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Effect of *In vitro* Exposure to Mercury Chloride on Phosphatase Enzymes in Yellowfin Sea Bream (*Acanthopagrus latus*)

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Abstract: The objective of this paper was to measure the acid and alkaline phosphatase (ALP and ACP) beside lipase and total protein activities and to analyses the "*In vitro*" effect of mercury on enzyme activity of yellowfin sea bream. According to the previously LC_{50} test, fish divided in 5 treatments. ALP and ACP, were determined by enzymatic methods with the automate apparatus Auto-analyzer. Total protein activities were determined with Photometric method in Pars-Azmoon reagent kit. Both ALP and ACP activities exhibited highly significant analysis of variance ($P<0.001$) with lower considerable values than those of the control group in ALP and higher considerable values than those of the control group in ACP. No significant changes occurred in the activities of the Lipase ($P<0.05$), however it was decreased. Values recorded for activity of total protein show high significance depletion ($P<0.001$) with mercury exposed. Results of the present investigation indicated that the sub- acute mercury concentrations tested may cause several changes in the metabolic and enzymatic parameters of the studied fish.

Key words: Mercury · Pollution · Phosphatase enzymes · *Acanthopagrus latus*

INTRODUCTION

Exposure to heavy metals, including mercury, induces formation of highly oxidative chemical species like peroxide or superoxide groups in the cell [1], which generate different types of cell damage, The cell reacts to these aggressions by activating different enzymes directly related to detoxification and the reestablishment of the redox balance in the cell [2].

Mercury inhibits enzyme activity and provokes cell damage. It has a high affinity to lipids, allowing movement across cell membranes and can interfere with cell metabolism [3]. The principal toxic effects of mercury involve interactions with a large number of cellular processes, including the formation of complexes with free thiols and protein thiol groups, which may lead to oxidative stress [4].

Antioxidant systems are located within different cellular compartments. These enzymes are found virtually in all tissues of vertebrates, but show in general, high activity in the liver, a major organ for xenobiotics uptake and enzymatic transformation of ROS [5] 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 contaminants such as metals and other xenobiotics [6].

Induction of oxidative stress with mercury makes an important contribution to molecular mechanism for liver injury [7]. Recent studies confirm that mercury causes severe oxidative damages [8], thus mercury is proved to be a potential oxidant in the category of environmental factors.

Enzymes catalyze physiological reactions by decreasing the activation energy level that the reactants (substrates) must reach for the reaction to occur.

The influence of pollutants on enzymatic activity of fish is one of the most important biochemical parameters which are affected under exposure of toxicants. In exposure to a toxicant, enzyme activity appears to be increased or it may be inhibited due to the active site being either denatured or distorted. Since some enzymes catalyze some steps in the metabolism of carbohydrates and protein, they are present in most tissues. The increase or decrease in enzyme level in a very accurate index for

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diagnostic of quantity and quality of toxicant. For example, such effects have been observed after chronic exposure to low doses or acute exposure to high doses of mercury. The hazardous influence of mercury on fish can be measured accurately by detection of enzymes and other biochemical indicators. Although most similar study has been conducted on terrestrial vertebrates, there are a few studies related to the effects of mercury on enzyme activity in fish [4, 7, 9].

In the current study, oxidative stress enzymatic biomarkers were measured in order to investigate patterns of response in these enzymes and to quantify the extent of alterations caused by the mercury compounds, so the objective of this paper was to characterize the effect of *In vitro* mercury exposure on acid and alkaline phosphatase beside lipase and total protein activities of important economic fish, yellowfin sea bream, to estimate its potential use as a stress biomarker of mercury pollution.

MATERIALS AND METHODS

***In vitro* Test:** In laboratory of the Mariculture Research Station of the South Iranian Aquaculture Research Center, Mahshahr, Iran, forty five Fish were maintained in a 15 tank with seawater re-circulatory system equipped with physical/chemical filters and with aeration. All samples were acclimated for one weeks in a 15 aerated fiberglass tank containing 46 ppt saltwater maintained at 25°C under a constant 12:12 L:D photoperiod. Acclimatized Fish were fed daily with a live feed (fresh shrimp) and daily we check water quality and water parameters.

