IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Human Journals
Research Article
January 2018 Vol.:8, Issue:3

Impact of Lead Nitrate on the Haematological, Biochemical and Immunological Response of the Freshwater Fish, *Cirrhinus mrigala*

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INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
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Nimmy MV, Pawlin Vasanthi Joseph*

Department of Zoology Nirmala College for Women (Autonomous) Coimbatore-641018 Tamilnadu, India

Submission: 29 December 2017
Accepted: 5 January 2018
Published: 30 January 2018

Keywords: *Cirrhinus mrigala*, hematological parameters, biochemical indices, IgM, lead nitrate, platelet count, erythrocyte indices.

ABSTRACT

When wastewater is discharged into receiving water bodies without removal of heavy metals, it may be harmful to both human and aquatic life as they are non-degradable and persistent. The aim of the present work is to determine the effects of subchronic doses of inorganic Lead nitrate on the hematological parameters, biochemical indices and the immunological response of the inland fish *Cirrhinus mrigala*. The LC50 at 96 hrs was determined by the Probit analysis method (Finney 1971). The experiment was designed to expose the fish to different subchronic doses of lead nitrate (4mg/l, 6mg/l, and 8mg/l). One trough served as the control. Each trough contained ten fishes and the experiment was conducted in triplicate. The hematological profile of the control and treated fish included Haemoglobin, RBC, WBC, PCV, platelet counts in the whole blood of *Cirrhinus mrigala*. Erythrocyte indices of fish viz., MCV, MCH and MCHC were also calculated. Glucose, Protein, Albumin and Globulin were estimated in the whole blood. The IgM was derived by the ELISA technique. A significant decrease was observed in the RBC count, Haemoglobin, PCV, MCV, MCH, MCHC, proteins, albumin and globulin and the significant increase was observed in WBC count, platelet count at higher concentration, Glucose and IgM. It is abundantly clear that metals induce an early response in the fish as evidenced by alterations both at structural and functional levels of different organs, thereby affecting the innate immune system of exposed fish and increasing susceptibility to diseases.

INTRODUCTION

When wastewater is discharged into receiving water bodies without removal of heavy metals, it may be harmful to both human and aquatic life as they are non-degradable and persistent. Heavy metals are considered to be the following elements: Copper, Silver, Zinc, Cadmium, Gold, Mercury, Lead, Chromium, Iron, Nickel, Tin, Arsenic, Selenium, Molybdenum, Cobalt, Manganese, and Aluminum.

The capacity of heavy metals to form stable and irreversible co-ordination complexes with oxygen, sulfur and nitrogen donor atoms of the ligands of biologically active macromolecules is responsible for the toxicity. These contamination cause chemical, physical and biological deterioration of the water when the natural purifying capacity of the receiving water is exceeded and they either individually or in combination bring in sub-lethal effects at a cellular, organelle and individual level (Adeyemo *et al.*, 2008).

Their accumulation in the tissue is mainly dependent on water concentrations of metals and exposure period; although some other environmental factors such as water temperature, oxygen concentration, pH, hardness, salinity, alkalinity and dissolved organic carbon may affect and play significant roles in metal's accumulation and toxicity to fish (Jitar *et al.*, 2014).

Heavy metals are produced from a variety of natural and anthropogenic sources (Bauvais *et al.*, 2015). They are one of the most toxic persistent and widespread contaminants in aquatic systems and their concentrations are growing at an alarming rate (Malik *et al.*, 2010). The major industries located in Coimbatore are textile, dyeing, electroplating, motor and pump set, foundry and metal casting industries (Gandhimathi and Meenambal, 2012). In Coimbatore city, effluents from most of the industries are directly discharged into the soil, road canals and the rivers without any proper treatment (Gandhimathi and Meenambal, 2011).

Relatively low levels of exposure to lead that may not have any immunotoxic effects on a mature organism can, if experienced during the critical period of immune system development, result in immune dysfunction later in life. Several reports have indicated that lead can cause neurological, hematological, gastrointestinal, reproductive, circulatory, immunological, histopathological and histochemical changes all of them related to the dose and time of exposure to lead (Reglero *et al.*, 2009; Mirhashemi *et al.*, 2010).

