Original Article

Response of Melanomacrophage Centers in Yellowfin Seabream (*Acanthopagrus latus*) Immune Organs as an Immunohistological Biomarker in Short-term Exposure to Phenanthrene

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ABSTRACT

Background: The aim of this study was to assess the changes in plasma levels of antioxidant enzymes and melano-macrophage centers (MMCs) in immune tissues as biomarkers of yellowfin seabream (*Acanthopagrus latus*) exposed to phenanthrene (Phe) for 14 days.

Methods: The research was carried out at Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran in Jan 2016. Thirty-six *immature* fish were intraperitoneally injected with coconut oil (10 µl/g-bw) containing Phe (70mg/kg-bw) and compared with control group (without injection). Then tissue and blood samples were obtained at 1, 4, 7 and 14 d after injection.

Results: The SOD and CAT activity showed a significant decrease in fish exposed to Phe up to day 7, then activity increased at day 14 in Phe-treated fish (P<0.05). The size and number of MMCs in treated fish in spleen and head kidney were higher than control.

Conclusion: Changes in these parameters (SOD, CAT, and MMCs) might be used as useful biomarkers for *evaluating immunosuppressive* Phe in fish.

Keywords: Antioxidant Enzymes, Melano-Macrophage Centers, Phenanthrene, Yellowfin Seabream.

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are one of the most dangerous organic pollutants that can result in alterations of immune mechanisms of animals [1]. Phenanthrene (Phe), a model PAH, can accumulate in tissue and lead to oxidative stress [2]. Recently, a concentration of Phe varied from 15.438 to 632.682 ng/g in the sediment, and from 0.37 to 11.10 µg/L in surface water, in the north of the Persian Gulf [3]. It consequently could cause a destructive effect such as decreased survival in aquatic animals exposed to contaminated marine environments. Although there are great studies on the toxicity of PAHs, less information has been existed regarding the immunotoxicity of Phe on fish compared with other PAHs such as benzo (a) pyrene.

Monocytes can leave the blood and migrate into tissues where they are transformed into macrophages. Macrophages are digested and ingested cells that belong to the non-specific immune system. Melano-macrophage centers (MMCs) are packed *phagocytic cells containing* abundant *pigments* such as lipofuscin, melanin, and hemosiderin. They are aggregated in the stroma of hematopoietic tissues such as head kidney and spleen of teleost [4]. The increase in size and number of MMCs are used as biomarkers for water quality and the health status of fish [5]. Changes in antioxidant defense such as superoxide dismutase (SOD), catalase (CAT) have been suggested as a useful biomarker in the assessment of oxidative stress due to the imbalance between the *antioxidant* capacity and the production of ROS in animals exposed to PAHs [6].

The purposes of present study were to focus on the effect of Phe on the occurrence of MMCs in spleen and head kidney as target organs of one of the most economically important fish in the Persian

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Gulf, yellowfin sea bream *(Acanthopagrus latus)*; and to investigate the changes of antioxidant enzymes in plasma after short-term exposure of Phe.

MATERIALS AND METHODS

Chemicals

Phe (98% pure) and other chemicals were bought from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Fish Maintenance and Experimental Design

The research was carried out at Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran in Jan 2016. Some 36 *immature yellowfin seabream (*81.11 ± 12.1 g mean body weight and 15.78 ±0.2 cm mean body length) were collected from Musa Creek in the northwest of the Persian Gulf. Fish were kept in 300-L tanks filled with UV-treated running seawater (temperature 25 ± 1 °C, dissolved oxygen 7.12 ± 0.10 mg/l, pH 7.2 ± 0.06) for 10 d to acclimate to laboratory conditions. Animals were fed twice-daily using commercial dry pellets (Dibaq-Diprotg S.A., Segovia, Spain) up to 24 h before the experiment. Water renewed daily at about 50%. The chemical characteristics of the water were maintained constant during the experiment and fish were kept under photoperiod (12/12 h light/dark cycle).

Yellowfin seabream was exposed to Phe, dissolved in corn oil **(10µl g^{-1} body weight)**, by intraperitoneal (IP) injection of doses resulting in 0 (vehicle control) and 70 mg/ kg- bw of Phe. Fish were anesthetized with a 2-phenoxyethanol solution (0.2%) and weighed before injection. Choosing of Phe concentration was based on the previous studies on the PAHs toxicological effects on fish [7, 8] and reported levels of PAHs in sediment samples from the Persian Gulf [9]. In addition, the dose of Phe, used in the present study, purposed to display an actuated biological response in an acute exposure and a rapid peak in effects. Similar biological response could occur due to exposure of fish to PAHs in the environment. Phe-treated group was compared with control group (without injection) and samplings were performed at different time intervals (1, 4, 7 and 14 d).

