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RESEARCH PAPER

The Effect of Benzo[*a*]pyrene on Xenobiotic Metabolizing Cytochrome P4501A (CYP1A) Expression in Spotted Scat, *Scatophagus argus* (Linnaeus, 1766)

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Abstract

Low molecular weight Polycyclic Aromatic Hydrocarbons (PAHs) are widely concerned due to their significant health risks such as carcinogenicity, reproductive- and endocrine-disrupting effects, immunotoxicity and neurotoxicity on human and marine animals. The induction pattern of xenobiotic metabolizing cytochrome P4501A (CYP1A1) gene was studied on 150 spotted scat *Scatophagus argus* (weight 32±3 g) captured from Northern Persian Gulf and exposed to 0, 5, 10, 15, 20 mg kg^{-1} of benzo[a]pyrene in 100L aquarium. The induction of CYP1A1 was quantified using reverse-transcription real-time PCR from treated fish liver samples. The results revealed that the expression of CYP1A1 gene in *S. argus* liver is time-dependent and directly correlated to the concentration of B[a] P, therefore the study provides the basis for further use of CYP1A1 of this commercially ad ecologically important species as a biomarker.

Introduction

Environmental studies related to pollutants are mainly based on chemical assessments (the measurement of pollutant concentrations in water and sediments) and biological monitoring (the measurement of contamination in the organism's tissues) in the natural ecosystems (Abdallah and Abdallah, 2008). However, chemical analysis is relatively easy and inexpensive; do not provide adequate data on the bioavailability of contaminants to organisms. Furthermore, fluctuations in physiochemical factors cause high variations in physicochemical monitoring measurements (Giusti, 2001). In contrast, the biological monitoring approach using appropriate biomarkers provides more reliable data on the bioavailability of contaminants and the probable effects on organisms (Amiard et al., 2006).

The marine environment has been exposed to different contaminations for the last several decades subsequent to increased human activities and industrialization. Hydrocarbon contamination is a significant stress on marine ecosystems. Polycyclic Aromatic Hydrocarbons (PAHs) are from aquatic pollutants which proved to have many adverse effects on marine animals and biomagnifying through the food webs (Jönsson et al., 2006). Their adverse effects are more significant when organisms expose to the continuous influx from the anthropogenic origin (Jönsson et al., 2006). There are major concerns about

low molecular weight PAHs which cause acute toxicity, especially the promutagenic/procarcinogenic compounds such as benzo[a]pyrene.

Cytochrome P450 (CYP) superfamily, is a group of divers hemoprotein families containing heme as a cofactor. CYPs act as monooxygenase in diverse intracellular as well as extracellular compounds in many animals and plants. Many members of this superfamily were identified during the last decades. To date 137 P450 coding gene from 18 families have been reported from fishes (Lee et al., 2018).

P450 enzymes involved in detoxifying extracellular compounds such as pharmaceuticals, carcinogen chemicals, xenobiotics, as well as the metabolism of intracellular substances such as steroids, lipids, vitamins, and prostaglandins (Uno et al., 2012). CYP1 family in fish comprised of 4 subfamilies: CYP1A, CYP1B, CYP1C and CYP1D. This family is important due its role in catabolism and oxidation of cyclic structures including polycyclic aromatic hydrocarbons (PAHs), therefore they play important role in detoxifying and prevention of DNA adducts. CYP1A in fish is the most important in detoxification and can be used as a useful biomarker of the aquatic environment (Brammell et al., 2010; Jung et al., 2011). CYP1A is one of the most sensitive biomarkers of organic pollutants especially oil pollutants (Uno et al., 2012). CYP1A protein induction has been altered in correlation to PAHs exposure in much teleost fish (Brzuzan et al., 2005; Moore et al., 2003; Rees and Li, 2004). Therefore, a measurement of CYP1A expression can be considered as a biomarker of exposure of PAH contaminations.

