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Iron exposure modifies acetylcholinesterase activity in zebrafish (*Danio rerio*) tissues: distinct susceptibility of tissues to iron overload

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Abstract Iron is one the most abundant metals on the earth being essential for living organisms even though its free form can be toxic. The overload of this metal may be related with some disorders, like Alzheimer and Parkinson diseases, and hemochromatosis in the liver. The aim of the present study was to evaluate the effects of iron on acetylcholinesterase (AChE) activity in brain and liver of zebrafish and to investigate the possible correlation with the iron content in these tissues. Different corresponding concentrations of iron were tested using in vitro (0.018, 0.268, and 2.6 mM) and in vivo (1, 15, and 150 mg/l) assays. The in vitro studies showed that iron promoted a significant increase in AChE activity in brain (52%) and liver (53%) at the higher concentration (2.6 mM). In the in vivo assays, a significant increase in this enzyme activity was observed in the presence of 15 mg/l in both, brain (62%) and liver tissue (70%). Semiquantitative RT-PCR did not reveal significant changes in acetylthiocholinesterase mRNA levels. Moreover, we observed that iron content was significantly increased in liver tissue when exposed to 15 (226%) and 150 mg/l (200%). These results indicate that iron can promote significant alterations in AChE activity which probably is not directly related to the iron content in zebrafish tissues.

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Introduction

Iron is one of the most abundant metals on the earth (Bury and Grosell 2003). This metal is vital for all living organisms being essential for multiple metabolic processes including oxygen transport, electron transport (Crichton 2001), DNA synthesis, and as cofactor for many important proteins (Zecca et al. 2004). Although iron is essential for the cell functioning, its free form can be toxic (Donovan et al. 2002). Iron toxicity is largely based on Fenton chemistry where iron reacts with reactive oxygen intermediates, including hydrogen peroxide (H2O2) and the superoxide anion (O_{2}_), both byproducts of aerobic metabolism, to produce highly reactive free radical species such as the hydroxyl radical (OH_).

Brain cells have relatively low antioxidant defenses (Ward et al. 1994) and are especially susceptible to metal toxicity (Crichton et al. 2002). Studies using samples of *postmortem* patients' brain have demonstrated that iron is present in some protein aggregates observed in Alzheimer and Parkinson (Zecca et al. 2004) and in the striatum in Huntington′s disease (Dexter et al. 1991; Dexter et al. 1992; Chen et al. 1993; Lumsden et al. 2007). Additionally, iron overload disorder, hemochromatosis, is one of the most common genetic disorders in individuals and is associated in humans with mutations in various genes, responsible for different forms of the disease (Donovan et al. 2002; Feder et al. 1996; Njajou et al. 2001).

The establishment of the relationship between behavioral changes and iron accumulation in brain regions has been a challenge for many researchers (Fredriksson et al. 1999; Fredriksson et al. 2001; Guo 2004; Lima et al. 2005a, b, 2007). Studies demonstrated that neonatal rats subjected to iron treatment presented in adulthood a recognition memory deficits and that this cognitive impairment was prevented with the administration of iron chelating (Lima et al. 2005b). Moreover, studies have been shown that changes in locomotor activity promoted by iron could be related with the increase in iron content in specific brain areas (Fredriksson and Archer 2003).

Acetylcholine (ACh) is a classical neurotransmitter involved in learning and memory process, control of motor tonus, and autonomic functions (Herlenius and Lagercrantz 2004). After released, ACh is rapidly removed from the synaptic cleft by acetylcholinesterase (AChE, EC 3.1.1.7), which belongs to the family of type B carboxylesterases, cleaving ACh into choline and acetate. The measurement of AChE activity in organisms is widely used as a specific biomarker of toxicity (Roex et al. 2003). Several studies demonstrated the inhibitory effect of toxic agents on AChE activity in zebrafish brain, for example to cadmium and zinc (Senger et al. 2006), to ethanol (Gerlai et al. 2006; Rico et al. 2007), and to methanol (Rico et al. 2006).

Zebrafish (*Danio rerio*) is a small freshwater teleost widely used as a vertebrate model of developmental, neurobiological, toxicological, and pharmacological studies (Rubinstein 2003; Guo 2004; Goldsmith 2004; Hill et al. 2005). This fish presents a unique situation among vertebrates, because AChE is the only ACh-hydrolyzing enzyme in this organism (Behra et al. 2002). In addition, it has been shown that the cholinergic system is widely distributed in the zebrafish brain (Park et al. 2008).

