

Research Paper:

The Effects of Lead Toxicity on the Hematological Parameters Common Carp (*Cyprinus Carpio*) at Varying Salinity Levels



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ABSTRACT

Background: Lead (Pb) is an important metal used in industries, mining and agriculture. It is equally important to the aquatic ecosystems and has negative impact on the health of aquatic organisms.

Methods: The LC₅₀ of lead toxicity at varying salinities was first determined for 96 hours. Then juvenile fish were transferred to tanks individually and were exposed to 0, 15, 30, 60 and 120 mg/l of Pb at varying salt concentration of 0, 0.1, 0.2 or 0.4 g/l. The blood samples were taken after the 4th day and the hematological parameters were evaluated.

Results: Red Blood Cells (RBC) and White Blood Cells (WBC) were decreased in almost all lead treated groups. The Hb levels decreased consistent with increasing lead concentrations (P<0.05). The mean corpuscular volume, hemoglobin and the concentration were not affected by the lead concentrations compared to those for the control group. The WBC and RBC counts showed an inverse correlation with the lead concentrations in the Pb-exposed fish. A direct correlation was also observed in the lead concentration and the heterophile rate (P<0.05).

Conclusion: The results showed that salinity did not affect the hematological parameters, but the LC₅₀ values decreased with increasing salinity.

Keywords: Hematological parameters; Common carp; Heavy metal; Erythrocytes; Leukocytes

Introduction

Lead is a common environmental pollutant used in many industries and enters the aquatic environment with effluents from mines, smelters, and chemical factories manufacturing pigments, paints, plastics, etc. [1, 2]. Lead toxicity has been reported in liver, muscles, kidneys, brain and gonads in common carp [3]. Moreover, the toxicity has been reported in this fish muscles and liver in Gorgan bay and Gomishan marsh, in Iran [4]. Also, lead has been detected in fish food in many situations [5]. Lead has caused pathological alterations in fish erythrocyte [6], cellular membranes [7],

and often induces anemia in fish [8, 9]. This heavy metal has an extremely high affinity for erythrocytes [10, 11]. In fish and other aquatic animals, lead is responsible for direct erythrocytic injuries, basophilic stripping and inhibition of a key enzyme involved in hemoglobin formation, i.e. delta-aminolevulinic acid (ALA-D) [12]. Further, lymphocytosis and neutropenia have been reported in eel, *Anguilla*, at 0.3 mg/l Pb [13].

Similarly, Olanike et al. [8] observed a rise in lymphocyte percentage and a decline in neutrophils accompanied by a decrease in White Blood Cells (WBC) in African catfish (*Clarias gariepinus*) that were subjected to 25-250 mg/l of Pb for four days. Conversely, two

other studies [14-16] have reported an increase in WBC in lead-exposed fish including common carps. Another study showed that leukocyte counts in Pb-exposed carps increased shortly followed by a decrease in WBC counts and lymphocytosis [16].

It is believed that Pb-induced tissue damage may activate the immune response in fish [13]. Salinity is one of the important factors in the aquatic ecosystem that affects fish growth and survival [17], and may cause a variety of physiological stress responses [18]. Alteration in water salinity also affects the Pb availability and subsequent toxicity by competing with metal ions for binding to sites on many biological molecules [19]. Ijeoma et al. [20] reported that in the fresh water at a salinity level of 2 ppt the Pb toxicity was higher than that observed for salinity at 12-18 ppt.

The bioavailability of Pb to aquatic organisms is dependent on the physical and chemical forms of this heavy metal [21, 22]. In this context, a decrease in the salinity level increases the toxicity associated with Pb bound to the assayed sediments. This increase permits a more readily transport of Pb through the plasma membranes and causes an increase in Metallothionein (MTs) [23]. Various physiological parameters have been used to investigate the sublethal toxicity of heavy metals in freshwater fish. One practical parameter is hematology, which provides a measure of the fish physiology relative to changes in the aquatic environment. Therefore, we conducted this study to examine the effects of varying salinity levels on lead toxicity in common carp, followed by evaluating the hematological parameters.

