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Short Communication

Heat Shock Proteins and Cytochrome *P450 1a1* Expression as Biomarkers of Exposure to Bisphenol A in *Catla catla*

Mehwish Faheem[1]*, Maleeha Rafeeq[1], Aisha Majeed[1] and Saba Khaliq[2]

[1]*Department of Zoology, Government College University, Lahore*
[2]*Department of Physiology and Cell Biology, University of Health Sciences, Lahore*

ABSTRACT

Biomarkers are extensively used to evaluate the early adverse effects of a toxicant exposure in an organism. Freshwater is exposed to many anthropogenic compounds that adversely affect aquatic life especially, fish population. Effect of acute exposure of low concentrations of BPA (10, 100, 1000 µg/l) on liver *cyp1a1* and heat shock proteins mRNA level was evaluated using real time qPCR. Results revealed that exposure to bisphenol A caused decrease expression of *cyp1a1* in a concentration dependent manner. Exposure to graded concentrations of bisphenol-A resulted in significant increase in mRNA expression of heat shock proteins (*hsp*70 and *hsp*90). In the light of present results, expression of *cyp1a1* or heat shock proteins may be used as biomarkers to assess the presence of BPA in the environment.

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MR and AM carried out the experiment and prepared the manuscript under supervision of SK and MF.

Key words
Bisphenol-A (BPA), Biomarkers, Heat shock proteins, cyp1a1, *Catla catla*

Biomarkers of exposure are early physiological and biochemical alterations that occur as a response to exposure of a contaminant (Hook *et al*., 2014). They provide early warning signals about the presence of a contaminant that can be further studied up to population level and provide a critical evidence about the relationship between a toxicant and its detrimental effects on an individual or a population (van der Oost *et al*., 2003; Handy *et al*., 2003). Biomarkers of exposure are usually specific to chemicals with similar mode of action and therefore, can be used more generally in environmental monitoring.

Bisphenol A (BPA) is a known plasticizer and used in production of plastic and a variety of plastic products (Staples *et al*., 2002), epoxy resins, dental sealants (as dimethacrylate; BIS-DMA), thermal paper, personal care products and medical equipment (Mendum, *et al*., 2011; Liao and Kannan, 2014). As fish inhabits water bodies, they are directly exposed to all anthropogenic chemicals and are one of the most important indicator for assessing the health status of water bodies. Similarly, heat shock proteins and cytochrome P450 levels can be used as biomarkers of exposure to different compounds like estrogenic endocrine disrupting chemicals (EDCs) in fish (Kroon *et al*., 2017).

All natural and anthropogenic chemicals undergo biotransformation in liver yielding metabolites that are non-toxic and easily excreted. Thus, residual time and efficacy of any chemical depends upon its metabolism (Akdogan and Sen, 2010). Cytochrome P450 is a group of cytoplasmic heme-proteins that are important phase I biotransformation enzymes (Nelson, 2011).

EDCs can effect teleost physiology through multiple pathways. Pathways mediated through aryl hydrocarbon receptor (AhR) are well studied because AhR pathway regulate the transcription of genes encoding several metabolic enzymes like hepatic cytochrome P450 (Cheng and Klaassen, 2008). Many studies have reported that expression of cyp450 are regulated through AhR (Lin *et al*., 2003; Gao *et al*., 2011). Out of many cytochromes, CYP1A1 is a major oxidative enzyme and can be affected by EDCs, especially compounds with estrogenic activity (Wong *et al*., 2000).

Heat shock proteins (HSPs) are induced by stress and can be used as potential biomarker for environmental monitoring (Ait-Aissa *et al*., 2000; Bierkens, 2000). HSPs are important because they respond rapidly to a variety of stressors. Most HSPs lack intron sequences (Deane and Woo, 2011), therefore the mRNA is rapidly translated into nascent protein within minutes of exposure to a stressor (Silver and Noble, 2012). Thus, assessment of mRNA level is a reliable predictor of HSP level (Silver and Noble, 2012). Previous studies reported alteration in HSP level by exposure to various anthropogenic chemicals e.g. heavy metals, chlorpyrifos and Polycyclic aromatic hydrocarbon (PAHs) (Gupta *et al*., 2010). Expression of HSPs has also

* Corresponding author: mehwishfaheem@gcu.edu.pk
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Table I. Primer sequences amplicon lengths and accession numbers of genes studied.

