The cytotoxic effects of different polycyclic aromatic hydrocarbons on the antioxidant function of the cultivated hepatocytes from *Acanthopagrus arabicus*: a comparative study Insert Title here

Samira Jalalipour
Master of Science, Faculty of Oceanography, Khorramshahr University of Marine Sciences and Technologies, Khorramshahr, Iran, Email: dr.samira.jalalipoour761@gmail.com

ABSTRACT

The present study aimed to compare the toxicity of different polycyclic aromatic hydrocarbons, including naphthalene, phenanthrene, pyrene, and benzo(a)pyrene (B(a)P), on viability and the antioxidant function of cultivated hepatocytes from Arabian sea bream (Acanthopagrus arabicus) in vitro. Cultivated hepatocytes from A. arabicus were exposed to different but equal concentrations of four pollutants, including 0 (control), 10-2, 10-1, 1, 10, 102 and 103 µg/ml (in 0.01% DMSO) for 48 h. The results showed that the sensitivity of cultivated hepatocytes to all four pollutants increased in a dose-dependent manner; although, the toxicity of low concentrations of all four pollutants did not show a significant difference with the control cells. Cultured hepatocytes exposed to the highest concentrations of all studied pollutants showed the lowest levels of total antioxidant capacity (TAC) and glutathione content (GSH) and the highest levels of lipid peroxidation (LPO) and alanine transaminase (ALT) activity after 48 h. Naphthalene and B(a)P showed the highest and lowest toxicity, respectively. The level of cytotoxicity, cell viability and the level of antioxidant enzymes as well as liver damage indicator enzymes in the present study were mainly affected by naphthalene followed by phenanthrene, and pyrene and B(a)P had less effect. In conclusion, although all PAHs are able to suppress the antioxidant defense system, PAH compounds with lower molecular weight and fewer benzene rings have a more potential to inhibit antioxidants and increase the level of lipid peroxidation due to their more ability to pass through cell membranes.

Keywords: *Naphthalene, Phenanthrene, Pyrene, Benzo(a)pyrene, Acanthopagrus arabicus, total antioxidant capacity, liver enzymes, glutathione*

1. *INTRODUCTION*

The progress of industries has led to an increased environmental pollution by natural or artificial pollutants resulting from human activities. The fact that the final destination of most pollutants is aquatic ecosystems has caused many concerns. Pollutants cause damage to biodiversity and ecosystems due to their ability to accumulate and magnify in living organisms [1].

Oil compounds are among the most important organic substances that enter the marine environment mainly through shipping activities, urban and industrial wastewater discharges, oil discoveries, oil spills, fossil fuels and natural leaks [2]. Polycyclic aromatic hydrocarbons (PAHs) are among the persistent organic oil pollutants in the environment, whose carcinogenic, mutagenic and teratogenic properties have been proven [3]. These compounds are easily absorbed by the organism due to their high solubility in lipid membranes [4]. Naphthalene, phenanthrene, pyrene and benzo(a)pyrene are among the most important PAHs with two, three, four and five hydrocarbon rings, respectively. These compounds are able to dissolve in the entire water column, which are absorbed by the suspended particles in the water or enter the sediments.

PAHs are dissolved in the water column and absorbed by the suspended particles in the water or enter the sediments. They can also accumulate in crabs, crabs, molluscs and fish, where they remain for some time or even turn into active carcinogenic metabolites [3]. These compounds have a high tendency to bioaccumulate in the liver tissue and cause oxidative stress [5]. Considering that liver cells have the ability to detoxify toxins and pollutants and/or convert them into less harmful metabolites, the liver is the most important indicator to evaluate the toxicity of environmental pollutants in terrestrial and aquatic organisms [6].

There is a balance between the production and removal of reactive oxygen species (ROS) in natural conditions. Lack of balance causes accumulation of oxygen or nitrogen species (RNS) which in turn lead to oxidative stress in living organisms. Living organisms are equipped with antioxidant defense system to deal with oxidative stress. However, the activity level of antioxidant parameters in liver tissue is higher than other tissues [7]. Therefore, the present study aimed to compare the toxicity of different hydrocarbons with various number of benzene rings including naphthalene, phenanthrene, pyrene and benzo(a)pyrene on antioxidant factors of liver cells from Arabian sea bream (*Acanthopagrus arabicus*) including catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), the level of lipid peroxidation (LPO) and liver enzymes including alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were used.

2. MATERIALS AND METHODS

2.1 Fish preparation and maintenance

In the present study, 9 healthy immature Arabian sea bream (*A. arabicus*) (220 ± 35 gr body weight and 15 ± 3.5 cm body length) were caught from the sugar cane estuary in the northwest of the Persian Gulf and transported alive in a tank equipped with oxygen capsules to the laboratory. Before the start of the experiment, the fish were acclimatized to laboratory conditions to reduce stress and were fed twice a day during this period.

2.2 Tissue sampling and cell culture

After anesthetizing the fish with 2-phenoxyethanol solution (0.2%), the fish body surface was sterilized with 70% ethanol. Then, the fish were dissected and their livers were separated under sterile conditions. The liver tissue was then moved to phosphate buffered saline (PBS) without Ca^{2+} (containing 100 units of penicillin, 20 mg/ml streptomycin, 10 µg/ml enrofloxacin, 25 µg/ml amphotericin B) and incubated at room temperature for 30 min. This solution was renewed 3 more times. The liver was then divided into small pieces in a fresh phosphate buffered saline (PBS) solution. Then, 0.1% collagenase IV solution in PBS was added to the tissue pieces to digest the tissues completely and incubated at room temperature for 20 min. Finally, the digested tissues were passed through a 70 µm nylon cell filter (Corning®) to ensure complete separation of cells [8].

