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BIOCHEMICAL MARKERS OF OXIDATIVE STRESS IN MUGIL CEPHALUS EXPOSED TO CADMIUM, COPPER, LEAD AND ZINC

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ABSTRACT

Oxidative damage and antioxidant properties were studied in *Mugil cephalus* exposed to cadmium, copper, lead and zinc in chronic toxicity test as pollution biomarkers. The elevated thiobarbituric acid reactive substances (TBA-RS) levels observed under exposure to heavy metals, leading to the oxidative damage resulting in lipid peroxidation (LPX). Increased activities of antioxidants, catalase (CAT) and glutathione-S-transferase (GST) under long term exposures to heavy metals are more prominent to metal stress suggesting activation of physiological mechanism to scavenge the ROS produced. Decreased values of reduced glutathione (GSH) on long exposures to cadmium and lead indicate utilization of this antioxidant, either to scavenge oxyradical or act in combination with other enzymes. The acetylcholinesterase activity was found to be decreased during metal exposures. The results suggest that heavy metal does alter the active oxygen metabolism by modulating antioxidant enzyme activities, which can be used as biomarker to detect sublethal effects of pollution.

KEY WORDS

Oxidative stress, antioxidants, reactive oxygen species, heavy metals, lipid peroxidation.

INTRODUCTION

Aquatic pollution started long back but intensified during the last few decades, and now the situation has become alarming, especially in India[1]. Metals are natural components of the aquatic environment, but their levels have increased due to anthropogenic activities[2]. The most basic property of heavy metal is that they are bioavailable and are indestructible having toxic effects on living organisms when they exceed a certain concentration limit[3]. Tolerance to heavy metals in metal accumulating organisms is linked to their ability to bind incoming metals, thereby controlling their intracellular availability. The binding of inappropriate metals to metal-sensitive sites such as mitochondria is often interpreted as a failure of detoxification mechanisms and could be an indicator of metal-induced stress[4]. The measurement of cellular and sub cellular responses to chemical contaminants in sentinel organisms are used as bio-indicators from aquatic environment allowing early detection of biological effects as well as assessment of the extent of contamination of pollutants[6].

Heavy metals deplete glutathione and protein bound sulfhydryl groups, resulting in enhanced production of Reactive Oxygen Species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals[7]. The sequential reduction of oxygen leads to generation of superoxide anion and hydrogen peroxide[8]. Superoxide anion also rapidly reacts with nitric oxide, yielding yet another reactive species peroxynitrite. All of these ROS have the potential to trigger cellular death[9]. ROS are considered as crucial mediators for the metal-triggered tissue injuries and apoptosis[7]. To prevent oxidation induced damage, there must be effective antioxidation systems in the organisms. Some components of these systems involve reduced glutathione (GSH) and certain antioxidant enzymes including free radical scavenging enzymes, such as Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidases (GPX) and Glutathione Reductase (GR). Other associated enzymes are the Glyoxalase I (GI), Glyoxalase II (GII) and Glutathione *S*-Transferase (GST). Under oxidative stress conditions, ROS can be reduced by GSH, with the concomitant formation of the oxidized disulphide, oxidized glutathione (GSSG)[10]. Oxidative stress is also of ecological significance, particularly in the aquatic environment, which provides a sink for many pollutants that are capable of causing oxidative stress [11]. Changes in the activity of enzymes and other biomarkers are the possible tools for aquatic toxicological research[12].

Fish being a source of protein to man, they play an important role in energy flow, nutrient cycling and maintaining community balances in these ecosystem[13]. Thus utility of fish for assessing environmental conditions in aquatic ecosystem has gained prominence in recent years[14]. Fish are considered to act as suitable biomonitors for environmental pollution and they are exposed to the heavy metals *in vitro* and the effect of metals on fish is studied[15].

The antioxidant defense system is being increasingly studied because of its potential utility to provide biochemical biomarkers that could be used in environmental monitoring system[11]. Biomarkers in environmental monitoring confer significant advantages over traditional chemical measurements because measured

biological effects can be meaningfully linked to environmental consequences so that environmental concerns can be directly addressed[16]. Hence in the present study the biochemical parameters were assessed through exposing *Mugil cephalus* to cadmium, copper, lead and zinc under longterm toxicity tests.

