

Effect of Inorganic Mercury on Hematological and Antioxidant Parameters on Olive Flounder *Paralichthys olivaceus*

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Abstract

The effects of inorganic mercury on hematological parameters and hepatic oxidative stress enzyme activity were studied in olive flounder *Paralichthys olivaceus*. Fish were injected twice intraperitoneally with mercuric chloride (2, 4, or 8 mg Hg/kg BW). The major hematological findings were significant decreases in the red blood cell count, hematocrit value, and hemoglobin level in olive flounder exposed to 8 mg Hg/kg BW. Remarkably low levels of calcium and chloride, and reduced osmolality, were also observed at 8 mg Hg/kg BW. In hepatic tissue, significant increases in glutathione peroxidase and catalase activity were observed above 4 mg Hg/kg BW. Inorganic mercury also increased glutathione S-transferase and glutathione reductase activity at 8 mg Hg/kg BW in hepatic tissue. The present findings suggest that exposure to a low concentration (≥ 4 mg Hg/kg BW) of inorganic mercury can cause significant changes in hematological and antioxidant parameters.

Key words: *Paralichthys olivaceus*, Inorganic mercury, Hematological parameter, Antioxidant enzyme

Introduction

Toxic heavy metals are increasingly being released into the environment as a result of industrialization. Mercury is a nonessential element that can have severe, toxic effects on aquatic animals when present in excessive amounts (Nriagu and Pacyna, 1988; Fitzgerald and Clarkson, 1991). Most of the mercury in water, sediments, or the biota is in the form of inorganic mercury salts or organic forms. Mercury has always been present at varying levels in environmental media and the biota, and all mercury is, in a sense, naturally occurring; that is, mercury is not a substance of human origin. Anthropogenic activities are thought to redistribute mercury from its original matrix through the atmosphere to other environmental media. Numerous studies have shown that the amount of mercury being deposited from the atmosphere has increased since the onset of the industrial age (Johansson et al., 1991; Nater and Grigal, 1992; Swain et al., 1992). Some mercury deposits arise

from natural sources while others are derived from anthropogenic activities.

The absorption, distribution, metabolism, and excretion of mercury depend on its form and oxidation state (Goyer, 1991). Organic mercurials are more readily absorbed than inorganic forms. An oxidation-reduction cycle is involved in the metabolism of mercury and its compounds in animals, including humans. Mercury poisoning causes necrosis in epithelial cells, epithelial hyperplasia, and Na⁺-K⁺ ATPase inhibition in fishes (Bouquegneau, 1977; Lock et al., 1981). During early embryonic development in zebrafish, elevated mercury levels cause reduced survival time and increased hatching time (Dave and Xiu, 1991). The tissue distribution of inorganic mercury in fishes varies with the route of administration (water, oral, and intraperitoneal); however, the liver and kidneys tend to accumulate the highest quantity of this metal overall (Weisbart,

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1973). Nevertheless, data on oxidative stress due to mercury exposure are lacking in fish.

Therefore, the objective of this study was to evaluate the effects of inorganic mercury delivered via an intraperitoneal injection on hematological parameters and hepatic antioxidant enzyme activity in olive flounder *Paralichthys olivaceus*.

Materials and Methods

Experimental fish

Olive flounders *Paralichthys olivaceus* were obtained from a local fish farm on Jeju Island, Korea. The fish were acclimatized for 3 weeks under laboratory conditions and their health status was evaluated prior to mercury exposure (Table 1). During the acclimation period, the fish were fed a commercial diet twice daily and maintained on a 12-h:12-h light/dark cycle at all times. After acclimatization, several fish (body length, 19.3 ± 1.2; body weight [BW], 53 ± 2.9 g) were selected for further study.

Exposure conditions

Mercury exposure took place in 0.5-ton fiberglass-reinforced plastic tanks containing 25 fish per treatment group. Each tank received a flow of 7 L min⁻¹ with continuous aeration. Mercury(II) chloride (Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) immediately before intraperitoneal injection. The fish were injected with 2, 4, or 8 mg Hg/kg BW as mercury chloride. The first injection was given 3 weeks after acclimatization; the second was given 1 week after the first treatment. The control group was subjected to the same regimen; however, they were injected with

an equal volume of PBS. Blood and hepatic tissue samples were taken to examine several hematological and antioxidant parameters at 1 and 2 weeks post injection.