Fish were randomly divided into five equal groups (15 per group) and each tank was randomly assigned to one of five experimental treatments filled with the appropriate concentration of an aqueous solution of Hg. According to the LC_{50} test [10], the Yellowfin sea bream were exposed to nominal mercury concentrations of 0, 10, 20, 40 and 80 μg l respectively and maintained for three weeks with aeration. Conditions within each experimental tank were monitored daily with the temperature 25°C ± 1, pH 7.8 ± 0.1 and salinity 46 ± 1 ppt under a natural photoperiod (12hL:12hD) in controlled condition.

Serum Analysis: Laboratory experiments were performed using 80-liter aquaria, at 20°C, under natural photoperiod and constant aeration. Serum samples were assayed in duplicate. Ultra pure water was used for all serum dilutions and standard preparations and duplicate readings were recorded for standards and serum samples. Biochemical analyses was done with the automate apparatus Autoanalyser, Metrolab 2300 plus, Argentina (Random Access Clinical Analyzer). All enzyme assays were performed in duplicate. Enzyme activity units (IU), defined as micromoles of substrate converted to product at assay temperature per minute, were expressed per liter and mg/dl of serum protein (specific activity).

Alkaline Phosphatase (ALP) and Acid Phosphatase (ACP): Alkaline phosphatase (ALP) and Acid phosphatase (ACP), were determined by enzymatic methods with the automate apparatus Auto-analyzer, Metrolab 2300 plus, Argentina (Random Access Clinical Analyzer) with Darman Kave & Pars Azmoon kit at 37°C and 410 nm & 405 nm, respectively. The limit of detection (LOD) of the procedures was 3 IU/L. Acid and alkaline phosphatase in serum (two point method) were determined by the procedure described by Vassault [11]. Intra-assay and Inter-assay coefficients of variation for ACP were of 2.60 and 2.80%, respectively. Intra-assay and Inter-assay Mean ± SD were 8.4 ± 0.29 and 8.7 ± 0.25 U/L, respectively. Intra-assay and Inter-assay coefficients of variation for ALP were of 1.50 and 1.60%, respectively. Intra-assay and Inter-assay Mean ± SD were 114 ± 1.71 and 120 ± 1.93 U/L, respectively.

Lipase: Lipase activities were determined with photometric method in Pars-Azmoon Diagnostics Infinity reagent kit (Procedure No. 1 50 24) at 580 nm for detection. The limit of detection (LOD) of the procedure was 3 IU/L [12].

Total Protein: Serum total protein levels were determined using Pars Azmoon, Iran (1 500 028) kit, with bovine serum albumin serving as standard at 546 nm and 37C. The limit of detection (LOD) of the procedure was 5 mg/dl. Intra-assay and Inter-assay coefficients of variation were of 0.91% and 1.06% respectively. Intra-assay and Inter-assay Mean ± SD were 5.27 ± 0.05 and 5.24 ± 0.06 g/dl, respectively [12].

Statistical Analysis: One-way analysis of variance ANOVA with Duncan Post Hoc was used to determine significant differences to evaluate the effect of mercury on parameters. To investigate associations between bioaccumulation and its effects, Pearson correlation coefficients (r) were calculated between mercury concentrations and enzymatic parameters. Multiple regressions were used to determine the relationship between mercury concentration and blood parameters.

The differences between means were analyzed at the 5% probability level (p value of less than 0.05 was considered as statistically significant). Data are reported as means ± standard deviation ($\bar{x} \pm$ SD).

RESULTS

Results of pure enzyme activity analysis are presented in Table 1. No significant changes occurred in the activities of the Lipase. ALP activities exhibited lower considerable values than those of the control group, On the other hand, ACP activities were significantly higher than those of the control group. Values recorded for activity of the ALP and ACP enzymes were about two times lower and higher than those of the control group respectively.

Looking at the differences among blank and treated Fish in Fig. 1, it is evident that except ALP, there is an increase in the enzyme/protein activities of other enzymes. ACP activities exhibited higher significance values than those of the control group, beside considerable decrease of ALP.