In spite of its disadvantages to the global environment, oil still is an essential commodity. The Persian Gulf surrounded by five of ten leading oil producer countries, which are the biggest oil exporters of the world. Therefore, due to the huge amount of oil-related explorations, drillings, refinement and shipping, and regional instability, the oil spills and oil contamination is not uncommon. Many researchers reported the Persian Gulf as one of the most polluted marine environments of the world (Al-Saleh and Al-Doush, 2002; Munawar et al., 2002; Sinaei and Mashinchian, 2014; Tolosa et al., 2005). Therefore, the development of a biomarker using a widespread species is essential for monitoring short-time and long-time exposure to endocrine-disrupting xenobiotics, especially oil derived contaminants.

Spotted scat (*Scatophagus argus*) is a euryhalaine herbivorous teleost from family Scatophagidae widely distributed in shallow coastal waters, brackish estuaries, mangroves and river mouths from Indo-Pacific region to Japan and southeast Australia. This species is adapted to tolerate different fluctuations in salinity, turbidity, temperature and dissolved oxygen. Therefore, meets desired charachteristics as an aquarium and aquaculture species, in addition to ecotoxicological examinations. *S. argus* is a commercially important foodfish in its range, valuable aquaculture fish in china and desirable brackish aquarium fish worldwide. This species is usually kept in fresh water in aquaria. Their spines have reported to induce toxicity to human and marine animals and are used in traditional Chinese medicine (Ghafari et al.,2013 Tang et al., 1987. Froese, et al., 2018)

Spotted scat *S. argus* is one of the model organisms of pollution which has been used for toxicology studies(Shaw and Panigrahi 1990; Sinaie et al., 2010; Velusamy et al., 2014). This species can be used as a model for biomonitoring of contamination in the region. We conducted the present research to study the CYP1A1 gene expression pattern in response to exposure to benzo[a]pyrene in the liver of spotted scot *S. argus* over a period of 72 h.

Materials and Methods:

Collection and Acclimation and of Samples

Spotted scot fish samples (32±3 gr mean weight) were collected from Bushehr coastal waters (north Persian Gulf, Iran). 150 specimens were then transferred to the laboratory avoiding physical stress and acclimated for a week in the 1000L (experimental fiberglass tank with 22-24°C and 14-10 light-dark photoperiod, salinity of 42 ppt and pH=8. After a week of acclimation, total of 150 (weight 32±3 g) were randomly distributed into 200L glass aquaria filled with 100 L of water at the stocking rate of 10 fish per tank. Each experimental treatment was randomly assigned to three replicates. 0.2 g of B[a] P (Sigma, Canada) was dissolved in 10 mL safflower oil. B[a] P doses of 0, 5, 10, 15, 20 mg kg^{-1} was injected. Daily, 50% of the water was renewed. Animals were fed to satiation twice per day, with the exception of the day before sampling. Fish were sampled after1, 3, 7 and 21 days of exposure and were sacrificed by usingt MS-222. The liver tissue was extracted and preserved in liquid Nitrogen.

RNA Extraction and First-strand

After homogenization of liver tissue, RNA was isolated using High Pure RNA Tissue kit (Roche, Germany), according to the manufacturer's protocols. The purity and concentration of the total RNA were then examined by measuring the absorbance at 260 and 280 nm (A260/280) in a Biophotometre (WTW). RNA integrity and DNA contamination were also verified in a 1% agarose gel, stained with Sybergreen(Lee et al., 2005). The first-strand cDNA was reverse transcribed from 500 ng of total RNA and10mmol Randum hexamer Primer using the cDNA Synthesis Kit (Cinagen, IRAN) and stored at −20°C.

Quantitative Real-time PCR (qRT-PCR)

Degenerate primers were designed using previously reported conserved sequences of CYP1A for teleost after multiple alignments. Online primer design

tool, Primer3 (htt p://primer3.ut.ee/) was used as a target to design gene specific primers. To ensure specificity for the intended target gene, the selected primers were then analyzed using Primer-BLAST. A total of 3 primers were designed for the CYP1A gene (Table 1). The reverse transcriptase chain reaction (RT-PCR) method was used to clone CYP1A.

