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Effect of Pentachlorophenol on Testicular Steroidogenesis during Different Reproductive Phases of the Catfish, *Heteropneustes fossilis*

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Abstract

Endocrine disruptor chemical may result in decline of fish population and affect other animals and human beings after entering into food chain. This study is important in order to generate baseline data for its toxicity in fish and to make an inference needed to evaluate the toxic potency of Pentachlorophenol (PCP). During pre-spawning phase, testes of PCP exposed group (32 µg/l/day for 28 days) showed a decreased in the values of testosterone while an increase in the E2 level was observed which indicates estrogenic potential of PCP whereas, in spawning phase, testes of PCP exposed group showed a significant increase in the testosterone level and decrease in the E2 level. Increase in Estradiol level was found in testes of exposed fish during pre-spawning phase might be due to the effect of PCP as endocrine disruptor and estrogenic as well. Increase in Testosterone level during spawning phase could be due to increased spermatogenesis during this phase. Study with dose and duration dependent exposure may explain further understanding.

Introduction

Pentachlorophenol (PCP) was extensively used for decades in agriculture and industry Zheng et al., 2012). It is an organic chlorinated compound used worldwide for preserving utility poles, railroad, and as insecticides, pesticides, and biocides for controlling agricultural and household pests (Cooper and Jones, 2008). Deleterious effects of PCP such as developmental toxicity, liver defects, genetic toxicity, and endocrine disrupting activity have been studied in livestocks (Morales et al., 2014). The pollution of PCP may pose a severe threat to the survival of aquatic species, and it is crucial to analyze its ecological risk. The adverse effects of PCP have been reported by many workers which included immunotoxicity, carcinogenicity, oxidative stress and metabolic disorders Chen et al., 2015). The PCP has been demonstrated to display estrogenic, anti-estrogenic and anti-androgenic in vitro

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Orton et al., 2009; and disturbance in the hypothalamic-pituitary-gonadal (HPG) axis in vivo study Zhang et al., 2008). The proteins in HPG axis often showed inconsistent values in response to the change of time and concentration of exogenous toxicant Liu et al., 2009). There are studies lacking on the distribution of steroid hormones which play important role in regulating reproductive status and spawning behavior (Bruton, 1996). The PCP has been reported to cause DNA damage, endocrine disruption (Zhang et al., 2014), impairments of ovaries, follicular atresia (Sawle et al., 2010), morphological deformities (Cheng et al., 2015), altered activities of antioxidant enzymes, changes in serum testosterone (Zhang et al., 2014), anti-estrogenicity (Zhao et al., 2006), reproductive organ damage (Zhang et al., 2014), immunotoxicity (Shelley et al., 2009) and mutation (Yin et al., 2009) in fish species. Many endocrine disruptors inhibit and/or induce sex steroid biosynthesis consequently linked to reproductive abnormalities in animals Reeder et al., 2005). Testicular steroids can induce spermiation and certain steroid glucuronides function as sex pheromones implying importance in breeding and captive reproduction (Chaube, et al 2018). Fish is used widely as a bio-indicator of water pollution due to rapid responses with high sensitivity to changes in their usual physiological functions such as molecular, biochemical, cellular, hormonal, or behavioral responses (van der Oost et al., 2003). The freshwater catfish, *H. fossilis* is an edible, economically important fish,

and is ideal for wastewater aquaculture. The present study will be useful in understanding the impact of PCP on metabolic and reproductive functions encountered by freshwater fishes. Steroids are chief hormonal messengers synthesized mainly by gonads and play key role in sexual differentiation, metabolism, osmoregulation and germ cell growth, maturation and release, these are degraded largely in the liver to form water soluble metabolites, which are excreted out (Chaube, et al., 2018). The objective of this study was to investigate possible impact of PCP on steroid profile of testes of the catfish, *H. fossilis*. The present analysis can provide valuable insights with regard to the functions of the steroids in the male fish exposed to the PCP.