Considering the ambiguous role of iron described above and the important function that cholinergic system plays in the central and peripheral nervous systems, the aim of this work was to evaluate the in vitro and in vivo effects of different concentrations of iron on brain and liver AChE activity from zebrafish. Furthermore, we also analyzed a possible relation between the iron concentrations overload in these organs and the AChE activity.

Materials and methods

Animals

Adult wild-type zebrafish (*Danio* rerio) of both sexes were obtained from commercial supplier (Delphis, RS, Brazil) and acclimated for at least 2 weeks in a 50-l thermo stated aquarium. The fish were kept on a 12-h light/dark cycle (lights on at 7:00 am) at a temperature of 25 ± 2°C, and the animals were fed with commercial fish pellet twice a day. The use and maintenance of zebrafish were according to the National Institute of Health Guide for Care and Use of Laboratory Animals, being healthy and free of any signs of disease. The Ethics Committee of Pontifícia

Universidade Católica do Rio Grande do Sul (PUC-RS) approved the protocol under the number 08/00025–CEUA.

Chemicals

Trizma Base, ethylenedioxy–diethylene–dinitrilo–tetraacetic acid (EDTA), ethylene glycol bis(beta amino ethylether)-N,N,N′,N′-tetraacetic acid (EGTA), sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma (St. Louis, USA). Ferrous sulfate was from analytical grade.

In vitro and in vivo treatments

The iron doses and time of treatment for in vitro and in vivo assays were chosen based on acceptable iron levels normally present in treated water (0,3 mg/l) and water effluents as previously described (Lima and Pedrozo 2001). For in vitro assay, the brain and liver homogenized samples were directly added to the reaction medium containing iron at final concentrations in the range of 0.018, 0.268, and 2.6 mM, preincubated for 10 min, and maintained throughout the enzyme assay. For the control group, the enzyme assay was performed in the absence of iron. For in vivo treatments, corresponding concentrations of iron used at in vitro assays, 1, 15, and 150 mg/l, were administered by placing the fishes in tanks containing 2 l water for 24 h. Afterwards, the animals were euthanized by decapitation and whole brain and liver were removed for the determination of AChE activity.

Determination of AChE activity

Brain and liver tissues were homogenized on ice in 60 volumes (v/w) of Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor-driven Teflon-glass homogenizer. The rate of acetylthiocholine hydrolysis (0.8 mM) was determined in a final volume of 2 ml assays solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB according to the method previously described (Ellman et al. 1961). For the AChE activity, a pool of 3 brains or livers was considered as a sample. Brain samples containing 10 μg of protein and liver samples containing 5 μg of protein were added at the reaction medium described previously and pre-incubated at 25°C for 10 min. The reaction assay began with the substrate addition, and the rate of acetylthiocholine hydrolysis was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (30 s intervals). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of acetylthiocholine. The linearity of absorbance related to time and protein concentration was previously determined. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. Four different experiments were performed, and the assays were run in triplicate.

Iron measurement

The iron accumulation was quantified in the water of the tanks and also in brain and liver after 24 h of treatment with iron at different concentrations (1, 15, and 150 mg/l). The tissues were removed and homogenized in 0.5 mL deionized water and centrifuged at 10,000*g*. The iron content was measured at the supernatant using commercial kit Labtest (Minas Gerais, Brasil). The water samples were sent to the LAPA (Laboratório de Processos Ambientais, PUC-RS; Porto Alegre; Brazil) to quantify the iron concentration.

Molecular analysis

Forward (5′-CCAAAAGAATAGAGATGCCATGG ACG-3′) and reverse (5′-TGTGATGTTAAGCAGA CGAGGCAGG-3′) *AChE* primers and the optimal conditions for RT-PCR experiments were used according to Rico et al. (2007). The *β-actin* primers forward (5′-GTCCCTGTACGCCTCTGGTCG-3′) and reverse (5′-GCCGGACTCATCGTACTCCTG-3′) were used as described previously (Chen et al. 2004).

