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BISPHENOL A INDUCES OXIDATIVE STRESS (OS) AND HEPATOTOXICITY IN *HETEROPNEUSTES FOSSILIS*

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Abstract
Bisphenol A (BPA) is a renowned endocrine disruptor and upon the exposure to it influences both toxic and estrogenic effects. This present study explored how BPA exposure affected oxidative stress biomarkers and liver injury in a fresh water fish, *Heteropneustes fossilis*. It also explores a correlation between antioxidant enzyme activities and the pathological symptoms of liver. After acclimatization, the fresh water fish *H.fossilis* (irrespective of the sex and age) were divided into four groups. The fish, were treated with various sublethal concentrations ($1/10^{th}$, $1/20^{th}$) and ($1/30^{th}$) of Bisphenol A for 28 days and was studied for several hepatic antioxidative enzymes, lipid peroxidation and histopathological structures. Our results displayed that sublethal concentrations of BPA exposure to *H.fossilis* significantly increased oxidative stress by increasing lipid peroxidation and antioxidant enzymes like SOD and CAT levels in the hepatic tissue in dose dependent manner. However, GPx levels were significantly decreased in all experimental animals. The outcomes point out that elevation SOD and CAT activities and LPO level and the decrease in GPx activities in fish could be due to higher levels of BPA-induced reactive oxygen species (ROS).The results of current study advocate that BPA generate ROS cause harmful effects on the hepatic tissue and antioxidant defense system. We conclude that exposure to sublethal concentrations of BPA in *H.fossilis* resulted in a significant liver damage due to high free radical production and the disruption in defense mechanism in the hepatic tissue. This study will be beneficial for upcoming research in explaining the detailed effects of BPA in other fish species.

KEYWORDS: Bisphenol-A, Histopathology, Oxidative stress, *H.fossilis.*

INTRODUCTION

The liver is the most important spot of energy metabolism and biotransformation reactions. Hence it is one of the most thoroughly investigated organs among vertebrate species including fish (Reddy and Singh, 2011;Reddy 2012 a, b). It also plays major roles in storage, food digestion, and the synthesis of blood clotting factors, vitellogenin (Vtg), and many hormones (Hinton *et al.,* 2001). Because of these multiple roles and biotransformation reactions, it is the most common targets for both cytotoxicity and carcinogenicity (Hinton *et al.,* 2001; Gu and Manautou, 2012). Most of the earlier research used morphological, biochemical, and molecular approaches to assess the fundamental integrity, efficiency, and clinical symptoms of the liver. Nonetheless, the assessment of histopathological examination is one of the most common and routine methods used in fish toxicological studies as it provide a direct evidence pertinent to the extent of overall health (Reddy,2012a,b: Reddy, and Rawat 2013; Corbett *et al,* 2014**;** Reddy, and Kusum, 2013; Sangeeta Pal and Reddy, 2018).

BisphenolA (BPA) is a monomer and omnipresent synthetic chemical substance. It is mostly used in plastic and in the inner coatings of food and beverage containers (Hassan, et al, 2012;Sangeeta Pal and Reddy, 2018). Several laboratory tests (both in vivo and in vitro) reveal the toxic effect of BPA and point out its possibility in inducing endocrine disruption in many vertebrates including fish (Rubin, 2011;Thilagavathi *et al,* 2018; Pandey *et al,* 2018; Ben-Jonathan, 2019). Several studies confirmed detrimental effects of BPA on the liver, and kidney (Hassan, *et al,* 2012; Sangeeta Pal and Reddy, 2018;Esplugas, *et al,* 2018; Qiu, *et al,* 2019; Faheem, *et al,* 2019). Most of experiments on BPA have concentrated on their endocrine disrupting and antagonistic effects on the reproductive system. Nevertheless, only some degree of data concerning the effects of these chemicals on other tissues likes liver and kidney.

