Journal of Aquatic Biology & Fisheries, Vol. 2(1) 2014: 122-128

EFFECT OF BISPHENOL A ON OXIDATIVE METABOLISM IN A FRESHWATER TELEOST, *ANABAS TESTUDINEUS*(BLOCH)

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Received on: 01.10.2013, accepted on: 10.11.2013

Abstract: In this present study, an attempt was made to understand the role of bisphenol A (BPA) on the activities of hepatic mitochondrial Na^+ K^+ ATPase, Ca^{2+} ATPase, malate dehydrogenase [MDH] and isocitrate dehydrogenase [ICDH] in a freshwater fish, *Anabas testudineus*. The results revealed that sub lethal exposure of BPA at different concentrations (2.5, 5.0 and 7.5 mg/l) for a period of 15, 30 and 45 days significantly influenced the activities of the mitochondrial enzymes in *A. testudineus*. Exposure of 2.5, 5.0 and 7.5 mg/ lBPA significantly decreased the activity of hepatic mitochondrial Na^+ K^+ ATPase and Ca^{2+} ATPase for 15, 30 and 45 days.Since the activity of ATPases was decreased, it is proposed that BPA influences mitochondrial membrane potential, a key factor responsible for mitochondrial metabolism.Exposure to BPA(2.5, 5.0 and 7.5 mg/l) significantly decreased the activity of ICDH for 15, 30 and 45 days.The MDH level significantly decreased following 2.5 and 5.0mg/l BPAexposure for a period of 15, 30 and 45 days. While the MDH activity decreased after 7.5mg/l BPA exposure for15 and 30 days, but in 45 days of exposure, the MDH activity was restored. The electropherograms revealed the absence of some proteins and also the appearance of some new proteins having appropriate molecular weights when compared to control. Hence based on the results of present study, it is concluded that BPA may exert its influence on oxidative metabolism in *A. testudineus* probably by disrupting the action of some hormones, which control mitochondrial metabolism.

Key words: Bisphenol A, Na^+ K^+ ATPase, Ca^{2+} ATPase, Isocitrate dehydrogenase, Malate dehydrogenase, Oxidative metabolism.

INTRODUCTION

In recent years, a growing body of scientific research indicates that chemicals in the environment may interfere with the normal function of the endocrine system of animals. These compounds may be man-made such as industrial chemicals, crop protection chemicals, synthetic hormones or natural chemicals like phytoestrogens (Arukwe, 2001; Swan *et al*., 2003). BisphenolA (BPA) is an endocrine disruptor and is a compound widely used in the production of polycarbonate and other plastics with an annual production exceeding 420000 tons (Alexander *et al.,* 1988). Bisphenol A is present in certain food contact materials because it is used in the production of polycarbonate and epoxy-phenolic resins. According to Pritchett *et al.* (2002) humans are likely to be more efficient at breaking down BPA due to relatively larger liver. The targets of the toxic effects of BPA are probably mitochondria and mitochondrial respiration (Nakagawa and Tayama, 2000). The sodium-potassium activated adenosine triphosphatase [Na^+K^+ ATPase] is the enzyme responsible for the active transport of sodium and potassium ions to maintain the ionic gradient, Na^+ coupled transport of nutrients into cells, osmotic balance and regulation of cell volume (Zanatta *et al.,* 2001). The role of environmental chemicals on mitochondria and mitochondrial metabolism has not been studied in detail in fish. The present study an attempt was made to screen the effect of BPA on the activity of mitochondrial Na^+ K^+ ATPase and Ca^{2+} ATPase in the liver and gill and the activity of hepatic mitochondrial enzymes such as malate dehydrogenase [MDH] and isocitrate dehydrogenase [ICDH] in a freshwater fish, *Anabas testudineus*.

MATERIALS AND METHODS

The fish, *A. testudineus* used in the experiments were collected from local suppliers, brought to the laboratory andkept in large cement tanks with aerated and dechlorinated tap water. The fish were fed with 40% protein feed. Prior to the experiment, adult healthy fish of body weight 40±2g were selected and divided into 10 groups of six each in separate tanks. The first group of fish was served as control. Fish in groups II, III and IV were exposed to 2.5mg BPA/l of water for the periods 15, 30 and 45 days respectively. Similarly the groups of fish V, VI and VII were exposed to 5.0 mg BPA/l of water and groups of VIII,IX and X were exposed to 7.5mg BPA/l of water for a period of 15, 30 and 45 days respectively. Freshwater sample having the specified doses of BPA was replaced in every three days. After stipulated periods of exposure, fish were sampled and killed by spinal concussion. Then, liver and gill were excised straight away and frozen immediately at $^{-}80^{0}$C [NBS, USA] for enzymes assay.