Total RNA was isolated from zebrafish brain using TRIzol reagent (Invitrogen) in accordance with manufacturer instructions. RNA was quantified by spectrophotometer, and all samples were adjusted to 160 ng/μl. cDNA species were synthesized using SuperScript III™ First-Strand (Synthesis System for RT-PCR) Invitrogen Kit following the suppliers. One microliter of RT reaction mix was used as a template for each PCR. PCR for *AChE* was performed in a total volume of 25 μl using 0.08 μM of each primer,

0.2 μM dNTP, 2 mM $MgCl_2$, and 1 U Taq DNA polymerase (Invitrogen). PCR for *β-actin* gene was performed in a total volume of 20 μl using 0.1 μM of each primer, 0.2 μM dNTP, 2 mM $MgCl_2$, and 0.5 U Taq DNA polymerase (Invitrogen). PCR was conducted at 1 min at 94°C, 1 min at 60°C (*AChE*) and at 54°C (*β-actin*), and 1 min at 72°C for 35 cycles. A postextension cycle at 72°C was performed for 10 min. For each set of PCR, a negative control was included. PCR products were analyzed on 1% agarose gel containing GelRed® and visualized with ultraviolet light. The low DNA Mass Ladder (Invitrogen) was used as molecular marker, and normalization was performed employing *β-actin* as a constitutive gene. PCR parameters, concerning $MgCl_2$, cDNA template concentration, and PCR cycles (25–45 cycles), were first optimized (Fig. 3c), and reactions were performed using optimal conditions that allowed product detection within the linear phase of band densitometry analyzed. The band intensities were measured by optical densitometry analysis, and the optical density ratios (*AChE*/*β-actin*) were established for each treatment using the Kodak 1D Image Analysis Software.

Protein determination

Protein was measured using Coomassie Blue as color reagent and bovine serum albumin as standard (Bradford 1976).

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test and expressed as means ± SD. Differences were considered significant for $P \leq 0.05$.

Results

The effect of different iron concentrations on AChE activity from zebrafish brain and liver was demonstrated by in vitro (0.018, 0.268, and 2.6 mM) and in vivo (1, 15, and 150 mg/l) studies. In the in vitro experiments, iron treatment is able to promote a significant increase in brain (52%) (Fig. 1a) and liver (53%) (Fig. 1b) AChE activity only at higher concentration (2.6 mM). In the in vivo analysis, we verified that 24 h of iron treatment at dose of 15 mg/l increased the AChE activity in both, brain (62%) (Fig. 2a) and liver (70%) (Fig. 2b).

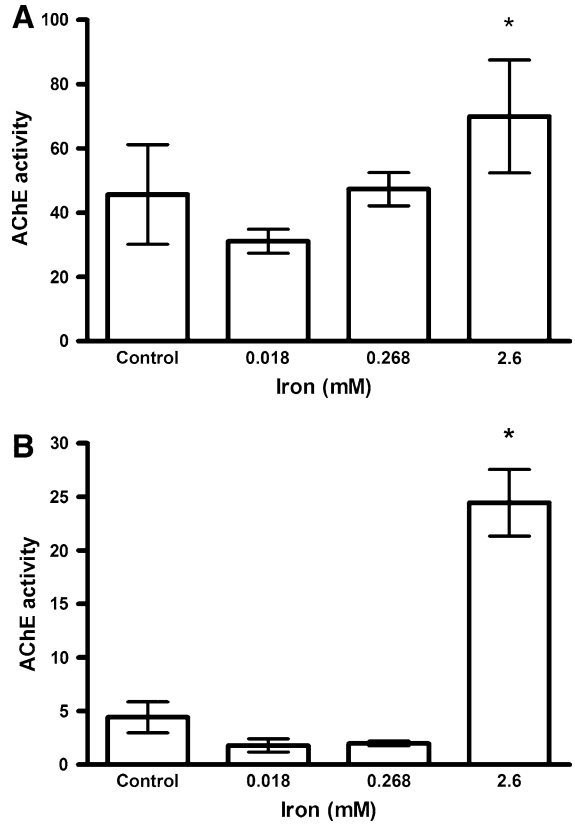

Fig. 1 In vitro effects of varying concentrations of iron (0,018, 0,268, or 2,6 mM) on zebrafish **a** brain AChE activity (45 ± 15 control values) and **b** liver AChE activity (4.4 ± 1.4 control values). Bars represent the mean ± SD of at least five different experiments, each one performed in triplicate. The AChE activity was expressed as μmol of thiocholine released per hour per milligram of protein. Data were analyzed statistically by one-way ANOVA followed by Tukey multiple range test. *$P < 0.05$ denotes a significant difference from the control group

In order to verify the effect of iron on AChE transcriptional control, semiquantitative RT-PCR experiments were conducted. Although iron treatment altered the AChE activity, our results showed that AChE transcript levels did not significantly change (Fig. 3a, b).

