

Original Article**Effect of Phenanthrene on the Tissue Structure of Liver and Aminotransferase Enzymes in Yellowfin Seabream (*Acanthopagrus latus*)**

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ABSTARCT

Background: Polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene (Phe) represent one of the most abundant forms of organic pollutants. The aim of this study was to assess changes in plasma levels of aminotransferase enzymes, total protein and liver tissue as biomarkers of yellowfin seabream (*Acanthopagrus latus*) exposed to Phe for 14 d.

Methods: The research was carried out in January 2016 at Khorramshahr University of Marine Sciences and Technology, Khorramshahr, Iran. Some 72 fish were injected with 2, 20, 40 and 70 mg/kg of Phe. Then tissue and blood samples were obtained at 1, 4, 7 and 14 d after injection.

Results: Exposure of fish to Phe resulted in a significant increase of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and decrease of total protein after 7 d of the experiment ($P < 0.05$). The main histopathological alteration was showed in different sampling days including nucleus margination, hypertrophy, vacuolation, melanomacrophages aggregates, sinusoid dilation, degeneration and picnotic nucleus. Degree of tissue change (DTC) of liver was recorded in the Phe-exposed fish from normal range to moderate changes.

Conclusion: The studied biomarkers such as changes in concentrations of ALT, AST and total protein as well as tissue damages in liver may be served as beneficial biomarker to assess Phe toxicity in yellowfin seabream.

Keywords: Aminotransferase Enzymes, Live, Phenanthrene, Yellowfin Seabream.

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of hydrophobic organic compounds in aquatic environments that tend to accumulate on a broad range of marine animals and represent destructive effects such as mortality, growth reduction, reproductive disorders, genetic mutations and carcinogenesis [1]. Phenanthrene (Phe) is a constituent of polycyclic aromatic hydrocarbon with a low molecular weight, composed of three fused benzene rings mainly derived from pyrogenic sources [2]. Many researchers have completed toxicological studies of PAHs [1, 2]; however, studies have limited to estimating impacts of Phe on a suite of biomarkers in teleost. Therefore, it is necessary to determine the interrelation between biological responses and the toxicity of Phe in fish.

Biochemical parameters in the blood have been used to detect tissue damages and to assess

the health status of organisms. Transaminases and total proteins have been proved suitable stress biomarkers in fish [3]. Histopathological studies serve to evaluate relations between exposure to pollutants and several biological responses and use as a useful bioindicator in detecting direct effects of chemical compounds [4]. The liver is a target of toxic chemicals, which can disrupt its functions. Histopathological biomarkers in the liver can be used as indicators of the general health of the fish and mirror effects of exposure to pollutants [4].

Yellowfin seabream (*Acanthopagrus latus*) seems to be suitable for monitoring of pollution exposure. It is one of the most important fish in Iran aquaculture [3].

We aimed to study the effects of Phe on plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein and liver histological alterations of the yellowfin seabream (*A. latus*) for 14 d. Alterations

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can serve as early biological markers for evaluation of polluted aquatic ecosystems.

MATERIAL AND METHODS

Chemicals

Phe (98% pure) was bought from Sigma. AST, ALT and total protein kits were purchased from Pars Azmun, (Tehran, Iran). Other chemicals were purchased from Merck (Germany). The research was carried out in January 2016 at Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran.

Fish Maintenance and Experimental Design

Seventy two *immature yellowfin seabream* (81.11 ± 12.1 g mean body weight and 15.78 ± 0.2 cm mean body length) were obtained from Musa creek in the northwest of the Persian Gulf with trawling. All samples were placed in 300 L tanks for 10 d to adapt to experimental condition. Fish were fed twice daily using *commercial dry pellets* (*Dibaq- Diprotg S.A., Segovia, Spain*) at 2% of the body weight until 24 h before sampling. Water in the tanks changed daily at about 50% during the experimental period. During the exposure period, temperature, pH and dissolved oxygen concentration in water in the tanks ranged from 25 ± 1 °C, 7.2 ± 0.06 and 7.12 ± 0.10 mg/l, respectively. Factors were measured twice daily.

