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Quantitative precipitation tests for Anti-Avidin during experimental plumbism in Clarias Batrachus, Linn.

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Abstract

Fishes exposed to different short-term and chronic concentrations of lead acetate [Pb(CH3COO)2] solution were sensitized with Avidin , as standardized antigen. The antibodies developed against avidin were quantified by precipitation tests using spectrophotometric methods and were tried to be correlated to quantity of lead accumulated in the blood. Anti-avidin was found to be elevated after 48 hrs. of treatment (from 67.41± 1.436 to 146.8±1.544µ) while decreased after 7 days (from 69.38 ± 1.567 to 36.36 ± 1.862µ and chronic exposure (from 76.69 ± 2.907µ to 24.53 ± 1.082µ significantly. The co- relation coefficient (r) was highly significant after 7th day and chronic exposures. ANOVA tests reveal a dose- dependant effect on the immune system during short-term exposure while a time dependent response during chronic exposure along with lead accumulation in the blood..

Key Words: APDC, Immunity, Antibodies, Antigens, Avidin, Anti-avidin, Lead.

Introduction

According to WHO environmental health criteria (No. 180-1996), because of their environmental conditions, fish are an excellent model for studying the effects of water and sediment-borne pollutants. There are several other good reasons for studying immunotoxicity in fish: as many of the diseases are related to environmental quality, various pollutants have immunotoxic potential, and many of the diseases have an immune component. Fishes are easy to obtain, there is an extensive body of knowledge, and their economic interest facilitates the finding of research resources. At present immunotoxicology in fish is not as sophisticated as that in mammals. Screening and functional tests are being developed in the laboratory but cannot yet be applied for field and laboratory studies. Our model fish, Clarias batrachus fulfills almost all the criteria outlined above for immunotoxic studies. In our earlier studies we have reported some of the screening and functional tests (Rout and Naik, 1996, 1998b, 1998c) that could just open the gate to understand the mechanism of immunotoxicity by lead. Furthermore, we also have tried to understand the DNA, RNA and Protien relations in different organs including those involved in haemopoiesis and formation of lymphocytes (Rout et.al. 1997a). Lead interacting with activities of immunologially important enzymes was also under our investigation (Rout, Choudhury and Naik, 1998a).

In the present study our aim is to detect and quantify precipitating antibody for a specific antigen like Avidin. Again Anti-avidin has to be correlated to lead-induced immune supression and the degree of association is to be understood.

2. 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 (CH3-COO)2], Johnson and Sons, Ltd., London, 1990] were chooseH after obtaining LC50 (500 ppm) and LT50 (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.

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 LT50 (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 (Fig. 1) following APHA (1980) carefully in each day during replacement of water.

2.2. Development and estimation of anti-avidin

10 mg. packs of Avidin were procured from SISCO. Research Laboratory (SRL) Mumbai and dissolved in 4 ml, of physiological saline to 1.0 ml of 10% potash alum. 10% sodium carbonate solution (0.5 ml, is then added to bring the PH between 5.8 and 6.8. Thus total 5 ml. contains 10 mg. (=10,000 µg.) of avidin- adjuvant complex. Each fish was then injected intra-peritonealy with 0.1 ml. of avidin-adjuvant carefully before 48 Hrs. of test. The fishes thus developed antibody against avidin called anti-avidin.

Collection and preparation of antiserum was done following plumer (1987) and Rastogi (1996). 2 ml. of avidin was pipetted out into a test tube and slowly 2 ml. of saturated ammonium sulphate solution was added, left on ice for about 30 minutes with occasional stirring. Then it was, centrifuged at 2500g. for 15 mins. Supernatant was removed and the pellet was resuspended in a known volume of the buffered saline. The extinction was measured at 280 nm. (Systronics- 108) on a suitably diluted sample and calculated by assuming 1 mg./ml. of protein present 1 mg/ml of protein present has an extinction of 1.40 at. 280 nm. Then avidin was added to a series of tubes in following quantities: 0, 10, 20, 50, 100, 200, 400, 500, 1000 and the PBS was added to bring the final volume to 0.45 ml. Then 50 ul of antiserum was added to each test tube, thoroughly mixed and incubated at 37 C for 1 Hr., then 4 C for overnight. The tubes were then centrifuged at 300 g for 5 minutes and supernatant was removed. The precipitate was washed with ice-cold buffer saline and dissolved in 0.1 mol/litre NaOH.

Extinction of each tube was read at 280 nm and a graph was plotted between OD against amount of Antigen added. Finally the antibody count per ml. of antiserum was determined.