After the complete detachment of the cells, the cell suspension was centrifuged at 200 rpm for 8 min. Then the supernatant was removed and the RPMI 1640 culture medium (containing 15% bovine serum albumin (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, 5 mM NaHCo3 and 0.5% ITS (insulin, transferrin, selenium)) was added to the cell pellet and centrifuged again. The above steps were performed once more.

In order to determine the cell viability, cell counting was performed using trypan blue exclusion test. This test is based on the fact that living cells remain transparent due to the blocking of the vital dye trypan blue (Sigma-Aldrich.UK). Instead, trypan blue can enter dead cells and turn them blue. 10 µl of cell suspension was mixed with 10 µl of trypan blue dye solution (0.1 vol% trypan blue in 0.15 M PBS) on a hemocytometer slide, and the number of stained (dead) cells and unstained (living) cells were counted using an inverted microscope. Then the cell viability percentage was calculated using the following formula [8].

Cell viability percentage= 100 x total number of counted cells/number of dead cells

After ensuring that more than 90% of the cells are alive, RPMI 1640 culture medium containing 20% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 mM NaHCo3 and 0.5% ITS was added to the cell pellet in 25 cm^3 flasks and incubated at 27°C for two weeks. The total number of cells in a 25 cm^3 flasks was adjusted to 4 x 10^6 cells. The cells gradually started to increase in the incubator and attached to the bottom of the flask. When the density of cells in the flask reached more than 70%, cell passage was performed to prevent cell death [8].

2.3 Cytotoxicity of naphthalene, phenanthrene, pyrene and benzo(a)pyrene

The cytotoxicity of naphthalene, phenanthrene, pyrene and B(a)P and the effect of these compounds on the liver cells viability were measured using the MTT method [8]. The MTT test is a colorimetric method based on the reduction and breaking of yellow tetrazolium crystals by succinate dehydrogenase enzyme and the production of insoluble blue formazan crystals. The succinate dehydrogenase enzyme is located in the

mitochondria of living cells. Therefore, the production rate of insoluble formazan crystals is higher in more metabolically active cells [9].

To prepare MTT solution with a concentration of 5 mg/ml, 50 mg of MTT powder was dissolved in 10 ml of 0.15 M PBS. This was a stock solution. The stock solution was diluted 10 times with PBS to obtain a working solution with a concentration of 0.5 mg/ml.

The same concentrations of all four studied pollutants including 0 (control), 10^{-2}, 10^{-1}, 1, 10, 10^{2} and 10^{3} µg/ml (dissolved in 0.01% DMSO) were added to a 96-well microplate containing $1x10^{6}$ cells/well. The selection of the concentration range was based on the reported amount of these compounds in the aquatic environment as well as the values used in previous studies [10-13]. After 24 h, cells were stained with MTT solution (0.5 mg/ml) and incubated for 3 to 5 h at 30°C. Then, the supernatant was replaced with 100 µl of DMSO solution and thoroughly mixed for 5 min. Finally, the results were read by a microplate reader at 570 nm and the amount of cytotoxicity was calculated using the following formula [9]:

Cytotoxicity percentage= Mean optical absorbance of treated cells/Mean absorbance of control cells × 100

Finally, the cells were transferred to 24-well microplates (10^{6} cells/well) and incubated at 30°C for 24 h. After cell adherence, the supernatant was replaced with a fresh culture medium containing different concentrations of studied pollutants (0 (control), 10^{-2}, 10^{-1}, 1, 10, 10^{2} and 10^{3} µg/ml (dissolved in 0.01% DMSO)) and incubated at 30°C for 48 h. Each treatment was run with 10 replicates. The control cells were not exposed to any of the pollutants or any other chemicals. Cell sampling was conducted after 48 h.

2.4 Biochemical analyses

2.4.1 Measurement of antioxidant parameters

2.4.1.1 Measurement of total antioxidant capacity (TAC)

Total antioxidant capacity is often used to evaluate the overall response of all antioxidant parameters of an organism against free radicals produced [14]. In the present study, the total antioxidant capacity was measured according to Benzie and Strain [14] based on the ability of the antioxidant system for iron reduction (Ferric Reducing Antioxidant Power (FRAP)). In this method, the rate of conversion of 3-valent iron (Ferric) to 2-valent iron (Ferrous) was measured using colorimetric method. First, the FRAP reaction mixture (pH: 7.2) (containing 2.5 ml of a 10 mmol/l TPTZ (2,4,6-tripyridyl-s-triazine; Sigma) solution in 40 mmol/l hydrochloric acid, 2.5 ml of 20 mmol/L iron chloride, and 25 ml of 0.3 mmol/l acetate buffer) was prepared. Then, 1.5 ml of FRAP working solution was mixed with 50 µl of cell suspension or standard solution (1 mmol/l of iron sulfate (FeSO4)). After 10 min, the optical absorbance was recorded at 593 nm at 37 °C. The result was presented as as µmol equivalent of FeSO4.

2.4.1.2 Measurement of glutathione content

In the present study, the glutathione content was measured according to Paglia and Valentine [15]. First, 10% trichloroacetic acid was added to the samples and the resulting suspension was centrifuged at 10,000 g for 10 min at 4°C. Then, 50 µl of the supernatant was transferred to a 96-well microplate and 230 µl of 0.4 M TRIS (pH=8.9) and 20 µl of 2.5 mM DTNB in 25% methanol were added to each well. The optical absorbance of the samples was read at 415 nm and the concentration of GSH was expressed as µg/mg of protein.