MATERIAL AND METHODS

Fingerlings of *Mugil cephalus* of mean 1.5 ±0.4cm in length and 0.13 ±0.02g in weight. Collected juveniles were immediately transported to the laboratory in air filled plastic bags and acclimatized fish fingerlings in 200 L Fiberglass Reinforced Plastics (FRP) tanks with aerated natural filtered seawater. Stock solutions of cadmium, copper, lead and zinc were freshly prepared by dissolving the proper metal salts of cadmium chloride hemi (pentahydrate), copper (II) chloride, lead (II) nitrate, and zinc sulfate in deionized (double distilled) water. Fresh stock solutions were prepared daily. These solutions were serially diluted to get the experimental concentration for the toxicity test. The experimental method includes static renewal (24 hour renewal) test [17]. Five concentrations in a geometric series including control were prepared for the test for 30 days in chronic toxicity test [18]. Toxicant and seawater were replaced on daily basis. Test animals were fed three times during the test. Maximum-allowable control mortality was 20 per cent for 30 days for chronic [18].

SAMPLE PREPARATION

At the final stages of the chronic toxicity test, the tissue samples of survived test animals were pooled and made in duplicates. For the analysis of lipid peroxidation marker and antioxidant enzyme activities, 1g tissue was homogenized in chilled pestle and mortar with 5ml homogenization buffer (0.25Msucrose, 10 mMTris, 1 mMEDTA, and pH 7.4) and centrifuged at 5,000 rpm for 15 minutes at 4°C. The resulting supernatant was the homogenate which was used for the estimation of various biochemical assays.

LIPID PEROXIDATION (LPO)

Lipid peroxidation level was assayed by measuring Malondialdehyde (MDA), a decomposed product of polyunsaturated fatty acids. Hydroperoxides were determined by the thiobarbituric acid reaction and was measured at 532 nm in the UV-Spectrophotometer[19]. The amount of Thiobarbituric Acid Reactive Substance (TBARS) was calculated by using an extinction coefficient of 1.56 x 105/M/cm and expressed as nmol TBARS formed /mg protein.

GLUTATHIONE S-TRANSFERASE (GST)

Activity of Glutathione *S*-transferase (GST) was assayed at 340 nm by measuring the increase in absorbance using 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate[20]. The results were expressed as nM of GSH and CDNB conjugate formed /min/mg protein. Values expressed as nanomoles of reduced glutathione and CDNB conjugate formed/min/mg protein.

CATALASE (CAT)

Catalase (CAT) activity was measured at 240 nm by determining the decay of hydrogen peroxide levels and was expressed as µmol of hydrogen peroxide consumed /min/mg/protein[21].

REDUCED GLUTATHIONE (GSH)

The reduced glutathione (GSH) was measured at 412 nm using 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent [22]. The values were expressed as µmol of GSH oxidized/mg protein.

ACETYLCHOLINESTERASE ACTIVITY (AChE)

Acetylcholine esterase activity (AChE) activity was determined using Ellman's reagent, DTNB (5, 5'-dithio-bis (2-

nitrobenzoic acid); 0.5mM) and acetylthiocholine iodide (ACTI) as substrate [23, 24, 25]. The rate of change of absorbance at 412 nm was recorded over 1.5 minutes at 25°C.

TOTAL PROTEIN

The protein concentration of each of the sample extract was determined measured at 750 nm in UV-Spectrophotometer[26].

RESULTS

The level of total protein in cadmium exposure significantly (*P*<0.001) decreased in 80 and 160 µg/l. Glutathione-*S*-transferase (GST) exhibited a significant (*P*<0.001) increase in the activity. Reduced glutathione (GSH) level significantly (*P*<0.001) decreased in the 30 days of exposure compared to control in all the concentrations. Catalase (CAT) and lipid peroxidation (LPO) showed similar trend of significant increase in linear increase in the cadmium concentration. The activity of acetylcholinesterase (AChE) significantly (*P*<0.001) decreased throughout the exposure concentration. The total level of protein in *M.cephalus* exposed to copper showed no significance, there was no linear decrease or increase in the level. Same trend was also found for GST (Table 1).

The level of GSH was significantly (*P*<0.001) increased in 160 µg/l copper concentration. CAT activity was also found increased in the same concentration. AChE activity was found to decrease significantly (*P*<0.001) in 40, 80 and 160 µg/l. LPO significantly had no linear activity of increasing or decreasing trend, the LPO level was significantly increased in 160 µg/l.

