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REPRODUCTIVE TOXICITY AND ENDOCRINE DISRUPTION INDUCED BY BISPHENOL A IN A FRESHWATER FISH *ANABAS TESTUDINEUS* (BLOCH)

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Abstract: Aquatic pollution has increased substantially in the last decades due to an exponential increase in the generation of industrial, agricultural, commercial and domestic wastes. Several pollutants are endocrine disruptors, which interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for development, behavior, fertility, and maintenance of homeostasis. The present study aims at investigating the reproductive toxicity and endocrine disrupting potential of Bisphenol A in afreshwater fish, *Anabas testudineus*. Exposure to BPA resulted in many patho-physiological changes which were restored back to normalcy upon maintaining the fish in unpolluted water without BPA; but the histological aberrations in gonads persisted, strongly indicating a permanent damage in reproductive machinery. BPA also up regulates the expression of aromatase in both male and female fish, which also evidences the induction of reproductive toxicity and endocrine disruption.

Key words: Aquatic pollution, endocrine disruptor, polycarbonates, patho-physiological aberrations, steroidogenic pathway.

INTRODUCTION

Accumulating evidence indicates that the exposure to many naturally occurring and synthetic agents that have the potential to disrupt normal endocrine processes regulating cell growth, homeostasis and development may adversely impact human health. These substances are deemed "endocrine disrupting compounds," or EDCs. They interfere with the synthesis, storage, release, transport, elimination or receptor binding efficiencies of endogenous hormones (Cooper, 1997). One of the most common chemicals that behave as an endocrine disruptor is Bisphenol A [2, 2-bis (4-hydroxyphenyl) propane] or BPA. It is widely used to produce polycarbonates, food and beverage can linings, dental sealants etc. Previous studies on the effect of BPA on various aspects of the freshwater fish,*Anabas testudineus* showed that BPA causes alterations in the sex steroid levels, induces vitellogenesis and ovo-testis formation in males and also causes oxidative damage and ionic imbalance. The present study aims at investigating the effect of BPA on various physiological, biochemical, histological and molecular aspects in a freshwater teleost *Anabas testudineus* to analyze its reproductive toxicity and endocrine disrupting potential.

MATERIALS & METHODS

Experimental design

The common freshwater fish, *A. testudineus* was used as the experimental model. Fish weighing 30 ± 5g were acclimatized to the lab conditions by maintaining them in 40L tanks for 15 days.The

fish were divided into 10 groups, consisting of eight fish per group. The first group of fish served as control and was kept in normal de-chlorinated tap water. The LD 50 value of BPA for *Anabas testudineus*is 10mg/l. So sub-lethal concentrations 2.5, 5.0 and 7.5 mg/l were selected as experimental doses. Fish belonging to groups II, III and IV were exposed to 2.5mg BPA/l of water for the periods 10, 20 and 30 days respectively. Similarly, the groups V, VI and VII were exposed to 5mg BPA/l of water and groups of VIII, IX and X were exposed to 7.5mg BPA/l of water for a period of 10, 20 and 30 days respectively. Water was replaced on alternate days and appropriate doses of BPA were introduced. After treatment, the fish were sacrificed, liver and gonads were removed, a portion of these tissues were fixed in Bouin's fluid for histological analysis and the remaining portions were stored at $^{-}80^{0}$C for further studies. The remaining fish after treatment were subjected to restoration studies by maintaining them in unpolluted water for 30, 60, 75, 100 and 125 days. The estimation of various physical and physiological parameters and qualitative and quantitative hepatic protein profiling of the control, treated and restored fish were carried out using standard protocols. Histological analysis was done to assess the effect of BPA on gonadal architecture. Molecular analysis based on the expression of aromatase in gonads was also carried out to evaluate the endocrine disrupting potential of BPA.

Comparison of physical and physiological parameters

After 10, 20 and 30 days of treatment with different sub-lethal doses, the various physical and physiological parameters such as body weight and hue, liver weight and texture and hepato-somatic index of control, treated & restored models were compared.

Protein Estimation

100mg of liver tissue from control, treated and restored samples were ground with 1ml ice cold PBS (pH 7.2) using a tissue homogenizer, centrifuged at 10,000rpm for 10 minutes at 4^{0}C, and the supernatant was used for estimating the amount of protein in each sample using the standard protocol of Bradford(Bradford, 1976).

