



# Interactions of oxidative DNA damage and CYP1A gene expression with the liver enzymes in Klunzinger's mullet exposed to benzo[a]pyrene

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## ABSTRACT

Benzo[a]pyrene (B[a]P) is an important contaminant whose liver biotransformation is dependent on the species, the route of exposure and the concentration. The goal of this study was to assess the interactions of oxidative DNA damage and CYP1A gene expression with the liver enzymes in Klunzinger's mullet (*Liza klunzingeri*) exposed to benzo[a]pyrene. Sublethal doses of B[a]P (5, 10 and 50 mg/kg) were intraperitoneally administered to the fish for 14 days. The alterations in antioxidant enzymes' activity (SOD, CAT, and GPX), hepatic enzymes' activity (ALT, AST and ALP), DNA damage (measured by comet assay and cellProfiler software) and CYP1A gene expression in the fish liver were studied on the 1st, 3rd, 7th and 14th days. The determination of these parameters in the liver showed that most of these parameters significantly increased mostly in a time-dependent manner. Multiple regression analysis showed that DNA damage and CYP1A gene expression had positive correlations with the liver enzymes in this fish species intraperitoneally exposed to these concentrations. Moreover, these interactions indicated that these parameters are sensitive biomarkers for the exposure to B[a]P in Klunzinger's mullet. However, other possible factors and B[a]P metabolites should be considered in future studies for better elucidating the biotransformation mechanisms and introducing better biomarkers of B[a]P.

## 1. Introduction

Benzo[a]pyrene (B[a]P) as a representative compound of polycyclic aromatic hydrocarbons (PAHs) is a ubiquitous and persistent organic pollutant [1]. This global environmental contaminant is one of the most commonly tested compounds in ecotoxicology [2,3]. The PAHs in aquatic environments can arise from natural sources and anthropogenic activities [4]. The uptake of the PAHs in aquatic organisms can occur through contact with contaminated food, water and sediments [5,6]. Fishes are especially susceptible to water pollution, such as PAHs [7,8]. Pollutant compounds may interrupt many physiological and biochemical pathways in the body of fish, therefore fish respond more sensitively to pollutants than mammals and many other organisms [7,9,10]. In Fish, hepatic phase I and II metabolism including biotransformation enzymes and the cytochrome P450 (CYP) superfamily are the major pathway for the biotransformation of the B[a]P and other PAHs [11,12]. During the biotransformation of B[a]P that dominates toxicokinetics, carcinogenic metabolites are produced and may reach higher concentrations in organisms than their parent compounds [13,14]. Also, Reactive oxygen species (ROS) are continuously generated in this process [15,16]. Previous studies showed that intermediates compounds produced during B[a]P metabolism in the phase I and II can

react with DNA, leading to DNA strand breaks [17,18] This damage can be measured and analyzed using single-cell gel electrophoresis and CellProfiler software [19].

It is well known that ROS can deplete endogenous antioxidants by induced oxidative stress [20]. Changing the oxidation-reduction (redox) state in an organism can lead to various cell damage such as lipid peroxidation, nucleic acid oxidation and protein oxidation [21]. Although the cellular oxidative damage, resulting from the exposure to PAHs and association between oxidative stress and disease, is well documented in fish and other aquatic organisms [22–24], the mechanistic links between PAHs metabolism and the occurrence of oxidative damage need for more research [23,25]. Toxicity of oxyradicals is neutralized by an integrated array of antioxidant defenses and free radical scavengers, the levels of which may change in response to oxidative pressure [23]. However, it is often difficult to predict how antioxidants respond to chemical stress by a single index reflecting the overall susceptibility to oxidative stress. Therefore, investigations on an effective and feasible approach to measure the effect of PAH oxyradicals on fish body functions is necessary to integrate the effect of PAHs antioxidants, DNA damage, and gene expression.