Yellowfin seabream was exposed to Phe, dissolved in corn oil ($10 \mu\text{l g}^{-1}$ body weight), by intraperitoneal (IP) injection of doses resulting in 0 (vehicle control), 2, 20, 40 and 70 mg/kg- bw of Phe. Fish were anesthetized with a 2-phenoxyethanol solution (0.2%) and weighed before injection. Doses were selected based on earlier studies done with other fish species to provoke biological responses [5-7] and reported levels of PAHs in sediment samples from the Persian Gulf [8]. Phe-treated groups were compared with control group (without injection) at different times. Fish were sampled at 1, 4, 7 and 14 d after injection from each group.

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 fish livers were separated and fixed in 15% formalin buffer for 48 h.

Biochemical Analysis

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined by commercial diagnostic kits (Pars Azmun, Tehran, Iran) and were recorded using Auto-analyzer (Technicon RA1000, New York, USA) at 340 nm and 37 °C. Total protein was determined using Bradford reagent and bovine serum albumin as the protein standard (Pars Azmun, Tehran, Iran).

Histopathologic Study

Livers were dehydrated in ascending concentrations of ethanol and embedded in paraffin using a tissue processor (Tissue Tek Rotary, Rx-11B) then sections were prepared at 5 μm thickness by an RMZZ45 rotary microtome (Leica, Wetzlar, Germany). The liver sections were stained with hematoxylin and eosin (H&E) [9] and evaluated by a light microscope (Olympus-CH40, Japan), and digital images were performed with Dino Capture software (FDP2, New Taipei City, Taiwan).

The degree of tissue change (DTC) method as explained by Poleksic and Mitrovic-Tutundzic [10] was modified and used to prove the severity of lesions and the possibility of recovery in liver of the exposed fish. Liver lesions were progressively classified in three stages of tissue damage. Elisions, which did not alter the normal functioning of the liver and possibility of repair, were classified as stage I lesions. Elisions, which were the most severe and harmful for the normal liver function, were classified as stage II lesions but these lesions are repairable. Lesions, which were very severe and induced irreparable liver damage even with improved water quality, were classified as stage III elisions [11]. DTC was measured for each animal as $\text{DTC} = (1 \times \Sigma \text{I}) + (10 \times \Sigma \text{II}) + (100 \times \Sigma \text{III})$, where I, II, and III correspond to the number of stages of alterations 1, 2, and 3, respectively. Mean DTC values of 0–10 indicate normal function of the liver, 11–20 slight damage to the liver, 21–50 moderate changes of the liver, 50–100 severe lesions of the liver, and >100 irreversible damage to the liver.

Statistical Analysis

All results were noted as mean \pm SD. The

treated groups were compared with the control groups and data were calculated using one-way ANOVA followed by Turkey's post hoc test by (Chicago, IL, USA) 16.0 software at $P < 0.05$ level.

Ethical Considerations

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

RESULTS

During sampling, no mortality was found in control, solvent control and experimental groups during the experiment. The body size of yellowfin seabream did not differ significantly among the experimental groups (body length: 15.78 ± 0.2 cm, wet weight: 81.11 ± 12.1 g). No significant difference was reported in all parameters between control and vehicle control.

