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RESEARCH ARTICLE

"AnEvaluation of Lead (Pb) Toxicity in Developing Zebrafish(Daniorerio) Embryos"

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Abstract

Lead (Pb) toxicity is known to cause an array of defects including developmental, cellular and neurological damage. Although numerous studies have attempted to characterize the mechanism underlying lead toxicity, the exact molecular targets remain unclear.Additionally, there is a discrepancy among different studies regarding the concentrations of lead at which developmental defects arise. The present study was conducted to characterize the developmental malformations induced in Zebrafish embryos following a brief exposure to lead. The study also attempted to identify changes in protein profiles in Zebrafish embryos following lead exposure to identify putative molecular targets of lead toxicity. Embryos were treated with varying concentrations of lead (100ppb,500ppb, 1000ppb) at 5 hours post fertilization (hpf) until 24hpf. Morhological observations of various developmental parameters such as embryo viability, hatching rate, heart beats, eye diameter, straightening of body axis were carried out at 48hpf, 72hpf and 96hpf. Proteins extracted from embryos at these stages were subjected to SDS-PAGE and silver staining to observe changes in protein band patterns following lead treatment. An increase in hatching rate, heart beats and eye diameter was notedat 48hpf following treatment. Lead treatment was also seen to induce subtle changes in electrophoretic protein band patterns as compared to controls.

INTRODUCTION

The desirable characteristics of lead - a malleable heavy metal resistant to corrosion - have resulted in its extensive use, especially in building construction, batteries, piping, paints, solders etc. for the past several millennia and it continues to remain a ubiquitous metal even today. Studies in the past two decades have established that lead is an environmental pollutant that is known to cause an array of defects including developmental, cellular and neurological (reviewed in Kali and Flora, 2005)The effects of lead toxicity are more severe upon exposure at very early stages of development (Xing et al., 2009). Thus the acceptable dose levels for lead in adults do not hold true for children, where lead produces lasting neurological damage at doses which are well below those that produce toxicity in adults (Rodier, 2004). Of most concern among the effects of low-level Pb exposure is the occurrence of reduced cognitive capacity in children exposed early in life (Bellinger et al., 1991). Lead is a potent neurotoxin that damages the nervous system and causes brain disorders (Hsiang and Diaz, 2011). Alterations in the properties of glutamatergic, cholinergic, and dopaminergic neurotransmitter function and signal transduction have been reported(Bielarczyk et al., 1994; Lasley and Gilbert, 2002; Fortune and Lurie, 2009)wherein Pb exposure leads to perturbation in the GABAergic and glutamatergic systems in adult rat brains (Struzynska and Sulkowski, 2004).

Lead toxicity has been assessed in a number of experimental animal models and almost all yield similar deleterious results. In one such study, zebrafish embryos were treated with a sub-lethal dose of lead acetate immediately after fertilization till 72hpf (hours post fertilization) and 120hpf. Lead was seen to affect neural morphogenesis and dendrite branching (Peterson et al., 2011; Zhang et al., 2011). Similar studies demonstrate lead interference with enzymes of the heme synthetic pathwayand immunomodulatory effects of lead, especially on the functioning of testicular macrophages(Patrick, 2006; Barbhuiya et al., 2013), among many others. Despite the exhaustive research on effects of lead in a number of animal models, the precise mechanism of action continues to elude us although putative targets have been suggested. Zebrafish is a universally recognized model organism for toxicological studies, useful due to its similarity to humans in terms of gene homology and similar vertebrate body plan. Coupled with its ease of availability, small size and transparent embryos, it served as a cost-effective, low maintenance system for the present study (Hill et al., 2005; Chakraborty et al., 2009).

MATERIALS AND METHODS

2.1 MAINTENANCE AND BREEDING OF ZEBRAFISH

Wild type zebrafish were procured from local fish breeders and maintained in aerated standard glass tanks in dechlorinated tap water at a temperature of 28±2 ºC and a pH of 6.5±1. Males and females were maintained in separate tanks under an artificial 14:10 hour light: dark cycle and were fed with standard food pellets twice a day.

For breeding purposes, male and female zebrafish were placed in specially constructed breeding tanks overnight in the ratio 1:2. Eggs laid the following morning with the first flash of light were aspirated using blunt-ended droppers and transferred to petridishes containing Hanks embryo medium.