Although the biotransformation of B[a]P occurs in many aquatic organisms, their most effect is seen in the liver of fish [26]. In this case,

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the key point is that the liver biotransformation of B[a]P is dependent on the species [27,28], the route of exposure and the concentration [6]. Since PAHs, such as B[a]P, do not tend to be bio-accumulated in tissues, the direct quantification of these compounds in fish tissues may not be an exact estimate of B[a]P exposure and its uptake [6]. Although intraperitoneal injection is not a common route of exposure to B[a]P in nature, it can be useful to determine the action mechanism induced by B[a]P and interpret the data obtained in the field conditions [23,29].

In the present study, the sub-lethal toxicities of B[a]P were studied in Klunzinger's mullet (*Liza klunzingeri*) as an in vivo model by the measurement of dose-dependent changes of hepatic enzymes' activity, antioxidant enzyme activity, P450 gene expression, and DNA oxidative damages. The main aim of this study is to get new insights into the complex relationship of oxidative damage to DNA and CYP1A gene expression with liver enzymes in Klunzinger's mullet exposed to B[a]P.

## 2. Materials and methods

### 2.1. Experimental animals and conditions

Sexually immature Klunzinger's mullet (*Liza klunzingeri*) were caught in the coastal water of the Persian Gulf (Khuzestan, Iran). Fish (weighing  $23 \pm 2$  g and  $10 \pm 1$  cm in length) were acclimatized to laboratory conditions in tanks (250 L) for two weeks (flow-through tank with filtered seawater at room temperature, pH: 8.2; salinity: 36‰; O<sub>2</sub>: 4.2–4.6 ml/L; NO<sub>3</sub>: 0.1–0.5  $\mu$ mol/L; NH<sub>4</sub>: 0.1–0.3  $\mu$ mol/L; organic carbon: 3–5 mg/L). The fish were fed (2% of body weight) with live food (rotifer obtained from the Marine Fish Research Center in Imam Khomeini Port). The water of tanks was changed every 3 days, and any dead fish were removed. Before starting the experiment, antibiotic (oxytetracycline (7 ppm)) and fungicide (formalin (25 ppm)) were used in fish tanks [30,31].

### 2.2. Experimental design and sampling

The fish (n = 75) were divided into five groups including three exposure groups and two control groups (without vehicle group) with three replicates. After anesthetizing the fish with MS22 (40 mg L<sup>-1</sup>) and weighing, B[a]P (5, 10, 50 mg/kg of body weight dissolved in corn oil) was intraperitoneally injected [32] weekly (divided into two doses) for two weeks. Sampling was carried out on the 1st, 3rd, 7th and 14th days, and the blood was collected from the caudal vein using heparinized syringes and transferred into ice-chilled vials. The blood was centrifuged (200 g, 15 min) and collected plasma was stored at  $-80$  °C. Then, the fish was sacrificed by decapitation and the livers were dissected out (All experiments were performed according to Khorramshahr University of Marine Science and Technology Animal Scientific Procedure Act.). One part of the livers was immediately frozen in liquid nitrogen and stored at  $-80$  °C. Other parts of the liver were kept in crayotubes containing DMSO and PBS (1:9), and frozen in liquid nitrogen.

### 2.3. Antioxidant capacities

All antioxidant enzyme activities were measured by the spectrophotometry at 25 °C. Frozen liver samples were homogenized in 0.1 M sodium phosphate buffer (pH 7.5) with the ratio of 1:10 w/v and centrifuged at 12,000 g at 4 °C for 15 min, and the supernatants were used for assaying SOD, CAT and GPx and GST activities. The total protein content in each sample was determined according to the Lowry method [33]. The SOD activity was measured by commercially available kits (ZellBio GmbH, Germany) using an indirect inhibition assay of nitroblue tetrazolium (NBT) reduction method [34]. All data were expressed as the unit of SOD activity per mg of protein. The GPx and CAT activities were examined according to the method of Hafeman [35] and Aebi [36], respectively.

### 2.4. Hepatic enzymes assay

The level of aspartate aminotransferase (AST) and alanine transaminase (ALT) enzymes in the serum samples were analyzed using an auto-analyzer (Technicon, RA1000, USA) and commercial kits (Pars Azmoon, Tehran, Iran) according to the method of Reitman and Frankel [37]. Also the level of alkaline phosphatase (ALP) was assayed by the method of Babson [38].

