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Effect of ferrous sulphate on aspartate and alanine aminotransferases of brain of *Tilapia mossambica*

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ABSTRACT

Iron in the form of ferrous sulphate coming from sources such as mines, writing inks, blue pigments, dyeing industries, photography, medicine, deodorizers, disinfectants, fungicides and molluscides, etc. contributes in elevating ferrous sulphate of water bodies. The present study investigated the action of ferrous sulphate on the local fish *Tilapia mossambica*. Tilapia exposed to 0.001 g/L ferrous sulphate for 30 days showed reduction of cytosolic AST and ALT activities of cerebral cortex by 35.4% and 29.1%, respectively, while exposure to 0.01% ferrous sulphate promoted 49.2% and 38.4% reduction of AST and ALT activities. Similarly mitochondrial AST and ALT activities reduced by 50% and 34.8%, respectively, on exposure to 0.001 g/L ferrous sulphate while 0.01 g/L ferrous sulphate promoted 51% and 47.8% reductions of AST and ALT activities at the end of 30 days, suggesting interference in the glutamate and protein metabolism of Tilapia brain.

1. Introduction

Ferrous sulphate or ferrous sulphate heptahydrate occurs in nature as the mineral melanterite, either crystalline or fibrous, and appears to have formed by the oxidation of pyrite or marcasite. It is a constituent of mine effluents and acid mine drainage from mines (Maltby et al., 1987; Singh, 1987). When dissolved in water it readily gets degraded to form Fe (III) and sulphuric acid. The iron (III) hydroxide is precipitated out of solution as reddish brown precipitate normally known as ferric hydroxide that increases the turbidity of water (Adams et al., 2004). It is also used in preparations of writing inks, blue pigments, textile dyes, medicines, deodorizers, disinfectants, fungicides and molluscides, reducing agents, feed additives, fertilizer additives and soil acidifiers to prevent ferric chlorosis or iron deficiency (HCN:CUOEL, 2004).

Accidental spillage or discharges of untreated effluents or sludge containing iron into natural water bodies may have harmful effects on the fish population and other forms of aquatic life (Van Anholt et al., 2002; Sotero-Santos et al., 2007). On exposure to water contaminated with iron, the fish tend to accumulate iron in their tissues. Bioaccumulation of iron in fish is well documented (Patin, 1984; Mohamed et al., 1990; Lappivaara et al., 1999; Wepener et al., 2001; Burger et al., 2002). The increase of iron load in animal tissues may impair normal physiological processes of fish leading to serious health problems for fish and consumers at large. Lappivaara and Marttinen (2005) reported that waterborne iron promotes accumulation of iron in liver and gills with decreased haemocrit value, decreased levels of catecoholamine and β-estradiole levels in plasma of white fish. Further, they observed that fish shows lowered responses to the external threat which is likely to promote decline in local fish population. The lowered responses of the fish to external threats indicate neuronal dysfunction. Therefore, it was necessary to investigate the action of ferrous sulphate on the local fish *Tilapia mossambica*, with reference to aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities of brain as these enzymes of brain are involved in glutamate metabolism (Peng et al., 1998) and the impairment of it could lead to neuronal dysfunction.

2. Materials and methods

2.1. Biological material

The fish species, *T. mossambica*, were obtained from Dhaujem farm, Old Goa, India and maintained in glass aquaria of 1000 L capacity containing well-aerated unchlorinated ground water. Fish weighing 11.60 ± 2.18 g were transferred to a 100 L glass aquarium (180 × 90 × 90 cm) for 20 days for conditioning and fed with commercial dry feed pellets (Fish Dry Pellets; Waterbabies Products, Madgaon, Goa, India). The aquarium water was renewed daily, was aerated with an aquarium air pump (Jumbo-Jet, Super-8300, made in India) and the natural photoperiod of

Abbrevations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; AR, analytical reagent; L:D, light:dark; LC, lethal concentration; EC, effective concentration; DNPH, dinitrophenyl hydrazine; SD, standard deviation; NADH, nicotinamide adenine dinucleotide reduced form; ROS, reactive oxygen species.

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13:11 h (L: D) was maintained. The conditions for acclimatization and tests were maintained at: temperature 26 ± 2 °C, pH 7.10 ± 0.05 and dissolved oxygen 8.15 ± 55 mg/L. Following acclimatization to laboratory conditions for 20 days, the healthy male fish were used. Experiments were performed according to the guidelines of committee for the purpose of Control and Supervision of Animals in India and were approved by the Institutional Animal Ethics Committee of Goa University, Goa, India.