Biochemical Analysis

Isolation of mitochondria from liver was carried out according to the method prescribed by Irving and Watson (1976). The homogenate prepared to measure ATPases activity was similar to that described by Zaugg (1982). The protein content was estimated by Bradford method using BSA as standard (Bradford, 1976).The activity of MDH was determined by the method of Mehler*et al.* [1948]. The enzyme ICDH was assayed by the method of Ochoa (1955). Electrophoresis was performed under denaturing and discontinuous conditions on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel using a mini vertical gel unit (Hoefer, USA) by the method of Laemmli (1970). For assaying all the above enzymes, 100mg tissue was homogenized in 1ml SET buffer andcentrifuged at 10,000g for 10 min and the pellet was taken, washed thrice and suspended in the same medium.

Assay of $Na^{+}K^{+}$ and Ca^{2+}ATPases

Experiment and control cocktails were prepared separately. Experimental cocktails of Na^{+} K^{+} ATPase contained tris, NaN_3, EDTA, NaCl, KCl and ATP. Ca^{2+} ATPase cocktail contained $CaCl_2$ instead of NaCl and KCl. The tubes were kept and incubated at room temperature for 15 minutes, after which the reaction was terminated by adding 1 ml of 10% TCA. The tubes were kept on ice for 30 minutes, after that they were centrifuged at 2000 rpm for 10 minutes.After centrifugation, the supernatant fraction was analyzed for inorganic phosphate at 640 nm in a UV visible spectrophotometer [Perkin Elmer, USA]. The enzyme activity was expressed as nanomol phosphate liberated / min / mg protein. The protein content was estimated by Bradford method using BSA as standard.

Assay of ICDH

The reaction mixture in a quartz cell [d=1cm] consisted of 300ml glycylglycine buffer, 100ml $MnCl_2$ and 200ml $_{DL}$-Isocitrate. The aliquot was made up to 2.8ml by adding double distilled water. One hundred microlitre tissue homogenate containing enzyme was added to the reaction mixture. The reaction was started by the addition of $NADP^{+}$ and the change in absorbance was noted at 340nm in a UV-visible spectrophotometer [Perkin Elmer, USA] against a blank containing all components except $NADP^{+}$, at intervals of 15 seconds for 1 min.

Assay of MDH

The reaction mixture in a quartz cell [d=1cm] contained 300ml glycylglycine buffer and 25ml of oxaloacetate. The aliquot was made up to 2.8ml by adding double distilled water. One hundred-microlitre tissue homogenate-containing enzyme was added to the reaction mixture. The reaction was started by the addition of NADH and the change in absorbance was noted at 340nm in a UV-visible spectrophotometer [Perkin Elmer, USA] against a blank containing all components except NADH, at intervals of 15 seconds for 1 min.

Electrophoresis

Electrophoresis was performed under denaturing and discontinuous condition of 10% sodium dodecyl sulfate [SDS] polyacrylamide gel using a mini vertical gel unit [Hoefer, USA]. Known molecular weight protein ranging from 250-15

kDa was used as molecular markers [Rainbow marker, Amersham, USA]. The liver and gill homogenate samples loaded in the wells were electrophorised at a constant current of 120V for stacking and running gel in an electrophoresis buffer consisting of 0.1% SDS, 0.05M Tris and 0.384M glycine buffer, pH 8.3 for about 3hr. The gels were stained with 0.25% Coomassie brilliant blue R 250 in a mélange of 40% methanol and 7% acetic acid over night, firstly destained with destain I solution of 40% methanol and 7% acetic acid for about half an hour and then destained with destain II solution of 7% acetic acid and 5% methanol to get the best quality protein bands. Stained gels were then fixed in detain II containing 1% glycerol (Westermeier, 1997). The appropriate molecular weights of resolved proteins were determined by comparison with known standards.

Chemicals were of analytical grade and purchased from Sigma chemicals USA. Data differences were determined by one- way ANOVA. Significant level used was 0.05. The groups that were not significantly different in Duncan's (1955) multiple range test were considered homogeneous. Statistical analysis was performed by the use of SPSS 10.0 software.

RESULTS

In the present study, exposure of fish to 2.5 mg/l and 5.0 mg/l of BPA for 15, 30 and 45 days significantly reduced the activity of mitochondrial Na^+K^+ and Ca^{2+}ATPases activity in liver in time dependent manner, except hepatic Ca^{2+} ATPase activity, there is no significant change after 15 days of exposure in 2.5mg/l of exposed fish. (Figs. 1, 2). The least activity for all the enzymes was recorded after 45 days of exposure in both tissues.

