Int. J. Aquat. Biol. (2019) 7(6): 368-373
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Physiological, Biochemical and Neurochemical responses of *Cirrhinus mrigala* upon short term exposure to Cerium oxide

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Abstract: The current study was performed to ascertain the biochemical and physiological impact of cerium oxide (CeO_2) on freshwater fish *Cirrhinus mrigala*, which are widely consumed. Biochemical, neurochemical and physiological modifications were evaluated and LC_{50} of CeO_2 was found to be 22 ppm, observed for 24 h. Further $1/10^{th}$ of the LC_{50} concentration of CeO_2 (2.2 ppm) was used for short term investigation at 96 h. The results demonstrate an increase in physiological levels of serum lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) compared to control groups. Studies revealed variations in oxidative stress markers with a significant reduction in the serum superoxide dismutase (SOD) and catalase (CAT) activities and an increase in malondialdehyde (MDA). The study reported the increase in brain glutamate concentrations indicating possible brain tissue damage. The above analysis highlights the potential toxicological impact of CeO_2 on freshwater fish and their ecosystem.

Article history:
Received 10 September 2019
Accepted 21 December 2019
Available online 25 December 2019

Keywords:
Biochemistry
Cirrhinus mrigala
Neurochemical response
Physiology

Introduction

The existence of xenobiotics, chemicals and their derivatives in the environment and ecosystem due to their agricultural and industrial applications are well-documented. The majority of these chemicals in the form of heavy metals and their derivatives are not degradable and have the possibility of endogenous binding molecules as they enter the biological system may lead to unfavorable toxic effects (Amdur et al., 1991). The existence of these metals in a biological system can bring about undesirable modifications in a cell which may be circulated to the tissue or the organ (Bernet et al., 1991). The eventuality of these metals to accumulate in the aquatic ecosystem, particularly fish, has a detrimental impact on the food chain. Human populations who consume aquatic products as a primary source of food are also part of the food chain, thus generating a cause of concern in public health (Di Giulio and Hinton, 2008). The impact and bioaccumulation of metals and their derivatives in the organs of aquatic animals like fish depend on a variety of factors like proximity of the animals from the pollutants, metabolic activity and membrane transport potential, and climatic conditions (Abhijith et al., 2016).

Cerium oxide is extensively used as diesel fuel additive exploiting their potential of oxygen storing, and their applications can also be found in television tubes, UV absorbents, and glass industries (Khan et al., 2011), besides their uses also found in biomedical industry like to retinal neurodegeneration and protection against radiation caused injury (Celardo et al., 2011). In this study, we used freshwater fish *Cirrhinus mrigala* for the measurement of acute toxicity of cerium oxide. It is bottom-dwelling fish that is widely consumed in the Indian subcontinent. LC_{50} concentration is a vital indicator of the extent of resistance of an organism to metal and its derivatives (Reda et al., 2010). Biochemical and physiological changes of exposed fish give the precise extent of damage caused by these metals (Banaee et al., 2019b). Toxic stress leads to oxidative damage by the production of ROS, consequently with the exhaustion of natural antioxidants (Banaee et al., 2019a).

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DOI: https://doi.org/10.22034/ijab.v7i6.657

Quantification of oxidative stress markers such as SOD, catalase and lipid peroxidation (LPO) demonstrates the biochemical variations due to toxicity. Alterations in brain neurotransmitter glutamate is assessed given that neurons are susceptible to ROS, ensuing in variations of neurotransmitter and causing neurodegenerative disorders due to oxidative damage (Li et al., 2010). Thus in the current research, we evaluated the biochemical, neurochemical (glutamate) and oxidative stress imparted to freshwater fish *C. mrigala* upon short term exposure (96 h) to cerium oxide.

Materials and Methods

Cerium oxide (CeO_2) sample preparation and inoculation: 22 g/L of the solution was prepared by dissolving CeO_2 in double-distilled water. The groups of fishes were exposed to CeO_2 for a short period of 96 h (acute toxicity). Concentrations of sample 2.2 mg/L based on $1/10^{th}$ of LC_{50}concentration (22 mg/L) were introduced into 100 L aquariums containing 50 fishes each. The fishes with similar size and weight were kept in the groups. Every 24 h, the water was changed and freshly prepared solution of CeO_2 was introduced to the aquariums. The control group of 50 fishes was also maintained under the comparable physical conditions like the treated group fishes. Food was withdrawn 24 h before sacrifice to circumvent postprandial variations. Control and treated fish (2.2 mg/L) were taken for further investigation. Zero mortality was observed during 96 h of exposure time.