2.2. Test substance

Ferrous sulphate heptahydrate (CAS registry number: 7782-63-0, EU number: 231-753-5, AR grade purity 99.5%, common names: Green vitriol, Copperas, Melanterite) was used as a toxicant. LC_{50} for different exposure periods was determined by using probit analysis (Finney, 1971). The EC_{50} value of ferrous sulphate for *T. mossambica* was 0.3 g/L for 96 h. Considering the EC_{50} value, the sublethal doses of 0.001 g/L and 0.01 g/L were chosen for the present study. Similar concentrations of iron were found in a few water bodies in Goa (unpublished data). A set of five fish each for respective doses and exposure periods (15 days and 30 days) was used for both control and experiments. Thirty minutes prior to the termination of exposure period escape test was performed for each fish of every set. Escape test constituted of elucidation of escape response by tactile stimulation with the rod (4 mm diameter) which was presented randomly from both side of the caudal fin of a stationary fish, followed by an attempt to capture by hand trapping. This phenomenon was repeated three times for every fish with a gap of 5 min after each test. Escape response was noted in terms of fish being caught which served as no escape response or fish not being caught which served as successful escape response. At the end of exposure period fish were cold narcotised and dissected open to collect their cerebral cortices for enzyme assays and histological analysis.

2.3. Enzyme assay

For estimation of cytosolic enzymes, the frozen tissue was homogenized at 0 °C in 0.32 M sucrose solution containing 5 mM Hepes buffer (0.1 g/10 ml; pH 7.4). The homogenate was freeze centrifuged (4 °C) for 20 min at 12,000*g*. Undiluted supernatants were used for enzyme assay. Whereas for estimation of mitochondrial enzymes, cerebral cortices were homogenized in nine volumes of 0.32 M sucrose. Homogenate was centrifuged at 1000*g* for 10 min. The pellet thus obtained was washed twice and applied on a discontinuous sucrose gradient consisting of 0.8, 1.0, 1.2 and 1.4 M sucrose. After centrifugation at 75,000*g* for 2 h, the mitochondrial pellet was obtained at the bottom of the tube, which was used as sample source for estimation of mitochondrial enzymes. Aminotransferases were measured according to 2, 4 dinitrophenyl hydrazine (2, 4-DNPH) method. The incubation mixture for ALT contained 500 µl of alanine-α-ketoglutarate, 100 µl of enzyme extract, 500 µl of 2, 4-DNPH and 5 ml of 0.4 N NaOH. The incubation mixture for AST contained 500 µl of aspartate-α-ketoglutarate, 100 µl of enzyme extract, 500 µl of 2, 4-DNPH and 5 ml of 0.4 N NaOH. Optical density of corresponding brown coloured hydrazone formed in alkaline medium was read at 505 nm. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.4. Histological investigation

For histopathological evaluation, cerebral cortices were embedded in paraffin and sections were cut on microtome at 8-µm. Sections were stained with hematoxylene and eosin. Histopathological changes were evaluated by light microscopy.

