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Highlighted article

Brain cholinesterase response in the snakehead fish (*Channa striata*) after field exposure to diazinon ☆

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ABSTRACT

The snakehead *Channa striata* is an economically important air-breathing fish species in the Mekong delta of Vietnam. Rice paddies, which are disturbed by the frequent application of agro-chemicals, are among the preferred habitats for this species during the rainy season. Diazinon is one of most commonly used chemicals in rice paddies. In the present study, exposure of adult snakehead fish to a single diazinon application in cages within a rice field resulted in long-term brain cholinesterase inhibition, while the water concentration of this insecticide fell below the detection limit within 3 days. In addition, incubation of brain homogenates with 2-PAM caused reactivation of the cholinesterase diazinon complex to within 80% of the control level. These experiments also showed that chemical ageing of the diazinon cholinesterase binding occurred, which may explain the long-term effects of this pesticide.

1. Introduction

The snakehead fish, *Channa striata* (Bloch, 1793), is indigenous to the Mekong River Delta (MRD) (Khoa and Huong, 1993), where unfarmed populations have traditionally provided an important and preferred source of protein for the local human population. This species is highly adaptable and is found in a variety of water bodies in the delta region (Khoa and Huong, 1993; Lee and Ng, 1994). It reproduces in shallow water and for this reason rice paddies provide an ideal habitat for reproduction (Amilhat and Lorenzen, 2005), where it also encounters agro-chemicals. Although actual snakehead population statistics do not exist, harvest figures are in decline (Edwards et al., 1997) and unpublished figures (Dr. Vo Tong Anh, Angiang University, Vietnam, personal communication) based on extensive farmer interviews suggest that populations of wild fish in the region have been reduced to as little as 20% of the 1980 level. One of the cited causal factors for this decline is the rapid increase in pesticide usage that has occurred over the same period in the region (Edwards et al., 1997).

One of the most frequently used pesticides is diazinon, which is sold in the delta under 14 different trade names and is used for insect control in a variety of crops including rice. For rice, the recommended application rate is 300–500 g/ha in up to two applications per crop at day 25 and again on days 45 or day 60 after sowing. The snakehead has been shown to be very sensitive to diazinon. With a 96 h LC50 of less than 1 mg/L (Cong et al., 2006), it is considerably more sensitive than other common paddy fish such as *Anabas testudineus*, *Channa punctatus*, and *Barbodes gonionotus* (Rahman et al., 2002). Brain cholinesterase (ChE) activity in this species is also sensitive to diazinon and in laboratory studies prolonged inhibition for more than 30 days after exposure has been shown after exposure to low environmentally realistic concentrations (Cong et al., 2006). However, extrapolation of this kind of laboratory data to field situations is notoriously difficult, and it is clear that field experiments are necessary to determine whether this kind of long-term inhibition occurs after spraying rice fields and before ChE activity levels can be used as a biomarker in field monitoring (Mayer et al., 1992).

The sensitivity of brain ChE to inhibitors has been widely used as a biomarker for diagnosing exposure to organophosphate and carbamate pesticides and also to indicate effects of these insecticides (Coppage et al., 1975; Peakall, 1992). However, ChE activity varies among fish species (Chuiko et al., 2003) and also between fish populations (Gibson et al., 1969). In birds it has been found to be sex dependent, which has not been shown to be the case in fish (Chuiko et al., 1997; Beauvais et al., 2002), including

☆ All experiments were carried out according to the national and institutional guidelines for the protection of animal welfare.