Histopathologic Study

Histopathological changes were recorded in the liver (Table 1, Figures 1-4). No alterations were observed in the liver of the control, vehicle control and fish treated with 2 mg/kg of Phe in all sampling days and other concentrations in 1 and 14 d except fish treated with 70 mg/kg of Phe in 14 d, which recorded relatively less number of damages. In contrast, the structure of the liver of Phe-treated fish was altered in 4 and 7 d. Examination of liver of control fish represented the presence of hepatocytes with central *round nucleus* and polygonal shape and sinusoids which occupied spaces *between* the plates of *hepatocytes*. Various findings like nuclear hypertrophy, nucleus in a lateral position, cellular hypertrophy, cytoplasmic vacuolation, dilation in sinusoids, cytoplasmic degeneration, picnotic nucleus, melanomacrophages aggregates were observed in the liver of fish exposed to Phe. The severity of liver damages was more pronounced in fish exposed to the highest concentration of Phe.

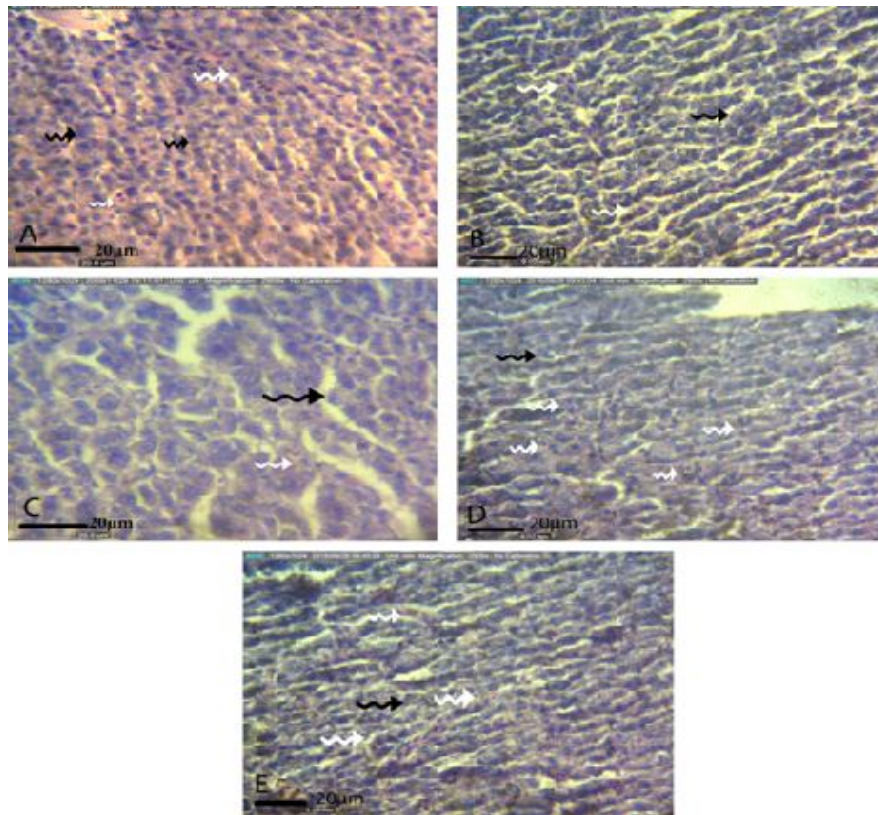


Figure 1. Photomicrographs of the liver tissue structure in control and Phe-exposed fish (*A. latus*) in a day after exposure: (A) normal tissue; (B) *A. latus* exposed to 2 mg/kg of Phe; (C) *A. latus* exposed to 20 mg/kg of Phe; (D) *A. latus* exposed to 40 mg/kg of Phe and (E) *A. latus* exposed to 70 mg/kg of Phe. Hepatocytes (black convoluted arrows), sinusoids (white convoluted arrows). A, B, C, D and E (H&E; $\times 2900$).

