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Experimental Approaches of Hematotoxic and Immunotoxic Effects of Mercury Chloride on Yellowfin Sea Bream (*Acanthopagrus latus*)

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Abstract: Fish blood is sensitive to pollution-induced xenobiotics and changes in the hematological and immunological parameters may lead to hematotoxic and immunotoxic effects. The objectives of current study were to determine the *in vitro* effects of mercuric chloride ($HgCl_2$) on hematological and immunological features of yellowfin sea bream. In laboratory, fish were maintained in a seawater re-circulatory system. Numbers of blood leukocytes was counted using a hemocytometer_Neubauer and the light microscope. The leukocyte differential count was made in peripheral blood smears, giving the neutrophil value of differential neutrophil and the mononuclear value of differential lymphocytes plus monocyte and eosinophil (100 leukocytes count). Hematocrit values (Ht%) were measured with microhematocrit centrifuge. Hemoglobin levels (Hb mg/100) were colorimetrically determined by measuring the formation of cyanomethemoglobin. Result declared significance increase of Hb, Ht and differential monocyte within higher considerable values than those of the control group, beside significance decrease of leukocyte count, differential lymphocyte and eosinophyle ($P>0.05$) with lower considerable values than those of the control group. The major findings of this study were that the sub-acute mercury concentrations tested may cause several changes in the hematological and immunological parameters of the studied fish, so estimation of these indices, could provide a useful indicator of pollution of water bodies.

Key words: Hematology · Immunology · Mercury · *Acanthopagrus latus*

INTRODUCTION

Several biochemical and physiological responses occur when a fish exposed to the xenobiotics, if fish can not tolerate and acclimatized it may lead to toxicity [1]. The measurement of physiological parameters is a suitable method generally used in aquatic toxicology and biomonitoring programs. Physiological changes induced by xenobiotics are also apparent at the biochemical and physiological level, such as in the carbohydrate and protein metabolism and in hematology. In cases whereas these alternations are adaptive they are referred to as stress responses, while they are considered effects when they have a negative cause on the physiological condition or even survival of the fish [2]. The intensity and duration of these responses and/or effects are affected by several factors, including the concentration of the contaminant, duration of exposure and the fish species [3]. Other researches have confirmed this found, for example, changes in hematocrit, hemoglobin, plasma glucose and lactate levels in Cd-exposed fish [4].

Physiological stress indicators such as some hematological and blood parameters could be useful to evaluate the effects of contaminants such as heavy metals in fish [5]. Hematological indices are more often used when clinical diagnoses of fish physiology are used to determine sub chronic concentrations of contaminants [6]. Blood indices are considered pathophysiological parameters of the whole body and therefore are important in diagnosing the structural and functional status of fish exposed to xenobiotics [7]. Moreover, hematological indices provide quite frequently and routinely accepted methods in aquaculture to evaluate the interactions between dietary levels of nutrients [8].

Fish blood is sensitive to pollution-induced stress and changes in the hematological and metabolic parameters can be used as toxicity indices of xenobiotics [9]. Although fish blood indices have been increasingly examined in ecosystem monitoring programs as valuable parameters of physiological changes in the presence of xenobiotics, the lack of basic knowledge about the blood response to stressors

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mainly from tropical species is the most important leakage to using these indices in environmental monitoring programs [10].

Many experimental and epidemiological reports have confirmed the immunotoxicity of heavy metals in a variety of marine biota. Several studies on different condition of *in vitro* and *in vivo* experiments have confirmed that the immune system as a whole is affected by exposure to these xenobiotics [11]. Though, the extent of immune dysfunction is dependent on the type of pollutant, duration of exposure, species and even the strain of animal used. Furthermore, many of these studies have tested exposures to concentrations that are unrealistic under field conditions.

Although the hematotoxicity and immunotoxicity of mercury is well established, evaluation of their potential toxicity in marine biota is complicated by variables that could modulate the immune response to contaminants, so the objectives of current study were to determine the in vitro effects of mercuric chloride ($HgCl_2$) on hematological and immunological features of yellowfin sea bream to aim toxicity of mercury pollution.

MATERIALS AND METHODS

Experimental Design: Forty five fish, all immature male in same size (120 g final body weight average) were maintained in laboratory to the Mariculture Research Station of the South Iranian Aquaculture Research Center, Mahshahr, Iran, in 15 tanks (300-L) 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 water quality and water parameters were daily checked.

