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Effects of pyrene exposure on immune response and oxidative stress in the pearl oyster, *Pinctada martensii*

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ABSTRACT

Pyrene is a polycyclic aromatic hydrocarbon (PAH) commonly observed in aquatic ecosystems, which originates primarily from the incomplete combustion of fossil fuels and the use of petroleum compounds. Pyrene can cause the immune disturbance and oxidative stress, result in immunotoxicity, DNA damage, reduce reproduction significantly, and induce behavioral changes. Marine bivalves are commonly used as bioindicators for marine pollution, and hemolymph is a metabolite transfer medium for PAH pollutant. However, the vital immune indicator responses of pearl oyster *Pinctada martensii* hemolymph exposed to pyrene is still unclear. Thus, the immunotoxic responses of pyrene on the hemolymph of the *Pinctada martensii* were investigated in this study. After exposure to pyrene for 7 days, the total number of hemocytes (THC), cell membrane stability (CMS), phagocytic activity (PA) and total glutathione (GSH_T) all decreased significantly. Pyrene also caused a significant increase in lipid peroxidation (LPO). Median effective concentrations (EC_{50}) of pyrene on THC (4.5 μg L^{-1}) and LPO (5.2 μg L^{-1}) were lower than those for CMS (13.8 μg L^{-1}), PA (12.1 μg L^{-1}) and GSH_T (7.2 μg L^{-1}), which indicates that THC and LPO were more sensitive. Additionally, a clear dose-effect relationship indicated that pyrene stimulated a marked immune response, as well as oxidative stress in *P. martensii*, which demonstrates the subtle effects of pyrene exposure on marine invertebrates and the potential associated risk.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants in the marine environment, which possess immunotoxic characteristics, carcinogenic and mutagenic [7,56], such as which can disrupt the immune defenses of *Crassostrea gigas* [5], *Pecten maximus* [32], *Haliotis diversicolor* [27], *Mytilus edulis* [33] and *Venerupis philippinarum* [48]. PAHs occur in crude petroleum oil or as a result of incomplete combustion and have been considered priority pollutants by the U.S. Environmental Protection Agency [38]. Pyrene is a model PAH, which has been commonly found in aquatic ecosystems and has been detected in higher concentrations than other PAHs at some creosote and pyrogenic discharge sites [15,39,55,80]. Avio et al. [4] reported that a marked accumulation of pyrene in the hemolymph and gills of the mussel *Mytilus galloprovincialis*, as well as a particular concentration in the digestive tissues. The cellular effects of pyrene included alterations of immunological responses, peroxisome proliferation, antioxidant system, and neurotoxic effects. Additionally, pyrene can cause severe oxidative stress and DNA damage [34], reduce reproduction significantly [49] and stimulate alterations in behavior [57] in marine organisms. Additionally, we have detected the concentrations of pyrene in surface water and sediment samples in the southwestern region of Hainan Province [43,44]. Pyrene is one of the key polycyclic aromatic hydrocarbon (PAH) contaminants in estuarine and coastal areas [57] and has become ubiquitous in the marine environment, it is necessary to assess the relevant biological endpoints which will provide further information on the

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immunotoxicity of pyrene in bivalves.

Marine bivalves are commonly used as sentinel organisms for monitoring marine pollution [41] because of their longevity, sessile lifestyle, filter-feeding behavior and tolerance of harsh environmental conditions [71]. Bivalves also exhibit relatively sensitive biological responses to stress, high amounts of bioaccumulation, and low rates of metabolizing PAHs [78]. *P. martensii* is distributed throughout the Guangdong, Guangxi, Hainan, and Taiwan provinces of China and along the Japanese coast and is one of the most commercially important pearl-producing bivalves in southern China [40]. In addition, bivalves have been widely used in previous immunotoxicity response studies [1,54,75]. The host defense system of bivalve consists of both cellular and humoral components, the former includes circulating hemocytes which can kill microbes through phagocytosis and cytotoxic reactions, while the latter includes lectins, lysosomal enzymes and antimicrobial peptides [83]. The cellular defense mainly relies on the hemocytes through infiltration, aggregation, encapsulation, cytotoxic reactions, and phagocytosis of foreign particles [22]. In addition, hemolymph is a transfer medium for xenobiotic compounds, hemocytes constitute the major immune defense of mollusks [14] and are susceptible to the toxic effects of xenobiotics [59]. While the gonad, gill and hepatopancreas are the target tissues for PAH toxicity, contaminants reach these organs via the hemolymph circulatory system [30]. However, no report exists currently on the immunotoxic effects of pyrene on the immune response and oxidative stress of the commercially important bivalve *P. martensii*.