### 2.5. CYP1A gene expression

The total RNA was extracted from thawed liver samples (25 mg) in controls and treatment groups using an YTA RNA Extraction kit (Yekta Tajhiz Azma®, Tehran, Iran), according to the manufacturer's instructions. The quantity and quality of the RNA extracted were determined by NanoDrop spectrophotometry (Thermo Scientific NanoDrop 2000, USA) (wavelengths, 260 and 280 nm) and the agarose gel electrophoresis. The RNA extracted (1  $\mu$ g) was reversely transcribed into cDNA with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, K1622, USA), following the manufacturer's instructions. All cDNA reactions were diluted 10-fold before Q-PCR amplification. Specific primers used to amplify the sequence of CYP1A by PCR were as the following: forward, 5'-CACTGCACCACAAAAGACAC-3'; reverse, 5'-GGATGATGGCTCTTTCCACA -3'; The reference gene was  $\beta$ -actin protein of *Liza aurata* with the following primers: forward, 5'-TTCCTCG GTATGGAGTCCT-3'; reverse, 5'-TGGGGCAATGATCTTGATCTT-3' [39]. Quantitative real-time PCR was carried out in final volumes by using an ABI 7300 thermal cycler (Applied Biosystems, USA) with Maxima SYBR Green/ROX qPCR Master Mix (2 $\times$ ) (K0222; Fermentas International Inc., Canada) for the detection. The sample amplifying was performed in the reaction mixture (25  $\mu$ L) containing 0.3  $\mu$ M of forward and reverse primers, 12.5  $\mu$ L of Maxima SYBR Green/ROX qPCR Master Mix (2 $\times$ ), DNA template  $\leq$  500 ng/reaction, and nuclease-free water. For each primer pair, the NTC (no template) was used. Instrument setting for all amplification reactions was as the following profile: incubation at 95 °C for 1 min, followed by 40 cycles of 95 °C for 1 min, 58 °C for 10 s and 72 °C for 12 s, according to the melting curve analysis. Gene expression was shown as a fold change between the level of expression in the control and treatment groups and calculated using the  $2^{-\Delta\Delta Ct}$  method [40].

### 2.6. Comet assay

Alkaline version of comet assay was performed according to Ardeshtir et al [19]. Briefly, liver samples were homogenized in PBS and centrifuged (142 g for 10 min at 4 °C). The pellets were dispersed in PBS (1 ml) and the samples were centrifuged again (93 g for 7 min at 4 °C). Then, the pellet was washed gently with PBS and resuspended in PBS (1 ml) and passed through a 100  $\mu$ m nylon cell strainer (BD; Franklin Lakes, NJ) and kept on ice. Cellular viability was assessed by trypan blue dye exclusion and always was more than 95%. Prepared isolated cells were embedded in 50 mL low melting agarose (75%) and layered on conventional slides, predipped in 1% normal-melting-point agarose. The slides were immersed in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris-HCl, 1% Triton X-100 and dimethylsulfoxide 10%, with final pH of 10) and kept for 12–18 h at 4 °C to lyse the cells and allow DNA unfolding. The electrophoresis (25 V; 300 mA, 25 min) treatments were performed in an ice bath and darkness by electrophoresis buffer [NaOH (0.4 g/mL) and EDTA (0.7 g/mL) were added to 500 mL dH<sub>2</sub>O with final pH of 13]. After that, the slides were washed in neutralization buffer (Tris base solution (0.048 g/mL) with pH 7.5 and fixed in cold-pure ethanol. Then the slides were dried at room temperature and stained with SyberGreen (1  $\mu$ L/ml stain was dissolved in TE buffer) immediately before the observation. The comets were observed and photographed by an Axioscop-40 FL fluorescent microscope (Carl Zeiss, Jena, Germany) equipped with MRc-5

CCD camera. For the analysis of comet morphology, CellProfiler® software 2.0 (revision: 10997 for Windows) was used according to previous studies [19,41].

