Effect Of Chronic Diethylhexylphthalate Exposure On Antioxidant Enzyme Activities In Zebrafish (*Danio rerio*)

[1*]S. Senthilnathan [2]P. Hemalatha, [3] M. Sellappan, [4] M. Rajasekara Pandian
[1]Guest Faculty, [2]Research Scholar, [3]Assistant Professor, [4]Head and Assistant Professor
[1]Departmennt of Zoology, [2]Reasearch scholar in Zoology, [3]Department of Zoology, [4]Department of Zoology
[1]Arignar Anna Government Arts College, Namakkal, [2]Manonmaniam Sundaranar University, Tirunelveli,
[3]Arignar Anna Government Arts College, Namakkal, [4]Arignar Anna Government Arts college, Namakkal.

*Corresponding Author
Dr. S. Senthilnathan
Department of Zoology
Arignar Anna Government Arts College,
Namakkal - 637002

Abstract

Diethylhexylphthalate (DEHP) in the waterways could cause metabolic changes in the fishes. Only few studies have documented the chronic toxicity of DEHP in rats, mice and humans. The present study focused on the changes elicited by DEHP on the enzyme activity Superoxide dismutase and Catalase of the various organs (gill, liver, muscle, testis and ovary) of male and female Zebrafish. The observation registered in this study reflects that antioxidant enzymes activities were significantly enhanced in all the tissues (gill, liver, muscle, testis and ovary) when compared to control (untreated DEHP). This could be due to the detoxification mechanism exhibited by the Zebrafish on exposure of DEHP.

KEYWORDS:
Diethylhexylphthalate, Zebrafish, Superoxide dismutase, Catalase, Antioxidant defense

I. INTRODUCTION

In India, DEHP is used as a perfume binder in the incense stick industry*;* hence workers in this industry are usually occupationally exposed to DEHP, as it involves manual rolling of a paste made by mixing DEHP with perfumed saw dust using bare hands (Sonde *et al.*, 2000). Release into the environment occurs primarily as a result of production and manufacturing of DEHP itself and during the use and disposal of products containing DEHP (Giam and Wong, 1987*;* Joblings *et al.*, 1995). Releases are also expected to be primarily to water or to soil as a result of leaching from land-fills (Silva *et al.*, 2004) and human exposure to phthalates is primarily due to ingestion of food contaminated from environmental sources (Kavlock *et al.*, 2002a, 2002b, 2002c).

Over the years, it is becoming increasingly recognized that humans are not exposed to single chemicals. Rather, humans are exposed sub-sequentially, by various routes of exposure, to a large number of chemicals from a variety of sources over varying periods of time. The magnitude of the problem is immense. In our daily living, exposures to mixture of chemicals are ubiquitous in the air, food and water. Currently, there is scientific and public concern about potential human health risks from exposure to phthalates and diesters of phthalic acid (Contzen Pereira and Vaman Rao, 2006). These concerns stem from studies viewing that most of the U.S. general population is expose to phthalates (Silva *et al.*, 2004). Knowledge on the physiological action of the toxicants helps to predict important sub-lethal effects and analyses of biochemistry, hematology and histopathology may be used to determine the mode of action of the toxicant. Fish health may thus reflect, and be a reliable indication of the health status of a specific aquatic ecosystem (Burkepile *et al.*, 2000).

The antioxidant defense (AD) system of organisms provides a means of dealing with oxidative stress and includes several enzymes and vitamins (Filho, 1996; Rudeva, 1997; Kelly *et al.*, 1998; Marcon and Filho, 1999). A primary role of the AD system is protecting cellular components from ROS damage (Kelly *et al.*, 1998). The antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase, act to remove oxygen radicals produced within the cell (Filho, 1996; Filho *et al.*, 1993; Michiels *et al.*, 1994; Kelly *et al.*, 1998). Superoxide dismutase occurs in two forms: (1) a cytosolic form that has copper and zinc in its active site (CuZnSOD) and (2) a mitochondrial form that has manganese in its active site (MnSOD) (McCord and Fridovich, 1969). Both forms of SOD protect the cell from potential ROS damage by converting superoxide anions to H_2O_2 and H_2O (McCord and Fridovich, 1969).