Exposure of fish to 7.5 mg/l of BPA for 15 days did not evoke any significant change, 30 and 45 days of exposure significantly reduced the activity of mitochondrial Na^+K^+ and Ca^{2+}ATPases in liver (Fig. 3). Here also the least activity was noted at 45 days of BPA exposure.

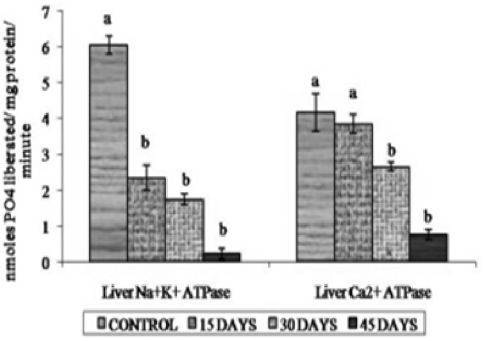


Fig. 1. Effect of BPA(2.5 mg/l) on ATPase activity in the liver of *A.testudineus* for control, 15, 30 and 45 days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different subscript letters (a,b) were significantly different ($p<0.05$).

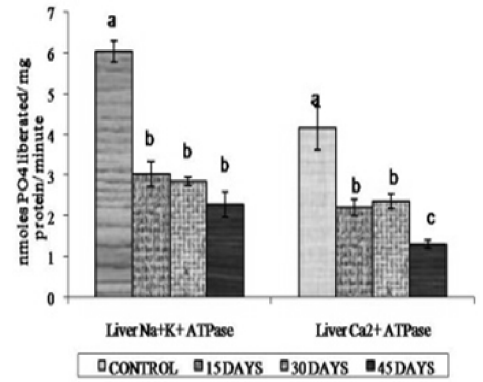


Fig. 2. Effect of BPA(5.0 mg/l) on ATPase ATPase activity in the liver of *A.testudineus* for control,15,30 and 45 days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different subscript letters (a,b,c) were significantly different ($p<0.05$).

Hepatic mitochondrial enzymes activity

Isocitrate dehydrogenase activity in fish liver exposed to BPA (2.5, 5.0 and 7.5mg/l) for 15, 30 and 45 days are shown in Fig.4. There was a significant decrease in ICDH activity in liver tissue after 15, 30 and 45 days of exposure to 2.5mg/l BPA. ICDH activity did not change significantly after 15 days of exposure to 5.0 and 7.5mg/l BPA. However, significant decrease in

ICDH activity occurred after 30 and 45 days of exposure to 5.0 and 7.5mg/l BPA.

Malate dehydrogenase activity exposed to BPA (2.5, 5.0 and 7.5mg/l) for 15, 30 and 45 days are shown in Fig.5. There was a significant decrease in MDH activity in liver tissue after 15, 30 and 45 days of exposure to 2.5 and 5.0mg/l BPA. Exposure to 7.5mg/l BPA significantly decreased MDH activity after 15 and 30 days but there was no significant change in MDH activity at 45 days of exposure.

Electrophoresis

The electropherograms revealed the absence of some proteins and also the appearance of some new proteins having appropriate molecular weights when compared to control.

The electropherogram of liver extract revealed appearance and disappearance of some protein bands. A protein band with molecular weight 95 kDa appeared after 15 and 45 days of exposure and also a protein band with molecular weight 87 kDa appeared after 45 days of exposure [Fig.6 A]. A protein band having molecular weight 45 kDaintensified after 15 days, disappeared after 30 days and reappeared after 45 days of exposure.

The electropherogram of liver mitochondrial extract clearly showed the presence of two protein bands in mitochondrial extract with molecular weight 32 kDa and 50 kDa. The intensity of these proteins gradually decreased in 15, 30 and 45 days of exposure as compared to control [Fig. 6 B]. The decrease in the concentration of these protein bands after BPA exposure may be due to the decreased activity of mitochondrial enzymes studied.

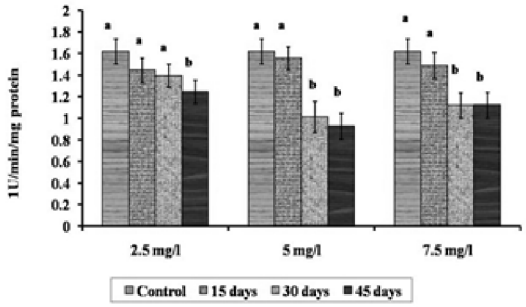


Fig. 4. Effect of BPAon ICDH activity in the liver of *A. testudineus* for control, 15, 30 and 45days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different subscript letters (a,b) were significantly different ($p<0.05$).

