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A preliminary study on the expression level of P_{450} gene in liver and gill tissues of Persian sturgeon (*Acipenser persicus* Borodin, 1987) exposed to water soluble fractions of crude oil

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Abstract

The present study was conducted to investigate the expression level of cytochrome P_{450} enzyme in detoxification of water soluble fractions (WSFs) of crude oil in Persian sturgeon, *Acipenser persicus*. Fish were exposed to WSFs at concentrations of 0 (control), 10, 20, and 50% of the LC_{50} for 14 days, and then, the expression level of P_{450} gene was assayed in gill and liver samples. According to the results, the expression level of P_{450} gene increased in both the gill and liver tissues of fish when exposed to sub lethal concentrations of WSFs. High level of expression in treatment of 50% WSFs was observed compared to the control ($p<0.05$). However, no significant differences were observed between treatments of 10%, 20%, and 50% WSFs. Additionally, P450 was expressed higher in the gill tissue than the liver. The present research demonstrates that P_{450} gene expression can be used as a molecular biomarker to assay the severity of oil pollution in the Caspian Sea.

Keywords: Crude oil, Cytochrome P_{450}, Pollution, Persian sturgeon

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Introduction

The Persian sturgeon is one of the commercial and ecological sturgeon fish composing the majority of sturgeon catch in southern parts of Caspian Sea. Unfortunately, the population of all sturgeon species has declined in Caspian Sea over the last decades (Khodorevskaya *et al*., 1997; Ivanov *et al*., 1999; Bahmani *et al*., 2000; Jabbarzadeh Shiadeh *et al*., 2000; Khodorevskaya *et al*., 2009; Nazeri *et al*., 2019). Moghim *et al*. (2006) have reported a decline of 80–90% for Persian sturgeon populations during last 30–40 years. In many cases, the population decline is attributed to some problems, including loss of habitat and spawning grounds, illegal and overfishing, and also water pollution (De Meulenaer and Raymakers, 1996; Kiabi *et al*., 1999; Nasrollahzadeh, 2010). It was well recognized that water pollutants adversely affect all aspects of life in sea such as growth, osmoregulation, reproduction, etc. (Hanson *et al*., 2007; Olsvik *et al*., 2010; Suvetha *et al*., 2010; Xing *et al*., 2012; Katuli *et al*., 2014). In Caspian Sea, a considerable amount of pollutants such as pesticides, heavy metals, crude oil, and other chemicals are released annually into the water due to agricultural and industrial wastewater activates (Hajirezaee *et al*., 2016; Hajirezaee *et al*., 2017).

Over the last decades, crude oil exploration, production, and related industries have caused Caspian Sea to be polluted by oil derivatives, including alkanes, cycloalkanes, and aromatic compounds. Furthermore, oil loading, transportation, and tankers accidents in sea have intensified the problem (Dumont, 1995; Kaplin, 1995; Dumont, 1998; Abilov *et al*., 1999; Clauer *et al*., 2000; Tolosa *et al*., 2004). The main sources of pollution of the Caspian Sea are considered to be offshore oil production and land-based sources, especially the Volga River (Karpinsky, 1992). The most production of oil in the Republic of Azerbaijan is from offshore resources.

The fate of crude oil in the sea is mainly determined by evaporation, dissolution, emulsification, precipitation, and degradation process. Acute toxicity studies reported that fish are generally the marine organisms susceptible to the water soluble fraction (WSF) of crude oil (Wolfe, 2013). In the present study, we investigated the expression level of cytochrome enzyme, P450 (CYP1) in detoxification of WSFs in Persian sturgeon, *Acipenser persicus* for the first time. The cytochrome P_{450} enzyme not only plays a key role in the degradation of endogenous substrates, such as steroids, fatty acids, and hormone, but also it is important for the metabolism and detoxification of xenobiotics such as drugs, aromatic hydrocarbons, pesticides, and water soluble fractions (WSFs) of crude oil (Goksøyr, 1995; Kennedy and Farrell, 2005; Arukwe *et al*., 2008; Jager *et al*., 2016). Therefore, in the present research we examined acute and chronic toxicity of WSF and P450 gene expression as a molecular biomarker in Persian sturgeon.