Materials and Methods

Chemicals

PCP (Crystalline, 99% pure) was purchased from Acros Organics (Geel, Belgium). All other chemicals were of analytical grade and purchased locally. The PCP was dissolved in ethanol and then diluted with water to obtain the required concentrations 32 µg/l (1/10th of LC_{50}).

Animal collection and maintenance

The experiments were performed in accordance with local/national guidelines for experimentation in animals and all care was taken to prevent cruelty of any kind. The freshwater catfish *H. fossilis* were purchased from the local market in Varanasi and used for the experiment. They were acclimated to laboratory conditions for one week prior to the experiments. They were maintained in the laboratory under normal photo period (13.0L:11.0D) and temperature (25±2^{0}C) until use for experiments. The fish were fed with boiled egg daily *ad libitum.*

Experimental setup

The acclimatized adult male fish classified into two groups (15 fish per each): first group control, second group PCP treated (28 days with 32 µg/l/day) in pre-spawning and spawning phase. In the present study, the amount of PCP exposures was 32 µg/l and the exposure concentrations were determined by performing LC_{50} experiment Dubey and Verma, 2019, 2021; Dubey et. al., 2022). The conditions of the experiment were as that of acclimatization with changing all the containers water and concentrations of PCP every day.

Steroid extraction

The tissues were homogenized separately in 4 volume cold PBS (0.02 M, phosphate buffered-saline pH 7.4) with an ultrasonic homogenizer at 0^{0}C for 5–10 seconds. The homogenate was centrifuged at 5000 rpm for 20 min at 4^{0}C and extracted with 3 volume diethyl ether, three times. The ether phase was pooled, evaporated and dried under N_2 gas, and stored at -20^{0}C. The incubation medium was directly extracted with diethyl-ether, as described above. The ether phase was pooled group-wise, evaporated, dried under N_2 and stored at -20^{0}C. For the hormone assay, both tissue supernatant and corresponding incubation medium were pooled to make a sample.The supernatant was collected and steroid extracted in 3 volume diethyl ether, 3 times. Organic fractions were pooled, evaporated and dried under N_2 gas and stored at -20°C till further

estimation. Prepared the extracted steroid in solution form by adding 100 µl methanol to steroid extract.

A. Testosterone assay

Testosterone was assayed using an ELISA kit according to the manufacturer's instructions. Briefly, 25 µl each of standard (0, 20, 100, 400 and 1600 pg/ml) and samples were pipetted into the anti-TIgG-coated plate well. The immunoreaction was started by adding100 µl of T-HRP conjugate solution to each well, followed by incubation at 37^{0}C for 1 hour. The content from each plate was removed and washed with 300 µl of distilled water, 5–6 times. Water was completely drained out from each

well. Next 100 µl of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate was dispensed into each well and incubated at 25°C for 15 min in dark. Color development was stopped by adding 100 µl of stop solution (0.15 M, sulphuric acid). Absorbance was taken at 450 nm using a Multiscan microplate reader (Thermo Electron Corporation, USA).

B. Estradiol-17β (E2) assay

E2 was assayed using an ELISA kit according to the manufacturer's instructions. Briefly, 25 µl each of standard (0, 20, 120, 300, 600 and 2000 pg/ml) and samples were dispensed into the anti-E2-IgG-coated plate well. The immunoreaction was started by adding 200 µl of E2-HRP conjugate solution to each well, followed by incubation at 37°C for 2 hour. The content from each plate was removed and washed with 300 µl of distilled water, 5–6 times. Water was completely drained out from each well. Next 100 µl of 3, 3', 5, 5'-tetramethyl benzidine substrate was dispensed into each well and incubated at 25°C for 30 min in dark. Color development was stopped by adding 100 µl of stop solution (0.15 M, sulphuric acid). Absorbance was taken at 450 nm using a Multiscan microplate reader (Thermo Electron Corporation, USA).

Statistical Analysis

The data were expressed as means ± standard error mean (SEM). Comparisons of means (control and treated fish) were done by Student's t-test. The result was considered significant at 5% level ($p < 0.05$).