Gene name	Primer sequence (5'-3')	Product size	Accession number
Cytochrome p450 1a1	TGTGTCTGAGAGCCTTGTGG GGCTCAGATGTGGGTTGTTT	164bp	JX480500.2
Heat shock protein 70	TGAGATCGACTCGCTGTACG CCACCAACCAACACAATGTC	170bp	KC599207.1
Heat shock protein 90	GAACTCATCCCCGATCTGAA CCGAATTGACCGATCATAGAA	171bp	KC800801.1
TATA box binding protein (TBP)	AACAGCTTGTCCCTCCTGGA CAGGAGTGATGGGGGTCATA	213bp	KX371090.1
GAPDH	ATCACAGCCACGCAGAAGACC CAGGAATGACTTTGCCCACAGC	126bp	JX480499.1

been used as biomarker of exposure in various fish species and other aquatic organisms (Vijayan *et al*., 1998; Chen *et al*., 2011; Jin *et al*., 2010). The results of these studies showed that heat shock proteins are sensitive markers of chemical exposure and use of molecular approaches towards this end can be very promising.

Gene expression studies are now increasingly used to determine protein and mRNA levels and can be good prognostic parameters for predicting protein metabolism (Chen *et al*., 2011). Moreover, measuring mRNA level is sensitive method and provide mechanistic approach to evaluate toxic endpoints. Therefore, in the present study, cytochrome P4501A1 and heat shock protein 70 and 90 expression was studied as potential biomarkers of exposure to BPA in a commercial edible fish, *Catla catla*.

Materials and methods

A stock solution of BPA was prepared in ethanol (2mg/ml), exposure concentrations (10, 100, 1000µg/l) were prepared by dissolving stock solution in aquarium water. Female *C. ctala* were purchased from a commercial fish farm. After acclimatization for two weeks, fish were divided into four groups (n=3) in replicate and exposed to various concentrations (10, 100 and 1000 µg/l) of BPA for 14 days. Toxicant solution was replaced every other day. At the end of exposure period, fish were anesthetized with clove oil and length and weight were measured. Fish were humanely dissected and liver was removed, snap frozen in liquid nitrogen and stored at -80°C until further analysis.

RNA was isolated from frozen tissues using Trizol reagent, cDNA was prepared with 1µg of total RNA using Reverse aid MMLV cDNA synthesis kit (Thermo Fischer Scientific). Geometric mean of tata box binding protein and *glyceraldehyde-3-phosphate* was used as reference control following Faheem *et al*. (2018). Gene sequence was obtained from National Center for Biotechnology Information (NCBI) and primers for all genes were designed using primer 3plus software. Primer sequence, gene accession number and product size is listed in Table I. Real time q-PCR was performed on CFX 96 (Bio-Rad, Hercules, CA, USA) with Syber green fluorescent label. A melt curve analysis was performed at the end of reaction to ensure amplification of the single product and PCR product was run on 1.5 % agarose gel. Ct values generated by software were converted to fold change relative to control using $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001).

Gene expression analysis is expressed as mean±S.E.M. Data was checked for homogeneityof varience through Shapiro-Wilk test. To evaluate significant difference among groups ($p<0.05$), one way analysis of variance was used followed by Tukey's post hoc test. All statistical analysis was performed using IBM SPSS (Version: 20).

Results and discussion

Primers of genes were specific in binding and yielded a single band of desired product size (Fig. 1). Expression of *cytochrome P450 1a1* gene relative to control is shown in Figure 2. The cytochrome P450 enzymes metabolize toxic compounds and CYP1A1 is most important as it is involved in metabolism of estrogens (Whyte *et al*., 2000). In the present study, effect of BPA was studied on mRNA expression of *cyp1a1* in liver of *Catla catla* which is endemic to Pakistan and commercially important species. Graded concentration of BPA (10,100,1000µg/l) for 14 days resulted in marked decrease in *cyp1a1* transcript level in a concentration dependent manner. Similar decreases in *cyp1a1* level were reported in Atlantic cod exposed to 50 µg/l of BPA for 21 days (Olsvik *et al*., 2009). Similarly, a dietary exposure of bisphenol A (5 and 50 mg/kg bw) for 21 days in *Sparus aurata* juveniles resulted in significant down regulation of *cyp1a1* level. In present study, down-regulation of *cyp1a1* recorded in all treatment groups (Fig. 2A) is in agreement with available data on the cross-talk between the reproductive system and the detoxification pathway (Jeong *et al*., 2001) and is supported by studies

showing an inverse relationship between plasma E2 levels and EROD activity during the reproductive cycle (Arukwe and Goksøyr, 1997). Faheem *et al*. (2017) recently reported that exposure of same concentrations of BPA to *Catla catla* females resulted in increased plasma estradiol concentration. The decrease in *cyp1a1* transcript level may be the cause of increased E2 levels reported by Faheem *et al*. (2017), as elevated E2 concentrations are known to be associated with decreases in CYP1A1 activity (Whyte *et al*., 2000). These studies and results of present study confirmed that estrogenic compounds like BPA inhibits the detoxification process and therefore, mRNA level of key gene (cyp1A1) can be used as potential biomarker to confirm presence of estrogenic compounds in water bodies.

