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Toxic effects upon exposure to polycyclic aromatic hydrocarbon (chrysene) in scallop *Chlamys farreri* during the reproduction period

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ABSTRACT

This study aims to investigate potential toxic effects of chrysene (CHR) on mature scallop *Chlamys farreri* during the reproduction period, using indicators of antioxidant defences and oxidative stress. Scallops were exposed to 0.2, 0.8 and 3.2 μg/L waterborne CHR for 21 days, at day 10 scallops were induced to spawn. At days 1, 3, 6, 10, 11, 15 and 21, aryl hydrocarbon hydroxylase (AHH), 7-ethoxyresorufin-*O*-deethylase (EROD), glutathione-*s*-transferase (GST), glutathione (GSH), superoxide dismutase (SOD), lipid peroxidation (LPO), protein carbonyl (PC) and DNA strand breaks in digestive glands were examined by separately analysing male and female scallops. During the pre-spawn period, Levels of enzymatic activities and oxidative stress were all induced by the exposure to CHR for females and males. GST activity presented a good time- and dose-dependent relationship only in males, and GSH content showed a dose-dependent manner in both sexes. During the post-spawn period, different trends were observed, while PC contents maintained growth in time- and dose-dependent manner. Overall, males were more sensitive than females to CHR exposure in enzyme activities, and correspondingly, females suffered from more serious oxidative damages. Both GSH and PC contents seemed to be potential biomarkers for PAH exposure. These results will offer the information on toxicity of CHR in this species, and ensure the influence of gender and reproductive status on PAH detoxification metabolism.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) originate mainly from incomplete combustion, industrial and urban runoff, and oil spills, with the highest concentrations detected in marine coastal areas (Lüchmann et al., 2014). In 2008 the European Food Safety Authority (EFSA) concluded that the sum of the four specific PAH compounds, namely, benzo[a]anthracene (BaA), benzo[a]pyrene (BaP), benzo[b]fluoranthene (BbF) and chrysene (CHR), was the most suitable indicator for assessing PAHs in food to minimise the health risk from dietary PAHs exposure (EFSA, 2008). CHR is found at about the same level as BaP in products of incomplete combustion, vehicle exhaust and cigarette fumes (van Lipzig et al., 2005). Structurally, CHR is a symmetrical PAH consisting of four condensed benzene rings, and has two highly reactive Bay-regions where the main carcinogenic species can be formed. In spite of not acting as a complete carcinogen, CHR could cause increased incidence of liver tumours in mice (Levin et al., 1978). In addition, several hydroxylated CHR metabolites have been found to be estrogenic (Fertuck et al., 2001) or anti-estrogenic (Tran et al., 1996). Among 16 priority PAHs, CHR occurs at the highest content in bivalve molluscs and other aquatic animals from polluted seawaters (Guillen et al., 1997). However, sound scientific research is presently unavailable as to CHR on molluscs.

PAHs exert the toxic effects on the organism following biotransformation to toxic metabolites. The transformation processes generally involve Phase I and Phase II reactions in mollusc, for which the digestive gland is often studied since it is the primary organ of detoxification metabolism, as well as the major active site of biotransformation (Livingstone, 1998). In phase I, the xenobiotics metabolism is characterized by oxidation, reduction or hydrolysis of compounds, and in phase II, their products are conjugated with endogenous metabolites. Reactive oxygen species (ROS) can be

Abbreviations: AHH, aryl hydrocarbon hydroxylase; AhR, aryl hydrocarbon receptor; BaA, benzo[a]anthracene; BaP, benzo[a]pyrene; BbF, benzo[b]fluoranthene; CDNB, 1-chloro-2,4-dinitrobenzene; CHR, chrysene; DNPH, 2,4-dinitrophenylhydrazide; E2, estradiol; ECOD, ethoxycoumarin-*O*-deethylase; EFSA, European Food Safety Authority; ER, estrogen receptor; EROD, 7-ethoxyresorufin-*O*-deethylase; GSH, glutathione; GST, glutathione-*s*-transferase; LPO, lipid peroxidation; PAHs, polycyclic aromatic hydrocarbons; PC, protein carbonyl; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, trichloroacetic acid.

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generated through non-redox cycling by up-regulating antioxidant systems, such as antioxidant enzymes (Livingstone, 2003). Nevertheless, failure of the antioxidant defences to detoxify the excess ROS production can result in an oxidative damage including protein oxidation, lipid peroxidation (LPO) and DNA damage (Altenburger et al., 2003). In recent research the multi-biomarker approach has gained considerable interest in both laboratory and field studies, and can both signal exposure to contaminants and quantify their impact on living organisms. Phase I enzymes aryl hydrocarbon hydroxylase (AHH) and 7-ethoxyresorufin *O*-deethylase (EROD) and phase II enzymes glutathione *S*-transferase (GST) have been commonly used as indicators of the exposure to pharmaceuticals. Among the antioxidant systems, reduced glutathione (GSH) and superoxide dismutase (SOD) have been demonstrated to be useful in evaluating oxidative stress in a range of aquatic organisms responding to numerous contaminants. The analyses of protein oxidation, LPO and DNA damage have been extensively applied to describe cellular effects induced by xenobiotics. In these studies, the gender of each animal included in the pooled samples was not determined, when evaluating biological indicators for the long-term biomonitoring. According to Tairova et al. (2012), male eelpouts *Zoarces viviparus* contained higher concentrations of PAH metabolites than females. Therefore, only a few studies on detoxification metabolism considered the impact of intrinsic parameters such as sex (Chu et al., 2003). In addition, age, length, feeding behaviours, state of health or reproductive cycle of organisms can affect bioaccumulation pattern of xenobiotics in tissues, all of which in return can impact on the metabolic detoxification. Our previous studies on scallops *Chlamys farreri* in spawning period showed that there was a quick reduction of PAH bioaccumulation in tissues after spawning (Xiu et al., 2015). A striking change of pollutants in tissues might influence enzyme activities related to detoxification metabolism, but few references exist on the relationship between reproductive behaviour and biomarkers in bivalves.