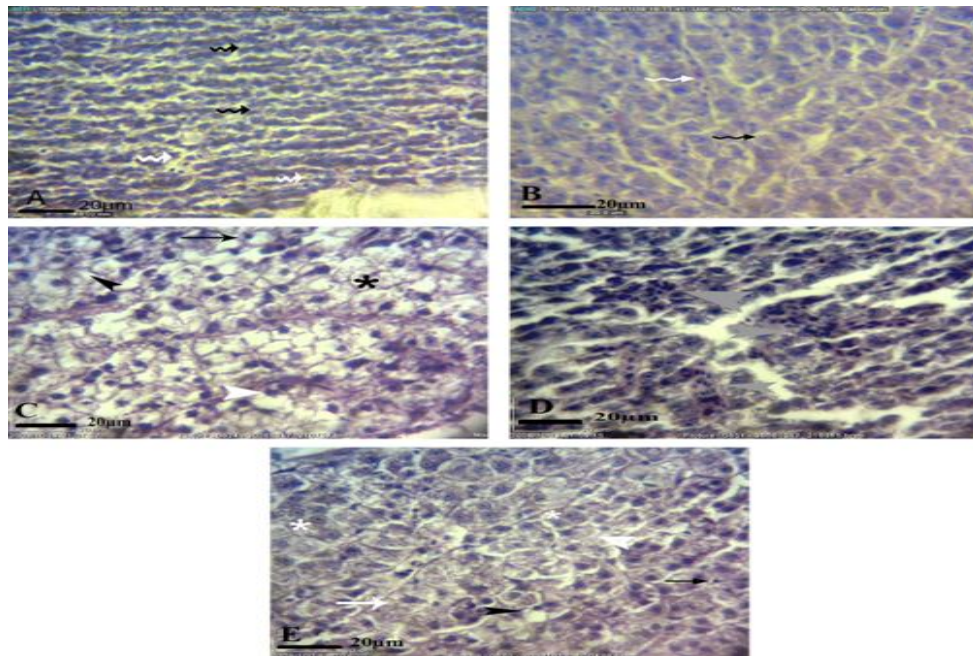


Figure 2. Photomicrographs of the liver tissue structure in control and Phe-exposed fish (*A. latus*) in 4 d after exposure: (A) normal tissue; (B) *A. latus* exposed to 2 mg/kg of Phe; (C) *A. latus* exposed to 20 mg/kg of Phe; (D) *A. latus* exposed to 40 mg/kg of Phe and (E) *A. latus* exposed to 70 mg/kg of Phe. Hepatocytes (black convoluted arrows), sinusoids (white convoluted arrows), nuclear hypertrophy (white*), nucleus in a lateral position (white arrowhead), cellular hypertrophy (white arrow), cytoplasmic vacuolation (black arrowhead), dilation in sinusoids (gray arrowhead), cytoplasmic degeneration (black*), pycnotic nucleus (black arrow). A, B, C, D and E (H&E; $\times 2900$).