Materials and methods

Preparation of water soluble fractions (WSFs) of crude oil

The crude oil was mixed with well-filtered water (the same water source used for fish) in a special container at ratio of 1:9. Then, the emulsion was shaken for 23 h by shaker and remained for 1 h until the soluble phase was separated. The lower part, which is soluble phase or WSFs, was isolated, and used as stock solution over the course of the experiment *(Anderson et al*., 1974).

Determination of acute toxicity and mean lethal toxicity (LC_{50}) of WSFS

Fish were obtained from Shahid Beheshti Sturgeon Hatchery Center, Rasht. The experiments were conducted in Sturgeon Reference Centre (Gene Bank), Rasht. A semi-static method was used to determine the acute toxicity and toxicity limit of crude oil on Persian sturgeon (mean weight: 5±0.5 g). In this method, fish were kept in 30×30×40 cm aquariums (10 fish per aquarium) and exposed to different concentrations of WSF solution including 27, 29.25, 31.5, 33.75, 36, and 38.25 mg L^{-1}, and the last group was considered as control. The experiment was carried out in 3 replications. During the experiment, all the aquariums were aerated and the temperature was kept in the range of 18 to 20 ºC. In each treatment, the WSF solution of the aquariums was renewed every 24 h with a fresh solution at same concentration. Additionally, the dead fish were removed from the aquarium and counted at the same time. After 96 h, the total number of dead fish in each aquarium was counted. Finally, using the obtained numbers, a LC_{50} value of 33.95 mg L^{-1} was calculated via Probit regression analysis with SPSS software, version 20 (Finney, 1952).

Exposure experiment

The experiment was conducted in 4 experimental treatments with three replicates. Persian sturgeon with stocking density of 10 fish per aquarium were exposed to WSFs at concentrations of 0 (control), 10, 20, and 50% of LC_{50} for 14 days and after this period, the expression level of P450 gene was assayed in gill and liver samples. During the 14 days experiment, the water quality parameters (e.g., temperature, dissolved oxygen, pH, turbidity, etc.) were held in normal range.

Gill and liver sampling

Liver and gill tissue samples were obtained after killing fish using high concentration of $MS\text{-}_{222}$ solution (anesthetic agent made in SIGMA factory). Then samples were placed into RNAase-free microtubes and stored at -80 ºC until the onset of RNA extraction and gene expression analysis.

RNA extraction and cDNA synthesis

RNA extraction of gill and liver tissues was carried out using acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (1987). For total RNA extraction, 50-100 mg of each tissue was homogenated in 1.0 ml Biozole reagent (Bio flux; China) for 15 min at room temperature until the cell digestion was

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completed and the nucleoproteins became separated. Then, the chloroform was added to homogenized solution at ratio of 1:5. After 15 times shaking, the homogenate was incubated at room temperature for 5 min., and then centrifuged at 12000 rpm at 4 °C. In the next step, 100 µl Phenol-Chloroform-Isoamyl alcohol (5:1:1) was added to supernatant and after shaking the solution for 15s, it was centrifuged at 12000 rpm at 4 °C. The supernatant was decanted and the residual was washed with 75% alcohol. The concentration of RNA was measured by spectrophotometer (DR 2800, HACH Germany) at 260/280 nm. Moreover, the RNA quality was evaluated by electrophoresis on a 1.5% agarose gel and stained by ethidium bromide (Miandare *et al*., 2013).

First-strand cDNAs was synthesized from 4 µl of total RNA using a Fermentas cDNA synthesis Kit (Bio RI, Bioflux-Bioer) for RT-PCR, following the manufacturer’s instructions. The quality of synthesized cDNA was evaluated by PCR using the primers of β-actin gene.

Primer design for qPCR

The qPCR primers were designed based on gene sequence of P450 gene for Persian sturgeon in GenBank of NCBI database. Table 1 displays the sequence of designed primers. The β-actine gene was used as reference gene in order to standardize the expression levels of gene.

Quantitative real-time PCR (qPCR)

Real-time PCR analysis was carried out by an iCycler [(Bio-Rad, USA) CFX] using commercial kit (Bio flux-Bioer Technology Co., China) following the manufacturer’s instructions. The obtained data were analyzed using Bio-Rad CFX Manager software version 1.6. The relative gene expression was calculated by the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

Data analysis

All data were presented as mean±standard error of mean (SEM). The Kolmogorov–Smirnov test was used to evaluate the normality of the data. As data were not normally distributed, Levene’s test was used to compare P450 gene expression in gill and liver tissues relative to β-actine. All statistical analyses were performed using SPSS software V.20. The significant differences between groups were considered at $p<0.05$.

