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Effects of nonylphenol on cholinesterase and carboxylesterase activities in male guppies (*Poecilia reticulata*)

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ABSTRACT

Compared to the estrogenic effects of 4-nonylphenol (NP), there is little data available on other potential toxic effects of NP in aquatic animals. The effects of NP on cholinesterase (ChE) and carboxylesterase (CbE) activities of male guppies exposed to 10, 60, 150, or 300 $\mu g\,L^{-1}$ NP were examined after 1, 2, 4, and 7 days of treatment. A significant muscle ChE inhibition, that used acetylthiocholine iodide as a substrate, was noted in male guppies in all NP treatment groups after a 4-day exposure, and 60 and 150 $\mu g\,L^{-1}$ of NP treatment groups after a 7-day exposure. All guppies exposed to 300 $\mu g\,L^{-1}$ NP died during the 7-day treatment. However, there was no significant inhibition of muscle ChE that used butyrylthiocholine iodide as a substrate in male guppies for any NP treatments in different exposure times. There were no CbE activity differences in livers of male guppies among NP treatment groups after different exposure times. This is the first report showing the ChE activity inhibition by NP in fish. Further mechanistic studies are needed to define how NP directly or indirectly alters ChE activities at molecular level. The implication of ChE inhibition of NP on potential impacts of aquatic animals also warrants further research.

1. Introduction

4-Nonylphenol (NP) has been widely used in many industrial applications as well as household cleaning products. It has been found worldwide in wastewater discharges, sewage-treatment-plant effluents, natural water, and sediments (Bennie, 1999; Ying et al., 2002). NP is a ubiquitous, persistent, and easily bioaccumulated pollutant (Ying et al., 2002), and its potential effects are found in many aquatic animals both acutely and chronically (Nimrod and Benson, 1996; Lussier et al., 2000; Staples et al., 2004). In the past, most published studies have mainly focused on the estrogenic effects of NP as an endocrine disruptor both *in vivo* and *in vitro* (Nimrod and Benson, 1996; Bandiera, 2006). Compared to the estrogenic effects of NP, there is little data available on other potential toxic effects of NP in aquatic animals.

In fact, NP may have damaging effects on important physiological processes without involvement of estrogen receptors (Khan et al., 2003). For example, acetylcholinesterase (AChE) activity of rat pheochromocytoma PC12 cells incubated with NP for 24 h was found to be significantly inhibited (Talorete et al., 2001). In general, the immediate result of AChE inhibition will cause an increase in acetylcholine that may result in a decrease in cholinergic receptor number, which is a compensatory response to an acetylcholine buildup. Interestingly, Jones et al. (1998) did report that NP exposure could cause a decrease in brain muscarinic cholinergic receptors in three species of trout *in vitro*. However, there is little information on effects of NP on ChE activities of aquatic animals *in vivo*. Lee and Choi (2006) reported that there was no significant difference in the AChE activities of *Chironomus riparius* larva after 24 h of NP exposure between 10 and 100 $\mu g\,L^{-1}$. On the other hand, Li (2008) found a significant decrease in the ChE activities of planarian exposed to NP at 500 $\mu g\,L^{-1}$ after 48 h treatment. Considering the important role of ChE in animal's behavior and physiology (Soreq and Seidman, 2001), the effects of ChE inhibition by NP in aquatic vertebrates are still scarce and warrant further research.

The guppy has long been used as a popular animal model in ecological and evolutionary research as well as in behavioral studies. Recently, it is also widely applied as a test organism both *in situ* and laboratory bioassays, because it is a readily available and easily handled small body-sized species with a short life cycle (Castro et al., 2004; Araújo et al., 2006). In addition, several studies combining various biochemical measurements in guppies were very useful for studying environmental pollution problems (Pierson, 1981; Frasco and Guilhermino, 2002; Larrson et al., 2002; Castro et al., 2004).