The relative expression of the CYP1A gene in liver were measured by qPCR. Real-time PCR was performed with 6.25 µl of 2xSYBER Mix(Cinnagen, IRAN), 0.5µl of 10 pmol μl^{-1} of each forward and reverse primers, 2.5 units of Taq polymerase, 0.5µl of template cDNA and 4.65 µl of DNase free water using following conditions: initial denaturation for 2min at 94°C, 40 cycles of denaturation at 94°C for 15 seconds, annealing for 30 seconds at 60°C, extension at 72° for 30 seconds. At the end of each run a melting curve analysis was performed (from 55°C to 95°C) to determine the formation of specific products. Fish 18sRNA gene was used as a reference to normalize the level of relative expression between samples and negative controls with no cDNA in each reaction. The relative expression of the target genes was calculated by the $2^{-\Delta\Delta Ct}$ method, considering efficiencies close to 100% (Livak and Schmittgen, 2001).

Statistical Analyses

Analysis of obtained sequences was performed using BLAST, followed by alignment, calculation of genetic distance, genetic identity and constructing phylogenetic tree by using Mega 5.0. Statistical analysis was performed using Microsoft Office Excel ver. 2010 and SPSS ver. 22, as Kolmogorov–Smirnov test was used for normality of data, one-way ANOVA was used for testing homogeneity of variance, and Duncan's Multiple Range Test was used to detect differences between means and the effects of doses and time on gene expression (P<0.05).

Results

The results of the sequencing of PCR products showed an 879 bp sequence (Figure 1) by using CYPAR2 and CYPAF4 primers. The obtained sequence as the result of using CYPAR2, CYPAF2 did not result in an acceptable clear sequence.

Homology test using BLAST program revealed correct amplification of sequences, and alignment of obtained sequences with other similar sequences from other fish showed the highest similarity to *Dicentrarchus labrax* -Family Moronidae- CYP1A (Table 2).

Phylogenetic dendrogram of the CYP1A gene of *S. argus* as well as those of other fishes were constructed (Figure 2).

The expression of CYP1A gene in all *S. argus* exposed to different doses of B[a]P increased significantly from the first day of exposure compared to the control group. Comparison of different treatments showed the expression levels of the CYP1A gene varied significantly between different doses of exposure and times (P<0.05). The expression of CYP1A was significantly correlated to the dose of exposure to B[a] P. The highest expression was observed in 20 mg kg^{-1} exposure dose (Figure 3).

The expression of CYP1A reached its peak at the first 24 hours subsequent to exposure (16 fold compared to control) followed by a decrease in the next days. At the day 3, no significant differential gene expression at two exposure levels of 15 and 20 mg kg^{-1} was observed, however at 7th day after exposure, a significant increase of the gene expression was observed in all doses in contrast to control group and the first-day level (P<0.05) (Figure 4). Decreased expression levels were observed in all doses on day 21. The correlation coefficient between the exposed dose and the decrease in the expression level at day 7 and 21was very high (r>0.9), but relatively lower at day 1 and 3, however the

Table1. Properties of primer used in RT-PCR for CYP1A cloning

Gene	Primer name	Primer sequence 5-3	Tm(°C)
18sRNA Housekeeping gene	18s F1	AGGCCCTGTAATTGGAATGAGT	62
	18s F2	GACTCTTTCGAGGCCCTGTAAT	62
	18s R	TTTTCAAAGTAAACGCTTCGGA	62
CYP1A (*S. argus*)	ARTF	CAGTGATGAGTTTGGTCAAGTGG	62
	ARTR	TCTCATCTGACACCTGGACGTTA	62