## 2.7. Statistical analysis

Before all statistical analyses, the data were tested for normality via the Kolmogorov-Smirnov test and quantitative data were presented as mean  $\pm$  standard deviation. One-way and two-way ANOVA with Multiple Comparisons Test were used to compare the different groups, followed by Tukey's test. Multiple regression analyses were performed on all measured variables in treatment groups to investigate possible correlations between various biological responses. The significance level was set at  $P < 0.05$ . All analyses were performed using OriginPro 2016, Version b9.3.226.

## 3. Results

### 3.1. Antioxidant capacities

Results showed that B[a]P exposure could significantly increase SOD activity compared to the control group, especially on the 7<sup>th</sup> and 14<sup>th</sup> days in the fish liver ( $P < 0.05$ ) (Fig. 1). However, there was no significant difference between the three exposure groups in this case ( $P > 0.05$ ) (Fig. 1). The CAT activity significantly decreased in the 1<sup>st</sup> day and significantly increased on the 3<sup>rd</sup> to 14<sup>th</sup> days ( $P < 0.05$ ) (Fig. 1). There was significant difference in CAT activity between three groups exposed to B[a]P including 10 mg/kg > 50 mg/kg > 5 mg/kg ( $P < 0.05$ ) (Fig. 1). The GPX activity significantly increased on the 7<sup>th</sup> and 14<sup>th</sup> days after exposure to 10 mg/kg B[a]P ( $P < 0.05$ ) (Fig. 1), whereas its activity increased on the 3<sup>rd</sup> to 14<sup>th</sup> days after exposure to 50 mg/kg B[a]P ( $P < 0.05$ ) (Fig. 1). The GPX activity was significantly higher than other doses ( $P < 0.05$ ) (Fig. 1) after exposure to 50 mg/kg B[a]P.

### 3.2. Hepatic enzymes assay

B[a]P exposure caused a significant increase in the fish liver ALT and AST in the three exposure groups on the 1<sup>st</sup> to 14<sup>th</sup> days ( $P < 0.05$ ) (Fig. 2). However, the increase of ALT levels depends on the increased doses of B[a]P ( $P < 0.05$ ) (Fig. 2). After the exposure of fish to 5 mg/kg and 10 mg/kg of B[a]P, ALP level significantly increased ( $P < 0.05$ ) (Fig. 2). However, the ALP level significantly decreased after the exposure to the high dose of B[a]P on the 14<sup>th</sup> day compared to the control group ( $P < 0.05$ ) (Fig. 2).

### 3.3. CYP1A gene expression

The results of CYP1A gene expression showed that this expression significantly increased after the exposure to B[a]P compared to the control group (Fig. 3) ( $P < 0.05$ ). This increase was completely in a time- and dose- depend manner, especially in the high dose (Fig. 3) ( $P < 0.05$ ).

### 3.4. Comet assay

The measurement of comet tails in different B[a]P exposure groups showed that this parameter significantly increased on the 3<sup>rd</sup> to 14<sup>th</sup> days ( $P < 0.05$ ) (Fig. 4). The highest comet tail was observed after exposure to 50 mg/kg B[a]P ( $P < 0.05$ ) (Fig. 4).

## 4. Discussion

In recent years, concerns about the harmful effects of environmental pollution such as PAHs on aquatic wildlife have arisen. In a general scenario, these contaminants in aquatic organisms, just like