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the snakehead (Cong et al., 2006). Other biological variables shown to affect ChE activity include age and size (Beauvais et al., 2002; Phillips et al., 2002; Flammarion et al., 2002). In addition, environmental parameters such as temperature have been shown to influence ChE activity in some species (Chuiko et al., 1997; Hogan, 1970), although this is not the case in the snakehead (Cong et al., 2006). Since this kind of baseline information of ChE activity is still incomplete in the snakehead and since the availability of suitable unexposed populations for use as controls are difficult to find, it is desirable to develop procedures for using the same individual fish as its own control. This is possible using enzyme reactivation techniques. Here, enzyme activity is measured after exposure to the insecticide, after which it is reactivated to a level close to its pre-exposure level and measured again. With organophosphate pesticides such as diazinon, this can be achieved with pyridine-2-aldoxime methiodide (2-PAM) (Wilson and Ginsburg, 1958). Since a variety of reactivation incubation times and concentrations exist in the literature and since high concentrations of 2-PAM can in fact cause ChE inhibition instead of reactivation (Escartin and Porte, 1997; Monserrat and Bianchini, 2000), methods must be developed for individual fish species.

In the present study, the time course of effects of diazinon on brain ChE activity of snakehead in a typical MRD rice field was investigated. In addition, the experiment was also designed to reveal the usefulness of 2-PAM in monitoring the exposure of snakehead fish to this pesticide.

2. Material and methods

2.1. Test animals

The adult snakehead (*C. striata*) used in this study originated from a single parent pair caught in a pesticide-free pond in the MRD and were stocked in 600 L fiberglass tanks as mentioned previously (Cong et al., 2006). The fish larvae were reared at a density of 200 individuals per tank and fed *Tubifex sp* daily at about 5% of body weight up to an age of about 75 days, after which they were fed marine fish meat until experimentation. The tanks were continuously aerated and cleaned daily before feeding and 4 h after feeding, to remove uneaten food and feces by changing about 50% of the water.

2.2. Insecticide

The insecticide used was a commercial product, trade name BASUDIN 50EC containing 50% [6-methyl-2-(1-methylethyl)-4-pyrimidinyl] ester by weight, common name diazinon, purchased from An Giang Plant Protection and Services Company (Long Xuyen City, An Giang Province, Vietnam).

2.3. Experimental designs

Before the field experiment, concentrations of 2-PAM (Eastgate, White Lund, Morecamble, England) from 0.1 to 0.3 mM were pre-tested in the laboratory for its capacity to reactivate ChE activity after diazinon exposure. 2-PAM incubation times were also tested from 10 to 150 min at 37 °C. This preliminary study revealed that reactivation was not affected by incubation time and 0.3 mM of 2-PAM gave highly significant reactivation of the ChE activity to approximately 83% of the control activity. This concentration of 2-PAM was used to treat sample in field study with an incubation time of 20 min.

For the field study, a rice field was chosen whose water supply could be isolated from surrounding fields. The chosen field was bordered by a water supply canal and a 0.5 m high dyke planted with unsprayed fruit trees. The remaining two sides were bordered by rice fields with a lower water level as they did not contain fish. These sides were enclosed by conventional 20 cm high by 30 cm wide mud dykes, which almost eliminate water exchange between fields. The chosen rice field, which is conventionally farmed and of a typical size for the area, is $45 \times 66\,m^2$ and is located at An Binh village, Ninh Kieu district, Can Tho City, Vietnam. A local rice variety was sown at a density of 15 kg seed per $1000\,m^2$. This variety is typically harvested 100 days after sowing. Over the experimental period, the rice field water level was measured every second day in the morning (7:00–8:00) and in the afternoon (14:00–15:00). It varied from 4.5 to 17 cm at the morning measurement and 4.5 to 9.5 cm in the afternoon. There was no rainfall during the experimental period.

Thirty-nine days after sowing, three areas $1.2 \times 1.2\,m^2$ along the central axis of the rice field separated by approximately 15 m were cleared of rice plants and a cage $1 \times 1 \times 0.6\,m^3$ made of nylon mesh placed in each of these areas. At the same time, three cages with the same separation were placed in the adjacent canal. Water depth at this time was approximately 10 and 25 cm for the rice field and canal, respectively. Cages were left for 1 day to allow suspended solid to settle from the water and 20 snakehead fish (live weight 17.5 ± 1.4 g, total length 43.9 ± 10.1 cm) from the laboratory stock were placed into each cage. At the same time, three 600 L fiberglass tanks were set up in the laboratory with clean water to run with as identical conditions as possible over the same period. Twenty snakehead fish were placed into each tank. These tanks were cleaned daily by changing 50% of their water and continuously aerated throughout. Fish in both the laboratory and in the field cages were fed daily at approximately 5% of live weight with fresh marine fish meat.