Blood and Tissue Sampling

specimens were euthanized with 2-phenoxy ethanol (2%), **weighed** and the peripheral blood samples then were taken from the caudal vein of three fish per group by heparinized syringes at different time intervals (1, 4, 7 and 14 d), centrifuged (6000 rpm for 10 min) to separate plasma and then plasma samples were frozen at -80 °C until use. Then spleen and head kidney **were separated and** fixed in 15% formalin buffer for 48 h.

Aggregation of Mmcs in Spleen and Head Kidney

Tissues were processed and embedded in paraffin, sectioned at 4 to 6 µm, and stained with hematoxylin and eosin (H&E). Slides were viewed by light microscopy and MMCs counted using 10× magnification. In each tissue section, similar anatomical locations were viewed for counting and measuring the size of MMCs.

Determination of SOD and CAT Activity

Protein concentration was determined using Bradford reagent and bovine serum albumin as the protein standard [10]. The SOD activity in the plasma was assayed by the method of pyrogallol auto-oxidation by superoxide radicals and expressed as U/mg protein [11]. CAT activity was determined by monitoring the decrease in absorbance of H_2O_2 at 240 nm and expressed as µmol/ min /mg protein [12].

Statistical Analysis

Normality data were examined with Shapiro-Wilk test and Bartlett's tests were applied to assess data for homoscedasticity of variance. The mean ± SD for each experimental group (n = 3) was detected and data were subjected to one-way analysis of variance (ANOVA) using the SPSS 16.0 (Chicago, IL, USA) software. Differences between means were assessed by Turkey's post hoc test. The significance level for all tests was $P<0.05$.

Ethical Considerations

This research was performed according to convention of animal rights (approved by the Ethics Committee of Khorramshahr University of Marine Science and Technology). We tried to use fish without causing them unnecessary suffering if it could be avoided.

RESULTS

No abnormal behaviors were observed with respect to the swimming, movements, and mortality rates among the fish. No significant difference was reported in all parameters between control and vehicle control.

Histological Findings

The number and the size of MMCs in both tissues were significantly higher in Phe-treated fish than

controls in all sampling days (P<0.05) (Figure 1). No significant difference was observed in the number and the size of MMCs in both tissues between controls and Phe-treated fish in day 1 (P>0.05).

The spleen of control fish consisted of red pulp and white pulp. The head kidney of control fish showed a normal tissue structure which consisted of lymphoid tissue, melano-macrophage centers, and blood cells. Melanomacrophage centers were observed in both tissues of Phe-exposed fish (Figure 2).

