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RESEARCH ARTICLE

TRACING OUT CORRELATION BETWEEN BLOOD LEAD AND HEAMATOLOGICAL PARAMETERS IN *Clarias batrachus, Linn*. DURING EXPERIMENTAL PLUMBISM

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ABSTRACT

Clarias batrachus, Linn. a common Indian cat fish was treated with various sub acute concentrations of Lead acetate for three days and seven days. A third set carried chronic exposure to 15 ppm lead acetate for 105 days. Different blood parameters studied included total RBC and total WBC counts, gm % of haemoglobin, % cytotoxicity, haematocrait, mean corpuscular haemoglobin (MCH). Differential count of WBCs were also done for the respective groups. Lead accumulation in the blood was estimated by Atomic Absorption Spectrophotometer (AAS). A Co-relation Co-efficient ('r') between lead content of blood and Mean values of these parameters was worked out. The r –values are found to be highly significant.

INTRODUCTION

According to WHO guidelines on immunotoxicology (1996), the fishes are excellent models for studying effects of water and sediment borne pollutants. Besides, these are several other good reasons for studying immunotoxicity in fish: (i) many of their disease are related to environmental quality (ii) various environmental pollutants have immunotoxic potential and (iii) many of the disease have an immune component. Moreover, there is concern about the health status of aquatic ecosystems in relation to pollution, and fish will useful target species for developing biomarkers in two tier tests that include screening tests with conventional haematology and other functional tests. The WHO comments on above tests and assays on fishes indicate that- Immunological biomarker in fish have great potential may have not yet been fully explored, probably owing to practical limitations of lack of specificity and predictivity. As leucocytes play a major role in specific and non-specific humoral and cellular immune responses, this parameters is used as a measure of status of the defence systems, in particular in tier-I testing Zelikoff *et al.* (2002). Another possible parameter is haematocrait, however it has no known specificity for any immune function, although it may be considered as a general indicator of stress. Estimation of haemoglobin in some cases may be done. But a good indicator of haematological tests may be carried out on study of cell viability (≈ cell death) at a particular time period to relate the environmental toxicant with status of blood. In the earlier studies it has been seen that absorption and accumulation of lead in the blood is both dose dependant as well as dependant on the period of exposure (Rout and Naik 2013). Hence blood is a good indicator of lead toxicosis and effect of lead on haematological parameters is thus evident.

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II. MATERIAL AND METHODS

2.1 Experimental design

Large-sized (120-200 gms.) fishes were collected from culture ponds of village Deopada in Bhadrak district of Odisha and aclimatized for seven days in the laboratory aquaria as reported earlier (Rout and Naik 1996). For short-term studies, different subacute concentrations of lead acetate [Pb $(CH_3\text{-}COO)_2$], Johnson and Sons, Ltd., London, 1990] were choosen after obtaining LC_{50} (500 ppm) and LT_{50} (45 days for25pm, 40 days for 50 ppm, 37days for 75 ppm., 35 days for 100 ppm, 30days for 125ppm and 28 days for 150ppm. The cases however are slightly different than the others such as Shamshun Nehar *et al.* (2010) and Ahmad khan *et al.* (2011) in their won conditions. For 3 days and 7 days of treatment seven fishes each were selected and kept in separate aquaria demarcated for control, 25 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm and 150 ppm of lead acetate. For chronic treatment 15 ppm of lead acetate was chosen because of relatively high LT_{50} (150 days), suitable for chronic studies. Eight fishes taken for experiment each in separate aquaria and eight for control. The study periods involve 1st, 15th, 30th, 45th, 60th, 75th, 90th and 105th days respectively with the control for comparison. All the experiments were repeated ten times and the physico-chemical profiles were monitored following APHA (2000) carefully in each day during replacement of water (Rout and Naik 2000).

