Effects of estrogen mimics, bisphenol-A and butylhydroxy anisole on the reproduction of Indian major carp, *Labeo rohita*

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Abstract

Background/Objectives: The estrogen mimics are predominantly come into the freshwater bodies and affecting the fish reproduction by increasing anthropological usages like cosmetics, pesticides, detergents, plastics and other pharmaceutical compounds. In the present study, an attempt has been made to study the effect of estrogen mimics, Bisphenol-A (BPA) and Butylhydroxyanisole (BHA) on the reproduction of Indian major carp *L. rohita.*

Methods: Fishes were exposed to BPA and BHA at 5µM concentration for 30 days. The ovary, testis and liver was chosen to measure the Gonadosomatic index (GSI), Hepatosomatic index (HSI), histology, total protein, lipid, and acid and alkaline phosphatase enzymes activity. All the parameters showed the minimum of three to four fold reduction in the treated fishes when compare with control in its function. The estrogen mimics might play an effective role in the ovarian follicle steroid synthesis. The Present study carp oocytes were incubated with follicle growth inducing steroid precursor, pregnenolone with or without BPA and BHA to find the interference of estrogen mimics in the normal steroidogenesis.

Results: The pregnenolone treated eggs showed the secretion of progesterone and 17α,20β-P production when the incubation medium analysed by TLC. The BPA and BHA treated group incubations recorded the 21-OHprogesterone. This changes might be due to the shift of enzyme from 17α-hydroxylase to 21-hydroxylase in the presence of BPA and BHA.

Conclusion : From this study, it is confirm that the estrogen mimics adversely affect the process of reproduction in the Indian major carp *L. rohita* at various levels.

***Keywords*:** Endocrine disruptors, Estrogen mimics, BPA, BHA, Indian carp

1. Introduction

In the past 40years, substantial evidence has surfaced on the hormone-like effects of environmental chemicals such as pesticides and industrial chemicals in wildlife and humans. The endocrine and reproductive effects of these chemicals are believed to be due to their ability to: a. mimic the effect of endogenous hormones, b. antagonize the effect of hormones, c. disrupt the synthesis and metabolism of hormones, and d. disrupt the synthesis and metabolism of hormone receptors. Endocrine disrupting chemicals (EDCs) can cause adverse effects by interfering in endogenous hormones and blocking the chemical signals or giving wrong signals [1]. There are number of ways that chemicals can disrupt hormones, it is estimated that more than 70,000 chemicals are in commercial use, which can disrupt the endocrine system [2].

Many xenobiotic compounds introduced into the environment by human activity have shown adversely affect both humans and other species. EDCs are the by-products and wastes from plastics, cosmetics, pharmaceuticals and food industries contain estrogen mimics such as Bisphenol-A (BPA) and Butylhydroxyanisole (BHA). Aviation crop dusters handling DDT were found to have reduced sperm counts, and workers at a plant producing the insecticide kepone were reported to have lost their libido,

became impotent and had low sperm counts. Subsequently, experiments conducted in lab animals demonstrated unambiguously the estrogenic activity of these pesticides. Man-made compounds used in the manufacture of plastics were accidentally found to be estrogenic because they fouled experiments conducted in laboratories studying natural estrogens. For example, polystyrene tubes released nonylphenol, and polycarbonate flasks released BPA. Alkyl phenols are used in the synthesis of detergents (Alkyl Phenol Polyethoxylates) and as antioxidants. These detergents are not estrogenic; however, upon degradation during sewage treatment they may release estrogenic alkyl phenols. The surfactant nonoxynol is used as intravaginal spermicide and condom lubricant. When administered to lab animals it is metabolized to free nonylphenol and it can bind with steroid receptors as endogenous steroids. BPA was found to contaminate the contents of canned foods; these tin cans are lined with lacquers such as polycarbonate. BPA is also used in dental sealants and composites. Other xenoestrogens recently identified among chemicals used in large volumes are the plasticizers benzylbutylphthalate, dibutylphthalate, the antioxidant butylhydroxyanisole, the rubber additive p-phenyl phenol and the disinfectant O-Phenyl phenol. These compounds act cumulatively at different species and at different organs [3].

Bisphenol A (2-2-bis [4-hydroxy phenyl] propane) is used widely in the production of polycarbonates, epoxy resins and flame-retardants. In addition very low (mg/l) concentration of BPA were detected in various aquatic environment [4, 5]. Phenolic antioxidants such as alkylphenols are used in the manufacture of plastics and to protect petroleum against oxidative degumming [6]. Some phenolic antioxidants such as butylated hydroxytoluene and BHA are used to prolong the shelf life of foodstuffs and to reduce nutritional losses by retarding oxidation. In addition to the estrogenic alkylphenol antioxidants. BHA is estrogenic [7] and is effective to control oxidation of short chain fatty acids such as coconut and palm oils [8].