Water temperature and dissolved oxygen (DO) were measured in the morning (7:00–8:00 AM) and afternoon (14:00–15:00 PM) every second day during experimentation, using an Orion 830A DO meter (Thermo, Beverly, MA, USA), whereas pH was measured only in the morning using a Portamess 911 pH meter (Knick, Berlin, Germany).

The rice field was sprayed at day 7 after placing the fish into the cages, corresponding to day 46 after sowing. The field was sprayed with 500 g/ha of diazinon following the farmer's normal practice with a carried spraying system. A water sample was collected from each of the cages and from the laboratory tanks at several intervals during experimentation for diazinon concentration measurements. These were collected immediately after introduction of the fish, at day 7 (1 h after spraying) and at each of the sampling times at days 10, 14, 21 and 28. In all, 1 L of water was collected for each sample in brown glass bottles. These samples were kept on ice from sampling and until return to the laboratory, where they were frozen at −20 °C until analysis. The water concentration of diazinon was determined following the method described Parfitt (2000) using gas chromatography.

Fish were sampled from all sites at day 7 immediately prior to spraying and thereafter at the same time as the water concentration samples described above. At each sampling time, four fishes were removed randomly from each cage or fiberglass tank. These fish were kept on ice for transportation to the laboratory, where they were immediately processed for ChE activity measurements.

2.4. Sample preparation

All steps up to and including homogenization were performed on ice. The whole brain was dissected out and placed in a pre-weighed Eppendorf before measuring its weight. The brain tissue was homogenized and diluted in 0.1 M phosphate buffer (pH 7.4) at a concentration of approximately 60 mg fresh tissue per milliliter using a glass homogenizer (Uniform, Jencons PLC, Leighton Buzzard, UK). The homogenizer was rinsed with acetone (Merck, Darmstadt, Germany) and distilled water between samples.

Each fish brain homogenate was divided in two Eppendorfs, 0.5 mL for each. To one of these aliquots, 0.5 mL of 0.1 M phosphate buffer pH 7.4 was added, while 0.5 mL of 0.6 mM 2-PAM in the same buffer was added to the other aliquot. The homogenates were then mixed at 2500 rpm per min for 2 min using a minishaker (MS2, IKA, Wilmington, USA). The final concentration was approximately 30 mg/L fresh brain tissue and 0.3 mM of 2-PAM for those aliquots diluted with 0.6 mM of 2-PAM. Aliquots with 2-PAM were incubated at 37 °C for 20 min in a temperature-controlled water bath (Memmert WB 14, Schutzart DIN 40050-IP 20, Germany).

2.5. Cholinesterase assay

Brain homogenates were centrifuged at 2000 rpm and 4 °C for 20 min (Centrifuge 4k15, Sigma Osterode am Harz, Germany). The pellet was discharged and the supernatant kept on ice for ChE analysis within 6 h. ChE activity was measured according to the method described by Ellman et al. (1961) with minor modifications described in Cong et al. (2006). Enzyme ChE activity was detected using an U2800 Spectrophotometer (HITACHI, Japan) for 200 s at 412 nm. The r^2 value of the absorbance curves used to calculate the rate coefficients were in all cases higher than 0.9. The results of these measurements were expressed as a rate (Delta absorbance per minute) and the ChE activity was calculated.

2.6. Data analysis

Data were analyzed with one-way ANOVA and Dunnett's post-hoc test for multiple comparisons. Student *t*-test was applied for comparison of ChE activity between samples after 2-PAM reactivation and their initial activity. Data were checked for normality and variance homogeneity prior to statistical analysis. A chi-squared test was applied for data that did not meet the normality and variance homogeneity requirement. The analyses were performed using SPSS for windows (Ver 10.0; SPSS, Chicago, IL, USA).