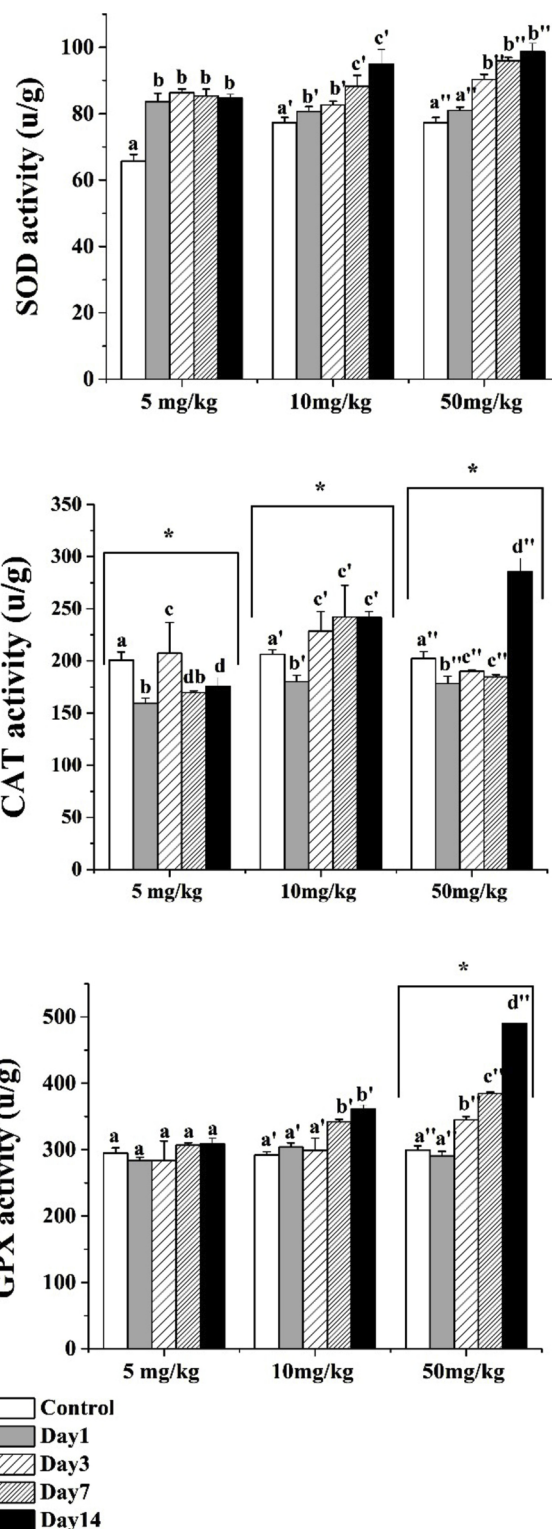


Fig. 1. Antioxidant enzymes activity (SOD, CAT and GPX) in the liver of Klunzinger's mullet (*Liza klunzingeri*), exposed to the sublethal doses of B[a]P for 14 days. Different lower cases or capital letters indicate significant differences in each group, and the star indicates a significant difference between three exposure groups ( $P < 0.05$ ). The values are given as mean  $\pm$  SD.

mammalian, can stimulate ROS production, antioxidant defense and oxidative damage [42]. In aquatic organisms such as fish, this pathway is much less known in these aspects, particularly in toxicology events and the relationship of oxidative damage with the disease [43,44].