A number of environmental contaminants make their way into surface waters. Fish species often serve as valuable sentries for environmental toxins. BisphenolA (BPA) has come about a communal health hassle because of its

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uninterrupted introduction into the environment through food and drinking water. Aquatic organisms, together with fish, are frequently exposed to changing the concentrations of this toxic chemical at various phases of their life cycle.Fish and other vertebrate species possess both enzymatic and non-enzymatic antioxidant arrangements as protection against oxidative stress (Reddy, 2016; Reddy, 2017; Patil Anil and Reddy, 2017; Srivastava, and Reddy, 2017; Sun *et al,* 2018).Oxidative stress (OS) is fundamentally a difference between the productions of reactive oxygen species (ROS), including superoxide (O−2), (O2−), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO^-) and the ability of the body to neutralize or clear their destructive effects.Reactive oxygen species (ROS) damage several cellular constituents such as unsaturated lipids, proteins, and nucleic acids. A huge quantity of ROS is produced by scavenger proteins or by dysfunction of the mitochondrial respiratory chain pathway causes oxidative stress (Ooe *et al,* 2005). The oxidation of nucleic acid, lipid, and protein is alleged to effect in the origin of several ailments, including cancer, infertility, and neurodegenerative diseases (Langseth 1995; Ooe *et al,* 2005; Tiwari and Vanage, 2017; Tadros, and Vij, 2019).Therefore, in the present study, we decided to assess the effects of BPA on the oxidative stress of hepatic tissue of freshwater fish, *Heteropneustes fossilis.* We intended to calculate the sublethal effects of BPA on the parameters of oxidative status like (catalytic activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) and measuring levels of malondialdehyde (MDA) in liver homogenate. Lastly, microscopic examination of liver sections from all studied groups was also performed.

MATERIALS AND METHODS

Chemical reagents

BisphenolA (BPA): Bisphenol-A*2, 2-Bis* (*4-hydroxyphenyl*) *propane* (CAS Number 61788-97-4 with 90% pure was acquired from Novel Chem, Vadodara, and Gujarat, India.

Dosage: The experiment was performed on the sublethal doses based on our earlier publication (Sangeeta Pal and Reddy, 2018). As BPA is not soluble in water, it was dissolved in olive oil.

Ethics statement: Fishes were treated kindly and with respect to lessening of suffering for the care and use of laboratory animals. The Institutional Animal Ethics Committee (IAEC) permitted the experimental protocol.

Experimental Design: Healthy adult fish, *Heteropneustes fossilis* irrespective of sex and approximately of the same size and weight were acquired from local market (weight of 27.24 ± 1.35g and total length of 17.58 ± 0.74cm) and were acclimatized to the laboratory backgrounds for 15 days. The fishes were exposed to sublethal concentrations of BPA (0.714 mg/L (1/10th), 1.428 mg/L (1/20th and 2.142 mg/L (1/30th) i.e. (Group II, III and IV) on every alternate day basis for 28 days using ten fishes in each aquarium. On 29th day, four fishes from each aquarium were randomly picked and dissected for hepatic tissue. Immediately it was cleaned in 0.75% saline solution, blotted with tissue paper, kept in Teflon tubes, and finally stored at −200C for later analysis.

***Histopathology*:**Fragments of hepatic tissue were fixed in buffered 10% formalin. Subsequently tissues were processed in graded ethanol series, cleared in xylene and finally embedded in paraffin wax (melting point 60oC). The embedded block were sectioned at 6µ on a rotary microtome, mounted on glass slides, dried and stained with haematoxylin and eosin (H&E). Sections were examined under a high-resolution light microscope (NIKON ECLIPSE E 400, USA) and photographed by using digital camera attached to the microscope.

Tissue Preparation: Hepatic tissue was taken out from the freezer and soaked with tissue paper. The tissues were weighed maximum up to 0.1 g and homogenized in 2 mL of 0.5 M (pH 7.4) Tris-HCl buffer by using REMI Lab Homogenizer (RQ-127A/D) and finally centrifuged at 8,000 rpm for 25 minutes at 00C. The supernatant was collected in Teflon tubes and immediately used for examining total protein, activities of antioxidant enzymes, and markers of oxidative stress. Each sample was tested in triplicate.

Measurement of Oxidative stress biomarkers: MDA levels were estimated by the thiobarbituric acid reaction as described by Ohkawa *et al.* (1979).

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Assessment of antioxidant enzymes: Catalase (CAT) activity was calculated based on the spectrophotometric method described by (Aebi, 1984). Superoxide dismutase (SOD) was measured by the procedure described by Das *et al.*, (2000). Glutathione peroxidase (GPx) activity was measured based on the procedure given by Paglia and Valentine, (1967).