Materials and Methods

Fish and Treatment: The experiments were conducted in the fall of 2015 on healthy common carp ($n=210$), weighing 21 ± 2 grams. The fish were obtained from the hatchery pond of the Inland Fisheries Institute in Ahvaz, Iran. They were acclimated to the laboratory setting for 2 weeks in an aerated flow-through tank, at $24\pm 1^\circ$ C, and dissolved oxygen

saturation 70%-80%, supplied, at the density of 10 g/l. The Pb concentrations were set at 15, 30, 60 and 120 mg/l at each salinity level. Fish were fed daily with standard food (Faradaneh Co. Ahvaz, Iran) at the rate of 1% of the stock mass during the acclimation period.

Acute Toxicity Experiments at Varying Salinity Levels: All ethical considerations regarding the fish treatment were observed as set by the university guidelines. In addition we followed the Organization for Economic Cooperation and Development (OECD) Guideline No. 203 test conditions. The juvenile Common carp ($n=210$) were exposed to acute toxicity tests at varying salinity levels. The experimental Pb solutions were prepared, using the material Pb ($C_2H_3O_2$)₂; Kimia Goštar Pouyesh Co., Ahvaz, Iran). The concentrations of lead tested at varying salinity levels are shown in Table 1. The lead concentrations were selected according to a pilot study and 96 h LC_{50} value for common carp, reported as $15\mu g/l$ in water by the U.S. Environmental Protection Agency (USEPA) (Table 1). Ten fish were kept in each tank and the mortality rate was monitored daily for the initial four consecutive days. Thereafter, the 96 h LC_{50} was conducted, using probit regression analysis [24] (Table 2).

Hematological Parameters: Based on the LC_{50} results of lead test at varying salinity levels, four Pb concentrations were selected for the evaluation of hematological parameters: 15, 30, 60 and 120 mg/l at identical salinities used for the LC_{50} test, i.e. 0, 0.1, 0.2 and 0.4 g/l of Pb added to pure water. The fish were kept in groups of 10 per tank. Control group was subjected to the same treatment except for the Pb exposure. Blood samples were collected from the caudal peduncle vein, using heparinized needles, and kept in heparinized eppendorf tubes after 96 h of Pb exposure [24].

The following hematological parameters were evaluated and compared among the fish groups: hematocrit (Ht), erythrocytes count (RBC: Red Blood Cells), Blood Hemoglobin concentration (Hb), Packed Cell Value (PCV), White Blood Cell (WBC), and the differential WBC counts.

Table 1. Lead Concentration at varying salinities

Salinity (ppt)	Cyprinus carpio																					
	0			0.1			0.2			0.4												
Concentration lead (mg/l)	0	3.75	7.5	15	30	60	0	7.5	15	30	60	0	15	30	60	120	0	30	60	90	120	150
Fish number	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Table 2. Lethal concentrations of Lead vs. Salinity over time

Salinity	Lead (Pb)	Exposure Time (hr)			
	Lethal Concentration (mg/l)	24	48	72	96
0 ppt	LC ₁₀	9.17	6.26	5.20	3.5
	LC ₃₀	16.85	11.85	9.51	7.1
	LC ₅₀	25.56	18.42	14.45	11.56
	LC ₇₀	38.88	28.64	21.96	18.85
	LC ₉₀	71.24	54.15	40.194	37.98
	LC ₁₀₀	164.32	130.41	92.53	101.02
0.1 ppt	LC ₁₀	42.19	30.2	12.53	8.53
	LC ₃₀	51.93	40.23	23.7	16.15
	LC ₅₀	59.96	49.09	36.84	25.116
	LC ₇₀	69.22	59.89	57.27	39.06
	LC ₉₀	85.2	79.79	108.3	73.91
	LC ₁₀₀	113.46	118.55	260.82	178.17
0.2 ppt	LC ₁₀	113.94	59.18	43.4	40.93
	LC ₃₀	211.51	127.55	84.81	59.26
	LC ₅₀	324.62	217.1	134.88	76.57
	LC ₇₀	498.23	369.51	214.53	98.94
	LC ₉₀	924.6	796.2	419.22	143.25
	LC ₁₀₀	2171.4	2298	1056.6	238.71
0.4 ppt	LC ₁₀	125.37	85.75	74.42	59.19
	LC ₃₀	234.59	129.23	103.47	77.8
	LC ₅₀	362.04	171.67	130.01	97.46
	LC ₇₀	558.72	228.06	163.35	122.07
	LC ₉₀	1045.2	343.67	227.12	169
	LC ₁₀₀	2481.6	605.4	357.9	264.73

Additionally, erythrocyte indices were calculated, including Mean Cell Volume (MCV), Mean Cell Hemoglobin (MCH), and Mean Corpuscular Hemoglobin Concentration (MCHC). The PCV was measured, using the microhematocrit method in heparinized capillaries after centrifugation at 11,000 rpm for 5 min. The RBC and WBC counts

were determined, using a hemocytometer unit with the blood sample diluted 200 times with natheric solution.

