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BISPHENOL A-INDUCED OXIDATIVE AND OSMOTIC STRESS IN THE JUVENILES OF MOZAMBIQUE TILAPIA, *OREOCHROMIS MOSSAMBICUS* (PETERS)

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Abstract: Bisphenol A (BPA) is a wide spread contaminant of the aquatic ecosystems. Low levels of BPA have also been to found to cause biological effects and its mode of action appears to mimic that of the female hormone estrogen. The aim of the present study was to determine the role of BPA on oxidative stress and osmoregulation in juvenile *Oreochromis mossambicus*. Oxidative stress was analysed by assaying the activity of Superoxide dismutase (SOD), Catalse (CAT), Glutathione peroxidase (GPx), Glutathione-S-transferase and non-enzymatic scavenger glutathione content in the liver and the activity of branchial Na^+ K^+ and Ca^{2+}ATPases for osmotic stress in the juvenile fish. The juvenile *O. mossambicus* were exposed to three sub lethal doses (2, 4 and 6mg/l) of BPA for 7, 14 and 21 days. All three doses of BPA exposure significantly inhibited the activity of SOD, CAT and GPx and elevated the activity of the GST and the level of non-enzymatic content glutathione in juvenile fish. The activity of Na^+ K^+ and Ca^{2+} ATPases also significantly reduced after exposure of all sub lethal doses of BPA. Alterations in antioxidant enzymes and ATPase activities indicated that BPA exposure is capable of inducing oxidative and osmotic stress in juvenile *O. mossambicus*.

Key words: *Bisphenol A, Osmoregulation, Tilapia, Oxidative stress, Osmotic stress, Antioxidants*

INTRODUCTION

Bisphenol A (BPA) is an industrial chemical widely used in the production of polycarbonates, epoxy resins and flame retardants. It has also been detected in marine and freshwater habitats around the world at considerable concentrations. BPA exhibits estrogenic property by activating estrogen receptor, androgen receptor, thyroid hormone receptor, peroxisome proliferator activated receptor-γ, and other endocrine-relevant signaling pathways (Watson *et al*., 2011). Endocrine disruptors can stimulate the production of reactive oxygen species (ROS), which results in oxidative damage, to aquatic organisms (Livingstone, 2001). Enzymatic and non enzymatic antioxidants constitute an important biological defense against environmental pro-oxidants by countering the impact of ROS. Cellular ROS accumulation can lead to oxidative stress in an individual and result in various types of tissue damage and diseases when the dysfunction of antioxidation occurs with overproduction of reactive oxygen intermediate species (Janssen *et al*., 1993). Therefore, oxidative stress indices and antioxidant parameters are considered potential biomarkers that have been used as rapid screening tools to assess different environmental stress impacts. ATPases play an important role in maintenance of functional integrity of plasma membrane and in several intracellular functions and are considered to be a sensitive indicator of toxicity (Yadwad *et al*., 1990). Na^+ K^+ ATPase and Ca^{2+}ATPases are membrane bound ATPases. Na^+ K^+ ATPase transports Na^+ and K^+ ions and plays a central role in whole body osmoregulation purposes (Sancho *et al*., 2003). Ca^{2+} ATPase present in the gill membranes are probably involved in Ca^{2+} transport. Exposure of BPA produced oxidative stress in the liver of *Anabas testudineus* by influencing the antioxidant enzyme (Aruna *et al*., 2015) and it also influenced the activity of mitochondrial antioxidant

enzymes in liver and gill of *A. testudineus* (Gireesh and Sunny, 2013). BPA has been the subject of considerable aquatic toxicity studies in recent years because of its effects on wildlife, since the aquatic environment could receive discharges of BPA from production, processing and sewage treatment plant effluents. The present study aims to evaluate the effect of endocrine disruptor, BPA on the activities of hepatic antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione-S-transferase (GST) and non-enzymatic glutathione content and the activities of branchial Na^+K^+ ATPase and Ca^{2+}ATPases in the juveniles of freshwater fish, *O. mossambicus*.

MATERIALS & METHODS

Experimental design

The juvenile fishes of *O. mossambicus* used in the experiments were collected from local fish suppliers and acclimated to the laboratory conditions for a period of two weeks in large cement tanks containing well aerated dechlorinated tap water at an optimum temperature of 26±2^{0}C in natural photoperiod. The juveniles were fed with fish feed prepared in the laboratory. Prior to the experiment, juveniles of body weight 6±1g were selected and divided into 10 groups of twenty four each in separate glass tanks. The first group of fish were maintained in dechlorinated tap water and served as control. Fish in group II, III and IV were exposed to 2mg BPA/l of water for the periods 7, 14 and 21 days respectively. Similarly, the groups of fish V, VI and VII were exposed to 4mg BPA/l of water and groups of VIII, IX and X were exposed to 6mg BPA/l of water for a period of 7, 14 and 21 days respectively. Water sample having the specified doses of BPA were replaced in each tank every third day.