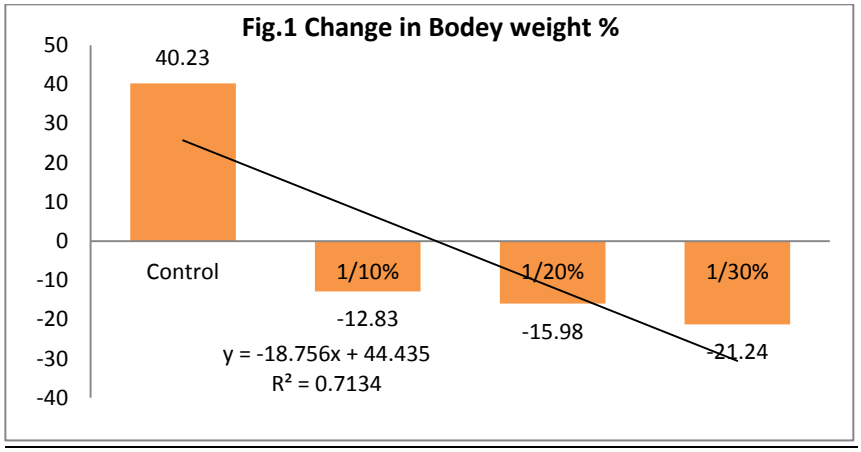
***Statistical analysis*:** The entire results were presented as mean ± standard error (SE). The outcomes of oxidative stress biomarkers in all experimental groups were tested and compared with the control group using student 'T' test (Fisher, (1955).

RESULTS

No fish mortality was observed during the experimental period. Water parameters of aquarium were examined and regularly kept constant. The initial and final body weights of male fish of all experimental groups are presented in Table 1. There was a significant difference in the body weight in BPA exposed fish compared to control. Social and swimming behavioral alterations were not recorded and fish were survived all through the experimentation. BPA caused significant effect on weight loss, highest (-21.4%) was observed on 28 days in Group IV treated with 1/30% of BPA compared to control fish in which a significant weight gain (40.23%) was noticed. An increase of 40.23% in body weight in control fish was noticed in 28 days of experiment. However, a significant decrease in the body weight percentage was noticed in all experimental groups treated with various sub lethal doses of BPA. It was -12.83,-15.98 and -21.24 in Group II, Group III, and Group IV respectively.

T**able.1.Effects of BPA on body weight (gm) of *H.fossilis***

	Control	BPA (1/10%)	BPA (1/20%)	BPA (1/30%)
Initial body weight (gm)	27.24 ± 0.87	26.89 ± 0.68	27.9± 1.24	28.33 ± 1.2
Final body weight (gm)	38.2 ± 1.1	23.44 ± 0.87	23.4 ± 1.14	22.31 ± 0.72
Change%	40.23	-12.83	-15.98	-21.24



Histopathological changes in the hepatic tissue after BPA exposure: Light microscopic study of control fish liver revealed normal hepatic architecture with the central vein of normal hepatocytes holding central rounded nuclei. Liver sections after exposure to various sublethal concentrations of BPA displayed several structural anomalies in a dose-dependent manner. Fish exposed to 1/10% of BPA (Group II) showed vacuolation, necrosis, degeneration, ruptured central vein and few broken hepatocytes. Aggregation of melanomacrophages (MMC) and higher amount of glycogen granules were also seen.