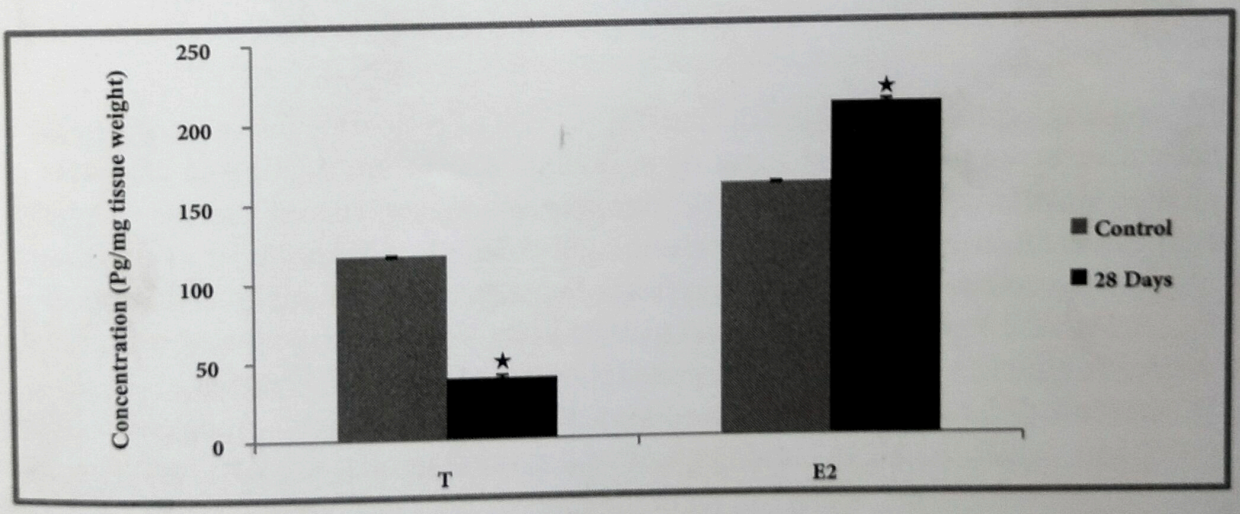

Fig. 1: Estimation of Testosterone (T) and estradiol-17β (E2) in the testes of the catfish *H. fossilis* exposed to 32µg/l/ day PCP for 28 days during the pre-spawning phase of reproduction. (Asterisk shows significant difference from Control at P<0.05; Student's t-Test) Data were expressed as mean ± SEM