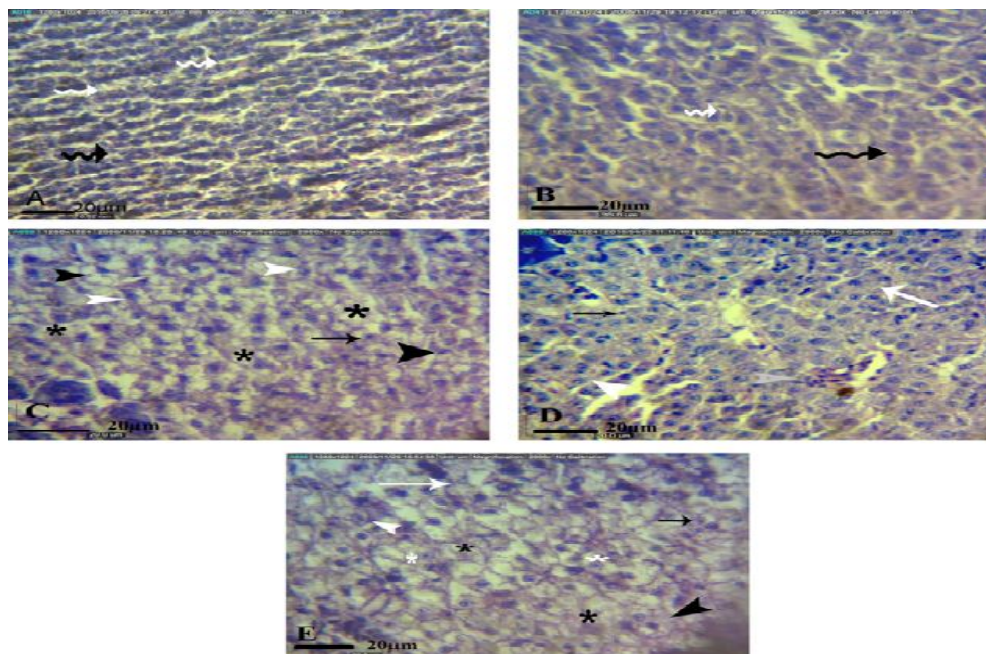


Figure 3. Photomicrographs of the liver tissue structure in control and Phe-exposed fish (*A. latus*) in 7 d after exposure: (A) normal tissue; (B) *A. latus* exposed to 2 mg/kg of Phe; (C) *A. latus* exposed to 20 mg/kg of Phe; (D) *A. latus* exposed to 40 mg/kg of Phe and (E) *A. latus* exposed to 70 mg/kg of Phe. Hepatocytes (black convoluted arrows), sinusoids (white convoluted arrows), nuclear hypertrophy (white*), nucleus in a lateral position (white arrowhead), cellular hypertrophy (white arrow), cytoplasmic vacuolation (black arrowhead), dilation in sinusoids (gray arrowhead), cytoplasmic degeneration (black*), pycnotic nucleus (black arrow). A, B, C, D and E (H&E; $\times 2900$).

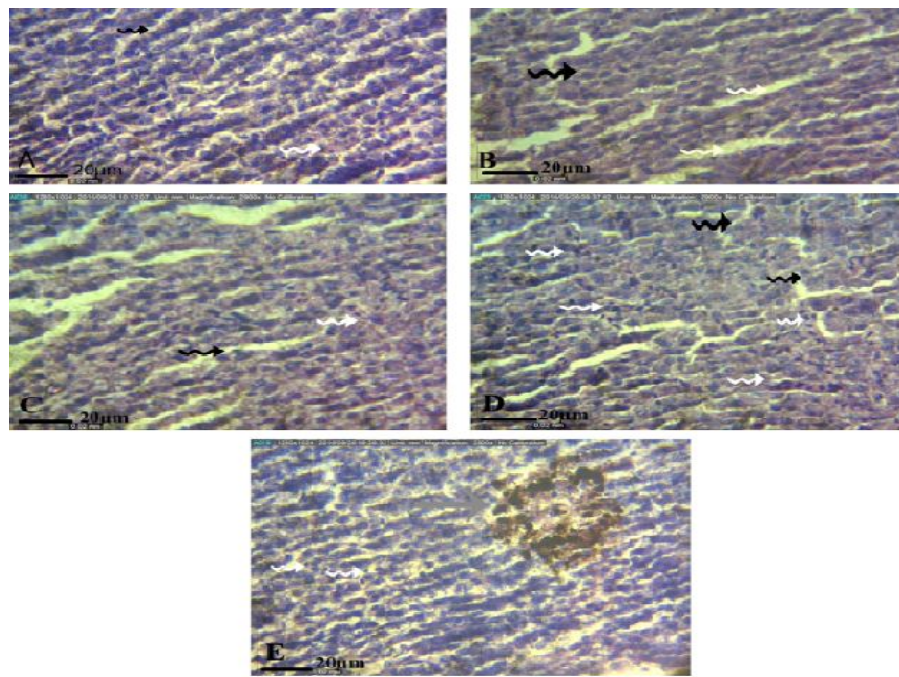


Figure 4. Photomicrographs of the liver tissue structure in control and Phe-exposed fish (*A. latus*) in 14 d after exposure: (A) normal tissue; (B) *A. latus* exposed to 2 mg/kg of Phe; (C) *A. latus* exposed to 20 mg/kg of Phe; (D) *A. latus* exposed to 40 mg/kg of Phe and (E) *A. latus* exposed to 70 mg/kg of Phe. Hepatocytes (black convoluted arrows), sinusoids (white convoluted arrows), melanomacrophages aggregates (gray arrow). A, B, C, D and E (H&E; ×2900).