2.3 Estimation of lead

Lead was estimated by Atomic Absorption spectrometry as described earlier (Rout et.al. 1997a) using APDC (Ammonium Pyrrolidinedicarbonate, Aldrich, 1990) following Australian standard methods of Analysis (AS-241. 1980). After collection of blood by heparinized syringe with caudal puncture samples were kept in 4 C. During analysis 1.5 ml. of blood was dispersed to the centrifuge tube and complexed with 0.25 ml of 2% APDC. Then the complex was extracted into 1.5 ml of n- butyl acetate by proper shaking. Lead was estimated in the organic phase by AAS . (Purkin elmer- 31)within one hour at 510 nm.

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

2.4 Stastitical analysis

The data obtained were expressed in terms of Mean± SEM, Unbiased student's t-test was performed following Sanders(1994). Correlation between lead accumulation respective change in anti-avidin concentration was analyzed by parson's co-relation coefficient.

One-tailed ANOVA tests were carried on to find the effectiveness of dose variation against anti-avidin content in short-term exposure while in chronic case/s the same is done to show the effectiveness of variation in exposure period.

3. Results

Anti-avidin content after 3 days of exposure is found to be increased significantly from 67.41 ± 1.436µg to 146.8 ± 1.544µg a1ongwith accumulation of lead in the blood from 4.24 ± 0.340µg in control up to 13.44 ± 0.55µg in fishes, exposed to 150 ppm of lead acetate. The correlation coefficient between lead accumulation and anti-avidin content is highly significant (Table-1). The ANOVA test for effectiveness of variation of dose of lead acetate has shown highly significant value of F and about 0.86 of total variances are related to change in concentration of lead acetate (Table-2).

After 7 days of treatment production of anti-avidin is significantly decreased from 69.35±1.567µg to 36.36 ±1.882µg with increased accumulation of lead from 4.05 ± 0.365µg to 20.42 ±1.015µg/dl.The co-relation coefficient 'r' between decrease in antibody content lead accumulation is significant at each concentration (Table -3). The ANOVA tests also show effectiveness of variation in doses of lead acetate and production of antibodies (F=51.236). Around 0.81 of total variations tested to be related to change in concentration of lead acetate (Table- 4).

The results of chronic exposure to 15 ppm lead acetate is shown in table- 5.After initial elevation in anti-avidin content (from 69.45±2.877 µg/ml to 76.69±2.907µg/ml), the production falls constantly throughout the period of exposure and reaches at 24.53 + 1.082 µg/ml (almost two and half fold decreases) in 105 th day. The co-relation coefficient 'r1 between antiavidin a lead accumulation at every set of experiment is highly significant. ANOVA tests also justify a time dependent decreases in anti-avidin production (F= 85.0) during chronic exposure.

4. Discussion

After 3 days of exposure in various concentrations of lead acetate the production of anti-avidin increased from 6.53% to 26.04% (Fig.2-4|)depending upon the dose. This short of dose-dependent response is also reported earlier in case of C-reactive protein (Rout and Naik - 1998 c), a non-specific immune profile which increases with inflammation caused by a toxic substance like lead. As here Avidin is a specific antigen derived from egg albumin of birds, it thus triggers the production of a specific antibody like anti-avidin. Here it must be noted that the fishes are injected with antigen before they are released into the lead exposure medium along with the control. Hence the stimulation of primary humoral immune system is needed to be discussed. In animals the primary response is carried out by B-lymphocytes which get cloned after sensitized by an antigen and thus produce antibodies

against the specific antigen. Whether lead is mitogenic or not it is not reported. But the elevation of CRP and Histamine as reported earlier (Rout and Naik, 1996, 1998b, 1998c) are mitogenic in nature. One may thus speculate that after the fish is sensitized with an antigen and released immediately to a toxic medium the non-specific immune profiles may stimulate the specific one which at initial period is independent of the toxin (lead) as outlined by Roit (1998) and Falus (1994) . However the common hypothesis of lead's interferance with antibody formation is not verified as in this case the r- value remain insignificant in four subsets out of six studied (Table - 1).

Again lead is always a cumulative poison as its impact on body is not immediately manifested (Parikh 1990) ; (WHO 1995). This can be well understood with inhibition of antiavidin production after atleast 7 days of treatment in Clarias batrachus . After seven days the production .of anti-avidin is decreased from 950% up to 25.36% depending of doses of exposure, while during chronic 15ppm exposure depending upon period of exposure it was inhibited from 4.77% to 25.79% (Fig. 3 and Fig. 4), The results support earlier works as reported by Koller (1973, 1990) Koller and Kovacic (1974). Trust et.al (1990).