When exposed to higher concentrations, organs of aquatic animals may accumulate heavy metals (Pelgrom *et al.,* 1995; Grosell *et al.,* 1996; Kalay *et al.,* 1999; Mazon *et al.,* 2002 and Ashraf, 2005). Heavy metals are taken up and accumulated by aquatic organisms both from the surrounding medium and via food sources. Deep-sea carnivorous fish, in particular, accumulate metals in their muscles *via* the food chain. Studies have shown that when the water quality is affected by toxicants, any physiological changes will be reflected in the values of one or more of the hematological parameters (Vanvuren, 1986).

The aim of the present work is to determine the effects of subchronic doses of inorganic Lead nitrate on the hematological parameters, biochemical indices and the immunological response of the inland fish *Cirrhinus mrigala*.

MATERIALS AND METHODS

The toxicant used in the static bioassay was lead nitrate in tap water. Fingerlings of *Cirrhinus mrigala* having the size 10 – 12cm were randomly distributed in plastic troughs of 20 liters capacity. One plastic trough served as the control and the other troughs were provided with different concentrations of lead nitrate namely 32mg/l, 34mg/l, 36mg/l, 38mg/l, 40mg/l and 42mg/l. Ten fishes were placed in each trough and mortality was recorded after 24 hrs, 48 hrs, 72 hrs, and 96 hrs. The LC_{50} at 96 hrs was determined by the Probit analysis method (Finney 1971). To determine the sublethal concentration of lead nitrate, $1/10^{th}$ of the concentration of LC_{50} value for 96 hours was taken.

The experiment was designed to expose the fish to different subchronic doses of lead nitrate (4mg/l, 6mg/l, and 8mg/l). One trough served as the control. Each trough contained ten fishes and the experiment was conducted in triplicate. The duration of the experiment was for 30 days. Haematological, biochemical and immunological parameters were determined after 15 and 30 days.

HAEMATOLOGICAL PARAMETERS

For the hematological profile of the control and treated fish, Haemoglobin, Haematocrit, RBC, WBC, PCV, platelet counts were measured in the whole blood of *Cirrhinus mrigala*. Erythrocyte indices of fish viz., MCV, MCH and MCHC were also calculated.

Total Erythrocyte Count

Calculation

$$\text{Number of RBC /ml} = \frac{\text{Number of cells counted} \times \text{dilution factor}}{\text{Area counted} \times \text{depth of fluid}}$$

Total Leucocyte Count

Calculation

$$Number\ of\ WBC\ /cumm = \frac{Number of\ WBC\ counted \times dilution\ factor}{Area\ counted \times depth\ of\ fluid}$$

Haemoglobin estimation

When blood is added to 0.1N HCl, hemoglobin is converted into brown colored acid haematin. The resulting color after dilution is compared with a standard reference of haemoglobinometer. The level of the fluid at its lowest meniscus is noted and the reading corresponding to the scale in gm% is recorded.

Red blood cell indices

Mean Corpuscular Volume (MCV)

Calculation

$$\text{MCV} = \frac{\text{Haematocrit}(\%)}{\text{RBC count in million}}$$

Mean Corpuscular Haemoglobin (MCH)

Calculation

$$\text{MCH} = \frac{\text{haemoglobin}}{\text{RBC count}} \times 10$$

Mean Corpuscular Haemoglobin Concentration (MCHC)

Calculation

$$\text{MCHC} = \frac{\text{haemoglobin in g/l}}{\text{hematocrit \%}}$$

Haematocrit Value (Packed Cell Volume-PCV)

Calculation

$$\frac{\text{Volume of blood}}{\text{volume of packed RBC}} \quad \text{x } 100 = \%$$

BIOCHEMICAL PARAMETERS

Glucose, Protein, Albumin and Globulin were estimated in the whole blood of *Cirrhinus mrigala* using standard methods.