Among sentinel species, bivalve molluscs have been recommended as appropriate indicator of water environmental quality. The scallop *C. farreri* is an economically and ecologically important bivalve species native in the China Seas. The objective of our research to investigate the response of detoxification systems (AHH, EROD, GST, GSH and SOD), as well as oxidative damages (protein oxidation, lipid peroxidation and DNA damage) in the digestive gland of females and males, *C. farreri* exposed to CHR, during the reproduction period. This study may provide deeper insight into toxic effects of CHR on *C. farreri*, and ascertain the impact of gender and reproductive status on PAH detoxification metabolism.

2. Materials and methods

2.1. Chemicals

CHR (chrysene, CAS#218-01-9) was purchased from Supelco (Bellefonte, PA, USA). All chemicals for sample preparation were of chromatogram grade and obtained from Sigma (St. Louis, USA) and E. Merck (Darmstadt, Germany). In addition, biochemicals for toxic effects were of analytical grade.

2.2. Animals and treatments

Healthy sexually mature scallops *C. farreri*, aged 2 years, were collected from the Shazikou shellfish farm (Yellow Sea, Qingdao, China). Only scallops with the similar shell length (6.19 ± 0.60 cm) were selected for experiments. They were held in laboratory tanks at ambient seawater temperature (18–20 °C) for one week before the exposure test. The seawater was continuously aerated, and salinity, temperature and pH were, respectively, maintained at 31‰, 19(±1) °C and 8.1. The water was renewed completely every 24 h. The scallops were fed daily with dried powder of *Spirulina platensis* (30 mg for each individual).

The background concentration of CHR in seawater from Taiping Bay was analyzed by HPLC before the experiment, and the concentration was 0.126 ng/L. In treatment aquaria, scallops were exposed to 0.2, 0.8 and 3.2 μg/L CHR, which are relevant with the environmental concentrations, to mimic the natural contaminant concentrations of PAHs. One group was served as control without any pollutant additive. There were triplicates for each level and 72 scallops in each aquarium. CHR was first dissolved in acetone. The final acetone concentration was 0.001% in all tanks including the control ones (the acetone test has been done in a preliminary experiment with the result that there was no influence of acetone on scallops). Experimental conditions (salinity, pH, temperature, scallop density and feeding) were the same as those used for acclimation, and the exposure media were renewed daily. We determined the CHR concentration of exposure groups every day before renewing the water during the experiment. The analysis of CHR concentration was as below, CHR: 0.17 ± 0.06 μg/L, 0.77 ± 0.04 μg/L, and 3.04 ± 0.08 μg/L.

The exposure experiment lasted for 21 days, including 10 days for pre-spawn experiment and 11 days for post-spawn experiment. After sampling of day 10, scallops from each group were immediately induced to spawn. During the experimental period, there was no mortality of scallops at any concentrations of CHR and the control groups. Scallops were sampled 1, 3, 6, 10, 11, 15 and 21 days after the end of the acclimatization period. Eight scallops were sampled for each day and concentration, including controls. The whole soft tissues of scallops were excised and frozen immediately at −80 °C for subsequent examination.

2.3. Enzyme extraction and measurements

The digestive glands of scallops were homogenized in an ice-cold buffer containing 20 mM Tris–HCl, 1.5 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol (v:v) (pH 7.6) at 0 °C. Samples were centrifuged for 3 min at 4 °C, 12000*g* to remove tissue debris firstly, then the obtained supernatants were centrifuged again for 25 min at 4 °C, 3000*g*. The supernatants were collected for analysing the activity of AHH, EROD, GST, SOD, GSH and the contents of protein.

AHH activity was determined as described by Willett et al. (1999) with modifications. The 1.0 mL incubation mixture consisted of 50 mM Tris–HCl, pH 7.6, 0.1 mM NADPH, and 500 mg of microsomal protein. Samples were preincubated at 30 °C and the reaction was initiated by the addition of 60 μM B[a]P. Samples were incubated for 30 min and stopped with the addition of 1 mL cold acetone followed by 3.25 mL hexane. Samples were vortexed, 2 mL of the organic layer was drawn and extracted with 5 mL aqueous NaOH, and fluorescence was determined with a spectrofluorometer (Molecular Spectroscopy LS 55 from Instruments, P.E., MA, USA) at 396/522 nm (excitation/emission). The spectrofluorometer was calibrated using an authentic 3-OH B[a]P standard.

EROD activity was measured according to the modified method of Pohl and Fouts (1980). The reaction mixture contained 100 μL supernatant, 10 μL 0.2 mM O^7-ethylresorufin, 10 μL 6 mM NADPH and 1.88 mL phosphate buffer (0.125 M, pH 7.7, containing Na_2EDTA, 0.05 M, 2–4 °C), allowed to proceed for 10 min at RT, and stopped by the addition of 0.5 mL carbinol. Incubation vials were centrifuged to remove precipitated microsomal protein, and supernatants were transferred to vials for measurement of resorufin concentrations in a luminescence spectrometer (Model LS55, PerkinElmer of U.K.) at an excitation wavelength of 560 nm and an emission wavelength of 580 nm. Resorufin was identified and concentrations were calculated by comparison to retention times and responses of resorufin standards. Blanks corresponded to