2.4.1.3 Measurement of lipid peroxidation (LPO)

Reactive oxygen species often lead to the peroxidation of unsaturated fatty acids and their conversion to active aldehydes such as malondialdehyde (MDA). Therefore, MDA is considered as an indicator of lipid peroxidation. In the present study, MDA concentration was measured by spectrophotometric method according to Buege and Aust [16]. First, 2 ml of thiobarbituric acid (TBA)-trichloroacetic acid (TCA)-HCL reagent solution (0.37% (w/v) TBA, 15% (w/v) TCA and 0.25% (w/v) HCL)) was added to each well containing 1 ml of cell suspension in a 96-well microplate. After heating for 30 min, the solution was cooled in an ice bath and then centrifuged at 1000g for 10 min. After adding 1 ml of n-butanol, the solution was vigorously shaken and finally the optical density (OD) was read at 535 nm. The results were reported as nmol TBARS/mg protein.

2.4.2 Evaluation of liver index enzymes

2.4.2.1 Aspartate aminotransferase (AST) enzyme

In the present study, the level of aspartate aminotransferase enzyme was measured using the calorimetric method using the Pars Azmoun commercial kit according to Reitman and Frankel [17]. The conversion of amino acid aspartate to amino acid oxaloacetate by SGOT enzyme substrate is the basis of this method. A

brown colored complex was produced by adding a colored reagent in an alkaline environment. Then, the optical density (OD) was read at 505 nm.

2.4.2.2 Alanine transferase (ALT) enzyme

In the present study, the alanine transferase enzyme level was measured using the calorimetric method based on Reitman and Frankel [17] using the Pars Azmoun commercial kit. Based on this method, amino acid alanine is converted to amino acid pyruvic by SGPT enzyme, which produces a brown colored complex after adding a color reagent in alkaline medium. Then, the optical density (OD) was read at 505 nm.

2.4.2.3 Alkaline phosphatase (ALP) enzyme

In the present study, the level of alkaline phosphatase enzyme was measured by spectrophotometric method according to Krogdahl et al. [18] using the Pars Azmoun commercial kit. This method was based on the hydrolysis of the substrate solution consisting of p-nitrophenyl phosphate and buffer by an enzyme in the presence of magnesium and producing a yellow-colored product called p-nitrophenol. Then, the optical density (OD) was read at 405 nm.

2.5 Data analysis

In the present study, all experiments were run in 10 replicates. All data were presented as mean ± standard deviation. Data analysis and comparison between groups were conducted using one-way ANOVA method followed by Tukey's test. A confidence level above 95% ($P<0.05$) was considered.

3. RESULTS

3.1 Hepatocytes primary culture

The cell viability after isolation and before incubation (Fig. 1) was determined using trypan blue exclusion test to be more than 90%. The liver cells gradually started to adhere to the culture plate and form colonies after three days of incubation (Fig. 1). The cultivated hepatocytes were epithelioid like, polygonal or spindle shaped after adhesion (Fig. 1). Liver cells reached 90% monolayer density after 15 days of incubation (Fig. 1).

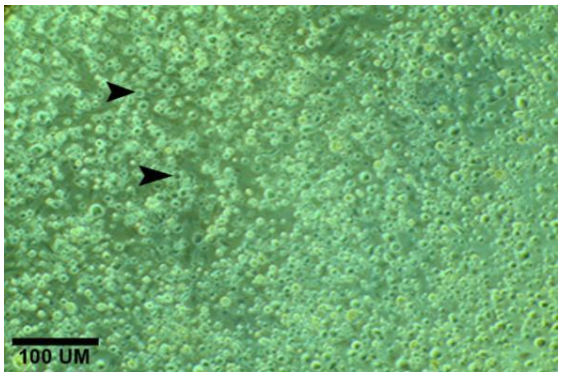

Fig. 1. Micrographs of cultivated hepatocytes from *Acanthopagrus arabicus*

3.2 MTT results

The studied PAHs (including naphthalene, phenanthrene, pyrene and B(a)P) used in the present study with the following concentrations: 0 (control), 10^{-2}, 10^{-1}, 1, 10, 10^{2} and 10^{3} µg/ml. The toxicity level and the cell viability are presented in Fig. 2. According to the MTT results, the toxicity of studied PAHs decreased with the increase in the number of benzene rings. Accordingly, naphthalene showed the highest toxicity and the lowest percentage of the cell viability was measured in naphthalene treated cells ($P < 0.05$; Fig. 2). The toxicity of benzoalphapyrene was significantly lower than other studied compounds, and the percentage of survival of cells treated with this pollutant was significantly higher than the other studied pollutant ($P < 0.05$; Fig. 2).

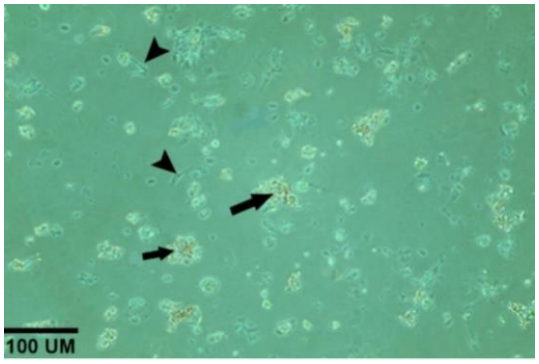

Fig. 2. The percentage of cell viability and cytotoxicity of pollutants in cultivated hepatocytes from *Acanthopagrus arabicus*

3.3 Evaluation of antioxidant parameters

3.3.1 Measurement of total antioxidant capacity (TAC)

Fig. 3 shows the amounts of the total antioxidant capacity in control and pollutants (naphthalene, phenanthrene, pyrene and B(a)P) treated cells after 48 h of exposure. According to the results, the amount of TAC in cells treated with the lowest concentration of all studied pollutants (10^{-2} µg/ml) was not significantly different from the control group ($P < 0.05$; Fig. 3). TAC decreased in cells treated with the other concentrations of pollutants. Total antioxidant capacity in cells treated with naphthalene, especially with high concentrations, was significantly higher than other pollutants ($P < 0.05$; Fig. 3). The amount of TAC in cells treated with different pollutants was as follows: Naphthalene < Phenanthrene < Pyrene < B(a)P.