Table 1: In vitro pure enzyme activities of yellowfin sea bream exposed to mercury

	ALP (U/ L)	ACP(U/ L)	LIPASE (mg/dl)
Control	197.43±27.60^{a}	15.33±2.65^{bc}	9.73±1.19^{a}
10 µg	1 125.33±29.39^{b}	21.50±5.43^{b}	8.93±1.02^{a}
20 µg	1 86.55±42.91^{bc}	20.83±3.86^{b}	9.03±0.96^{a}
40 µg	1 77.83±10.87^{c}	14.25±3.16^{c}	8.7±1.83^{a}
80 µg	1 87.33±44.57^{bc}	31.00±8.6^{a}	8.45±0.33^{a}

Table 2: In vitro correlation of enzyme activities of yellowfin sea bream with mercury chloride

	ALP	ACP	LIPASE	Protein
Pearson correlation (r)	0.54**	0.55**	0.31	0.67**
Sig (p)	0.002	0.002	0.09	0.00

** Correlation is significant at the 0.01 level

Table 3: In vitro curve fit linear regression of enzyme activities of yellowfin sea bream with mercury

	ALP	ACP	LIPASE	Protein
R square (r^2)	0.29	0.30	0.096	0.45
F	11.8	12.1	2.99	23.7
Sig (p)	0.001	0.001	0.09	0.00

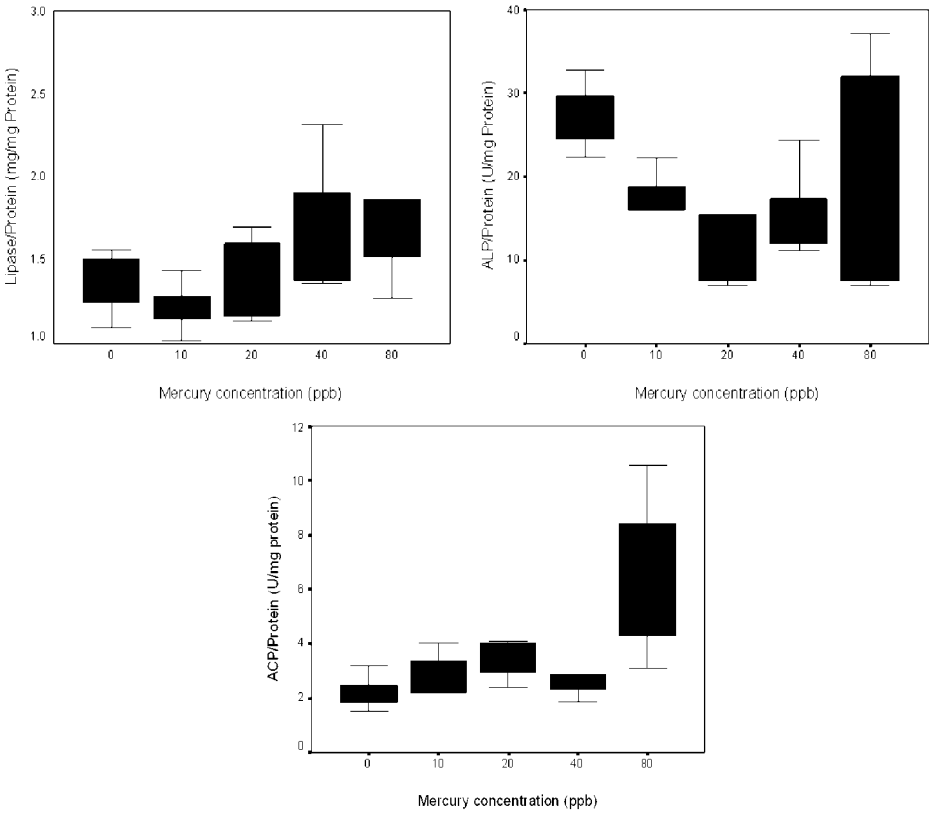

Fig. 1: Enzyme/protein response (ALP, ACP and Lipase) of the yellowfin sea bream during in vitro exposed to different concentration of mercury chloride (box plots contain mean and standard deviation). Values of specific enzyme activity are expressed in (U/mg Protein) except Lipase (mg/mg Protein)

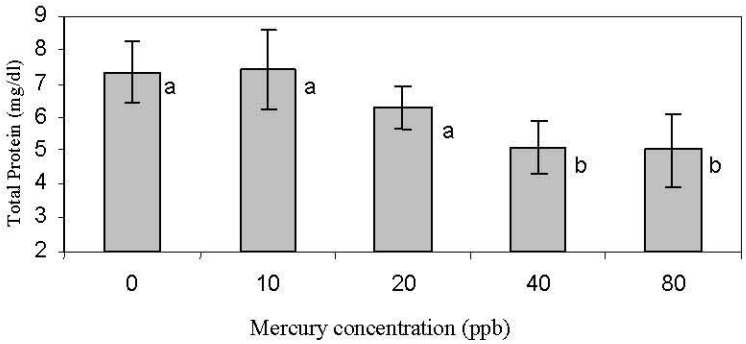

Fig. 2: Total protein response in the yellowfin sea bream during in vitro exposed to different concentration of mercury chloride