More recently, the pharmacological concept of concentration addition (CA) has been applied to the assessment of estrogenic mixtures. The validity assessment of CA in aquatic habitat is a means to predict toxicity of multi-component mixtures in various assays with fish, daphnia, algae and bacteria [9, 10, 11, 12, 13, 14]. There is considerable evidence that CA may also be used to predict the effects of mixtures of estrogenic chemicals. The validity of this approach has been demonstrated *in vitro,* using assay such as the yeast estrogenicity screen (YES) and the human breast cancer cell proliferation assay (E–SCREEN) [3, 15,16]. Studies have revealed the capacity of the components of the mixture to contribute to the overall effect by acting in relation to their potency even at low- effect concentrations.

Studies revealed a single chemical might produce neurotoxic, estrogenic and antiandrogenic effects. It has been hypothesized that endocrine disruptors may play a role in the decrease in the quantity and quality of human semen during the last 50years, as well as in the increased incidence of testicular cancer and cryptorchidism in males and breast cancer in both females and males [17]. Research on wildlife populations has demonstrated that EDCs profoundly impair animal reproduction and development. Birds with deformed beaks, female birds that nests with females and male alligators with underdeveloped penises all have high levels of endocrine disruptors. Fish egg does not develop when exposed to even low levels of disrupting chemicals and ultimately it causes population imbalance. In humans scientists are attempting to determine the relation, if any, between low sperm count and exposure to endocrine disruptors especially the estrogen mimics and androgen blockers [18]. However, the physiological effects of the estrogen mimics were not much known in aquatic vertebrates. Hence, an attempt has been made to study the effect of estrogen mimics, BPA and BHA on the reproduction of Indian carp, *L. rohita.*

2. Materials and methods

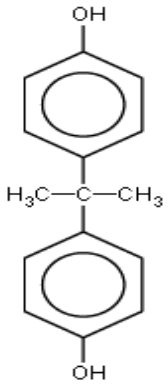
2.1 Collection and maintenance of fish

The freshwater teleost, Indian Major carp *L. rohita* used in the present work was collected from Poondi reservoir during the month of March and April 2013. The fish were acclimatized for three weeks

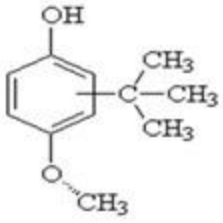
under lab condition before starting the experiment. The fishes used in the experiments were one year old and showing the vitellogenic period. Adult male were identified with its anal narrow vent with slender body and adult female were identified with distended anal vent with broad abdomen. The fishes were kept in the larger cement tanks having the measurement of 6`x4`x5` holding 800 liter water. During the time of acclimatization and experiment the fishes were fed with rice barn and groundnut oil cake. All the fish tanks were provided with aerators and the water was changed in alternate days.

2.2. Estrogen mimics

The estrogenic xenobiotics, Bisphenol-A (2, 2- Di (4-hydroxyphynyl) propane) and Butylhydroxyanisole used in this experiment were obtained from S.D. Fine Chem ltd, Mumbai.



Bisphenol-A



Butylhydroxyanisole

2.3. Experimental design

The experimental fish were treated with BPA and BHA at the concentration of 5µM in water tanks for 30 days. Earlier study [17] suggested the 5µM as the safe concentration for *in vitro* studies. However, the same concentration has been chosen for present *in vivo* study. Since the BPA and BHA were dissolved in ethanol (10µl) and subjected into the desired concentration, the same amount of ethanol given to the control fish. After 30 days, fishes were dissected out and the liver, ovary, testis and brain tissues were removed, weighed and stored in -70^{o}C for further analysis. The parameters chosen for the evaluation are HSI, GSI, Histology, Protein, Lipid, Acid Phosphatase, Alkaline Phosphatase, *in vitro* study on the oocytes and steroid estimation by thin-layer chromatography (TLC).

The HSI and GSI were calculated with the ratio of total weight of the body with the organ weight. HSI=Wt. of liver/Body wt. x100. GSI=Wt. of gonad/Body wt. x 100. The histological observation was made in liver, ovary and testis by the 5µ microtome sections with heamatoxilin-eosin stain. Biochemical estimation of total protein by Biuret and Dumas method [19] and total lipid by Phospho-vanillin Method [20]. Enzymatic assays of acid phosphatase (ALP) and alkaline phosphatae (AKP) were measured by using King's Method [21] in gonads and liver.