ChEs are a class of serine hydrolases and generally classified into two isoenzymes: AChE and butyrylcholinesterase (BChE) also

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called pseudocholinesterase or non-specific ChE. Augustinsson (1949) first reported a high level of AChE activity in guppy muscle. Van der Wel and Welling (1989) demonstrated the contribution of BChE to total ChE activity was only about 1% in guppy. Garcia et al. (2000) also characterized ChE activities of guppy muscle and confirmed that AChE was the main ChE form in guppy muscle tissue. Interestingly, Castro et al. (2004) found that the guppy AChE activities were inhibited after 96 h *in situ* exposure in an acid mine drainage effluent, whereas mosquitofish AChE activities were not affected. This result suggests that guppy AChE is a sensitive biomarker to environmental pollutants.

The objective of this study was to examine the effects of short-term exposure to NP on the ChE and carboxylesterase (CbE) activities of male guppies. Some environmental pollutants found to inhibit ChE activity may also inhibit CbE activity (Chanda et al., 2002). CbEs include several related isozymes widely distributed in living organisms. The physiological roles of these CbEs are still unclear but might be related to lipid metabolism and steroidogenesis (Jewell and Miller, 1998). Therefore, measurements of muscle ChE, head ChE, and liver CbE activities were simultaneously performed on male guppies exposed to 10, 60, 150, or 300 $\mu g L^{-1}$ NP after 1, 2, 4, or 7 days in the present study.

2. Materials and methods

2.1. Chemicals and reagents

NP (94%) was obtained from Riedel-de Haën (Sigma-Aldrich, Germany). 3-Aminobenzoic acid ethyl ester (MS222) and all other chemicals used in enzyme assay were obtained from Sigma Chemical Company (USA). Acetone (HPLC grade) was purchased from Mallinckrodt (Phillipsburg, USA). NP was dissolved in acetone for preparing the test stock solution.

2.2. Animals and study design

Adult male guppies were obtained from a local supplier and acclimated in the laboratory to a temperature of 28 ± 2 °C and a 12-h photoperiod for at least 7 days before the experiments. The guppies were fed daily with commercial dry flake food (TetraGuppy, Tetra, Germany). To eliminate the risk of leached potential endocrine disruptors, plastic materials were avoided in all aquaria and plumbing. Dechlorinated tap water was stored in a stainless-steel tank before use. The fish were exposed to either NP at nominal concentrations of 10, 60, 150, or 300 $\mu g L^{-1}$, or to acetone alone at a nominal concentration of 30 $\mu g L^{-1}$ as solvent control for different exposure periods (1, 2, 4, or 7 days). Each group of five male guppies was kept in 5 L of water in a glass beaker. Each fish was fed an average of 3–4 mg of commercial dry flake food in each beaker just 30 min before daily test-water replacement. The test water was replaced every 24 h in all groups to maintain treatment concentrations. The fish were inspected daily for mortality and dead fish were immediately removed for the entire experimental period.

Based on the results of different time treatment experiments, 4-day treatments were repeated two more times at nominal concentrations of 150, 300, or 900 NP $\mu g L^{-1}$, or to acetone alone at a nominal concentration of 30 $\mu g L^{-1}$ as solvent control to further examine effects of NP on muscle ChE of guppy and to estimate acute toxicity of NP for male guppies. For these two repeated 4-day treatment experiments, their experimental conditions followed the same experimental protocol as mentioned above. All Guppies exposed to 900 NP $\mu g L^{-1}$ died within 4 days; therefore, head and muscle AChE and liver CbE were only measured in fish exposed to 150 and 300 NP $\mu g L^{-1}$ from these two repeated 4-day treatment experiments.

2.3. Tissue preparation

After 1, 2, 4, or 7 days of treatment exposure, all fish were anesthetized in MS222 (300 $\mu g L^{-1}$) for 15 min. After whole-body weighing, the head was cut off just behind the operculum; sample of liver, testis, and muscle were removed for each guppy. Liver and testis wet weight were measured for each guppy. The gonadosomatic (GSI) and hepatosomatic (HSI) indices were calculated as the testis weight to fish whole-body weight and liver weight to fish whole-body weight, respectively. Muscle, head, and liver samples were stored at −80 °C until biochemical assay within 1 week.