SDS PAGE

Qualitative hepatic protein profiling was performed according to the Laemmli method (Laemmli, 1970). Before electrophoresis, liver supernatants were diluted 4:3 with reducing sample buffer (125mmol l^{-1}Tris HCl pH 6.8, 0.2mmol l^{-1} dithiothreitol, 4% SDS, 40% glycerol, 0.04% Bromophenol Blue, 5% â-mercaptoethanol) and boiled for 3 min. 30µg protein sample was loaded into each welland electrophoresis was carried out at 25mA (constant current) in the mini vertical gel unit (Mini VE, Amersham Biosciences, U.S.A).. The gel was stained with Coomassie Brilliant Blue R-250. Molecular masses were determined by comparing the mobility of the polypeptides in sample with those of standard proteins (SDS-PAGE Molecular Weight Standards, Broad Range, 161-0317, Bio-Rad). The protein bands in gel were visualized and photographed using gel documentation system (Gelstan, Medicare, Germany).

Histopathological analysis

The gonads of control,treated and restored fish were fixed in Bouin's fluid. After fixation, the gonads were passed through alcohol series 30 to 100% for dehydration and finally cleared in benzene. Tissues were then impregnated with paraffin wax at 58^{0}C and embedded. After embedding, block sectioning was carried out using ultra microtome (Leica, Germany). Sections were spread on clean glass slides coated with Mayer's albumin. The sections were then stained with haematoxylin and counter stained with eosin. The stained slides were rendered transparent with xylene and mounted in DPX. The sections were observed under binocular research microscope attached with CCD camera (Leica, Germany). Appropriate sections and portions of tissues were photographed for the purpose of description and comparison using Leica image analyzer software. A minimum of 10 representative sections per gonad were analyzed.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

100mg gonad from both sex of control and treated fish were homogenised in 1ml Trizol® Reagent and centrifuged at 12000g for 10min at 4°C. The homogenates were incubated at room temperature for 5min, and 0.2ml chloroform was added for phase separation.The samples were vigorously shaken for 15 seconds and then incubated for 10min at room temperature. The samples after incubation were centrifuged at 12,000g for 15min at 4°C. The aqueous phase was collected and transferred to a fresh tube. RNA was precipitated by adding isopropyl alcohol (0.5 ml per millilitre Trizol® used in the initial homogenisation) and incubated at room temperature for 10min. Samples were again centrifuged at 12,000g for 10min at 4°C. The RNA pellet was washed once with 75% ethanol, air-dried and finally re-dissolved in RNase-free milliQ water. The purity of the extracted total RNA was determined by UV spectrophotometry (A_{260}/A_{280} ratio) and total RNA was diluted to 1ìg ìl^{-1}in RNase-free milliQ water.

2ìg total RNA was converted to cDNA usingreverse primer for aromatase (antisense 5'TGGCTGATGCTCTGCTGAGG3') and reverse primer ofâ-actin (antisense 5'AGGGACAACACTGCCTGGAT3') according to the manufacturer's instructions. Reverse transcription was performed in a 20ìl reaction mixture with a finalconcentration of 1.5mM 10X PCR buffer (500mM KCl, 100mM Tris-HCl (pH 9.0),15mM $MgCl_2$), 3.5mM $MgCl_2$, 1mM dNTP, 1U RNase inhibitor, 20U reverse transcriptase, 0.75mM of the arom reverse primer, and 0.75mM â-actin reverse primer at roomtemperature for 10 min and then at 42°C for 60 min. PCR amplification of cDNA was carried out using gene specific primer pairs based on the cDNA sequences of carp aromatase and â-actin. The forward and reverse primersfor aromatasegene were (sense, 5'ATCGGATCCCTGCCTGTGAC3') and (antisense, 5'TGGCTGATGCTCTGCTGAGG3') respectively and forward andreverse primers for â-actinwere (sense 5' CCAAAGCCAACAGGGAGAA3') and (antisense 5'AGGGACAACACTGCCTGGAT3') respectively. PCR amplification was carried out in mastercycler (Eppendorf, Germany) in a 20ìl reactionusing 1.25U of Taq DNA Polymerase, 1XPCR Buffer, 1.5mmol l^{-1} $MgCl_2$, 0.2mmol l^{-1} dNTP, 0.1ìmol l^{-1} of each primer, and 2-5 ìl of template cDNA. PCR conditions consisted of initial denaturation at 95°C for 5 min, followed by 35cycles of denaturation at 94°C for 30s, annealing at 68.9^0 C (for aromatase primers) and 65.45^{0}C (for â-actin primers)for 30s and extension at 72°C for 1 min. A final elongation step was performed at 72°C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis with ethidium bromide. (Mini VE Amersham Biosciences,USA).The PCR products werevisualized and photographed using gel-documentation system (Gelstan, Medicare, Germany).

