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Zinc Neurotoxicity Inflicts Mitochondrial Dysfunction in the Brain of *Clarias batrachus* L.: Implication in fish death

Zinc Toxicity induces Piscine Brain Mitochondrial Dysfunction

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Abstract
The present *in vivo* study was designed to investigate the neurotoxic impact of zinc on mitochondrial function mediated by reactive oxygen species (ROS) generation in the brain of *Clarias batrachus*. Though the neurotoxic impact has been studied in several animal models, the impact was completely unknown in the piscine model in relation to mitochondrial dysfunction. The fishes were exposed to 10% and 20% of the derived 96 h LC_{50} value, 9.45 and 18.90 mg L^{-1}, respectively, and sampled on 20, 40 and 60 days. Exposure of fish brain to zinc demonstrated partial inactivation of complex II, III and IV activities, reduction in mitochondrial membrane potential, energy depletion accompanied by enhanced ROS generation. Concluding the results of our current investigation, we suggest that brain mitochondrial dysfunction can be an important contributor for fish death in addition to the other established mechanisms.

Index Term: Mitochondrial electron transport chain, zinc, neurotoxicity, mitochondrial dysfunction

I INTRODUCTION

Zinc (Zn) is an essential trace element but also a potential toxicant in excess amount to living organisms. In unpolluted areas, zinc is found in water at nanomolar levels, reaching micromolar and higher values in metal-contaminated environments [1,2,3].Excessive Zn can inhibit physiological activities of aquatic organisms including fishes and even be fetal [4,5,6]. Several lines of evidence exist that suggest mitochondria and energy metabolism as subcellular targets for the toxic actions of Zn [7,8]. Zinc can inhibit glycolysis [9], the tricarboxylic acid cycle [10], and complexes in the electron transport chain [11,12,13,14]. It has also been shown that Zn dissipates mitochondrial membrane potential, decreases oxygen consumption and enhances reactive oxygen species (ROS) accumulation [7]. However, it remains unclear whether similar impact occurs in fish brain mitochondria as there exists no report that highlight the impact of Zn neurotoxicity on mitochondrial function especially on individual mitochondrial electron transport chain complexes in piscine brain.

In the present study the piscine model of *Clarias batrachus* was chosen as *Clarias batrachus* is naturally cultivated in India in the marshy lands and aquatic bodies adjacent to the industrial settlements where the concentration of Zn in polluted water bodies often varies in higher concentrations and acts like a toxicant in water. *Clarias batrachus* being bottom dwellers become exposed to metals (like zinc, lead, nickel, chromium, mercury) that are present in sediments of fresh water bodies as a result of industrial discharge. So it was considered worthwhile to study the impact of Zn neurotoxicity in piscine model as such metal exposure often contributes in mass killing of fishes in polluted water bodies.

II MATERIALS AND METHODS

Animal use protocols have been approved by the University of Kalyani Animal Care Committee in accordance with national guidelines. Healthy adult specimens of *Clarias batrachus* L. (60 ± 3.20 g body weight, 16.3 ± 1.751 cm total length) were collected from a local hatchery and were acclimatized to laboratory experimental condition for 2 weeks in dechlorinated tap water in large glass aquaria in the laboratory. They were fed *ad libitum* on alternate days and the water with requisite Zn salt was renewed after every 48 h, leaving no faecal matter, unconsumed food or dead fish, if any. Prior to the commencement of the experiment, 96 h median lethal concentration (96 h LC_{50}) of zinc sulphate (E. Merck, India) was estimated by probit analysis [15].

Adult *Clarias batrachus* were exposed to $ZnSo_4.7H_2O$ treated water at 10% (9.45 mg L^{-1} Zn) and 20% (18.90 mg L^{-1} Zn) of the 96 h LC_{50} value (94.5 mg L^{-1} Zn). Eight fishes were randomly assigned for each aquarium containing 30 l of $ZnSo_4.7H_2O$ treated water, prepared in tap water (having dissolved O_2 6.5 mg L^{-1}, pH 7.12, water hardness 23.5 mg L^{-1} and water temperature 25 ± 2°C). Identical groups of eight fishes each were kept in separate aquaria containing 30 L of plain dechlorinated tap water (without zinc salt) as controls. After each of the exposure periods of 20, 40 and 60 days, fishes from the respective experimental, as well as control aquaria were sacrificed and the brain tissues were utilized for various biochemical experiments. Atomic absorption spectrometry was used to measure the actual concentration of Zn in experimental water during the exposure periods of 20, 40 and 60 days and was found very near to the desired concentration levels. The brain mitochondrial fractions were prepared and were utilized for mitochondrial biochemical assays.