So far, as literature regarding fish immunology are a few, at this stage it is known that fishes posses Predominantly IgM-the immunoglobin responsible for agglutination and precipitation properties. (Douglas and Anderson- 1974). The etiological components and their role in pathogenesis are poorly understood (WHO- 1996). The immunoglobulin M (1 gm) in mammalian systems is a pentameric structure. Again each unit is supposed to bind two antigenic determinant and thus one molecule is supposed to bind ten antigenic determinants. The five four-chain subunits are linked by two dis-ulfide "bonds, In addition there is a J-chain with eight cysteine residues, six of which are involved in the formation of intra-chain disulphides. These dis-ulfide bridges are main areas of attack by ionic lead (Pb) as earlier reported by several workers (Chaurasia et.al 1997, Rout and Naik.l998a). The reaction leads to removaa. of a thiol group (-SH) from the protein by lead and thus disruption of the original configuration of the protein. The antibodies thus get precipitated.

Conclusion:

The results hold good as agglutination tests previously were found to be of similar observations. IG M of the fish may not be "same as in mammalian cases, but so far the interaction of lead with sulphide linkage is true , the findings hold ok. However, more emphasis should be given on the detailed structure of fish immunoglobulins and their nature.

References

1. *AS: Australian Standard Method of Analysis AS-241.(1980), Sydney .*
2. *Chourasia SS, Panda S And Kar.A,(1997) inhibits type-I iodiopathy-ronine S' monodeiodinase in the Indian rock pigeon Columba livia; A possible involvement of essential thiol groups J. Biosci. 22 (2) pp. 247-254.*
3. *Douglas P, and Andesron E (1974): In Fish immunology (Edrs). S. Sniesikoaa H.R. Oxford TFH, publications Inc. Ltd., London.*
4. *Falus. A (1994): Histamine modulates acute phase response at multiple points. Immunology Today 15(12) pp. 596.*
5. *Koller, L.D. (1973) Immunosupression Produced by Lead, cadmium and mercury, Amer. J. Vert. Res. 34 (11) p. 1457-1458.*
6. *Koller LD (1990). The immunotoxic effects of lead-exposed laboratory animals. Ann. N. Y. Acad Sci., 587 p.160-167..*
7. *Koller, Ld and Rovacic. A (1974) Decreased antibody formation in mice exposed to lead: Nature 250: p. 148-150.*
8. *Parikh CK(1996): A text book of toxicology and medical jurisprudence , 3rd Edn. CBS Publication, New Delhi pp. 763.*
9. *Plumer. D.T. (1988): An Introduction to _ Practical biochemistry, 3rd Edn. Tatamc. Graw Hill publication, New Delhi, p. 112-113.*
10. *Rastogi SC (1996): Immunodiagnostics, Principles and practice. New Age International Publications, New Delhi, pp. 30-32.*
11. *Rqit I M (1988) : Essential Immunoldgy: ELBS Publication, London.*
12. *Rout PC and Naik BN (1996), Immunotoxic studies on Clarias bachachusr linn, during experimental Plumbism- J. Appl. Zool. Res. 7(2) p. 185-186,*
13. *Rout Prafulla Chandra, Jena Sushresumita, Khatua AK, Naik BN and Baral AK, (1997)a: Evaluation of DNA, RNA and protien relations with response to lead accumulation in different tissues of Clarias batrachus, Linn, dirung experiemntal Plumbism. J . Appl, Zool. Res. 8(1) p. 91-93.*
14. *Rout Prafulla Chandra, Naik BN and Choudhury S(1997b): Lead accumulation in various tissues of Clasias batrachus during experimental plumbism. J. Appl. ZooT"!Res. 8(2) p.157-159.*
15. *Rout Prafulla Chandra, Naik BN (1998)a: activities of two importent enzymes in blood stream of clarias batrachus during experimental plumbism J.Appl. zoo I". Res. 9(1&2). P.58-59.*
16. *Rout Prafulla Chandra and Naik BN(1998)b. Immunotoxic studies on clarias batrachus during subacute lead toxicity.J.Natcon 10(1) p.97-99.*
17. *Rout Prafulla Chandra and Naik BN (1998)c: (Presented): Elevation of blood histataine and C-reative protien during short-term lead (Pb) exposure in Clarias batrachus ; National symposium^n End-ocrinologyand its relevance to Biotechnology, Dept of Zoology and International centre of Biotechnology. Utkal University, Bhubaneswar, April, 24-27.*
18. *Sanders, D.H, (1994): Stastistics: a fresh Approach, Me. GrGw Hill Publication, New york.*
19. *Trust KA,Miller MW, RINGELMAN JK AND ORME IM (1990): Effect of injected lead on antibody Production in malads (Anas platyrhynchas) J. Wild life. Dis. 26 (3) P. 316-322.*
20. *WHO(1995): Inonganic lead. Environmental health criteria. 165. published by WHO. Geneva.*
21. *WHO (1996): Principles and methods for assessing direct immurotoxicity associated with exposure to chemicals. Environmental health- criteria 180: Published by UNEP, ILO, WHO Geneva.*