2.2 TREATMENT AND FIXATION OF EMBRYOS

Prior to lead treatment, the embryos were staged using developmental milestones documented by Kimmel (1995) and their development was studied so as to determine any deviations in these milestones as compared to literature due to environmental conditions, temperature, geographical location, etc. Following this, the embryos were segregated into control and treated groups with the latter being subjected to lead nitrate (MERCK, India) concentrations of 100ppb, 500ppb and 1000ppb. The concentrations chosen were sublethal, well below those that induced overt toxicity as per earlier documented studies (Peterson et al., 2011). The embryos were exposed to lead concentrations at the blastula stage till 24hpf, at which point the lead was washed off and the embryos were transferred to Hanks embryo medium.

At regular time points *i.e.* 24hpf, 48hpf, 72hpf and 96hpf, a batch of embryos (n=10, number of sets=20) were withdrawn from culture and fixed overnight in 4% paraformaldehyde (SDFCL, India) at 4 ºC. Post fixation, the embryos were washed in 2X PBS solution at room temperature, then subsequently transferred to methanol (MERCK, India). The embryos were then stored in fresh methanol at -20 ºC till further use in morphological studies.

2.3 PHYSIOLOGICAL AND MORPHOLOGICAL EVALUATION

Physiological parameters such as mortality, hatching and heartbeats were assessed at 24hpf, 48hpf, 72hpf and 96hpf. For assessment of morphological parameters, fixed embryos were mounted in DPX (di-n-butylphthalate in xylene) (SDFCL, India) on glass slides and photographed at 4X and 10X magnification with a light microscope- attached camera (Lawrence & Mayo PH100 with a 0.46X magnification).

2.4 PROTEIN EXTRACTION AND ELECTROPHORESIS

Proteins were extracted at 48hpf and 72hpf using previously documented regents and protocols (Westerfield, 1995).Quantitation of proteins was carried out by the Folin-Lowry method using BSA (bovine serum albumin) (LOBA Chemie, India) as a standard. 15µg protein was resolved by SDS-PAGE on a 7.5% gel at 100V. Proteins were visualized via silver staining. The images were captured using gel doc software (AlphaDigiDoc RT2).

RESULTS

3.1 EFFECT OF LEAD ON MORTALITY, HEART RATE AND HATCHING

The zebrafish embryos were treated with lead concentrations of 100ppb, 500ppb and 1000ppb for a period of 24 hours. As compared to the control group, lead treatment was seen to induce an increase in percent mortality. The increase in percent mortality however, was not statistically significant at any dose of lead (Table 1).

Assessment of the heart rate of embryos treated with lead, relative to control group, revealed a statistically significant increase in heart rate. At 24hpf, the increase was found to be statistically significant for all three doses of lead. The effect was normalized by 48hpf where the increase in heart rate was found to be statistically significant only at 100ppb of lead (Table 2).

In developing zebrafish, embryo hatching is typically observed between 48hpf and 72hpf. The hatching percentage at 48hpf was found to be 38.6% for control groups, 34.4% at 100ppb, 50% at 500ppb and 82% at 1000ppb of lead. While an increase in hatching percentage was observed in the treated embryos as compared to control embryos at

48hpf, the increase was not statistically significant. By 72hpf, hatching was complete in both, the treated and control groups.

3.2 EFFECT OF LEAD ON ZEBRAFISH MORPHOMETRY

Methanol-fixed embryos from the treated and control groups were air-dried, mounted on glass slides in DPX and photographed (Figures 1 and 2), following which morphometric measurements such as eye diameter, yolk sac diameter and embryo length were taken. Treated embryos showed an increase in eye diameter as compared to control embryos at 48hpf. The increase was found to be significant at 1000ppb of lead. At 72hpf, there was an increase in eye diameter at 100ppb and 1000ppb of lead, along with the decrease in diameter at 500ppb of lead. These measurements however, were not statistically significant (Table 3).

Yolk sac diameter showed a relative increase in the treated groups compared to the control group. This increase however, was not to statistically significant levels (Table 3).

There was no significant difference seen in embryo lengths of embryos treated with lead against control embryos at 48hpf or 72hpf (Table 3).