Estimation of Glucose (Phosphomolybdate method)

Calculation:

mg of glucose / 100ml of blood = mg of glucose in standard X (OD of test / OD of Standard) X 100 / 0.2

Estimation of Protein (Lowry method)

Calculation:

The protein is estimated by a standard graph by plotting the values of the standard and the unknown value is calculated

Estimation of Albumin (Bromocresol green method)

Calculation

Subtract the A620 of the Blank (0 g/dL) from the A620 of each Standard and plot the A620 against standard concentrations. Use the standard curve to determine the sample albumin concentration. Conversion factors for albumin:

0.1 g/dL = 15 µ M = 0.1% = 1,000 ppm

$$\text{Albumin}(\text{gm/dl}) = \frac{\text{A test}}{\text{A standard}} \times \text{cocentration of standard}$$

Estimation of Globulin (Bromocresol green method)

Calculation

The serum globulin level (gm/dl) was calculated using the formulae:

Serum globulin = Serum total protein – serum albumin

IMMUNOLOGICAL PARAMETER

Immunoglobulin M: The Ig M is derived by the ELISA technique.

Principle

Test sample is added to the microtitre plate, if there is presence of Ag or Ab in the test sample, there will be Ag-Ab reactions (with immobilized Ab or Ag). Later enzyme-labeled antibody is added in the reaction mixture, which will combine with either test antigen or Fc portion of test antibody.

The enzyme system consists of;

1. An Enzyme: horseradish peroxidase, an alkaline phosphatase which is labeled or linked, to a specific antibody.

2. A specific substrate:

- O-Phenyl-diamine-dihydrochloride for peroxidase
- P Nitrophenyl Phosphate- for Alkaline Phosphatase

A substrate is added after the antigen-antibody reaction. The enzyme catalyzes (usually hydrolyzes) the substrate to give a color endpoint (yellow compound in case of alkaline phosphatase). The intensity of the color is proportional to the amount of antibody or antigen present in the test sample, which can be quantified using ELISA reader.

STATISTICAL ANALYSIS

Student's t-test, one-way ANOVA and two-way ANOVA was done for all the parameters of the study using SPSS16.0 software

RESULTS AND DISCUSSION

50% of mortality was observed at a concentration of 40mg/lit of lead nitrate (Table 1). The log concentration using Probit analysis (Finney, 1997) is 1.60.

RBC

The control RBC count after 15 days of treatment is 1.30 ± 0.08 ($P<0.01$). A significant decrease in RBC content has been observed in all the treatments (1.20 ± 0.14, 1.10 ± 0.08 and 1.02 ± 0.05, $P<0.01$) (Table 3). After 30 days the control sample had a value of 1.6 ± 0.08. A significant decrease has been observed in all the treatments (1.30 ± 0.21, 1.10 ± 0.08 and 1.02 ± 0.05) (Table 4).

WBC

The control WBC count after 15 days is 501.00 ± 2.16 ($P<0.01$). A significant increase in WBC content has been observed in all the treatments (504.00 ± 2.16, 752.00 ± 0.81 and 780.00 ± 2.16, $P<0.01$) (Table 3). After 30 days the control had a value of 453.00 ± 4.32. A significant increase has been observed in all the treatments (500.00 ± 3.55, 612.00 ± 2.58 and 850.00 ± 7.11) (Table 4).

Haemoglobin

The control Haemoglobin content after 15 days is 3.80 ± 0.08 ($P<0.001$). A significant decrease in Haemoglobin content has been observed in all the treatments (3.70 ± 0.14, 3.30 ± 0.14 and 3.00 ± 0.08, ($P<0.001$) (Table 3). After 30 days the control sample had a value of 4.80 ± 0.14. A significant decrease has been observed in 4mg and 6mg treated fishes (4.00 ± 0.14 and 3.30 ± 0.12 $P<0.001$). The 8mg treated fish showed an increase (3.80 ± 0.16) (Table 4).

PCV

The control PCV after 15 days is 11.40 ± 0.08 ($P<0.01$). A significant decrease in PCV has been observed in all the treatments (11.10 ± 0.14, 9.90 ± 0.08 and 9.00 ± 0.08, $P<0.01$) (Table 3). After 30 days the control sample had a value of 14.40 ± 0.14. A significant decrease has been observed in all the treatments (12.10 ± 0.14, 9.80 ± 0.08 and 9.30 ± 0.16) (Table 4).

MCV

The control MCV after 15 days is 92.50 ± 0.21 (P<0.01). A significant decrease in MCV content has been observed in all the treatments (90.10 ± 0.08, 90.00 ± 0.14, 87.60 ± 0.08 P<0.001) (Table 3). After 30 days the control sample had a value of 92.00 ± 0.16. A significant decrease has been observed in all the treatments (90.60 ± 0.16, 89.90 ± 0.14, 90.00 ± 0.28) (Table 4).