2.4. In vitro studies

The female fish used for the study showed peri-vitellogenic oocytes (end of vitellogenesis). The ovaries were dissected out and cut into smaller fragments containing approximately 100 oocytes for in vitro study. The ovarian fragments were introduced into culture vials each containing 3ml of the medium. The culture medium was prepared by dissolving 7.3 gm NaCl, 0.18 gm KCl, 0.07 gm $MgSO_4$, 0.18 gm $MgCl_2$, 0.29 gm $CaCl_2$, 0.95 gm HEPES and 1.0 gm Glucose in 1 litre of distilled water and maintained at 18^{0}C, and pH at 7.2.

Pregnenolone used as steroid precursor in the culture vials towards the oocyte incubation. Oocytes were incubated towards final maturation (resumption of meiosis) with two different concentrations of Pregnenolone i.e. 1µg/ml, 0.1µg/ml. In the experimental incubation vials 20µl of 5µM

concentration of estrogen mimics Bisphenol-A (BPA) and Butyl hydroxy anisole (BHA) was added along with Pregnenolone. Three replicas were maintained for each concentration to get the concordant result. The incubation was maintained for 24 hours at 18^0C in a modified BOD incubator. The incubated medium was stored in separate vials at -70^0C for further processing to know the synthesis of steroids.

2.5. Steroid extraction

The oocyte incubated medium was extracted thrice with cyclohexane and ethylacetate, 1:1 solvent. The dried extract was then dissolved in 50 µl of dichloromethane and methanol (9:1).

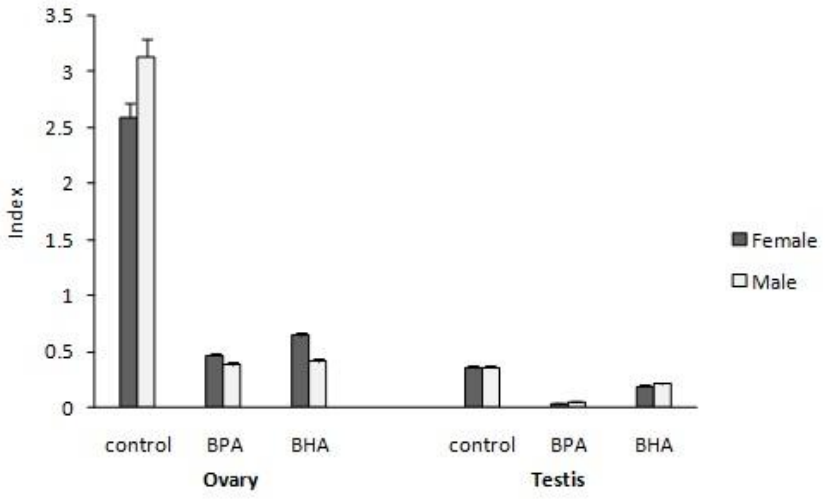
2.6. Thin layer chromatography

The steroid extracts were separated by TLC using Merck $60F_{254}$ 20/20 cm Silica plates. About 5µl of the redissolved oocyte extract was loaded on the plate and developed in a saturated atmosphere of the solvent Benzene:Acetone (80:20) at room temperature. The bands exhibited by the steroids were visualized and analysed under UV lamp along with reference steroids and with Rf values.

3. Results

The weight of the ovary was measured along with the body weight and calculated the GSI. Both BPA and BHA treated fish showed the significant decrease in the ovary weight when compared with control fish. BPA compare with the BHA, the BPA treated fish ovary more reduced than the BHA. It confirms the gonadal deformities in the estrogen mimis treatment (Fig. 1). Similarly, the weight of the testis also showed the reduction in its weight (Fig. 1). HSI was measured in the male and female fish treated with BPA and BHA. Both BPA and BHA treated fish showed the reduction in its size and its weight. About 50% of weight reduction observed in both male and female fishes treated with the estrogen mimics (Fig. 1).

Figure 1: Influence of BPA and BHA on the GSI and HSI of *L. rohita*.