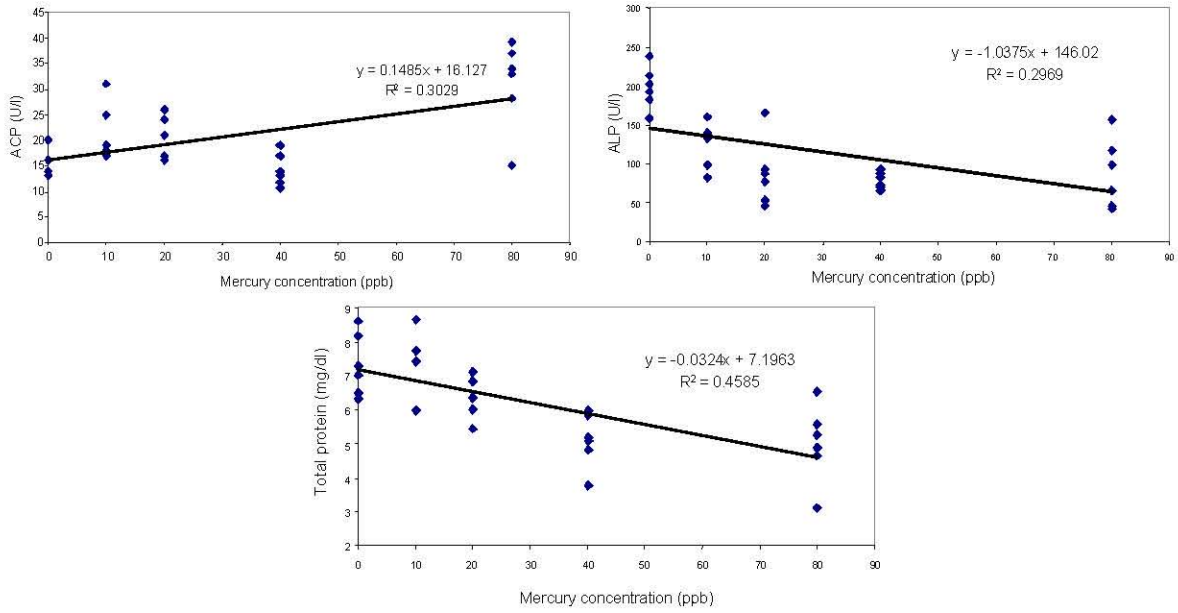

Fig. 3: Regressions model (Y = a ± bX) of ALP, ACP and Total protein of the yellowfin sea bream during in vitro exposed to different concentration of mercury chloride

During *In vitro* results, the correlation between mercury with enzyme parameters and protein was statistically tested by analyzing the data obtained during the mercury exposed. Only the Lipase level had not statistically significant and other parameter show significant correlation ($P<0.05$) with mercury exposed, all correlation was negative except ACP (Table 2). Correlation test of same enzyme (Phosphatase) with each other imply that there was significantly negative correlation (r -0.40, p 0.02).

Curve estimation regressions data were used to determine the relationship between mercury concentration and Total protein, ALP, ACP and Lipase content. Only the Lipase level had not statistically significant and other parameter show significant linear regression ($P<0.05$) with mercury (Table 3). Regressions model $Y = a \pm bX$ of significant parameter are in Fig. 3.

DISCUSSION

Acid (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) (ACP and ALP, respectively) catalyse the hydrolysis of various phosphate-containing compounds and act as transphosphorylases at acid and alkaline pHs, respectively. Acid phosphatases act as marker enzymes for the detection of lysosomes in cell fractions and can be altered by the presence of xenobiotics [13], whilst alkaline phosphatases are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells. Both enzymatic activities have been studied in several organisms and the influence of heavy metals has been reported [14]. These enzymatic activities are involved in a variety of metabolic processes, such as molecule permeability, growth and cell differentiation and steroidogenesis [15]. For fish, in laboratory conditions,

liver alkaline phosphatase activity changes in response to waterborne metal making it useful as indicator of heavy metal exposure [15].

