ensure optimum water quality and maintain diazinon concentrations. Water temperature in each tank was maintained at 21 ± 2°C using heaters, dissolved oxygen was maintained at 7.3 ± 0.2 mg/L, and the pH was 7.5 ± 0.2. The MSU Institutional Animal Care and Use Committee (IACUC) approved fish holding and experimental protocols.

Exposure

Prior to initiation of the chronic exposure study, a pilot study was conducted first to determine the short term effect of the sub-lethal dosages by measuring ChE inhibition in the plasma. The target dosage was selected as the dosage required to maintain the level of ChE inhibition below 25% to ensure that the fish would survive the duration of the chronic experiment. Based on the results from the pilot study, 0.01, and 0.1 mg/L diazinon were selected for use in the chronic study.

After 10 days of acclimation period, four fish were placed into each tank filled with well water. Fish were either exposed to the ethanol vehicle, 0.01 mg/L diazinon, or 0.1 mg/L diazinon. Final concentration of ethanol was 0.001%. To prevent stress from daily handling, fish remained in the same tank for the duration of the experiment. Each day approximately 80% of the water in each tank was replaced with clean water followed by 10 min of continuous flow of clean water. Following cessation of flow, fresh diazinon was added daily. Fish status was monitored, and any change or abnormal behavior was recorded during the duration of the treatment. In addition, water quality was monitored though out the experiment.

Blood collection

On day 30, fish were placed individually in a mixed solution of anesthetic (500 mg/L tricaine methanesulfonate MS-222). Fish were then wrapped and cleaned with paper towel to remove mucus before collecting blood. Blood (3 ml) was collected through caudal vein puncture into a Vacutainer heparin glass tube and placed on ice. Blood was then centrifuged for 10 minutes at 16.1g to obtain the plasma which was collected and stored at -80°C until assay.

ChE Assay

For determination of ChE activity, plasma was diluted (12.5 µl serum/ml final concentration) in cold 0.05 M Tris-HCl buffer (pH 7.4). The activity was measured spectrophotometrically using a modification [29] of Ellman et al [30] with acetylthiocholine as the substrate (1mM final concentration) and 5,5'-dithiobis (nitrobenzoic acid) as the chromogen. Both acetylcholinesterase and butyrylcholinesterase will hydrolyze acetylthiocholine. Plasma protein concentration was quantified with the Folin phenol reagent using bovine serum albumin as a standard [31]. Specific activity was calculated as nmoles product produced min^{-1} mg $protein^{-1}$.

Statistical methods

Data were analyzed by analysis of variance (ANOVA) using SAS statistical package (SAS Institute Inc., Cary, NC) followed by mean separation by least squared means. Data from four fish were pooled into each replication for statistical analysis. Data are presented as mean ± standard error and the level of significance was $p \leq 0.05$.

Results

Fish swam normally during acclimation. After one week of exposure, fish produced excess mucous. By the beginning of the second of week exposure, fish became motionless and developed lighter skin color (Table 1). The fish did not demonstrate a change in appetite, and no deaths occurred during the study.

Table 1: Clinical signs of alligator gar chronically exposed to diazinon for 30 days.

Indices	Control	0.01 mg/L	0.1 mg/L
Death	None	None	None
Swimming	Active	Motionless	Motionless
Skin Color	Normal	Lighter	Lighter
Mucous	Normal	Increased	Increased

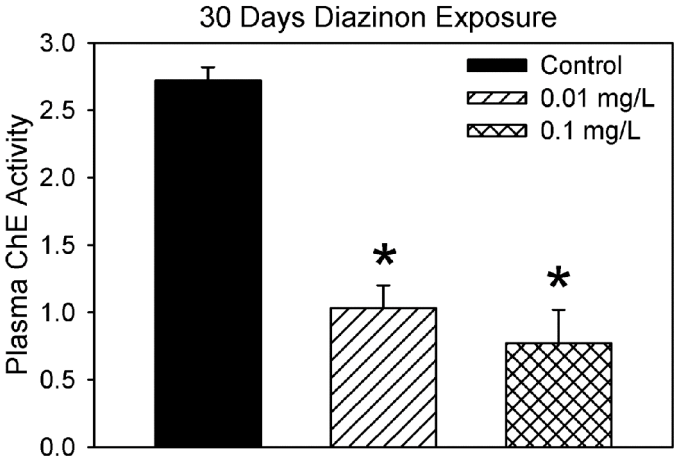

Figure 1: Effect of diazinon on ChE activity (nmoles min^{-1} mg $protein^{-1}$) in plasma of alligator gar exposed daily to either 0.01 or 0.1 mg/L diazinon for 30 days (n=2-3). Asterisks (*) indicate statistically different from control ($p \leq 0.05$).

The effect of chronic diazinon exposure on plasma ChE activity is presented in Figure 1. Plasma ChE activity was significantly reduced in both treatment groups as compared to controls. Exposure induced a dose-response type pattern of ChE inhibition with 62% and 72% inhibition with 0.01 and 0.1 mg/L diazinon, respectively. There were not any significant differences in ChE activity between the two diazinon treatments.

Discussion

Long-term exposure to sub-lethal concentrations of diazinon significantly inhibit ChE activity in alligator gar. Several studies have reported similar effects on ChE activity in different fish species following exposure to diazinon and to other pesticides [6-8,26,32-36]. However, the majority of these studies reported the effects on either brain, muscle, or liver ChE following short term acute exposure to high levels of chemical. In contrast, our study reports that persistent exposure to low and high diazinon levels for 30 days significantly inhibits plasma ChE by 62% and 72%, respectively. Our data are similar to previous work [13] which reported that exposure of rainbow trout to 0.1 mg/L diazinon for 28 days resulted in a ~70% inhibition in plasma ChE activity.

The presence of chemicals in the aquatic environment have had a significant impact on fish health [24,25,37,38]. Exposure to organophosphate pesticides, such as diazinon, can inhibit ChE activity and it is frequently assumed that decreased activity of ChE is a biomarker of exposure to these organophosphates. A study conducted to assess the effects of various pollutants on spotted gar (*Lepisosteus oculatus*) in the lower Mississippi River Basin showed a significant decrease in ChE activity in brain and liver samples [7]. However, other environmental contaminants such as cadmium and other heavy metals can also effect ChE activity [7,10,15,39-44]. Therefore, inhibition of plasma ChE activity could serve as a biomarker for multiple pollutants in the aquatic environment.

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