Hematological parameter analysis

Blood samples were collected within 35-40 s through the caudal vein of the fish in 0.5-mL disposable heparinized syringes using a 30-gauge needle. The syringes were kept at 4°C until the blood parameters were completely studied. The total red blood cell (RBC) count, hemoglobin (Hb) concentration, and hematocrit (Ht) value were determined immediately. The blood samples were centrifuged to separate erythrocytes from serum at 3,000 g for 15 min at 4°C. Total RBC counts were made according to Klontz (1979) using modified Yokoyama diluting fluid and a Spencer Bright-Line hemocytometer. The Hb concentration was determined using the Drabkin and Austin cyanmethemoglobin technique (Kit 525; Sigma). The Ht value was determined by the microhematocrit centrifugation technique. The serum samples were analyzed for inorganic phosphorus by the ultraviolet method (Kit 360; Sigma) and for calcium (Kit 588; Sigma), magnesium (Kit 595; Sigma), and chloride (Kit 461; Sigma) by the colorimetric method. Plasma osmolality was measured directly using 20 µL of sample on a Micro-Osmometer (Model 3300; Advanced Instruments, Inc., Norwood, MA, USA).

Antioxidant enzyme analysis

Liver tissues were excised and homogenized with 5 volumes of ice-cold homogenization buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DL-dithiothreitol, and 150 mM NaCl) with several strokes using a Teflon pestle (099CK4424; Glas-Col, Terre Haute, IN, USA). The homogenate was centrifuged at 12,000 g for 20 min under refrigeration and the obtained supernatants were stored at -80°C for analysis. Glutathione peroxidase (GPx) activity was measured according to the method of Paglia and Valentine (1967) using cumene hydroperoxide as the substrate. To measure glutathione-S-transferase (GST) activity, the 1-chloro-2,4-dinitrobenzene (CDNB) method was used (Habig et al., 1974). Glutathione reductase (GR) activity was assessed by monitoring the oxidation of NADPH initiated by oxidized glutathione addition at 37°C (Cribb et al., 1989). Catalase (CAT) activity was measured as described by Johansson and Borg (1988).

Statistics

Statistical analyses were performed using the SPSS/PC+ statistical package (SPSS Inc., Chicago, IL, USA). Significant differences between groups were identified using one-way ANOVA and Duncan's test for multiple comparisons or Student's *t*-test for two groups (Duncan, 1955). The significance level was set at $P < 0.05$.

Table 1. Chemical components of seawater used in the acclimation period of olive flounder *Paralichthys olivaceus*

Parameter	Value
Temperature (°C)	18.21 ± 0.30
pH	8.10 ± 0.23
Salinity (‰)	31.82 ± 0.52
Ammonia (µg-at/L)	13.65 ± 1.34
Nitrite (µg-at/L)	2.28 ± 0.26
Nitrate (µg-at/L)	9.82 ± 1.12
Phosphate (µg-at/L)	6.42 ± 0.78
SS (mg/L)	4.84 ± 0.18
DO (mg/L)	6.95 ± 0.43
COD (mg/L)	1.08 ± 0.04

Results

Hematological properties

The RBC count, Hb concentration, and Ht value of olive flounders exposed to different levels of inorganic mercury are summarized in Table 2. The major hematological findings were a significant decrease in the RBC count and Hb concentration in olive flounders exposed to >4 mg Hg/kg BW compared with the control group after 2 weeks ($P < 0.05$). The Ht value following inorganic mercury exposure for 2 weeks was also decreased in fish administered 8 mg Hg/kg BW compared with the control group ($P < 0.05$).

Inorganic components

The blood serum inorganic components of olive flounder treated with inorganic mercury are shown in Table 3. No significant differences were observed in serum phosphorous or

magnesium among the treatment groups. However, a clear decreasing trend was noted in serum calcium and chloride at 8 and ≥ 4 mg Hg/kg BW, respectively, compared to the controls after 2 weeks ($P < 0.05$). The control group maintained a normal blood osmolality (between 242 and 248 mOsm/kg). However, at 1 and 2 weeks, the blood osmolality in the fish exposed to >4 mg Hg/kg BW had significantly decreased compared to the controls ($P < 0.05$).