MCH

The control MCH after 15 days is 30.80 ± 0.08 (P<0.01). A significant decrease in MCH content has been observed in all the treatments (30.10 ± 0.14, 30.00 ± 0.16, 29.20 ± 0.14 P<0.001) (Table 3). After 30 days the control sample had a value of 30.70 ± 0.14. A significant decrease has been observed in all the treatments (30.20 ± 0.16, 30.00 ± 0.08, 29.80 ± 0.14) (Table 4).

MCHC

The control MCHC after 15 days is 33.30 ± 0.14 (P<0.001). A significant decrease in MCHC content has been observed in all the treatments (33.10 ± 0.08, 33.00 ± 0.14, 32.00 ± 0.08 P<0.001) (Table 3). After 30 days the control sample had a value of 33.30 ± 0.08. A significant decrease has been observed in all the treatments (32.00 ± 0.14, 31.20 ± 0.14, 31.00 ± 0.08) (Table 4).

Platelet

The control Platelet count after 15 days is 23.00 ± 244.94 (P<0.001). A significant decrease in Platelet content has been observed in the 4mg and 6mg treatments platelet count (19.00 ± 141.42, 16.00 ± 244.94, P<0.001). There is a significant increase in platelet count 8mg treatment (90.00 ± 216.02) (Table 3). After 30 days the control sample had a value of 17.00 ± 424.26. A significant decrease has been observed in the 4mg and 6mg treatments (15.00 ± 368.55 and 14.00 ± 496.65 P<0.001). A significant increase in 8mg treatment (95.00 ± 216.02) (Table 4) was observed.

Glucose

The control Glucose content after 15 days is 22.00 ± 0.81 (P<0.01). A significant increase in glucose content has been observed in all the treatments (34.00 ± 1.41, 40.00 ± 1.41and 41.00 ± 0.81 P<0.001) (Table 5). After 30 days the control sample had a value of 50.00 ± 3.55. A significant increase has been observed in all the treatments (50.00 ± 4.08, 59.00 ± 4.24 and 64.00 ± 3.26) (Table 6).

Protein

The control protein content after 15 days is 2.90 ± 0.14 (P<0.01). A significant decrease in protein content has been observed in all the treatments (2.60 ± 0.08, 1.10 ± 0.08 and 1.70 ± 0.08 P<0.001) (Table 5). After 30 days the control sample had a value of 2.20 ± 0.14. A significant decrease has been observed in all the treatments (1.80 ± 0.14, 1.70 ± 0.16 and 1.40 ± 0.21) (Table 6).

Albumin

The control albumin content after 15 days is 1.50 ± 0.14 (P<0.001). In the treated fishes a significant decrease in albumin content has been observed in the 4mg and 6mg treatments (1.30 ± 0.08 and 0.65 ± 0.01 P<0.001). A significant increase has been observed in 8mg (1.10 ± 0.142) (Table 5). After 30 days the control sample had a value of 1.10 ± 0.08. A significant decrease has been observed in all the treatments (0.80 ± 0.08, 0.78 ± 0.03 and 0.63 ± 0.03) (Table 6).

Globulin

The control globulin content after 15 days is 1.40 ± 0.08 (P<0.001). A significant decrease in globulin content has been observed in all the treatments (1.30 ± 0.14, 0.60 ± 0.08 and 0.400 ± 0.08 P<0.001) (Table 5). After 30 days the control sample had a value of 1.10 ± 0.14. A significant decrease has been observed in all the treatments (1.00 ± 0.08, 0.62 ± 0.03 and 0.51 ± 0.03) (Table 6).

Immunoglobulin M

The control IgM concentration after 15 days is 7.20 ± 0.08 (P<0.001). A significant increase in IgM concentration has been observed in all the treatments (8.17 ± 0.09, 8.80 ± 0.08 and

11.0 ± 0.81 P<0.001) (Table 7). After 30 days the control sample had a value of 8.30 ± 0.28. A significant increase has been observed in all the treatments (12.30 ± 0.16, 13.40 ± 0.24 and 15.00 ± 0.21).

