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BIOCHEMICAL EFFECTS OF BISPHENOL A (BPA) IN ASIAN STINGING CAT FISH *HETEROPNEUSTES FOSSILIS*

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ABSTRACT:

Bisphenol A (BPA) is one of the major industrial worldwide pollutants, and has been extensively detected in surface waters, residues and biota.In the present study, female*Heteropneustes fossilis*was exposed to various sublethal concentration of BPA and histopathological and biochemical responses were studied. The median lethal concentration of BPA to *Heteropneustes fossilis*was calculated by probit analysis method and the value was found as 7.1443 mg/l for 96hrs. Fish were exposed to different sublethal concentrations i.e. to 0.714 mg/L ($1/10^{th}$), 1.428 mg/L ($1/20^{th}$) and 2.142 mg/L ($1/30^{th}$) (Group II, III and IV) using ten specimens in each aquarium for 28 days.At the end of the experiment, fish were dissected and ovary was taken out for histopathological observations. Blood sample was collected by severing the caudal peduncle and processed for the estimation of total plasma protein content, glucose, Aspartate aminotransferase (AST/GOT, EC 2.6.1.1), Alanine transaminase (ALT/GPT, EC 2.6.1.2) and17β-Estradiol. The results shown that BPA was reasonably toxic to the fish *Heteropneustes fossilis* and toxic effects are depending on concentration dependent. The treatment of sub lethal concentrations of BPA induced substantial alterations in the structural design of ovary. The structural changes mainly include necrosis, increased oocyte atresia, perifollicular cell hyperplasia/hypertrophy, decreased vitellogenesis, and changes in gonadal stagings. BPA treatment showed a significant reduction in plasma protein levels. Exposure to sublethal concentrations of BPA showed a significant and gradual increase in AST (*SGOT),* ALT (SGPT*)* and plasma E2 levels all experimental groups. Overall, our study suggests that the incorporation of sublethal concentrations of BPA can alter endocrine system and reproductive function in adult fish and permits further research.

***Keywords**: Bisphenol A (BPA), Heteropneustes fossilis, ovary, Biochemical changes.*

INTRODUCTION:

BPA is one of the major environmental contaminants and endocrine disruptor that has become a major concern in recent times. Aquatic environs have become major sinks for various waste products include -ing BPA. Previous studies have confirmed severe effects of various anthropogenic toxicants including BPA on various organs of fish (Faheem et al., 2016, Reddy, P.B, 2017, Ratn, A. et al, 2018). The global production of BPA is about 3 billion kg each year and over 100 tons released into the atmosphere by yearly production (Vandenberg et al., 2009). BPA is found in wastew- ater from manufacturing units, as it is not totally removed during wastewater treatment. This wastewater containing BPA can be a source of contamination of the aquatic environment (Staples et al., 1998; Kang et al., 2006). BPA act as anti-androgens, anti-estrogens, and steroidogenic enzyme inhibitors that interfere with steroid action/ production as the mechanism to alter reproductive fitness. According to a report of U.S. Environmental Protection Agency (USEPA), one million pounds of BPA are discharged into the environment per annum and found in municipal wastewater in low quantities (Erler C, Novak J, 2010). The results of on-going laboratory research also raised the concern about the probable threats of BPA (Bisphenol A)as an endocrine disruptor as it closely mimics the structure and function of the hormone estradiol by binding to and activating he same estrogen receptor as the natural hormone (Takayanagi, S., et al, 2006,Takahashi, M., et al, 2018). Most of them are highly persistent in the environment and can accumulate in body tissues. Bisphenol A is a synthetic endocrine disrupting chemical (EDC). Earlier, studies have confirmed the severe effects of various anthropogenic toxicants including BPA on various organs of fish (Faheem et al., 2016, Ratn, A. et al, 2018).The effects of EDCs differ from those of usual pollutants are that such effects are not all the time immediate. Furthermore, exposure to EDCs during developmental stages can produce permanent structural changes in anatomy and the results of which might not become visible until adulthood (Frye, C., et al, 2012).Present study examined whether bisphenol-A (BPA) can alter fish reproduction by changing endocrine function or not.