M.cephalus exposed to lead in short-term chronic toxicity test showed that all the biochemical components and antioxidative enzymes of the oxidative stress showed significant changes in the tissues exposed concentration of lead. *M.cephalus* exposed to zinc showed no linear variation in the level of protein. The Activity of GST and CAT increased but was not linear. Significant increase was brought about by 74, 118 and 188 µg/l of zinc in the level of GSH. AChE activity increased with no linearity, significant (*P*<0.05) effect was produced by 188 µg/l of zinc concentration. The LPO level showed significant (*P*<0.05) increase in 29 and 118 µg/l, significant (*P*<0.01) increase in the level of LPO in the 188 µg/l (Table 1).

Table 1

Biochemical alterations in M.cephalus exposed to cadmium, copper, lead and zinc in short-term chronic toxicity test

Metal	Concentration (µg/l)	Protein[a]	GST[b]	GSH[c]	CAT[d]	AchE[e]	MDA[f]
Cd	0	14.14 ±0.61	3.80 ±0.14	112.10 ±1.52	416.96 ±10.28	19.95 ±0.52	10.78 ±0.46
	10	13.41±0.11	4.25 ±0.21	93.68 ±6.18**	467.22 ±8.15	17.73 ±2.82**	19.23 ±1.93
	20	12.86 ±0.23	4.75 ±0.21	81.75 ±1.48***	517.97 ±18.12*	13.40 ±0.61***	23.01 ±2.59*
	40	11.96 ±0.46**	5.40 ±0.28**	76.68 ±2.56***	535.67 ±36.61*	6.73 ±0.32***	28.08 ±3.41**
	80	10.69 ±0.59***	6.40 ±0.42***	73.05 ±3.70***	613.45 ±36.23**	5.34 ±0.31***	36.52 ±1.85**
	160	9.97 ±0.21***	7.95 ±0.35***	65.00 ±1.34***	1047.15 ±38.18***	5.04 ±0.06***	52.68 ±3.84***
Cu	0	15.96 ±0.21	3.32 ±0.09	107.27 ±4.19	390.85 ±8.58	17.69 ±0.03	15.49 ±1.48
	10	16.83 ±0.66	3.16 ±0.10	99.08 ±4.40	399.49 ±8.26	14.20 ±2.91	18.30 ±0.81
	20	17.91 ±0.19	3.00 ±0.02	99.74 ±5.28	416.79 ±9.69	9.61 ±0.38**	16.63 ±1.58
	40	17.17 ±0.56	3.15 ±0.17	116.06 ±3.19	426.83 ±11.37	4.93 ±0.09***	20.89 ±0.58*
	80	16.52 ±0.71	3.46 ±0.07	126.45 ±4.10*	419.70 ±9.23	4.04 ±0.13***	19.91 ±0.90
	160	15.07 ±1.29	3.96 ±0.47	147.80 ±7.13***	492.78 ±44.84**	3.96 ±0.37***	24.23 ±2.21**
Pb	0	15.98 ±0.15	3.25 ±0.08	94.89 ±0.32	397.63 ±3.69	17.67 ±0.43	19.06 ±0.18
	51	15.21 ±0.67	2.98 ±0.05	89.70 ±1.63	386.65 ±2.88	15.61 ±1.91	20.54 ±1.01
	76	15.03 ±0.08	2.72 ±0.09*	82.77 ±2.52**	383.91 ±3.83	11.45 ±0.27**	25.37 ±1.89
	114	14.75 ±0.08*	2.18 ±0.16***	82.78 ±1.26**	374.60 ±3.71*	5.73 ±0.05***	28.98 ±1.97**
	171	14.01 ±0.02**	1.83 ±0.09***	77.50 ±1.08***	371.13 ±2.77**	4.76 ±0.05***	33.42 ±2.18***
	256	13.14 ±0.31***	1.46 ±0.18***	73.67 ±2.07***	357.51 ±9.03***	4.55 ±0.71***	40.06 ±2.79***
Zn	0	15.91 ±0.27	2.67 ±0.35	110.38 ±8.77	177.67 ±3.46	8.86 ±0.06	19.87 ±1.58
	29	16.75 ±0.45	2.86 ±0.23	123.96 ±4.83	178.30 ±7.25	7.99 ±0.50	24.14 ±1.16*
	46	17.76 ±0.37*	2.90 ±0.40	130.83 ±3.35	179.75 ±5.80	7.46 ±0.94	23.99 ±0.62
	74	16.94 ±0.81	3.39 ±0.16	137.70 ±1.05*	180.58 ±8.67	7.52 ±0.70	23.63 ±1.13
	118	15.94 ±0.16	3.05 ±0.13	138.56 ±9.47*	179.76 ±9.31	7.10 ±0.05	25.18 ±0.64*
	188	16.06 ±0.17	3.69 ±0.46	141.03 ±6.18*	180.39 ±7.30	6.76 ±0.20*	26.91 ±1.82**