Antioxidant system is located within different cellular compartments. These enzymes are found virtually in all tissue of vertebrates, but show in general high activity in the liver. A major organ of xenobiotics uptake and enzymatic transformation of ROS (Lamaire *et al.*, 1992) and eventually leak to blood. Some of these enzymes, like aminotransferase and phosphatase group, can constitute good molecular bioindicators for oxidative stress and can also indicate the magnitude of response in populations chronically exposed to contaminate such as metals and other xenobiotics (Livingstone *et al.*, 1994). Keeping this in view, the aim of this study was to assay the enzyme activity of SOD, CAT of the gill, liver, muscle, testis, and ovary of the both male and female of Zebrafish.

II. MATERIALS AND METHODS

Fish maintenance in aquarium

Fifteen days old fingerlings of Zebra fish were collected from Krishna Ornamental fish farm, Tiruchirappalli, Tamil Nadu, India. These fingerlings were reared in aquarium tanks for a period of 180 days at standard environmental conditions (28 °C and 14 hour photoperiod). At the end of the 60th day, ten Zebrafish from each of the treatments and control were collected. The Gill, liver, muscle, testis and ovary of zebrafish were dissected and subjected to enzyme assay, SOD and CAT. About 0.4 mg of tissue sample was homogenized in 800µl Tris-Hcl buffer (100mM, pH-7.4) using a glass homogenizer. Then the homogenate was centrifuged at 10000 rpm for 15 min at 4 ^{o}C, and the supernatant was collected for antioxidant enzymes. CAT activity was determined according to the method of Sinhas (1972) and SOD activity was assayed based on a modified method of Marklund and Marklund (1974).

Statistical Analysis

Oneway analysis of variance (ANOVA) was performed using SPSS version 16.0 to assess the intergroup differences followed by Duncan Multiple Range Test (DMRT).

III. RESULTS AND DISCUSSION

Effect of DEHP exposure to male Zebrafish for SOD assay

The data displayed in (Table 1) reveal that DEHP has induced significant variation in mean SOD concentration of gill, liver, muscle and testis of male Zebrafish. DEHP at 2.5ppm, 5ppm and 10ppm registered mean SOD concentration of 0.725±0.017 µmol/mg, 0.893±0.127 µmol/mg and 0.930±0.109 µmol/mg, respectively. On the other hand, control fishes registered mean SOD concentration of 0.267±0.145 µmol/mg. ($F=456.88$, $P<0.001$). Liver mean SOD concentration significantly increased ($F=304.735$, $P<0.001$) on exposure of Zebrafish to DEHP (2.5ppm: 0.326±0.011 µmol/mg; 5ppm: 0.551±0.020 µmol/mg, 10ppm: 0.787±0.014 µmol/mg) when compared to the control (0.207±0.010 µmol/mg) Similarly muscle mean SOD concentration significantly ($F=1.196$, $P<0.001$) elevated an exposure of Zebrafish DEHP (2.5ppm: 4.288±0.014 µmol/mg; 5ppm: 5.249±0.013 µmol/mg; 10ppm: 7.117±0.011 µmol/mg) when compared to the control 3.211±0.020 µmol/mg. Testis mean SOD concentration significantly ($F=6.426$, $P<0.001$) elevated on exposure of Zebrafish to DEHP (2.5ppm: 4.261 ± 0.074 µmol/mg; 5ppm : 6.462 ± 0.011 µmol/mg; 10ppm: 9.244±0.008 µmol/mg) when compared to the control (2.158±0.007 µmol/mg).

Table.1 Variation in the mean SOD concentration in various tissues of male Zebrafish dexposed to DEHP

DEHP Treatment	Gill (µmol/mg protein)	6Liver (µmol/mg protein)	Muscle (µmol/mg protein)	Testis (µmol/mg protein)
Control	0.267±0.145 [d]	0.207±0.010 [d]	3.211±0.020 [d]	2.158±0.007 [d]
2.5ppm	0.725±0.017 [c]	0.326±0.011 [c]	4.288±0.014 [c]	4.261±0.074 [c]
5ppm	0.893±0.127 [b]	0.551±0.020 [b]	5.249±0.013 [b]	6.462±0.011 [b]
10ppm	0.930±0.109[a]	0.787±0.014 [a]	7.177±0.011 [a]	9.244±0.008 [a]
F	456.88***	304.735***	1.196***	6.426***

***Significant at P < 0.001. In a column, figures having dissimilar letters differ significantly according to Duncan Multiple Range Test (DMRT). Values are expressed as mean ± standard error.