Sample collection: The cardiac puncture was adopted to draw blood from the fish. The blood drawing syringes were precoated with heparin, an anticoagulant. The samples (Abhijith et al., 2016) for biochemical evaluation were transferred to EDTA vials and centrifuged at 9392 g for 20 min at 4ºC. The brain tissue (Raju et al., 2004) sample for estimation of neurotransmitter (glutamate) was immediately dissected and placed in ice. The brain tissue during analysis was homogenized with 8 volumes of cold Tris buffer saline (10 mM Tris-HCl, 10 mM sucrose, 0.7% NaCl, 0.1 mM EDTA of pH 7.2 at 4ºC (Raju et al., 2004). The homogenate was then centrifuged at 3000 rpm for 12 min at 4ºC, and the supernatant was obtained for further evaluation.

Biochemical variable measurement: Blood glucose level was measured by preparing a reaction mixture of 0.1 mL of plasma, 4 ml of o-toluidine (Cooper and McDaniel, 1970), which was then mixed and incubated for 10 min on a boiling water bath. The samples were cooled and the absorbance was measured at 630 nm in a UV spectrophotometer. The glucose concentration was expressed in mg/dL. Plasma protein concentration was assessed by lowry's method (Lowry et al., 1951). 100 µl of plasma, 900 µl of double distilled water and 4 ml of Lowry reagent were mixed well and incubated for 10 min at room temperature, to the reaction mixture, 0.5 ml of FC reagent was added and incubated for 20 min at room temperature. The sample was then measured at 720 nm and expressed in µg/ml.

Analysis of serum transaminases: Serum L aspartate aminotransferase (L-AST) and L-alanine aminotransferase (L-ALT) activities were demonstrated at 37ºC by a colorimetric method (Reitman and Franckel, 1957) and enzyme activity was expressed as IU/L. Lactate dehydrogenase (LDH) was analyzed by the method developed by Anon (1984) and activity was expressed in IU/L.

Oxidative stress markers and glutamate analysis: Superoxide dismutase (SOD) was measured (Das et al., 2000) by the inhibition of superoxide led nitrite formation from hydroxylamine hydrochloride and the absorbance was measured at 540 nm and activity was expressed as U/mg of protein. Catalase activity was determined based on the formation of a stable complex of hydrogen peroxide with ammonium molybdate and absorbance was read at 405 nm and expressed as U/mg (Goth, 1991). Lipid peroxidation was determined by taking malondialdehyde (MDA) as standard (Buege and Aust, 1978). The reaction mixture of TCA-TBA HCl reagent and the sample was boiled for 10 min, cooled and then centrifuged at 10000 g and the supernatant was used for quantification at 535 nm and expressed as nmol/mg. Glutamate levels were demonstrated by multiple development of paper chromatography (Raju et al., 2004). The supernatant

Table 1. Alterations in the biochemical variables of *Cirrhinus mrigala* exposed to CeO2 during short term exposure.

Biochemical variables	Exposure duration (in h)							
	24		48		72		96	
	C	E	C	E	C	E	C	E
Glucose (mmol/L)	5.42±0.51	8.37±0.45	5.53±0.82	20.11±1.33*	5.72±0.63	24.72±1.54*	5.81±0.05	26.55±1.43*
Protein (g/L)	8.66±0.47	14.24±0.31*	8.63±0.13	13.12±0.18	8.78±0.75	11.57±0.92*	8.58±0.03	6.33±0.24*
LDH (IU/L)	4.07±0.51	8.46±0.27*	4.28±0.15	8.07±1.37*	4.92±038	7.86±0.95*	4.88±0.41	8.17±1.06*
AST (IU/L)	14.91±1.05	17.62±1.09*	14.53±0.92	18.43±0.39*	14.34±0.44	19.53±1.05*	15.01±0.48	20.17±0.96*
ALT (IU/L)	18.25±0.91	22.58±1.15*	18.91±1.16	22.93±0.24*	19.07±0.86	23.41±0.87*	18.95±0.36	23.58±1.15*

All values are expressed as mean± SE of three individual samples,* $P<0.05$ is significant (Control: C. Experimental: E)

Table 2. Alterations in the ROS variables of *Cirrhinus mrigala* exposed to Cerium oxide during short term exposure.