The lowest amount of TAC was measured in cells treated with the highest concentration of all studied pollutants (10^{3} µg/ml) after 48 h of exposure ($P < 0.05$; Fig. 3A). 10^{3} µg/ml of naphthalene, phenanthrene, pyrene and benzo(a)pyrene resulted in 91, 89, 65 and 59% reduction of TAC, respectively ($P < 0.05$; Fig. 3).

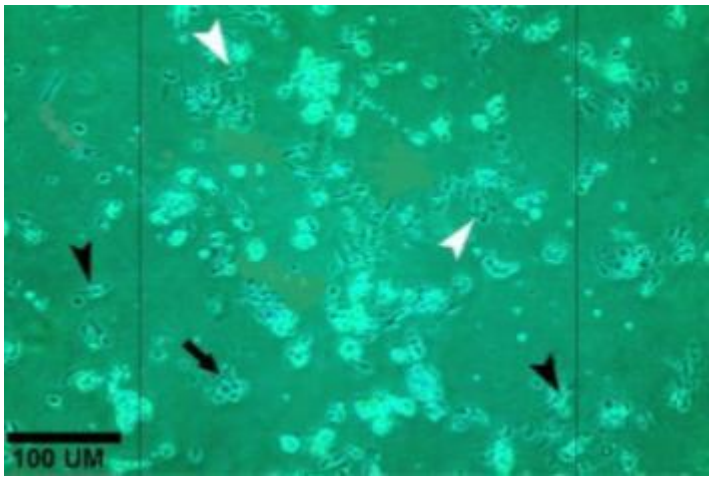

Fig. 3. Total antioxidant capacity. Data are shown as mean ± standard deviation. Small letters indicate significant differences between different pollutants in each concentration group ($P<0.05$)

3.3.2 Glutathione content

Fig. 3 shows the level of glutathione content of control cells and cells treated with different concentrations of studied pollutants (naphthalene, phenanthrene, pyrene and B(a)P) after 48 h of exposure. According to Fig. 3, the lowest concentrations of all studied pollutants (10^{-2} and 10^{-1} µg/ml) did not change glutathione content of treated hepatocytes ($P>0.05$; Fig. 3). Other concentrations of naphthalene and phenanthrene led to a significant decrease in glutathione content of treated liver cells compared to the control ($P<0.05$; Fig. 3); although, no significant difference in glutathione content was measured among cells treated with different

concentrations of these two pollutants (except for the highest concentration) (P>0.05; Fig. 3). Exposure of hepatocytes with 10^3 µg/ml of naphthalene and phenanthrene led to a 62% and 43% decrease in glutathione content, respectively (P<0.05; Fig. 3). On the other hand, only the highest concentration of pyrene and B(a)P (10^3 µg/ml) led to a significant reduction of glutathione content of treated liver cells (P<0.05; Fig. 3).

3.3.3 Lipid peroxidation

According to Fig. 3C, lipid peroxidation in cells treated with low concentrations (10^{-2}, 10^{-1} and 1 µg/ml) of studied compounds (naphthalene, phenanthrene, pyrene and B(a)P) was not significantly different with the control (P > 0.05; Fig. 3C). Although MDA concentration in cells treated with 10 µg/ml naphthalene was higher than others, it was not significantly different from cells treated with other pollutants (except B(a)P) (P > 0.05; Fig. 3C). The level of lipid peroxidation was significantly higher in cells treated with the highest concentration of studied pollutants (10^3 µg/ml) (P<0.05; Fig. 3C). The highest amount of MDA was recorded in the cells treated with high concentrations of naphthalene (10^2 and 10^3 µg/ml), which was significantly more than the others (P<0.05; Fig. 3C).

Fig. 3. Total antioxidant capacity (A), glutathione content (B) and lipid peroxidation (C) in cultivated hepatocytes from *Acanthupagrus arabicus* after 48 exposures to different concentrations of PAHs. Data are shown as mean ± standard deviation. Small letters indicate significant differences between different pollutants in each concentration group (P<0.05).

3.4 The activity of liver enzymes

3.4.1 Alanine aminotransferase activity

According to Fig. 4, the ALT activity in the cultivated hepatocytes exposed to 10^{-2} µg/ml of the studied PAHs showed no change compared to the control (P > 0.05; Fig. 4). Other concentrations of naphthalene and phenanthrene significantly increased the ALT activity in liver cells compared to other groups (P > 0.05; Fig. 4). The highest amount of ALT activity was measured in liver cells treated with different concentrations of naphthalene (P < 0.05; Fig. 4). The highest concentration of naphthalene (10^3 µg/ml) caused a 53% increase in the activity of ALT in treated cells (P < 0.05; Fig. 4). On the other hand, only the highest concentrations of pyrene (10^2 and 10^3 µg/ml) led to a significant increase of the activity of ALT in treated cells, after 48 h of exposure (P > 0.05; Fig. 4). The lowest level of ALT activity was recorded in liver cells treated with all concentrations of B(a)P (except its highest concentration) (P < 0.05; Fig. 4).

