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Effects of phenanthrene on hepatic enzymatic activities in tilapia (*Oreochromis niloticus* ♀ × *O. aureus* ♂)

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Abstract

The effects of phenanthrene (Phe) on hepatosomatic index (HSI) and hepatic enzymatic activities in hybrid tilapia (*Oreochromis niloticus* ♀ × *O. aureus* ♂) were investigated via the static freshwater exposure at dosage of 50, 100, and 400 μg/L for 4–14 d. Compared with the control group, HSI was significantly decreased ($P < 0.05$) at 400 μg/L at day 14. Increased enzymatic activities ($P < 0.05$) were observed for catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) at either 100 or 400 μg/L at day 8 and 14, as well as for CAT at 50 μg/L at day 14, except for GPx at 400 μg/L at day 8. Ethoxyresorufin *O*-deethylase (EROD) activity was significantly increased ($P < 0.05$) at all dosage at day 4 as well as at 50 μg/L at day 8, but significantly decreased at either 100 or 400 μg/L at day 14 ($P < 0.05$). Glutathione-*S*-transferase (GST) activity was not affected. The results suggest that CAT, GPx, SOD and EROD, as well as HSI in tilapia may be used as the biomarkers or indexes for evaluating or monitoring the pollution of polycyclic aromatic hydrocarbons (PAHs) such as Phe.

Key words: phenanthrene; hepatic enzyme; biomarker; hybrid tilapia (*Oreochromis niloticus* ♀ × *O. aureus* ♂)
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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants coming from the incomplete combustion of fuel, oil spilling and so on. In the aquatic environments, PAHs are widely found in sediment, water and fish body (Moon *et al*., 2001; Wu *et al*., 2003; Chen *et al*., 2004; Zhang *et al*., 2004). Phenanthrene (Phe) is a representative environmental pollutant of PAHs with three aromatic rings. It has a chemical formula of $C_{14}H_{10}$ with a "bay-region" and a "K-region", which is associated with PAHs' carcinogenesis and included in the 16 US EPA priority PAHs (Smith *et al*., 1989). Phe is widely distributed in the aquatic environments and ranged from 14.6 to 1460 μg/L (Vrana *et al*., 2001; Anyakora *et al*., 2005).

Recently, acute or chronic toxicity studies have been carried out to obtain biomarkers at morphology, behavior, histology and molecular levels to assess the ecological risk of environmental contaminants (Van der Oost *et al*., 2003). Biomarkers are meaningful for environmental monitoring because they respond quickly and often highly specific to chemical stressors. Toxicological studies with PAHs show that petroleum hydrocarbons can cause structural damage to the respiratory lamellae of the gills (Correa and Garcia, 1990; Prasad, 1991); PAHs and their metabolites can elicit toxicity through the generation of reactive oxygen species (ROS) or covalently binding to cellular macromolecules such as DNA, RNA and protein (Van der Oost *et al*., 2003; Yin *et al*., 2007). The activities and expression levels of metabolic and anti-oxidative enzymes were often used as molecular biomarkers to evaluate the influence of PAHs on the biochemical pathways and enzymatic function in fish (Whyte *et al*., 2000; Sun *et al*., 2006; Correia *et al*., 2007).

Hybrid tilapia (*Oreochromis niloticus* ♀ × *O. aureus* ♂) is a euryhaline fish, which can survive in relatively badly polluted brackish and freshwater environment. Its omnivorous characteristics means that the fish is exposed to pollutants not only directly from contaminated water, but also from the food chain. Therefore, it is possible to use this species as a potential indicator organism for aquatic environmental contaminants including PHAs. In the present study, the tilapia juveniles were exposed to Phe via a static freshwater system for 4–14 d, and hepatosomatic index (HSI), as well as the activities of hepatic catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), ethoxyresorufin-*O*-deethylase (EROD) and glutathione-*S*-transferase (GST) were examined, to evaluate the toxic effects of Phe and the action mechanism, and then obtain biomarkers for assessing Phe pollution as well. The results can also provide information for using fish as a biomonitor for environmental pollution of PAHs.

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1 Materials and methods

1.1 Experimental animals and chemicals

Tilapia juveniles (*Oreochromis niloticus* ♀ × *O. aureus* ♂) in freshwater were obtained from a local aquaculture company. They were acclimated to the laboratory condition for 2 weeks prior to experiment.

Phenanthrene (> 98% purity, Aldrich) was initially dissolved in little absolute ethanol and then diluted with freshwater to 10 mg/mL stock solution, and finally to the exposure concentrations during experimentation.