Sequentially, we evaluated a possible correlation of the iron amount and AChE actions, quantifying the iron accumulation in zebrafish brain and liver tissue after 24 h of in vivo treatment at the same doses used to test the AChE activity. The iron amount was not

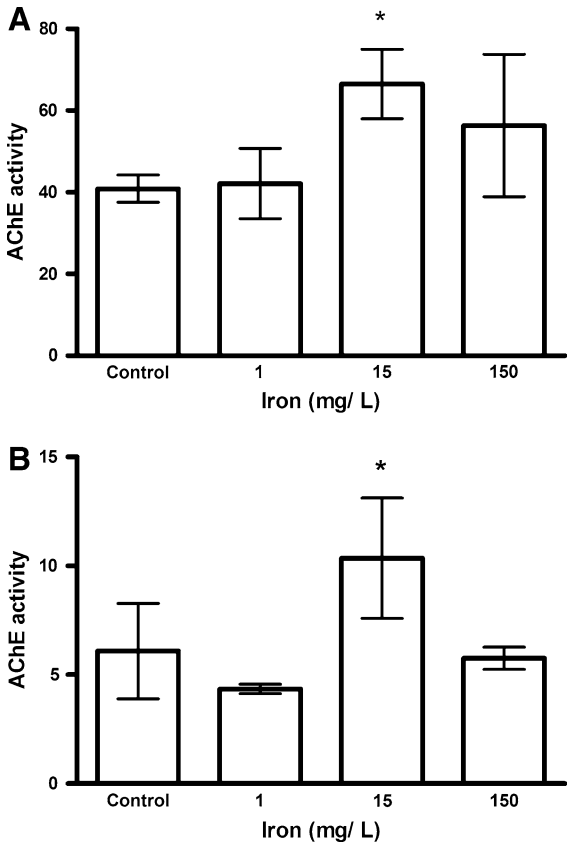

Fig. 2 In vivo effects of 24-h acute treatment with iron (1, 15 or 150 mg/l) on zebrafish **a** brain AChE activity (41 ± 3.6 control values) and **b** liver AChE activity (6 ± 2.2 control values). *Bars* represent the mean ± SD of at least five different experiments, each one performed in triplicate. The AChE activity was expressed as μmol of thiocholine released per hour per milligram of protein. Data were analyzed statistically by one-way ANOVA followed by Tukey multiple range test. $*P < 0.05$ denotes a significant difference from the control group

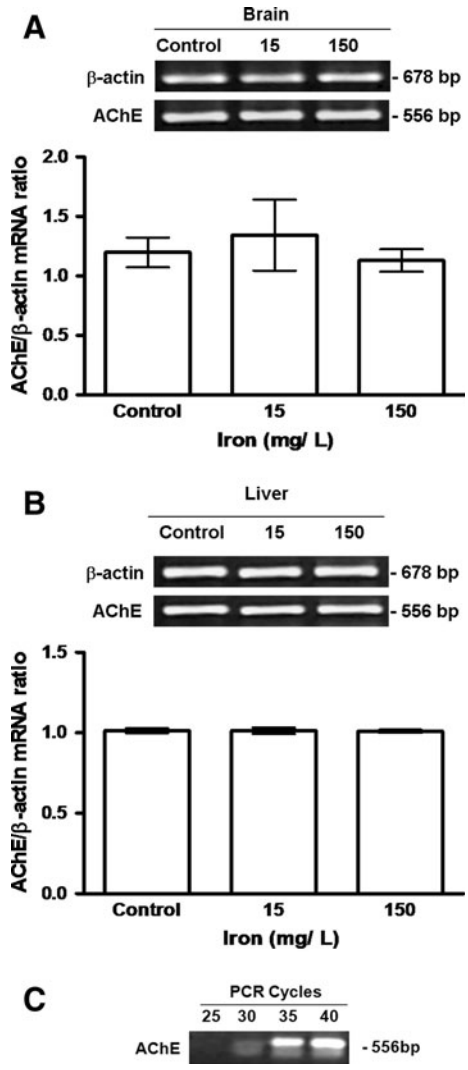

Fig. 3 Effect of 24-h iron exposure (15 and 150 mg/l) on AChE transcripts from zebrafish **a** brain and **b** liver. The PCR products were subjected to electrophoresis on a 1% agarose gel, using *β-actin* as constitutive gene. The figure shows a representative gel, and the optical density ratios (*AChE/β-actin*) expressed as arbitrary units, determined by four independent experiments, with entirely consistent results. Data were analyzed statistically by one-way ANOVA followed by Tukey multiple range test. The calibration curve obtained with different PCR cycles that allowed product detection within the linear phase of band densitometry analyzed is shown (**c**)