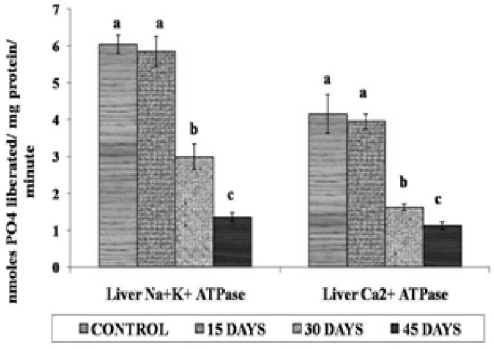


Fig. 3. Effect of BPA(7.5 mg/l) on ATPase activityin the liver of *A. testudineus* for control,15,30 and 45days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different subscript letters (a,b,c) were significantly different ($p<0.05$).

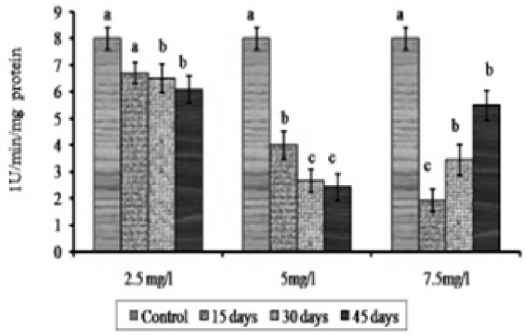
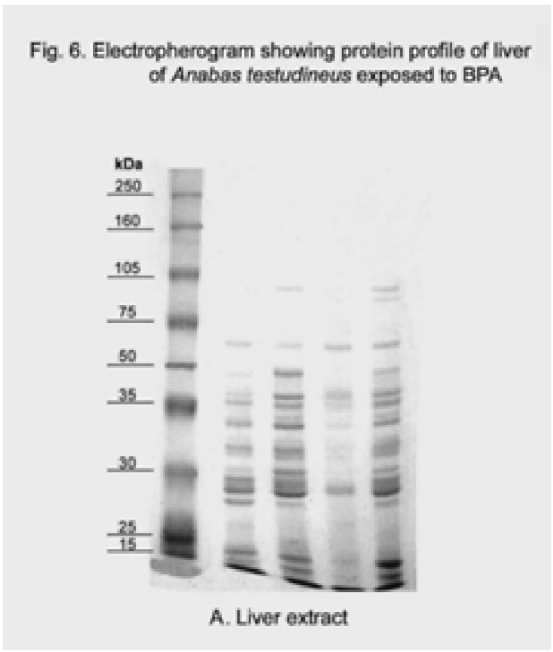
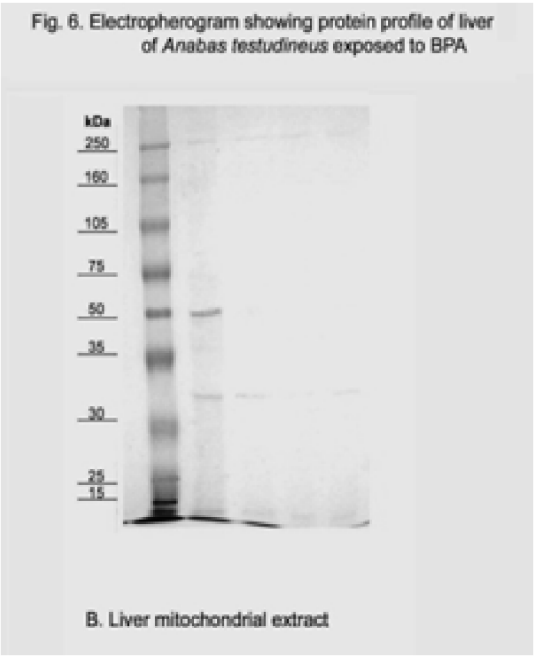


Fig. 5. Effect of BPA on MDH activity in the liver of *A. testudineus* for control, 15, 30 and 45days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different subscript letters (a,b,c) were significantly different ($p<0.05$).





DISCUSSION

The results of the present study clearly revealed that exposure of BPA significantly reduced the activity of mitochondrial Na^+K^+ ATPase, Ca^{2+} ATPase, MDH and ICDH activity in liver of the fish, *A. testudineus*. The decreased activities of mitochondrial enzymes by BPA indicate the negative influence of BPA on the oxidative metabolism of the fish studied.