The total protein concentration of the liver was decreased in the treated fish as compared to control fish. The BPA was more affected than the BHA. BPA adversely affected the production of protein concentration of the liver in the treated fish (Fig.2). The total protein concentration of the ovary and testis was decreased about 15 times less in the treated fish as compared to control fish in both BPA and BHA treated fishes. Similarly, BPA and BHA were decreased the lipid accumulation in the liver. The total lipid concentration is reduced by the EDCs in both gonads of ovary and testis. In ovary, BHA reduced the lipid accumulation by one third and the BPA reduced one half when compare with the control (Fig. 3). The lipid reduction is the direct documentation for the vitellogenesis process in the ovary.

Figure 2: Influence of BPA and BHA on the total protein levels of liver and gonadal tissues of *L. rohita*.

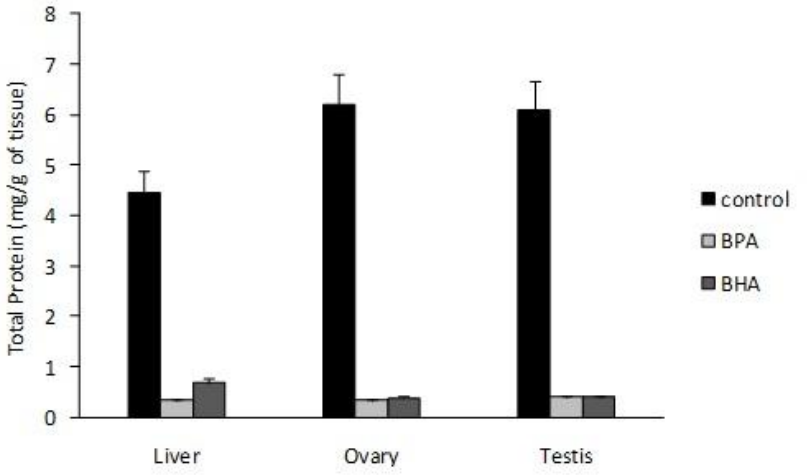
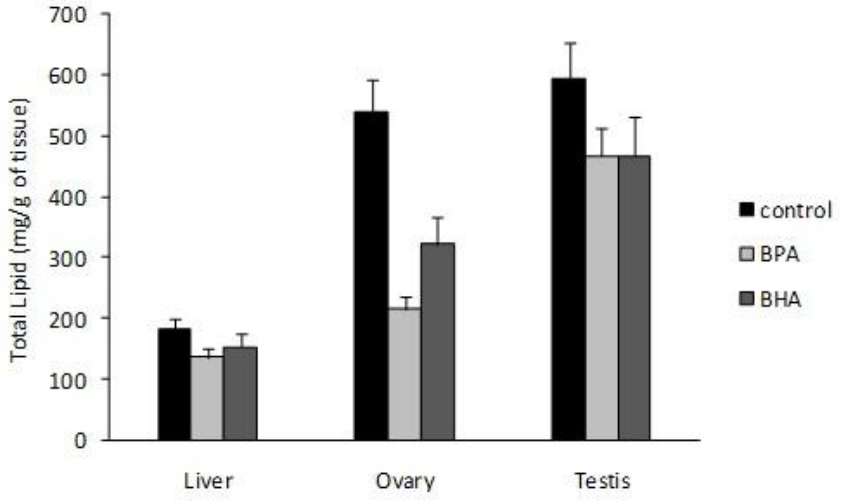
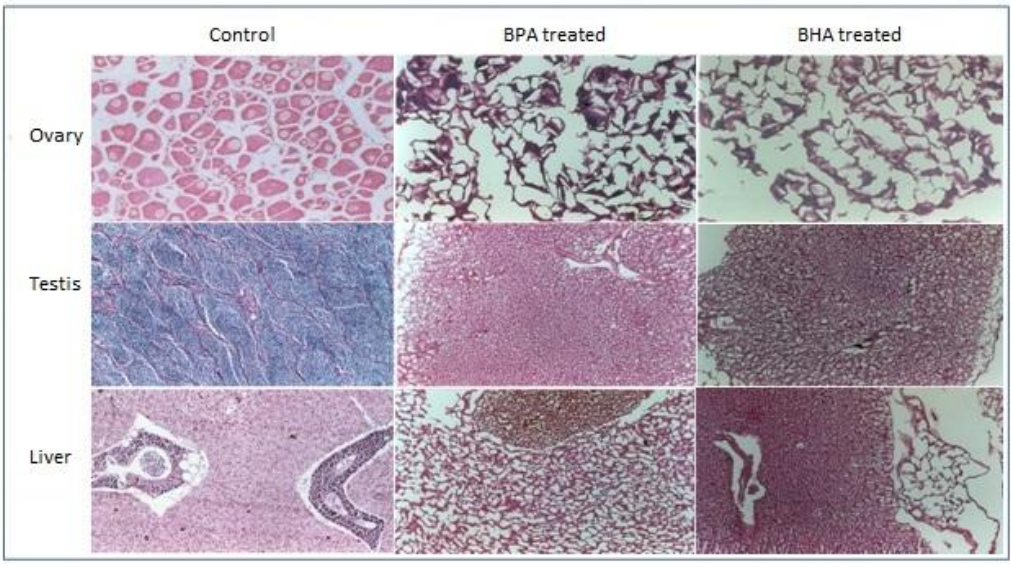