Effect of DEHP exposure to female Zebrafish for SOD assay

Similar pattern of change in mean SOD concentration was evinced in DEHP exposed female Zebrafish (Table 2). Mean SOD concentration of gill significantly (F=57.560, P<0.001) elevated in DEHP treated fishes (2.5ppm: 0.390±0.018 µmol/mg*;* 5ppm: 0.650±0.021 µmol/mg*;* 10ppm: 0.931±0.011 µmol/mg) when compared to the control (0.251±0.072 µmol/mg) DEHP untreated female. Zebrafish recorded liver mean SOD concentration of 0.277±0.012 µmol/mg which was found to the significantly higher (F=1.782, P<0.001) (2.5ppm: 1.878±0.014 µmol/mg*;* 5ppm: 3.730±0.017 µmol/mg*;* 10ppm 4.740±0.014 µmol/mg) than the DEHP unexposed ones. Muscle SOD concentration significantly increased (F=2.276, P<0.001) (2.5ppm: 23.613±0.012 µmol/mg*;* 5ppm: 24.235±0.017 µmol/mg, 10ppm: 27.389±0.016 µmol/mg) in the DEHP exposed Zebrafish when compared with untreated ones (21.811±0.015 µmol/mg). Similar pattern of variation in mean SOD concentration was evinced in the ovary of Zebrafish exposed to DEHP (2.5ppm: 7.222±0.008 µmol/mg*;* 5ppm: 9.155±0.007 µmol/mg*;* 10ppm: 11.187±0.012 µmol/mg) when compared to the control (6.194±0.021 µmol/mg) (F=2.634, P<0.001).

Table 2.Variation in the mean SOD concentration in various tissues of female Zebrafish exposed to DEHP

DEHP Treatment	Gill (µmol/mg protein)	Liver (µmol/mg protein)	Muscle (µmol/mg protein)	Ovary (µmol/mg protein)
CONTROL	0.251±0.072[d]	0.277±0.012[d]	21.811±0.015[d]	6.194±0.021[d]
2.5ppm	0.390±0.018[c]	1.878±0.014[c]	23.613±0.012[c]	7.222±0.008[c]
5ppm	0.650±0.021[b]	3.730±0.017[b]	24.235±0.017[b]	9.155±0.007[b]
10ppm	0.931±0.011[a]	4.747±0.014[a]	27.389±0.016[a]	11.187±0.012[a]
F	57.560***	1.782***	2.276***	2.634***

***Significant at P < 0.001. In a column, figures having dissimilar letters differ significantly according to Duncan New Multiple Range Test (DMRT). Values are expressed as mean ± standard error.

Effect of DEHP exposure to male Zebrafish for CAT assay

Mean CAT concentration in gill of male Zebrafish enhanced significantly (F=1.516, P<0.001) an exposure to DEHP (2.5ppm: 7.372±0.009 µmol/mg*;* 5ppm: 8.783±0.019 µmol/mg*;* 10ppm: 11.125 ± 0.019 µmol/mg). On contrary, control fishes registered gill mean CAT concentration of 5.789±0.022 µmol/mg (table 3). Similarly, liver mean CAT concentration also significantly elevated (F=263.93, P<0.001) in male Zebrafish exposed to DEHP (2.5ppm: 24.253±0.008 µmol/mg*;* 5ppm27.189±0.009 µmol/mg*;* 10ppm:

29.157±0.012 µmol/mg) when compared to the control (24.738±0.279 µmol/mg). Muscle mean CAT concentration also exhibited similar pattern of increase in DEHP exposed fishes (2.5ppm: 33.223±0.066 µmol/mg; 5ppm: 35.494±0.208 µmol/mg; 10ppm: 38.223±0.075 µmol/mg) when compared to the control (28.375±0.014 µmol/mg) (F=1.297, P<0.001). Testis mean CAT concentration of DEHP treated Zebrafish also significantly enhanced (2.5ppm: 18.444 µmol/mg; 5ppm: 23.333±0.014 µmol/mg; 10ppm: 24.241±0.013 µmol/mg) when compared to the untreated ones (12.254±0.082) (F=1.667, P<0.001).

Table 3. Variation in the mean CAT concentration in various tissues of male Zebrafish exposed to DEHP

DEHP Treatment	Gill (µmol/mg protein)	Liver (µmol/mg protein)	Muscle (µmol/mg protein)	Testis (µmol/mg protein)
Control	5.789±0.022^{d}	24.738±0.279^{d}	28.375±0.014^{d}	12.254±0.082^{d}
2.5ppm	7.372±0.009^{c}	24.253±0.008^{c}	33.223±0.066^{c}	18.444±0.007^{c}
5ppm	8.783±0.019^{b}	27.189±0.009^{b}	35.494±0.208^{b}	23.333±0.014^{b}
10ppm	11.125±0.019^{a}	29.157±0.012^{a}	38.223±0.075^{a}	24.241±0.013^{a}
F	1.516***	263.93***	1.297***	1.667***

***Significant at P < 0.001. In a column, figures having dissimilar letters differ significantly according to Duncan Multiple Range Test (DMRT). Values are expressed as mean ± standard error.