Mitochondria have long been recognized as the generators of energy for the cell. Like any other power source, however, mitochondria are highly vulnerable to inhibition or uncoupling of the energy harnessing process and run a high risk for catastrophic damage to the cell. The exquisite structural and functional characteristics of mitochondria provide a number of primary targets for xenobiotic-induced bioenergetic failure. They also provide opportunities for selective delivery of drugs to the mitochondria. In light of the large number of natural, commercial, pharmaceutical, and environmental chemicals that manifest their toxicity by interfering with mitochondrial bioenergetics, it is important to understand the underlying mechanisms (Wallace and Starkov, 2000). Mitochondrial ATPase is involved in the phosphorylation of ADP for the synthesis of ATP. The inhibitory activity of Na^+K^+ and Ca^{2+} ATPase following BPA exposure may be because of decreased phosphorylation in the liver of *A. testudineus*. It has been recently reported that warm acclimation decreased oxidative capacity of isolated mitochondrial preparationswas decreased in of rainbow trout (Kraffe *et al*., 2007).

The respiratory parameters of a freshwater teleost, *Oreochromismossambicus* were studied under sub lethal intoxication of methyl parathion, an endocrine disruptor. The rate of oxygen consumption by whole fish and selected tissues decreased during a 48-hr time course study. The activities of the respiratory enzymes succinate dehydrogenase, malate dehydrogenase, and cytochrome-c oxidase also decreased considerably under methyl parathion exposure in muscle, gill, liver, and brain tissues. These results suggest that methyl parathion has a profound effect on the oxidative metabolism of the fish which results in low ATP turnover,

possibly due to its influence on the respiratory center of the brain.

Many insecticidal activities occur due to the opening and closing of specific ion channel proteins embedded within the plasma membrane. The voltage-gated sodium channel is the well established target of a variety of insecticides including DDT, pyrethroids, N-alkylamides, and the recently introduced oxadiazine in doxacarb (Zlotkin, 1999; Lapied *et al.,* 2001). A large subtype diversity of cholinergic or GABA cell membrane receptors are also altered by other classes of insecticidally active molecules such as neonicotinoids and phenylpyrazoles (Bloomquist 2001; Nauen *et al*., 2001). Besides these most extensively known insecticidal targets, the mitochondria, which is responsible for most ATP production, is also targeted by pesticides (Schuler and Casida, 2001). The disruption of energy metabolism usually results of either an inhibition of the electron transport system or an uncoupling of the transport system from ATP production. Some compounds block the production of ATP through an inhibition of the electron transport system and causes a decrease in oxygen consumption by the mitochondria.

Others reported the involvement of the ATPases activity (Buckman and Reynolds, 2001), Ca^{2+} influx through mitochondrial Ca^{2+}uniporter [Duchen et al., 1998], mitochondrial membrane anion channels (Aon *et al*., 2003), and free radicals released from the matrix side of mitochondria (Aon *et al*., 2003). This may be true in the case of the present study where the fish exposed to BPA significantly altered mitochondrial energy metabolism by influencing the membrane potential through Na^+ K^+ ATPase and Ca^{2+} ATPase activity. Since the activity of ATPases was decreased, it is proposed that BPA influences mitochondrial membrane potential a key factor responsible for mitochondrial metabolism. It is still not clear how change in mitochondrial membrane potential in a small number of mitochondria might influence the overall function of the cell.

It has been recently reported that the fluctuations in mitochondrial membrane potential reflect an intermediate unstable state of mitochondria, which may lead to or reflect mitochondrial dysfunction (Vergun and Reynolds, 2004). These fluctuations were not the consequence of oxidative stress or the high conductance mitochondrial permeability transition. They reported that this phenomenon was triggered by Ca^{2+} and can be potently inhibited by adenine nucleotides.

The results of present study was strongly supported by the findings of Ramakritina *et al*. (2005) who reported that pollutants affect fish mainly on energy metabolism. Energy metabolism plays a key role when the animal is forced to expend more energy to mitigate toxic stress. Toxicants are known to affect the oxidative metabolism. In the current study, ATPases, MDH and ICDH activities of liver and gill of *A. testudineus* was depleted with increasing sub lethal concentrations of BPA as well as duration of exposure. The decreased activity of the enzymes' was confirmed by electrophoretic analysis of liver mitochondrial extract. Based on the data of the present work, it is proposed that fish chronically exposed to BPA appeared to affect oxidative metabolism by influencing the mitochondrial enzymes.

ACKNOWLEDGEMENT

We acknowledge the financial support from UGC and KSCSTE, Government of Kerala

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