Reactive oxygen species (ROS) are a challenge to aerobic life [50]. Bivalve mollusks can generate reactive oxygen species (ROS) by biotransformation [68]. Under normal conditions, the ROS are kept at a low level. However, increases in the cellular ROS level can cause a shift in the balance between oxidation and antioxidation, and induce oxidative damage to membrane lipids, proteins and DNA [37]. Studies have shown that metabolites and reactive intermediates such as diol epoxides, radical cations and redox active o-quinones of PAHs are characterized by high redox potential. This can stimulate the formation of ROS, and react with DNA to form adducts that result in mutations [64]. A variety of parameters such as granulocyte percentage, hemocyte concentration, phagocytosis, membrane stability, ROS production and oxidative burst have been used to monitor biological responses to contaminants in the marine environment [65]. Among these, hemocyte concentration and phagocytosis were most often used as biological parameters of immunotoxicity in bivalves exposed to environmental xenobiotic compounds [18,20]. Additionally, measuring malondialdehyde (MDA) is considered an indirect way to assess lipid peroxidation (LPO), which indicates the damage to cellular membrane lipids caused by ROS [77]. Detoxification activity and antioxidant defense were determined by glutathione (GSH) assay [76].

This study investigated the effects of pyrene on immune responses (including THC, CMS and PA) and oxidative stress (including GSH_T and LPO) in the hemolymph of *P. martensii*. It's very important to analyze the dose-response relationships of hemolymph exposed to pyrene, which can reveal a possible relationship between oxidative stress and the inhibition of hemocyte function, demonstrates potential biomarkers, and provides early warnings for marine chemical pollution.

2. Materials and methods

2.1. Pearl oyster collection and acclimation

One hundred twenty *P. martensii* (60–80 mm shell height) were hand-picked from a trawl at a local aquaculture site in Li'an Bay in Hainan, China in November 2012. Oysters were immediately transferred to the laboratory and housed in a 1000 L tank containing 800 L of seawater from the sampling site. There are no industries around Li'an Bay, so it is considered to be a clean site. The pearl oysters were cultured in a pearl farm, and the selected batch of *P. martensii* exhibited a high level of uniformity. Therefore only a few pearl oysters were dissected, and examined for spermatozoa and oocytes under the microscope. These specimens were verified to be of similar gonad maturation and at a pre-spawning stage of gametogenesis [51]. At the time of collection, ambient seawater temperature was 21 ± 1 °C. Pearl oysters were acclimated to laboratory conditions for one week in static tanks containing recirculated and filtered seawater (32‰ salinity, 21 ± 1 °C). During the acclimation period, the oysters were fed twice a week with the algal concentrate (*Isochrysis*) Instant Algae® (approx. 2.7×10^8 cells per oyster) and water was changed daily. No mortalities were observed during the whole acclimation period.

2.2. Experimental design

Pyrene was purchased from Sigma (St. Louis, Missouri, USA), and dissolved in acetone to a concentration of 1 mg mL^{-1}. The total volume of stock solution was 30 mL, and it was stored in the dark at −20 °C. These working solutions were diluted to reach the final tested concentrations of 4, 8, 16, 32 and 64 μg L^{-1} and the final concentrations of acetone were lower than 0.01% of the tank volume. The experiment was performed in a 20 L glass tank with 15 oysters placed in each tank. The collected pearl oysters were divided into seven total groups, which included one acetone solvent control (0.01%) and one seawater control group with three replicates.

The pearl oysters were quickly transferred to the tanks and then exposed to a treatment for 7 days. To maintain water quality and constant pyrene concentrations, seawater (32‰ salinity, 21 ± 1 °C) was pumped from Li'an Bay and filtered through a sand filter with 300 μm mesh before use. The seawater was also changed with new pyrene-fortified seawater every 24 h. The solvent blank and seawater blank were also included in the experiment and water was completely changed every 24 h. Oysters were not feed and were aerated during the exposure period, all other conditions were kept in the same conditions as those used for acclimation.

No mortalities were observed in any of the tanks after 7 days. To eliminate differences between individual oysters, hemolymph was collected from 9 pearl oysters in each tank. For each oyster, approximately 0.1 mL or less of hemolymph was drawn from the posterior adductor muscle via a 1 mL syringe and then transferred to an Eppendorf tube with the same volume of physiological saline (0.02 M HEPES, 0.4 M NaCl, 0.1 M $MgSO_4$, 0.01 M KCl, 0.01 M $CaCl_2$; pH 7.4) [32]. Then, the nine samples from each treatment were blended. Parameters tested included THC, protein content, CMS, PA, GSH_T and LPO. The hemolymph samples used to test CMS and PA tests were stored on ice to minimize cell aggregation, while the hemolymph used for GSH_T and LPO were stored at −80 °C until tested.

2.3. Pyrene analysis

To confirm variations in pyrene concentration over 24 h, 1 L of water was collected from the tanks immediately after the initial pyrene fortification and after 24 h of exposure, but prior to changing out the seawater. Pyrene concentration was determined according to the procedure described by Li et al. [43,44]. 500 mL of seawater was collected using 1 L amber Duran bottles. Pyrene was extracted by passing 500 mL of sample through a C18 solid phase cartridge at a flow rate of 5 mL min^{-1} and eluting with 10 mL of methylene chloride/n-hexane (v/v = 1:1). The eluents were dried