As we know proteins are a major constituent in the metabolism of animals and heavy metals may be involved in the normal working of these molecules, therefore it is important to detection of alterations in protein metabolism induced by metal exposure for further information. Alterations that may occur are the increased synthesis or breakdown of proteins and the inhibition or activation of certain enzymes. These changes can be observed in the total protein content, free amino acid (FAA) concentration and the activity of proteases and phosphatase such as alkaline phosphatase (ALP) and Acid phosphatase (ACP) [16, 17], so in current study Portion of ACP and ALP to protein were discussed. Our results show the same result and endorsement exact increase and decrease of ACP and ALP, respectively.

Karan *et al.* [18] found increased levels of ACP from serum when exposed the carp *C. carpio* to copper sulphate for 14 days. Poleksic´ and Karan [19] investigated the effects of the herbicide Trifluralin on carp *Cyprinus carpio* and suggested that a rapid up take from water joint to arapid metabolization could explain the increase in ACP activity.

Ghorpade *et al.* [20] find that ACP levels were significantly increased in a plasticizer (Diethyl phthalate) treated fish. This process of rapid uptake could explain also the increased levels of ACP activity measured during the present study. The increase in the activities of functional enzymes in the yellowfin sea bream exposed to sub-acute mercury levels could be attributed to cell membranous system damage leading to changes in membrane permeability and inter cellular metabolism as reported by Pelgrom *et al.* [21]. This increase is due to increased lysosomal activity in the liver andmuscle. It is apparent that mercury causes increased ACP activity by interacting with lysosomes. The high value found for ACP activity indicates that it plays a functional lysosomal role [22]. Lysosomes play an important role in the function of eukaryotic cells, many environmental contaminates including heavy metals and organic xenobiotics are known to be sequestered in lysosomes and their stability reduced in vertebrates with increasing contamination.

Metals could alter the structure, permeability and integrity of lysosomal membranes resulting in the diffusion of their enzyme into cytosol [12]. The measurement of alkaline phosphatase activity is generally carried out in clinical and ecotoxicological studies. In ecotoxicology, this enzyme may serve as an indicator of intoxication because of its sensitivity to metallic salts [23]. The activities of alkaline phosphatase was markedly inhibited due to high concentrations of Hg and Cd and marked differences in electrophoretic patterns of proteins [24].

ALP activity is known to be involved in bone formation and in transport membrane activities. This enzyme is also involved in shell deposition in Bivalves [22]. In the blue crab, *Callinectes sapidus*, ALP activity is involved in the modulation of the osmoregulatory response. The low levels found for ALP activity suggest that membranes are an ineffective barrier to most molecular substances in this species [25].

In the current study, mercury may affect intestinal functions by inhibiting the enzyme activity during absorption processes; especially serum alkaline phosphatase is mainly originated from intestines, liver and bones. Liver is among the principal target organ for mercury intoxication. Mercury is one of the agents which disturb cell lipid membranes, leading to the release of hydrolases [26]. It seems that mercury has a bouncing effect on alkaline phosphatase activity. ALP is basically a membrane bound enzyme and any perturbation in the membrane property caused by interaction with mercury could lead to alteration in ALP activity. Mercury being a lipophilic substance, could interact directly with the plasma membrane and bring about alteration in its functions.

The clear increase in lipid oxidation and its markers may also be due to the decrease in antioxidant enzyme activities. Accordingly, the antioxidant enzyme levels have been found to be decreased significantly in this study and a similar type of response has alsobeen observed in fish such as *Geophagus brasiliensis* in response to oxidative stress [27]. Maintenance of high constitutive levels of antioxidant enzymes is essential to prevent oxyradical-mediated lipid peroxidation [28]. Long term exposure of fish to pollutants may also be a possible reason for the decrease in antioxidant enzyme levels. Detoxification enzyme variations also exert negative effects such as increased susceptibility to reactive oxygen species formation, increased energetic demand, proliferation of cells, etc [30].

Results of the present investigation indicated that the sub- acute mercury concentrations tested may cause several changes in the metabolic and enzymatic parameters of the studied fish. The major findings of this study are that mercury is a toxic substance in yellowfin sea bream, with change enzyme activities in serum of fish exposed to various concentrations.

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