RESULTS AND DISCUSSION

Comparison of physical and physiological parameters

The body weight of treated models decreased and the body colour changed from dark grey to pale white. The colour of the liver also changed from red to brown. Comparison of hepato-somatic index of control and treated ones showed no significant difference, indicating a proportionate decrease in liver weight with decrease in body weight.

Liver, being the main detoxifying organ has undergone physiological changes resulting in change in its normal colour and texture. The liver receives the major load of toxic materials in such stress conditions. The treated ones also showed increased mucous secretion, which is a stress response behavior.

The restored models showed an increase in body and liver weight, improvement in body hue and liver colour and decreased mucus secretion.

The metabolic system of the fish could have regained its normalcy upon withdrawal of BPA, and the results support this possibility.

Protein estimation

The treated ones showed an increase in the concentration of liver proteins as compared to

the controls. The hepatic protein content of the restored fish was found to be similar to control values upon withdrawal of BPA. (Fig: 1 &2)

The increase in protein content of the treated models may be attributed to the expressionof genes that code for stress proteins; which got down-regulated upon withdrawal of BPA.

SDS PAGE

Marked up-regulation of proteins was noted in treated fish in a dose-dependent manner (Fig: 3).The up-regulated bands disappeared gradually during the restoration period.The complete absence of BPA leads to a gradual down-regulation of the gene coding for the stress proteins. Even though BPA is completely withdrawn, the heavy xenobiotic load persistently remains in the system and the liver detoxifies it gradually. As the initial concentration increases, the time taken for complete detoxification also increases.

The results of the present study also points out the potential of BPA in regulating the expression of genes in *A. testudineus*. Studies in the common mangrove killifish, *Kryptolebias marmoratus* (formerly *Rivulus marmoratus*) have shown that exposure to BPA induces changes in the expression of genes associated with oestrogen signaling, including cytochrome P450 aromatase A and B and ERá but not ERâ or androgen receptor (Lee *et al*., 2006). Similarly, studies on carp, *C. carpio* exposed to BPA employing gene arrays have reported alterations in the expression of many of the same genes induced by exposure to natural and synthetic oestrogens (oestradiol and ethinyloestradiol), but also some unique genes too. Studies on *K. marmoratus,* following exposure to BPA also demonstrated differential expression of large sets of genes and, in particular, genes belonging to the Gene Ontologies representing catalytic activity and binding (Lee *et al*., 2007). These studies do support the findings of the present study.

Histopathological analysis

The gonads of the control fish showed normal histological architecture. The ovary of the control fish showed large number of oocytes in the pre-vitellogenic (PRV), vitellogenic (VT) and post-vitellogenic (PSVT) stages of development and the testis of the control fish showed intact seminiferous tubules (ST) with more number of spermatozoa (SV) (Fig 4 &5). The ovary of treated female fish showed reduction in the number of pre-vitellogenic and vitellogenic oocytes, increased number of intensely vacuolated (VCO) post-vitellogenic oocytes, atretic oocytes (ATO), disrupted ovarian wall (DOT), and presence of melano-macrophage centres (MMCs) (Fig: 6, 7& 8). The testis of the treated male fish showed various abnormalities reduced number of spermatozoa, disruption of testicular wall (DTW), disorganized seminiferous tubules (DST), interstitial fibrosis (IF)and presence of MMCs. (Fig: 9)

The histology of gonads of the restored fish showed persistence of such aberrations even though BPA was completely withdrawn from the surroundings. The ovary of restored female fish showed disrupted ovarian wall, presence of MMCs, reduction in the number of pre-vitellogenic and vitellogenic oocytes, increased number of intensely vacuolated post-vitellogenic oocytes etc.The testis of restored male fish showed vacant, shrunken seminiferous tubules (DST) with reduced number of spermatozoa, disruption of testicular wall, interstitial fibrosis and presence of MMCs (Fig: 10-14).