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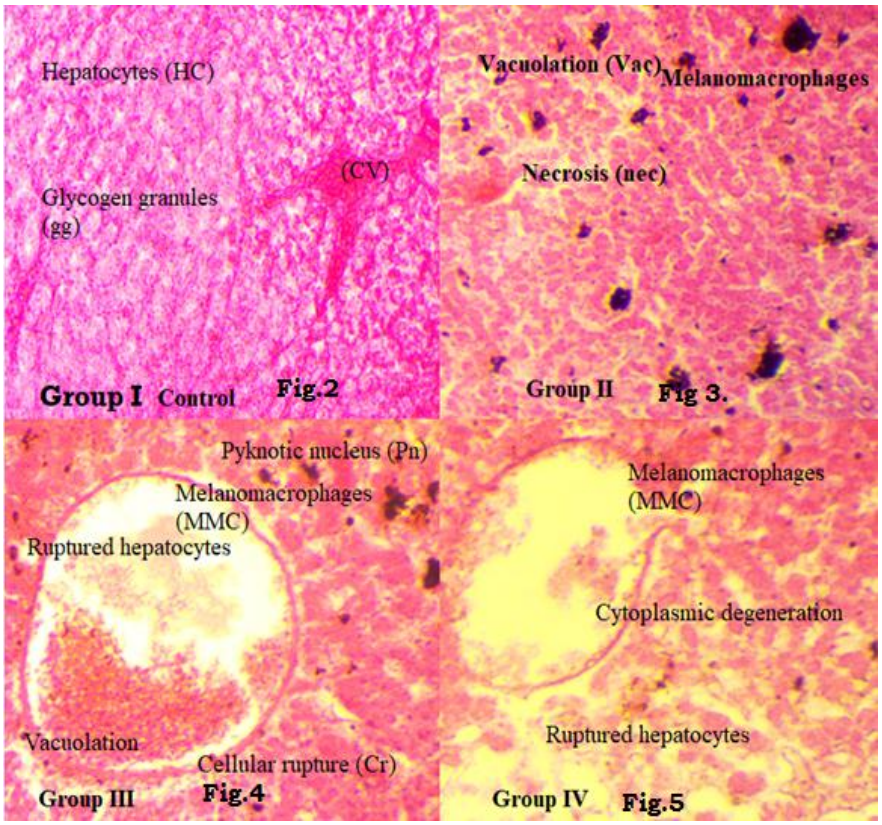

Fig.2-5. Effects of sub lethal concentration of BPA on histopathology of liver of *Heteropneustes fossilis*. Fig.2. Group I Control. (x 1000) Fig. 3.Histology of liver of *Heteropneustes fossilis* treated with 1/10% of BPA showing vacuolation, acute cellular swelling, and hypertrophy.Fig.4.Histology of liver of *Heteropneustes fossilis* treated with 1/20% of BPA showing vacuolation, ruptured hepatocytes and aggregation of melanomacrophages (MMC).Fig.5.Histology of liver of *Heteropneustes fossilis* treated with 1/30% of BPA showing secondary infections in between hepatocytes. (All sections are H&E Stained x400).

Liver of fish exposed to 1/20% of BPA (Group III) displayed nuclear and cytoplasmic degeneration, cellular rupture, and congestion of blood vessels. Diffusion of massive number of macrophage was also seen. In the fish of group, IV exposed to 1/30% of BPA shown extreme damage with pyknotic nucleus and cellular rupture. Hepatocytes wereatrophied as compared to other groups. The histopathological signs resulted in the sub-lethal treatment of BPA evidently implies that the BPA is capable to modify the liver histology in fish.

Antioxidant enzymes activities: The experimental results of lipid peroxidation and antioxidant enzymes are presented in Table 2 and Fig 6-9. The SOD activities of both SOD and CAT were significantly increased while GPx activity was reduced significantly in BPA treated fishes in dose dependent manner. The thiobarbituric acid reaction (MDA) values showed a significant ($p<0.05$) increase in all BPA treated fishes. The increase was gradual along with the increase of BPA concentration. Correlation analysis among lipid peroxidation and antioxidant enzyme activity in the liver of different experimental groups of *H.fossilis* are shown in Figures 6-10. The SOD activity showed $R^2 = 0.997$, CAT showed $R^2 = 0.917$, and GPx showed $R^2 = 0.999$ compared to the control group. The correlation values of TBARS level exhibited $R^2 = 0.928$.

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Table 2. Antioxidant enzymes activity in liver of fish exposed to sub-lethal concentration of BPA in H.fossilis

Parameters	Control	BPA (1/10%)	BPA (1/20%)	BPA (1/30%)
SOD	09.86 ± 0.78	10.99 ± 0.88	12.41 ± 0.96	13.75 ± 1.3
CAT	17.11 ± 0.89	18.14 ± 1.38	22.84 ± 1.84	29.35 ± 0.87
GPX	27.66 ± 1.81	23.68 ± 0.81	19.97 ± 0.92	16.26 ± 2.23
MDA (LPO)	41.88 ± 2.79	49.68 ± 3.24	51.67 ± 3.24	63.62 ± 3.02

SOD, CAT (units/mg^{1} protein), MDA (LPO) (nmol/TBARS formed mg protein^{1}min^{1}), GPx (n mol/NADPH oxidized mg1 protein min^{1}).TAC (µM/mg protein).Each value is mean ± SE (n ¼ 6), N insignificant difference ($p > 0.05$) compared to control.