3.3 EFFECT OF LEAD ON ZEBRAFISH EMBRYO PROTEIN PROFILES

Figure 3 indicates the results of SDS-PAGE carried out using proteins extracted at 48hpf and 72hpf from control and lead-treated embryos. At 48hpf, the proteins with molecular weights of 51.53 KDa, 54.88KDa and 59.82 KDa show a decrease in intensity at 500ppb of lead as compared to the control sample while the 76.41 KDa molecular weight protein showed an increase in intensity at 500ppb of lead, suggesting a change in levels of these proteins as a result of lead treatment.

The abundance of protein of molecular weight 14.92 KDa at 48hpf suggests that it is probably yolk protein which is seen to disappear with successive stages and ceases to be visible at 72hpf. Further, the 43.23 KDa molecular weight protein seen at 72hpf but not at 48hpf is suggestive of another stage-specific zebrafish embryo protein.

At 72hpf, the proteins of molecular weights 51.53 KDa and 59.52 KDa show a decrease in intensity with 100ppb and 500ppb lead treatment, similar to 48hpf. At 1000ppb of lead, there is an increase in intensity of the higher molecular weight protein bands though the poor resolution of these proteins renders their assessment inadequate

Table 1. Percentage mortality of zebrafish embryos following lead exposure

	Control	100ppb	500ppb	1000ppb
24hpf	26.44 ± 6.72	22.51 ± 7.79	30.4 ± 10.63	32.35 ± 12.17
48hpf	12.97 ± 11.14	12.28 ± 10.45	23.66 ± 4	18.65 ± 10.25
72hpf	42.81 ± 13.66	52.15 ± 17.24	51.32 ± 16.1	78.0 ± 12.22

Table 2. Effect of lead exposure on heart beats of zebrafish embryos

	Control	100ppb	500ppb	1000ppb
24hpf	30.34 ± 1.79	**36.06 ± 1.09***	39.31 ± 1.09*	**39.94 ± 1.18***
48hpf	52.9 ± 1.12	**55.7 ± 0.54***	53.75 ± 0.98	53.65 ± 0.42
72hpf	53.43 ± 1.19	50.4 ± 1.71	50.05 ± 1.17	48.94 ± 2.22

Values indicate heart beats per 15 seconds.

*Statistically significant at α=0.05 as compared to vehicle control

Table 3. Morphometric measurements (mm) of Control and lead-treated embryos

		Control	100ppb	500ppb	1000ppb
EYE DIAMETER	48hpf	0.391 ± 0.023	0.412 ± 0.089	0.431 ± 0.009	**0.456 ± 0.01***
	72hpf	0.513 ± 0.031	0.530 ± 0.024	0.473 ± 0.007	0.561 ± 0.029
YOLK SAC	48hpf	1.041 ± 0.056	1.062 ± 0.016	1.072 ± 0.018	1.17 ± 0.108
	72hpf	0.815 ± 0.071	0.868 ± 0.068	0.916 ± 0.047	0.935 ± 0.064
FULL LENGTH	48hpf	0.139 ± 0.011	0.165 ± 0.001	0.146 ± 0.003	0.15 ± 0.004
	72hpf	0.158 ± 0.005	0.157 ± 0.004	0.151 ± 0.006	0.152 ± 0.002