ANOVA

The One Way ANOVA for 15 days and 30 days of treatment is significant (P<0.001) (Table 8 and 9). The Two Way ANOVA is significant at 1% level (Table 10 and11) for all the parameters studied.

Table 1: Percentage (%) Mortality in *Cirrhinus mrigala* treated with different concentrations of Lead nitrate.

Sr. No.	No. of fishes	Toxicant concentration in mg/l	Mortality in Test Animals	
			96Hrs	%
1	10	32	0	0
2	10	34	1	10
3	10	36	2	20
4	10	38	3	30
5	10	40	5	50
6	10	42	6	60

Table 2: LC 50 Value of Lead nitrate and the 95% confidence limit in *Cirrhinus mrigala*

LC 50 (Log concentration)	95% Confidence		Probit Equation	Chi-square
	Lower limit	Upper limit		
1.60	1.53	1.63	Y= -22.33+17.012x	0.988

Table 3: Haematological parameters of *Cirrhinus mrigala* after 15 days of exposure to Lead nitrate.

Concentration	RBC in millions/cu mm	WBC in No. of cells/cu mm	HB in gm%	PCV in gm%	MCV in microns	MCH	MCHC	PLATELET count in No. of cells/cu mm
CONTROL	1.30±0.08	501.00±2.16	3.80±0.08	11.40±0.08	92.50±0.21	30.80+0.08	33.30±0.14	23.00±244.94
4mg/l	1.20±0.14	504.00±2.16	3.70±0.14	11.10±0.14	90.10±0.08	30.10±0.14	33.10±0.08	19.00±141.42
6mg/l	1.10±0.08	752.00±0.81	3.30±0.14	9.90±0.08	90.00±0.14	30.00±0.16	33.00±0.14	16.00±244.94
8mg/l	1.02±0.05	780.00±2.16	3.00±0.08	9.00±0.08	87.60±0.08	29.20±0.14	32.00±0.08	90.00±216.02

Values are expressed as mean ± SD, Comparison between Control vs different concentration *- significant at 5% level **- Significant at 1% level. NS - not significant.

Table 4: Haematological parameters content of *Cirrhinus mrigala* after 30 days of exposure to Lead nitrate.

Concentration	RBC in millions/cu mm	WBC in No. of cells/cu mm	HB in gm%	PCV in gm%	MCV in microns	MCH	MCHC	PLATELET count in No. of cells/cu mm
CONTROL	1.6±0.08	453.00 ±4.32	4.80± 0.14	14.40±0.14	92.00±0.16	30.70±0.14	33.30±0.08	17.00±424.26
4mg/l	1.30±0.21	500.00 ±3.55	4.00± 0.14	12.10±0.14	90.60±0.16	30.20±0.16	32.00±0.14	15.00±368.55
6mg/l	1.10±0.08	612.00 ±2.58	3.30± 0.21	9.80±0.08	89.90±0.14	30.00±0.08	31.20±0.14	14.00±496.65
8mg/l	1.02±0.05	850.00 ±7.11	3.80± 0.16	9.30±0.16	90.00±0.28	29.80±0.14	31.00±0.08	95.00±216.02

Values are expressed as mean ± SD, Comparison between Control vs different concentration *- significant at 5% level **- Significant at 1% level. NS - not significant

Table 5: Biochemical parameters in the serum of *Cirrhinus mrigala* after 15 days of exposure to Lead nitrate

Concentration	GLUCOSE	PROTEIN	ALBUMIN	GLOBULIN
CONTROL	22.00±0.81	2.90±0.14	1.50±0.14	1.40±0.08
4mg/l	34.00±1.41	2.60±0.08	1.30±0.08	1.30±0.14
6mg/l	40.00±1.41	1.10±0.08	0.65±0.01	0.60±0.08
8mg/l	41.00±0.81	1.70±0.08	1.10±0.142	0.400±0.08

Values are expressed as mean ± SD, Comparison between Control vs different concentration *- significant at 5% level **- Significant at 1% level. NS - not significant.