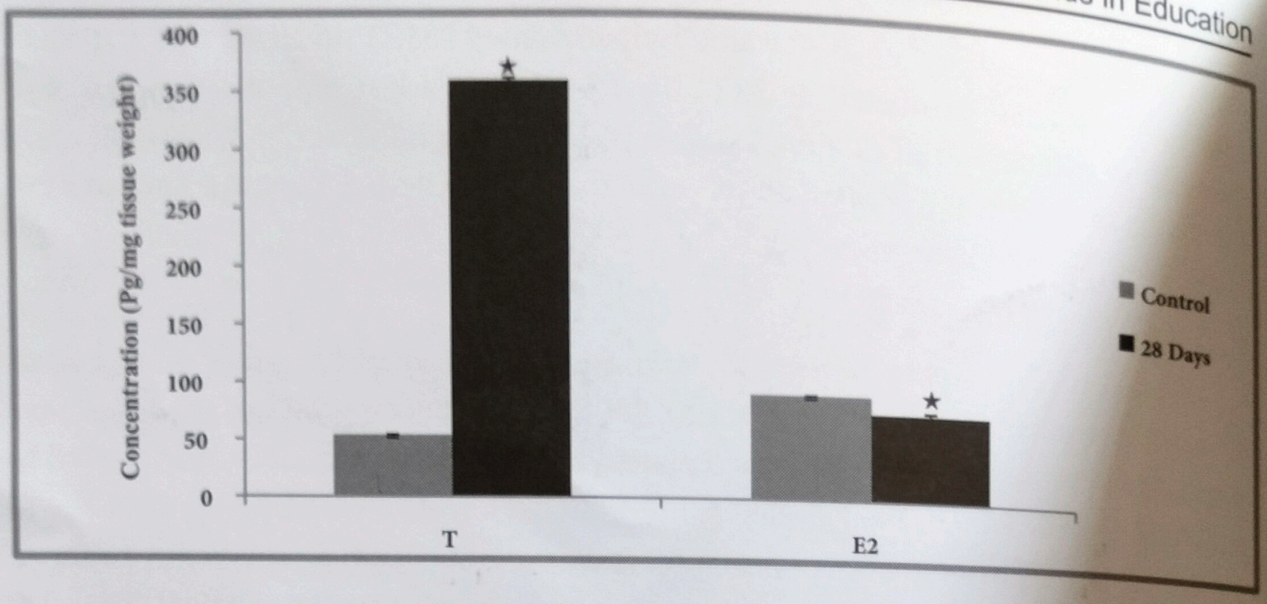

Fig. 2: Estimation of Testosterone (T) and estradiol-17β (E2) in the testes of the catfish *H. fossilis* exposed to 32µg/l/ day PCP for 28 days during the spawning phase of reproduction. (Asterisk shows significant difference from Control at P<0.05; Student's t-Test). Data were expressed as mean ± SEM.

Results

During pre-spawning phase, testes of PCP exposed group (28 days with 32 µg/l/day) showed a decrease in the values of testosterone while an increase in the E2 level was observed. In spawning phase, testes of PCP exposed group (28 days with 32 µg/l/day) showed a significant increase in the testosterone level while decrease in the E2 level was observed.

Discussion

Amongst vertebrates, fishes are the most at the risk of endocrine disruption since their habitat receives the greatest input of pollutants due to anthropogenic activities or natural weathering processes. Fishes are, therefore, considered the most suitable animal model in endocrine disruption-related research. To date most of the studies elucidating toxicity of xenobiotics on fishes focus on two of the major endocrine axes, the stress and reproductive (Hontela, 1998). It is reported that sex steroids are synthesized in the brain, renal and gonadal tissue of teleosts (Petersen et al., 2015). Further in studies it is revealed that androgen biosynthesis is under the control of the HPG axis which perceives endogenous and environmental cues to modulate sex steroid synthesis from cholesterol (Miller and Bose, 2011). Steroidogenesis produces the estrogen, E2, and the androgen (Carnevali et al., 2018). The steroidogenic pathway is highly conserved across vertebrate taxa and is a major target of endocrine disruption (Feswick et al., 2014). Initial spermatogenesis is under FSH control, while later spermiogenic stages are regulated by pituitary LH (Itoh et al., 1988). Some studies relate reproductive cycle in adult fish with fluctuating levels of androgen synthesis

(Weltzien et al., 2002). Seasonality was reported in plasma testosterone levels (Borg, 1994) and is directly correlated to spermatogenesis (Rinchard et al., 2001). During late spermatogenesis, a reduction in testosterone is attributed to a shift in the steroidogenic pathway towards 17,20β-P or similar Mullerian inhibiting hormone (MIH) production (Yaron and Levavi-Sivan, 2011). Androgens also have roles in lipid transport and oxidation (Hoffman et al., 2008), β lymphocyte differentiation (Moens, et al., 2007), xenobiotic clearance (Martyniuk and Denslow, 2012) and protein metabolism (Dorts et al., 2009). Androgens also regulate endothelial function, inflammation and oxidative stress in fishes (Si et al., 2014), although the specific mechanisms through which they accomplish this, is currently unknown.

Several xenobiotic compounds that are reported to disrupt various physiological activities fall in the list of endocrine-disrupting chemicals since minute alteration in hormone levels, their receptors or post-receptor signals can cause significant changes in the functions of target cells and tissues (Colborn et al., 1993).

The PCP is highly persistent with the potential to modulate several biological processes that have an impact on growth and development. It is often detected in the aquatic environment (Crosby et al., 1981). Wu et al (2001) showed that in channel catfish testes, estrogens, participate in the regulation of male gamete development and fertility. In vertebrates ranging from sharks to mammals, testicular tissues have been shown to contain aromatase, the rate-limiting enzyme for estrogen formation (Gist et al., 2007).