*Statistically significant at α=0.05 as compared to control

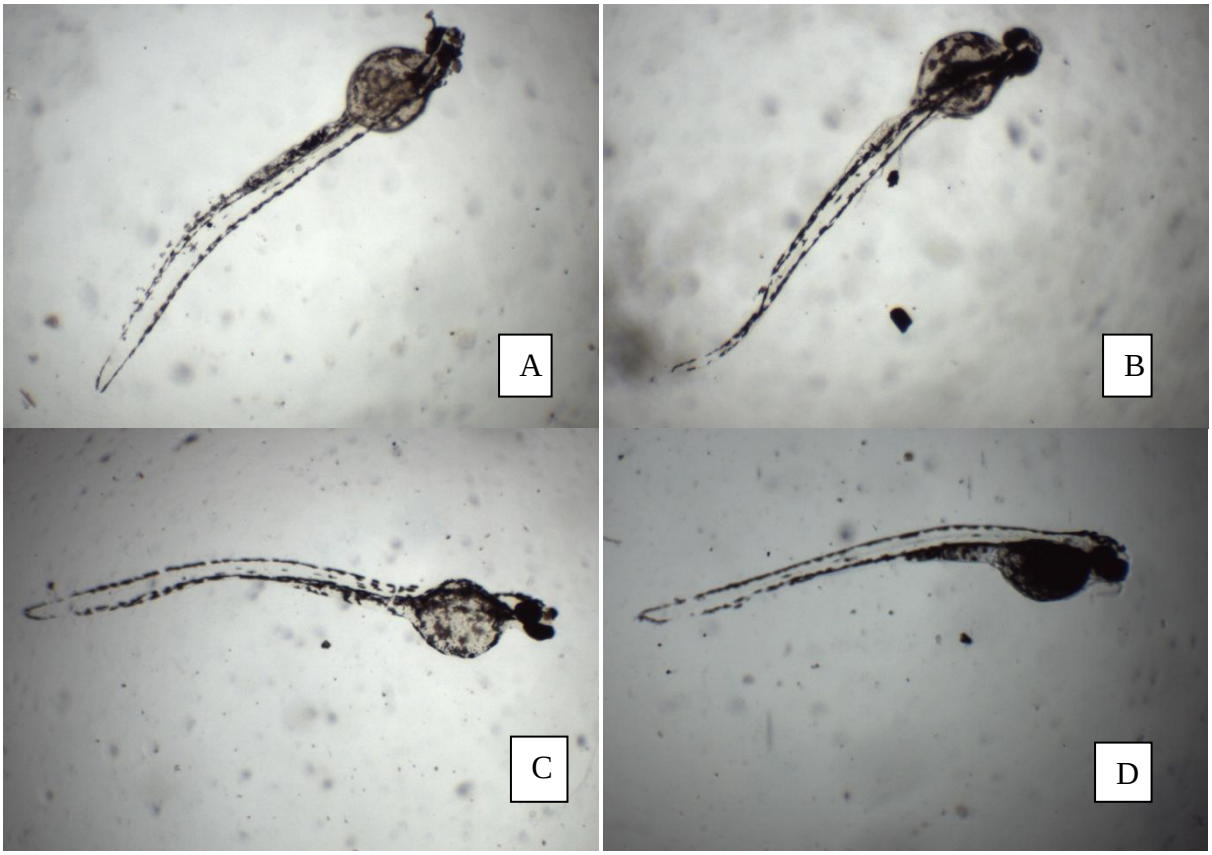
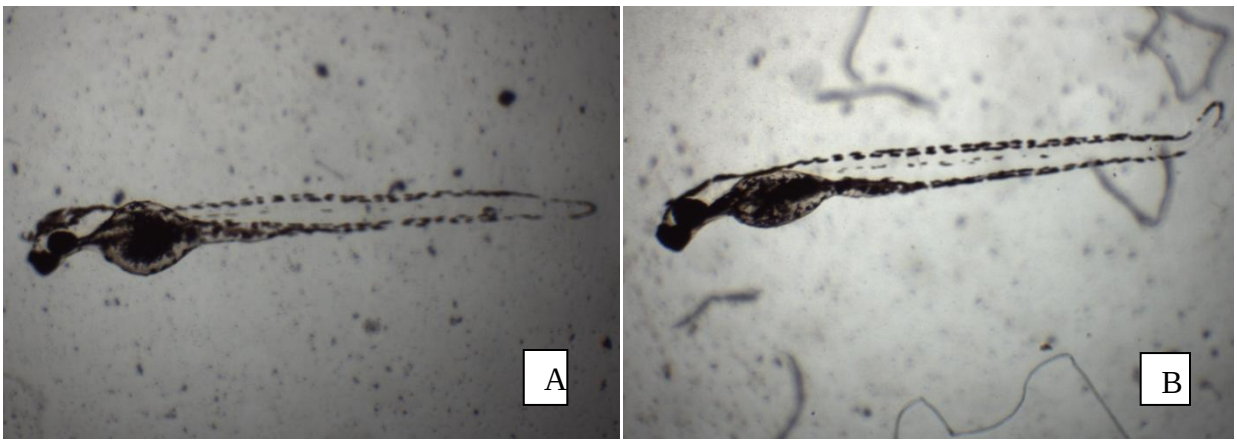


Figure 1.Embryo morphology at 48hpf. A, Untreated control embryo; B, 100ppb lead-treated; C, 500ppb lead-treated; D, 1000ppb lead-treated. Mag X 40.



