DEVELOPMENTAL AND BIOCHEMICAL TOXICITIES INDUCED BY ECOLOGICALLY RELEVANT CONCENTRATIONS OF ARSENIC, CADMIUM, AND LEAD ON ZEBRAFISH EMBRYOS

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Heavy metals like As, Cd, and Pb at trace concentrations cause non-negligible effects on the environment. Limited literature in this regard formed the base of the present study to investigate the toxicities induced by ecologically relevant concentrations of As, Cd, and Pb incorporating the zebrafish embryo-toxicity model. The zebrafish embryos at 2 hpf were exposed to a series of environmentally relevant concentrations of As, Cd, and Pb for 24 to 96 hrs adhering to the OECD guideline no. 236. Mortality (%), hatchability (%), and LC_{50} were determined. Zebrafish embryos exposed to sublethal concentrations of As^{3+} (4.6 µg L^{-1}), Cd^{2+} (0.3 µg L^{-1}), and Pb^{2+} (3.0 µg L^{-1}) were utilized to determine the developmental toxicities, activities of liver damage marker enzymes (ALP, GOT, GPT, and LDH), total protein, and creatinine levels. Exposure to trace levels of As, Cd, and Pb severely affected the survival and hatchability of zebrafish embryos. The LC_{50} values of As^{3+}, Cd^{2+}, and Pb^{2+} were recorded as 46.366 ± 0.186, 264.841 ± 0.18, and 2978.43 ± 0.199 µg L^{-1}, respectively at 96 hpf. Sublethal doses of As^{3+} and Cd^{2+} produced marked reductions in the eye area and total body length whereas, Pb^{2+} produced a notable rise in the yolk sac area of zebrafish embryos compared to the control at 96 hpf. Sublethal As^{3+} exposure significantly elevated the activity of ALP ($p=0.012$) and GPT ($p=0.008$) whereas, sublethal Cd^{2+} and Pb^{2+} treatments significantly diminished the activities of LDH and GOT, respectively ($p<0.05$) compared to the control at 144 hpf. All tested heavy metals at sublethal doses gave rise to marked reductions in the total protein content ($p >0.05$) compared to the control at 144 hpf. Accordingly, the present study implies the detrimental consequences of exposure to trace levels of heavy metals on growth and development and the feasibility of the zebrafish embryo toxicity model as an early warning indicator of aquatic heavy metal contamination. Nevertheless, thorough investigations are warranted to elucidate the underlying mechanisms of heavy metal-induced toxicities.

Keywords: Heavy metals; Zebrafish embryo-toxicity model; Developmental toxicity; Liver damage marker enzymes; Total protein content

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INTRODUCTION

Human exposure to toxic heavy metals is a major public health concern worldwide. Among various heavy metals, arsenic (As), cadmium (Cd), and lead (Pb) are ranked as the most toxic metals and are known to affect different body organs through their acute and chronic toxic effects. Heavy metals released into the environment either naturally or anthropologically ultimately end up in surface water bodies through surface runoff thereby aggravating the contamination of aquatic systems. Aquatic heavy metal contamination is a paramount health concern as drinking water is a prime source of heavy metal exposure. According to the World Health Organization (WHO), the maximum permissible levels of As, Cd, and Pb in drinking water are 10, 3, and 10 µg L^{-1}, respectively (Herschy, 2012). Nevertheless, elevated levels of As, Cd, and Pb have been identified in certain freshwater ecosystems, particularly in South Asian countries. Elevated levels of Cd, iron (Fe), and Pb were reported in some major reservoirs in the North Central Province (NCP) and certain tributaries of the river Mahaveli in Sri Lanka. Further, naturally occurring groundwater extracted from certain parts of the country such as Mannar, Mulativu, Puttalam, and Jaffna was found to contain elevated levels of As (Bandara *et al*., 2008).

Toxicities induced by heavy metals require elaborative research findings due to the lack of comprehensive studies investigating the different toxicities and their underlying mechanisms. Toxicological model organisms would provide a broad understating of the heavy metal-induced toxicities. Among various animal models used in ecotoxicology, zebrafish (*Danio rerio*) has become a robust model organism owing to its salient characteristic features.

The deleterious effects that arise in aquatic organisms including zebrafish in response to exposure to As, Cd, and Pb have been reported in previous literature. However, a limited number of studies have furnished the toxicities incurred by As, Cd, and Pb at ecologically relevant concentrations on the growth, development, and biochemical parameters of zebrafish embryos. Hence, the present study was developed to shed light on the toxicities induced by ecologically relevant concentrations of As, Cd, and Pb using the zebrafish embryo toxicity model.

