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Short Communication

Non-Monotonic Endocrine-Disrupting Effects of Bisphenol-A on Vitellogenin Expression in Juvenile Freshwater Cyprinid, *Catla catla*

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ABSTRACT

Endocrine-disrupting chemicals *e.g.* bisphenol-A can alter fish reproduction. Vitellogenin (*vtg)* is the egg yolk precursor and used as a biomarker for estrogenic endocrine disruption. To elucidate the endocrine-disrupting effect of bisphenol-A, juvenile *Catla catla* was exposed to graded concentrations of bisphenol-A (10,100,1000µg/l) for 14 days. BPA exposure strongly elevates *vtg* mRNA level in fish exposed to 100µg/l but at 1000 µg/l exposure of BPA, *vtg* level decreased compared to 100µg/l exposed fish. These results showed that BPA has estrogenic action and cause endocrine disruption in juvenile *C. catla* at environmentally relevant concentration. Moreover, these results also depict the non-monotonic, biphasic dose response to BPA.

Many chemicals released into the environment have the potential to disturb the endocrine system of organisms (Diamanti-Kandarakis *et al*., 2009). These chemicals are termed as endocrine-disturbing chemicals (EDCs) and can interfere with synthesis, release, circulation and metabolism of endogenous hormones which in turn lead to reproductive abnormalities. Because water bodies tend to contain a variety of EDCs from agricultural, municipal and industrial discharge, fish inhabiting such aquatic environments are exposed to all waterborne contaminants during brief periods or for entire lifespans, and are therefore considered more vulnerable to these EDCs (Eggen *et al*., 2003; Goksøyr, 2006).

Vitellogenin induction is used as a biomarker in assessing endocrine disruption in aquatic environment, particularly by estrogenic compounds (Matozzo *et al*., 2008). Vitellogenin (*vtg)* is a precursor of egg-yolk protein, synthesized in liver of female fish under influence of endogenous estrogen (Thomas-Jones *et al*., 2003). Male and juvenile fish also has *vtg* gene but is not expressed due to the absence of substantial levels of circulating estrogens (Harries *et al*., 1997). Therefore, induction of *vtg* mRNA in male and juvenile fish is considered as biomarker of endocrine disruption by environmental estrogens (Sumpter and Jobling, 1995; Kime, 1999).

Bisphenol-A (BPA) is an estrogenic endocrine-disrupting chemical (EDC) that gained much attention over the past decade. It is commercially important and widely used chemical (Vandenberg, 2014). It is a monomer used in the production of polycarbonate plastic and epoxy resins which in turn are used to make a large variety of plastic products including lining of food beverage containers (Staples *et al*., 1998, 2002). BPA is ubiquitous in aquatic environment and a number of reproductive and developmental effects have been reported in fish (reviewed in Bhandari *et al*., 2015).

Large number of studies reported estrogenic effects of BPA in fish, but to the best of our knowledge, no study is present concerning the effects of BPA on *Catla catla*. In this respect, a dose-response study was performed in order to determine the estrogenic potential of BPA and to establish the threshold for BPA induction of *vtg* in *C. catla*. In the present study, the estrogenic potential of BPA was determined by measuring mRNA expression of *vtg* in liver of juvenile *C. catla*.

Materials and methods

Juvenile *Catla catla* were purchased from a commercial fish farm located at suburbs of Lahore, Pakistan. Fish were acclimatized in cement ponds for two weeks under natural photoperiods. After acclimatization, fish were divided into four groups (10 fish per group). Three groups were exposed to graded concentration (10, 100, 1000µg/l) of BPA for 14 days, and the fourth group was vehicle control. BPA stock solution was prepared in ethanol and control group was exposed to maximum level of ethanol used for BPA dilutions. A fresh toxicant solution was added every other day after renewal of 75

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% water. Experiments and fish handling was performed according to OECD guidelines for fish toxicity (OECD, 1992). After 14 days, fish was anesthetized with clove oil and length and weight of fish measured. Fish were humanly sacrificed, liver was removed and snap frozen in liquid nitrogen and stored at -80 ^{0}C.

