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RESEARCH ARTICLE

Bisphenol-A (BPA) Alters Plasma Thyroid Hormones and Sex Steroids in Female Pakistani Major Carp (*Catla catla; Cyprinidae)*

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ABSTRACT

In teleosts, steroid synthesis is mainly controlled by hypothalamus-pituitary-gonadal (HPG) axis. A number of compounds released into aquatic environment that have potential to interfere with fish endocrine system. These endocrine disturbing chemicals (EDCs) can disturb piscine endocrine system at every level by interference with HPG axis. Bisphenol-A (BPA) is an estrogenic endocrine disrupting chemical used in production of polycarbonate plastic and epoxy resins. The present study was aimed to investigate the effects of BPA on level of steroid and thyroid hormones in a fresh water cyprinid, *Catla catla*. Virgin female *C. catla*, two years of age, were exposed to graded concentration of BPA (10,100 and 1000µg/l) for 14 days, after stipulated time fish were bled and hormone titers were estimated. Plasma level of estradiol significantly increased in response to 100 and 1000µg/l BPA exposure. A significant decrease in plasma testosterone, triiodothyroxin (T3), thyroxin (T4) was recorded after 14 days BPA exposure. Plasma follicle-stimulating hormone (FSH) levels showed significant increase only at 10µg/l BPA exposure. Significant increase in plasma luteinizing hormone (LH) was observed in fish exposed to 1000µg/l of BPA. Change in plasma sex hormone and gonadotropin levels may cause subsequent reproductive dysfunction by interfering with the feedback regulatory mechanisms of the HPG-axis.

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INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are discharged into the environment through anthropogenic activities. These alter the normal function of the endocrine-signaling pathways in organisms by acting either as antagonists or agonists of their receptors (Vandenberg *et al*., 2009). EDCs can affect normal reproduction and development of an organism by altering an array of hormonal signals (Zhang *et al*., 2008). EDCs from agricultural, municipal and industrial wastewater are most often discharged to aquatic environments, where these contaminants can persist, accumulate, and impact aquatic life inhabiting such environments. Thus, fish are routinely exposed to many waterborne contaminants during critical periods of their development or entire lifespans, and are therefore considered more vulnerable to these EDCs (Goksøyr, 2006).

Bisphenol A (BPA), a known EDC, is used extensively as a raw material in the manufacture of plastics and many plastic products. It is also used in the manufacture of epoxy resins that line food and drinking containers and pipes for drinking water (vom Saal and Hughes, 2005). BPA mimics the activity of endogenous estrogen (17 β-estradiol [E2]), via interaction with either α and/or β estrogen receptors (ERs). It binds weakly to the estrogen receptor (1000-fold less than 17β-estradiol) and also has anti-androgenic activity (Sohoni and Sumpter, 1998). *In vitro* studies with BPA revealed that it has the potential to bind weakly to the thyroid hormone receptors thereby suppressing the T3-stimulated transcriptional activity (Moriyama *et al*., 2002).

The balance of sex steroids is important in fish as they regulate sexual differentiation, maturation and gamete growth; and thyroid hormones exert essential functions in the regulation of reproduction, embryologic development, metabolism and metamorphosis (Di Giulio and Hinton, 2008). Therefore, based on the economic, ecological, and societal importance, *Catla catla* was used as test species. *C. catla* is one of the most commercially

important freshwater fish in Pakistan, and most popular edible fish in Indo-Pak subcontinent. It constitutes a major aquaculture species, In Pakistan, *C. catla* is cultured on a large scale with indigenous major carps (*Labeo rohita* and *Cirrhinus mrigala*) and exotic Chinese carps in a polyculture system (Lone *et al*., 2009). Laboratory studies with *C. catla* can be used to depict the effects of BPA on this species in natural water bodies and currently, there is no information, whatsoever, on the effects of BPA on steroid concentrations in *C. catla*.

The primary goal of this study was to evaluate the effects of an estrogen mimic, BPA on the brain-pituitary-thyroid and gonadal axis by measuring serum levels of key hormones involved in reproduction and metabolism so that we could elucidate a potential mechanism of action of BPA and its downstream effectors on circulating hormone levels.

MATERIALS AND METHODS

Exposure to Bisphenol-A: Two-year-old, virgin *C. catla* females in their first reproductive cycle (mean length, 39.9±1.10 cm; mean weight, 1023.53±99.97 g) were purchased from a commercial fish farm (latitude 31° 58′N, longitude 74°13′E) located in the suburbs, 40 km from Lahore, Pakistan. Fish were collected by cast nets from ponds, brought to the laboratory alive, and acclimatized for 15 days in concrete tanks. During acclimatization, fish were fed with commercial carp pallet diet (Oryza Orgaics) twice a day. Physico-chemical parameters such as water temperature, dissolved oxygen, electrical conductivity and hardness, were recorded after every other day during acclimation and exposure.