In fish, increasingly the monitoring of ovarian growth and structure and sex steroid hormone concentrations are proving to be useful tools to study the effects of endocrine disrupting chemicals in wild populations(Hecker,M.,etal,2002). Therefore, to assess probable effects of Bisphenol A (BPA), the present study was designed to study histopathological changes of ovary and changes in Serum Estrogen (17β-estradiol) hormone concentrations in a catfish, *H.fossilis*.

MATERIALS & METHOD:

Healthy adult fish were handpicked during spawning season of 2016 from local commercial aqua culture ponds and were acclimatized to the laboratory settings for 15 days.Bisphenol-A*2, 2-Bis (4-hydroxyphenyl) propane (CAS* Number: 80-05-7)97% pure was obtained from Shreeji Pharma International, Vadodara, Gujarat, India. Groups of 10 healthy fishes (mean wt. 36.78g) were exposed to different concentrations of Bisphenol A to calculate the medium lethal concentration LC_{50} value using probit analysis method (Finney, D. J, 1952).The LC_{50} of BPA for *H.fossilis* in this experiment was found to be 7.142 mg/L. The fish was exposed to different sublethal concentrations of BPA (0.714 mg/L (1/10th), 1.428 mg/L (1/20th and 2.142 mg/L (1/30th) i.e. (Group II, III and IV) for 28 days using ten fishes in each aquarium. At the end of experiment, fish was dissected and ovary was taken out for histopathological examination. Blood samples were collected from the fishes of all experimental groups by caudal vein puncture using Ethylene diamine tetra acetic acid (EDTA) as an anticoagulant.

Histopathology: Fixed ovarian tissue was administered for in graded ethanol series, cleared in xylene and finally embedded in paraffin wax (melting point 60^{o}C). The embedded block were sectioned at 6µ on a rotary microtome, mounted on glass slides, dried and stained with haematoxylin and eosin (H&E). Sections were examined under a light microscope (NIKON ECLIPSE E 400, USA) and photographed by using digital camera attached to the microscope.

Biochemical studies: The total Protein content of the BPA exposed tissue samples were estimated according to modified standard method by (Lowery *et al.*, 1951). Blood glucose was determined using the Folin Malmros micro procedure as modified by (Murrell, L.R. and Nace, P.F., 1958). Both Aspartate aminotransferase/ serum glutamic oxaloacetic transaminase (AST/ GOT, EC 2.6.1.1) was assayed by the method as prescribed by Friedemann, et al (1943) and Alanine aminotransferase/ serum glutamic pyruvic transaminase (ALT/GPT, EC 2.6.1.2) was assayed by the method prescribed by Mohun, A.F, and Cook, I.J.Y. (1957).

Hormone assay: Serum Estrogen (17β-estradiol) assay was performed by using specific RIA as reported by Jyotsna, U.R. and Medhamurthy, R., (2009).

RESULT & DISCUSSION:

Ovarian histopathology

Histopathological studies were performed to examine the internal damage in the ovary of BPA exposed fish. The ovaries of control and experimental fishes were at a similar stage in the beginning of the experiment. A number of deformities were observed in the ovary sections of treatment groups when compared with the control. Fish exposed to 1/10th(Group II) sub lethal concentration of BPA displayed increased oocyte atresia, perifollicular cell hyperplasia/ hypertrophy and decreased vitellogenesis. Gonadal stagingvacuolation with spaces, necrosis, degeneration, ruptured wall with reduced lumen were also noticed. Ovary of fish exposed to 1/20thof LC50 value, (Group III) revealedatretic follicles, karyoplasmic clumping, necrosis, and ruptured zona radiata. The ovary of fish of group IV exposed to of 1/30th of BPA showed more extreme structural anomalies as compared to Group II and III. Degeneration of oocytes, atretic follicles, and damaged zona radiata were clearly seen. (PLATE I).Fish from all experimental groups showed oocytes with irregularities in shape and disintegrated cytoplasm in contrast with control fish, which had normal oocyte appearance.