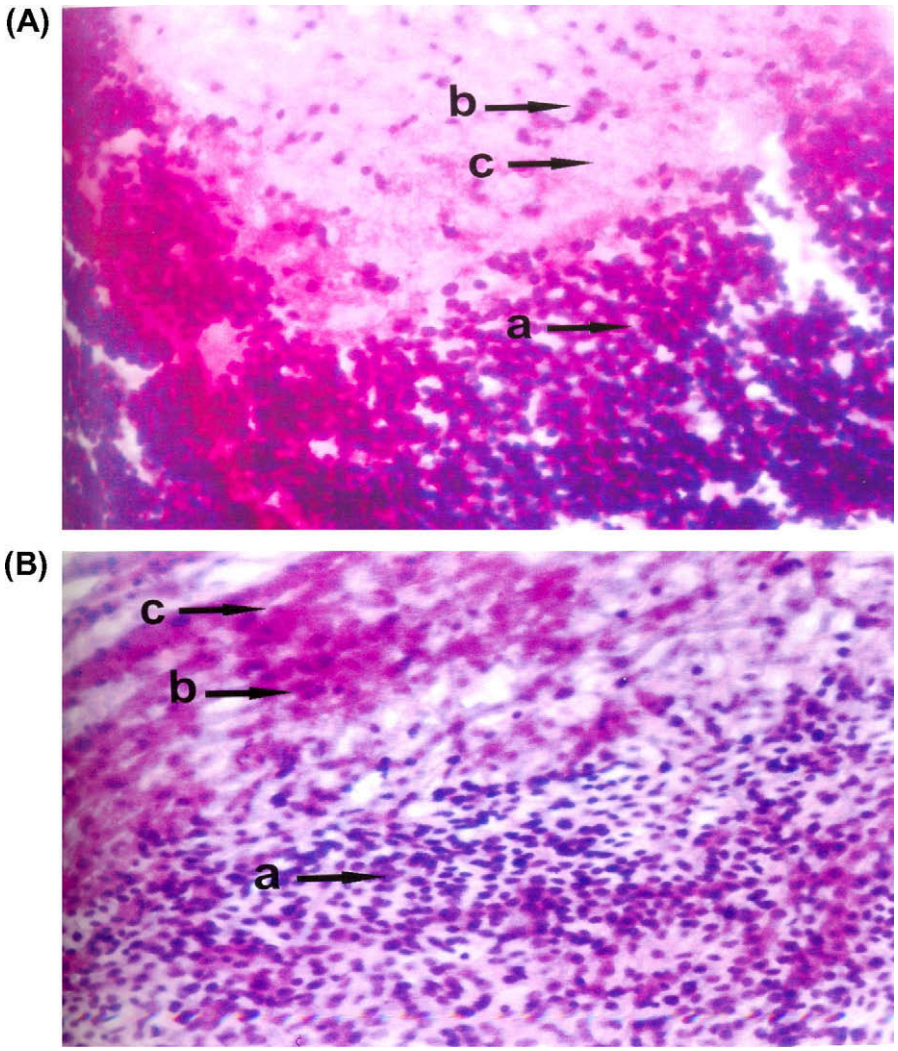

Fig. 1. (40×) (A): Section of cerebral cortex of control fish and (B): section of cerebral cortex of fish exposed to 1 mg/L of ferrous sulphate for 30 days of exposure period (a), granular cells; (b), Purkinje cells and (c), mossy mat of fibers.