Effect of DEHP exposure to female Zebrafish for SOD assay

Mean CAT concentration of gill female Zebrafish also exhibited a similar treated on exposure to DEHP (Table 4). DEHP at 2.5ppm, 5ppm and 10ppm recorded mean CAT concentration of 0.847±0.087 µmol/mg, 2.219±0.015 µmol/mg and 3.302±0.024 µmol/mg, respectively. On the other hand, control female Zebrafishses recorded mean CAT concentration of 0.804±0.014 µmol/mg. Thus significant variation in gill mean CAT concentration was evident between DEHP treated and untreated ones (F=0.657, P<0.001). Liver mean CAT concentration (F=2.301, P<0.001) also elicited significantly increase in DEHP treated fishes (2.5ppm: 35.756±0.009 µmol/mg; 5ppm: 36.506±0.021 µmol/mg; 10ppm: 39.236±0.009 µmol/mg) when compared to the untreated ones (33.606±0.016 µmol/mg).Muscle mean CAT concentration also significantly (F=4.335, P<0.001) enhanced in DEHP treated fishes (2.5ppm: 66.695±0.015 µmol/mg; 5ppm: 67.748±0.016 µmol/mg; 10ppm: 71.161±0.007 µmol/mg) when compared to the control (65.574±0.010 µmol/mg). Ovarian mean CAT concentration also elicited similar response in DEHP treated female fishes (2.5ppm: 0.543±0.017 µmol/mg; 5ppm: 0.676±0.007 µmol/mg; 10ppm: 0.884±0.015 µmol/mg) when compared to the untreated ones (0.311±0.015 µmol/mg) (F=283.161, P<0.001).

Table 4. Variation in the mean CAT concentration in various tissues of female Zebrafish exposed to DEHP

DEHP Treatment	Gill (µmol/mg protein)	Liver (µmol/mg protein)	Muscle (µmol/mg protein)	Ovary (µmol/mg protein)
Control	0.804±0.014^{d}	33.606±0.016^{d}	64.574±0.010^{d}	0.311±0.015^{d}
2.5ppm	0.847±0.087^{c}	35.756±0.009^{c}	66.695±0.015^{c}	0.543±0.017^{c}
5ppm	2.219±0.015^{b}	36.506±0.021^{b}	67.748±0.016^{b}	0.676±0.007^{b}
10ppm	3.302±0.024^{a}	39.236±0.009^{a}	71.161±0.007^{a}	0.884±0.015^{a}
F	0.657***	2.301***	4.335***	283.161***
P	0.001	0.001	0.001	0.001

***Significant at P < 0.001. In a column, figures having dissimilar letters differ significantly according to Duncan Multiple Range Test (DMRT). Values are expressed as mean ± standard error

SOD and CAT play important roles in protecting the cell against the potentially toxic effects of experimental pollutants (Kuthan *et al.*, 1986). SOD, catalyzes the dismutation of the superoxide ion (o_2^-) to hydrogen peroxide and oxygen molecule during energy processes. The reaction diminishes the caustic oxidative processes in cells. The level of antioxidant enzymes have been extensively used as an early warming indicator of lake pollution (Lin *et al.*, 1998). The present result is in good accord with the findings of Neeraj kumar *et al.* (2011) who have confirmed that activities of anti-oxidative enzymes was significantly (P<0.01) influenced by endosulfan in a dose dependent manner in Tilapia *Oreochromis mossambicus*. They have noticed significant (P<0.01) increase in the activity of CAT, SOD and GST in gill and liver of Tilapia**.** Superoxide dismutase is an antioxidant enzyme involved in the elimination of ROS (reactive oxygen species). The antioxidant enzymes that make up the antioxidant defense system are expected to be intrinsically linked and dependent upon the activity of one another. Therefore, one could expect to see correlative changes in the activity of SOD and CAT (Filho *et al.*, 1993). Thus such pattern was observed in their study and correlative activity among catalase and superoxide dismutase has been evinced. The present findings in agreement with Ezemonye and Erineku (2011) who have reported significant (P<0.05) dose dependent increase in the specific activity of SOD and CAT relative to controls in the liver of *Hoplobatrachus occipitalis* exposed cadmium (0.25, 0.5, 100 and 2.00 mg L^{-1}) for 28 days. SOD and CAT are important antioxidants and play a crucial role in counteracting oxidative stress. The increase in SOD and CAT observed in the study of Gupta *et al.* (1991) who have reported that cells increase the production of antioxidant enzymes such as SOD, CAT and glutathione peroxidase in order to circumvent oxidative stress.