The brain mitochondrial fraction was isolated following the method of Berman and Hastings 1999, with minor modifications [16]. The activity of complex I (NADH dehydrogenase) was assayed by using ferricyanide as the electron acceptor as adapted from Hatefi ,1978 [17]. The complex II activity (succinate-coenzyme Q_{10} oxidoreductase) and complex III (coenzyme Q_{10} – cytochrome c oxidoreductase) were measured by following the methods of Fisher et al. 1985 [18] and Rustin et al. 1994 [19] respectively. The activity of complex IV (cytochrome c oxidase) was assayed by noticing the oxidation rate of reduced cytochrome c (ferrocytochrome c) at 550 nm as mentioned in Wharton and Tzagoloff, 1967 [20]. The mitochondrial membrane potential was measured with 5 µM JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolylcarbocyanine iodide, CS0760, Sigma-Aldrich) as mentioned in Proost et al. 2008 [21]. Mitochondrial ATP production was carried out using luciferin-luciferase bioluminescent assay following the method of Hays et al. 2003 [22]. DCFH-DA dye was used for measurement of reactive oxygen species (ROS) production as mentioned in the method of Dreiem et al. 2005 [23]. For all parameters mean ± SE were calculated. All data were subjected to analysis of variance and Duncan's Multiple Range Test (DMRT) was used to determine the significant differences among means at 5% level of significance (Duncan 1955)[24]. The control values of the biochemical variables of 20, 40 and 60 days duration are nearly the same, within 5% variation.

III RESULTS AND DISCUSSION

The extent of mitochondrial dysfunction in the piscine brain of *Clarias batrachus* was evidenced by the inhibition of complex II, complex III and complex IV activities of the mitochondrial electron transport chain. In mitochondrial complex II measurement, with respect to duration and concentrations of treatment, the combination of 60 days treatment with Zn 20% (of 96 h LC_{50}) exposure recorded the highest loss of complex II activity (-37% vs. control, $p<0.05$, Fig.1B), closely followed by 60 days treatment with Zn 10% (of 96 h LC_{50}) exposure (-35% vs. control, $p<0.05$, Fig.1B).In 40 days treatment duration, both the low and high concentrations of Zn treatment inflicted extensive inhibition of complex II activity with inhibition of -24% and -32% with respect to control values (no variation among controls of 20, 40 and 60 days duration) ($p<0.05$, Fig.1B). For complex III measurement, a different pattern of inhibition was observed with only the high concentration of Zn (20% of 96 h LC_{50}) lowering the complex III activity at both 40 days and 60 days of Zn treatment (-20% vs. 40 day control and -33% vs. day control respectively, $p<0.05$, Fig.1C). This observation of inhibition of complex II and complex III activities is in accordance to earlier studies, however, not in piscine models. The study by Lemire et al. 2008 [25] reports the inhibition of complex II activity in the hepatocytes while Dineley et al. 2003 [7] clearly depicts the dysfunction of complex III in rat brain mitochondria. The inhibition of complex III also receives support from several other studies in bovine heart mitochondria [26,13]. In case of complex IV activity, only 60 days treatment period at both low and high doses of Zn exposure inflicted severe inhibition of complex IV (-30% vs. control and -40% vs. control respectively, $p<0.05$, Fig.1D) that was unnoticed in 20 and 40 days treatment period. This observation of inhibition of complex IV is not in accordance with the report by Dineley et al. 2003 [7], however, this finding receives support from several other reports that prove the inhibition of complex IV in different working models [25,14].

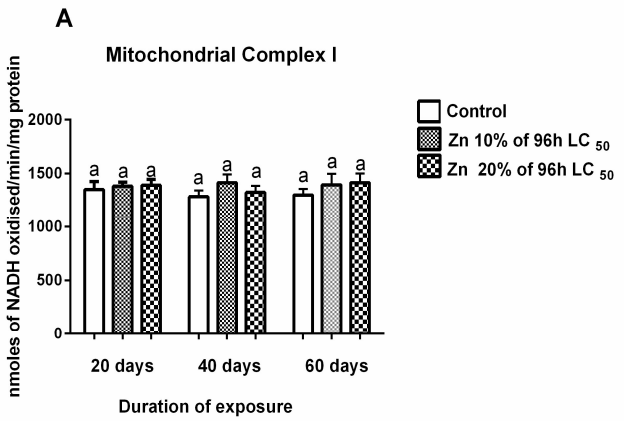
In mitochondrial membrane potential, high dose of Zn treatment (20% of 96 h LC_{50}) reduced the membrane potential at both 40 and 60 days of treatment with a loss of -26% (vs. 40 day control) and -37% (vs. 60 day control) respectively, $p<0.05$ (Fig.2A). Zn treatment at low concentration (10% of 96 h LC_{50}) failed to alter mitochondrial membrane potential at 20 and 40 days treatment period though reducing the membrane potential at 60 days exposure period (-25% vs 60 day control, $p<0.05$, Fig.2A). The inhibition of mitochondrial electron transport at different levels as earlier mentioned might have resulted in dissipation of mitochondrial membrane potential along with lowering of mitochondrial energy production as observed in our study. Maximum loss of mitochondrial ATP production was observed at 60 days treatment period at Zn 20% (of 96 h LC_{50}) exposure with -36% lowering of energy level compared to control (Fig.2B) while in 40 days treatment period the lowering of energy production was by 17% (compared to 40 day control, $p<0.05$, Fig.2B).However, in 40 days treatment period only Zn 20% (of 96 h LC_{50}) exposure was able to inflict loss of ATP production. This loss of mitochondrial energy receives support