After acclimatization, fish were divided into four groups of 10 fish per group. One group served as the control, and three groups were exposed to graded concentrations (10, 100 and 1000µg/l) of bisphenol-A (Sigma-Aldrich, St. Louis, MO, USA). BPA was dissolved in ethanol (2mg/ml). The control group was exposed to the maximal level of ethanol (0.5ml/l) used for BPA dilution. The experiment was conducted in a semi-static condition, following OECD guideline number 203 (OECD, 1992). Fish were exposed to BPA for 14 days, ¾ water was replaced every other day and fresh toxicant solutions were added after water renewal (Faheem *et al*., 2016).

Sample collection: After 14 days, fish from each treatment group were removed from the tanks and anesthetized immediately with clove oil (Faheem *et al*., 2016). Anesthetic was prepared fresh by dissolving clove oil into absolute alcohol (Merck, Germany) in a ratio of 1:2. This solution was used as a stock for mixing with water to achieve the desired concentration (5ppm). Fish were removed from the anesthetic chamber when completely sedated. Total body weight and total body length were measured. Blood was collected from the caudal peduncle using EDTA-washed syringes (3 ml, equipped with a 21- gauge needle) and transferred into ice-chilled vials. Plasma was separated by centrifugation at 1000 x g for 10 min, and aliquoted and frozen at -80°C until analysis. All samples were collected before noon so as to minimize variability due to diurnal fluctuations in plasma steroid concentrations. Sex of fish was determined after dissection, plasma of only female fish was used for present study (n=5). After dissection, organs were removed for further histological and gene expression studies.

Hormone assay: Plasma levels of sex steroids (estradiol and testosterone), gonadotropins (FSH and LH) and thyroid hormones (T3, T4 and TSH) were measured for five fish per group. Commercially available enzyme-linked immunosorbent assay (ELISA) kits (for E2, testosterone, FSH and LH; Biobasic, USA) and radioimmunoassay (RIA) kits for thyroid hormone were used according to the instructions from the manufacturer. Each sample set was run in duplicates with set of standards and negative controls. A calibration curve of standards was made at the end of each sample set.

Statistical analysis: All values 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 for each endpoint relative to the control group. The level of significance was set at $P<0.05$.

RESULTS

No mortality was recorded for any treatment groups during the exposure period. No statistically significant difference was observed in individual weight and size of fish among controls and the treated groups.

Plasma steroid hormones: After 14 days of treatment, plasma 17β-estradiol concentrations were significantly increased in 100µg/l and 1000 µg/l BPA treated groups compared to control. At the lowest concentration employed (10µg/l) of BPA, estradiol showed a tendency to decrease. (Fig. 1A). Plasma testosterone decreased in a concentration dependent manner and a significant decrease was observed in the plasma of fish exposed to the greatest concentration of BPA (1000µg/l) (Fig. 1B). The sex steroid ratio (E2/T) in female *C. catla* increased in a concentration-dependent manner. A significant increase was also observed in females exposed to 1000µg BPA/l (Fig. 1C).

Plasma thyroid hormones: Fourteen days of BPA treatment resulted in altered plasma concentrations of thyroid hormones in *Catla catla*. Plasma T3 exhibited a loose negative dose-response curve (Fig.2A). Plasma concentration of T4 increased in the 10µg/l of BPA treatment group; however, at 100 and 1000µg/l the T4 concentration decreased compared to controls (Fig. 2B). Measured concentrations of TSH in plasma were higher in BPA treated fish, but the difference was not significant relative to the controls (Fig. 2C).

Plasma gonadotropin hormones: Follicle-stimulating hormone increased significantly in fish exposed to the lowest concentration of BPA (10µg/l); and as the exposure concentration increased, FSH returned to the control level (Fig. 3A). With the increase in concentration of BPA, plasma LH level increased but significant increase was only observed in fish exposed to the highest concentration (1000µg/l) of BPA (Fig. 3B).