2.2 Analysis of blood samples for lead

It was followed according to Australian standard (AS2411 - 1980) for AAS. After collection the heparinized blood samples were kept at 4°C. During analysis 3ml of thoroughly mixed blood sample were immediately dispensed to the centrifuge tube. The lead in blood was made complexes with APDC by adding 0.5 ml of 2% APDC to it and extracted into 3 ml of n-butyl acetate by proper shaking. Lead was determined in the organic phase by AAS within one hour.

Calculation was done by the help of calibration curve using standard solution.

Lead content = $\frac{\text{OD of the sample}}{\text{Standard Value}}$ µg/gm/ml of the tissue.

2.3. Haematological methods

The methods of Sahoo (1991) for haematological studies were followed.

2.3.1. Collection of blood

Blood from the fishes was collected by caudal puncture with an injection syringe (Needlle No.22). The needle and the syringe were primarily ringed by 10% sodium-heparin to prevent any chance of clotting of the blood.

2.3.2. TC of RBC & WBC

(a) R. B. C.: Blood was taken out from the previously collected glass Petri dish ringed with sodium heparin sucking through micropipette up to 0.5 mark. Then Haym's fluid was suck up to, the mark 101 and mixed thoroughly by steady rotation. One drop of blood was then passed into the cover slip of haemocytometer previously ringed and dried with spirit. After settlement it was taken for counting under microscope. Counting was done carefully in the 1st/ 5th, 13th/ 21st and 25th smallest squares. The RBC laying on the lower and right side of the square are to be added to the total while these lying on the upper one left side were rejected.

Calculation: Total no of RBCs present in mm^3 (a) R. B. C. Blood was taken out from the previously collected glass Petri dish ringed with sodium heparin sucking through micropipette up to 0.5 mark. Then Haym's fluid was suck up to the mark 101 and mixed thoroughly by steady rotation. One drop of blood was then passed into the cover slip of haemacytometer previously ringed and dried with spirit. After settlement it was taken for counting under microscope., Counting was done carefully in the 1st/ 5th, 13th/ 21st and 25th smallest squares. The RBC laying on the lower and right side of the square are to be added to the total while these lying on the upper one left side were rejected.

Calculation: Total no of RBCs present in mm^3 = No. of RBC count in 5 squares x 10000

(b) W.B.C. Cleaned apparatus were used. The blood was sucked upto the 0.5 mark of the pepette/ then genetian vio-let solution was sucked upto the mark 11 of the pepette. The pepette was then rotated slowly so that the fluid mixed with the blood properly. Then dilution ws kept for 5 minutes/ so that whole of the WBC diluted. 1-2 drops of the fluid were removed from the pipette. The pipette was then touched just near the edge of the coverslip/ so that the counting chamber is properly flooded. The slide was kept for sometimes for settling of WBC in the fluid. Counting was then started by putting this slide under microscope. The WBCs were counted in the 4 squares of the conting chamber of the slide. The WBC which are on the boarder line were not taken into consideration. WBCs were recognised by the retractile appearance and the slight colouration given to them by staining.

Number of WBC/mm= $\frac{\text{No of cells counted x 10 x dilution}}{\text{No of squares counted (= 4)}}$

2.3.3. D.C. of WBC

Taken a drop on the end of a clean slide, place the slide on a smooth surface holding it steady with the left hand. Held a second slide or drawing slide at 45° just in front of the drop of the blood, draw this slide in such a way that the blood spreads along its edge. Pushed the second or drawing slide forward at 45° on the first slide without exerting any pressure, a film of blood will be made on the first slide, the blood film was dried in air. The blood film was stained with Leishman's stain or Wright's stain. After one minute distilled water added carefully to the stain. The water and stain were mixed well by sucking the mixture in and out of a pipette. After a time a greenish metallic scum forms on the surface of the mixture. Allow the diluted stain to act from 7 to 10 minutes. The stain was drained off and washed film for 10 seconds with distilled water. The film should be rose pink in colour. The slide was dried and examined it under the microscope for white blood corpuscles. First examined the stained film under low power of microscope to get an idea of leucocytes. Then it was examined under high power or oil immersion lens after thinly smearing the stained film with oil. In a longitudinal strip of the film count the various kinds of white blood corpuscles from top to the bottom. Count 100 WBC noting on a paper by tally method and calculated the percentage.