Fish Maintenance: Fish were randomly divided into five equal groups (15 fish per any group) and each tank was randomly assigned to one of five experimental treatments filled with the appropriate concentration of an aqueous solution of Hg (standard solution for atomic absorbance spectrophotometer) in dechlorinated tap water.

The Yellowfin sea bream were exposed to nominal mercury concentrations of 0 μg l (tank 1), 10 μg l (tank 2), 20 μg l (tank 3), 40 μg l (tank 4), 80 μg l (tank 5) and maintained for three weeks with aeration. These sub-lethal doses were chosen on the basis of preliminary toxicity tests and determinations of LC_{50} 96h for this species, suggestive of inducing toxic effects but not lethally so [12].

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 room. Water was oxygen saturated through constant aeration in a static system. Voluntary feed intake was near to maintenance ration at the time of the maintenance. Fish were fed two times a day (08:30 and 17:30 h) but were starved for 48 h prior to the start of the experiment and throughout its duration. Fecal remains and food residues were removed by suction every other day. The food supply was provided to each predator fish with fresh prawn, collected from creeks without pollutants sources.

Hematology Analysis: Determinations of the number of CBC tests were performed immediately on fresh blood.

Numbers of blood leukocytes was performed by diluting heparinized blood with Giemsa stain at 1:30 dilution and cells were counted using a hemocytometer Neubauer under the light microscope [13].

The leukocyte differential count was made in peripheral blood smears stained by Merck Giemsa [14], giving the neutrophils value of differential neutrophils (100 leukocytes count) and the mononuclear value of differential lymphocytes plus monocyte and eosinophile (100 leukocytes count).

Giemsa staining enabled the specific identification of lymphocytes from other types of leukocytes. Lymphocyte numbers were determined by direct counting under the microscope using a Neubauer chamber. By means of a suspension based in methylene blue, circulating lymphocytes of sea bream can be distinguished from erythrocytes and thrombocytes have morphological features which differentiate the two cell types. Criteria for identification of lymphocytes were as given in Hibiya [15].

Hematocrit values (Ht%) were immediately determined after sampling by placing fresh blood in glass capillary tubes and centrifuged for 5 min at 10,500 rpm in a microhematocrit centrifuge (Hettich, Germany) then measuring the packed cell volume [16]; Hematocrit readings were performed with the aid of a microhematocrit reader.

Hemoglobin levels (Hb mg/l) were colorimetrically determined by measuring the formation of cyanomethemoglobin according to Lee *et al.* [17].

Mean corpuscular hemoglobin concentration (MCHC) were calculated from RBC, Ht and Hb according to Lee *et al.* [17] as MCHC (mg l^{-1}) = Hb ($mgdl^{-1}$) / Ht (ratio).

Statistical Procedure: 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

With respect to *in vitro* raw data, the Kolmogrov-Smirnov normality test was significant at a P<0.05, for all parameters. Results of *in vitro* hematological and immunological activity analysis are presented in table 1. All *in vitro* activities exhibited significant analysis of variance (P<0.05), but the statistical analysis did not reveal any significant difference between control groups with MCHC and differential monocyte. *In vitro* result declared significance increase of Hb, Ht and differential monocyte within higher considerable values than those of the control group, beside significance decrease of leukocyte count, differential lymphocyte and eosinophyle (P>0.05) with lower considerable values than those of the control group.

During *in vitro* results, the correlation between mercury with all parameters was statistically tested by analyzing the data obtained during the mercury exposed. Only the Ht, MCHC and monocyte levels had not statistically significant and other parameter show significant correlation (P<0.05) with mercury exposed, that among Hb and neutrophil correlation was positive and lymphocyte, eosinophil and leukocyte correlation was negative (Table 2).

Curve estimation regressions data were used to determine the relationship between mercury concentration and Hb, Ht, MCHC, leucosyte, lymphocyte, monocyte, neutrophil and eosinophils activity. Only the Ht, monocyte and MCHC levels had not statistically significant and other parameter show significant linear regression (P<0.05) with mercury (Table 3). Regressions model Y = a±bX of significant parameter are in Fig. 1.