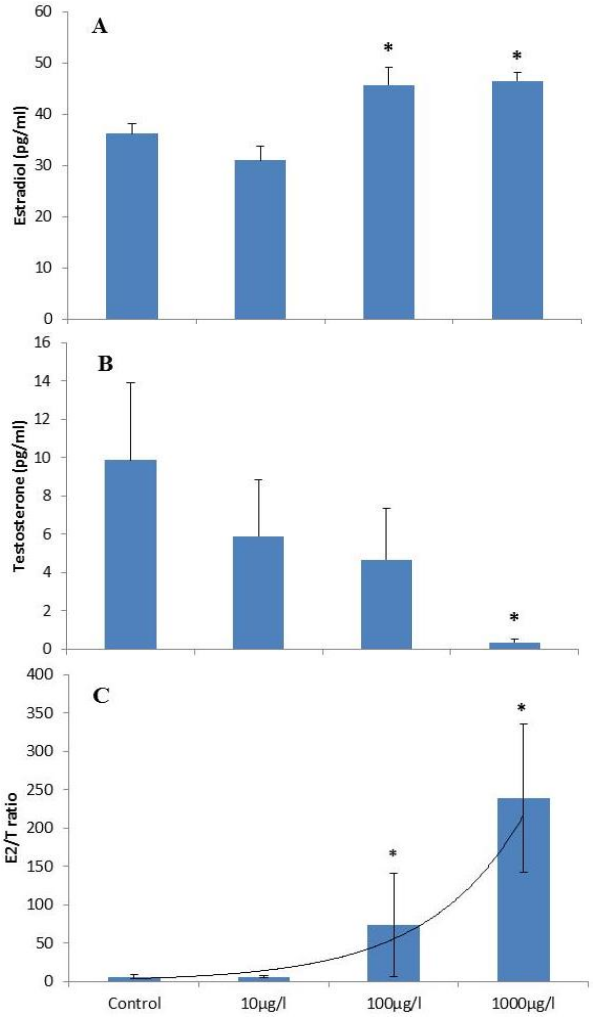

Fig. 1: Plasma concentrations of sex steroids and their ratio in female *C. catla* after exposure to BPA (10, 100 and 1000µg/l) for 14 days. (A) Estradiol (E2), (B) Testosterone (T). (C) E2/T ratio. Data is expressed as the mean±SEM. n=5. A significance difference when compared to the control is indicated by *P<0.05.

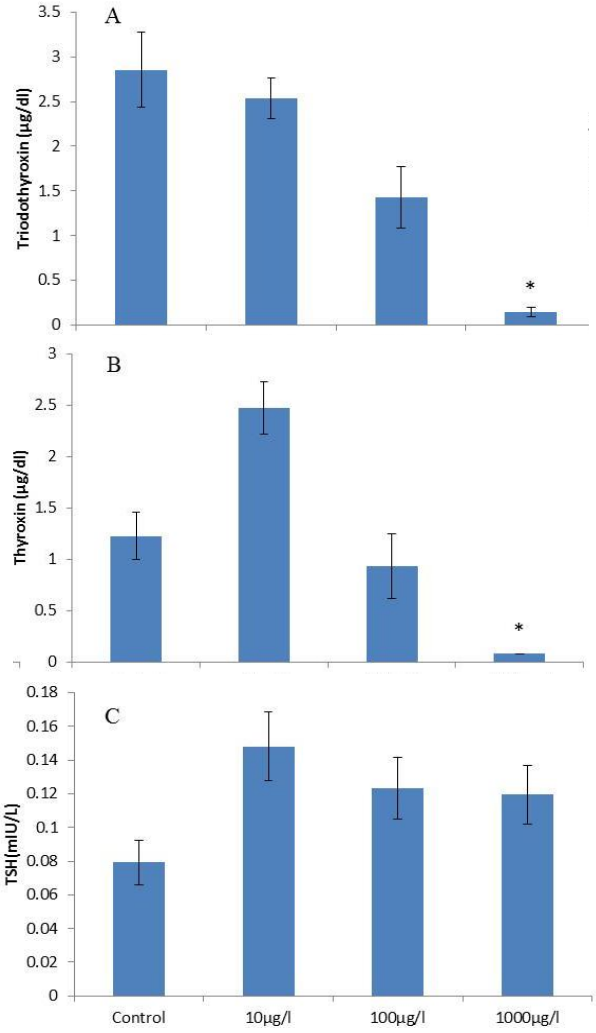

Fig. 2: Plasma concentrations of thyroid hormones in female *C. catla* after exposure to BPA (10, 100 and 1000µg/l) for 14 days. (A) Triiodothyroxin (T3), (B) Thyroxin (T4). (C) Thyroid stimulating hormone (TSH). Data is expressed as the mean±SEM. n=5. A significance difference when compared to the control is indicated by *P<0.05.