Table 6: Biochemical parameters in the serum of *Cirrhinus mrigala* after 30 days of exposure to Lead nitrate

Concentration	GLUCOSE	PROTEIN	ALBUMIN	GLOBULIN
CONTROL	50.00±3.55	2.20±0.14	1.10±0.08	1.10±0.14
4mg/l	50.00±4.08	1.80±0.14	0.80±0.08	1.00±0.08
6mg/l	59.00±4.24	1.70±0.16	0.78±0.03	0.62±0.03
8mg/l	64.00±3.26	1.40±0.21	0.63±0.03	0.51±0.03

Values are expressed as mean ± SD, Comparison between Control vs different concentration *- significant at 5% level **- Significant at 1% level. NS - not significant.

TABLE 7: IgM concentration of *Cirrhinus mrigala* after 15 days and 30 days of exposure to Lead nitrate.

Concentration	15 days	30 days
CONTROL	7.20±0.08**	8.30±0.28**
4mg/l	8.17±0.09**	12.30±0.16**
6mg/l	8.80±0.08**	13.40±0.24**
8mg/l	11.00±0.81**	15.00±0.21**

Values are expressed as mean ± SD, Comparison between Control vs different concentration *- significant at 5% level **- Significant at 1% level. NS - not significant.

TABLE 8: One way ANOVA for Haematological, Biochemical and Immunological parameters of *Cirrhinus mrigala* after 15 days of exposure to Lead nitrate

Parameters	N	F-value	P-value
RBC	4	6.45**	<0.001
WBC	4	25392.27**	<0.001
Haemoglobin	4	41.00**	<0.001
PCV	4	492.00**	<0.001
MCV	4	800.67**	<0.001
MCH	4	93.64**	<0.001
MCHC	4	101.00**	<0.001
Platelet	4	2976.79**	<0.001
Glucose	4	228.75**	<0.001
Protein	4	273.00**	<0.001
Albumin	4	44.38**	<0.001
Globulin	4	99.67**	<0.001
IgM	4	60.29**	<0.001

**significant at 1% level

TABLE 9: One way ANOVA for Haematological, Biochemical and Immunological parameters of *Cirrhinus mrigala* after 30 days of exposure to Lead nitrate

Parameters	N	F-value	P-value
RBC	4	2.680ns	<0.090
WBC	4	5665.970**	<0.001
Haemoglobin	4	54.94**	<0.001
PCV	4	1197.09**	<0.001
MCV	4	97.650**	<0.001
MCH	4	32.550**	<0.001
MCHC	4	326.75**	<0.001
Platelet	4	289.74**	<0.001
Glucose	4	13.31**	<0.001
Protein	4	15.41**	<0.001
Albumin	4	39.250**	<0.001
Globulin	4	45.09**	<0.001
IgM	4	612.25**	<0.001

**significant at 1% level; ns – not significant

Table 10: TWO WAY ANOVA for Haematological parameters analyzed during the experimental period of 15 and 30 days for different concentration of Lead nitrate.

Parameters	Source of variation	Sum of squares	df	Mean Square	F	P
WBC	**Days**	8.820	1	8.820	622.588	.000**
	Treatment	69.220	3	23.073	1628.706	.000**
	days*treat	11.380	3	3.793	267.765	.000**
RBC	**Days**	2.645	1	2.645	90.686	.000**
	Treatment	49.375	3	16.458	564.286	.000**
	days*treat	9.895	3	3.298	113.086	.000**
HB	**Days**	2.205	1	2.205	105.840	.000**
	Treatment	5.055	3	1.685	80.880	.000**
	days*treat	1.255	3	.418	20.080	.000**
PCV	**Days**	8.820	1	8.820	622.588	.000**
	Treatment	69.220	3	23.073	1628.706	.000**
	days*treat	11.380	3	3.793	267.765	.000**
MCV	**Days**	2.645	1	2.645	90.686	.000**
	Treatment	49.375	3	16.458	564.286	.000**
	days*treat	9.895	3	3.298	113.086	.000**
MCH	**Days**	0.180	1	0.180	9.818	.005**
	Treatment	6.360	3	2.120	115.636	.000**
	days*treat	.580	3	.193	10.545	.000**
MCHC	**Days**	7.605	1	7.605	570.375	.000**
	Treatment	13.815	3	4.605	345.375	.000**
	days*treat	3.295	3	1.098	82.375	.000**
PLATELET	**Days**	60775312.500	1	60775312.500	610.935	.000**
	Treatment	506600937.500	3	168866979.167	1697.511	.000**
	days*treat	42525937.500	3	14175312.500	142.495	.000**

Table 11: TWO WAY ANOVA for Biochemical and Immunological parameters analysed during the experimental period of 15 and 30 days for different concentrations of Lead nitrate.