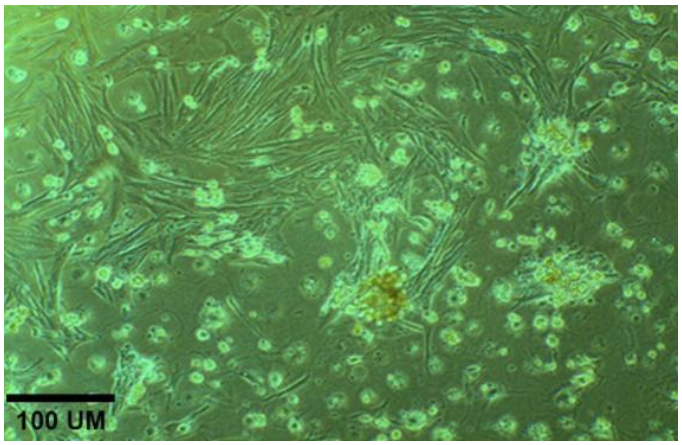

Fig. 4. The activity of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase enzymes in cultivated hepatocytes from *Acanthupagrus arabicus* after 48 exposures to different concentrations of PAHs. Data are shown as mean ± standard deviation. Small letters indicate significant differences between different pollutants in each concentration group (P<0.05)

3.4.2 Aspartate aminotransferase activity

According to Fig. 4B, exposure to low concentrations of studied PAHs (10^{-2} and 10^{-1} µg/ml) did not change the activity of AST (P > 0.05; Fig. 4B). Other concentrations of naphthalene and phenanthrene led to a significant increase in the activity of this enzyme in treated cells (P < 0.05; Fig. 4B). The highest level of AST activity was measured in hepatocytes treated with 10^3 µg/ml of naphthalene after 48 hours of exposure (P < 0.05; Fig. 4B). Although, the AST amount in the liver cells treated with pyrene and B(a)P was significantly

lower than naphthalene and phenanthrene after 48 hours of exposure; however, It did not show a significant difference with the control ($P > 0.05$; Fig. 4B).

3.4.3 Alkaline phosphatase activity

The ALP activity in the treated cultivated hepatocytes did not show a significant change compared to the control ($P > 0.05$; Fig. 4C); however, the highest concentration of naphthalene and phenanthrene (10^3 µg/ml) led to an increase ALP activity in treated liver cells ($P < 0.05$; Fig. 4C).

Fig. 4. The activity of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase enzymes in cultivated hepatocytes from *Acanthupagrus arabicus* after 48 exposures to different concentrations of PAHs. Data are shown as mean ± standard deviation. Small letters indicate significant differences between different pollutants in each concentration group ($P<0.05$).

4. DISCUSSION

The main goal of the present study was to compare the toxic effects of four PAH compounds with various molecular weights and different number of benzene rings, including naphthalene (with two benzene rings), phenanthrene (with three benzene rings), pyrene (with four benzene rings) and B(a)P (with five Benzene ring). Tuvikene [19] stated that PAHs with four to six benzene rings have greater toxicity potential than smaller PAHs. On the other hand, recently, many researchers believe that two- and three-ring PAHs have a greater ability to pass through cell membranes and therefore are more cytotoxic; whereas, four- to six-ring PAHs have more genotoxic potential [20, 21]. In the current study, the effect of naphthalene, phenanthrene, pyrene and B(a)P on cultivated hepatocytes from *A. arabicus* was assessed by evaluating antioxidant markers including TAC, glutathione content and lipid peroxidation and liver cell function markers (ALT, AST and ALP).

In the present study, although all four pollutants resulted in decrease in cell viability and function, naphthalene followed by phenanthrene were more toxic compared to pyrene and B(a)P. B(a)P had the lowest cytotoxicity compared to the other three studied PAHs and only the highest concentrations of B(a)P caused a significant change in toxicity and cell viability. Schirmer et al. [10] stated that among the sixteen PAHs studied, the cytotoxic effect of naphthalene on RTgill-W1 cell line from rainbow trout was significantly more than fluoranthene and pyrene after short-term exposure. On the other hand, B(a)P with the experimental concentrations was not cytotoxic at all [10]. According to Schirmer et al. [10], more water solubility and lipophilicity and easier passage through cell membranes are the main factors of most cytotoxicity of two- and three-ring PAHs. Mitchelmore and Chipman [22] also reported that there was no significant decrease in the viability of cultivated hepatocytes from rainbow trout exposed to concentrations of less than 200 µM B(a)P. Derakhshesh et al. [13] stated that B(a)P affected the viability and function of hepatocytes from *Epinephlus coioides* in concentrations of more than 2 x 10^3 µmol. Wessel et al. [23] also did not observed significant cytotoxicity in cultivated cells from *Solea solea* after 24 h exposure to 0.1 to 25 µmol of B(a)P. Schirmer et al. [24] reported that exposure to 288 µmol of B(a)P resulted in significant cytotoxicity in RTL-W1 hepatocytes from rainbow trout.

Pollutants such as PAHs are able to produce O^{-2} and H2O2 through biotransformation. If these two products are not metabolized, it leads to oxidative stress. Superoxide dismutase is an important antioxidant enzyme that catalyzes the inhibitor of lipid peroxidation, thereby preventing lipid peroxidation, and also converts superoxide radicals into H2O2 and O2. Other antioxidant enzymes such as catalase and glutathione peroxidase also convert the resulting H2O2 into water. In general, antioxidants play an important role in protecting the organism against oxidative stress. On the other hand, measuring all antioxidant components is very complicated, time-consuming and expensive. Measurement of total antioxidant capacity (including a set of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase, as well as macromolecules such as albumin, ceruloplasmin and ferritin) shows the cumulative effect of all antioxidants in plasma and body fluids, which is far more reliable than measuring antioxidant parameters individually [25].