ROS variables	Exposure duration (in h)							
	24		48		72		96	
	C	E	C	E	C	E	C	E
SOD (U/mL protein)	5.11±0.43	3.37±0.26*	5.23±0.12	4.04±0.35*	5.01±0.27	4.17±0.33*	5.93±0.65	3.53±0.41
CAT (µmol/ml protein/min)	5.54±0.12	3.44±0.26*	5.98±0.11	3.97±0.25*	5.43±0.33	2.68±0.42*	4.95±0.73	3.74±0.32*
nmol of MDA/ mg protein)	6.28±0.25	7.34±0.15*	6.77±0.27	7.08±0.58	6.14±0.15	7.34±1.12*	5.99±0.43	7.93±0.68*

All values are expressed as mean± SE of three individual samples,* $P<0.05$ is significant.

was evaporated at 70ºC and mixed with 100 ml double distilled water. The two mM glutamate standard solution with the sample was spotted on Whatman no.1 chromatography paper which is then allowed to develop on the mobile phase (Butanol: acetic acid: water 12:3:5 v/v). The chromatogram is developed again, following which the papers are dried and sprayed with ninhydrin and incubated at 100ºC for 4 min. The bands which reveal glutamate corresponding to the standard is cut and eluted in 75% ethanol with 0.005% $CuSO_4$. The absorbance is measured against the blank at 515 nm in a spectrophotometer. The concentration is expressed as µmol/g of glutamate.

Statistical analysis: All data were statistically analyzed and represented as Mean±SE. In all experiments, the level of statistical significance was set for $P<0.05$. The significance was calculated by using an independent sample *t*-test by MS-Excel.

Results

The fish were divided into treated (2.2 ppm of CeO_2) and control groups and studied for 24, 48, 72 and 96 h. The blood analysis of CeO_2 treated *C. mrigala* demonstrates a remarkable raise in glucose levels (Table 1) throughout the 4 days of experiment, however major difference of 5.81±0.05 / 26.55±1.43 was found to be on the 4th day (96 h). The lowering in plasma protein level (Table 1) was significantly ($P<0.05$) demonstrated in treated groups (2.2 ppm of CeO_2) on the 4th day (8.58±0.03 / 6.33±0.24) compared to control group. In the current analysis, there was a marked rise in both AST and ALT concentration of treated groups on all the four days in comparison to the control groups. Lactate dehydrogenase (LDH) is an isoenzyme playing a vital task in glycolysis, which is also considered as important biomarker of organ and tissue damage. Table 1 reports the increased activity of LDH on treatment of *C. mrigala* fish to CeO_2 (2.2 ppm).

Figure 1 shows the increase in brain glutamate concentration levels in treated fish (8.35±0.16) when compared to the control groups (14.53±0.82) on the 4th day. The results revealed decrease in SOD and CAT in the treated groups compared to the control one on all the days of sample inoculation. The LPO analysis in *C. mrigala* exposed to 2.2 ppm of CeO_2, suggested a remarkable raise in MDA levels in treated fish compared to the control fish (Table 2).

Discussions

In the current study, there was a considerable increase in glucose concentration of treated fish compared to the untreated fish. This can be credited to stimulation of stress resulting in hyperglycemia (Simakani et al.,

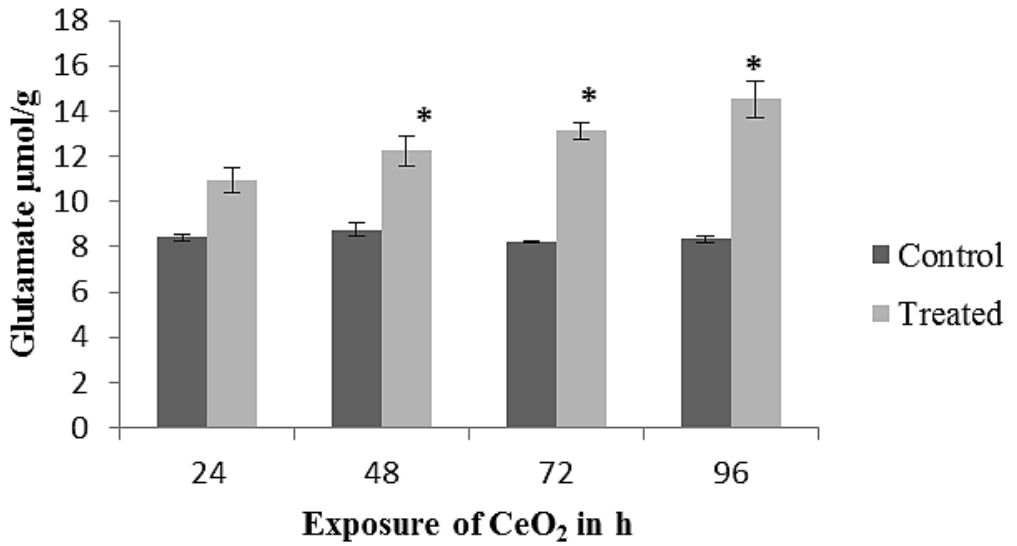

Figure 1. Alterations in the brain glutamate level of *Cirrhinus mrigala* exposed to CeO_2 upon short term exposure (All values are expressed as mean±SE of three individual samples,* P<0.05 is significant)