Antioxidant enzymes

The hepatic GPx, GST, GR, and CAT activity levels in fish exposed to inorganic mercury are presented in Fig. 1. GPx, GST, GR, and CAT activity in olive flounders treated with inorganic mercury at a concentration of >2 mg Hg/kg BW increased in a dose- and time-dependent manner. GPx and CAT activity in olive flounders exposed to inorganic mercury at concentrations ≥ 4 mg Hg/kg BW increased significantly compared to the controls after 2 weeks ($P < 0.05$). A significant in-

Table 2. Hematological parameters of olive flounder *Paralichthys olivaceus* exposed to the various inorganic mercury concentrations

Hematological parameters	Exposure period (week)	Mercury dose (mg Hg/kg BW)			
		0	2	4	8
RBC count ($\times 10^4/\text{mm}^3$)	1	152.5 \pm 5.1 ^a	148.2 \pm 6.9 ^a	141.6 \pm 7.5 ^{ab}	134.8 \pm 4.2 ^b
	2	147.3 \pm 4.2 ^a	150.5 \pm 4.1 ^a	136.4 \pm 5.5 ^b	129.2 \pm 3.7 ^b
Hb (g/dL)	1	9.4 \pm 0.9 ^a	9.8 \pm 1.1 ^a	7.9 \pm 0.6 ^{ab}	6.8 \pm 1.0 ^{bc}
	2	9.6 \pm 0.7 ^a	8.7 \pm 1.2 ^{ab}	7.1 \pm 0.5 ^{bc}	5.9 \pm 0.9 ^c
Ht (%)	1	18.1 \pm 2.1 ^a	17.1 \pm 1.4 ^a	15.7 \pm 0.9 ^{ab}	14.1 \pm 1.1 ^b
	2	17.7 \pm 1.8 ^a	16.5 \pm 1.2 ^a	15.1 \pm 1.6 ^{ab}	13.7 \pm 0.8 ^b

All data are expressed as mean \pm SE. Values with different superscripts in each parameter are significantly differences ($P < 0.05$) as determined by Duncan's multiple range test. RBC, red blood cell count; Hb, hemoglobin; Ht, hematocrit.

Table 3. Inorganic components and osmolality of olive flounder *Paralichthys olivaceus* exposed to various inorganic mercury concentrations

Inorganic components	Exposure period (week)	Mercury dose (mg Hg/kg BW)			
		0	2	4	8
Phosphorous (mmol/L)	1	2.52 \pm 0.15 ^a	2.43 \pm 0.09 ^a	2.58 \pm 0.09 ^a	2.47 \pm 0.12 ^a
	2	2.56 \pm 0.28 ^a	2.62 \pm 0.21 ^a	2.38 \pm 0.35 ^a	2.42 \pm 0.16 ^a
Calcium (mmol/L)	1	2.72 \pm 0.12 ^a	2.80 \pm 0.37 ^a	2.63 \pm 0.14 ^a	2.03 \pm 0.11 ^b
	2	2.69 \pm 0.25 ^a	2.76 \pm 0.25 ^a	2.32 \pm 0.21 ^{ab}	1.93 \pm 0.22 ^b
Magnesium (mmol/L)	1	1.45 \pm 0.15 ^a	1.56 \pm 0.30 ^a	1.67 \pm 0.22 ^a	1.51 \pm 0.16 ^a
	2	1.62 \pm 0.23 ^a	1.49 \pm 0.25 ^a	1.58 \pm 0.19 ^a	1.48 \pm 0.32 ^a
Chloride (mmol/L)	1	139.2 \pm 4.8 ^a	136.3 \pm 5.3 ^a	129.7 \pm 5.8 ^{ab}	123.5 \pm 5.4 ^{bc}
	2	141.4 \pm 7.2 ^a	134.8 \pm 4.6 ^a	122.2 \pm 4.2 ^{bc}	117.6 \pm 4.6 ^c
Osmol (mOsm/kg)	1	242.6 \pm 7.5 ^a	245.6 \pm 6.5 ^a	226.6 \pm 4.5 ^b	203.6 \pm 6.3 ^c
	2	248.4 \pm 8.3 ^a	234.4 \pm 7.2 ^{ab}	218.4 \pm 6.4 ^{bc}	199.4 \pm 5.6 ^c

All data are expressed as mean \pm SE. Values with different superscripts in each organic components are significantly differences ($P < 0.05$) as determined by Duncan's multiple range test.