Mustafa Kaplan *et al.* (2009) found significant decrease in kidney SOD and CAT activities in cadmium exposed rat when compared to unexposed ones. The same authors have also reported increased SOD and CAT activities of liver in melatonin ($CdCl_2$: 200µg/ml+0.02% melatonin) for 3 months. Further $CdCl_2$ (200µg/ml) + 0.08% melotonin exposed rats for a period of 3 months and 7 days elicited increased SOD activity in the kidney of rat but did not induce any change in CAT activity. Kidney tissue contains same antioxidant enzymes such as SOD and CAT to protect itself from the hazardous effects of oxidative attack. Guluzar Atli and Mustafa Canli (2007) showed a significant increase in CAT activity in the liver of *Oreochromis niloticus* exposed to 10µM Cd. On the other hand, lowest liver CAT activity was evinced at 10µM Zn exposure. The liver was found to be stronger into the face of oxidative stress than the other tissues and a uniform organ with the highest antioxidant enzyme activities (SOD and CAT). It is well known that xenobiotics can generate reactive oxygen species such as hydrogen peroxide and superoxide anion, which in turn are responsible for cell and tissue damage (Roche and Boge, 1993*;* Filho *et al.*, 2001*;* Pinto *et al.*, 2003). Antioxidant defense mechanisms including CAT have a considerable importance for fish because it is induced to protect them from free radicals produced due to oxidative stress and other factors. In this study, the increase in CAT activity may be related to increased oxidative stress caused by DEHP exposures. Similar response was observed in Liver CAT, SOD and GST activity in liver of rainbow trout (*Oncorhynchus mykiss*) exposed to sublethal concentration of carbosulphan (25 µg L^{-1}) for a period of 60 days.

Biomarkers such as protein level, enzyme activity and DNA can be used to measure the interaction between biological systems and chemical, physical or biological environmental agents (Watson and Mutti, 2004*;* Hernandez *et al.*, 2010). The liver is the primary organ for detoxification of xenobiotics and excretion of toxic substances in fish (El-Naggar *et al.*, 2009). The important function of the liver is to clean of any pollutant from the blood, but these pollutants could subsequently lead to structural damage in liver (Pathan *et al.*, 2010) as seen in the present study. Liver antioxidant enzymes are used to determine if the liver is functioning normally or if it has an injury or disease. Studies showed that enzymatic techniques are inexpensive and reliable to determine the toxicities of pollutants on marine animals in the living environment (Telli Karakoc *et al.*, 1997*;* Sunmonu and Oloyede, 2006*;* Hegazi *et al.*, 2010).

Oxidative stress occurs if the activity of the antioxidant defence systems such as SOD, CAT and GPx (glutathione peroxidase) enzymes change by environmental pollution induces the production of reactive oxygen species (Li *et al.*, 2011). SOD is a vital antioxidant defence enzyme in nearly all cells to catalyze the dismutation of superoxide into oxygen thus protecting the cell from superoxide toxicity. SOD enzyme is more sensitive to the lethal effects of superoxide generating chemicals (Gardner *et al.*, 1995). CAT is found in highest levels in the liver as a result of breaking down toxins present in the blood and processing metabolic products for degradation (Aebi, 1984*;* Chelikani *et al.*, 2004). The other study indicated that CAT and SOD activities were significantly higher in liver than in brain and gill of fish exposed to pesticide (Li *et al.*, 2011). The present results agree with earlier reports of increased antioxidant enzymes in fish exposed to environmental pollutants.

IV. Conclusion

The elevation in the enzyme activity observed in the present study could be due to the formation of reactive oxygen species such as hydrogen peroxide, superoxide anion (O_2^-), hydroxyl radicals (OH^-), which has to be neutralized by antioxidant enzymes. Due to their elevated reactivity, these species may damage lipids, proteins, carbohydrates and nucleic acids..

V. References

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