DISCUSSION

An alteration in the steroid sex hormone balance, in response to a toxicant, is considered to be a reliable biomarker of reproductive disturbance (Kime *et al.*, 1999). The results of the present study indicate that BPA can change steroid and thyroid hormone levels considerably in *Catla catla*. Exposure to graded concentrations of BPA showed elevations in plasma E2 levels in response to 100 and 1000µg/l of BPA after 14 days of exposure. This increase in plasma E2 suggests that BPA acts as an estrogen mimic. Increasing the exposure concentration of BPA caused a significant reduction in plasma testosterone levels of female *C. catla*. The increased level of E2 and subsequent decrease in testosterone may be due to an increase in aromatase activity that converts testosterone (from thecal cells) to E2 (granulosa cells). Liu *et al*. (2012) reported that BPA at 15µg/l caused a 0.43-fold (extremely significant) increase in ovarian aromatase transcript compared to the controls in the minnow. Mandich *et al.* (2007) also reported a decrease in plasma E2 levels in common carp exposed to 10µg/l of BPA and an increase in E2 levels when exposed to 100 and 1000µg/l. Our data support these results, but the increase at 1000µg/l is not as pronounced as reported by Mandich *et al.* (2007). The differences in study results may be due to species differences, BPA uptake rates, species-specific ER binding affinities, age, maturity and exposure period (Crain *et al*., 2007). In addition, the response of the fish will also be dependent on the season (photoperiod and temperature) in which the experiment was conducted.

A significant reduction in testosterone was observed in female *Catla catla* exposed to the greatest concentration of BPA. Mandich *et al*. (2007) reported a similar decrease in plasma testosterone levels both in females and males exposed to 1-1000µg/l of BPA. This reduction in testosterone may also have been due to the increased aromatase activity after BPA exposure which has been reported in many studies (Lee *et al*., 2006; Liu *et al*., 2012; Zhang *et al*., 2014). Consistent with this hypothesis of elevated aromatase activity, was the observation of increases in plasma concentrations of E2.

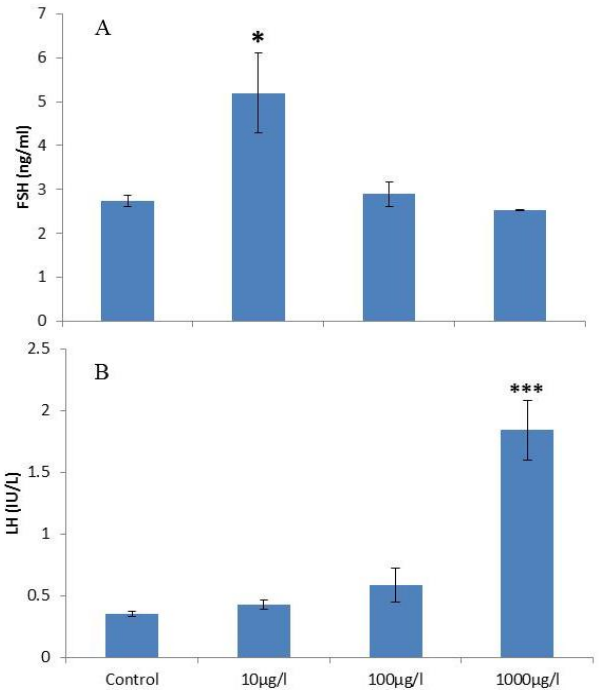

Fig. 3: Plasma concentrations of gonadotropins in female *C. catla* after exposure to BPA (10, 100 and 1000µg/l) for 14 days. (A) Follicle stimulating hormone (FSH), (B) Luteinizing hormone (LH). Data is expressed as the mean ± SEM. n=5. A significance difference when compared to the control is indicated by *P<0.05.

In adult male goldfish, T and 11-KT were significantly decreased upon BPA exposure at 0.6–11µg/l for 20 or 30 days (Hatef *et al*., 2012). Fang *et al.* (2016) reported similar significant decrease in plasma testosterone level of female zebrafish exposed to graded concentration (2, 20, 200 µg/l) BPA for 21 days. A similar trend in E2 levels was observed in female fish exposed to BPAF (bisphenol-A F, a fluorinated analog of BPA), and a decrease in testosterone was observed in zebrafish exposed to 1 mg/L BPAF (Yang *et al.*, 2016). All of these findings, along with those of the present study, are consistent with the actions of a weak estrogen on normal steroid hormone homeostasis.