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          10        20        30        40        50        60        70        80        90       100
TAATGTGATNTGTGGCATGTGTTTGGCCGACGCTATGACCACGATCACCAGGAGCTGCTCAGCTTGGTGAACCTCAGTGATGAGTTTGGTCAAGTGGTGG 100
GCAGTGGTAACCCTGCAGACTTCATCCCTATTNTTCAGTTTCTGCCCAGCACAACAATGAAGAAGTTTGTGAGCATCAATGCTCGTTTCAACACATTTGT 200
GCAAAAAATTGTCAGCGAGCACTATGGCACCTATGACAAGGACAACATTCGTGACATCACCGACTCCCTTATTGACCACTGTGAGGATAGGAAGTTAGAT 300
GAGAATTCTAACGTCCAGGTGTCAGATGAGAAGATTGTGGGAATCGTCAATGATCTGTTTGGAGCTGGTTTTGACACTATCTCCACTGCGCTGTCTTGGT 400
CAGTGATGTACATGGTGGCTTACCCTGAGATACAGGAAAGACTTTTTCAAGAATTGAAGGACAATGTGGGTGTGAATCGCACACCTCGTCTCTCTGACAA 500
ACAAAAATTACCCTTTCTGGAGGCCTTCATCCTTGAGATCTTCCGCCACTCTTCATTCTTGCCCTTCACTATCCCTCACTGCACGTCAAAAAACACATCT 600
CTGAATGGCTACTTCATTCCTAAAAACACCTGTGTCTTCATCAACCAGTGGCAGATCAACCATGACCCTGAACTCTGGAAAGATCCATCTTCCTTCAACC 700
CAGATCGTTTCCTGAGTGCTGATGGCGCTGAGATCAATAAGCTGGAGGGGGAGAAGATGATGGTTTTTGGCATGGGGAAGCGCCGCTGCATTGGCGAGGT 800
CATAGCACGAAATGAAGTTTTCCTCTTCTTGGCAATCCTTGTCCAGAAGCTGCACTTCNTGCAATCCCGAGAGCNGTGA                     879
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Figure 1. The nucleotide sequence of *S. argus* CYPA1 gene.

significant correlation was observed between the exposure concentration and the gene expression at all exposure levels in all days (P<0.05) (Figure 5). The CYP1A gene expression trend in *S. argus* was decreasing in all doses over time since the maximum expression level was found at the first 24 hours after exposure.

Discussion

The results of the present study showed the CYP1AF4/CYP1AR2 primers are appropriate for the cloning of CYP1A gene in *S. argus* fish. Using these primers and the described PCR conditions, an 876 bp nucleotide was obtained from 5′ region of CYP1A1 in *S. argus*. Molecular analysis of this sequence revealed a 90% identity to *Dicentrarchus labrax.* Phylogenetic analysis of the sequence demonstrated 75-80% similarity to other Osteichthyes (Figure 2), in the same phylogenetic cluster with *Dicentrarchus labrax*, *Terapon jorbua,* and *Lateolabrax japonicas.*

Considering the low expression level of CYP1A1 in the liver, the use of an inducer is required in order to increase the expression level. Therefore, *Scatophagus argus* samples were exposed to Pyrene, B[a] P, and BNF. The results showed that the expression level of CYP1A1 was increased under B[a] P exposure, facilitating the cloning of the gene. The expression of CYP1A genes is different in fishes, however generally PAHs highly induce CYP1A gene in many fish species. According to Oh et al. (2009) the expression of the CYP1A gene of goldfish *Carassius auratus* was accelerated using B[a]P and the cloning was performed. Campell and Devlin (1996) cloned the CYP1A gene in *Oncorkynchus tshawytscha* using BNF inducer. The cloning of the CYP1A gene has been performed in three Salmonid species using B[a]P exposure (Rees and Li, 2004). Zapata-Pe et al. (2012) used Pyrene to induce the expression of CYP1A gene in tilapia *Oreochromis niloticus*.