2.4. ChE activity measurement

Head or muscle samples were homogenized 1:10 (w/v) with 1 mM EDTA and 0.5% Triton X-100 in 0.1 M phosphate buffer (pH 7.5). The homogenates were centrifuged at 12,000*g* for 20 min at 4 °C and ChE activity was immediately measured in the supernatant using the colorometric method of Ellman (Ellman et al., 1961). The reaction was triggered by adding 10 μL of 75 mM substrate (acetylthiocholine iodide for head and muscle samples and butyrylthiocholine iodide for muscle samples) to 990 μL reaction mixture containing 50 μL of 0.01 M dithiobisnitrobenzoate (DTNB) and 50 μg of muscle or head protein (for acetylthiocholine iodide) or 200 μg of muscle protein (for butyrylthiocholine iodide) in 0.1 M phosphate buffer (pH 8.0). The rate of increase of optical density of the reaction medium was measured using a Hitachi UV/VIS spectrophotometer at 412 nm for 60 s at room temperature. Because of limited muscle sample volumes, ChE activities were measured in duplicate. Despite selective inhibitors not being used, ChE activities using acetylthiocholine iodide as a substrate were referred as AChE and butyrylthiocholine iodide as a substrate were referred as BChE. The enzyme activity was expressed as nmol min^{-1} mg^{-1} of proteins.

2.5. CbE activity measurement

Livers were homogenized 1:10 with 0.2 mM EDTA in 0.05 M Tris-HCl buffer (pH 8.0) and centrifuged at 12,000*g* for 20 min at 4 °C. The supernatant was immediately used for CbE activity measurement. CbE activity was determined with *p*-nitrophenyl acetate as substrate according to the method of Ljungquist and Augustinsson (1971). The reaction was triggered by adding 10 μL of 0.0 5 M *p*-nitrophenyl acetate (in ethanol) to 1 mL reaction mixture containing 50 μg liver proteins in 0.05 M Tris-HCl buffer (pH 8.0). The rate of increase of optical density of the reaction medium was measured using a Hitachi UV/VIS spectrophotometer at 405 nm for 60 s at room temperature. The CbE activities were measured in duplicate. The enzyme activity was calculated as nmol min^{-1} mg^{-1} of proteins.

2.6. Protein determination

The protein concentrations of the head, muscle or liver samples were measured using Bradford's method (Bradford, 1976), with bovine serum albumin as the standard. All protein measurements were performed in triplicate.

2.7. Statistical analysis

The acute toxicity of NP for male guppy was determined from daily mortality noted from different treatment experiments with concentrations ranging from 10 to 900 $\mu g L^{-1}$. The nominal concentrations lethal to 50% of the organisms (LC_{50}) for NP at 96 h were calculated using trimmed Spearman–Karber analysis with trimmed Spearman–Karber Program (version 1.5) obtained from Environmental Monitoring Systems Laboratory (USEPA, Cincinnati, Ohio).

For comparison reasons, data were expressed as ratio of change from the respective control value (taken as 100%) for ChE or CbE activities from different time treatments. No statistical test was conducted in these data because of limited sample sizes and only graphical expression was presented for these different time treatment experiments.

For the 4-day treatment experiments, three independent experiments were conducted. Differences among treatment groups were analyzed by the nonparametric Kruskal–Wallis test using the Minitab statistical program (Version 13.2). If a significant result was found, Mann–Whitney *U* test was used to determine which treatment groups were significantly different from the controls. Differences were considered significant if $P < 0.05$.

3. Results

3.1. General biological parameters from different time treatments

Two, one, and five fish died in 10, 150, and 300 $\mu g L^{-1}$ of NP during 7 days of exposure, respectively (Table 1). Therefore, there were only three NP treatment groups in the 7-day exposure experiment. There were no general dose-related trends in the body weight, GSI index or HSI index among different NP-treated guppies after 1, 2, 4, or 7 days of exposure.