Biochemical studies

Results are précised in Table.1. Exposure to graded concentrations of BPA showed significant reduction in plasma protein in all experimental groups while glucose levels in all treated groups (Table.1). However,the values of both *AST* (*SGOT), ALT* (*SGPT*) (Fig.5 and Table.1) and 17-estradiol (E2)clearly shown a gradual and significant increase in all experimental fish treated with BPA.

Developmental stage of the gonads is an endpoint usually required from histological analysis in monitoring programs and other reproductive investigations. Both natural andsyntheticchemical compounds (EDC) are identified to obstruct with metabolism and reproductive functions (Muthulakshmi, S., et al, 2018)and cause a severe threat to both human and animal health (Haq, I. and Raj, A., 2019.). It is very importantand difficult to describe developmental stage when comparing hormone levels and ovarian histopathology among experimental groups. (Dutta, H., 2017, Milton, J., et al, 2017, Karnatak, G., et al, 2018, Lal, B., 2018).In most of teleosts, ovary is a combined and is composed of ovarian follicles, oogonia and the surrounding follicular epithelium (Karnatak, G., et al, 2018, Lal, B., 2018). Microscopic observations clearly revealed that all fishes were at identical stage at the commencement of the experiment. Nevertheless, the sub lethal concentrations of BPA treatment induced many structural changes in dose dependent manner in all experimental groups. The major changes include oocytes disintegrated cytoplasm, cellular degeneration, nuclear hypertrophy, follicular atresia, egg debris, broken zona radiata and fibrosis. Microscopic examination clearly revealed that oocytes of various developmental stages get affected in a different way at various concentrations of BPA treatment. Though, earlier research investigations have shown reproductive toxicity of BPA, but the full mechanisms of action on HPG axis and steroidogenesis pathways in female *H.fossilis* have not been detailed. In the present study, we found that BPA causes adverse effects on ovarian growth by altering the HPG axis, which can lead to reproductive toxicity. Hence, it becomes apparent that steady sub-lethal doses of BPA can bring substantial alterations in the overall histological structures of the fish *Heteropneustes fossilis*.Nevertheless, unfortunately, there is no uniform method for presentation fish gonads (Blazer, V.S., 2002). Detained ovarian development has been described at some polluted sites (Johnson et al. 1988). Milton et al (2017) observed five different developmental stages were identified in *Channa gachuaas and in other teleosts*.In the present study, histopathological alterations of *Heteropneustes fossilis* exposed to sub lethal concentration of BPA may have inhibitory effects on the ovarian development, which can lead to reduced fecundity and abnormal offspring. Such impairments may interfere with the enzyme system in metabolism or destroy the secretion and function of hormone that regulate the ovarian growth and leads to decline reproductive activity (Kumar and Ali, 2014). Interruption of reproductive function will disturb the successful breeding in fishes and threaten the survival of the fishes. The histopathological anomalies shown in this study may be due to modification in expression pattern of various genes involved in reproductive pathway, which lead to negative effects on reproductive system of female fish (Faheem et al, 2017). **Biochemical studies**