In the present research, the expression of CYP1A1 was studied using an intraperitoneal injection of different doses of B[a] P as a biomarker of oil pollution. The results showed different patterns of expression/induction ratio over time. The results

Table 2. Genetic distances of CYP1A of *S. argus* in contrast to other fishes

Fish species	*Danio rerio*	*Anguilla anguilla*	*Acanthopagrus schlegelii*	*Chelon labrosus*	*Liza macrolepis*	*Mugil cephalus*	*Periophthalmus waltoni*	*Scatophagus argus*	*Dicentrarchus labrax*
Danio rerio	0.000								
Anguilla anguilla	0.346								
Acanthopagrus schlegelii	0.357	0.259							
Chelon labrosus	0.352	0.269	0.153						
Liza macrolepis	0.348	0.269	0.165	0.03					
Mugil cephalus	0.356	0.284	0.17	0.073	0.082				
Periophthalmus waltoni	0.402	0.336	0.268	0.263	0.272	0.272			
Scatophagus argus	0.355	0.265	0.152	0.157	0.163	0.176	0.246		
Dicentrarchus labrax	0.349	0.249	0.123	0.139	0.144	0.168	0.259	0.113	
Lateolabrax japonicus	0.345	0.248	0.115	0.126	0.136	0.15	0.248	0.115	0.082

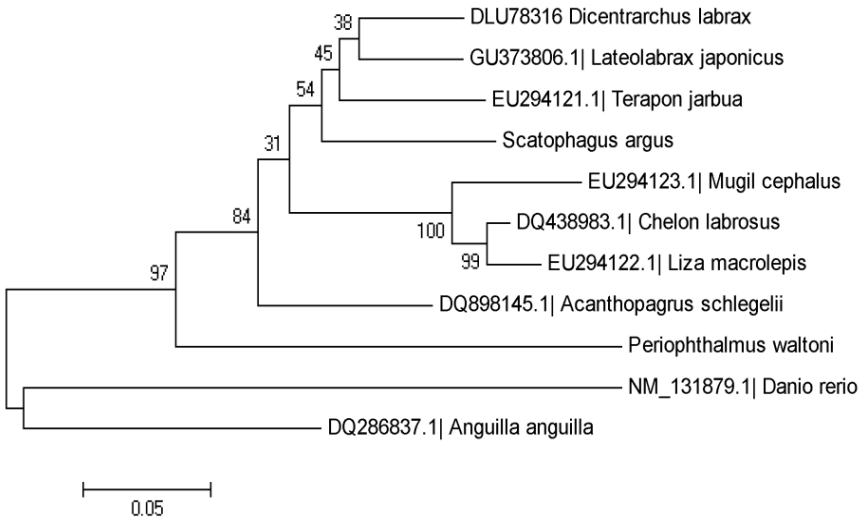

Figure 2. Phylogenetic tree of *S.argus* CYP1A and those of other fish species constructed by the neighbor-joining method.

showed a rapid acceleration of gene expression within the early 24 hours after administration, followed by a dose-dependent decrease in the gene expression. Many researchers documented the effect of increased concentration of oil pollutants on expression levels of CYP1A1 in fish, all confirmed the induction effect of petroleum hydrocarbons such as polychlorinated biphenyls (PCB), beta-naphthoflavone, zinc cobalt, benzopyrene, pesticides and 2, 3, 7, 8 -tetrachlorodibenzo-p-dioxin, and the increase in concentrations correlated to increase in expression levels of CYP1 genes.

In the present study, the increase in the expression was relatively lower in contrast to some experiments; for instance in rainbow trout, 50mg injection-induced 100 fold increase of CYPA1 gene induction at first 24