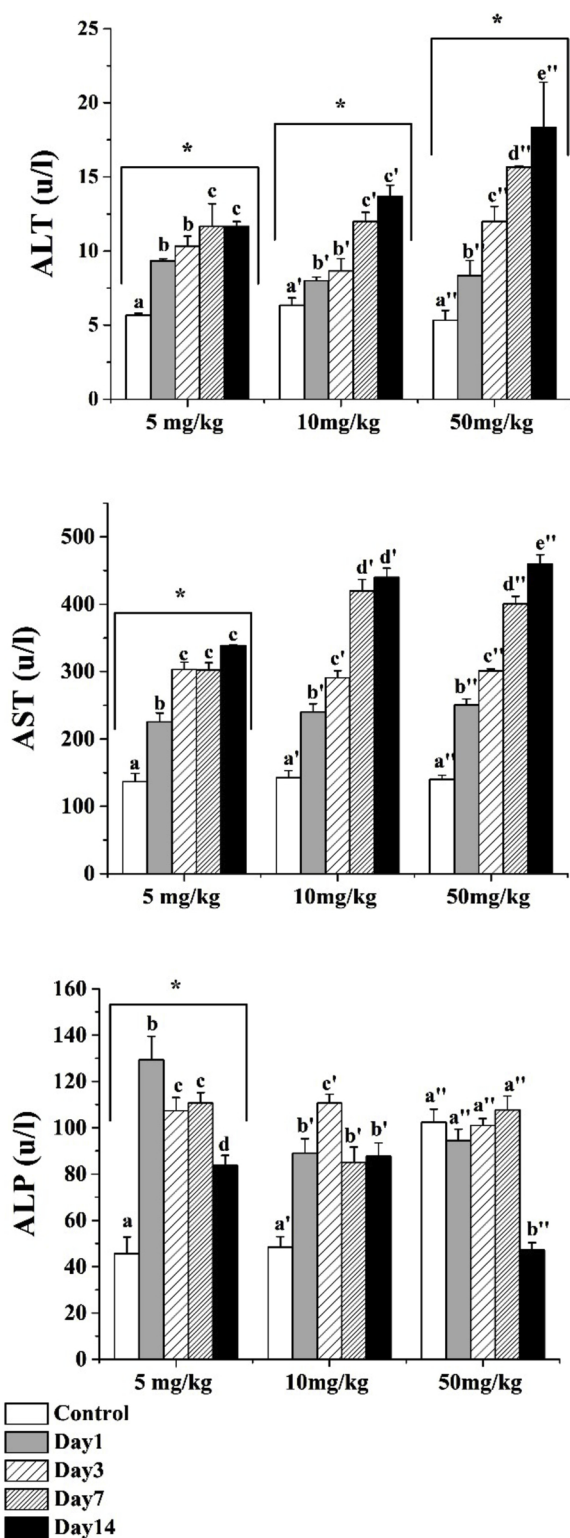


Fig. 2. Alterations in the liver enzymes (ALT, AST, and ALP) of Klunzinger's mullet (*Liza klunzingeri*) exposed to the sublethal doses of B[a]P for 14 days. Different lower cases or capital letters indicate significant differences in each group, and the star indicates a significant difference between three exposure groups ( $P < 0.05$ ). The values are given as mean  $\pm$  SD.

Therefore, the investigation of different fish species exposed to PAHs can assist us to find out the differences in their responses [45]. This study tried to investigate oxidative stress responses after exposure to sublethal doses of B[a]P in Klunzinger's mullet.

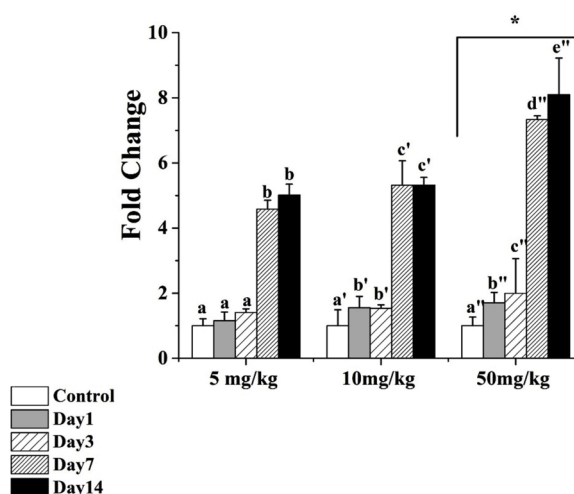


Fig. 3. CYP1A mRNA expression relative to  $\beta$ -actin in the liver of Klunzinger's mullet (*Liza klunzingeri*) exposed the sublethal doses of B[a]P for 14 days. Different lower cases or capital letters indicate significant differences in each group, and the star indicates a significant difference between the three exposure groups ( $P < 0.05$ ). The values are given as mean  $\pm$  SD.