Fig.6.SOD activity in liver of fish exposed to sub-lethal concentration of BPA in *H. fossilis*

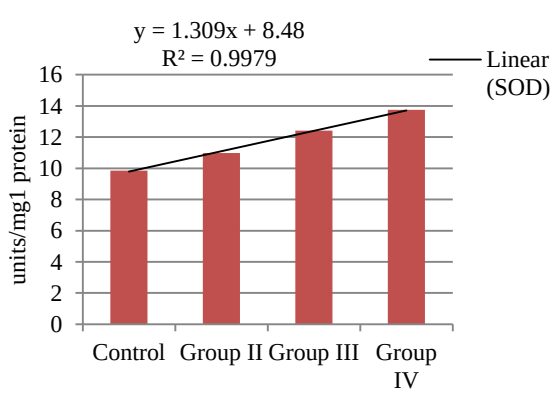


Fig.7. CAT activity in liver of fish exposed to sub-lethal concentration of BPA in *H. fossilis*

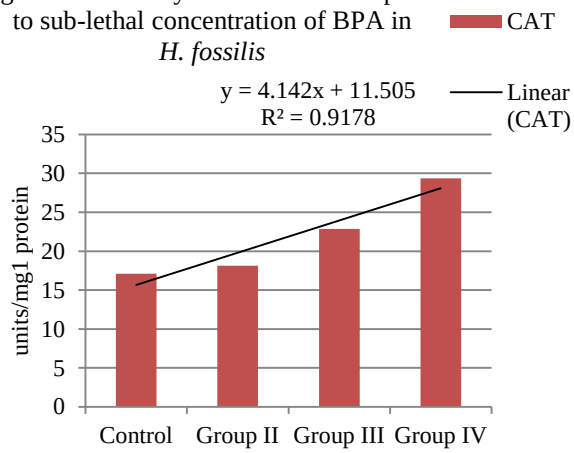


Fig. 8. GPx activity in liver of fish exposed to sub-lethal concentration of BPA in *H. fossilis*

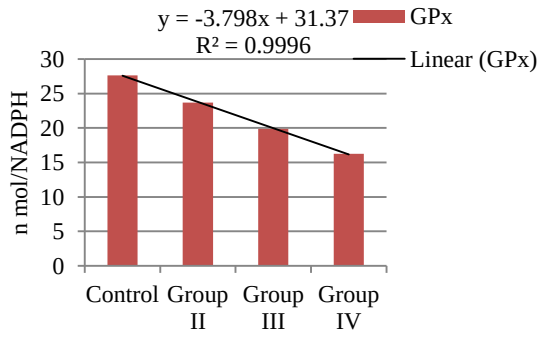
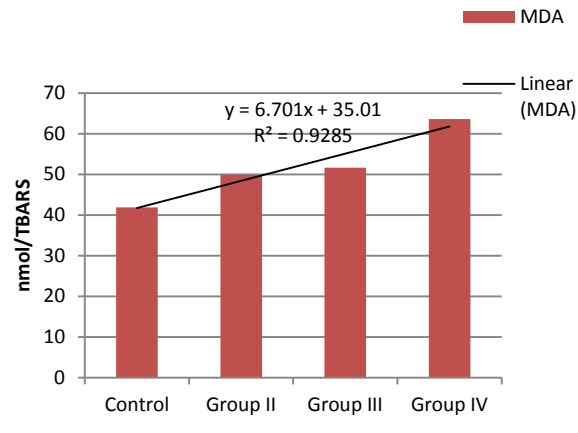


Fig. 9. MDA values in liver of fish exposed to sub-lethal concentration of BPA in *H. fossilis*



DISCUSSION

Pollution induced toxicity in fish caused worries in metabolic activities, which consequently decrease the growth rate and reproductive processes of fish species (Reddy, 2012 a, b). A number of industrial chemicals are released into

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aquatic bodies which distress the various developmental processes in all aquatic animals particularly fish. Toxicological experiments in fish play an essential role in ecological risk assessment and threat classification.Liver is the main target tissue as it is the primary site for detoxification and biotransformation reactions. The induction of oxidative stress conditions in various tissues of fish is considered as a promising defense mechanism (Lushchak, 2014).Assessment of both histopathological and antioxidant status are useful to define the effects of various industrial contaminants including BPA (Reddy, 2016; Reddy, 2017; Patil Anil and Reddy, 2017; Srivastava and Reddy, 2017; Sun*et al,* 2018). The current experimentation revealed that BPA exposure induced oxidative stress and altered the histoarchitecture of liver in freshwater fish, *H.fossilis.*