Table 1. Histological alterations of the liver in *A. latus* exposed to different concentrations of Phe at sampling d. Stage I: slight changes; Stage II: moderate changes; Stage III: irreversible damage.

Changes	Groups																
	Phe 2 mg/kg				Phe 20 mg/kg				Phe 40 mg/kg				Phe 70 mg/kg				
	Sampling days																
	1	4	7	14	1	4	7	14	1	4	7	14	1	4	7	14	
Stage I	Nuclear hypertrophy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+
	Nucleus in a lateral position	-	-	-	-	-	++	+	-	-	++	+	-	-	+++	++	-
	Cellular hypertrophy	-	-	-	-	-	++	+	-	-	++	+	-	-	++	+	-
	Cytoplasmic vacuolation	-	-	-	-	-	++	+	-	-	++	+	-	-	+++	++	-
	Melanomacrophages aggregates	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+	+
Stage II	dilation in sinusoids	-	-	-	-	-	-	-	-	-	++	+	-	-	++	+	-
	Nuclear vacuolation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Cytoplasmic degeneration	-	-	-	-	-	+	+	-	-	+	+	-	-	+	+	-
Stage III	Picnotic nucleus	-	-	-	-	-	+	+	-	-	+	+	-	-	++	+	-
	Necrosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: absent (0); rare (+); frequency (++); very frequency (+++)

The Mean DTC of the liver in Phe-exposed was ranged from 0.66 to 36. The mean amounts of DTC in the control fish and fish treated with 2 mg/kg of Phe in all sampling days as well as Phe-exposed fish on the first day of experiment were within the normal range. Nevertheless, at 4 and 7 d postexposure, the Mean DTC of the exposed fish liver suggested moderate organ damage. The

highest amounts of DTC in the liver were recorded in fish treated with 70 mg/kg of Phe at days 4 and 7. In addition, the DTC value of the liver was graded as normal tissue in Phe-treated fish after 14 d (Table. 2).

Different letters show significant difference between fish in different sampling days for the same groups ($P < 0.05$)

Table 2. Degree of tissue changes in the liver of *A. latous* exposed to Phe in different sampling days.

Groups	Days			
	1day	4d	7d	14d
Control	0	0	0	0
Coconut oil	0	0	0	0
Phe 2 mg/kg	0	0	0	0
Phe 20 mg/kg	0	24.33±1.5 ^a	24±2 ^a	0
Phe 40 mg/kg	0	23.67±0.6 ^a	25±1.7 ^a	0
Phe 70 mg/kg	0	26±1 ^b	26±2 ^b	0.66 ± 0.57 ^a

AST and ALT Assays

Alterations of the AST and ALT in control and treated fish are presented in Figure 5a, b. AST and ALT levels were significantly higher in fish exposed to different concentrations of Phe in all sampling days than controls ($P<0.05$). However, there were no significant difference ($P>0.05$) in AST and ALT levels between controls and fish treated with 2 mg/kg of Phe in all sampling days. Moreover, no significant difference was observed in AST level between controls and fish exposed to different concentrations of Phe and in ALT level between controls and fish treated with 20 and 40 mg/kg of Phe at the end of the experiment ($P>0.05$). The levels of the AST and ALT increased dose dependently in fish exposed to different concentrations of Phe after a day of exposure up to day 7. The concentration of both

factors then decreased in all treated fish two weeks after exposure. However, no significant difference was observed in the AST and ALT levels in fish treated with 2 mg/kg of Phe in all sampling days ($P>0.05$).