2.3.4. Estimation of Hb, MCH & Haematocrit

i) Haemoglobin: Haemoglobin concentration was estimated by Sahl's haemometer. For this the graduated tube is pulled out of distilled water and then with methylated alcohol. After drying/ with the help of a dropper 2cc of N/10 HC1 is transferred into the dry graduated tube. The previously collected heparinised blood was taken into the micropipette and transferred into the graduated tube. With constant stiring distilled water was added dropwise till the colour of the experiment type matched with the side tubes. Then the blood dilution mark was noted which is the percentage of haemoglobin observed in the blood.

ii) MCH: This is a calculated value and is calculated as: Haemoglobin in gms/100 cc of blood per RBC in millions/mm^3 of blood.

iii) Haematocrait: Blood collected with anticuagulant and kept in wintrob's tube and centrifused at 3000 rpm for 30 minutes. The volume occupied by the red cells when packed together is the PCV or haematocrait value

2.3.5. Cytotoxicity

The number of viable cells in a population of RBC was determined by Trypan blue (Sigma TO776) following Talwar (1983). Viable cells exclude the dye while non viable cells take up and thus appear blue in colour. I ml. of the blood was suspended in 1 ml of the apparatus and is cleaned with 1ml blood was suspended in 1ml of phosphate buffer soline (PBC). Trypan blue was prepared 0.1% W/V in saline (by dissolving 100 mg in 100 ml of 1% saline). To one part of the Trypan blue solution one part of the cell suspension was added and this was then loaded into a haemocytometer and the number of unstained and stained cells was counted separately.

Viable cell number = (Average number of viable cells) x 10^4 x 1/dilution.

% of viable cells = $\frac{\text{No of viable cells x 100}}{\text{No of viable cells + No of dead cells}}$

% of Cytotoxicity = 100 - % of viable cells.

2.4. Statistical methods

All the data obtained from the control and experimental fishes were statistically analyzed as follows:

2.4.1. ANOVA: One-way analysis of variance (ANOVA) for accumulation and kinetic studies, DNA, RNA and protein relations, activities of different enzymes, haematological studies and Immunological studies were performed with the respective F-values in all observations following Sanders (1994). All the ANOVA tables are separately attached by an annexure.

2.4.2. Parson's Correlation Coefficient (r): The r- values between doses of lead acetate with lead accumulation in blood as well as the lead accumulation and various parameters of the control groups & experimental were calculated with significance following Sanders (1994) and Chainy *et al.* (2008).

III. RESULTS

After 3 days of sub acute treatment, the blood parameters like total RBC, total WBC, Haematocrait and % of cytotoxicity increase (Table 1) with increase in doses of Lead Acetate but the gm % of Haemoglobin and MCH are reduced. The total RBC content is increased from 1.80 ±0.01 million / mm^3 in control to 2.25±0.01 million / mm^3 in 150 ppm lead acetate(F = 140.6). Similarly there is a dose dependant increase in total WBC content from 1.23±0.01 thousands/mm^3 to 5.52±0.23 thousands/mm^3 (F= 243.476). The gram percentage (gm%) of haemoglobin is nearly constant(From 30.33± 0.31 to 35.28±0.28). The mean corpuscular haemoglobin (MCH) is reduced from 394.5±1.35 to 313.11± 1.43(F=21.901). The percentage of cytotoxicity has been observed as a dose dependant matter which increased from 8.45±0.12 in control to 19.25 ±0.14 in 150 ppm lead acetate (F= 57.02). Lead accumulation in blood after 3 days fairly remains constant without any significance.

in both short term three day (Figure 1) and seven day (Figure 2) experiments.