The findings of the present study are supported by several published reports. BPA has also been shown to induce alterations in gonadal development and gamete quality in fish, at concentrations found in the environment. These effects include alterations in the progression of germ cell development in fathead minnow (1 µg BPA l^{-1}, Sohoni *et al*., 2001), alterations in the gonadal structure in male carp (from 1 µg BPA l^{-1}) and an enhancement of atresia in oocytes in female carp (1 µg BPA l^{-1}, Mandich *et al*., 2007). In addition, in brown trout (*Salmo trutta f. fario*), exposures to BPA has been shown to cause reduced sperm quality (1.75–2.40 µg BPA l^{-1}), delayed ovulation in females (1.75 µg BPA l^{-1}) and inhibition of ovulation in females (5 µg BPA l^{-1}). The effects observed for brown trout may have

population-level implications as they would lead to a delay in breeding, with the risk that offspring would be produced at less favorable seasonal periods. In carp, exposure to very high levels of BPA (1 mg l^{-1}) has also been shown to induce intersex (Mandich *et al*., 2007).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR analysis revealed that the gene for aromatase is up-regulated in the gonads of both male and female fish treated with BPA. The band for aromatase was224 bp and was seen prominent in the gonadal samples of both the sexes of fish treated with BPA. Presence of the internal reference gene â actin (409 bp) was seen in all the four samples.The up-regulation of aromatase in the treated models is a clear indication of the endocrine disrupting potential of BPA.

Cytochrome P450 aromatase is the enzyme complex responsible for the synthesis of estrogens by the aromatization of androgens.In teleost fish there are two isoforms of the aromatase gene, cyp19a and cyp19b, also termed CYP19A1 and CYP19A2 (Kazeto *et al*., 2001). They encode two structurally different proteins, P450aromA and P450aromB respectively, with similar affinities for the substrate (usually testosterone) but with different catalytic properties (P450aromB > P450aromA when activity is corrected by unit protein and time). Several endocrine disruptors have been shown to affect aromatase. Expression levels of P450aromA in the vast majority of the species studied are higher in females (ovary) than in males (testis). The higher expression of P450aromA in ovary versus testis and the subsequent high aromatase activity are responsible for the synthesis of estrogens and have been related with ovarian sex differentiation in several fish species (Devlin and Nagahama., 2002). There is growing evidence that some environmental contaminants, considered as endocrine disruptors (EDCs) have the ability to modulate and/or disrupt the endocrine system in vertebrates (Colborn *et al*., 1993). Among the possible biomarkers used to test the effects of EDCs, particularly xenoestrogens, vitellogenin production and CYP1A gene expression are the most widely assessed. More recently, the ability of several xenoestrogens to change aromatase gene expression and enzymatic activity has been demonstrated. In this regard, estrogenic compounds and synthetic estrogens have been shown to strongly up-regulate cyp19b gene expression in the zebrafish whereas all but a synthetic estrogen failed to induce any change in cyp19a gene expression (Kazeto *et al*., 2004). Enhancement of transcription of cyp19b after estrogen treatment has been shown in several fish species including tilapia (Tsai *et al*., 2000), black porgy (Lee *et al*., 2000), goldfish (Callard *et al*., 2001), and zebrafish (Kazeto *et al.* 2004). The fact that EREs are only present within the promoter sequenceof cyp19b and not cyp19a of all teleost species forwhich the promoter has to date been studied, is aplausible explanation for the non-responsiveness ofcyp19a to xenoestrogens and/or estrogens. This may indicate that the disruption of thereproductive axis could be mediated by a direct effecton cyp19b but not cyp19a and provide evidence thatERE is a functional transcription factor.