Table 1: Name, sequence, melting temperature (Tm) and product length of primers used in the present study to quantify P450 1A transcript of Persian sturgeon *Acipenser persicus* through Real-time PCR.

Gene	Tm	qPCR primers Forward/Reverse	Product length (bp)
P450	56	GTCATCTGTGCCATGTGCTT TCTTGTCGAAGGAGCGGTAG	237
Reference gene (β- actin)	56	TTGCCATCCAGGCTGTGCT TCTCGGCTGTGGTGAA	2015

Results

According to the results of qPCR, P_{450} gene was expressed in both gill and liver tissues. It was found that the level of expression varied slightly depending on the type of the tissue ($p<0.05$) (Fig. 1). The P_{450} gene showed significantly different expression levels in all treatments ($p<0.05$). All WSFs treatments showed higher P_{450} gene expression compared to the control ($p<0.05$). The highest P_{450} gene expression in both liver and gill tissues was observed in treatment of 50% WSFs compared to the control ($p<0.05$). Also, the expression level of P450 in treatment of 50% WSFs was higher in gill than in liver tissue ($p<0.05$). There were no significant differences in P450 gene expression between the liver and gill tissues in three treatments of 10%, 20%, and 50% WSFs ($p>0.05$).

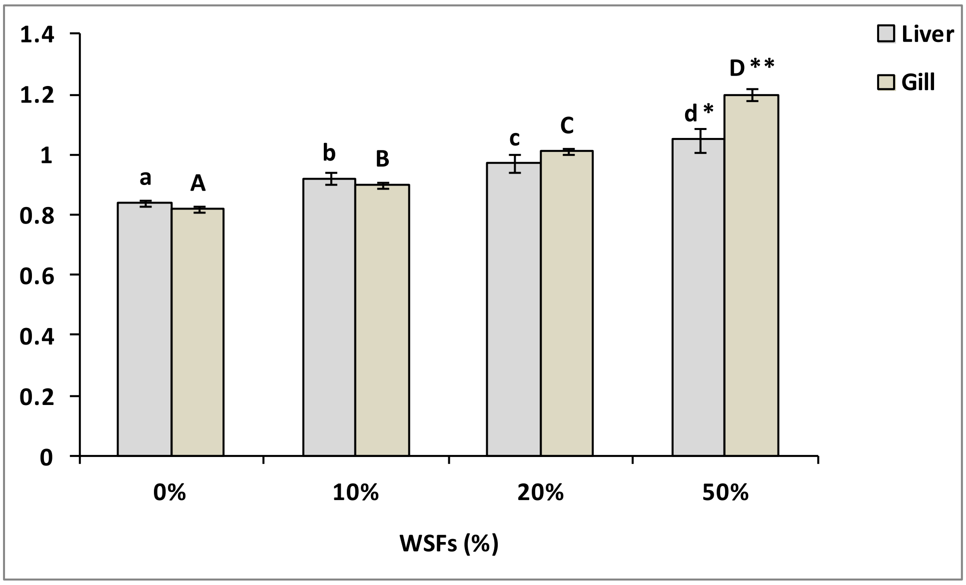

Figure 1: Relative P450 gene expression in liver and gill of Persian sturgeon, *Acipenser persicus* exposed to 0%, 10%, 20% and 50% WSFs for 14 days. In each tissue, bar assigned with different letters denote separately the significant differences during 14 days WSFs exposure ($p<0.05$). In each WSFs treatment, the significant differences in gene expression between gill and liver tissues are indicated with different symbols. Values are presented as the mean±SD.