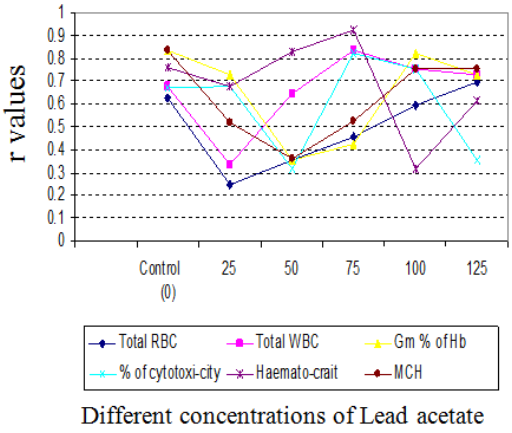

Fig.1. Correlation co-efficient (r) between Lead accumulation and Haematological parameters of *Clarias batrachus* after 3 days exposure to different concentrations of Lead Acetate

Table 1. Haematological parameters (Mean ± SEM) and F-values from ANOVA at different subacute concentrations of lead acetate on *Clarias batrachus* after 48 hrs (3 days) of treatment

Concentration of lead actetate(ppm)	Total RBC/mm^3 (10^6)	Total WBC/mm^3 (10^3)	gm % of Hb (100ml)	% of cytotoxicity (mm^3)	Haematocrait	MCH	Blood lead content (µg/dl)
CO	1.80	1.23	71.02	8.45	30.33	394.5	3.8
	±0.01	±0.01	±0.23	±0.12	±0.31	±1.35	±0.001
25	1.85	1.69	71.05	10.23	32.14	384.05	12.30
	±0.02	±0.12	±0.19	±0.25	±0.33	±2.28	±0.269
50	1.85	2.35	70.85	15.49	32.65	382.97	13.01
	±0.02	±0.23	±0.21	±0.31	±0.23	±1.45	±0.251
75	2.01	3.65	70.70	16.23	33.24	351.74	13.52
	±0.01	±0.82	±0.14	0.24	±0.28	±1.63	±0.512
100	1.98	4.38	70.75	18.25	35.26	357.32	14.13
	±0.02	±0.29	±0.12	±0.18	±0.14	±1.29	±0.132
125	2.13	5.28	70.62	18.20	36.21	331.54	13.21
	±0.03	±0.86	±0.21	±0.13	±0.28	±1.23	±0.213
150	2.25	5.52	70.45	19.25	35.28	313.11	12.69
	±0.01	±0.23	±0.24	±0.14	±0.27	±1.43	±0.619
F-value	140.6	243.476	91.972	35.06	57.02	21.901	0.58

Table 2. Haematological Prameters (Mean ± SEM) and F-values from ANOVA at different subacute concentrations of lead acetate in *Clarias batrachus* after 7- days treatment

Concentration of lead acetate (ppm)	Total RBC (10^6)	Total WBC (10^3)	gm % of Hb	% of cyto-toxicity	Haematocrait	MCH	Blood lead content (µg/dl)
0	1.93	2.01	71.25	8.23	31.39	369.17	3.7
	±0.04	±0.13	±0.25	±0.12	±0.24	1.22	±0.001
25	1.95	3.51	70.23	15.18	35.23	360.15	13.50
	±0.03	±0.14	±0.13	±0.21	±0.18	±1.21	±0.544
50	1.98	3.82	69.15	22.31	39.48	349.24	14.56
	±0.01	±0.21	±0.21	±0.23	±0.14	±1.48	±0.279
75	2.11	5.26	68.65	25.23	41.48	325.35	15.26
	±0.08	±0.31	±0.12	±0.14	±0.13	±1.36	±0.716
100	2.10	6.12	68.92	25.58	43.26	328.19	17.19
	±0.23	±0.23	±0.15	±0.27	±0.13	±1.38	±0.218
125	2.59	6.15	68.13	30.61	45.50	263.05	18.25
	±0.03	±0.41	±0.31	±0.23	±0.12	±1.12	±0.125
150	3.15	7.12	67.81	35.30	49.31	215.26	22.21
	±0.05	±0.33	±0.02	±0.12	±0.21	±2.15	±0.238
F-value	224.356	157.958	107.321	86.139	95.170	58.770	0.72