1.2 Experimental design and conditions

The experiment was consisted of three Phe exposed groups and one control group, each with triplicate glass aquarium (size 35 cm × 30 cm × 40 cm, 42 L). One day prior to the exposure, fish of approximately the same size were pooled into a plastic bucket, and then randomly distributed in 12 aquariums, each containing 10 fish with an average weight of 24.2 –26.5 g. Their sexes were indistinguishable by eye. The Phe exposure doses were at 50, 100, 400 μg/L. During 14 d experimental period, each aquarium was filled with 30 L freshwater with (treated group) or without (control) Phe, and half of the water was renewed every day to maintain relatively stable exposure concentration. Aquarium water was kept oxygen-saturated by aeration and water temperature was at 25–28°C. Photoperiod was 12 h light/12 h dark. Fish were fed twice a day to satiation with a commercial fish diet. No fish died during the course of experiment.

1.3 Sample collection

At day 4 and 8, three fish were randomly sampled from each tank, respectively. The remaining fish were sampled at day 14. After anaesthesia in 0.01% 2-phenoxyethanol (Sigma, USA), fish were weighted and dissected. Livers were quickly taken out and weighed, and then frozen in liquid nitrogen, and stored at –80°C for enzymatic activity analysis. Hepatosomatic Index (HSI) was calculated by LW/BW × 100%, where LW and BW were liver weight and body weight, respectively.

1.4 Measurement of enzymatic activity

Enzyme extracts from liver tissues and the measurements of CAT, GPx, SOD, and GST activities were conducted according to the user manual of test kits (Nanjing Jiecheng Bioengineering Institute, China). Briefly, tissues were homogenized (10%, *W/V*) in ice-cold 0.01 mol/L Tris-HCl buffer (0.01 mol/L tris, 0.0001 mol/L EDTA-Na_2, 0.01 mol/L sucrose, 0.8% NaCl, pH 7.4). After the homogenates were centrifuged at 1200 ×*g* for 15 min at 4°C, the remaining supernatant was collected for the measurement of protein content and enzymatic activities using the test kits. Protein contents were measured by the method introduced by Bradford (1976) using bovine serum albumin as standard. Enzymatic activity was assayed by spectrophotometry at 405 nm for CAT, at 412 nm for GST and GPx, at 550 nm for SOD, and was given in units per milligram of protein (U/mg protein). Each assay was carried out in triplicates.

In GST assay, 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) were used as substrates, where 1 unit of GST activity is the conversion of 1 μmol/(L·min) of GSH at 37°C as reported previously (Du *et al*., 2007a, 2007b). In CAT assay, the reaction between CAT and H_2O_2 was stopped by adding ammonium molybdate, and the latter reacted with H_2O_2 to form a slight yellow product, where 1 unit of CAT activity is the decomposition of 1 μmol/(L·s)) of H_2O_2 at 37°C. Since GPx can accelerate the reaction of GSH with H_2O_2 to produce oxidized GSH, 1 unit of GPx activity is the decrease of 1 μmol/(L·s) of GSH at 37°C. In SOD assay, xanthine oxidase and xanthine were used to generate superoxide radicals that react with hydroxylamine to form nitrite; SOD has an inhibitory action on superoxide radicals, and thus decreases the production of nitrite. Under the action of chromogenic reagent, mauve appeared from nitrite and the absorbance was then measured, where 1 unit of SOD activity is the absorbance at 50% inhibition in 1 mL reaction solution at 37°C.

EROD activity was determined using the kinetic fluorometric method as described by Willett *et al*. (1997) with slight modification. About 0.1 g liver tissue was homogenized in 1 mL ice-cold PBS buffer (0.125 mol/L Na_2HPO_4, 0.125 mol/L KH_2PO_4, 0.05 mmol/L EDTA-Na_2, pH 7.8), then centrifuged at 10000 ×*g* for 20 min at 4°C. The supernatant was immediately used for assay. The reaction mixture contained 1.88 mL PBS buffer, 0.2 nmol of 7-ethoryresomfin, 60 nmol NADPH and 100 μL supernatant in a final volume of 2 mL. The mixture was incubated at 20°C for 10 min, and the reaction was stopped by adding 0.5 mL of ice-cold methanol. EROD can catalyze 7-ethoryresomfin to form resomfin, and the fluorescence of resomfin was recorded with a spectrophotometer at 565 nm/584 nm excitation/emission wavelengths. Units of EROD activity were given as nmol of resomfin produced per min per milligram of protein.

1.5 Statistical analyses

All data are presented as means ± SEM (standard error of means). Differences between the control and Phe-exposed groups were analyzed by one-way ANOVA followed by Tukey's test. The level of significance was set at $P < 0.05$.