METHODOLOGY

The test solutions of As^{3+} (2.5, 5, 10, 20, and 40 µg L^{-1}), Cd^{2+} (0.75, 1.5, 3, 6, and 12 µg L^{-1}), and Pb^{2+} (2.5, 5, 10, 20, and 40 µg L^{-1}) were prepared using As_2O_3, $CdCl_2.H_2O$, and $Pb(NO_3)_2$ in distilled water. Standard dilution water was used as the negative control. Zebrafish maintenance, spawning, collection of eggs, and embryotoxicity assays were conducted as proposed by the OECD guideline 236[1]. The mortality and hatchability of zebrafish embryos were detected at 24, 48, 72, and 96 hours post fertilization (hpf). Eye area, heart rate, total body length, and yolk sac area were determined by exposing zebrafish embryos to sublethal concentrations of As^{3+} (4.6 µg L^{-1}: 10% of the LC_{50} for As^{3+} at 96 hpf), Cd^{2+} (0.3 µg L^{-1}: 0.1% of the LC_{50} for Cd^{2+} at 96 hpf), and Pb^{2+} (3.0 µg L^{-1}: 0.1% of the LC_{50} for Pb^{2+} at 96 hpf) at 96 hpf to assess the developmental toxicities. Embryo lysates were prepared using zebrafish embryos exposed to the sublethal concentration of As^{3+}, Cd^{2+}, and Pb^{2+} at 144 hpf. Embryos lysates were utilized to determine the activities of the liver damage marker

[1] http://www.oecd-ilibrary.org./

enzymes such as ALP (alkaline phosphatase), GOT (glutamic oxalacetic transaminase), GPT (glutamic pyruvic transaminase), and LDH (lactate dehydrogenase), and total protein content and creatinine level. The data obtained from three independent experiments were analyzed using the SPSS 20.0 package. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc was used to compare the significant differences between the experimental and the control groups. The significance for all analyses was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Zebrafish embryos at 2 hpf exposed to different concentrations of As^{3+}, Cd^{2+}, and Pb^{2+} indicated that the toxicity is time and concentration-dependent. The LC_{50} values of As^{3+}, Cd^{2+}, and Pb^{2+} reduced significantly throughout the exposure period and were recorded as 46.366 ± 0.186, 264.841 ± 0.18, and 2978.43 ± 0.199 µg L^{-1}, respectively at 96 hpf. The order of toxicity was observed as $As^{3+} > Cd^{2+} > Pb^{2+}$. The mortalities of zebrafish embryos upon exposure to different concentrations of As^{3+}, Cd^{2+}, and Pb^{2+} at 24, 48, 72, and 96 hpf are depicted in Figure 1. As^{3+} treatments, i.e. 10, 20, and 40 µg L^{-1} produced significantly high mortalities compared to the control at 96 hpf. Zebrafish embryos exposed to 6.0 and 12.0 µg L^{-1} of Cd^{2+} exhibited significantly high mortalities compared to the control at 96 hpf. The zebrafish embryos exposed to 20 and 40 µg L^{-1} of Pb^{2+} brought about marked elevations in embryo mortality compared to the control at 96 hpf and, the mortalities were recorded as 18.06 ± 0.577 % and 19.44 ± 1.764 %, respectively. The control treatment contained the lowest mortality throughout the exposure period. Moreover, the internal plate controls were devoid of embryo mortalities.

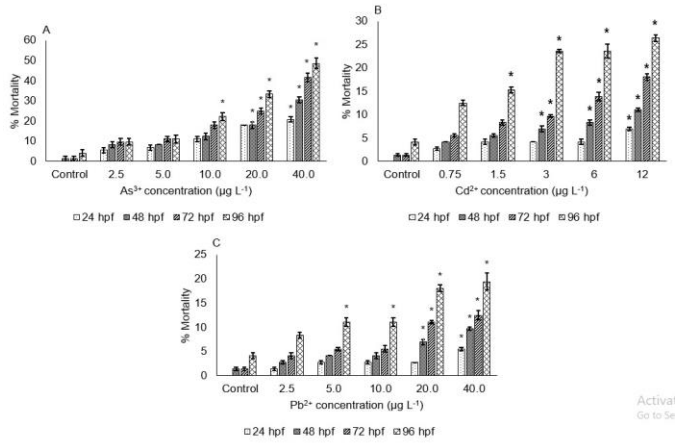

Figure 1. Graphical representations of % mortality of zebrafish embryos exposed to different concentrations of As^{3+} (A), Cd^{2+} (B), and Pb^{2+} (C) at 24, 48, 72, and 96-hour time intervals.