Parameters	Source of variation	Sum of squares	df	Mean Square	F	P
Glucose	**Days**	3698.000	1	3698.000	467.116	.000**
	treatment	1332.000	3	444.000	56.084	.000**
	days*treat	162.000	3	54.000	6.821	.002**
Protein	**Days**	.720	1	0.720	37.565	.000**
	treatment	7.060	3	2.353	122.783	.000**
	days*treat	2.440	3	0.813	42.435	.000**
Albumin	**days**	.778	1	0.778	99.402	.000**
	treatment	1.510	3	0.530	64.291	.000**
	days*treat	.514	3	0.171	21.873	.000**
Globulin	**days**	.110	1	0.110	12.781	.002**
	treatment	3.701	3	1.234	142.741	.000**
	days*treat	.275	3	0.092	10.590	.000**
Igm	**Days**	95.565	1	95.565	847.116	.000**
	Treatment	113.916	3	37.972	336.594	.000**
	days*treat	15.206	3	5.069	44.930	.000**

The red blood cells have the important function of hemoglobin transport which carries oxygen to all tissues in the body (Hibiya, 1982). The decreased red blood cell number following exposure to Cadmium could be the result of hemolysis or destruction of red blood cells. Due to metal toxicity, hemopoietic organs get affected and are unable to release normal RBCs in general circulation and thus can be held responsible for drastic decline. The reduced erythrocyte lifespan as well as slower erythropoiesis is responsible for the reduction in RBC number as a result of metal toxicity. Decrease in the red blood cells could also be the result of internal bleeding caused by the damaged kidney. Similar findings supporting the previous studies were recorded for Heteroclarias exposed to sublethal concentrations of Cadmium (Kori-Siakpere *et al*., 2006).

The significant increase in WBC count could be due to an increase in antibody production which helps in survival and recovery of the fish exposed to heavy metals (Joshi and Deep, 2002). In fish, the white blood cells respond to various stressors including infections and chemical irritants (Christensen *et al*., 1978). Total Leucocyte Count has been suggested due to stimulated lymphopoiesis and enhanced release of lymphocyte from lymphoid tissues. Such lymphocyte response in the presence of toxic substances is associated with pollutant-induced tissue damage and severe disturbance of non-specific immune system leading to increased production of leucocytes (Das and Mukherjee, 2003).

The decreased hemoglobin content might be due to suppression of haemopoietic activity of the kidney in addition to the increased removal of dysfunctional RBCs following exposure. According to Mustafa (2012), the reduction in Haemoglobin content in fish exposed to toxicant could also be due to the inhibitory effect of a toxic substance on the enzyme system responsible for the synthesis of Haemoglobin.

Metal can affect the hematological parameters of fish via osmotic changes which impose haemodilution or haemoconcentration (Sanchez dardon *et al.,* 1999). Spleen is responsible for these changes. Because it serves as potent blood storage in teleosts, sequestering blood cell under resting conditions and releasing them to circulating blood associated with various stress (Murugan, 2008). The PCV appears to be positively correlated with erythrocyte count. Fall in the number of red blood cells followed by PCV confirms anemia. The decrease in PCV may be attributed either to decreased cellular content and increased plasma content mainly in water (Walker *et al.,* 1969).

Houston and Keen, (1984) and Ikomi (2011) suggested that cadmium causes decrease in mean cell volume which is due to the result of the immature red blood cell from hematopoietic tissues. Immature cells are released to compensate the loss of red blood cells. Decrease in MCV level indicates hypochromic microcytic anemia (Shakoori *et al.,* 1996).

A significant decrease in MCH value has been reported in ammonia and toxic metal exposed fishes indicating microcystic anemia. (Atamanalp *et al.,* 2002). The reduction in values obtained for hematological parameters of treated fish in this study showed that the physiological activities of the treated fish were affected.

MCHC measurement is a diagnostic tool to assess the amount of RBC swelling. (Milligan *et al.,* 1982). A decrease in mean corpuscular volume (MCV), mean corpuscular hemoglobin

(MCH) was observed in all exposures which are directly related to hematocrit value, hemoglobin percentage and RBC count.