Antioxidant Enzymes: The enzymatic and non-enzymatic antioxidant defense activities perform a critical role in continuing redox homeostasis. The ROS produced during biotransformation of BPA occasionally may go beyond the capability of the intracellular antioxidant system.SOD is known to provide the first line enzymatic defense against the superoxide anion. The increase SOD activity implies a higher production of intracellular H_2O_2.CAT is accountable for the breakdown of H_2O_2 to water and oxygen to protect the cell from the harmful action of H_2O_2 and the hydroxyl radical. In the present study, increased activities of hepatic SOD, and CAT were noticed with increased concentration of BPA. The elevated levels of superoxide dismutase (SOD) in hepatic tissue possibly explained as a beneficial mechanism against BPA intoxication as in *Cyprinus carpio* which exposed to heavy metals (Vinodini, R. and Narayanan, M., 2009). Superoxide dismutase **(**SOD) is known to interfere in the first biotransformation of free radicles into either normal molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) while catalase (CAT) transforms it into H_2O and O_2. Yadav *et al,* (2015) found similar result in *H.fossilis*, exposed to sodium fluoride.Reddy, P (2016) also found higher level of SOD, and CAT and decreased GPx activities in pollution affected freshwater catfish, *Mystus tengara*. The excess production of hydrogen peroxide may increase the SOD activity, while the superoxide anion may be accountable for increased CAT activity (Modesto, and Martinez, 2010).CAT and GPx enzymes are considered as primary scavengers of H_2O_2.The reduction in activities of GPx in the present study might prompt the liver damage due to increased free radicals. Glutathione peroxidase (GPx) catalyzes the glutathione dependent reduction of hydroperoxides and is supposed to protect tissues against oxidative damage. In the present study, the level of hepatic glutathione peroxidase (GPx) was significantly decreased in all experimental animals. In the present study, deficiency of cysteine and reduced activities of GPx and GST might be responsible for reduced hepatic GPx (López-López *et al* 2011).

Nevertheless, due to lack of adequate CAT or GPx activity, more H_2O_2could be converted to toxic hydroxyl radicals, which caused oxidative stress. In our results, higher SOD and CAT activities were supplemented by the reduced GPx activities due to biotransformation process of BPA in the liver. The varied responses of the hepatic antioxidant enzymes to oxidative stress in the present study possibly due to tissue-specific and species specific antioxidant potentials (Oruc, *et al*,2004; Narra *et al,* 2017, Balaschand Tort, 2019).The changes (increase or decrease) in the activity of antioxidant enzymes upon the exposure of chemicals depend on the type, concentration, intensity and the experimental period, and the exposed organism (López-López *al.*,2011).

Lipid peroxidation: The present study confirmed that BPA caused hepatoxicity by increasing the activities of SOD CAT and MDA levels. The higher values of MDA in the liver of all BPA treated fish confirm enhanced oxidative stress in fish. The reactive oxygen species (ROS) formed during oxidative stress reacts with unsaturated fatty acids present in cell membranes and cause lipid peroxidation. Consequently, an increased lipid peroxidation can be used as biomarker of oxidative stress (Reddy, 2017; Patil Anil and Reddy, 2017; Srivastava, B. and Reddy, 2017). Though a majority of BPA is transformed into less toxic BPAG and BPAS in the liver, the remaining free BPA induces the production ROS through the enzymatic and non-enzymatic formation of phenoxyl radicals (Hassan, *et al*, 2012**;** Gassman, 2017**;** Faheem, and Lone, 2017**;** Qiu, *et al*, 2019). BPA induced increase in LPO and disruption of the cell membranes leading to inhibit the activity of membrane-bound enzymes has already been reported (Gassman, 2017).In an experiment, Chitra *et al* (2012) found an increased level of lipid peroxidation in time-dependent manner in the liver of BPA treated Fish, *Oreochromis mossambicus.* Faheem and Lone (2017) reported similar result in freshwater fish, *Ctenopharyngodon idella* treated with BPA. In another experiment**,** the common carp (*Cyprinus carpio*) upon exposure to 1000 µg/L BPA displayed higher levels of lipid peroxidation **(**Qiu *et al,* 2016). Recently, Hamed, and El-Sayed

(2019) noticed an enhancement of hepatic malondialdehyde (MDA) levels in Nile tilapia (*Oreochromis niloticus*) exposed to a sublethal concentration of BPA analogue pendimethalin (PM).