significantly different between control group and the treatments in brain homogenates (Fig. 4a). However, there was a significant increase in the iron concentration from liver of fish submitted to 15 (1.96 ± 0.9) and 150 mg/l (1.8 ± 0.9) when compared with the control group (0.6 ± 0.13) (Fig. 4b).

Discussion

In the present study, we evaluated the in vitro and in vivo effects of iron treatment at several concentrations on AChE activity from zebrafish brain and liver, as well as iron content in both tissues. Our results demonstrated that AChE activity was significantly modulated by higher concentrations of iron at in vitro assays in both organs evaluated. Despite observing an elevated enzyme activity by iron treatment, recent studies have shown that other metals, such zinc and cadmium, did not alter significantly the in vitro

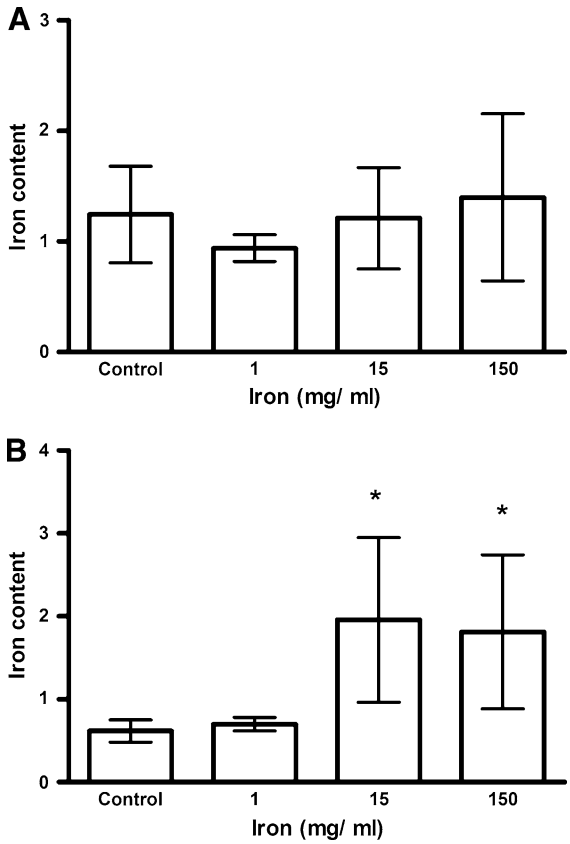

Fig. 4 Effect of 24-h iron exposure (15 and 150 mg/l) on iron content in zebrafish **a** brain tissue and (1.2 ± 0.4 control values) and **b** liver tissue (0.62 ± 0.13 control values). *Bars* represent the mean ± SD of at least five different experiments. Data were analyzed statistically by one-way ANOVA followed by Tukey multiple range test. $*P < 0.05$ denotes a significant difference from the control group

zebrafish brain AChE activity (Senger et al. 2006). In this context, Bolognesi et al. (2007) showed the in vitro ability of chelating metals, like iron and copper, to inhibit the AChE activity from humans. Taking into account this data, we could suggest that the effectual role of iron can be preceding directly on the enzyme without the influence of other biological systems, such as cell signaling pathways. Since AChE is anchored to the outer surface of the plasma membrane by a covalently attached glycosyl-phosphatidylinositol (GPI) structure, previous studies suggested that modifications in lipid membrane could be responsible for a change in the conformational state of the AChE molecule, which could induce the activation of AChE observed after long-term exposure to metals (Kaizer et al. 2005). In addiction, although no study described the effect promoted by metal exposure on hepatic AChE activity, in this study, we demonstrated that in vitro iron treatment was efficient to change this enzyme activity.