2 Results

After the hybrid tilapia (*O. niloticus* ♀ × *O. aureus* ♂) in freshwater were exposed to Phe at dosage of 50, 100, and 400 μg/L for 4–14 d, respectively, the effects of Phe on HSI and hepatic enzymatic activities were determined (Fig. 1).

Compared with the data of respective control group, the activity of GST was not influenced ($P > 0.05$, Fig. 1f); whereas HSI was significantly decreased by 33.22% ($P < 0.05$) at the highest dosage (400 μg/L) with the longest exposure time (14 d), but was not affected at lower dosage level (50–100 μg/L) or with shorter exposure time (4–8 d) (Fig. 1a). The activity of CAT were significantly increased by 67.43%–109.81% ($P < 0.05$) at 100–400 μg/L for 8–14 d and at 50 μg/L for 14 d (Fig. 1b). Similarly, GPx were significantly increased by 41.24%–79.34% ($P < 0.05$) at 100 μg/L for 8–14 d and at 400 μg/L for 14 d (Fig.1c), and SOD were significantly increased by 26.46%–64.49% ($P <$

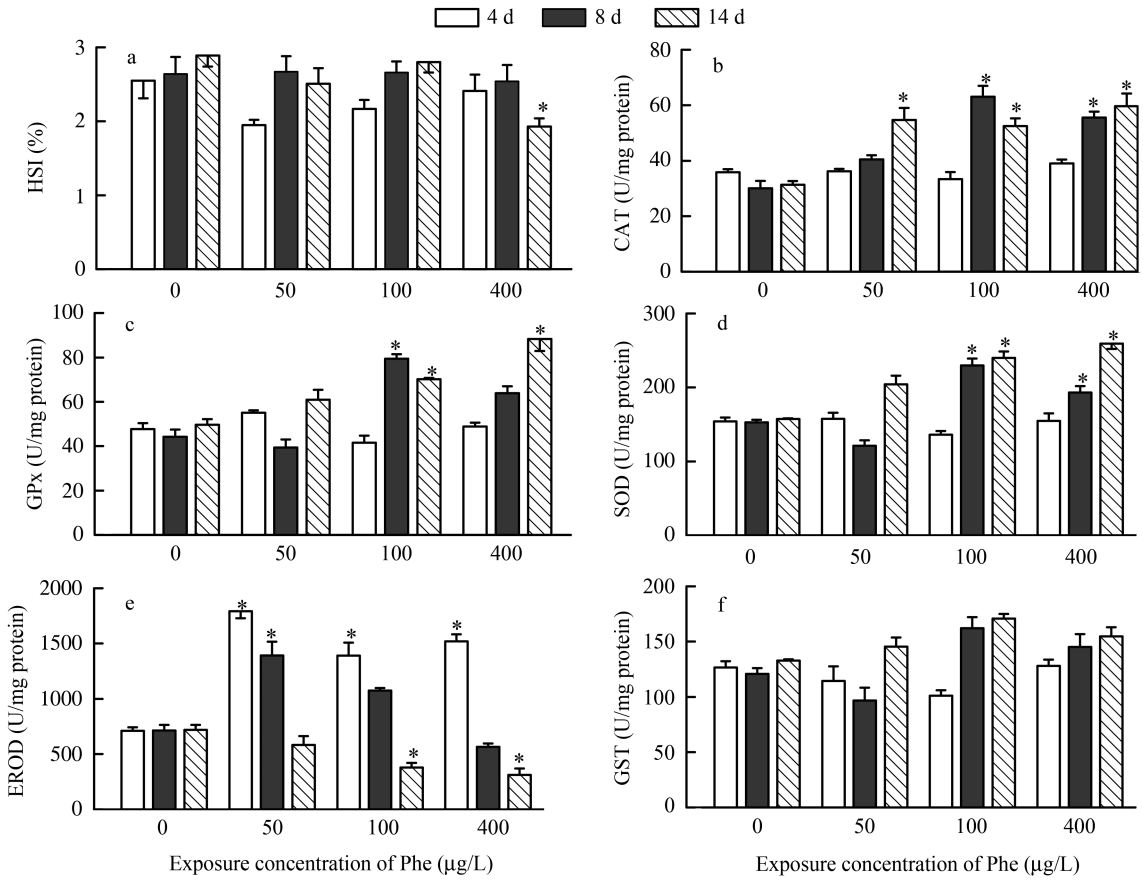

Fig. 1 Hepatosomatic index (HSI) (a) and hepatic enzymatic activities (U/mg protein) of catalase (CAT) (b), glutathione peroxidase (GPx) (c), superoxide dismutase (SOD) (d), ethoxyresorufin-*O*-deethylase (EROD) (e) and glutathione-*S*-transferase (GST) (f) in control and phenanthrene (Phe) exposed tilapia at 50, 100, and 400 μg/L for 4–14 d. Data are presented as mean ± SEM (n = 3 or 4). * $P < 0.05$ indicates significant different from the control (0 μg/L).