Table 1: *In vitro* hematological and immunological activities of yellowfin sea bream exposed to mercury chloride

	Control	10 µg l	20 µg l	40 µg 1	80 µg l
Hb (mg/l)	7.38±0.34^{b}	8.45±0.48^{a}	8.75±0.72^{a}	8.96±0.20^{a}	8.53±0.68^{a}
Ht (%)	21.33±2.80^{b}	26.00±3.74^{a}	27.00±2.60^{a}	27.50±3.01^{a}	26.33±1.50^{a}
MCHC(mg/l)	0.34±0.03^{a}	0.32±0.04^{a}	0.32±0.01^{a}	0.32±0.03^{a}	0.32±0.03^{a}
Leukocsyte (/ml)	11533±1001^{a}	9666±1150^{b}	9916±2312^{a}	10300±831^{a}	9133±1354^{b}
Lymphocyte (%)	77.50±2.66^{a}	69.83±5.07^{b}	72.66±4.08^{a}	71.66±4.17^{b}	69.50±4.41^{b}
Monocyte (%)	3.66±1.21^{a}	4.50±1.04^{a}	3.33±0.81^{a}	4.50±2.16^{a}	4.83±0.98^{a}
Neutrophil (%)	15.83±2.71^{b}	21.16±3.54^{a}	21.66±4.80^{a}	22.83±3.48^{a}	24.33±3.14^{a}
Eosinophils (%)	3.66±1.63^{a}	3.66±0.81^{a}	2.66±0.81^{ab}	2.83±1.16^{ab}	1.83±0.98^{b}

Table 2: In vitro correlation of hematological and immunological activities of yellowfin sea bream with mercury chloride

	Hb	Ht	MCHC	Leukocyte	Lymphocyte	Monocyte	Neutrophil	Eosinophils
r	0.37*	0.33	0.17	0.36*	0.38*	0.28	0.53*	0.51*
p	0.04	0.06	0.35	0.04	0.03	0.13	0.02	0.04

* Correlation is significant at the 0.05 level, ** Correlation is significant at the 0.01 level

Table 3: In vitro curve fit linear regression of hematological and immunological activities of yellowfin sea bream with mercury chloride

	Hb	Ht	MCHC	Leukocyte	Lymphocyte	Monocyte	Neutrophil	Eosinophils
(r^2)	0.13*	0.11	0.03	0.13*	0.14*	0.07	0.28*	0.26*
F	4.4	3.5	0.89	4.2	4.7	2.3	11.1	10.08
(*p*)	0.04	0.06	0.35	0.04	0.03	0.13	0.02	0.003