Almost similar results were observed after 7 day study of various haematological parameters (Table 2) except a sharp increase in cytotoxicity and blood lead content from 3.7 ± 0.001 µg/dl to22.21± 0.238 µg/dl (F = 0.72). Moreover there is a decline in haematocrait. The correlation coefficient (r) between lead content in blood and other haematological parameters were found to be highly significant

After three days of treatment to different concentrations of lead acetate, the neutrophyll percentage decreased from 62 to 40 of the DC (Figure 3). Similarly lymphocyte share is reduced from 27 % to 20%. On the other hand, there is significant enhancement of Eosinophyll (from 3 to 20%), Basophylls (from 1 to 7%), and Monocytes

Table 3. Haematological Parameters (Mean ± SEM) and F-values from ANOVA during 15 ppm Chronic treatment of lead acetate on *Clarias batrachus* up to 105 days

Periods of exposure (days)		Total RBC (10^6)	Total WBC (10^3)	% of cytotoxicity	gm % of Hb	Haematocrait	MCH	Blood lead content (µg/dl)
01	C	1.93	1.85	8.10	71.21	31.23	5.0	5.0
		±0.01	±0.11	±0.01	±0.05	±2.14	±0.187	±0.187
	E	1.90	3.13	12.10	71.00	31.42	10.11	10.11
		±0.01	±0.15	±0.12	±0.21	±1.28	±0.577	±0.577
15	C	1.89	2.14	8.51	71.15	31.21	4.83	4.83
		±0.02	±0.01	±0.02	±0.25	±2.16	±0.178	±0.178
	E	2.01	2.74	10.15	70.75	33.41	21.53	21.53
		±0.02	±0.13	±0.69	±0.31	±1.98	±0.758	±0.758
30	C	1.92	2.11	9.16	70.95	30.21	5.02	5.02
		0.02	±0.12	±0.03	±0.21	±1.29	±0.158	±0.158
	E	2.83	4.28	15.13	62.36	38.32	41.53	41.53
		±0.01	±0.12	±1.23	±0.25	±2.38	±1.561	±1.561
45	C	1.95	2.13	9.42	71.26	31.60	4.78	4.78
		±0.02	±0.16	±0.21	±0.25	±2.31	±0.129	±0.129
	E	3.21	5.21	22.35	58.87	46.21	55.07	55.07
		±0.01	±0.28	±1.13	±0.36	±2.84	±0.721	±0.721
60	C	1.93	1.86	8.31	71.28	30.69	4.98	4.98
		±0.01	±0.12	±0.05	±0.35	±1.74	±0.286	±0.286
	E	2.25	2.31	25.36	47.5	36.51	82.25	82.25
		±0.02	±0.21	±1.38	±0.17	±2.15	±1.406	±1.406
75	C	1.98	2.15	8.21	71.29	31.24	4.85	4.85
		±0.01	±0.12	±0.18	±0.08	±2.41	±0.183	±0.183
	E	1.64	6.33	29.62	34.31	26.31	111.29	111.29
		±0.01	±0.23	±2.15	±0.29	±4.16	±1.504	±1.504
90	C	1.92	1.97	9.16	72.28	30.23	4.85	4.85
		±0.01	±0.18	±0.02	±2.38	±1.41	±0.210	±0.210
	E	1.25	1.28	36.27	28.21	19.31	123.35	123.35
		±0.01	±0.21	1.38	±0.28	±2.82	±2.508	±2.508
105	C	1.95	1.98	8.29	71.28	33.12	4.98	4.98
		±0.01	±0.18	±0.04	±0.34	±2.25	±0.613	±0.613
	E	0.85	0.93	42.30	22.15	16.32	141.12	141.12
		±0.01	±0.31	±2.13	±0.32	±3.42	±2.413	±2.413
F-value	C	0.788	1.630	0.283	1.502	1.012	2.675	2.675
	E	1045.213	345.638	726.821	234.355	114.828	864.154	864.154