0.05) at 100–400 μg/L for 8–14 d exposure (Fig. 1d). For EROD, the effects of Phe differed with exposure dosage or duration time (Fig. 1e). The activity was significantly increased by 95.29%–152.68% ($P < 0.05$) at 50 –400 μg/L for 4 d and at 50 μg/L for 8 d exposure, but was significantly inhibited by 90.50%–130.31% ($P < 0.05$) at 100–400 μg/L for 14 d exposure.

3 Discussion

Tissue somatic indices are commonly reported in fishery studies because the indices can be easily determined and some indices such as HSI can be an excellent predictor of adverse health effect in fish (Adams and McLean, 1985). In this study, the HSI of tilapia was significantly decreased after 14 d of exposure to 400 μg/L Phe (Fig. 1a). Similar decrease of HSI was observed in Chinese rare minnow (*Gobiocypris rarus*) after 1 or 2 weeks of exposure to tributyltin (Zhou *et al*., 2002). In our previous experiments with *Siganus oramin*, however, both 4-*tert*-octylphenol and tributyltin increase the HSI level (Du *et al*., 2007a, 2007b). These suggest that toxicant effects on HSI may differ with fish species, exposure period or physiological status and so on (Du *et al*., 2007a). HSI may be used as a physiological index to evaluate the toxicity of Phe on fish at relatively higher concentration or longer exposure duration.

Under normal physiological status, the anti-oxidative defense systems including GSH, SOD, CAT, and GPx can be induced by a slight oxidative stress as a compensatory response, and thus the reactive oxygen species (ROS) can be removed to protect organisms from oxidative damage (Di Giulio *et al*., 1989; Livingstone, 2001). However, a severe oxidative stress will suppress the activities of anti-oxidative enzymes and lead to the oxidative damage. The increase of CAT, SOD, and GPx activities in Phe-exposed tilapia in this study suggests that the oxidative stress response still works well under the current conditions, and the increase of anti-oxidative enzymes may be a physiological adaptation for the elimination of ROS generation. Similar results have been observed in gilthead sea bream (*Sparus aurata*) and *Carassius auratus* exposed to Phe (Sun *et al*., 2006; Correia *et al*., 2007).

PAHs are typical inducers of cytochrome P450 related mixed function oxidases, and P4501A activity is generally measured as the activity of EROD because it can catalyze ethoryresomfin to produce resomfin (Feng *et al*., 1999). In this study, hepatic EROD was induced at all dosage levels with short exposure (4 d), but was inhibited at high dosage levels (100–400 μg/L) with long exposure (14 d). Similarly, an increase of EROD activity at low doses, but an inhibition at high concentrations, was observed in *S. aurata* in 4 d exposure (Correia *et al*., 2007), and hepatic EROD activity was significantly increased in *Liza aurata* by short (16 h) Phe exposure (Oliveira *et al*., 2007). The induction of CYP1A activity by PAHs seems to be

related to their ability to bind the aryl hydrocarbon receptor and to activate CYP1A gene transcription (Piskorska-Pliszczynska *et al*., 1986; Miller *et al*., 1999). The produce of ROS may damage hepatic cells and decrease EROD activity at comparatively high dosage for long exposure. Besides, PAHs and their metabolic products can disrupt the integrity of CYP1A and inactivate EROD activity (Stegeman and Hahn, 1994). These may explain the dosage- and duration-related effects of EROD after Phe exposure. The results suggest that EROD is a good biomarker for the early detection of toxic pollutants such as Phe.

4 Conclusions

This study demonstrated that Phe at 50–400 μg/L concentration level can cause adverse effects on tilapia, including the decrease of HSI at 400 μg/L after 14 d exposure, increase of hepatic activities of CAT, GPx, and SOD at 100–400 μg/L after 8–14 d exposure. The activity of EROD was significantly increased at 50–400 μg/L after 4 d and at 50 μg/L after 8 d exposure, but was significantly inhibited at 100–400 μg/L for 14 d exposure. The results suggest that the above biochemical indexes can be used as biomarkers to evaluate the toxic effects of Phe on fish. The hybrid tilapia is of potential to be used as a monitoring organism for freshwater and brackish environments.

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