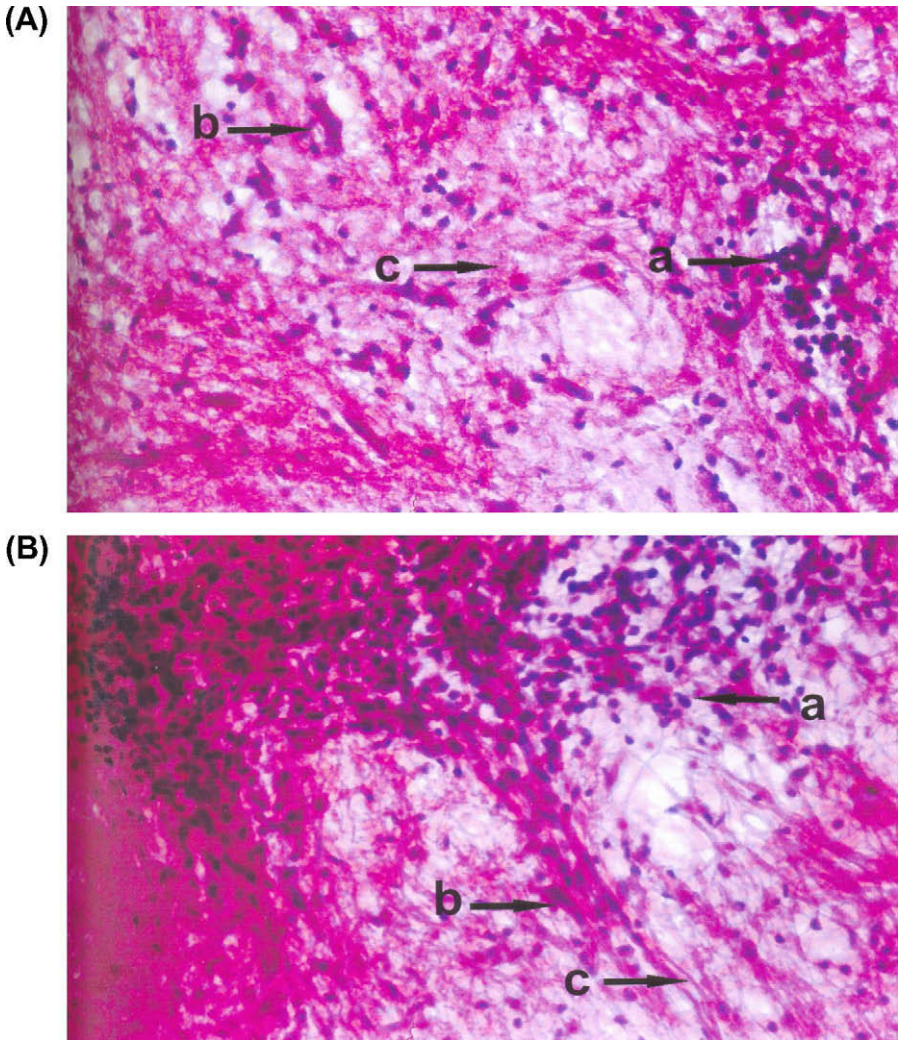

Fig. 2. (40×) (A): Section of cerebral cortex of fish exposed to 10 mg/L of ferrous sulphate for 15 days of exposure period and (B): section of cerebral cortex of fish exposed to 10 mg/L of ferrous sulphate for 30 days of exposure period (a), granular cells; (b), Purkinje cells and (c), mossy mat of fibers.

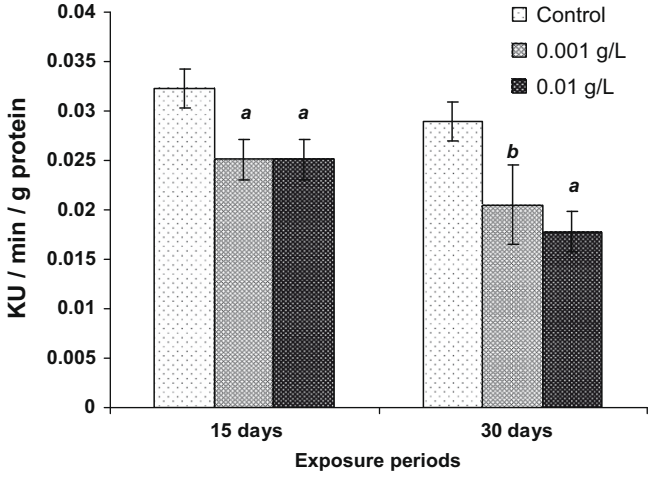

Fig. 3. Cytosolic ALT activity of cerebral cortex of Tilapia exposed to ferrous sulphate for 15 and 30 days. Data are mean ± SD for 10 independent experiments performed. (a) Different from control, $p < 0.001$; (b) $p < 0.05$ (Student's t-test).

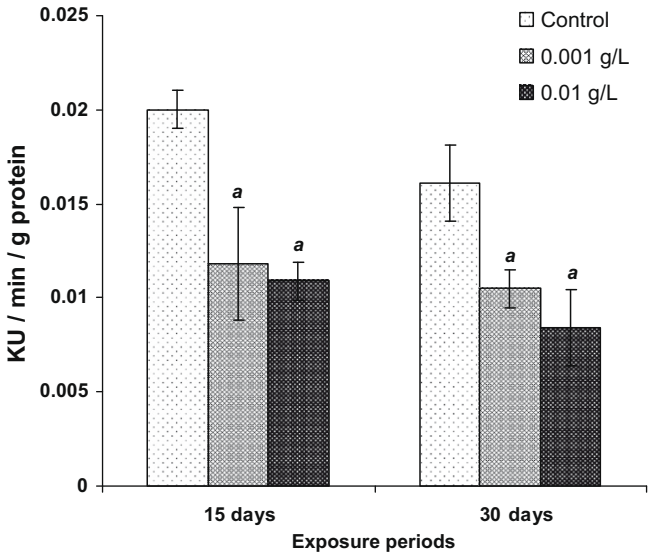

Fig. 4. Mitochondrial ALT activity of cerebral cortex of Tilapia exposed to ferrous sulphate for 15 and 30 days. Data are mean ± SD for 10 independent experiments performed. (a) Different from control, $p < 0.001$ (Student's t-test).

2.5. Statistical analysis

All values represented in graph are expressed as mean ± standard deviation (SD). Student *t*-test was used to compare the changes between control and treated sets and $p < 0.05$ was considered statistically significant.