Figure 3: Influence of BPA and BHA on the total lipid levels of liver and gonadal tissues of *L. rohita*.



Histological studies in the ovary of control fish is showing the normal development of ovarian follicles in stage-I oocytes. The ovarian lobules are well formed with the theca externa in the control fish. The BPA and BHA treated fish ovary showing the disintegrated ovarian follicles and absence of cell inclusions in the stage-I oocytes. Both the BPA and BHA treated fish ovary shows the absorption of follicles and disintegrated stromal tissues (Fig. 4). The male control fish is showing the normal development of semniferous tubules with active spermiation. The walls of the somniferous tubules are very clear and the Leydig cells are very prominently present in the interstitial region (Fig. 4). The BPA and BHA treated testis is showing the less development of testis and showing the early development of semniferous tubules. The semniferous tubules are vacuolated and not having the gamete development. The section is devoid of spermatogonial and spermatozoan cells. It indicates that the estrogen mimics, BPA and BHA are inhibited the development of testis and spermatogenesis process in the *L. rohita* testis. The liver of the control fish (Fig. 4) is showing the normal development of hepatocytes. The hepatic cells contain one or two vesicular nuclei, fat droplets, pigment granules, ribonucleoprotein, glycogen, mitochondria and golgi bodies. The nucleus is present in the centre of the hepatocystes. Each hepatic lobule is surrounded by the sinusoid. The BPA and BHA treated fish liver showing the disintegration of the hepatocytes and the vacuolization (Fig. 4). The BPA treated fish when compare with BHA, the BHA treated fish liver showing the less vacuolization and the disintegration is reduced.

Figure 4: Histological sections of ovary, testis and liver of *L. rohita* treated with BPA and BHA (x100). The detailed description is given in the result



The ACP and AKP activity of the BPA and BHA treated fish was drastically decreased as compared to control fish (Table 1). The result shows that the ACP in liver is higher than the AKP in normal animals. The differences between the BPA and BHA action was not noticed significant differences; both were affecting the ACP and AKP activity in liver and gonads of *L. rohita* (Table 1). The level of the decreased enzyme activity is more significant with 50% and above (Table 1).

Table 1: Effects of BPA and BHA on the enzymatic activities of ACP and AKP on the liver and gonads of *L. rohita*

Enzymes	Tissues	Control	BPA treated	BHA treated
Acid Phosphatase*	Liver	5.00	1.05	1.33
	Ovary	3.00	1.50	1.75
	Testis	0.83	0.25	0.40
Alkaline Phosphatase*	Liver	0.83	0.25	0.40
	Ovary	2.00	1.30	1.40
	Testis	2.00	0.60	0.60
*values in KA units and represent the mean of three replicates				

3.1. Steroid identification

The synthesis of the steroids was analyzed by incubating the fully grown oocytes with or without BPA and BHA. The detailed protocol followed in the experiment given in the material and method. The oocytes were incubated with the maturation inducing steroid precursor, pregnenolone. The ovarian follicular layer is synthesizing the maturational steroids, progestins from the pregnenolone. To check whether the estrogen mimics, BPA and BHA are involving in the interference of the steroid synthesis or not, the estrogen mimics were introduced along with the pregnenolone. After 24 hours of incubation, the incubation medium was analysed for steroids by TLC.

The standard steroids, 17α,20β-P; Testosterone (T); 20β-Progesterone (20β-P); Progesterone (P); Pregnanolone (P5); 17α-hydroxyprogesterone (17α-P); 17α,20β,21-trihydroxy-4-pregnen-3-one (17α,20β,21-P); and 17α -Pregnanolone (17P5) were loaded into the TLC sheet (Fig. 5) and its Rf values are tabulated in the Table 2. The figure 5 is showing the visualized bands and its description in detail. The oocytes incubated with the pregnenolone showed the synthesis of progesterone and the 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P). The pregnenolone with BPA and BHA added incubation showed the progesterone band and not the 17α,20β-P band. The g band (Fig. 5) visualized in all incubations but

the steroid is not identified with the available reference steroids. Along with the above results, the BPA subjected incubation alone showed the d band and the BHA subjected incubation alone showed the f band (Fig. 5). These d and f bands are not coincided with the reference steroids used in the TLC plate. It is considered to be an unknown steroid (Table 3) and it has to be further analyzed by HPLC.