Biochemical Analysis

The activity of Superoxide dismutase (SOD) (EC.1.15.1.1), Catalase (CAT) (EC.1.11.1.6), Glutathione peroxidase (GPx) (EC.1.11.1.9), Glutathione-S-Transferase (EC.2.5.1.18) and protein were determined using UV-Visible spectrophotometer (Perkin Elmer). SOD was estimated according to the protocol of Kakkar *et al.* (1984). Catalase activity was measured according to Maehly *et al.* (1954). GPx according to Lawrence *et al.* (1976) as modified by Agerguard and Jenson (1982). Glutathione content according to Benke *et al.* (1974). Branchial Na^+/K^+ and Ca^{2+}-ATPases activities will be assayed by the protocol of Gurnesy and Edelman (1986) and Fiske and Subbarow (1925). Protein content was estimated by Bradford method (1976). Chemicals were of analytical grade and purchased from Sigma chemicals USA.

For assaying all antioxidant enzymes 150mg liver tissue was homogenized in 2 ml sucrose solution (258mM) and centrifuged 10,000g for 10 min and the supernatant was taken as extract. For assaying ATPase enzymes, 50mg of each gill were homogenized in 2 ml SEI (75 mM Sucrose, 20 mM EDTA, 10 mM Imidazole) buffer and centrifuged at 1000 rpm for 10 min at 4^{0}C (Eppendorf, Germany) and the supernatant thus collected was used for measuring specific activity of ATPase.

Assay of SOD

The assay mixture contained 1.2 ml sodium pyrophoshphate buffer (0.052 M, pH 8.3), 0.1ml phenazine methosulphate (186µM), 0.3ml nitroblue tetrazolium, 0.2ml NADH (780µM), approximately diluted enzyme preparation and water in a volume of 3ml. The reaction was started by the addition of NADH. After incubation at 30^0 C for 90 seconds, the reaction mixture was stirred vigorously and shaken with 4ml n-butanol. The mixture was allowed to stand for 10 min, centrifuged at 2500 rpm for 10 min and butanol layer was carefully pipetted out. Colour intensity of the chromogen in butanol was measured at 560 nm against butanol (blank) using UV – Visible Spectrophotometer. One unit of enzyme activity required to inhibit the optical density at chromogen production by 50% in one min under the assay conditions. The specific activity was expressed as IU mg protein^{-1}.

Assay of CAT

The reaction mixture contained 3ml phosphate buffer (0.01 M, pH 7.0), 20µl H_2O_2 (2mM) and 0.1ml of approximately diluted extract. A system devoid of extract constituted blank. Decrease in absorbance was measured at 230 nm for 2 min.

The specific activity was expressed in terms of n moles of H_2O_2 decomposed/min/mg protein.

Assay of Gpx

The assay volume contained 2ml of 0.01 M phosphate buffer (pH-7.4) with 300µl EDTA, 300µl of 1mM sodium azide, 100µl of 0.1 M NADPH, 100µl of 1mM reduced glutathione and 200µl tissue extract. Blank contained all the reagents except tissue extract. Change in absorbance was measured at 340nm at 15 seconds interval for 1 min. Enzyme activity expressed as IU/mg protein.

Assay of tGSH

The reaction mixture contained 2 ml (0.3 M) phosphate buffer, 500µl, 0.04 % 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) and 200µl extract. A system devoid of extract served as blank. Change in absorbance was measured at 412 nm within 10 min. The concentration of tGSH was expressed as n mol/100 g tissue.

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

Experimental and control cocktails were prepared separately. Experimental cocktails of Na^+ K^+ ATPase contained 30 mM Tris, 10 mm NaN3, 1 mM EDTA, 130 mM NaCl, 10 mM KCl and 3 mM ATP. Ca^{2+} ATPase cocktail contained 100 mM $CaCl_2$ instead of NaCl and KCl. Control cocktail contained all the above reagents along with 3 mM Ouabain. The pH of the entire cocktail was adjusted to 7.4. The tubes were shaken and incubated at 30^{0}C for 15 min. The reaction was terminated by adding 1 ml of 10% TCA. Adenosine triphosphate was also added to the control. The tubes were kept on ice for 30 minutes, after that they were centrifuged at 2000 rpm for 10 minutes. The clear supernatant was used for the determination of inorganic phosphate (Fiske and Subbarow, 1925). Ammonium molybdate was added to this and kept for 10 min followed by ANSA (1- amino- 2- naphthol-4-sulphonicacid). The absorbance was measured at 640 nm with UV-Visible spectrophotometer (Perkin Elmer,USA). ATPase activity was calculated as the inorganic phosphate and expressed as nanomoles/min/mg protein. The protein content was estimated by Bradford method (1976) using BSA as standard.