3. Results and discussion

Tilapia exposed to ferrous sulphate, initially showed rapid swimming and frequent surfacing associated with coughing. However, exposure of Tilapia to ferrous sulphate for 15 and 30 days promoted lethargy in the fish. This was evident in the 'escape test' where exposed fish offered little or no resistance to capture while the control fish could easily swim away and avoid being caught. Similar observations are reported by Lappivaara and Marttinen (2005) in white fish, *Coregonus lavaretus* exposed to iron for 30 days. The weight of the cerebral cortex decreased by 10.0% and 15.6% after exposure of fish to 0.01 g/L ferrous sulphate for 15 and 30 days, respectively. After exposure to 0.001 and 0.01 g/L ferrous sulphate for 15 and 30 days, the corticular neurons showed swelling while Purkinje and granular cells exhibited dense cytoplasm (Figs. 1 and 2). The swelling of cells indicate osmotic stress mediated by ferrous sulphate. The deposition of iron in neurons and glial cells coupled with formation of free oxygen radicals may also promote impairment of membranes leading to disturbances in ionic homoeostasis thereby promoting osmotic stress leading to neuronal swelling. Iron facilitates the formation of highly toxic hydroxyl radicals ($^{\cdot}OH$) from H_2O_2 and superoxide (O_2^-) by the Fenton reaction and iron absorbed by the tissues is involved in generation of H_2O_2 and O_2^- by accelerating the non-enzymatic oxidation of molecules such as glutathione. The OH^- radicals cause lipid peroxidation by abstracting a hydrogen atom from a polyunsaturated acid side chain in a membrane lipid thereby promoting impairment of membrane function leading to osmotic flooding of water, cell swelling and eventual neuronal degeneration (Dexter and Jenner, 1996). Thus, the iron might have promoted, through the production of free radicals, degenerative changes in the neurons of Tilapia, hence the loss of weight of cerebral cortex.

Tilapia exposed to ferrous sulphate (0.001 g/L) for 15 and 30 days showed decline in cytosolic ALT activity of cerebral cortex by 22.3% ($p < 0.001$) and 29.1% ($p < 0.05$), respectively, while those exposed to 0.01 g/L exhibited decline in cytosolic ALT activity by 22.3% ($p < 0.001$) and 38.4% ($p < 0.001$), respectively (Fig. 3). Mitochondrial ALT activity of the cerebral cortex of Tilapia reduced significantly by 34.8% ($p < 0.001$) and 41% ($p < 0.001$) after exposure to 0.001 g/L ferrous sulphate for 15 days and 30 days, respectively. Exposure of Tilapia to 0.01 g/L of ferrous sulphate reduced mitochondrial ALT activity of cerebral cortex by 45.5% ($p < 0.001$) and 47.8% ($p < 0.001$) at the end of 15 and 30 days, respectively (Fig. 4).

Cytosolic AST activity of cerebral cortex of Tilapia declined by 17.3% ($p < 0.05$) and 35.4% ($p < 0.001$) on exposure to 0.001 g/L ferrous sulphate for 15 and 30 days, respectively, while 0.01 g/L of ferrous sulphate promoted reduction of cytosolic AST activity by 18.5% ($p < 0.05$) and 49.2% ($p < 0.001$) after 15 and 30 days, respectively (Fig. 5). Mitochondrial AST activity of cerebral cortex of Tilapia exposed to 0.001 g/L reduced by 32.7% ($p < 0.05$) and 50% ($p < 0.001$) after 15 and 30 days, respectively, while those exposed to 0.01 g/L ferrous sulphate showed 36.4% ($p < 0.001$) and 51% ($p < 0.001$) reductions of mitochondrial AST activity after 15 and 30 days, respectively (Fig. 6).

ALT and AST are native brain enzymes existing in cytosolic and mitochondrial forms. The significant reduction in the activities of these enzymes in cerebral cortices indicates reduction in glutamate metabolism. Particularly the observed lethargy could be due to the lesser production of glutamate, an essential neurotransmitter for excitatory responses. Besides, the decreased cytosolic AST activity could be due to decreased transport of NADH from cytosol to mitochondria leading to decreased metabolic rate as a corollary to the findings that increase in AST may be connected to increase in transport of NADH from cytosol to mitochondria (Netopilová et al., 2001). Reduction in ALT activity indicates reduction in protein metabolism (Abou El-Naga et al., 2005) particularly the transformation of alanine to pyruvate and vice versa. ALT is involved in synthesis of glutamate, which is a neurotransmitter in glutamergic nerves (Palailogos et al., 1988, 1989). Therefore, reduction in aminotransferase activities may be leading to lesser synthesis of glutamate, thereby causing neurological dysfunction (lethargic behaviour and reduced swimming activity). Some known facts on iron toxicity show that iron rich cells are more prone to oxidative damage (Varani et al., 1985). It also brings about inhibition of mitochondrial respiratory enzymes (Calabrese et al., 2005; Shamoto-Nagai et al.,