Total Protein Assays

The amount of total protein was significantly less in fish exposed to different concentrations of Phe in all sampling days compared to controls ($P<0.05$). The amount of total protein reduced dose dependently in fish exposed to different concentrations of Phe up to day 7. Then, the amount of total protein increased up to the end of the experiment (day14). However, there was no significant difference ($P>0.05$) in amount of total protein in fish treated with 2 mg/kg of Phe in all sampling days (Figure 5c).

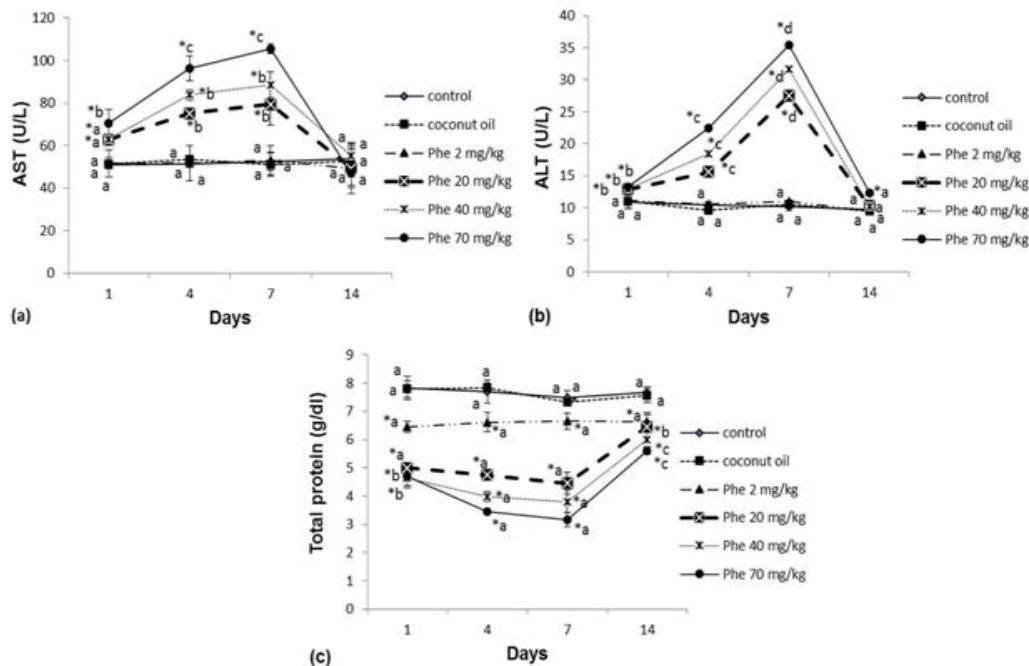


Figure 5. The amounts of AST (a), ALT (b) and total protein (c) in *A. latous* exposed to different concentrations of Phe (Mean±SD). Different letters show significant difference between fish in different sampling days for the same groups. The star (*) indicate the significant difference between control and treated fish ($P<0.05$).

DISCUSSION

In the present study, anomalies such as nuclear hypertrophy, nucleus in a lateral position, cellular hypertrophy, cytoplasmic vacuolation, melanomacrophages aggregates, and dilation in sinusoids, cytoplasmic degeneration, and picnotic nucleus were perceived in the liver of fish from exposure of Phe. These results were in accordance with those observed in *Chanos chanos* exposed to petroleum hydrocarbon [4], and Phe -treated *Clarias gariepinus* [2]. Damage levels in the current study, were elevated correspondingly with the increasing concentrations of the Phe. Hence, the highest hepatohistological changes were noted at fish treated with 70 mg/kg of Phe at 4 and 7 d.

Damages, observed in liver, classified as stages I & II. Hence, these elisions are reparable with improved water quality. The mean amounts of DTC in liver ranged from normal function to moderate changes in this fish depending on the concentration of Phe that did not disturb the normal function of the liver.