2018). Similar results were reported which are ascribed to the changes in carbohydrate metabolism, on account of glycogenolysis in freshwater fish (Ghelichpour and Mirghaed, 2019). Boost in glucose levels during metals exposure have also been reported due to synthesis of glucose from extra hepatic tissue amino acids (Almeida et al. 2001; Banaee et al., 2019c;). Many reports also related increase in plasma glucose levels to discharge of glucocorticoids and catecholamine`s from adrenal tissues of fish during stress incidents (Klaper et al., 2010).

The protein concentration decreased in the treated fish as duration of exposure was increased. The reduced protein levels are correlated to metal binding to blood and tissue proteins culminating in tissue injury and oxidative stress. It also causes changes in physicochemical properties which are evident in protein structure conformational change (Chen et al., 2011), and this might also be the basis of decline in protein concentration in this investigation. Toxicity of metals might also be the rationale for declined protein as it inhibits transcription and translational processes.

The detection of transaminases like aspartate transaminase (AST/GOT) and alanine transaminase (ALT/GPT) in blood have been clinically employed to identify with any tissue damage. Presence of these biomarkers can also be coupled to the enzyme inhibition in metabolic pathways upon exertion of stress. There was a notable increase in both ALT/AST concentrations in treated fishes. These can be attributed to the tissue and organ damage caused by exposure to CeO_2 and other toxicants (Abhijith et al., 2016). Accordingly, indicating the potential role of transaminases as chief biomarkers during exposure to toxicants and ensuing in metabolic stress. AST and ALT are indicators in liver function tests, as AST is synthesized by liver hepatocyte and typically found in liver and heart, while ALT primarily is present in liver and kidney. Subsequently, higher activity of these enzymes has been reported in fish exposed to pesticides (Rao, 2006). The raise in LDH can be ascribed to amplified glycolysis upon metabolic stress. Furthermore, the onset of anoxia is also an important reason for increased LDH activity. The prevalence of anaerobic conditions due to metal toxicity is also reported to rise in LDH activity (Min and Ju-Chan, 2008).

The result represents an increase in brain glutamate in treated fish. This might be coupled with excitotoxicity and excessive production of glutamate, which results in damage and death of nerve cells. The increased glutamate concentration makes alterations in the brain physicochemical environment which activates the glutamate receptors by allowing a high concentration of calcium ions to enter the cell (Manev et al., 1989).

The generation of ROS is the reason for oxidative stress, which agitates the biological processes by disconcerting homeostasis (Banaee et al., 2019a, b). This outcome is imbalance of equilibrium of the detoxification mechanism of ROS (Manke et al., 2013). Cells supply the range of enzymatic and non-enzymatic responses to scavenge the excessive generation of ROS. The pro-oxidant uniqueness of metals causes ROS generation during mitochondrial respiration and thus activating NADPH-like enzymes. Enzymes like SOD and CAT play a principal role in scavenging ROS formed during modifications of metabolic (Raisi et al., 2018) and physiological processes (Mirghaed et al., 2018). SOD has been reported to be the primary and instantaneous response to oxidative stress in a biological system (Winston and Di Giulio, 1991). This is owing to the generation O_2 and their conversion to H_2O_2, which may additionally lead to oxidation of cysteine in the antioxidant enzyme, thus reassuring CeO_2 toxicity.

Lipid peroxidation (LPO) is the primary culprit in the interruption of cell structure and function due to unwarranted generation of reactive oxygen species (Huang et al., 2003; Safari, 2016). The lipid peroxidation mechanism is assessed by measuring the malondialdehyde (MDA) concentration, which is one of the end products of the breakdown of lipids due to peroxidation. The observation suggests the existing cellular defense mechanism was not competent to ward off the oxidative damage.

All the above mentioned analysis suggests the bioaccumulation of CeO_2. The study proposes that the release of cerium oxide to the aquatic environment may lead to detrimental effects on aquatic animals and the human population who are reliant on aquatic products as source of diet.

Ethical approval: All experiments performed involving animals were following the ethical standards of the institution and fish from which biological samples were collected and held in the laboratory via approval IAEC Committee (permit CAF/ETHICS/648/2018). This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest: The authors declare that they have no conflict of interest.

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