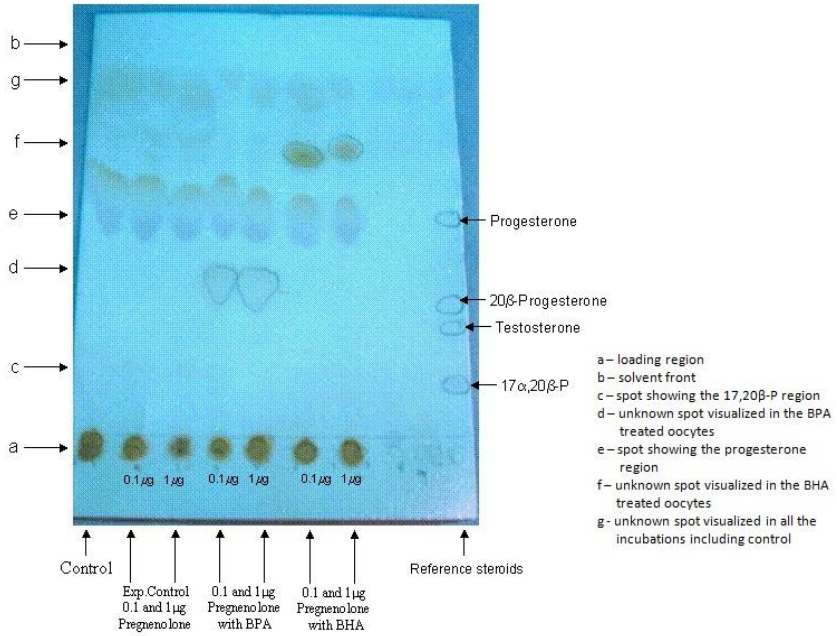
Table 2. Identification reference steroids used in the experiment to trace the steroid synthesis

S.No	Standard Steroids	Rf Value
1.	17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P)	0.2333
2.	Testosterone (T)	0.3666
3.	20β-Progesterone (20β-P)	0.4333
4.	Progesterone (P4)	0.6333
5.	Pregnanolone (P5)	Not exited
6.	17α-hydroxyprogesterone (17α-P)	0.42
7.	17α,20β,21-trihydroxy-4-pregnen-3-one (17α,20β,21-P)	0.2733
8.	17α -Pregnanolone (17P5)	Not exited

Table 3. Influence of BPA and BHA on the steroid synthesis of ovary

Incubation Treatement	**Presence of steroid bands in the TLC**								
	17α, 20β-P	**T**	**20β-P**	**P4**	**P5**	**17α-P**	**17α,20β, 21-P**	**17P5**	**Unknown bands**
Control				+					
Pregnenolone 0.1µg	+			+					
Pregnenolone 1.0µg	+			+					
Pregnenolone 0.1µg + BPA-5µM				+					+ [Rf: 0.3866]
Pregnenolone 1.0µg + BPA-5µM				+					+ [Rf: 0.3733]
Pregnenolone 0.1µg + BHA-5µM				+					+ [Rf: 0.72]
Pregnenolone 1.0µg + BHA-5µM				+					+ [Rf: 0.7466]

Figure 5: Effect of BPA and BHA on the synthesis of Steroids in the ovary of the *L. rohita*.



4. Discussion

The important of EDCs to animals and humans are dependent on the effects of the disruption on reproductive health and survival. The present study carried out to evaluate the effects of BPA and BHA on the reproductive development of *L. rohita.* The BPA and BHA are known to be the estrogen mimics which are the by-product of many domestic materials like plastics, soaps, detergents, lubricating materials, pharmaceutical products of condoms, and other cosmetics like perfumes etc. The present study highlights the estrogen mimics involvement in the reproductive functions and the steroid synthesis. The estrogenic mimics affected the vitellogenesis, development and reproduction capacity, it has been associated with reduced fecundity, reproductive failure and population level effects in a variety of aquatic organisms [22, 23, 24].