These hepatic damages might presumably be due to the primary function of the liver in the metabolism and excretion of toxicants that caused some morphological alterations in liver [4]. Vacuolations of hepatocytes are correlated with the inhibition of protein synthesis, glycogen depletion, and subnormal aggregation of neutral lipids and disaggregation of microtubules [12]. The large vacuole in the cell compels the nucleus in a lateral position and this condition is usually correlated with nuclear atrophy [13]. Dilatation of hepatic sinusoids is probably indicating the increased blood volume received by the liver to detoxify the organism [14].

An elevation in the density of the melanomacrophage aggregates is generally explained to noticeable hepatic damages [15], such as degenerative and necrotic processes or pyknotic nuclei. The presence of melanomacrophages in the liver of *A. latus* is strong evidence that this organ suffered structural and metabolic damage due to exposure to Phe. Hypertrophy of hepatocytes caused ultrastructural changes such as increased numbers of organelles mitochondria, lysosomes, endoplasmic reticulum system, glycogenosomes and peroxisomes due to increase in detoxification activities [16].

Then, in the present study, after 14 d exposure of Phe, the liver histology appeared relatively normal for all the groups. However, melanomacrophages aggregates were observed in fish treated with 70 mg/kg of Phe. The fish liver had the capacity of regeneration its tissue after removal of the Phe exposure due to Phe metabolism and its excretion through the bile or urine.

Based on the results, a significant decrease was detected in AST and ALT in fish exposed to 20, 40 and 70 mg/kg of Phe compared with the controls after 7 d. Increasing concentration of both enzymes in plasma presumably reflected a status of hepatic damage due to Phe toxicity. When the liver cells are destroyed, synthesis of liver enzymes are increased in cells, then membrane permeability is increased as a result of the cellular dysfunction, thus enzymes are released into the general blood circulation and enhanced in the blood [17]. Our results match within the case of *Epinephelus areolatus* exposed to benzo [a] pyrene for four weeks [1]. In addition, liver damage can liberate enzymes into the blood stream. In this study, significance difference between the control and fish exposed to 2 mg/kg of Phe were not noted in both enzymes for all sampling days ($P>0.05$). This considers that, in Phe 2 group, the Phe concentration was not high noticeably to destruct liver and other tissues.

The obtained results of plasma total protein showed a significant decrease in fish treated with Phe when compared with the control group after 7 d. The decrease in the plasma total protein in the present study might be due to cellular injury that happened in the liver of the fish exposed to Phe because the liver is a major organ of protein synthesis in organisms [18]. A decrease in the plasma protein content in common carp (*Cyprinus carpio*) exposed to pyrene in the highest concentration for 35 d [19].

In the present study, activities of enzymes in the plasma were decreased and plasma total protein in the Phe-exposed fish was increased after 14 d of exposure. These alterations could have been because of elimination of Phe in tissues due to Phe metabolism in the liver and Improvement of liver function after 14 d. Plasma total protein, ALT and AST levels accordance with the rate of histopathological lesions observed in this study. Phe removed slowly in fish,

Carassius auratus after 7 d depuration experiment [20]. In addition, PAHs in the tissue of fish have a short half-life of about 3 to 9 d [21].

CONCLUSION

Phe treatment induces liver damage as indicated by the increase of AST, ALT, and decrease of the total protein. Liver of Phe-exposed fish showed normal range to moderate damage. Effects of Phe were dose and time dependent. The 14 d of study period explained that the evaluation of biochemical parameters in the blood and tissue damages recorded useful biomarkers such as changes in concentrations of ALT, AST and total protein as well as tissue damages in liver to determine pollution levels of PAHs such as Phe in the yellowfin seabream under laboratory conditions. These alterations have led to the concern that Phe could be the *disruption of physiological systems* in fish.

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