from an earlier observation by Lemire et al. 2008 [25] where it was suggested that the loss of energy may lead to pathological conditions. The debilitating impact of Zn toxicity in piscine mitochondrial function may well be contributed by the significant enhancement of ROS generation under our experimental conditions. There was enhanced increase in ROS generation at both low and high doses of Zn exposure at 40 and 60 days treatment duration, though statistically significant increase in ROS was only noticed at high dose of Zn treatment at 40 and 60 days of treatment period (+32% vs. 40 day control and +58% vs. 60 day control respectively, $p<0.05$, Fig.2C). This finding of increased generation of ROS is in continuity to an earlier study by Bishop et al. 2007 [27].

IV CONCLUSION

Considering the information obtained from this study and from previous reports, it can be concluded that the Zn exposure induces toxicity in piscine brain mediated by ROS generation leading to mitochondrial dysfunction involving loss of complex II, III and IV activities, reducing mitochondrial membrane potential and affecting ATP generation. Though Zn is known to accumulate in different organs of the piscine body upon exposure including intestine and gills [28,29], neurotoxic effects of Zn on fish brain can also be an important contributor of fish death. It has been evidenced in several studies that Zn toxicity can induce cell death of neurons either through apoptosis or necrosis [30, 31, 32, 33], mitochondrial dysfunction as observed in the present study may contribute heavily for the cause of such neuronal cell death in piscine brain. In this connection, our piscine study primarily highlights for the first time the involvement of Zn in causing piscine neurotoxicity mediated by mitochondrial dysfunction that can lead to neuronal cell death.