Aminotransferases are the most sensitive and widely used liver enzymes as biomarkers of liver damage (Reddy, P.B, 2012). Under normal conditions, liver marker enzymes (ALT and AST) exist within the hepatocytes. However, when the liver is injured, these enzymes are leaked into the blood stream. The activities of SGPT and SGOT in the serum from all experimental fish treated with different sub lethal concentrations of BPA significantly elevated. Accordingly, much significant damage was appeared in the liver of *H.fossilis*, which was clearly evidenced in our histopathological observations. The higher level of SGPT and SGOT possibly due to cellular impermeability and damage of hepatocytes. Consequently, it leads to extensive liberation of SGPT in the blood serum of *H.fossilis* to combat BPA induced toxicity. The high level of SGPT and SGOT also points out distorted metabolism resulting from liver dysfunction or from cellular injuries in different tissues of experimental fish under the stress of BPA. Our findings are coinciding with the findings of Ajaz Ahmad Rather, (2015) under the pesticidal stress of Carbaryl and Parathion in *Clarias batrachus*. Kavya, K.S., et al., (2016) observed the parallel elevation patterns of SGPT and SGOT in Notopterus notopterus in response to change in saline concentration. In a study by Mishra, B.P., et al (2016) showed that exposure to sub lethal/lethal concentration of Rogor (pesticide) results in significant increase in SGPT and SGOT activities to combat the effect of pesticides toxicity. The levels of enzymes were compared with the degree of cell death or inflammation, which were seen histopathological observations. A recent study of Dar, S.A., et al (2018) shown elevated level of both aminotransferase enzymes in*Labeo rohita* treated with mebendazole (benzimidazole derivative). Another recent study on *Oreochromis niloticus* treated with mixtures of diflubenzuron and p-chloroaniline Mixtures of diflubenzuron and p-chloroaniline enhanced the activities of enzymes biomarkers on tilapia fish (Dantzger, D.D., 2018).

Plasma protein complement is extensively used approach for the identification of stress biomarkers (Banerjee, S., et al, 2017). Furthermore, differences in the plasma proteome have been linked with various physiological and pathophysiological conditions (Zhang, A.H., et al, 2013). In the present study, the total plasma protein content decreased significantly in all BPA treated groups, which may be because of renal excretion or impaired protein synthesis or due to damage of hepatocytes in liver. Another possible reason for a decrease in plasma protein content of exposed fish maybe due to breakdown of protein into amino acids first and possibly into nitrogen and other simple molecules. One more reason for the decrease in plasma protein content may be due to protein oxidation (oxidative stress) which leads to the production of protein carbonyls and protein peroxidation that leads to the production of nitrated protein (Banerjee, S., et al, 2017). Glucose is an essential fuel for the performance of specific tissues in fish, including the brain, gills and gonads. The blood glucose concentration is an indicator of general health condition of fish. Glucose is the major source of energy for most vertebrate organisms. All vertebrates including fish maintain a specific level of blood sugar. The level of plasma glucose is influenced by several factors suchas season, hormone production, food availability and temperature (Bartoňková, J., et al, 2007).The present investigation confirmed a gradual increase of blood glucose level in BPA treated fishes, which may be due to glycolsis. It happens as counter reaction by fish againstBPA induced stress for energy requirement. Madhusudan, S., et al (2003) confirmed that the increasing of glucose level is due to high secretion of hormones like catecholamines, glucocorticords and that lead to increasing of glycolysis resulting to high glucose level in blood. Nevertheless, at high concentrations, it affects the growth, development and survival of the fish. The chemical contaminants such as BPA transform the metabolism of carbohydrates, causing hyperglycemia by stimulating the glycogenolysis in fish (Levesque et al., 2002). Our results are in according to many researchers who were recorded in higher blood glucose levels in fishes exposed to various pollutants (Sayed et al., 2007; Osman et al., 2010, Mekkawy et al., 2010). This can be endorsed to the change in the activity of glucose-6-phosphate dehydrogenase and lactate dehydrogenase (Osman et al., 2010).