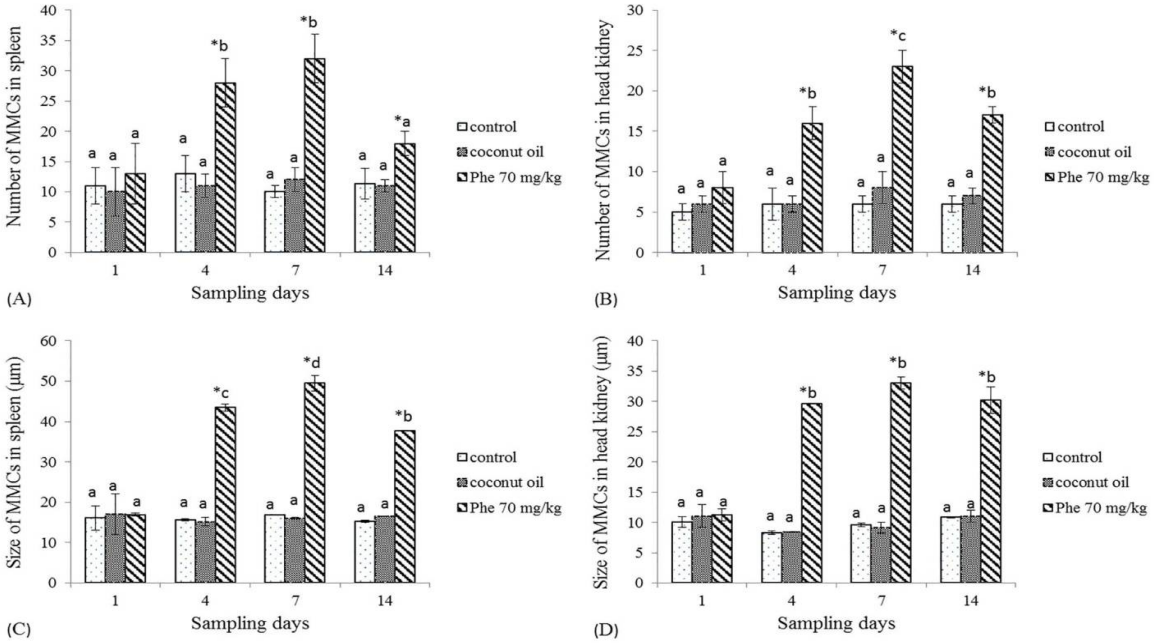

Figure 1. The number and the size of MMCs in spleen (A, C) and head kidney (B, D) in *A. latus* exposed to Phe (Mean ± SD), respectively. Different letters show a significant difference between fish in different sampling days for the same groups. The star (*) indicate the significant difference between control and treated fish (P<0.05).

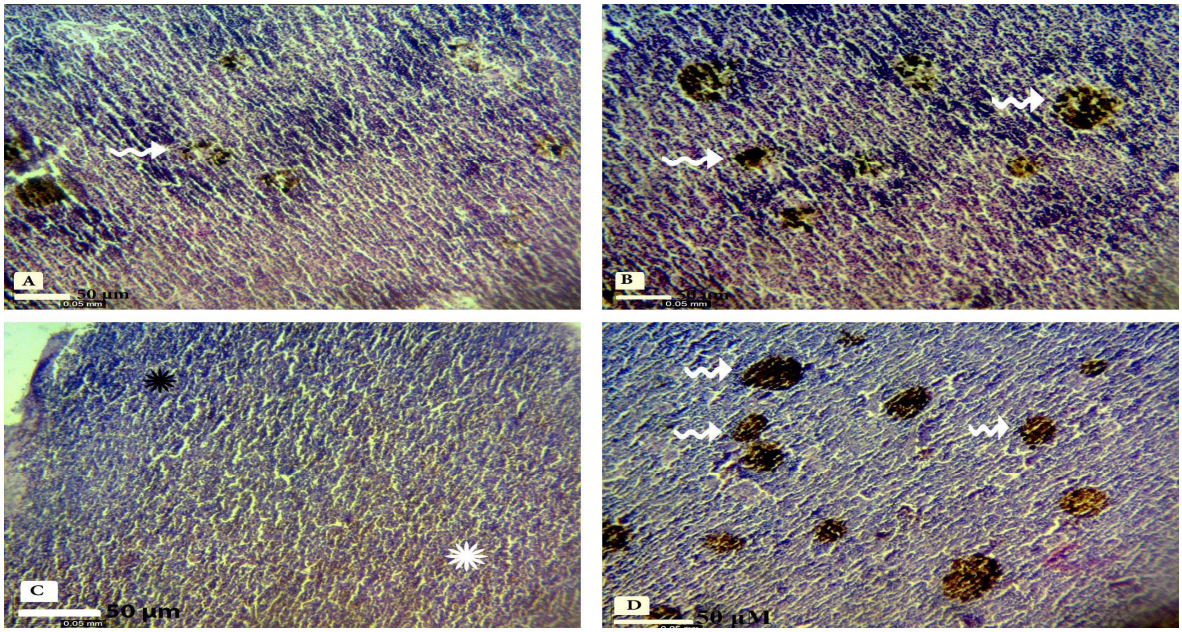

Figure 2. Photomicrographs of head kidney and spleen sections stained with hematoxylin and eosin: (A) Unexposed fish head kidney, (B) Phe exposed fish head kidney, (C) unexposed fish spleen, (D) Phe exposed fish spleen. Red pulp (white*), white pulp (black*), melanomacrophage centers (white convoluted arrows), (H&E; ×725).

SOD and CAT Assays

The SOD enzyme activity in plasma of Phe-exposed fish generally decreased compared to the control groups at days 4 and 7. CAT activity in plasma of Phe-exposed fish was significantly lower than control groups at 1, 4 and 7 d (P<0.05). The SOD and CAT enzyme activities decreased in Phe-exposed fish up to day 7 and then increased in Phe-treated fish at the end of the experiment (day 14) (Figure 3).