****values are significant at P<0.001, ** values are significant at P<0.01, * values are significant at P<0.05. One way ANOVA (Dunnetts multiple comparison test (α=0.05)); Values are the mean and standard deviation. a-mg protein /g tissue, b-(Glutathione-S-Transferase) GST activity nM of CDNB /min/mg protein, c- (Reduced Glutathione) µmol of GSH oxidized / mg protein , d- (Catalase) µmol of H_2O_2 Consumed/min/mg protein, e- (Acetylcholinesterase) nM/min/mg protein, f- (Lipid Peroxidation) Nm of MDA/ mg protein; The concentration column (mg/l) contains '0' indicating control in the test conducted in triplicate.*

DISCUSSION

Presence of low concentration of scavenging enzymes in the juveniles makes them susceptible to oxidative damage when attacked by reactive oxygen species (ROS)[27]. *M.cephalus* exposed to exposure concentrations experienced severe Oxidative Stress (OS) characterized by significant changes in the levels of OS biomarkers, which had also been observed in brain samples of the mullet[28]. Removal of H_2O_2 is an important strategy of marine organisms against oxidative stress[29]. Increased activities of CAT have been reported in several fish and invertebrate species [30], whereas inhibition of CAT has been suggested as a transitory response to acute pollution [31]. Concentration of LPO was significantly higher ($P< 0.05$) in higher concentrations of cadmium, copper, lead and zinc. CAT activity was reduced due to increased levels of exposure indicating the importance of antioxidant[32].

The reduction in protein content might be also due to the proteolysis process for energy production and utilization owing to the decreased food intake of crabs under stress[33]. The results obtained in our study show the existence changes in GSH content. These data may indicate a faster rate of GSH utilization or degradation, which could be responsible for the observed lower GSH content. Moreover, increase of GSH content may be related to prevention of oxidative challenge [34]. Aquatic organisms maintain high content of GSH in tissues and increased content has the function of protection[35]. High content of GSH could be a consequence of its increased synthesis due to high cysteine accessibility, which is necessary for GSH synthesis. GSH content increased after treatment with cadmium[36]. This could provide the first line of defense against the influence of toxic heavy metals.

General esterases are the good biomarkers to differentiate the levels of contaminants[37]. Maintenance of enzyme activities may serve as a quality criterion of the cells[38] and enzymatic changes may be regarded as important markers of the presence of hazardous substances[39]. Mullet (*Mugil* sp.) from contaminated Spanish areas revealed increased activities of antioxidant (catalase) and detoxifying Glutathione *S*-Transferase (GST) enzymes[41]. Channel catfish (*Ictalurus punctatus*) exposed to bleached kraft mill effluents resulted in a significant increase in catalase activity[42]. The metabolism of toxic compounds frequently results in the formation of ROS, which significantly contribute to their toxicity[8].

Changes in GST activity reflect detoxification process in fish exposed to toxic compounds[4,5]. Increase of GST was observed activity in fish exposed to cadmium, copper and zinc in the present study. This induction in GST activity could indicate a defense of fish against oxidative stress damage produced by adverse conditions. Increased levels of lipid peroxidation (LPO) have been observed in fish under experimental conditions, upon exposure to different xenobiotics[43]. There are evidences that heavy metals like those used in the studied, produced increased LPO levels in *M.cephalus*[44]. The concurrent use of several biomarkers is important to minimize misinterpretation in cases of complex situations of pollution[45].

CONCLUSION

M.cephalus inhabiting the intertidal coastal waters has been chosen to study oxidative stress responses in terms of TBA-RS content, arising from heavy metal pollution. Increased TBA-RS content associated with metal stress indicates that oxidation of lipids takes place after exposure, which could be representative of an emerging picture of potential ecological impacts on fish population. The other tissue specific responses to long term exposure to metals by these fish to TBA-RS and antioxidants such as SOD, CAT, GPX, GR, GST and GSH indicate interdependency of these antioxidants to scavenge the reactive oxygen

radicals. The result indicates that fish actively generate oxidative stress and antioxidant responses which can be used as biomarkers of pollution.

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