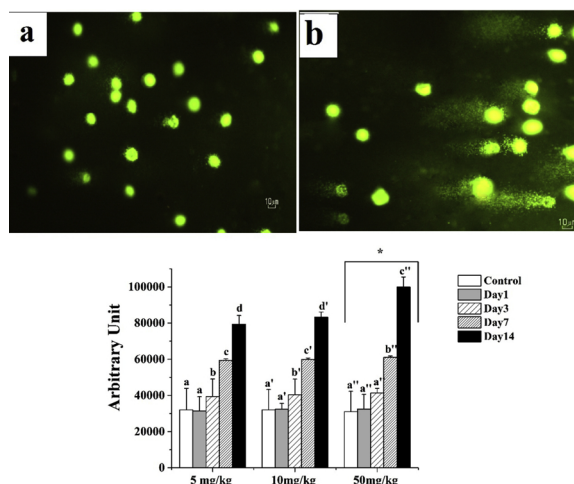


Fig. 4. Top: Comet assay. The liver cells from the Klunzinger's mullet (*Liza klunzingeri*) exposed to the sublethal doses of B[a]P were stained by SyberGreen. a: Control group. b: exposure to 50 mg/kg B[a]P on the 14<sup>th</sup> day. Bottom: Comparison of comet tails in the exposure and control groups. Different lower cases or capital letters indicate significant differences in each group, and the star indicates a significant difference among the three exposure groups ( $P < 0.05$ ). The values are given as mean  $\pm$  SD.

Results showed that SOD activity significantly increased after exposure to B[a]P compared to the control group. However, this increase was in a time-dependent manner and not dose-dependent manner. Moreover, increasing activity of CAT and GPX was observed after exposure to B[a]P. CAT activity increased obviously in a time- and dose-dependent manner (except on the 1st day). A key factor in controlling the cellular ROS level is antioxidant enzymes [46]. The sudden decrease in CAT activity on the first day indicated that the fish liver started to be faced with an oxidative challenge. Increasing antioxidant enzymes may result from the oxidative stress through excessive ROS production, especially a considerable amount of  $O_2^-$  and hydrogen peroxide ( $H_2O_2$ ) which need to be converted by these antioxidant enzymes [13]. Under combined hypoxia stress, the activities of this enzyme often increase because of the upregulation mediated by ROS to maintain cellular redox equilibrium [47]. After the exposure to PAHs, similar results were also found in Atlantic cod [48], European eel [49]. However, Yu

et al. [46] reported the decreased SOD activity in the liver of orange-spotted groupers (*E. coioides*) after the exposure to B[a]P [46]. These different results may be due to the inactivation effect of elevated ROS levels on SOD activity [46,50]. Although GPX activity increased after the exposure, increasing activity was weaker compared to CAT and SOD activities. The CAT and GPX are the catalyzer of the dismutation reaction of H<sub>2</sub>O<sub>2</sub> whereas the phospholipid hydroperoxide GPX has a cytosolic location, and therefore, its role is considered to be complementary, disposing of free radicals in their respective cell locations [51].

According to the results, intraperitoneal injection of B[a]P increases the liver enzymes (except ALP on the 14th day) compared to the control group. Many previous studies reported that these liver-specific enzymes are sensitive markers for hepatotoxicity and histopathologic changes [52]. The increased AST, ALT, and ALP activities represent liver damage resulting from the oxidative stress and the alteration in the permeability of the liver membrane [53]. It can be said that the severity of oxidative damage caused a significant decrease in ALP levels on the 14th day.

Among the P450 cytochrome family, the CYP1A subfamily is the most important case study [54,55]. This P450 cytochrome is induced by various contaminants including PAH, and therefore P450 activity is widely used as a biomarker for PAH effects on fish and other aquatic organisms [56,57]. In this study, the B[a]P exposure could significantly increase CYP1A expression, especially in high-dose exposure. After B[a]P exposure, the production of BDPEs or other metabolites could increase the activity and expression of cyp1a. In addition, this expression can be related to DNA damage [57]. The strong and significant correlations between cyp1a expression and DNA damage are evidence to support this claim (Fig. 5) ( $P < 0.05$ ).