Chemicals were of analytical grade and purchased from Sigma chemicals U.S.A. 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 tests were considered homogeneous. Statistical analysis was performed by the use of SPSS 22.0 software.

RESULTS

Heapatic antioxidant enzyme's activity

The present study revealed that BPA exposure significantly inhibited the activity of SOD, CAT and GPx and elevated the activity of GST and level of non enzymatic content glutathione in juvenile *O. mossambicus*. Superoxide dismutase activity in juvenile fish liver exposed to BPA (2, 4 and 6mg/l) for 7, 14 and 21 days are shown in Fig. 1. After exposure to 2mg/l BPA for 7, 14 and 21 days significantly decreased the activity of SOD. SOD activity was significantly decreased in a time dependent manner after exposure to 4 and 6mg/l BPA for 7, 14 and 21 days respectively.

Catalase activity was significantly inhibited after exposure to BPA (2, 4 and 6mg/l) for 7, 14 and 21 days (Fig.2). Exposure of 2mg/l BPA significantly decreased GPx activity for 14 and 21 days but the activity was unaffected after 7 days exposure (Fig.3). The activity of GPx was decreased significantly after exposure to 4 and 6 mg/l BPA for all the three periods (Fig.3)

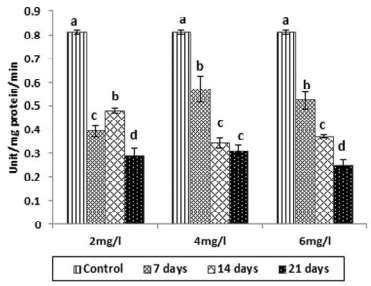

Fig.1. Effect of BPA on SOD activity in the liver of juvenile *O. mossambicus* for control, 7, 14 and 21 days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different superscript letters (a,b,c,d) were significantly different ($p<0.05$).

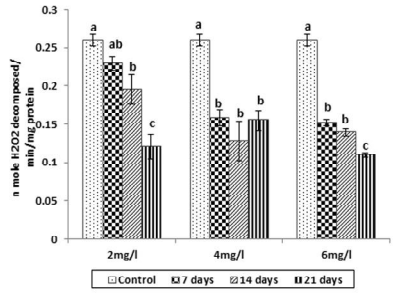

Fig.2. Effect of BPA on CAT activity in the liver of juvenile *O. mossambicus* for control, 7, 14 and 21 days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different superscript letters (a,b, c) were significantly different ($p<0.05$).

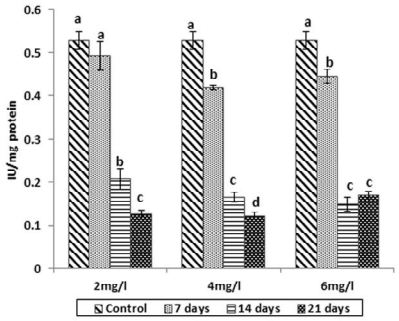

Fig.3.Effect of BPA on GPx activity in the liver of juvenile *O. mossambicus* for control, 7, 14 and 21 days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different superscript letters (a,b, c) were significantly different ($p<0.05$).

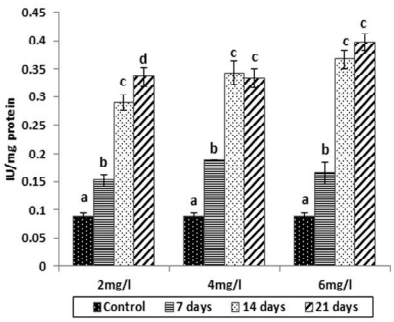

Fig.4. Effect of BPA on GST activity in the liver of juvenile *O. mossambicus* for control, 7, 14 and 21 days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different superscript letters (a,b,c,d) were significantly different ($p<0.05$).

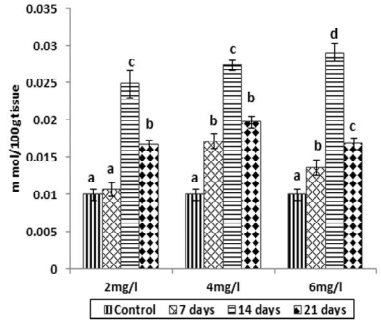

Fig.5. Effect of BPA on level of tGSH activity in the liver of juvenile *O. mossambicus* for control, 7, 14 and 21 days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different superscript letters (a,b,c,d) were significantly different ($p<0.05$).