Table 1
Anti-avidin and lead content in blood of Clarias batrachus after short-term i.e. 3 days exposure to different concentrations of lead acetate.

Concentrations of Lead Acetate (ppm)	Concentration of Anti-avidin (µg/ml)	Blood Lead (µg/dl)	Correlation co-efficient ®
Control (0)	67.41 ±1.436	3.8 ±0.001	0.324
25	87.31 ± 1.776	12.30 ±0.269	0.211
50	99.85 ± 1.208	13.01 ±0.251	0.821*
75	107.56 ±1.137	13.52 ±0.512	0.725†
100	127.66 ±2.433	14.13 ±0.132	0.825⊥
125	140.22 ± 2.399	13.21 ±0.213	0.325†
150	146.8 ±1.544	12.69 ±0.619	0.924*

*p<0.05 , ⊥p <0.01, †p <0.005

Table-2 :
ANOVA tests for effectiveness of dose variation of lead acetate on production of antibody after 3days (48 hrs) of exposure of clarias batrachus.

Sources of variation	Sum of squares	Degree of freedom	Variances	F	ω^2
Between groups	10801.4	6	1800.3	65.95	0.86
Within groups	1715.81	63	27.3		
Total	12517.21	69			

Table 3 :
Anti-avidin and lead content in blood of Clarias batrachus after sub-acute i.e. 7 days exposure to different concentrations of lead acetate.

Concentratons of Lead Acetate (ppm)	Concentration of Anti-avidin (µg/ml)	Blood Lead (µg/dl)	Correlation co-efficient®
Control (0)	69.38 ±1.567	3.7 ±0.001	0.624
25	56.92 ± 1.608	13.50 ±0.544	0.323⊥
50	55.89 ± 1.292	14.56 ±0.279	0.425†
75	49.90 ±1.599	15.26 ±0.716	0.426†
100	45.96 ±1.366	17.19 ±0.218	0.725*
125	40.94 ± 2.0	18.25 ±0.125	0.821*
150	36.36 ±1.822	22.21 ±0.238	0.722*

*p<0.05 , ⊥p <0.01, †p <0.005

Table-4
ANOVA tests for effectiveness of dose variation of lead acetate on production of antibody after 7-days of exposure of Clarias batrachus.

Sources of variation	Sum of squares	Degree freedom	Varia-nces	F	ω^2
Between groups	7384.5	6	1230.7	51.236	0.81
Within groups	1513.46	63	24.02		
Total	8897.96	69			

Table 5
Anti-avidin and lead content in blood of Clarias batrachus after chronic 105 days exposure to 15ppm of lead acetate.

No. of Days of Exposure		Concentration of Anti-avidin (µg/ml)	Blood Lead (µg/dl)	Correlation co-efficient®
01	C	69.45 ±2.877	5.0 ±0.187	0.924†
	E	76.69 ±2.907	10.11 ±0.577	
15	C	65.88 ±0.932	4.83 ±0.178	0.922*
	E	58.29 ±2.483	21.53 ±0.758	
30	C	65.85 ±1.036	5.02 ±0.158	0.928*
	E	54.4 ±2.448	41.53 ±1.561	
45	C	64.96 ±0.942	4.78 ±0.129	0.823*
	E	49.44 ±1.636	55.07 ±0.721	
60	C	66.22 ±0.898	4.98 ±0.286	0.853*
	E	44.89 ±1.191	82.25 ±1.406	
75	C	65.15 ±1.280	4.85 ±0.183	0.804*
	E	38.9 ±1.535	111.29 ±1.504	
90	C	65.20 ±1.148	4.85 ±0.210	0.809*
	E	28.37 ±1.224	123.35 ±2.508	
105	C	65.50 ±1.063	4.98 ±0.613	0.761*
	E	24.53 ±1.052	141.12 ±2.413	
F-	C	0.723	2.675	
	E	96.28	864.154	