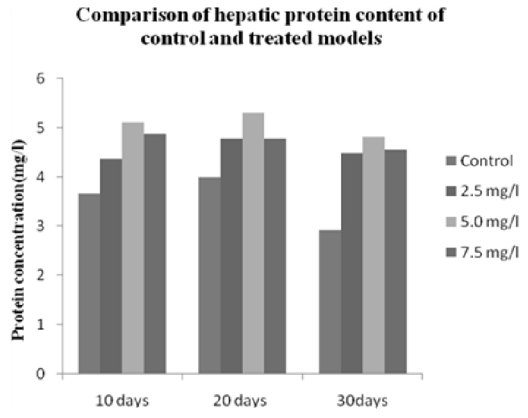


Fig. 1. Comparison of hepatic protein contentof control and treated fish

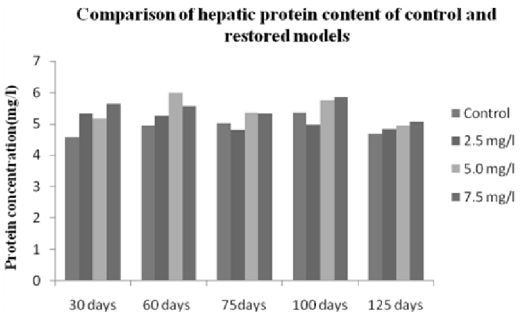


Fig. 2. Comparison of hepatic protein content of control and restored fish

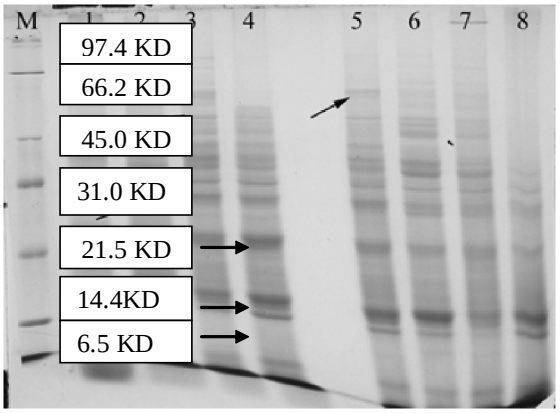
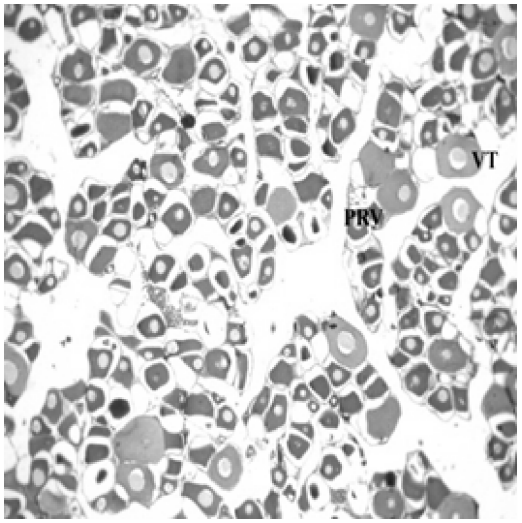
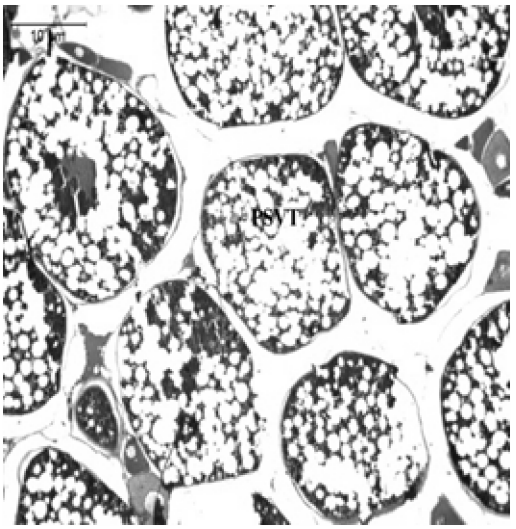


Fig. 3. Comparison of hepatic electrophoretic profile of control and treated fish

Loading pattern-M- Marker, **1**- Control, **2**- 2.5mg/l (20 days),**3**- 2.5mg/l (30 days),**4**- 5.0mg/l (20 days),**5**- 5.0mg/l (30 days), **6**- 7.5mg/l (10 days), **7**- 7.5mg/l (20 days),**8**- 7.5mg/l (30 days)