GST activity was significantly increased after exposure to 2, 4 and 6mg/l BPA for all three periods such as 7, 14 and 21 days (Fig.4). The amount of glutathione was significantly increased after 14 and 21 days after exposure to 2mg/l BPA. While 7 days of exposure did not produce any change. Exposure of 4 and 6mg/l BPA significantly increased glutathione content for 7, 14 and 21 days (Fig.5).

Branchial Na^+K^+ and Ca^{2+}ATPases activity

The activity of branchial Na^+K^+ATPase and Ca^{2+} ATPase was significantly inhibited by the exposure of BPA (2, 4 and 6mg/l) for 7, 14 and 21 days (Fig.6& 7).

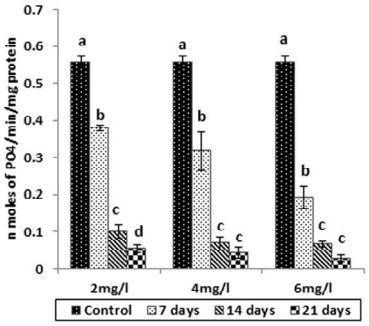

Fig.6.Effect of BPA on $Na^+ K^+$ ATPase activity in the gill of juvenile *O. mossambicus* for control, 7, 14 and 21 days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different superscript letters (a,b,c,d) were significantly different ($p<0.05$).

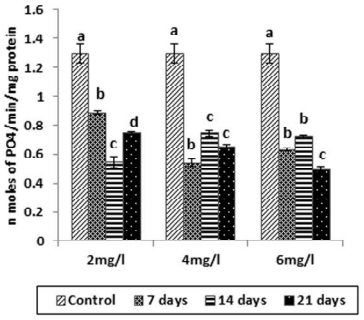

Fig.7. Effect of BPA on Ca^{2+}ATPase activity in the gill of juvenile *O. mossambicus* for control, 7, 14 and 21 days are plotted. The significant difference were analysed by one-way analysis of variance. Mean values of different superscript letters (a,b,c,d) were significantly different ($p<0.05$).

DISCUSSION

The results of the present study clearly revealed that BPA exposure leads to oxidative and osmotic stress in juvenile *O. mossambicus*. BPA exposure significantly reduced the activity of SOD, CAT and GPx and elevated the activity of GST and Glutathione content in the liver of juvenile fish. BPA exposure inhibited the activity of osmoregulatory enzymes such as $Na^+ K^+$ ATPase and Ca^{2+} ATPase in the gill of fish.

The activity of antioxidant may be increased or inhibited under chemical stress depending in the intensity and duration of stress applied as well as susceptibility of exposure species (Gad, 2011). In normal metabolism a balance exist between the generation of ROS and other pro-oxidants, and their detoxification and removal by antioxidant defense mechanism (Winston and Di Giulio, 1991).

Superoxide dismutase scavenges superoxide radical which appeared to be an important agent of toxicity of oxygen and this provides a defense against oxygen toxicity (Kadar *et al*., 2005). In this study juveniles and adult fish exposed to sub-lethal concentrations of BPA produced significant decrease in the activity of hepatic SOD compared with control group. Fall in the activity of SOD in liver might be due to inactivation by interaction with O_2 radicals or because of overproduction of H_2O_2, as SOD is inactivated by the products of its own reaction (Pigeolet *et al*., 1990). Significant decrease in the activity of SOD was reported in zebra fish embryos (Wu *et al*., 2011) and early life stages *Chalalburnus tarchi* following BPA exposure (Kankaya *et al*., 2015). Inhibition in SOD activity was also reported in the liver of *A. testudienus* after administration of aroclor 1254 (Amrutha, 2014) and sewage exposure (Soorya *et al*., 2012).

Catalase is one of the sensitive enzyme biomarkers and its activity is modulated by various factors including over production of superoxide radicals (Kono and Fridovich, 1982). In this study CAT activity was decreased in the liver of juvenile fish. Similar results were reported in Japanese sea bass fingerlings exposed to lower concentration of E_2 (Thilagam *et al*., 2010) and in liver of the one sided liver bearer, *Jenynsia multidenfala* exposed to endosulfan (Ballesteros *et al*., 2009). Similarly, herbicides exposure produced significant decrease in hepatic CAT activity on the teleost *Leporinus obtusidens and silver cat fish, Schilbe intermedius* (Moraes *et al*., 2009; Kathya *et al*., 2010).