Brandao *et al.,* (2009) and Oliveira Ribeiro *et al.,* (2002) found a reduction in immunological parameters and an increase in neutrophil and monocyte percentages were demonstrated in mercuric chloride exposed fishes. It is known that metals can induce abnormal responses in the immune system. Leucocyte counts after exposure to pollutants may be associated with a decrease in nonspecific immunity of the fishes.

Blood sugar levels are elevated in fish during acute exposure to a variety of compounds, including pesticides. Stressful stimuli elicit rapid secretion of both glucocorticoids and catecholamines from the adrenal tissue of fish; both hormones produce a rapid hyperglycemia (Singh and Srivastava, 1982). The hyperglycemia might be a result of glycogenolysis in muscle and liver causing a significant increase in blood glucose levels. Stressors induce some changes that alter the homeostasis of the animal (Schreck, 1981). The decrease in the specific activity of some enzymes like phosphofructokinase, lactate dehydrogenase and citrate kinase that decrease the capacity of glycolysis (Barnhart, 1969).

Proteins are indispensable constituents of the body and their metabolism is almost confined to the liver. Fall in serum protein level may be due to impaired function of kidney or due to reduced protein synthesis owing to liver cirrhosis (Garg *et al.,* 1989; Ravichandran *et al.,* 1994 and Kumari and Kumar, 1995). Das *et al.,* (2004) stated that reduction of protein content in serum occurs due to shrinkage and lysis of RBCs causing plasma dilution and/or protein catabolism where structural protein converts to energy. According to Radha *et al.,* (2005) the reduction of protein content may be due to increased proteolytic activity and decreased the anabolic activity of a protein as observed by Jenkins *et al.,* (2003).

Albumin is one of the important protein fractions, which play an important role in osmotic concentration of the blood. The adaptive changes in serum albumin level under toxic conditions are reported in few fish. Ibrahim (1992) reported reductions in the serum albumin levels in Tilapia nilotica. Sogorb *et al.,* 2002 reported the exposure could be taken as an adaptive immune response in the fish as serum albumin is reported to be capable of detoxifying the toxic substrates.

Serum globulin is the precursor for the synthesis of immunoglobulins and plays an important role in the immunity of the organisms towards diseased and toxic conditions. Hence, the

immunoglobulins are called as protective proteins. Reports on the serum levels of globulin in fish under toxic conditions are highly variable among the fishes. Mekkawy *et al., (*1996) reported significant reduction in the serum globulin level in Oreochromis niloticus and Chrysichthyes auratus exposed to atrazine. Dobsikova *et al.,* 2006; Khallaf-Allah, 1999; Banaee *et al., (*2011) and Yakeen and Fawole (2011) reported significant reductions in globulin levels indicated depressed respiratory metabolism in fish under toxic conditions.

According to Witeska (2005), a short-term exposure to high levels of heavy metals induce stress reaction in fish. White blood cells show that stress reduces the immune potential. This reduced immunological status persisted for at least several days after the removal of stressing agents from the water. IgM is the first antibody to appear in response to initial exposure to the antigen. The spleen is the major site of IgM production. IgM contributes to both innate and adaptive immunity in fish. IgM effector functions include complement activation which both lyses and opsonizes pathogens (Boshra *et al.,* 2004). IgM also mediates agglutination for phagocytosis and removing pathogens, and cellular cytotoxicity (Ye *et al.,* 2013).

CONCLUSION

In conclusion, the toxic effect of heavy metals in fish has been demonstrated in the present study. It is abundantly clear that metals induce an early response in the fish as evidenced by alterations both at structural and functional levels of different organs including enzymatic and genetic effects, thereby affecting the innate immune system of exposed fish and increasing susceptibility to diseases. It can be stated that fish biomarkers are necessary for monitoring environmentally induced alterations to assess the impact of xenobiotic compounds on fish. Also, it is recommended that treatment of all kinds of wastewaters, sewage and agricultural wastes must be conducted before discharge into the aquatic systems.

ACKNOWLEDGEMENT

With gratitude and respect, we record our indebtedness to the Department of Zoology, Nirmala College for women, Coimbatore, for providing all resources to carry out this research work.

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