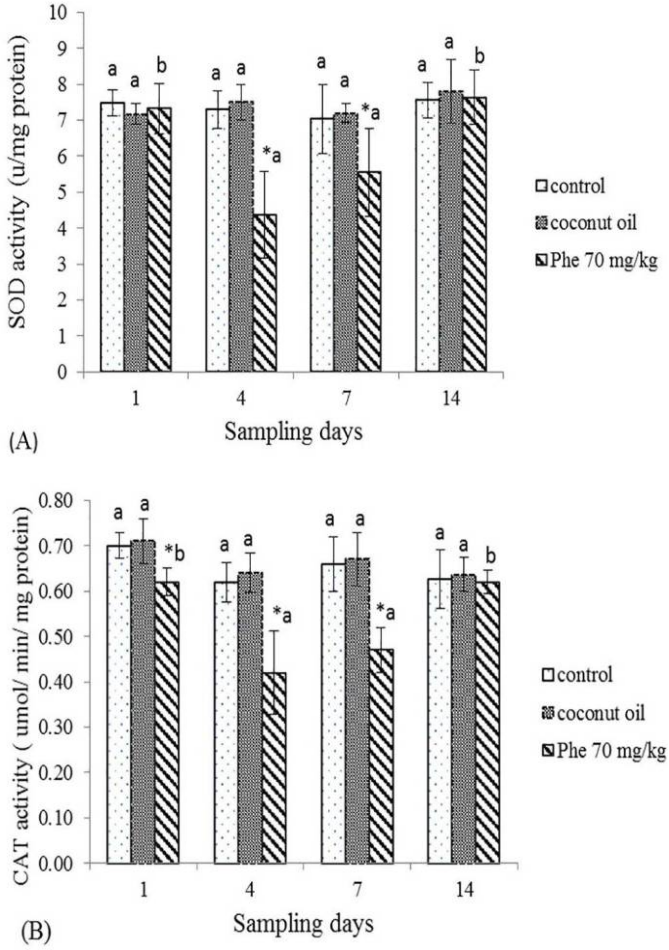

Figure 3. The amount of SOD (A) and CAT (B) activity in *A. latus* exposed to Phe (Mean ± SD). Different letters show a significant difference between fish in different sampling days for the same groups. The star (*) indicate the significant difference between control and treated fish (P<0.05).

DISCUSSION

In the present study, the size and number of MMCs in the head kidney and spleen showed a significant increase in Phe-treated fish compared to the control groups. The increase in MMCs might be due to phagocytosis of destroyed erythrocyte and detoxification processes under stress condition [13, 14]. Similarly, an increase in the size and number of MMCs in the kidney, spleen, and liver were reported [14] that exposed *Clarias gariepinus* to silver nanoparticles for 14 d. Increase in MMCs was suggested along with tissue changes due to oxidative stress lead to aggregation of lymphocytes.

On the other hand, unlike spleen MMC, kidney MMC was more influenced apparently by the deficiency of antioxidants [15]. It resulted in the accumulation of lipofuscin and hemosiderin due to increased lipid peroxidation and erythrocyte fragility, respectively [16, 17], leading to increasing in number and size of MMCs.

An increase in macrophage aggregates in treated fish may possibly be related to a higher content of cellular debris resulting from necrosis and degeneration of tissues [18]. The formation of MMCs in the spleen of *Oreochromis niloticus* exposed for 14 d to different concentrations of mercury chloride and observed that destroyed cells due to exposed to mercury could be caused the increase in phagocytosis by the melano-macrophage centers [19].

In the present study, the SOD and CAT activity showed a significant decrease in Phe-exposed fish by day 7. It could be caused an excess generation of superoxide anion and hydrogen peroxide which *exceeded* antioxidant system's capacity for neutralizing them [20]. Similar results were reported in scallop, *Chlamys farreri* after exposure to different concentrations of benzo (a) pyrene for 30 d [21].

In this study, SOD and CAT levels in Phe-exposed fish were similar to that in control at the end of the experiment. It appeared to be *due to* a balance between ROS production and ROS scavenging by antioxidant defense system after the elimination of Phe from tissues. Similarly, activities of antioxidant enzymes in fish, *Carassius auratus* exposed to Phe was returned to the control level after a 7-day recovery [2].

CONCLUSION

Phe is toxic to yellowfin seabream. Fish exposed to Phe showed an increase number and size of MMCs in the spleen and head kidney, indicating the immune role of the MMCs. The decrease in the level of antioxidant parameters (SOD and CAT) in the plasma of the fish exposed to Phe would suggest that these fish were experiencing oxidative stress. These parameters can be used as biomarkers in assessing the Phe toxicity.

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