In this study, DNA damage significantly increases in a time-dependent manner. The occurrence of DNA damage after the exposure to PAHs has been demonstrated in previous studies [58,59,57,60]. These

studies showed that the DNA damage can be caused by PAHs exposure, activated metabolites or by the ROS produced during biotransformation [57]. Correlation analysis between all measured parameters showed significant and positive relationships in most of these cases (Fig. 5) ( $P < 0.05$ ). These observations and using a lot of biomarkers can provide comprehensive data for understanding PAHs toxicology in fish.

In conclusion, the present study has shown that the fish exposed to the sublethal doses of B[a]P could increase antioxidant enzymes (including SOD, CAT and GPX) and liver biochemical enzymes (including ALT, AST and ALP). Also, CYP1a gene expression increased in a time-dependent manner in fish liver. In addition, the DNA damage detected in the liver cells after the exposure to sublethal doses of B[a]P was probably caused by reactive metabolites of this contaminant, indicating the efflux of these metabolites from the liver as the most metabolizing organ of fish. The strong and significant correlations between these parameters confirm that they are sensitive biomarkers in Klunzinger's mullet exposed to B[a]P. It may be some part of toxicokinetic pathways of the B[a]P in marine fish. For better elucidating the biotransformation mechanisms and introducing better biomarker of B[a]P, it is suggested that the focus on unknown PAH biotransformation pathways, such as the expression and activity of ABC transporters correlated to PAH, should be carried out in the future.

### Declaration of Competing Interests

The authors declare no conflict of interest.

### Declaration of Competing Interest

The authors report no declarations of interest.

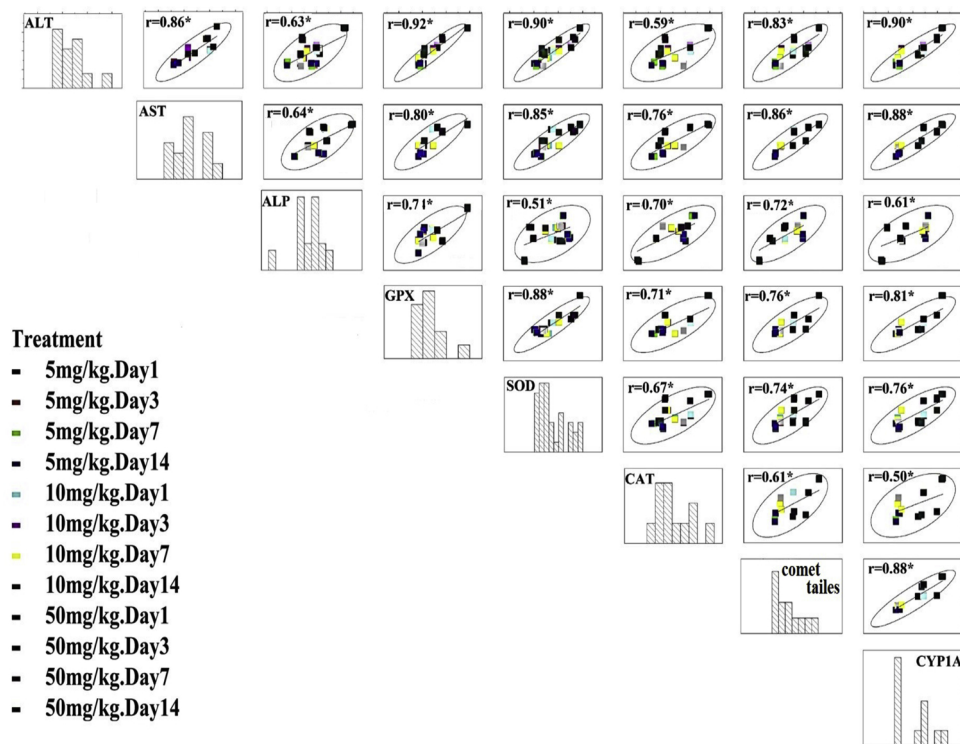


Fig. 5. Correlation coefficients between all liver parameters tested in Klunzinger's mullet (*Liza klunzingeri*) exposed to the sublethal concentrations of B[a]P. The sign (\*) shows  $P < 0.05$  based on Pearson's test.

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