Discussion

In order to assess the impact of pollutants in fish species, P450 gene expression alterations in response to xenobiotic were demonstrated (Zhang *et al*., 2012; Kumar and Denslow, 2016; Kühnert *et al*., 2017). The results obtained in this study showed that P_{450} gene is up-regulated in both the liver and gill tissues in response to WSFs exposure. This confirms that P_{450} enzyme is among the responses of the fish body to modify metabolic effects of WSFs. Moreover, it may be concluded that P450 is an early gene with the ability to induce the expression of other genes. Furthermore, our results were in agreement with those of the studies conducted by Safari *et al*. (2016) on Persian sturgeon, indicating the higher expression of P_{450} in response to endosulfan. Being transported to the

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nucleus, the receptor contaminant complex leads to expression of the gene (Billard *et al*., 2002). It has been shown that pollutant toxicity is related to its affinity to AhR. In addition, high affinity of pollutants to AhR induce P450 (Billiard *et al*., 2002). According Safari *et al*. (2016) P450 might be one of the first genes that activate later response genes, which can be considered as one more reason for a higher up-regulation on the primary day (Waisberg *et al*., 2003). Moreover, Paknejad *et al*. (2016) were evaluated cadmium impacts in Persian sturgeon. Their results were incontestable that cadmium causes some changes in two angiogenesis connected genes' expression in Persian sturgeon. Lari *et al*. (2015) were proposed that WSF of crude oil interrupt the systema respiratorium in Caspian roach (*Rutilus caspicus*) that lead to asphyxia can be a principal mechanism that concludes in fatal impact of WSF. Given the above, the P_{450} CYP1A enzyme can be considered as an effective candidate for assessment of environmental stresses, and several studies have indicated an induction of this enzyme activities with regard to the presence of anthropogenic sources of pollution, such as industries, urban wastewater, and harbors (Payne and Penrose, 1975; Stegeman, 1992; Stegeman, 1993; Søfteland *et al*., 2010; Zhang *et al*., 2012; Huang *et al*., 2014; Safari, 2014; Safari *et al*., 2016).

In this study, higher expression level of P_{450} was observed for WSFs treatment, showing the catalytic role of this protein in modulating the effects of various exogenous or endogenous agents, including WSFs in Persian sturgeon (Goksøyr and Förlin, 1992). Some studies have reported the increased expression level of P_{450} gene after exposure to environmental pollutants (Goksøyr, 1995; Dar *et al*., 2017; Zhang *et al*., 2017). In fact, the expression of P450 1A is adjusted by the Ah receptor (AhR).The transported receptor contaminant complex to the nucleus leads to expression of P_{450} gene (Billard *et al*., 2002). It has been shown that pollutant toxicity is related to its affinity to AhR. In addition, high affinity of pollutants to AhR induces P450 (Billard *et al*., 2002). Given the above, the P_{450} up-regulation in the contaminated Persian sturgeon may be mediated by WSF' high affinity to Ah receptor. Other studies have found decreases in P_{450} gene expression in fish exposure to high concentrations of pollutants (Søfteland *et al*., 2010; Zhang *et al*., 2012; Huang *et al*., 2014). These studies have concluded that the lower expression level of P_{450} gene may be due to the interaction of P_{450} protein with cortisol, as well as liver and gill tissue damages caused by high concentrations of PAHs and subsequent silence of P_{450} gene (Søfteland *et al*., 2010). In Gambusia, *Gambusia affinis* exposure to high concentrations of cadmium decreased the expression level of P450 gene (Huang *et al*., 2014). Moreover, the lower expression level of P450 gene was reported in *Ruditapes philippinarum* in response to 40 µg L^{-1} cadmium compared to 10 µg L^{-1} treatments (Zhang *et al*., 2012). It can be concluded that, P_{450} gene may be

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plays main role in the diverse environmental pollutants metabolizing.

In the current study, the P_{450} gene was expressed more in the gill tissue than the liver. These results are contrary to what were obtained for three-spined stickleback (*Gasterosteus aculeatus*) (Gao *et al*., 2011), killifish (*Fundulus heteroclitus*) (Zanette *et al*., 2009), Zebrafish (*Danio rerio*) (Jönsson *et al*., 2007), Rainbow trout (*Oncorhynchus mykiss*) (Jönsson *et al*., 2010) and brazialian guppy (*Poeciliavivipara*) (Dorrington *et al*., 2012), where higher expression of the gene was observed in the liver.

In conclusion, we reported the P_{450} gene expression induction in Persian sturgeon fingerlings in response to WSFs, indicating the key role of this gene in detoxification of WSFs in this species. Thus, P_{450} gene expression can be used as a molecular biomarker of crude oil pollution for Persian sturgeon.

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