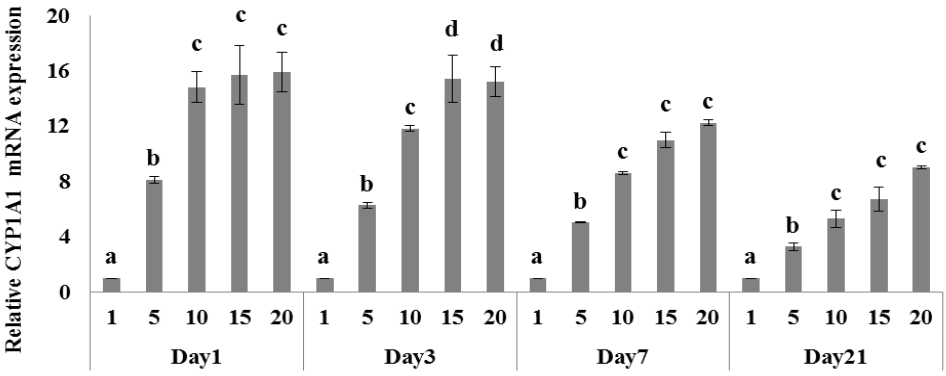

Figure 3. Relative mRNA expression of CYP1A1 in *S. argus* liver after exposure to different concentrations of benzo[a]pyrene. P<0.05.

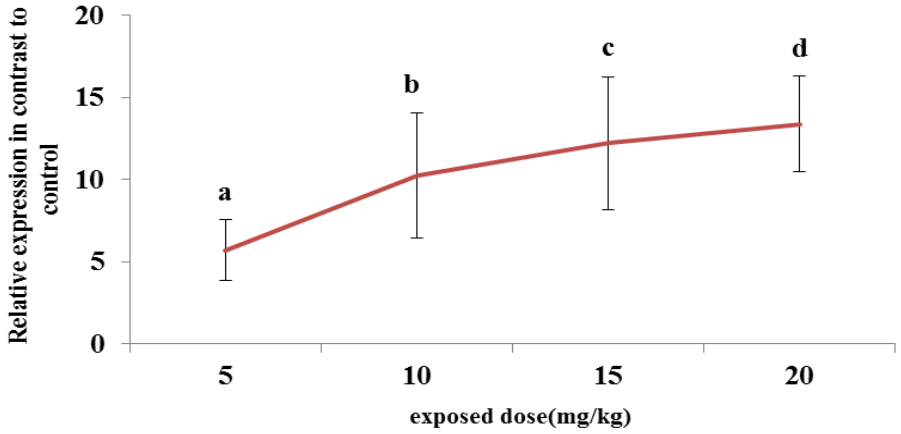

Figure 4. Relative expression of CYP1A1gene in the *S. argus* liver exposed to different doses of benzo[a] pyrene. P<0.05.

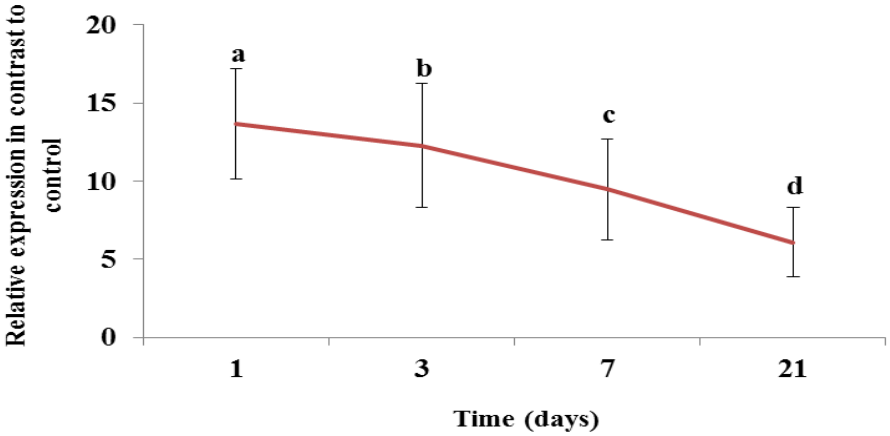

Figure 5 Correlation between the relative expression of CYP1A1gene in the *S. argus* liver and the time after exposure of benzo[a]pyrene. P<0.05.