*Correlation is significant at the 0.05 level

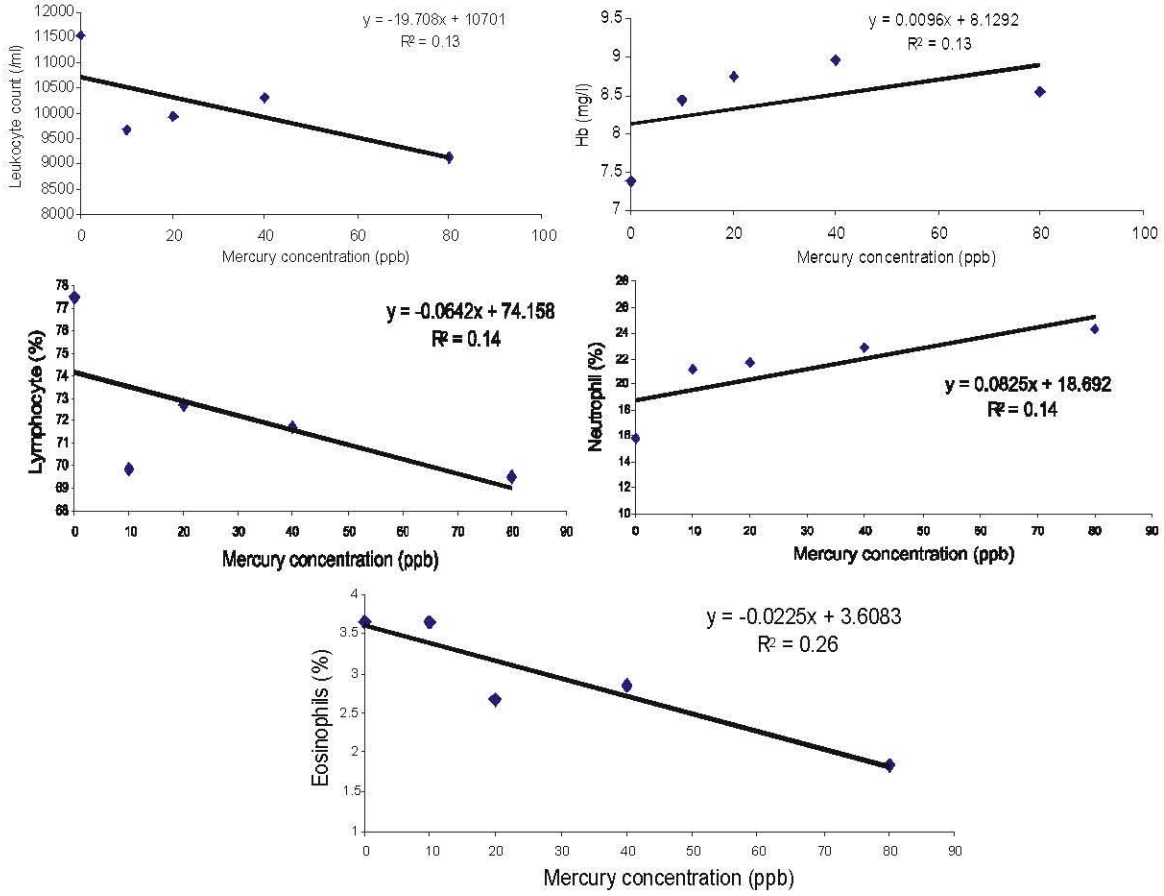

Fig. 1: Regressions model (Y = a±bX) of Hb, Leucosyte, Lymphocyte, Neutrophil and Eosinophils of the yellowfin sea bream during in vitro exposed to different concentration of mercury chloride

DISCUSSION

Hematotoxins can change quantitative and qualitative characteristics of blood cells to produce toxic symptoms. Hematotoxicity happen when some of these different blood components are present or structural anomalies occurring in blood components interfere with normal functioning.

Hematological parameters can be considered indicators of toxicity in fish studies [9]. There is little researches related to hematological responses in fish exposed chronic and sub-chronic by heavy metals. In this work a series of hematological and Immunological parameters were examined in *Acanthopagrus latus* after exposure to in vitro doses over a period of 3 weeks our result declared significance increase of both Hb and Ht within in vitro exposure.

Comparison of our blood values with those obtained previously show that, in African catfish, *Clarias gariepinus*, During the environmental Cu exposure significant increases in Hb and Ht will be predicable [18]. These results are similar to those found by Ghazaly [19]. Moreover, Wilson and Taylor [20] attributed the increase in the blood parameters to a shift of water from the plasma to the muscle cells, thereby increasing the hemo concentration. Palackova *et al.* [21] found an elevation in both parameters in carp fingerlings after 19 days exposure to Cd. Oliveira Ribeiro *et al.* [5] confirmed high significance increase of Ht and Hb exposed to methyl mercury and non significant increase to tributyltin chloride and inorganic lead in fish *Hoplias malabaricus.* The chemicals that stimulate blood cell/hemoglobin production, generally induce a hypoxic condition in fish that stimulated the spleen, which produces the blood cells in fish [22], to contract and release stored erythrocytes into the circulation.

The values observed for hematocrit and hemoglobin are relatively close to those of other tropical and nontropical species of fish such as *Ictalurus punctatus* (23.9%) [8] and *Colossoma macropomum* (20–23%) and

quite different from those of compared with other species [10], *Oncorhynchus mykiss* (35.6%) [23] and Rhandia quelen (35.3%) [24].

Oliveira Ribeiro *et al.* [5] find no significant effects in MCHC for all tested metals in fish Hoplias malabaricus. Carvalho and Fernandes [25] find no significant effects in MCHC fish Prochilodus scrofa on cooper toxicity, that are same with our non significant MCHC result.

Increased significantly the values of hematocrit after subchronic exposure, indicating the importance of the route of contamination. Results observed accord with those of Chowdhury *et al.* [26], who noted an increase of blood hematocrit and hemoglobin during environmental hypoxia and chronic or acute exposure to waterborne metals to increase blood oxygen carrying capacity when impairment of gas exchange occurs.

Phagocytosis plays a key role in both non-specific and specific immune responses of species and represents the first line of defence of the immune system against invading agents [27]. Although the immunotoxicity of heavy metals is well established, evaluation of their potential immunotoxicity in wildlife species is complicated by variables that could modulate the immune response to xenobiotics under field conditions. Our immunological result declared significantly decreased in leukocytes count, differential lymphocyte and eosinophyle with significantly increased in differential neutrophil and non significant increased in Monocyte.