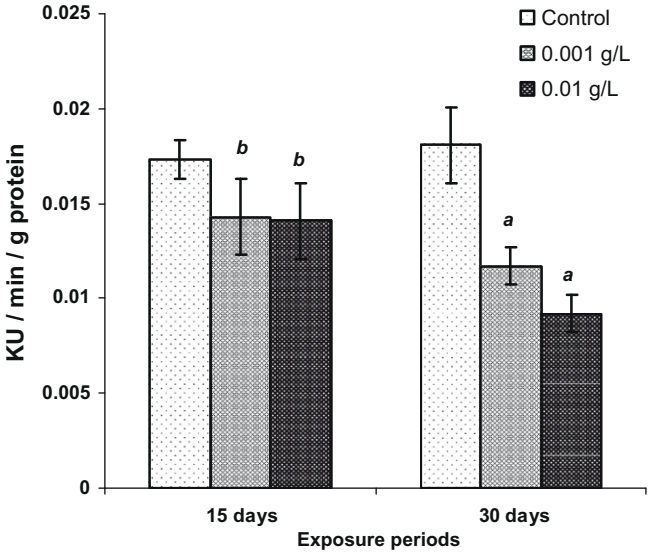

Fig. 5. Cytosolic AST activity of cerebral cortex of Tilapia exposed to ferrous sulphate for 15 and 30 days. Data are mean ± SD for 10 independent experiments performed. (a) Different from control, $p < 0.001$ and (b) $p < 0.05$ (Student's *t*-test).

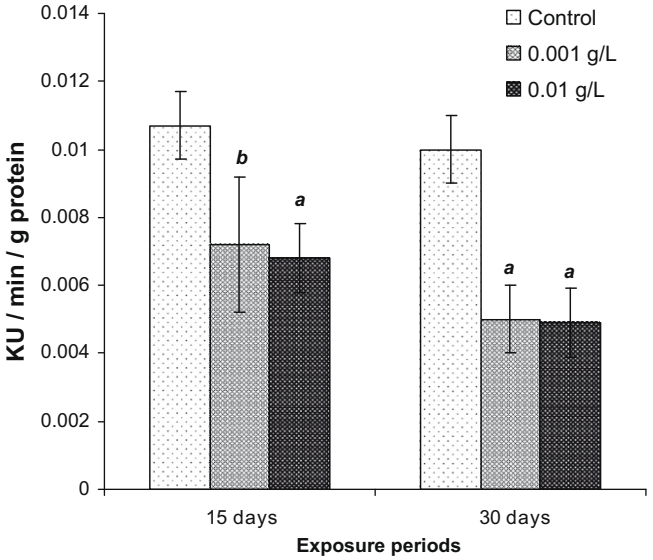

Fig. 6. Mitochondrial AST activity of cerebral cortex of Tilapia exposed to ferrous sulphate for 15 and 30 days. Data are mean ± SD for 10 independent experiments performed. (a) Different from control, $p < 0.001$ and (b) $p < 0.05$ (Student's *t*-test).

2006). Besides, it is also known that brain cells are relatively more susceptible to metal toxicity than other body cells as their antioxidant defenses are relatively low (Ward et al., 1994; Crichton et al., 2002). Even accumulation of trace physiological amounts of iron over a time affects gene expression responding to Fenton chemistry-triggered pathogenesis (Alexandrov et al., 2005; Gaasch et al., 2007). Therefore, it may be concluded that probable molecular mechanism involved in reduction in the aminotransferases may be due to direct inhibition of enzyme activity by iron-induced ROS and/or due to targeted genes which encode for these aminotransferase by the attack of ROS due to iron toxicity via Fenton chemistry-triggered pathogenesis thus bringing about less synthesis of these enzymes.

4. Conflict of interest

The authors declare that there are no conflicts of interest.

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