The increased LPO possibly due to decreased antioxidant defenses which were not completely able to scavenge them, thus leading to lipid peroxidation. GPx enzymes are responsible for detoxification of H_2O_2 and lipid peroxides at the membrane level into less reactive species using cellular GSH as substrate thus preventing the progressive formation of free radicals and provide cell important protection against oxidative stress and LPO. In the present study, the decreased GPx activity might be due to negative feedback from surplus of substrate or damage by oxidative stress, which consecutively might be causing a probable failure in antioxidant defense. Our results are in agreement with Minghong, *et al* (2011) in medaka (*Oryzias latipes*) upon chronic bisphenol A exposure and Kirici *et al*, (2017) that exposed freshwater fish *Capoeta umbla* to copper sulphate. Similar results were also obtained by Chitra and Sajitha (2014) in *Oreochromis mossambicus,* Wu *et al.* (2011) in zebrafish embryos, Rhee, and Rhee (2016) in the embryos of marine medaka and by Hamed, and El-Sayed (2019) in Nile tilapia, *Oreochromis niloticus* (L.).

Relationship between Lipid Peroxidation and Antioxidant Enzymes with Liver Pathology: The cells have various defense mechanisms against oxidative stress, including enzymatic scavengers (such as SOD, CAT and GPX) that protect the system from deleterious effects of ROS. Our data revealed that BPA caused marked oxidative impact by increasing the activities of antioxidant enzyme compared to their activities in the control group. BPA induced a significant increase in oxidative stress, which is accompanied by noticeable alterations in antioxidant enzymes and lipid peroxidation. The correlation between increase in SOD and CAT and a decrease GPx activity and the pathological symptoms of liver and lipid peroxidation was relatively evident. For catalase, and GPx activity, Significant opposite correlations were found between changes in activity of GPx and liver injury while it is positively correlated with the values of lipid peroxidation. Thus, the lowermost levels of antioxidant GPx enzymes were found in the experimental fishes with the most severe liver injury. In the present study, the production of higher amount of free radicals during the metabolism of BPA might exceed the capability of the antioxidant defense mechanisms and caused severe damage to the hepatic tissue. The results this study confirm that increased pathological conditions and increase in lipid peroxidation in BPA treated fish were accompanied by increased SOD and CAT but reduced activities of GPx. In consequence, it looks that reduced activity of GPx enzymes was associated with liver injury and that the amount of decrease in activity was related to the severity of liver injury and the level of lipid peroxidation.Altogether, it is admitted that BPA accelerate the production of reactive oxygen species (ROS) by inducing hepatic damage and mitochondrial dysfunction (Moon *et al.,* 2012; Asahi *et al.,* 2010). These data are in agreement with the previous results of Chitra *et al.* (2003) who illustrated that treatment of rats with BPA increases levels of ROS production.

Histopathological examination: The histopathological examination of hepatic tissue displayed varying extents of alterations including vacuolation, ruptured hepatocytes and aggregation of melanomacrophages (MMC)nuclear pyknosis and necrosis leading to total collapse and disintegration of hepatocytes. In some areas of the hepatic tissue, degeneration of hepatocytes, in close proximity to blood sinuses, were noticed. In consistent with the mentioned data, we observed histopathological changes in the liver indicating variable damage due to early life exposure to BPA (Sangita pal and Reddy, 2018). The results of our investigation are in agreement with other studies (Reddy, 2016; Reddy, 2017; Patil Anil and Reddy, 2017: Srivastava and Reddy, 2017; Sun*et al,* 2018).

CONCLUSIONS

In conclusion, the current investigation offers an insight into the mode of action of BPA-induced hepatotoxicity in *H.fossilis*. This study is valuable and permits further studies to clarify the mechanisms involved in the increased activity of SOD, CAT and lipid peroxidation. The microscopic examination of liver sections provided essential evidences for liver injury. Thus, BPA may induce production of ROS and oxidative stress to disrupt the structural architecture of liver. This study will be beneficial for upcoming research in explaining the detailed effects of BPA in other fish species.

Conflict of Interests

The authors declare that there is no conflict of interests.

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