In this study, the TAC level was reduced by all concentrations of studied pollutants, while only the highest concentrations of studied pollutants significantly reduced GSH content in treated cells. Several studies have reported the reduction of the components of the antioxidant system or the reduction of the total antioxidant capacity (TAC) when exposed to different PAH compounds. Similar to the present study, Derakhshesh et al. [13] reported that the levels of antioxidant enzymes such as superoxide dismutase and catalase in cultured hepatocytes of common grouper, *E. coioides*, decreased after 12 h of exposure to B(a)P. Yazdani [21] also reported a decrease in total antioxidant capacity and glutathione content in rainbow trout hepatocytes after 24

h of exposure to PAHs such as fluoranthene and B(a)P. Also, Pan et al. [26] reported that antioxidant enzymes such as superoxide dismutase and glutathione peroxidase in *Chlamys ferrari* are unable to fully metabolize O2 and H2O2 produced as a result of acute exposure to B(a)P and benzofluoranthene in a short period of time and were only able to slightly compensate the created oxidative stress. Therefore, O2 gradually accumulates in the environment and leads to an increase in the level of lipid peroxidation, followed by the induction of oxidative stress and damage to the cell morphology and function of cells. According to researches, antioxidant enzymes are not able to completely remove the intermediate products resulting from the metabolism of PAHs such as O2 and H2O2 [26, 27]. Monari et al. [28] also stated that the decrease in the activity of antioxidant enzymes when exposed to pollutants can be due to the increase in superoxide radicals produced in the living body. In the present study, with the increase in the concentration of studied pollutants, a decrease in total antioxidant capacity and glutathione level was observed after 48 h of exposure, which was associated with an increase in lipid peroxidation. Pan et al. [26] and Sole et al. [29] stated that the liver antioxidant system is usually able to control the stress caused by low concentrations of PAHs. But inhibition of liver antioxidant activity occurs when cells are exposed to higher concentrations of this pollutant. In general, organisms exposed to high concentrations of PAHs are not able to completely remove the pollutant from the cells, which resulted in oxidative stress in the organism [28, 29]. The decrease in the activity of antioxidant factors after exposure to PAHs is an important sign of disturbance in the antioxidant defense against oxidative damage caused by these pollutants [28].

In the present study, the lowest amount of total antioxidant capacity and glutathione content were observed in cells treated with naphthalene followed by phenanthrene. Similar to the results of the present study, Luís and Guilhermino [20] also reported that although both naphthalene and pyrene had toxic effects on common shrimp (*Palaemon serratus*) after 96 h of exposure, the ability of naphthalene to inhibit catalase, glutathione and glutathione peroxidase enzymes was significantly more than pyrene, while the genotoxic potential of pyrene was more than naphthalene. Corredor-Santamaría et al. [30] exposed *Aequidens metae* to different PAHs including naphthoflavone, naphthalene, phenanthrene and B(a)P for 72 h. they stated that the most genetic changes were observed in fish exposed to B(a)P, followed by naphthoflavone; while, naphthalene had the most inhibitory effect on the antioxidant system [30]. Pan et al. [26] also reported that the amount of catalase and superoxide dismutase enzymes in the hemolymph of *C. Ferrari* when exposed to benzofluoranthene was lower than B(a)P.

In the present study, exposure to higher concentrations (10^2 and 10^3 µg/ml) of studied PAHs led to a significant increase in the level of lipid peroxidation in cultured hepatocytes of *A. arabicus*. Increased production of reactive oxygen species and disruption of the enzymatic and non-enzymatic defense of the liver may cause the lipid peroxides activation [27]. Luís and Guilhermino [20] reported that levels of antioxidant enzymes such as catalase and glutathione peroxidase were significantly decreased and lipid peroxidation increased in common shrimp (*P. serratus*) exposed to naphthalene and pyrene. These researchers stated that due to the major role of catalase and glutathione in preventing lipid peroxidation, it seems logical to increase the level of LPO by reducing these enzymes. In the present study, the amount of total antioxidant capacity decreased significantly after exposure to the studied PAHs. Therefore, it seems that the decrease in the level of antioxidant defense in cultured liver cells after exposure to PAHs in the present study, led to the production of reactive oxygen species. On the other hand, the increase of free radicals led to the activation of lipid peroxides and lipid peroxidation, which in turn changes the fluidity and function of the cell membrane [26].

Although, all studied PAHs led to an increase in lipid peroxidation, the increase in lipid peroxidation level was significantly higher in cells exposed to naphthalene followed by phenanthrene. Luís and Guilhermino [20] reported that naphthalene had a more potential to increase lipid peroxidation levels in common shrimp (*P. serratus)* compared to pyrene. Pan et al. [26] also reported that the amount of lipid peroxidation in *Chlamys ferrari* exposed to benzofluoranthene was higher than B(a)P. Corredor-Santamaría et al. [30] also reported the highest level of lipid peroxidation in *Aequidens metae* exposed to naphthalene and phenanthrene. All these researchers believed that PAH compounds with lower molecular weight and fewer rings have a more potential for lipid peroxidation [20, 26, 30].