For insolating RNA tissue samples were ground in liquid nitrogen and total RNA was extracted from 100 mg of tissue using Trizol reagent (Sigma-Aldrich, USA) following manufacturer's instructions. Quantity and quality of RNA was checked using nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and ethidium bromide stained agarose gel respectively. Total RNA (1µg) was reverse transcribed with oligo-dT primers using Revert aid MMLV cDNA synthesis kit (Thermo-scientific). cDNA was diluted 1:10 for use in qRT-PCR.

Primers were designed using Primer3plus software. Primer sequence, annealing temperature and product size are listed in Table I. Validation of primer specificity was performed by conventional PCR and electrophoresing the PCR product on agarose gel to confirm a single band with the desired product size. Real-time PCR was performed using CFX 96 (Bio-Rad) with Syber green fluorescent label. In order to ensure amplification specificity, the melt curve of the PCR product was evaluated by heating from 60°C to 95 °C at the end of each reaction.

Ct value generated by software (CFX Manager Software, Version 3.1) at the end was used for further analysis. Baseline and threshold values were automatically set by the software. The Ct values for each of the gene were transformed into relative expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Data are expressed as means ± standard error of the mean. Data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test using IBM SPSS (Version: 20) to examine the effects of BPA exposure on *vtg* mRNA expression relative to the control group. The level of significance was set at $p < 0.05$.

Geometric mean of three most stable reference genes should be used as a reference control to accurately estimate mRNA transcript abundance (Vandesompele *et al*., 2002). Mean of three most stable reference genes, *gapdh, tbp*, and *18S* was used as internal control as described by Faheem *et al*. Unpublished.

Results and discussion

No mortality was observed during 14 day period in any treatment groups. Fold change in *vtg* concentration exposed to 10, 100 and 1000µg/l BPA is shown in Figure 1. *vtg* mRNA expression increased in fish liver exposed to 10, 100 and 1000µg/l BPA. At 100µg/l *vtg* expression increased many thousand folds compared to control, whereas at 1000 µg/l, *vtg* expression increased only 6 fold compared to control group. It appears from these results that 100µg/l is the optimum dose for *vtg* induction in juvenile *C. catla* in our experimental conditions.

Vitellogenin is a female-specific protein synthesized in the liver, transported through the blood to growing oocytes and accumulated in yolky eggs as a food reserve for embryos and early larval stages of fish. The analysis of *vtg* mRNA expression in the liver is a promising approach to monitoring estrogenic exposure (Bowman *et al*., 2000; Scholz *et al*., 2004). In particular, levels of mRNA rise rapidly after *vtg* gene induction, revealing recent exposure to

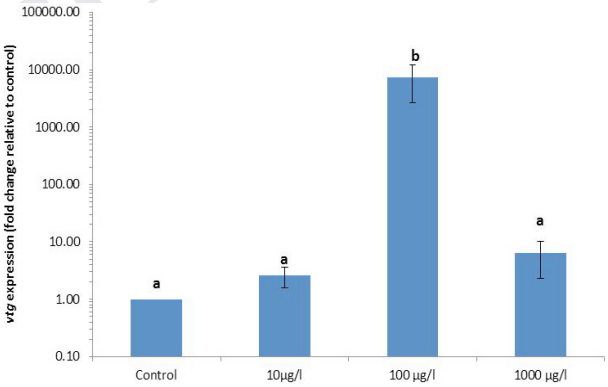

Fig. 1. Relative gene expression of the biomarker gene vitellogenin (*vtg*) normalized to mean of three selected genes (*gapdh, 18s, tbp*), in livar of juvenile *C. catla* after 14 days of exposure to graded concentrations of BPA. Different letters indicate significant differences among group. P<0.05.

Table I.- Primer sequences, amplicon lengths and annealing temperature of selected genes.

Genes	Primer sequence 5'to 3'	Amplicon size	Annealing temperature (°C)
gapdh	ATCA-CAGCCACGCAGAAGACC CAGGAATGACTTTGCCCACAGC	126	60
18S	CGGTGAACCTTGGTGACTCT CTTGGATGTGGTAGCCGTTT	189	60
tbp	AACAGCTTGTCCCTCCTGGA TCCAGGAGGGACAAGCTGTT	213	60
vtg	GTTGCTCTCCAGACCTTTGC GCAGAGCCTCCACCTTGTAG	180	60

estrogenic pollutants with elevated sensitivity (Bowman *et al*., 2000; Craft *et al*., 2004; Scholz *et al*., 2004) and it is one of the most studied estrogen-dependent processes in the reproduction of oviparous species.