The Hb level was measured in a spectrophotometer at 540 nm, using the cyanmet-hemoglobin method [24]. The derived parameters were calculated according to the following formulas (1, 2 & 3):

$$1. MCV = (Ht \times 10) / RBC$$

$$2. MCH = Hb / RBC$$

$$3. MCHC = (Hb \times 100) / Ht$$

Differential leukocyte counts were performed at 1000 magnification, using a Nikon Eclipse 300 light microscope on blood smears stained with Giemsa solution, and the results were presented in percentages. One hundred leukocytes were examined per blood smear [24].

Statistical Analyses: Statistical analyses were performed using SPSS V. 19 software and the data were presented as Mean \pm SD. The data were tested for normality (Kolmogorov–Smirnov test), and analyzed by one-way Analysis of Variance (ANOVA). The significant means were analyzed by Tukey's test and a $P < 0.05$ was considered to be statistically significant.

Results

Swimming at surface, erratic swimming (in vertical motions) and lethargy were the main behavioral changes observed in Pb-exposed fish throughout the experiment. These behavioral changes were mostly noted at high lead concentrations.

Acute Lead Toxicity vs. Salinity: All of the fish were kept in the tank at 60mg/l of lead concentration and salinity of 0 and 0.1 ppt (0 and 0.1 g/l). Another group of fish were kept at lead concentration 120mg/l and salinity of 0 and 0.1 ppt (0 and 0.1 g/l). LC_{50} showed that the increased in salinity led to an increase in toxicity (Table 2).

Hematological Parameters vs. Lead Exposure: Based on the data shown in Table 3, the RBC count did not show a significant change compared to that for the control group, with the exception of the fish kept at 60 ppm Pb and 0.1 ppt salinity. The WBC and Hb values decreased with the increasing lead concentrations. Further, most changes in PCV, MCV and MCHC values were seen at a salinity of 0.1, 0.2 or 0.4 ppt and Pb concentrations at 15 and 60 ppm.

The differential leukocyte count showed significant changes after exposure of the fish to certain Pb concentrations (Table 4). The leukocyte count in Pb-exposed fish gradually declined with the increasing lead concentration. The percentages of heterophiles were increased significantly at 0.4 ppt salinity, and 30, 60 and 120 ppm of Pb. Monocytes, basophils and eosinophils percentages were approximately less than 1%, except for those

determined at 0.1 ppt salinity and 60 ppm Pb concentrations (Tables 3 & 4).

Discussion

The present data showed that exposure of the fish to both salinity and lead caused variations in the hematological parameters. Johansson-Sjoberck and Larsson reported that reductions in RBC counts and Hb values accompanied by increased hematopoietic rates in rainbow trout after exposure to lead [25]. Moreover, hematological changes following exposure to lead have also been documented in other fish species. Examples of these are anemia in *Salvelinus fontinalis* [26], *Colisa fasciatus* [27], and *Salmo gairdneri* [25], and a significant elevation in the RBC count in *Salmo gairdneri* [28] and *Oreochromis aureus* [29]. Additional examples are RBC fragility and hemorrhage leading to hemolysis, damage to the hematopoietic tissues, and impairment in hemoglobin concentration [14]. Witeska et al. [16] showed that a short-term exposure of common carp to Pb resulted in moderate and transient alterations in WBCs, causing a temporary immune disturbance in the fish.

Also Malgorzata et al. [24] evaluated the effect of a short-term (3 h) exposure to 10mg/l of Pb (96 h; LC_{50}) on the hematological parameters in common carp. In that study, the RBC parameters fluctuated over the experimental period with increasing frequency in morphological anomalies. Their observations suggest that lead disturbed the immune response in carp. In our study, a short-term exposure of common carp to Pb resulted in moderate and transient alterations in WBCs, causing a temporary immune disturbance.