Thyroid hormones (THs) play an important role in development, growth and metabolism. BPA shares a structure similar to that of thyroid hormones, and acts as thyroid agonist or antagonist in amphibian models (Jung *et al*. 2007). In teleosts, synthesis and release of T4 are regulated by TSH, and TSH levels are maintained by T4 negative feedback (Di Giulio and Hinton, 2008). In the present study, it is likely that the decrease in T4 levels during 14 days of treatment in this study was due to a negative feedback effect on TSH secretion or direct effect on pituitary or hypothalamus. In fish, the biologically active form of thyroid hormone, T3, is produced by removal of an iodide group from the T4 molecule by the action of 5'-monodeiodinase (5.MDA) (Becker, 2001). Three types of deiodinases have been discovered in fish, and dysfunction of 5'MDA can reduce the conversion of T4 to T3. In rainbow trout *(O. mykiss*), 5'-MDA activity was significantly decreased by treatment with E2 (Okimoto *et al*., 1991). It is suspected that BPA acts in a similar manner, causing a reduction in 5'MDA activity that results in decreased conversion of T4 to T3. Decreased production of T3 by alteration of deiodinases can be possible factor in the reduced concentration of this hormone in *C. catla* during the experimental period. Our data are in agreement with the premise that E2 suppresses peripheral thyroid status by altering T3 production rates (Yamada *et al*., 1993) and the free T3 index (Cyr and Eales, 1996). Although at receptor level, BPA binds weakly as a ligand to Thyroid hormone receptor; it inhibits TR-mediated transcriptional activity and act as a T3 antagonist. (Moriyama *et al*., 2002; Freitas *et al*., 2010). In salmonids, E2 levels increase during later stages of ovarian development, which causes a reduction in TH levels in the circulation by exerting negative feedback (Yamada *et al*., 1993). McCormick *et al*. (2005) found that intraperitoneal administration of 0.5 to 150µg/g of 4-NP to juvenile Atlantic salmon led to a dose-dependent reduction in T4 levels, while only higher doses reduced plasma T3 levels; and E2 treatment reduced both hormones in the circulation. BPA act as a powerful inhibitor of T3 binding to human TH-binding proteins and in rodents it act as a weak ligand to liver TR. Therefore, as in mammals and other vertebrates, TH antagonism appears to operate not at the receptor level, but via the ability of the chemical to suppress TR-mediated transcription and consequently to reduce TR availability (Mathieu-Denoncourt *et al*., 2015).

The brain-pituitary-gonadal-axis is regulated by hormonal signaling and feedback loops. Estradiol-17β and other aromatizable steroid produced in the ovary regulate LH synthesis in the hypothalamus and secretion by the pituitary in immature fish through a positive feedback action (Dickey and Swanson, 1998). High concentrations of E2 are required for the induction of the pre-ovulatory LH surge, resulting in oocyte maturation (Peter and Yu, 1997). Estrogens also have a negative feedback effect on FSH levels (Breton *et al*., 1997; Saligaut *et al*., 1998). In the present study, we clearly saw the inhibition of FSH by increases in E2 concentrations after BPA exposure. At 10µg/l, a low level of plasma E2 resulted in high levels of circulating FSH; on the other hand, high circulating levels of E2 observed after higher BPA exposure caused a decrease in plasma FSH levels that is in accordance with the negative feedback mechanism exerted by E2. These data fit well with those of small numbers of studies that demonstrated that E2 inhibits either FSH synthesis (Breton *et al*., 1997) or secretion (Saligaut *et al*., 1998) in rainbow trout, or secretion in coho salmon (Dickey and Swanson, 1998). A non-significant increase in LH was observed in fish exposed to 10 or 100µg/l of BPA, while exposure to high concentrations of BPA induced a highly significant increase in LH concentrations. Fang *et al.* (2016) observed a significant reduction in plasma FSH and LH levels of female zebrafish exposed to 200µg/l BPA.

In summary, the exposure of sexually immature virgin *C. catla* to a widely distributed EDC, bisphenol-A, caused reproductive dysfunction by altering sex steroid homeostasis and possibly also by disturbing gonadotropin production that controls reproduction and gonad development. BPA also causes significant alterations in circulating thyroid hormone levels. BPA induced changes in the normal profile of endogenous hormones that may lead to adverse health effects and reproductive disorders such as a shift in spawning time, attenuated number of eggs per spawn, or even complete absence of spawning.

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Authors contribution: MF carried the experiment and prepared the manuscript under supervision of SK and KPL. ELISA was performed by HUA and MF. All authors critically revised the manuscript and approved the final manuscript.

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