Metabolism of many pollutants occurs in the liver; therefore, measuring the activity of liver enzymes (such as ALT, AST and ALP) is a suitable indicator to evaluate its function against the toxins [31]. In the present study, the activity of ALT increased in cells treated with different concentrations of naphthalene and phenanthrene, while only the highest concentration of pyrene and B(a)P increased the ALT level. On the other hand, none of the pollutants in the present study caused a significant change in the levels of AST and ALP enzymes. The amount of these enzymes is related to the permeability of the cell membrane [32]. Pollutants affect the structure and function of enzymes involved in the citric acid cycle, which leads to a decrease in their

activity. As a result, the activity of transaminase enzymes increases to compensate for this defect [33]. Therefore, the increase in ALT enzyme in the present study can be to compensate for the disturbance in the citric acid cycle as a result of the metabolic disturbance caused by PAHs and the need for more energy to deal with stress. Kim et al. [34] reported that AST activity increased significantly *Seabastes schlegeli* after exposure to different concentrations of B(a)P. Derakhshesh et al. [13] also reported a significant increase in the amount of AST and ALT enzymes along with a decrease in the total protein in the liver cells from *E. coioides* after exposure to B(a)P and nonylphenol.

In the present study, the increase in ALT activity was more in cells treated with naphthalene than other studied pollutants. Pan et al. [26] stated that two- and three-ring PAHs are more soluble in the cell membrane due to their lower molecular weight and are more toxic to the cell.

Hepatic ALP plays an important role in stimulating glycogen synthesis and deactivating phosphorylase enzymes. Increase of ALP level in the liver leads to the inhibition or reduction of oxidative phosphorylation in the respiratory chain. In the present study, the level of ALP enzyme did not show a significant change in exposure to the studied pollutants. It seems that it was due to the lack of significant change in the morphology of liver cells, especially in the lower concentrations of the studied pollutants.

In conclusion, it seems that the increase in the production of hydroxyl free radicals beyond the ability of antioxidants to remove them leads to oxidative stress and further reduction of antioxidant enzymes. Reducing the level of antioxidant factors in turn leads to an increase in lipid peroxidases and lipid peroxidation. On the other hand, PAHs with lower molecular weight and fewer rings are seem to be more toxic [26, 20, 30].

REFERENCES

[1] Livingstone, D.R., Lemaire, P., Matthews, A., Peters, L.D., Porte, C., Fitzpatrick, P.J., Förlin, L., Nasci, C., Fossato, V., Wootton, N. (1995). Assessment of the impact of organic pollutants on goby (*Zosterisessor ophiocephalus*) and mussel (*Mytilus galloprovincialis*) from the Venice Lagoon, Italy: biochemical studies. Marine Environmental Research, vol. 39, nos. 1-4, p. 235-240.

[2] Medeiros, P.M., Bicego, M.C., Castelao, R.M., Del Rosso, C., Fillmann, G., Zamboni, A.J. (2005). Natural and anthropogenic hydrocarbon inputs to sediments of Patos Lagoon Estuary. Brazil Environment International, vol. 31, p. 77-87.

[3] Di Giulio, R.T., Hinton, D.E. (2008). The textbook of fishes. CRC Press.

[4] Chomczynski P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques, vol. 15, p. 532 -534.

[5] Selck, H., Handy, R.D., Fernandes, T.F., Klaine, S.J., Petersen, E.J. (2016). Nanomaterials in the aquatic environment: a European Union United States perspective on the status of ecotoxicity testing research priorities, and challenges ahead. Environmental Toxicology and Chemistry, vol. 35, p. 1055-1067.

[6] Segner, H. (1998). Isolation and Primary culture of teleost hepatocytes. Comp. Comparative Biochemistry and Physiology Part A, vol. 120, p. 71-81.

[7] An, R., Li, Y., Niu, X., Yu, H. (2008). Responses of antioxidant enzymes in catfish exposed to lipid crystals from E-waste. International Journal of Environmental Research and Public Health, vol. 5, no. 2, p. 99-103.

[8] Kemnitzer, W., Drewe, J., Jiang, S., Zhang, H., Zhao, J., Crogan- Grundy, C. (2007). Discovery of 4-Aryl-4 H-chromenes as a New Series of Apoptosis Inducers Using a Cell- and Caspase- Based High-Throughput Screening Assay. 3. Structure- Activity Relationships of Fused Rings at the 7. 8- Positions. Journal of Medicinal Chemistry, vol. 50, no. 12, p. 2858-2864.

[9] Momeni, H.R., Soliemani Mehranjani, M., Abnosi, M.H., Nasimi, P. (2010). Evaluation of viability in adult mouse spinal cord slices by MTT. Iranian Journal of Biology, vol. 23, no. 2, p. 267-274.

[10] Schirmer, K., Dixon, D.G., Greenberg, B.M., Bols, N.C. (1998). Ability of 16 priority PAHs to be directly cytotoxic to a cell line from the rainbow trout gill. Toxicol, vol. 127, pp. 129-141.

[11] Castano, A., Becerril, C. (2004). In vitro assessment of DNA damage after short and long-term exposure to benzo[a]pyrene using PARD and the RTG-2 fish cell line. Mutation Research, vol. 552, p. 141-151.