This study demonstrated that the RBC counts decreased in all experimental groups except for the group that was treated at 0.1 ppt salinity and 100 ppm lead concentrations, in which the RBC level increased significantly (Table 3). In the latter group, the PCV was 35%, which might be due to the fish being in shortage of oxygen present in the water with low salinity and high lead concentrations. Similarly, the Hb value decreased with increasing lead concentration (Table 3). In addition, hemolysis was observed at high lead concentrations and low salinity levels. The most well-known effect of lead is the inhibition of δ -aminolevulinic acid dehydratase activity (ALA-D), an enzyme that catalyzes the formation of porphobilinogen from aminolevulinic acid. The disturbed hemoglobin synthesis due to the effect of lead on ALA-D might have also resulted in anemia in the fish [13].

Table 3. Changes in blood parameters on the 4th day of exposure to Lead at varying salinities

Blood Parameter	Salinity (ppt)	Lead (ppm)						
		Mean±SD			60		120	
		0	15	30	Mean±SD	Dead (%)	Mean±SD	Dead (%)
RBC (106/ μ l)	0	1.26±0.1	1.24±0.2	1.81±0.4	-	100	-	100
	0.1	1.58±0.1	1.33±0.4	1.26±0.2	8.45±0.3*	-	-	100
	0.2	1.73±0.3	1.07±0.7*	1.52±0.1	1.26±0.5	-	1.51±0.5	-
	0.4	1.88±0.5	1.38±0.6	1.72±0.2	1.50±0.1	-	1.12±0.2*	-
WBC (103/ μ l)	0	56±5.6	14.66±9.2*	18±8.5*	-	100	-	100
	0.1	59.5±16.3	42.66±12.2	45.33±44.1	26±2.8*	-	-	100
	0.2	64±5.6	21.33±23.4*	33.33±6.1	16±4*	-	20±17.0*	-
	0.4	66±42.4	16±17.4*	30.66±14.0	18.66±11.5*	-	13.33±2.3*	-
Hb (g/l)	0	125±2	105±16	97±4*	-	100	-	100
	0.1	141±15.5	112.7±6.4	109.7±13.9	65±43.8*	-	-	100
	0.2	139.5±27.6	140±24.5	123.7±5.5	111±11.1	-	111.3±15.0	-
	0.4	135.5±6.4	121±6.1	133±7.2	126.3±6.0	-	97.3±10.3*	-
PCV (%)	0	25±2.8	19.33±2.5	29.66±1.5	-	100	-	100
	0.1	23.5±0.7	20±0	19.66±2.1	35±1.4*	-	-	100
	0.2	24.5±2.1	36.66±5.8*	22.33±1.5	21.33±1.1	-	20.33±3.2	-
	0.4	26.5±2.1	34.66±4.6*	24.33±1.1	22.66±1.1	-	18.66±1.5	-
MCV (fl)	0	198.35±0.2	150.65±1.5	172.45±35.1	-	100	-	100
	0.1	146.53±4.0	150.49±5.1	155.66±4.5	432.33±177.1*	-	-	100
	0.2	144.37±12.7	492.48±367.5*	146.86±4.4	168.66±12.8	-	146.44±15.7	-
	0.4	140.48±7.6	276.54±97.9*	141.95±8.0	151.19±6.6	-	167.93±12.9	-
MCH (pg)	0	100±28.3	85.33±2.1	54.7±10.0	-	100	-	100
	0.1	88.5±3.5	84.66±7.4	86.66±0.6	89±79.2	-	-	100
	0.2	82.5±4.9	187±156.6*	80.66±4.2	87.33±9.0	-	80.33±11.0	-
	0.4	79.5±4.9	99.33±42.5	77.3±3.1	84.33±5.0	-	86.66±4.6	-
MCHC (g/l)	0	507.5±142.1	543.5±31	317.9±6.5	-	100	-	100
	0.1	600.5±84.1	563.33±32.1	554.97±9.3	188.37±132.8*	-	-	100
	0.2	512.75±12.6	388.6±89.1	382.9±282.1	519.5±28.0	-	548.95±26.4	-
	0.4	563.5±4.9	353.5±55.2	544.47±5.1	555.8±14.2	-	520.7±13.0	-

* Values significantly different from those found for the controls (Tukey's test at P<0.05)

100% Dead: Denotes no hematological parameters detected in the dead fish

Table 4. Changes in Leukocyte populations on the 4th day of exposure to lead at various salinities