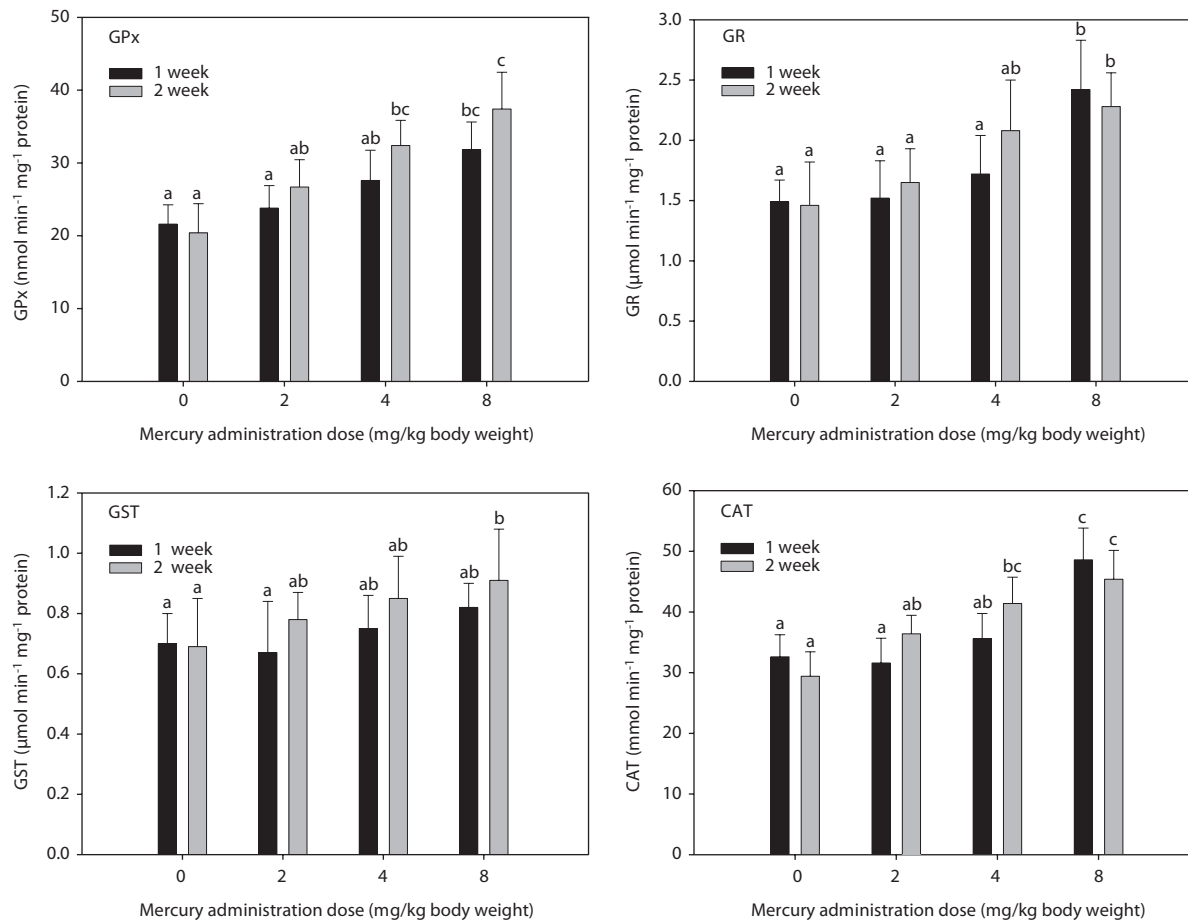


Fig. 1. Variations of glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR) and catalase (CAT) activities in the liver of olive flounder *Paralichthys olivaceus* exposed to the various inorganic mercury concentrations. All data are expressed as mean \pm SE. Different letters indicates significant differences ($P < 0.05$) between the groups.

crease in GST activity was observed at 8 mg Hg/kg BW after 2 weeks ($P < 0.05$). GR activity also increased at 8 mg Hg/kg BW after 1 week ($P < 0.05$).

Discussion

The blood properties of fish are suitable biomarkers for evaluating the potential risk of chemicals (Roche and Bogé, 1996). Past investigators have identified changes in several hematological variables as indicators of heavy metal exposure (Cyriac et al., 1989). The predominant hematological findings in this study were a significant decrease in the RBC count and Hb concentration in olive flounders exposed to inorganic mercury at >4 mg Hg/kg BW. The Ht value in fish following inorganic mercury exposure to 8 mg Hg/kg BW for 2 weeks also decreased. Chronic exposure of fish to heavy metals causes serve reductions in the RBC count, Ht, and Hb concentration (Tewari et al., 1987). Mercury has been shown to cause ane-

mia in fish by inhibiting Hb synthesis and shortening the life span of circulating erythrocytes (Houston et al., 1993). These results are in accordance with those of mercury-exposed *Dicentrarchus labrax* (Gwoździński et al., 1992), *Oreochromis aureus* (Allen, 1994), and *Aphanius dispar* (Hilmy et al., 1980), which exhibited higher mercury concentration-induced anemia. Our studies provide evidence that inorganic mercury affects hemolysis. The observed decline in RBC count, Hb concentration, and Ht presumably reflect hemolysis and/or irreparable damage to gill morphology and function (Gupta and Dua, 2002).