The effect of As^{3+}, Cd^{2+}, and Pb^{2+} treatments on the hatchability of zebrafish embryos is shown in Figure 2. Heavy metal exposure significantly reduced the hatchability of zebrafish embryos compared to the control with increasing concentration and time. The highest concentration of As^{3+} (40 µg L^{-1}), Cd^{2+} (12 µg L^{-1}), and Pb^{2+} (40 µg L^{-1}) employed produced 94.59%, 67.45%, and 30.91% reductions in hatchability, respectively at 96 hpf. Hatching is a process that includes both biochemical and behavioral aspects. Chorionase synthesized by the hatching glands is an essential enzyme required for the disintegration of chorion during hatching. Metal-induced interference of the transcription and translation attenuates the synthesis of proteins including chorionase. Moreover, the twist of the embryo further facilitates the breakdown of chorion to release the pro-larva. Impaired somite development due to heavy metals retards spontaneous muscle movement during hatching. Accordingly, the reduced hatching rates observed in the present study could be due to the heavy metal-induced attenuation of chorionase and reduced muscle movement (Jezierska *et al*., 2008).

Zebrafish embryos exposed to sublethal concentrations of As^{3+}, Cd^{2+}, and Pb^{2+} for 72 hrs were employed to determine the impact of heavy metals on heart rate. Heavy metal exposure has reduced the heart rate of zebrafish embryos but the apparent reductions were not significant compared to the

control (*p>0.05*). Nevertheless, alterations in the heart rate and cardiac activity appear in response to the metal intoxication.

Morphological parameters such as eye area, total body length, and yolk sac area were determined by exposing zebrafish embryos to sublethal concentrations of As^{3+}, Cd^{2+}, and Pb^{2+} for 96 hrs. Among the heavy metals tested, As^{3+} and Cd^{2+} exposures produced marked reductions in the eye area of zebrafish embryos compared to the control and the values were reported as 0.06 ± 0.001 mm^2 and 0.07 ± 0.016 mm^2, respectively (Figure 3B). As and Cd treatments significantly minimized the total body length of zebrafish embryos compared to the control but not Pb (Figure 3C). As shown in the figure 3D, heavy metal exposure has markedly elevated the yolk sac area of zebrafish embryos. Among them, the Pb treatment produced a notable raise in the yolk sac area of zebrafish embryos (0.33 ± 0.007 mm^2, *p=0.011*) compared to the control.

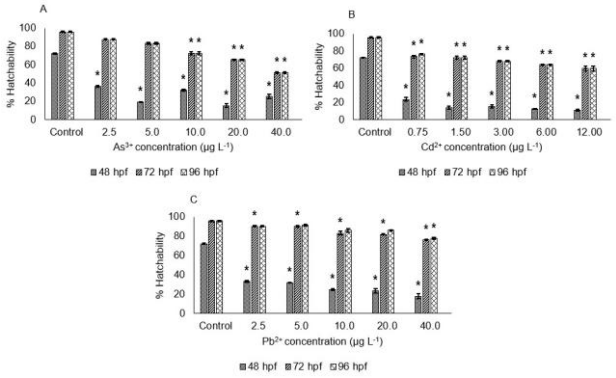

Figure 2. Graphical representations of % hatchability of zebrafish embryos exposed to different concentrations of As^{3+} (A), Cd^{2+} (B), and Pb^{2+} (C) at 24, 48, 72, and 96-hour time intervals.

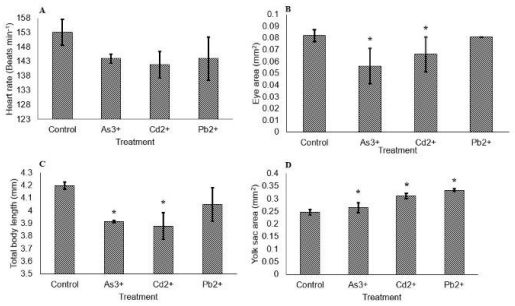

Figure 3. Heart rate (A), eye area (B), total body length (C), and yolk sac area (D) of zebrafish embryos exposed to sublethal concentrations of As^{3+} (4.6 µg L^{-1}), Cd^{2+} (0.3 µg L^{-1}), and Pb^{2+} (3.0 µg L^{-1}) at 96 hrs (*$p < 0.05$, mean ± SEM).