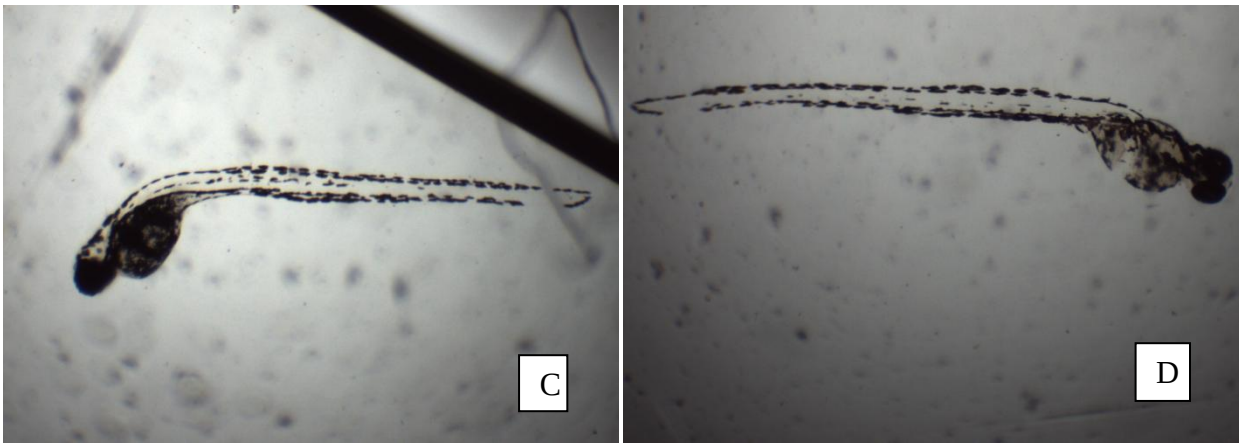


Figure 2. Embryo morphology at 72hpf. A, Untreated control embryo; B, 100ppb lead-treated; C, 500ppb lead-treated; D, 1000ppb lead-treated. Mag X 40.