Effects of BPA on sex hormones

Laboratory research offer better insight toward the understanding of the mode of action of BPA. Changes in the steroid sex hormone balance, in response to pollutants, are considered a reliable biomarker of reproductive disturbance (Kime et al., 1999). Ever-increasing data confirms that Endocrine disrupting chemicals (EDCs) like BPA can interact with oestrogen or androgen receptors and thus perform as agonists or antagonists of endogenous hormones (Whitehead, S.A. and Rice, S., 2006). Consequently, much data on the impact of BPA come from studies on aquatic vertebrates, fish in particular. In fish, steroid synthesis is mainly regulated by hypothalamus- pituitary-gonadal (HPG) axis. A variety of compounds including BPA is released into aquatic environment, which has potential to obstruct with fish endocrine system at minimum level, by interference with HPG axis (Faheem, M et al, 2017). Nevertheless, BPA effects fluctuate intensely as different vertebrate classes and species have different sensitivities to xenobiotics (Wang, Y., et al, 2018). Published facts advocate that BPA could act by using other mechanisms independent of ER. *In vitro*experiments 0.1 to 100 µmol/L BPA inhibited the binding of 3 nmol/L dihydrotestosterone to androgen receptors (ARs) in a dose-dependent manner (Ekman et al. 2012). Earlier studies based on both mammalian and fish are indicated that BPA could act as an antiandrogen, and the potency was similar for human (Reddy, A et al, 2018, Molina, A.M et al, 2018).

In the present experiment, the increase in plasma E2 in BPA treated fish indicates that BPA affected the sex steroid biosynthesis pathwayby the stimulating aromatase enzyme activity, to convert T to E2 (Simpson, E.R et al, 1994). BPA has affinity to bind to estrogen receptors (estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ)) (Takayanagi, S., et al, 2006). Mandich et al. (2007) also reported a decrease in plasma E2 levels in common carp exposed to 10 µg/l of BPA and an increase in E2 levels when exposed to 100 and 1000 µg/l. Our data is in support of these results, but the increase at 1000 µg/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 (Faheem et al, 2017). Moreover, the reaction and response of the fish will also be dependent on photoperiod and temperature in which the experiment was conducted. All of these findings, along with those of the present study, are reliable with the activities of a weak estrogen on normal steroid hormone homeostasis. Hormonal signaling pathways regulate brain-pituitary-gonadal-axis through feedback loops. Estradiol-17β steroid produced in the ovary regulate LH synthesis in the hypothalamus and secretion by the pituitary in immature fish through a positive feedback action. Higher levels of E2 are required for the initiation of the pre-ovulatory LH flow, resulting in oocyte maturation (Itskovitz, J., et al, 1991). Estrogens also have a negative feedback effect on FSH levels (Shaw, N.D., et al, 2010). In the present study, the high Estradiol-17β after BPA exposure may be due to inhibition of pituitary FSH. The low level of circulating E2 resulted in high levels of circulating FSH, while high circulating levels of E2 observed after higher BPA exposure might cause a decrease in plasma FSH levels through negative feedback mechanism. In précis, the exposure of sexually immature *H.fossilis* to bisphenol-A (BPA) triggered reproductive dysfunction by altering sex steroid homeostasis and gonad development (Faheem, M, et al, 2017, Samova, S., et al, 2018). 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. The increased level of E2 may be due to an increase in aromatase activity that converts testosterone (from thecal cells) to E2 (granulosa cells) (Faheem, M., et al, 2017, Sharma, N.K., et al, 2018).

CONCLUSION :

BPA has become a public health concern due to its extensive and continuous exposure through food and drinking water. Even though some authors questioned the outcome of some of the low-dose laboratory animal studies, but still some evidence have shown the possible linkage between BPA exposures and observed reproductive dysfunctions.*H. fossilis* is a hardy fish and capable of thriving against various inappropriate environmental conditions, the present research confirms that sub lethal concentrations of BPA induced various behavioral, biochemical and reproductive anomalies.