In the present study, the levels of *vtg* mRNA altered in juvenile fish exposed to 10, 100 and 1000µg/l BPA for 14 days. However, the increase was only statistically significant in100 µg/l treated fish. Significant increase in *vtg* level was reported in liver of juvenile Atlantic salmon exposed to BPA, 25 and 125 mg/kg body weight (Arukwe *et al*., 2000). Exposure of 50 µg/l of BPA for 21 days cause significant induction of *vtg* in Atlantic cod (Larsen *et al*., 2006). Similarly, BPA induced significant increase in *vtg* level of both male and female fathead Minnow (*Pimephales promelas*) exposed at concentrations of 160 and 640µg/l (Sohoni *et al*., 2001).

Induction of *vtg* at 10, 100 and 1000 µg/l BPA observed in the present study also suggests estrogenic activity of BPA at environmentally relevant concentrations. Other studies have shown *vtg* induction in a dose- and time-dependent manner. Dose dependent increase was observed in liver vtg mRNA level of male *Oryzias sinensis* exposed to BPA for six days in a range of 0.02 to 2 mg/l (Lee *et al*., 2007). *vtg* level increased significantly in common carp exposed to range of BPA (1,10,100,1000µg/l) for 14 days (Mandich *et al*., 2007) and a significant increase in medaka exposed to 1000µg/l of BPA for 21 days (Ishibashi *et al*., 2005). In rainbow trout exposure of 500µg/l of BPA for 12 days resulted in significant induction of *vtg* (Lindholst *et al*., 2000). All these studies reported that BPA is capable of *vtg* induction; however difference among studies can be due to difference in fish species used as model, species-specific estrogen receptor binding, water temperature and exposure time (Lindholst *et al*., 2000; Crain *et al*., 2007).

Mandich *et al*. (2007) reported dose dependent increase of vitellogenin in male and female *Cyprinus carpio* exposed for 14 days to gradient concentrations of BPA (1-1000µg/l). Our results are interesting as we observed the optimum increase in mRNA expression of *vtg* at 100 µg/l of BPA exposure, while at 1000µg/l of BPA increase in *vtg* mRNA expression is not significant compared to control. This means that BPA becomes less effective and toxic at concentrations above 100 µg/l. This is sometimes referred as a biphasic response. Our results at higher dose of BPA are contradictory to the findings of Mandich *et al*. (2007) who reported an increase of vitellogenin in *cyprinus carpio* exposed to 1000 µg/l. Recent studies from Zhang *et al*. (2016) showed similar inverted U-shaped response of BPA in rare minnow. BPA exposure of 1 and 15 µg/l significantly up-regulated *vtg* levels (1.09 and 1.13 folds, respectively) while BPA exposure at 225 µg/l causes down-regulation of *vtg* level. Virk *et al*. (2014) reported that common carp, a species related to *C. catla,* exposed to 100 µg/l of BPA had significantly higher plasma concentration of vitellogenin, while fish exposed to 1000µg/l BPA has lower plasma vitellogenin concentration. BPA showed inverted U-shaped kinetics regarding vitellogenin levels in common carp (Virk *et al*., 2014) which is also observed in the present study. Down-regulation of *vtg* observed at higher dose of BPA is due to the fact that at higher doses BPA become toxic to liver cells. In an earlier study, we evaluated the histopathological effects of BPA and found that 1000µg/l BPA exposure caused degenerative effects in liver and other vital organs of juvenile *C.catla* (Faheem *et al*., 2016a). NIEHS expert panel in 2007 also concluded that BPA can produce non-monotonic dose response curves (vom Saal *et al*., 2007). Vandenberg (2014) reported that non-monotonic dose-response curves are common with BPA and around 24% of *in-vitro* experiments with BPA showed non-monotonic response. *In vivo* studies with rodents also support the notion that BPA produce non-monotonic, biphasic responses (Xu *et al*., 2010; Jenkins *et al*., 2011; Angle *et al*., 2013).

Conclusion

Bisphenol-A exerts an estrogenic action and at environmentally relevant concentrations can induce *vtg* synthesis that cause potential harm to fish reproduction.

Statement of conflict of interest

The authors declare no conflict of interest regarding this paper.

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