In the present study testosterone levels was high in control group in pre spawning phase which was expected. Similar findings state that androgen levels are high moderately during the initiation of spermatogenesis (Rolland et al., 2009). Androgens of the testes increased during spermatogenesis and spermiation and play significant role in male reproduction (Amer et al., 2001).

Two androgen receptor (AR) subtypes (α and β) have been demonstrated in fish and they are all predominantly expressed in the gonad particularly expressed in sertoli and interstitial cells (Ikeuchi et al., 2001) suggesting that androgens develop biological activity via the testicular somatic cells. Similarly, the investigation also showed high levels of 17, 20β –DP in testes, levels being higher in pre spawning phase in testes than spawning phase. Baynes and Scott (1985) proposed that a major function of 17, 20β-DP in the male is to control sperm motility mediated by changes in K+ composition of seminal fluid. Another function of 17, 20β –DP is that it can be conjugated into glucuronide and conjugates and these water soluble conjugates have pheromonal role (Ikeuchi et al., 2001). In the catfish 17, 20β–DP may be involved in sperm maturation and spermiation and role in pheromone is a field to be investigated further. Steroid levels are generally low during the non-reproductive period, but increase gradually throughout gametogenesis and decline abruptly thereafter. The predominance of T, 11-

ketotestosterone (11-KT) and E2 in initiating and regulating seasonal reproductive events are most intensively studied (Zhou et al., 2007).

In catfish *H. fossilis* high E2 in testes in spawning phase was observed. Regarding the functional role of E2, it may promote or inhibit early spermatogonia renewal.

Song and Gutzeit, 2003). The E2 may regulate the regulation of the steroidogenesis (i.e. Star, 3β-HSD, aromatase A and B). Administration of E2 to maturing sea bream males resulted in the identification of numerous estrogen-dependent genes in the testes (Patino et al., 2006).

In spawning phase, testes of PCP exposed fish showed a significant increase in the testosterone level while a decrease in the E2 level. Increase in testosterone level during spawning phase might be due to increased spermatogenesis during this phase. Study with dose and duration dependent exposure is needed for its further understanding.

Endocrine disruption by PCP was monitored in adult zebrafish and rare minnow (*Gobiocypris rarus*) with elevated plasma thyroxine concentrations (Zha et al., 2007). Many synthetic chemicals have been shown to function as natural steroid hormones since they can interact with the estrogen receptor (ER) as agonists and elicit biological responses (Jin et al., 2012). For example, these endocrine disrupters can bind to ERα or ERβ and subsequently alter the normal expression of estrogen-responsive genes (Verderame et al., 2011). In zebrafish and rare minnow, the estrogen receptor (ER) mRNA was up-regulated in males and down-regulated in females, and vitellogenin mRNA induction and serum vitellogenin protein increase was reported in longer exposed females (Zhang et al., 2014). During pre-spawning phase, testes of PCP exposed *H. fossilis* showed a decrease in the values of testosterone and increase in estradiol level, could be due to the effect of PCP as endocrine disruptor and its estrogenic potential as well.

Conclusion

At present time, surface waters are polluting by various ways. Elevated levels of pollutants in aquatic systems have resulted from a number of activities including agriculture, urbanization, impoundments, mining and industrial activities. These effects reduced growth rates, impaired reproduction and sometimes death. Bioaccumulation and bio concentration of these in the food chain can put consumers, including humans at risk. PCP is an organic chlorinated compound used worldwide for preserving utility poles, railroad, and as insecticides, pesticides, and biocides for controlling agricultural and household pests (Cooper and Jones, 2008). During pre-spawning phase, testes of PCP exposed fish showed a decreased in the values of testosterone while an increase in the E2 level was observed which indicates estrogenic potential of PCP. In spawning phase, testes of PCP exposed group showed a significant increase in the testosterone level while decrease in the E2 level. The pollution of PCP

may pose a severe threat to the survival of aquatic species due to its endocrine disruptive activity.

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