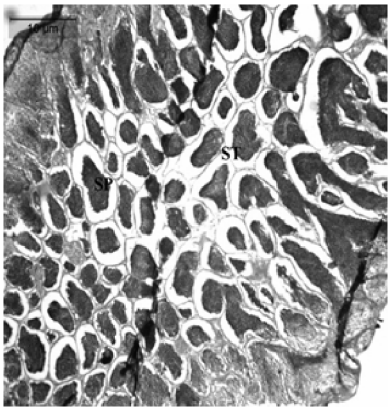




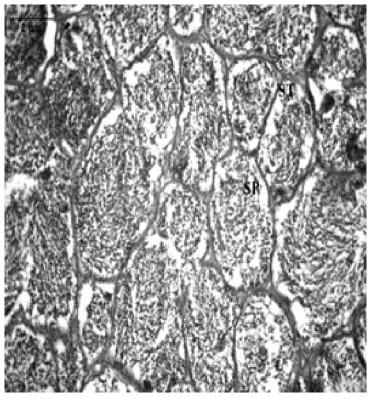
Control ovary

Control ovary

Fig. 4. Control ovary showing oocytes at various stages of development (X100)

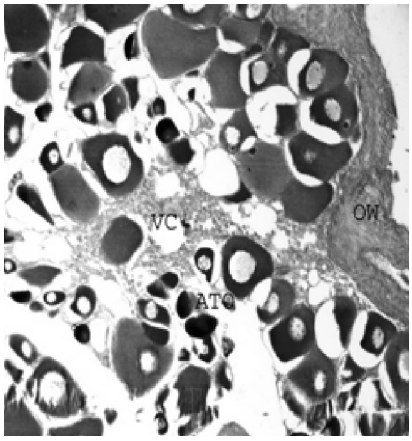


Control testis (X100)

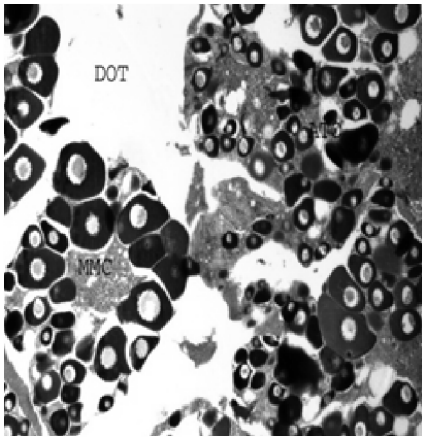


Control testis (X400)

Fig. 5. Control testis with seminiferous tubules(ST) containing spermatozoa (SP)

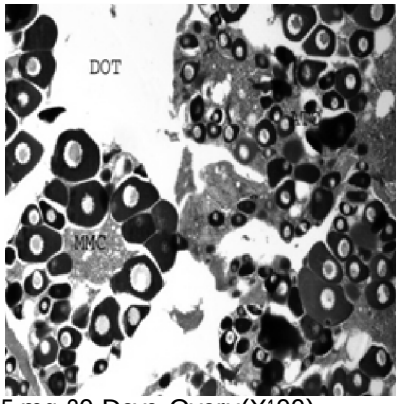


2.5mg-30 Days-Ovary

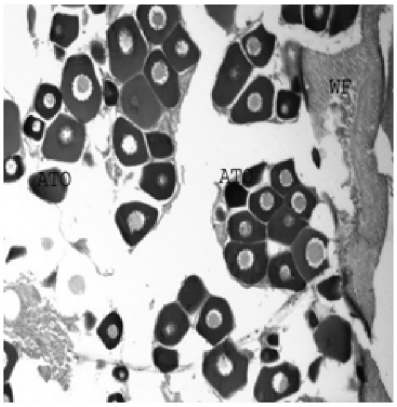


5.0 mg-20 Days-Ovary

Fig. 6. Disrupted ovary with atretic oocytes, vacuolation and disrupted ovarian wall(X100)

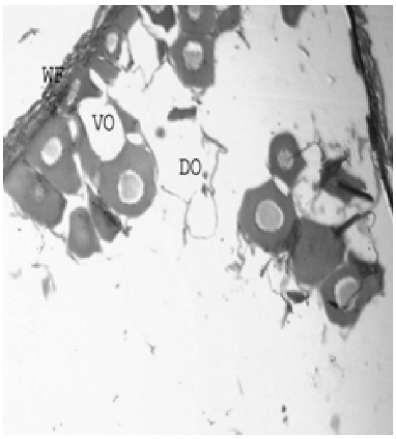


5 mg-30 Days-Ovary(X100)