C= Control, E= Experiment

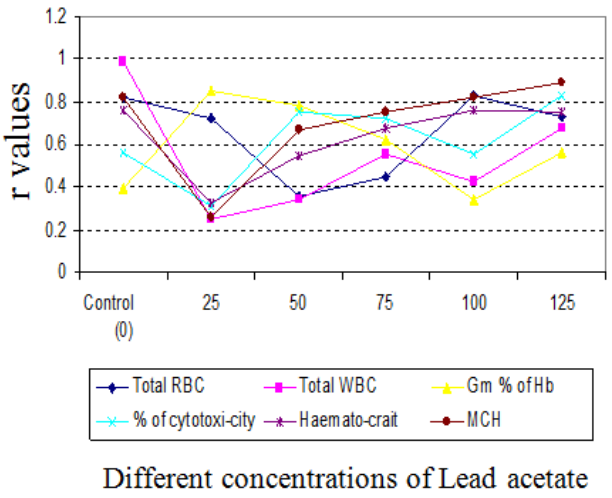

Fig. 2. Correlation co-efficient (r) between Lead accumulation and Haematological parameters of *Clarias batrachus* after 7 days exposure to different concentrations of Lead Acetate

(from 7 to 15%). Very similar results were found after seven day treatment in case of blood DC (Figure 4). The results are different in chronic 15ppm treatment for 105 days (Table 3). The blood lead concentration is significantly increased from 5.0±0.187µg/dl to 141.12 ±2.413µg/dl(F=864.154). The total RBC count declined from 1.93 + 0.01 million/mm3 to 0.85 + 0.01million/mm3.The total WBC count also follow a similar result from 1.85+0.11 to 0.93+0.31 thousands/mm3. Gram percrntage of haemoglobin declined from 71.21 + 0.05 to 22.15+3.42. The haematocrait enhanced MCH increased significantly along with percentage of cytotoxicity.

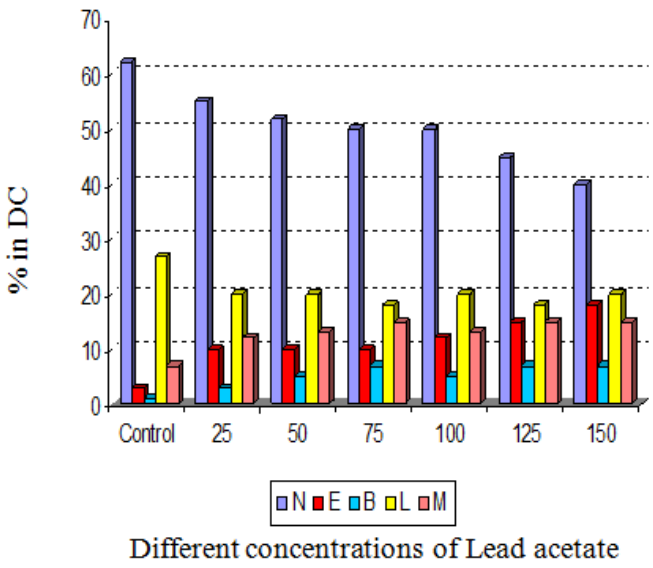

Fig.3. DC of WBC after 3 Days of different Concentrations of Lead Exposure on *Clarias batrachus*

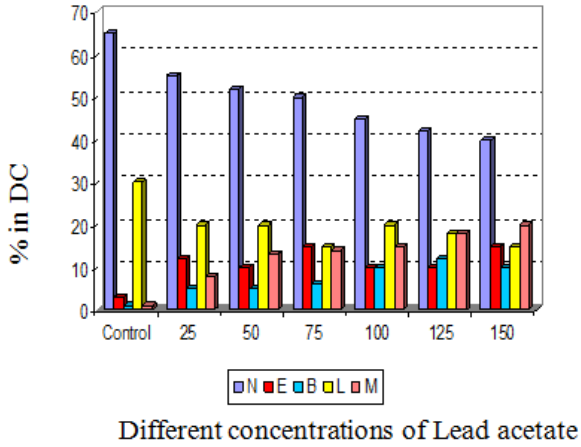

Fig.4. DC of WBC after 7 Days of different Concentrations of Lead Exposure on *Clarias batrachus*