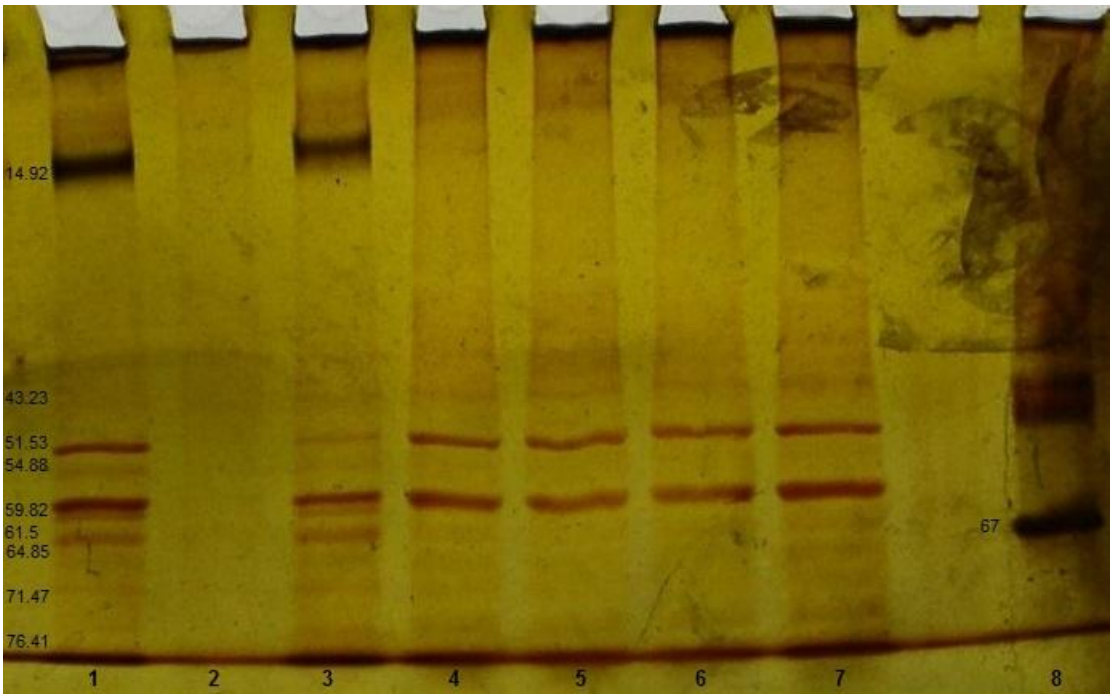


Figure 3. Silver-stained PAGE protein profiles of control and lead-treated zebrafish embryos. Lanes 1,2,3 depicting protein profiles of control embryos, 100ppb lead-treated and 500ppb lead-treated, respectively at 48hpf. Lanes 4,5,6,7 depicting protein profiles of control embryos, 100ppb lead-treated and 500ppb lead-treated, respectively at72hpf. Lane 8 depicting bovine serum albumin, the reference protein.

DISCUSSION

The study was undertaken with a view to characterize the normal development of zebrafish under the environmental conditions of our laboratory and to assess the effects of lead on morphology of the embryos. Along with this, another aspect of the study under consideration is the effect of lead on protein profile of zebrafish embryos. An alteration in protein profile is likely to indicate possible lines of investigation for further studies on the toxic mechanisms of lead.

Zebrafish is a tropical freshwater fish, belonging to minnow family Cypriniformes. Its similarity to humans in terms of its homology with genes and similar vertebrate body plan makes it an appropriate model for toxicity studies. Coupled with its ease of availability, small size and transparent embryos, it served as a cost-effective, low maintenance system that could be established with minimum efforts and proved useful to our study design.

Lead has long been recognized as a toxic agent that has a significant impact on human health. Lead is known to cause a number of adverse effects in humans such as kidney damage, hypertension, neurological damage *etc*. An increasing body of evidence has demonstrated that blood lead levels above 10ug/dL produce many behavioral deficits including lowered IQ, attention deficit hyperactivity disorder, etc (CDC Atlanta, 2005)

The lead concentrations chosen were sublethal doses, well below those that induced overt toxicity as per earlier documented studies(Peterson et al., 2011; Zhang et al., 2011).As such, the concentrations of lead nitrate used for exposure were 100ppb, 500ppb and 1000ppb.

The sublethal doses chosen showed no significant difference in embryo mortality at 24 hpf, 48 hpf or 72 hpf. Support for the results obtained can be cited from previous studies with lead which show that only those lead concentrations above 5000ppb show a significant difference in mortality by 120 hpf (Peterson et al., 2011).

The effect of lead on heart rate was assessed at 24 hpf, 48 hpf and 72 hpf while hatching was seen to occur at around 48 hpf and was completed by 72 hpf. Lead was seen to induce a significant increase in heart rate for all three doses at 24 hpf and for 100 ppb at 48 hpf. The increase at 24 hpf normalized by 48 hpf and 72 hpf which could be indicative of an adaptive response by the embryo to counter any adverse changes induced by lead. The increase in hatching percentage at 48 hpf in lead treated embryos was not statistically significant thus hatching appeared to be unaffected at the lead concentrations chosen for the study.

With respect to morphometric measurements, lead was seen to induce an increase in eye diameter to a statistically significant level at 1000ppb at 48 hpf. The moderate increase in yolk sac diameter and embryo length was not statistically significant.

Assessment of protein profiles of zebrafish embryos treated with lead reveals changes in band intensities which can be correlated with changes in protein levels as a result of lead exposure. A study depicted accumulation of an unprocessed protein RELN in lead treated protein extracts of embryos due to possible interference with protein degradation (Peterson et al., 2011). Our finding might have a similar mechanism resulting in the accumulation of a toxic unprocessed protein responsible for some of the observed morphological defects. Alternatively, the changes in protein profiles at higher lead concentrations of 500ppb and 1000ppb could be indicative of an up-regulation of protective proteins with a view to counter the adverse effects of lead on embryo development. The exact mechanism of lead toxicity is presently unknown and cannot be elucidated from this present study immediately; however, further characterization of these proteins is likely to reveal insights into the possible toxic mechanisms of lead.

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