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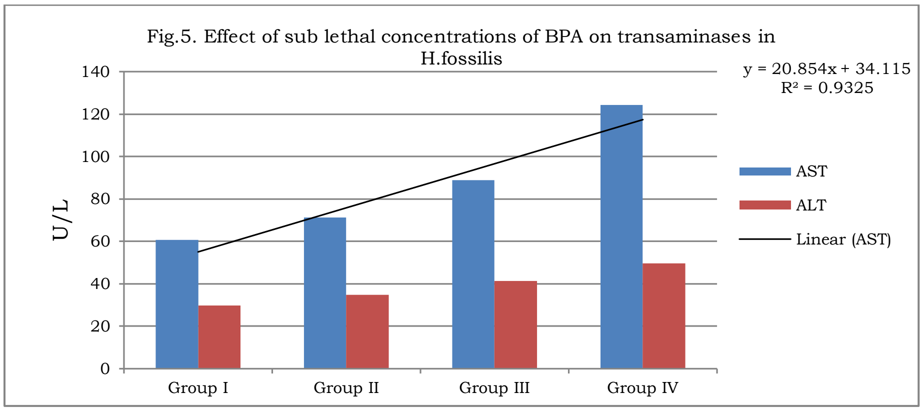
Table.1. Changes in the Biochemical and hormonal parameters in *H.fossilis* treated with sub lethal concentration of bisphenol A.

Parameter	Control (Group I)	Group II	Group III	Group III
Plasma protein(µg/ml)	9.77±0.95	8.34±0.24	6.56±0.63	4.58±0.61
Plasma Glucose (mg/dl)	98.2±2.11	106.8±2.87	124.5±3.69	143.5±4.56
AST (SGOT) (U/L)	60.61± 0.78	71.23± 1.13	88.94± 2.3	124.22± 3.4
ALT (SGPT) (U/L)	29.8± 0.31	34.8± 0.41	41.2 ± 0.51	49.6± 0.75
17-estradiol (E2) (ng/ml	2.4±0.41	2.7±0.52	4.9±0.58	6.1±0.81



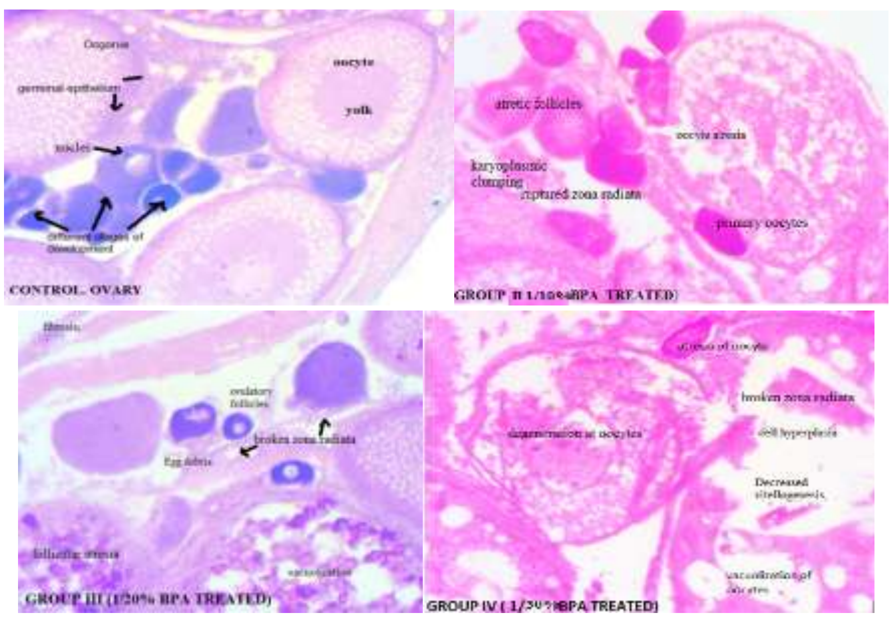


Plate I. FIG.1-4. Effects of sub lethal concentration of BPA on histopathology of ovary of *Heteropneustes fossilis*. A. Control fish show normal structure with oogonia, oocyte with different developmental changes. B. Ovary from Group II showing atretic oocytes, ruptured zona radiata and karyoplasmic clumping necrosis and nuclear hyper trophy C. Histology of ovary from Group III showing cellular degeneration, follicular atresia, egg debris, broken zona radiata and fibrosis. D. Histology of ovary from Group Iv showing degeneration, atrophy, germ cell syncytia, hypertrophy and pyknosis and vacuolated oocytes and decreased vitellogenesis (All sections are H&E Stained x400).