C= Control, E= Experiment *p<0.05, ⊥p <0.01, †p <0.005

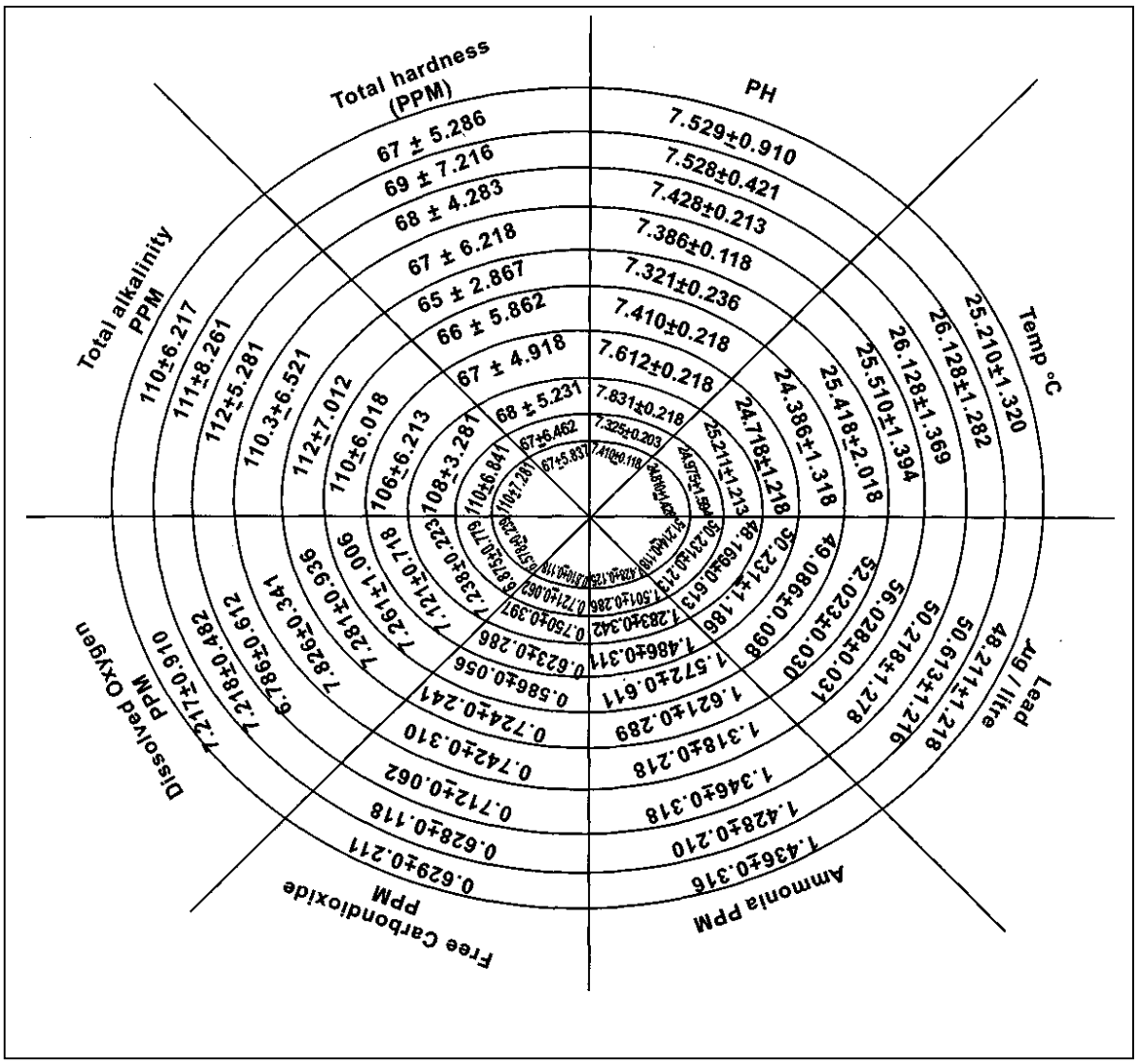

Fig . 1. Physico-Chemical profile of aquaria water used during the Experimental period (meant SEM). The Characters are shown for 1st, 3rd, 7th, 15th, 30th, 45th, 60th, 75th, 90th and 105th days respectively from the centre out wards.