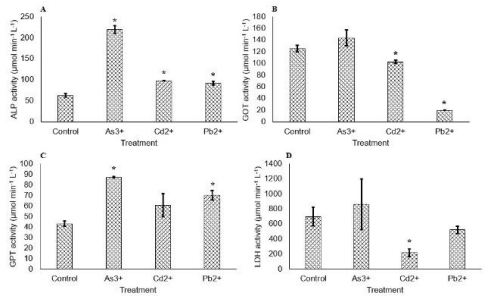

Figure 4. The activities of ALP (A), GOT (B), GPT (C), and LDH (D) of zebrafish embryos exposed to sublethal concentrations of As^{3+} (4.6 µg L^{-1}), Cd^{2+} (0.3 µg L^{-1}), and Pb^{2+} (3.0 µg L^{-1}) at 144 hrs (n=100, *$p < 0.05$, mean ± SEM).

In zebrafish embryos, the liver becomes fully perfused and functional from 72 hpf onwards. Hence, zebrafish embryos/larvae have been successfully incorporated to elucidate the biochemical and histopathological parameters related to hepatotoxicity. As shown in Figure 4A, exposure to sublethal concentrations of As^{3+}, Cd^{2+}, and Pb^{2+} significantly elevated the ALP activity of zebrafish embryos compared to the control at 144 hpf. Cd^{2+} (102.519 ± 3.112 µmol min^{-1} L^{-1}) and Pb^{2+} (19.519 ± 3.112 µmol min^{-1} L^{-1}) treatments significantly inhibited the GOT activity of zebrafish embryos compared to the control at 144 hpf (Figure 4B). GPT activity of zebrafish embryos rose in response to heavy metal exposure but notable elevations were made by As^{3+} ($p=0.008$) and Pb^{2+} ($p=0.03$) only (Figure 4C). The changes that appeared in the activities of hepatotoxicity biomarkers such as ALP, GOT, and GPT could possibly be due to the lesions that appeared in the liver as a result of heavy metal bioaccumulation. There was a marked reduction in the LDH activity of zebrafish embryos in response to Cd^{2+} exposure compared to the control (Figure 4D).

As revealed by Figure 5A, As^{3+} ($p=0.027$) and Pb^{2+} ($p=0.014$) treatments significantly enhanced the creatinine level of zebrafish embryos compared to the control at 144 hpf. The creatinine concentration is an indicator of kidney dysfunction and the rise of the creatinine content reflects the negative impacts of As^{3+} and Pb^{2+} on the kidney.

All the tested heavy metals produced notable reductions in the total protein content of zebrafish embryos compared to the control and the reductions were reported as 71.19%, 84.89%, and 69.67% for As^{3+}, Cd^{2+}, and Pb^{2+}, respectively.

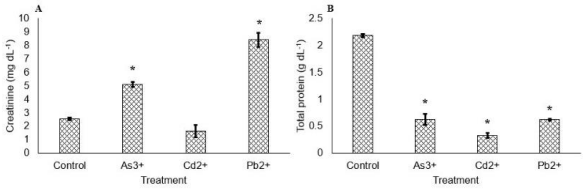

Figure 5. The creatinine (A) and total protein (B) content of zebrafish embryos exposed to sublethal concentrations of As^{3+} (4.6 µg L^{-1}), Cd^{2+} (0.3 µg L^{-1}), and Pb^{2+} (3.0 µg L^{-1}) at 144 hrs (n=100, $*p < 0.05$, mean ± SEM).

CONCLUSION

In conclusion, the present study explored the developmental and biochemical toxicities induced by environmentally relevant concentrations of As, Cd, and Pb using the zebrafish embryo toxicity model. As revealed by the results, it was evident that heavy metals like As, Cd, and Pb could induce adverse effects on the survival and development of zebrafish embryos even with trace amounts. Furthermore, exposure to environmentally relevant concentrations of As, Cd, and Pb were potent enough to induce alterations in the liver damage marker enzymes such as ALP, GOT, GPT, and LDH. In addition, creatinine and total protein content of zebrafish embryos were also altered in response to As, Cd, and Pb exposure. Overall, the present study implies the detrimental consequences of exposure to trace levels of heavy metals on growth and development and the feasibility of the zebrafish embryo toxicity model as an early warning indicator of aquatic heavy metal contamination. Nevertheless, thorough investigations are warranted to elucidate the underlying mechanisms of heavy metal-induced toxicities.

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