The development of the gonad was affected in the previtellogenic stage; Estrogen reduced the reproductive capacity of paired medaka [25]. Studies conducted in fathead minnows [26] exposed to BPA for 164 days and observed an inhibition of egg production in the 1.280µg/l group. These results suggested that the toxicity of BPA and BHA adversely reduces the reproduction success of *L. rohita*.

The BPA and BHA have an estrogenic potential to decrease the GSI. It is found that the nonylphenol, BPA and 17-beta estradiol affected the GSI of the *C. carpio* [27]. The estrogenic chemicals adversely affect the gonad development of fish. The studies shows that the decreased GSI and HSI in juvenile male summer flounder (*Paralichthys dentates*) exposure of 1,1,1,-trichloro-2-(P-chlorophenyl)-2-(O-chlorophenyl) ethane (O,P'-DDT), Octyl phenol and 1,1-dichloro-2,2-bis (P-chlorophenyl) ethane (P,P'DDE) treatment [28]. The chlorinated chemicals tested for the studies showed the reduction in the HSI. In agreement with the above studies, the present study also shows that the BPA and BHA are reducing the gonadal development with the effect of endocrine disruptors.

Histological studies showed the disintegrated testis and ovary in the present study in the Indian Carp, *L. rohita*. Indian carps are the major economically important species in India. The histological studies are the direct evident to show the internal changes occurring in the tissues. The GSI reduction is due to the vacuolization in testis, reduction in the seminiferous tubules, devoid of spermatogonial and spermatozoan cells, and reduction in the interstitial cells (Fig. 4). Similarly the ovarian lobules disintegrated when the fishes were subjected to BPA and BHA. The stage I ooctyes are predominant in the control fish whereas the treated fish overy the stage-I oocytes are absorbed in its cytoplasmic and germinal contents. It seems that the apoptosis like phenomenon occur in the treated fishes. The inter-

follicular spaces are developed in the ovary in the BPA and BHA treated fish shows the complete absorption occurs which completely arrested the development of the next stage of development.

The synthesis of protein level decreased in liver in the present study. Earlier study showed that the concentration of liver protein was decreased in *C. batrachus,* exposed carbaryl pesticide (1, 2 and 4mg/l) for 96hrs and 15 days treatment [29]. The total protein reflects the enzymes, transport elements, receptors, signal molecules and other vital molecules responsible for the functions of the gonads and liver. In the present study, both BPA and BHA decreased the total protein in the fish.

The estrogenic chemicals were affected the endocrine system of the fish by affecting the feedback mechanism for synchronizing the complete reproductive function. Acid and alkaline phosphatase activity decreased in liver, kidney and muscles of *C. gachua* exposure to endosulfan [30]. The alkaline phosphatase activity of testis is adversely affected in the treated fish. Linear alkyl benzene (LAS) affected the AKP activity in testis of the teleost fish *H. fossilis* (Bloch) [31]. These results have been associated with the effects of the present study and it confirms the phosphatase enzyme activity derailed the normal functions of gonadal and hepatic tissues both male and female fish.

The oocytes were incubated in the culture medium maintained for 24 hours alongwith the follicular layers. The oocyte follicular layers are synthesizing the maturation inducing steroids to induce maturation of oocytes. The follicular layer synthesizes the maturation inducing steroids under the influence of gonadotropins, LH and FSH secreted from the Pituitary. The various progestins are identified as the maturation inducing steroids in fishes. P, 17α-P, 17α,20β-P and 17α,20β,21-P are the most effective inducers of oocyte maturation [32, 33].

When the folliculated oocytes were incubated with the pregnenolone, the synthesis of progesterone and 17α,20β-P are found in the incubation medium by TLC procedure. It might be recorded as in the steroidogenic pathway (Fig.6).