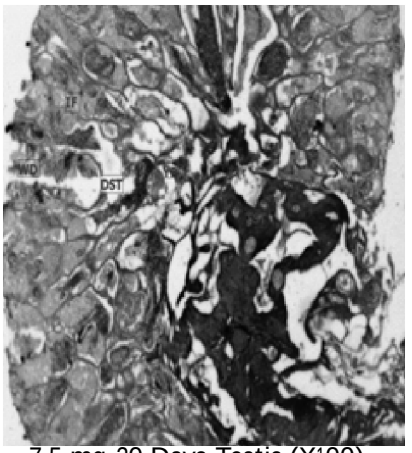
7.5mg-20 Days- Ovary (X200)

Fig. 7. Disrupted ovary with atretic oocytes, intense vacuolation & ovarian wall thickening



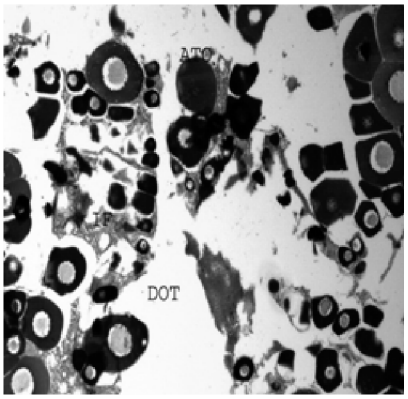
7.5mg-30 Days-Ovary(X100)

Fig. 8. Ovary with disrupted oocytes, intense vacuolation and ovarian wall thickening

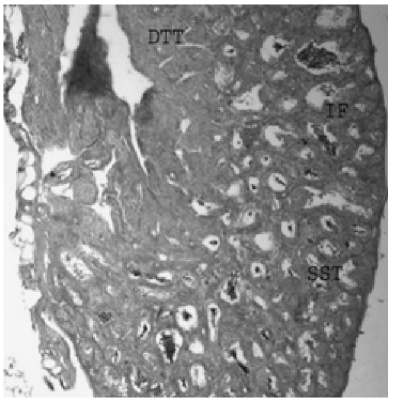


7.5 mg-30 Days-Testis (X100)

Fig. 9. Testis with disrupted and thread-like ST, wall damage, interstitial fibrosis

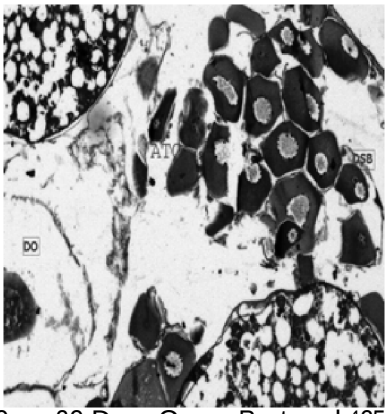


2.5mg-30 Days-Ovary-Restored-125

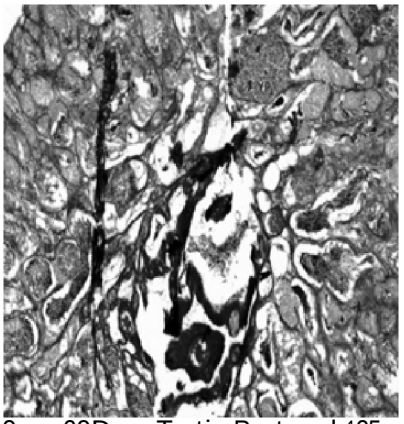


2.5mg -30 Days –Testis-Restored-125

Fig. 10. Persisting aberrations in the ovary and testis of restored models(X100)

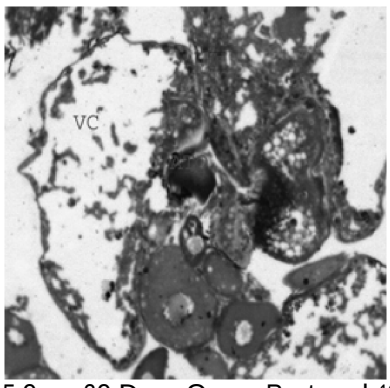


5.0mg-20 Days-Ovary-Restored-125

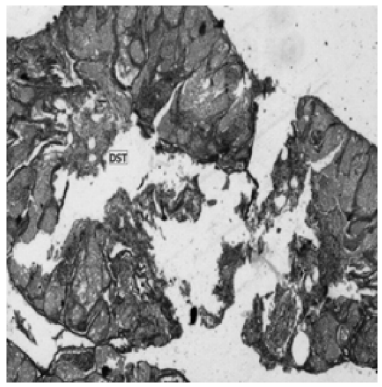


5.0mg-20Days-Testis-Restored-125

Fig. 11. Persisting aberrations in the ovary and testis of restored models (X100)