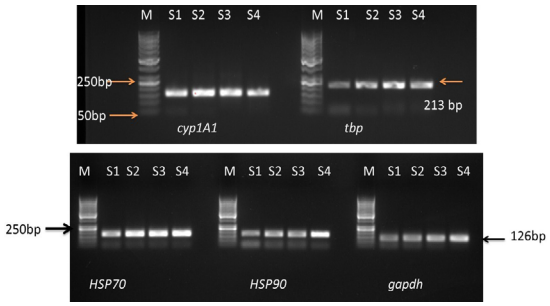

Fig. 1. Liver heat shock proteins (70 and 90), *cyp1A1* expression of adult female *Catla catla* exposed to graded concentration of BPA for 14 days. GAPDH, TBP used as reference control; M, marker; S1, control; S2, 10 µg/I BPA; S3, 100 µg/I BPA; S4, 1000 µg/I BPA.

Heat shock proteins (HSPs) are highly conserved cellular proteins present in all organisms studied to date and are known to function as stress proteins or molecular chaperones (Sung *et al*., 2011; Dang *et al*., 2018). HSP levels are mostly studied to assess the toxicity in organism both in laboratory conditions and at polluted environmental sites and therefore used as biomarkers to evaluate toxicity and overall ecological status of a water body (de Pomerai, 1996). In the present study, mRNA level of HSP 70 and HSP 90 was evaluated after 14 days' exposure to gradient concentrations of BPA. Expression of heat shock protein 70 and 90 (Fig. 2B). Exposure of 10µg/l BPA for 14 days caused 3 folds' significant increase in mRNA level of HSP 70 while 2.4-fold increase was recorded in fish exposed to 1000µg/l BPA for 14 days (Fig. 2B). Exposure of 10,100 and 1000µg/l BPA resulted in significantly higher expression of HSP 90 in liver of *Catla catla*. Pufferfish (*Takifugu obscurus)* exposed to 600 µg/l of bisphenol-A and 300 µg/l of 4-tert-octylphenol for 96 h resulted in significant upregulation of *hsp 70* and *hsp 90* after 24 h till 96 h (Kim *et al*., 2013). *Sparus aurata* juveniles fed with octylphenol for 21 days resulted in significant increased HSP70 mRNA expression compared to control (Traversi *et al*., 2014). Male and female black goby injected with nonylphenol and estradiol resulted in significantly increased expression of hsp70 mRNA after 72 hours (Maradonna and Carnevali, 2007). Many earlier studies also confirmed the elevated expression of HSP mRNA in fish exposed to pollutants hence, it can be used as a biomarker of exposure in fish (Rajeshkumar *et al*., 2013).

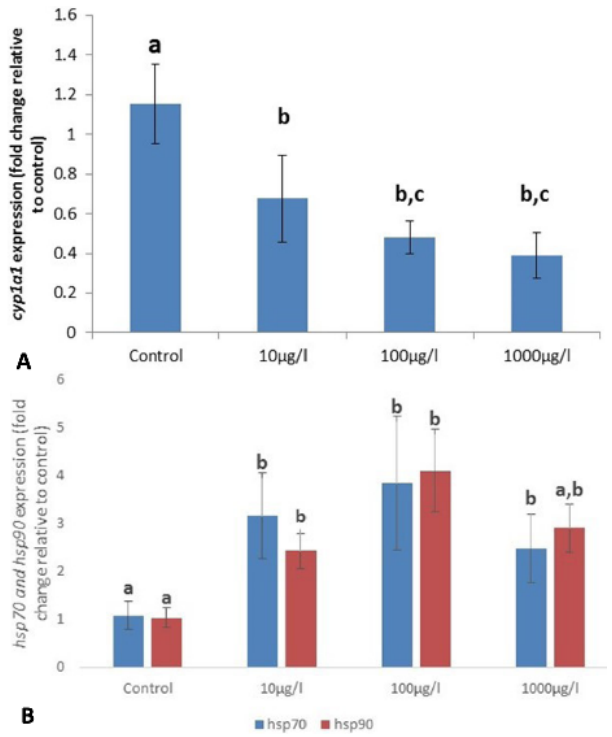

Fig. 2. Relative gene expression of the *cytochrome p450 1a1* gene normalized *tbp* and *gapdh* (A) and heat shock protein 70 and 90 normalized to mean of three selected gene (*gapdh, tbp*) (B) in liver of *Catla catla* (n=3) after 14 days of exposure to graded concentration of BPA. Data expressed as mean fold change ±S.E.M. Different letters indicate significant differences among groups. P<0.05.

Conclusion

In conclusion, the inhibition of *cyp1a1* and elevation of *hsp* transcript result in decreased detoxification ability which may lead to compromised health of organisms living in areas contaminated with environmental estrogens such as BPA. Our results offer baseline information which can be used for further research related to the EDCs and use of native carps like *Catla catla* as a bio-indicator for monitoring environmental health status.

Statement of conflict of Interest

The authors have declared no conflict of interest.

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