Leukocyte Population	Salinity (ppt)	Lead (ppm)						
		Mean±SD/ Sig.			60		120	
		0	15	30	Mean±SD/ Sig.	Dead %	Mean±SD/ Sig.	Dead %
Lymphocyte	0	86.33±5.5	49.33±17.9*	57±15.5*	-	100	-	100
	0.1	88.33±3.8	79.33±20.1*	93.5±4.9	37±18.4*	-	-	100
	0.2	93.33±1.5	24±12.7*	96.5±3.5	95±3.5	-	93±1	-
	0.4	91±4.6	41.66±20.4*	100±0*	100±0*	-	100±0*	-
Heterophil	0	13.66±5.5	47.33±15.1*	43±15.5*	-	100	-	100
	0.1	11.66±3.8	19.33±20.5*	6.5±4.9	42±42.4*	-	-	100
	0.2	6.66±1.5	76±12.7*	3.5±3.5	5±3.5	-	17±1*	-
	0.4	8.66±4.2	52±30.3*	0±0*	0±0*	-	0±0*	-
Monocyte	0	0±0	0±0	0±0	-	100	-	100
	0.1	0±0	0±0	0±0	19±26.9*	-	-	100
	0.2	0±0	0±0	0±0	0±0	-	0±0	-
	0.4	0.330±0.6	0±0	0±0	0±0	-	0±0	-
Basophil	0	<0.01	3.33±3.0*	<0.01	-	100	-	100
	0.1	<0.01	<0.01	<0.01	0.66±1.1	-	-	100
	0.2	<0.01	<0.01	<0.01	<0.01	-	<0.01	-
	0.4	<0.01	0.33±0.6	<0.01	<0.01	-	<0.01	-
Eosinophil	0	<0.01	<0.01	<0.01	-	100	-	100
	0.1	<0.01	<0.01	<0.01	<0.01	-	-	100
	0.2	<0.01	<0.01	<0.01	<0.01	-	<0.01	-
	0.4	<0.01	<0.01	<0.01	<0.01	-	<0.01	-

* Values significantly different from those found for the control group (Tukey's test, P<0.05)

100% Dead: Denotes no hematological parameters detected in the dead fish

Shah and Altindag reported that exposures to lethal concentrations of Hg and Cd resulted in a significant WBC reduction, while exposure to Pb caused a slight increase only. These findings suggest that the order of toxicity of these metals on the WBC of the fish are Hg > Cd > Pb [14]. In this study, we documented a significant decrease for WBCs as well (Table 3). We did not observe similar findings at varying salinity levels, which did not affect the hematological parameters in our study. Further, the LC₅₀ decreased with increasing salinity concentration. The rate of packed cell value did not change significantly compared to that in the control group, except in group 3. The MCV, MCH and MCHC

parameters did not vary significantly compared to that for the control group (Table 3).

In another study [16], transient leukopenia and neutropenia were observed and the reduced phagocytes' activity was similar to those found in the current study (Table 4). Phagocytosis in fish is the most important and potent immune mechanism [30]. A previous study reported a suppressor effect for high Pb levels (5.0-100.0 mg/l) on the activity of carp phagocytes in vitro [16]. However, Witeska and Wakulska [30] observed no significant effects for Pb on the fish at 1-10 mg/l. Similarly to our findings, Pb at 15-120 ppm caused a gradual decrease in leuko-

cyte count while enhancing heterophile and phagocytes' activities. Lastly, we did not observe significant changes in the basophils, eosinophils and monocytes activities.

Conclusions

There is evidence that both salinity and lead concentrations cause pathologic alterations to the hematological parameters in common carp. RBC and Hb levels were decreased with increases in lead concentration. Also, hemolysis was observed in the fish at high lead and low salinity concentrations. Our findings demonstrated that salinity did not affect the hematological parameters; however, the LC_{50} decreased with increasing salinity. Other studies also suggest the necessity for using various fish species in order to evaluate the dose-response versus Pb toxicity. This will help elucidate the sensitivity of different fish species to Pb toxicity.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article. The participants were informed about the purpose of the research and its implementation stages; they were also assured about the confidentiality of their information; Moreover, They were allowed to leave the study whenever they wish, and if desired, the results of the research would be available to them.

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Author's contributions

Conceptualization and methodology: Zahra Tulaby Dezfuly, Mojtaba Amir Aramoon and Mojtaba Alishahi; Formal analysis, investigation: Mostafa Halimi; Writing-original draft preparation and writing-review & editing: Roya Rahnama.

Conflict of interest

The authors declared no conflict of interests.

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