The in vivo results established that 15 mg/l of iron was able to promote a significant increase in AChE activity from zebrafish brain and liver. Interestingly, 150 mg/l of iron did not promote any modulation in AChE activity in both organs studied. It is possible to speculate that higher concentration of iron (150 mg/l) could trigger the mechanisms involved in the iron metabolism that confers protection against iron-induced damage. In this sense, it was already demonstrated that exposure of the mosquito *Aedes aegypti* cells to iron at low concentrations increases cytoplasmic iron, while higher iron levels result in a decline in cytoplasmic iron levels indicating that the excess of iron is removed from mosquito cells (Geiser et al. 2006).

There are several mechanisms that could regulate AChE after in vivo experiments, which include modifications at transcriptional and/or posttranslational levels. Our findings showed that the increased activity observed after iron exposure is not a consequence of increased AChE transcript levels. In this sense, it was already demonstrated that AChE from other sources was clearly regulated by a posttranslational event (Robitzki et al. 1997; Keller et al. 2001).

According to our data, Kaizer et al. (2005) also observed an increase in brain AChE activity from mice submitted to the aluminum treatment. Moreover, several studies have shown the influence of iron in some brain pathologies, like Alzheimer, Parkinson, and Huntington (Zecca et al. 2004; Chen et al. 1993; Dexter et al. 1991; Dexter et al. 1992; Lumsden et al. 2007). In this context, metal chelation has been suggested for the development of novel Alzheimer's disease therapeutics since two multifunctional compounds of carbohydrate showed a significant antioxidant capacity (Cuajungco et al. 2000; Storr et al. 2007). In another study, Oakley et al. (2007) showed that some primary changes in neuronal iron could lead to neurodegeneration in Parkinson, presenting the importance of iron in this disease.

Behavioral changes, such as learning, memory, and locomotor activity, are involved in neurodegenerative pathologies. Studies demonstrated that adult rats exposed to postnatal iron administration presented memory deficits related to iron load in some

brain areas (Schröder et al. 2001). Besides, postnatal treatment with iron and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can yet promote an iron overload-induced decrease locomotor activity (Fredriksson and Archer 2003). Fredriksson et al. (2001), verified that the concomitant postnatal treatment with MPTP and iron in rats contributed to functional and neurochemical deficits implicated in the neurodegenerative process of Parkinson disease. A previous study in fish demonstrated that liver iron stores were increased among fish treated with iron, suggesting that this metal could be accumulated in this zebrafish organ (Fraenkel et al. 2005). Considering that toxicological effects of iron could be related with its accumulation in several tissues (Schröder et al. 2001; Fredriksson and Archer 2003), we evaluated a possible correlation of iron content and AChE activity in zebrafish brain and liver. In our work, we demonstrated that there was no significant iron accumulation in brain tissues. Although both tissues presented a modulatory in vivo effect on AChE activity, our findings are an indicative that the iron effect over enzyme activity is not directly correlated with iron overload. A likely explanation to the enhancement on liver iron content could be due to a higher susceptibility of this organ for metal toxicity.

Degenerative and progressive neurological disorders like Alzheimer and Parkinson have been characterized by deficit in the cholinergic neurotransmission (Orhan et al. 2009). In this context, AChE inhibitors have been used in the treatment of both pathologies improving the cognitive symptoms and dementia-associated symptoms such as aggressiveness and the ability to perform activities of daily life (Anghelescu and Heuser 2007; Hasselbalch and Kampmann 2009). Furthermore, Nelson et al. (2009) showed that the treatment with AChE inhibitors was associated with a slower rate of cognitive decline in Alzheimer's disease (AD) and dementia with Lewy bodies (DLB). Recently, it has been shown that memory impairment in mice could be attributed to cholinergic synapse dysfunction (Watanabe et al. 2009). Because the cholinergic system can be involved in the development of neurodegenerative pathologies and metal chelation could be an effectiveness treatment for the progression of these disorders, we can suggest that iron-induced enhancement on AChE activity presented in our study could play a role, at least in part, to the neurochemical alterations presented in these pathologies.

In summary, our results have shown that iron treatment alters AChE activity from zebrafish brain and liver when tested in vitro and in vivo. The measurement of AChE activity is used worldwide as a biomarker of environmental contamination as demonstrated previously by significant variation of AChE activities attributed to neurotoxic substances like a high concentration of different metals (lead, cadmium, copper, manganese, and iron). Here, we observed that iron content seems not to be correlated with changes in AChE activity, but accumulation of iron in liver zebrafish seems to be attributed to distinct susceptibility between brain and liver to the iron exposure.

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