GPx degrades Hydrogen peroxides using reduced glutathione. GPx activity reduced in liver after exposure to BPA. Alzebeta Stara *et al.* (2012)

reported that chronic exposure of simazine caused significant decline in hepatic GPx activity in *Cyprinus carpio*. GPx activity decreased in liver, gill and muscle tissue of *O.mossambicus* exposed to diethylphthalate (Ummamaheswari and Senthilnathan, 2014). Ballesteros *et al.*(2009) observed inhibition of GPx activity in gill, intestine, liver and muscle of fish *Jenysia multidentata* exposed to 1.4 mg L^{-1}endosulfan for 24 hours. The low GPx activity in juvenile fish might be due to a direct BPA inhibition of enzyme synthesis or due to increased generation of hydrogen peroxide which may have inhibited the enzyme activity.

Glutathione – S- transferase is a multicomponent enzyme involved in the detoxification of many xenobiotics, which plays an important role in protecting tissues from oxidative stress (Fournier *et al*., 1992). The increase GST activity was suggested as a useful tool for biomintoring oxidative stress in channel cat fish (Di Giulio *et al*., 1993). In the present study exposure of BPA to juvenile fish produced increased activity of hepatic GST. The same results show in fingerlings and juveniles of Japanese Seabass exposed to 17β estradiol (Thilagam *et al*., 2010). GST activity elevated in gill, liver and muscle tissue of *O. mossambicus* exposed to butylphthalate (Ummamaheswari and Senthilnathan, 2014).

Glutathione, the major non – protein thiol of cells, is involved in the cellular defense against the toxic action of oxyradicals (Schulinga *et al*., 2002). In the present study, level of GSH in liver was increased in juvenile fish exposed to BPA. Nonylphenol exposure increased the content of GSH in Atlantic cod (Hasselberg *et al*., 2004a; b), largemouth bass, *Micropterus salmoides* (Hughes and Gallagher, 2004) and rainbow trout, *O. mykiss* (Uguz *et al*., 2003). Increase in glutathione content has been described as one of the protective mechanisms that fish adopt in the initial phase of exposure to aquatic pollutants (Stephenson *et al*., 2002). Antioxidant enzymes are commonly used useful biomarkers for oxidative stress (Living stone, 2003). However, the activities of these enzymes showed different toxicant responses than the higher, lower, and unchanged activities that have been reported in both laboratory and field studies in fish (Van der Oost *et al*., 2003).

The $Na^+ K^+$ ATPase and Ca^{2+}ATPases are involved in regulatory transportation of ions and are widely used as indices of osmoregulation in fish (Sunny and Oommen, 2001). In the present study, the activity of $Na^+ K^+$ ATPase was significantly reduced in gills resulting in hypo-osmoregulation. Darren *et al*. (2007) reported that exposure of higher doses of aroclor 1254 reduced the activity of $Na^+ K^+$ ATPase in the fish Atlantic salmon, *Salmmo salar*. According to Binitha (2009) the activity of $Na^+ K^+$ATPases activity was found to be altered by the exposure of sewage in *Anabas testudineus*. Also pollutants exert a biological effect on the ATPase system by partitioning the enzyme complex which cause an allosteric change that result in decreased $Na^+ K^+$ ATPase activity (Reddy *et al*., 1992). In the present study also this might be the reason for the decreased activity of $Na^+ K^+$ ATPase activity.

Ca^{2+} ATPase is also a significant enzyme in osmoregulation, functions to remove the Ca^{2+} ions from cytoplasm to protect the low Ca^{2+} levels (Watson and Beamish, 1981). Wong and Wong (2000) showed that the Ca^{2+} ATPase activity in the gill of *O. mossambicus* decreased in relation to cadmium exposure and altered Ca^{2+} levels in *Oreochromis niloticus* was due to the metal exposures (Atli, 2011) supported the decline of Ca^{2+} ATPase activity in the present study. The assessment of ATPase activity may therefore be used as an early warning signal of damage to the osmoregulatory and acid-based regulatory system in osmoregulatory organs such as gills and kidney (Atli and Canli, 2007).

CONCLUSIONS

The present study indicates that alteration in the activity of antioxidant and osmoregulatory enzymes following BPA exposure clearly reveals that juvenile fish experienced oxidative and osmotic stress. Antioxidant and osmoregulatory enzymes system can be used as a biomarker to provide information to clarify the mechanisms of BPA-induced toxicity during early fish stage and to evaluate the risk of BPA in aquatic environments.

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