hours (Levine and Oris, 1999), injection of the same dose to *Oncorhynchus tshawytscha* at first 24 hr showed a 160 time expression level of the gene (Campbell and Devlin, 1996); increase in CYP1A1 gene expression was 100 fold in the first day and accelerated to 300 fold at the third day (García-Tavera et al., 2013); the treatment of *Fundolus heteroclitus* fish with 30 pmol/g fish of PCB126 resulted in expression increase of CYP1A1 gene (10 fold in the liver and 800 fold in the brain) after 24 hrs. (Zanette et al., 2009); rainbow trout fish exposed to PCB126 showed a 100 and 120 fold expression increase in liver and gill respectively (Jönsson et al., 2010); however the expression increase in the present study was higher than some other studies: in B[a]P injected *Fundulus heteroclito*s there has been a 5-fold increase in CYP1A1 gene expression after 15 days (Wang et al., 2009). Roy et al. (2011) reported 2-15 times increased CYP1A1 gene expression level in sturgeons. These variations depend on the species and the type of the substances which is the species has been exposed to.

The gene expression levels have been found to be correlated to the concentration of exposed xenobiotics. In the study of B[a]P exposure on gene expression in tilapia, the activity ERAD was directly correlated to B[a]P dose (Zapata-Pérez et al., 2002). CYP1A1 gene expression was highly correlated to the administration dose of BNF and 3 MC in rainbow trout(r=0.99) (Campbell and Devlin, 1996). Roy et al. (2011) reported the dose-responsive increase of CYP1A1 expression in two sturgeon species exposed to TCDD and PCB126. For PCB126 minimum inducing dose was 0.01 ppb which induced 3-fold increased gene expression and maximum expression was induced by 1000bp dose which induced a 424-fold increase in the gene expression. For TCDD minimum inducing a dose of 0.001 ppb caused a 7.7-fold increase in the gene expression and up to 987-fold increase was reported at 10ppb dose. B[a]P injected *Oreochromis niloticus* showed a sharp increase of CYP1A1 expression in early 24h, then a decrease was observed within 72-120h after administration(García-Tavera et al., 2013). Administration of 1 nM of indigo and 10 nM of PCB126 to *Gasterosteus aculeatu*s induced gene expression increase of 100 and 120 fold respectively, followed by a decrease within 9 days(Gao et al., 2011). This trend of gene expression was reported in *Takifugu obscure*s in exposure to BNF (Kim et al., 2008). However in the study of Wang et al. (2009) on red sea bream (*Pagarus major*) under the administration of different doses of B[a]P, 3-fold increase of CYP1A1gene within early 24h was followed by subsequent more increase to 10-fold within 120h. The increase or decrease of CYP1 genes expression over time is different between species, however, they respond in a similar way, i.e. the increase of induction exposure of toxicant and subsequent reduction. An early robust increase in CYP1A1 mRNA following administration is mediated through the aryl hydrocarbon receptor (AhR). Due to the presence of two forms of AhRs in fish, the dose-response interactions are relatively different trough species.

Conclusion

The results of the present study showed that the expression of the CYP1A1 gene followed dose-response dependence to the injected dose of benzo[a]pyrene. A time-course study revealed a rapid increase of CYP1A1 gene expression within the early exposure phase (24h), followed by a decrease in expression level at all doses over time. However, up to 7th day a positive correlation was observed between the CYP1A1 gene expression and B[a] P doses, and no significant correlation was observed between induction of CYP1A1 and induced dose at 21st day. This toxicity response of fish to the administration of B[a] P may relate to disruption of metabolism and synthesis of proteins over time, as a result of this, the rapid decrease in the expression was observed in most doses. This pattern of primary increase and subsequent decrease in expression of CYP1 following administration of xenobiotics was reported in other studies. The results provide a basis for using the CYPA1 gene as a biomarker of exposure to PAHs in *S. argus*. This species is one of the model organisms in toxicology, thus the results given here are important for environmental and pharmaceutical researches at the molecular level.

Ethical Statement

All procedures were supervised by Research ethics committee of Vice Chancellor for Research and Technology of Persian Gulf University.

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Author Contribution

A.S.: Methodology, Lab experiments, Data Analysis, Writing review and Editing. A.G.: Methodology, Sampling, Lab experiments (Exposure, RNA extraction, cDNA syntheisis, PCR cloning).

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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