Lymphocytes are the most common circulating leukocyte found in fish. Monocytes are large leukocytes with an abundant blue-gray cytoplasm that lacks granules and are occasionally vacuolated, As in mammals, monocytes migrate to the tissues and became macrophages [28]. In fishes, the heterophil has been variably called heterophil or neutrophil depending on the size of cytoplasmic granules. Unlike avian and reptilian heterophils, fish heterophils contain large amounts of myeloperoxidase and their macrophages produce nitric oxide and reactive oxygen [28]. Most investigators have reported that neutrophils make up 25% of the fish leukocyte population [29]. The presence of peroxidase in fish leukocytes has been associated with bactericidal activity and functions as a defensive immune mechanism there is a lack of knowledge regarding the full function of fish eosinophils, but they seem to function in a similar manner to mammalian mucosal mast cells. The eosinophils have been associated with antigenic stimulation and parasitic infestations [30].

Our *in vitro* results show increase in differential monocyte and neutrophil and decrease in leukocyte count, differential lymphocyte and eosinophil that all were significant except monocyte. The monocytes and neutrophils increased and lymphocyte decrease during different stressors in cultured fish Oreochromis aureus [31].

It is believed that neutrophils and monocytes have phagocytic activity which might explain their increased percentage during infectious situations. Hlavek and Bulkley [32] found a transient neutrophilia in rainbow trout 24 h after treatment with malachite green, but this decreased after 4 days. These authors stated that the white cells changes in the trout exposed to malachite green were the result of a stress syndrome not specific to vertebrates and not due to leucocytotoxic effects of this chemical compound in the fish. Darwish *et al.* [33] also found an increase of neutrophil counts in channel catfish exposed to high doses of potassium permanganate. Changes in the differential leucocyte count are one of the most sensitive indicators of acute stress in fish [34].

Brandão *et al.* [35] find a reduction in some immunological parameters (platelet, leukocyte and lymphocyte counts) and the increase in neutrophil and monocyte percentages were demonstrated in $HgCl_2$ exposed.

It is known that mercury can induce abnormal responses in the immune system, including leukocyte count, a marker of cellular defense [36]. The increase in neutrophil and monocyte percentages, which represents the activity of the first and second lines of defense against the cellular damage, has been reported after mercury exposure [37].

Oliveira Ribeiro *et al.* [5] confirmed significant decrease of mononuclear (differential lymphocytes plus monocytes) and significant increased of differential neutrophil to methyl mercury and inorganic lead.

It is known that changes in leukocyte counts after exposure to pollutants may be associated to a decrease in nonspecific immunity of the fish. Oliveira Ribeiro *et al.* [5] showed decrease of leukocytes count to inorganic lead in fish *Hoplias malabaricus*.

According to Wedemeyer *et al.* [34] and Singh and Reddy [38] these findings are recognized as a sensitive indicator of stress in fish, as observed by Adhikari *et al.* [7] in L. rohita exposed to pesticides and by Chowdhury *et al.* [26] in O. mikiss chronically exposed to cadmium. The change of the leukocyte population could be related to the presence of tecidual damages such as necrosis, as previously observed in different organs exposed to mercury [39]. An immunosupression was observed in *Astyanax bimaculatus* exposed to TBT after similar doses intraperitoneally administered [40].

According to Wedemeyer *et al.* [34], the suppression of the immune system increases the susceptibility to diseases in fish, a very important aspect considering the presence of heavy metals in natural ecosystems as a result of human activities. However, result of leukocyte count in pollution exposure are different and some researcher show decrease of leukocyte count in exposure, like our study, (Reynolds *et al.* [31] for *Thomomys talpoides*; Lopes *et al.* [41] for (*Apodemus sylvaticus*).

Lohner *et al.* [42] find Leukopenia (reduced Leukocyte counts) and increase in both Neutrophils and Monocyte of Sunfish Populations (Lepomis sp.) in different creeks.

WBC abundance provides an indication of fish health and a high WBC count may indicate a subclinical infection. An extremely low WBC count indicates either suppression of circulating lymphocytes, a characteristic acute stress response, or that an active bacterial infection has induced leukocytolysis [43].

Due to the non differentiated counting of thrombocyte cells in the current work due to the difficulty to separate it from other white cells, which could be interfering in the leukocyte cells counting, this is one limitation of the use of this parameter to evaluate the effects of pollutants in fish. Although few studies have used dietary exposure to test the effects of contaminants in aquatic organisms [44], the present results showed that under experimental conditions blood parameters were sensitive to different aspects of heavy metals exposure.

In conclusion, the major findings of this study were that the sub-acute mercury concentrations tested may cause several changes in the hematological and immunological parameters of the studied fish, so estimation of these indices, could provide a useful indicator of pollution of water bodies.

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