Figure 1



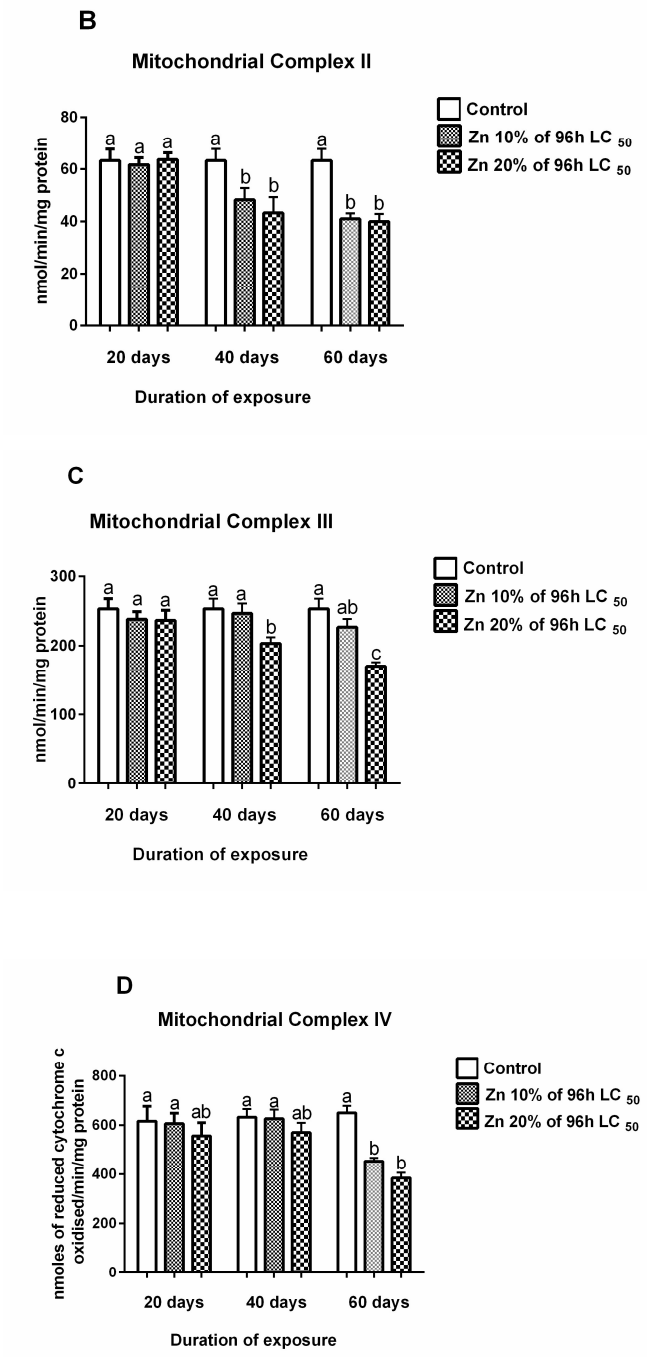


Fig. 1 Variation of (A) complex I (B) complex II (C) complex III and (D) complex IV of fish brain mitochondrial fraction exposed to 0% (0.00 mg L^{-1}), 10% (9.45 mg L^{-1}) and 20% (18.90 mg L^{-1}) of 96 h LC_{50} of Zn at different duration (20, 40, 60 days) of exposure Data are mean ± SEM of eight observations. Results of DMR Test have been represented by small letters. Common letter between any two bars indicate their similarity while two different letters indicate significant difference at 5% level.

Figure 2

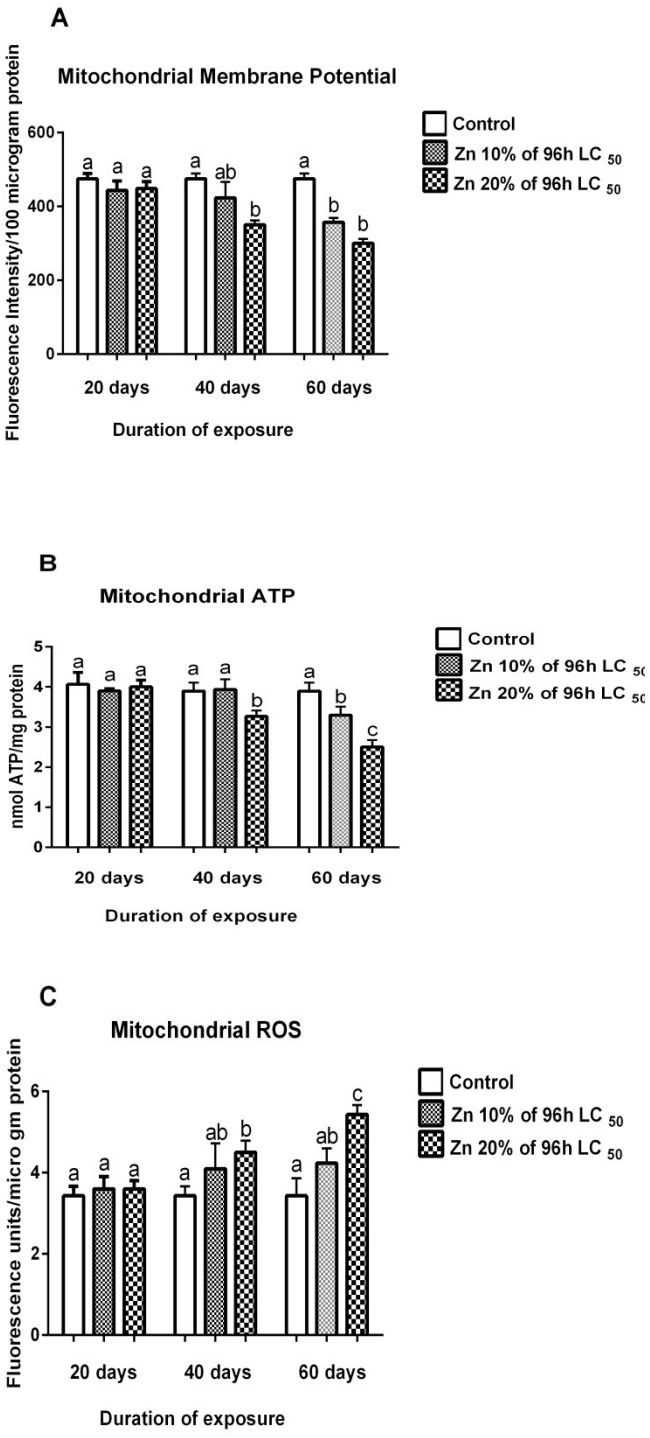


Fig. 2 Variation of (A) mitochondrial membrane potential (B) mitochondrial adenosine triphosphate (ATP) production and (C) mitochondrial reactive oxygen species (ROS) generation of fish brain mitochondrial fraction exposed to 0% (0.00 mg L^{-1}), 10% (9.45 mg L^{-1}) and 20% (18.90 mg L^{-1}) of 96 h LC_{50} of Zn at different duration (20, 40, 60 days) of exposure Data are mean ± SEM of eight observations. Results of DMR Test have been represented by small letters. Common letter between any two bars indicate their similarity while two different letters indicate significant difference at 5% level.

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