The correlation coefficient (r) between Blood lead content and different blood parameters is very highly significant (Figure 5). The differential count of WBCreveal that there is a clear case of neutropenia (Figure 6), eosinophillia basophillia, and monocytosis. The Lymphocyte percentage is almost constant. The comparison between Normal RBC of the controlled fish and Basophilic punctuated RBC of the experimental fishes is shown in Figure 7. The Blood smear of the experimental fishes show that the macrophases are granulated, the number of basophylls is increased so as the immature RBCs and degraded lymphocytes.

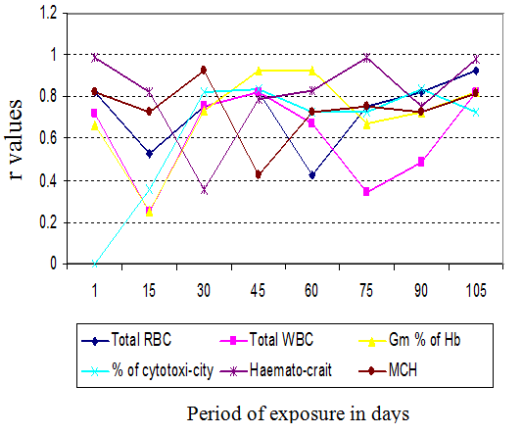

Fig. 5. Correlation co-efficient (r) between blood Lead and Haematological parameters of *Clarias batrachus* during chronic 105 days exposure to 15 ppm of Lead Acetate

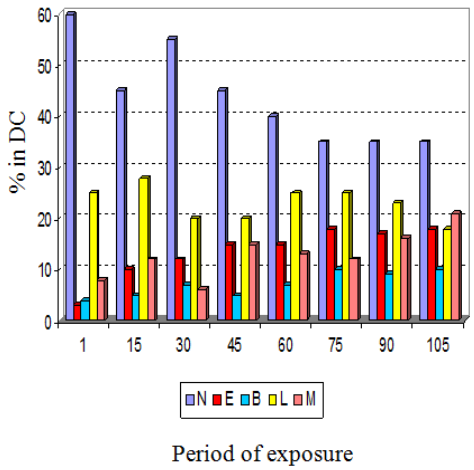

Fig.6. D.C. of WBC during chronic 105 days exposure of 15 ppm Lead Acetate on *Clarias batrachus*