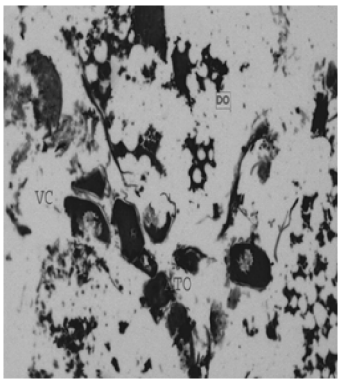


5.0mg-30 Days-Ovary-Restored-125

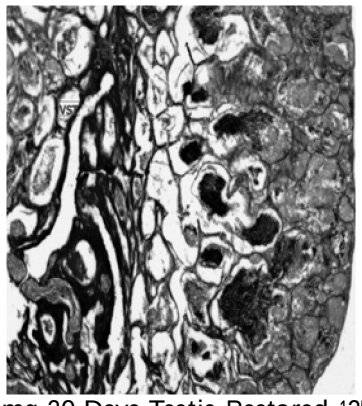


5.0mg-30Days-Testis-Restored-125

Fig. 12. Persisting aberrations in the ovary and testis of restored models (X100)

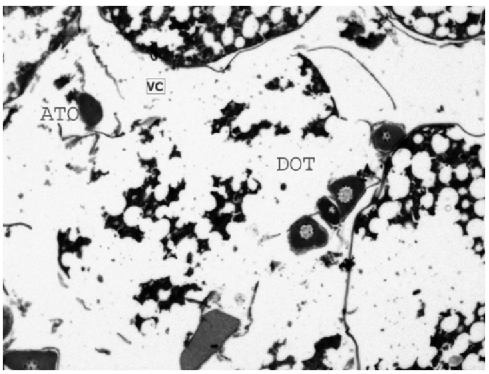


7.5mg-20 Days-Ovary-Restored-125



7.5mg-20 Days-Testis-Restored-125

Fig. 13. Persisting aberrations in the ovary and testis of restored models (X100)



7.5mg-30 Days-Ovary-Restored-125

Fig. 14. Persisting aberrations in the ovary of restored models (X400)

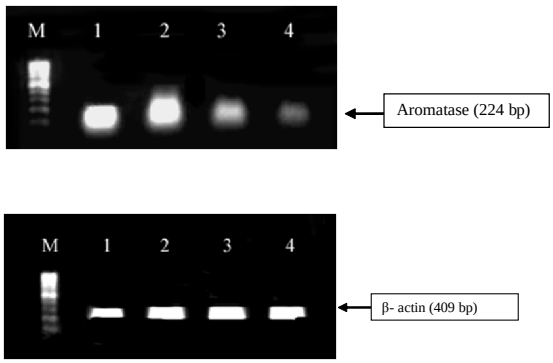


Fig. 15. Up-regulation of aromatase in treated models

M- Ladder, 1- Control ovary, 2- Treated ovary, 3- Treated testis, 4-Control testis

CONCLUSIONS

The study focused on analyzing the effect of BPA on various physiological, biochemical, histological and molecular aspects of the freshwater teleost,*Anabas testudineus*.Treatment with different sub-lethal doses of BPA resulted in decreased body weight and liver weight, reduced body hue and increased mucus secretion.Hepatic protein content was increased in treated models.The physiological and biochemical changes were restored to normalcy upon withdrawal of BPA.Hepatic protein profiling revealed appearance of new protein bands in treated models.Histopathological studies of the gonads of treated fish revealed variations from normal architecture, which were persistently seen even after the complete withdrawal of BPA.Aromatase gene was up-regulated in the gonads of both male and female fish treated with BPA, which strongly indicates the reproductive toxicity and endocrine disrupting potential of BPA.BPA functions as a potent reproductive toxicant and endocrine disruptor in aquatic organisms and also in higher life forms including humans. The potent threat of BPA contamination has to be assessed, monitoredand managed in an aquaculture environment for the propagation of economically valuable aquaculture species and other life forms.

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