Figure 6: Precursor pregnenolone conversion pathway for different progestins

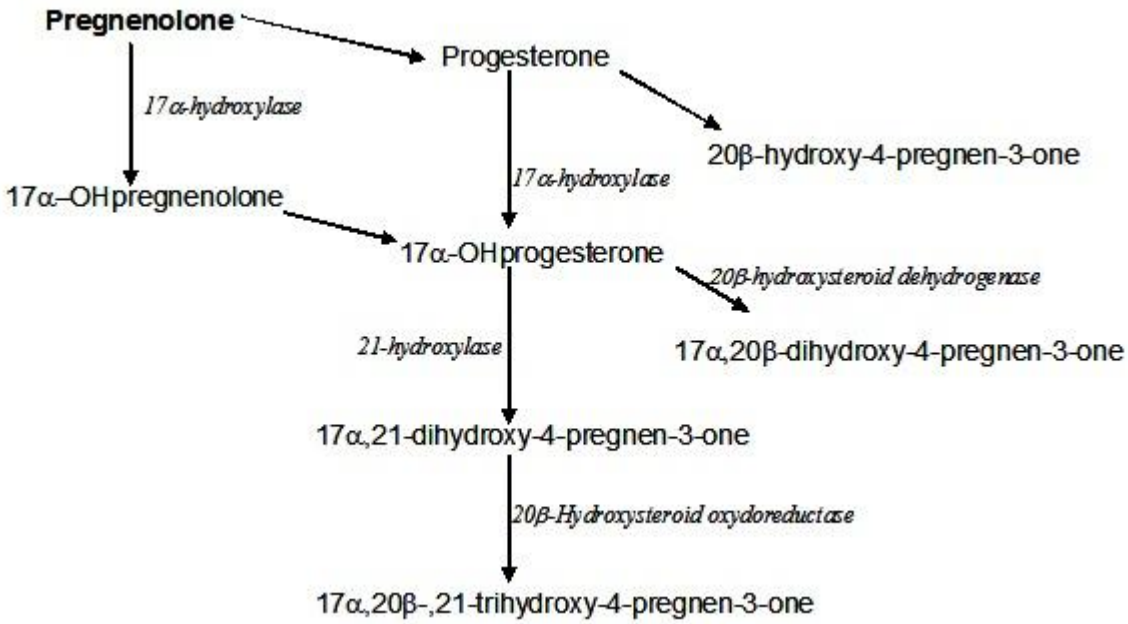
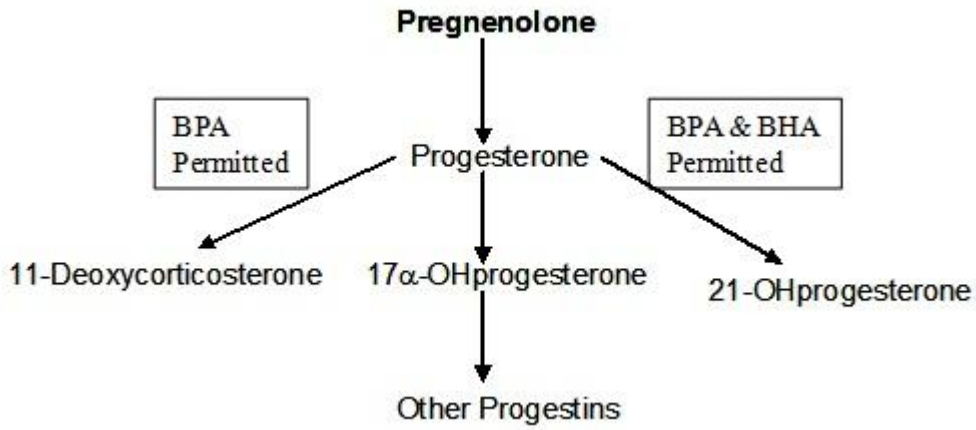


Figure 7: The BPA and BHA incubated oocytes showing the possible steroid synthesis.



The pregnenolone incubated oocytes showed progesterone and 17α,20β-P reveals that the enzymes of 17α-hydroxylase and the 20β-hydroxysteroid dehydrogenase are present in the ovarian follicles. When the same incubation added with the BPA and BHA not showed the production of 17α,20β-P, however the progesterone production recorded in both the concentrations. This is a first report showing that the estrogen mimics not affected the 17α-hydroxylase enzyme but affected the 20β-hydroxysteroid dehydrogenase enzyme (Fig. 5 and Table 3).

The unknown spots identified in the TLC from the oocytes incubated with BPA are 'd' and with BHA are 'f' (Fig. 5). The unknown spots were further proposed to postulate the steroid production in the incubation. The BPA and BHA incubation showed the 21-hydroxyprogesterone. When compare with the treatments, BPA permitted the synthesis of the steroid higher level than the BHA treated oocytes. This can be inferred that the 17α-P pathway diverted to form the 21-hydroxyprogesterone due to the shift of enzyme from 17α-hydroxylase to 21-hydroxylase. This is the first report in the estrogen mimics involvement in the ovarian steroidogenesis (Fig. 7). In addition, BPA produced the 11-deoxycorticosteroid (DOC) which normally occurs in adrenal tissue. The production of the DOC might be due to the progesteronal elimination pathway. However, the present experiment, conclusion could not be made to identify the steroid. It need further study to confirm the name of the steroid.

5. Acknowledgement

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