[12] Zhou, B., Liu, Ch., Wang, J., Lam, P.K., Wu, R.S.S. (2006). Primary cultures cells as sensitive in vitro model for assessment of toxic-comparison to hepatocytes and gill epithelia. Aquatic Toxicology, vol. 80, p. 109-118.
[13] Derakhshesh, N., Salamat, N., Movahedinia, A., Hashemitabar, M., Bayati, V. (2019). Exposure of liver cell culture from the orange-spotted grouper, Epinephelus coioides, to benzo[a]pyrene and light results in oxidative damage as measured by antioxidant enzymes. Chemosphere, vol. 226, p. 534-544.
[14] Benzie, F.F., Strain, J.J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Analytical Biochemistry, vol. 239, no. 1, p. 70-76.
[15] Paglia, D.E., Valentine, W.N. (1967). Studies on the quantitative and qualitive characterization of erythrocyte glutathione peroxidase. Journal of Laboratory and Clinical Medicine, vol. 70, p. 158-169.
[16] Buege, J.A., Aust, S.D. (1978). Microsomal lipid peroxidation. Methods in Enzymology, vol. 30, p. 302-310.
[17] Reitman, S., Frankel, S. (1957). A colorimertic method for the determination of oxaloacetic acid glutmic pyruvic transminase. In: Roy, S., Bhattacharya, S., 2006. Arsenic induced histopathology and synthesis of stress proteins in liver and kidney of *Channa punctatus*. Ecotoxicology and Environmental Safety, vol. 65, p. 218-229.
[18] Krogdahl, A., Mckellep, A.B., Baeverfjord, G. (2003). Effects of graded levels of standard soybean metal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). Aquaculture Nutrition, vol. 9, p. 361–371.
[19] Tuvikene, A. (1995). Responses of fish to polycyclic aromatic hydrocarbons (PAHs). Annales Zoologici Fennici, vol. 32, p. 295–309.
[20] Luís, L., Guilhermino, L. (2012). Short-term toxic effects of naphthalene and pyrene on the common prawn (*Palaemon serratus*) assessed by a multi-parameter laboratorial approach: mechanisms of toxicity and impairment of individual fitness. Biomarkers, vol. 17, no. 3, p. 275-85.
[21] Yazdani, M. (2018). Comparative toxicity of selected PAHs in rainbow trout hepatocytes: genotoxicity, oxidative stress and cytotoxicity. Drug and Chemical Toxicology. https://doi.org/10.1080/01480545.2018.1497054.
[22] Mitchelmore, C.L., Chipman, J.K. (1998). Detection of DNA strand breaks in brown trout (Salmo trutta) hepatocytes and blood cells using the single cell gel electrophoresis (comet) assay. Aquatic Toxicology, vol. 41, no. 1/2, p. 161–182
[23] Wessel, N., Ménard, D., Pichavant-Rafini, K., Ollivier, H., Le Goff, J., Burgeot, T., Akcha, F. (2010). The toxicity of benzo[a]pyrene on sole (*Solea solea*) hepatocytes: assessment of genotoxic and enzymatic effects. Polycyclic Aromatic Computation, vol. 30, no. 5, p. 346–354.
[24] Schirmer, K., Chan, A.G., Bols, N.C. (2000). Transitory metabolic disruption and cytotoxicity elicited by benzo[a]pyrene in two cell lines from rainbow trout liver. Journal of Biochemical and Molecular Toxicology, vol. 14, no. 5, p. 262–276
[25] Gad, A.S., Khadrawy, Y.A., El-Nekeety, A.A., Mohamed, S.R., Hassan, N.S., Abel- Wahhab, M.A. (2011). Antioxidant activity and hepatoprotective effects of whey protein and Spirulina in rats. Nutrition, vol. 27, no. 5, p. 582-589.
[26] Pan, L. Q., Ren, J., Liu, J. (2006). Responses of antioxidant systems and LPO level to benzo[a]pyrene and benzo[k]fluranthere in the haemolymph of the scallop *Chlamys ferrari*. Environmental Pollution, vol. 141, p. 443-451.
[27] Beytut, E., Aksakal, M. (2003). Effects of Dietary Vitamin E and Selenium on Antioxidative Defense Mechanisms in the Liver of Rats Treated with High Doses of Glucocorticoid. Biological Trace Element Research, vol. 91, p. 231-241.
[28] Monari, M., Foschi, J., Motozzo, V., Marin, M. G., Fabbri, M., Rosmini, R., Serrazantti, G.P. (2009). Investigation of EROD, CYP1A immunopositive proteins and SOD in haemocytes of *Chamelea gallina*

and their role in response to B[a]P. Comparative Biochemistry and Physiology Part C, vol. 149, p. 382-392.

[29] Sole, M., Buet, A., Ortiz, L., Maynou, F., Bayona, J.M., Albaiges, J. (2007). Biochemical responses in mussels exposed to the water-accommodated fraction of the "Prestige" fuel oil. Scientia Marina, vol. 71, p. 373-382.

[30] Corredor-Santamaría, W., Calderón-Delgado, I.C., Arbeli, Z., Navas, J.M.., Velasco-Santamaría, Y.M. (2023). Acute effects on hepatic biomarkers in the freshwater native fish *Aequidens metae* exposed to polycyclic aromatic hydrocarbons. Global Journal of Environmental Science and Management, vol. 9, no. 1, p. 1-14.

[31] Vutukuru, S.S., Prabhath, N.A., Raghavender, M., Yerramilli, A. (2007). Effect of arsenic and chromium on the serum amino-transferaseses activity in Indian major carp, *Labeo rohita*. International Journal of Environmental Research and Public Health, vol. 4, p. 224-227.

[32] Banaee, M., Sureda, A., Mirvaghefi, A., Ahmadi, K. (2011). Effects of diazon on biochemical parameters of blood in rainbow trout (*Oncorhynchus mykiss*). Pesticide Biochemistry and Physiology, vol. 99, p. 1-6.

[33] De la Torre, F.R., Salibian, A., Ferrari, L. (2000). Biomarkers assessment in juvenile *Cyprinus carpio* exposed to waterborne cadmium. Environmental Pollution, vol. 109, p. 277-282.

[34] Kim, S.G., Park, D.K., Jang, S.W., Lee, J.S., Kim, S.S., Chung, M.H. (2008). Effects of Dietary Bezo[a]pyrene on Growth and Hematological Parameters in Juvenile Rockfish, *Seabastes schlegeli* (Hilgendorf). Bulletin of Environmental Contamination and Toxicology, vol. 81, p. 470-474.