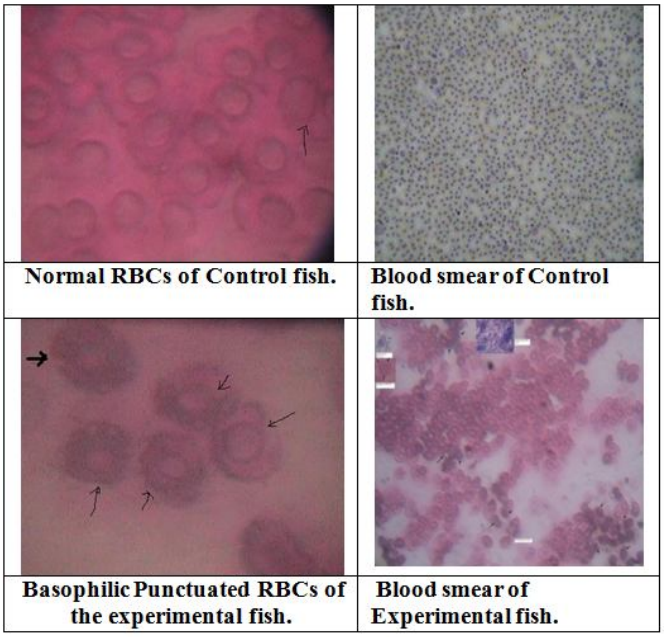

Fig.7. Comparison between the blood smears of control and experimental fishes

IV. DISCUSSIONS

In the previous sections it has been seen that absorption and accumulation of lead is both dose dependant as well as period of exposure. Hence blood is a good indicator of lead toxicosis and effect of lead on haematological parameters is thus evident. The principal clinical manifestations of lead toxicity on the haematopoetic system are anaemia but this occurs only with high levels of exposure (WHO 1996). The results also are very clear of this treatment. During subacute short-term exposure, the gm% of haemoglobin and MCH do not fall so low that the condition is said to be anaemic. Further, the increase in total count of RBC and WBC alongwith cytotoxicity is a compensatory way of cell break and death during exposure to a xenobiotic. This may be a primary response in the experimental animals (Karnakar and Saxsena 1990). The increase in cell number again indicates the immaturity of the cells and their vulnerability to lytic mechanisms. However, during chronic exposure 15 ppm lead acetate exposure after 45 days the fall in haematocrit along with RBC and WBC number indicate a fail in respiratory mechanisms as more than 25% cell death is accounted. Haemoglobin and MCH concentration also fall in the. As per definition, a haematocrit value of less than 35% or reduction of haemoglobin to less than 50% indicate Anaemic condition. Further more, a depressed MCH concentration show that Anaemia is microcytic (Havera *et al.,* 1992) in nature. This is due to an increase in plasma volune caused by disturbed water balance or inhibition “erythropoietin”, the hormones for erythropoiesis or decreased erythrocyte survival. This may lead hypertension as advocated by Howard (2001). The most favourable explaination in this regard is the failure of haemoglobin synthesis as many of its steps are inhibited by lead. Lead inhibits certain enzymes like Pyrixidine -5′- nucleotidase, ACA dehydratase, ferrochelatase, C Coproporphryl rinosenoxidase leading to basophilic stippling of RBCs and Sideroblastosis. It has also been shown that lead induces haem oxygenage activity thereby increasing the degradation of haemproteins, which may adversely affect a number of cell functions such as respiration and energy production. The results of total and differential count of leucocytes need special attention for discussion as there cells are on the main line of the vertebrate immune system. The white blood cells count is indicative of the immune status of a person as the verieties of cells involved in the defence of the body are leucocytes. The increase in total no of WBCs in all the 3 sets may be related to the increase in functions like Phagocytosis and anaphylaxis.

The results of D.C. of WBC show that in all the 3 sets of subacute and Chronic experiments, Neutrophils and Lymphocyte number decrease either with increase in dose or duration of exposure. In contrast Eosinophils, Basophils and Monocytes increase. This indicates the moderate to severe immune neutropenia associated with allergic status, abnormal liver disorder or chronic inflammatory processes. The reduction in neutrophil number is associated with inhibition of myeloperoxidase, Lysozymes, lactofemin and Hydrolases, which generally apply to poor defence against pyogenic bacteria. The increase in Eosinophils and Basophils increase the release of histamine perforin like Protein and C-reactive Protein, the common indices of allergy and anaphylaxis as advocated in earlier studies (Rout and Naik 1998). As the Lymphocytes are associated with both the cell mediated and humoral immune responses, their decrease in number may inhibit the defence mechanism of the body. The revealed monocytes constitute of immature macrophages constituting the mononuclear phagocyte system (MPS) which serve two major functions to ingest and destroy particulate matter by opsonisation when coated by complement or antibody. The other function involves the initial the recognition, Processing and presentation of antigen to the T-lymphocyte to elicit the scientific immune response. The many fold increase in number of monocytes suggest that these monocytes are either unable to mature and perform their function, or to clear the debrises due to successive cell death (as % of cytotoxicity increase) in the blood. The results are very much similar to exposure to mercury (Maheswari *et al*., 2008).

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