No significant differences in serum phosphorous or magnesium were detected among the treatment groups. However, a significant decrease in calcium and chloride was observed at 8 and ≥ 4 mg Hg/kg BW, respectively, at 2 weeks. Previous laboratory studies have documented the inhibitory effects of various metals on gill function in fish (Evans, 1987; Watson and Benson, 1987). Indeed, the gills of freshwater teleosts function as the primary site for the active absorption of ions

from the external medium and for the exchange of respiratory gases. Mercury can cause altered osmoregulation in both marine and freshwater fish. In this study, the blood osmolality of olive flounder exposed to >4 mg Hg/kg BW was significantly decreased compared to the controls. This result can be explained by direct mercury-induced osmoregulation failure. Lock et al. (1981) suggested that mercury caused osmoregulatory effects primarily by increasing gill permeability to water. Stinson and Mallatt (1989) reported increased permeability of the gills in lamprey in response to mercury poisoning.

The main enzymes that detoxify reactive oxygen species in all organisms are GPx (EC 1.11.1.9), peroxidase (EC 1.11.1.7), and CAT (EC 1.11.1.6), all of which are abundant in fish tissue (Di Giulio et al., 1993). These enzymes, which are induced by reactive oxygen species, may be useful indicators of oxidative stress. The phase II system and endogenous cellular glutathione have received relatively little attention as environmental indicators (Stein et al., 1992). The induction of antioxidants is a sensitive early warning signal of incipient oxidative stress (Benson and Di Giulio, 1992). GST has been identified in all organisms in which it has been investigated (Dierickx, 1984; Stenersen et al., 1987), and it seems likely that it is ubiquitous. GST activity is known to increase in rats exposed to polychlorinated biphenyls (Kamohara et al., 1984). The present findings corroborate the observations of Davies (1985) and Fair (1986) in fish exposed to chlorothalonil and benzo(a)pyrene, respectively.

Similarly, in a previous study, cadmium exposure in rainbow trout for 4 weeks caused an initial decrease followed by a net increase in hepatic GST (Förlin et al., 1986). In this study, hepatic GST activity in olive flounders toward CDNB after 2 weeks of exposure to 8 mg Hg/kg BW was markedly elevated. These results demonstrate that GST activity was significantly altered by treatment with inorganic mercury. The status of other antioxidant systems is variably affected by polycyclic aromatic hydrocarbon (PAH) exposure (Niyogi et al., 2001; Veignie et al., 2004).

In this study, hepatic GR activity was significantly higher in fish exposed to 8 mg Hg/kg BW after 1 week compared to the controls. Similar results were reported by Stephensen et al. (2003), who observed increased hepatic GR activity in fish exposed to PAHs under laboratory conditions as well as increased GR activity in fish inhabiting a PAH-polluted harbor. GPx is the most important peroxidase for the detoxification of hydroperoxides. It catalyzes the glutathione-dependent reduction of hydroperoxides and hydrogen peroxide (H₂O₂). GPx activity may be induced by environmental pollutants. Its activity increased together with GR in rainbow trout injected with tetrachlorobiphenyl (Otto and Moon, 1995) and carp exposed to copper, but not to paraquat (Matkovics et al., 1987). In this study, hepatic GPx and CAT activity in olive flounders exposed to inorganic mercury at ≥4 mg Hg/kg BW was significantly elevated compared to the controls after 2 weeks. GPx has been postulated to protect erythrocytes from damage by

H₂O₂ and to be responsible for the reduction of lipid hydroperoxides. Therefore, it is hypothesized that this enzyme protects tissue against oxidative damage due to lipid peroxidation. The liver is a major site of detoxification and the first target of ingested oxidants; thus, it is considered to be an important tissue in the study of the protective role of GPx in lipid peroxidation. CAT occurs primarily in peroxisomes. Its activity increased together with other peroxisomal enzymes in fish liver upon exposure to bleached kraft mill effluents, suggesting the induction of peroxisome proliferation (Mather-Mihaich and Di Giulio, 1991). Some pollutants may inhibit CAT activity. High concentrations of copper were shown to inhibit CAT activity in liver, gill, and muscle; a similar effect was induced by 100 ppm of ZnSO₄ in gill and muscle (Radi and Matkovics, 1988).

We conclude that exposure to a low concentration (≥4 mg Hg/kg BW) of inorganic mercury resulted in significant changes in several hematological and antioxidant parameters.

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