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Aluminum exposure alters behavioral parameters and increases acetylcholinesterase activity in zebrafish *(Danio rerio)* brain

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Abstract Aluminum is a metal that is known to impact fish species. The zebrafish has been used as an attractive model for toxicology and behavioral studies, being considered a model to study environmental exposures and human pathologies. In the present study, we have investigated the effect of aluminum exposure on brain acetylcholinesterase activity and behavioral parameters in zebrafish. In vivo exposure of zebrafish to 50 μg/L $AlCl_3$ for 96 h at pH 5.8 significantly increased (36%) acetylthiocholine hydrolysis in zebrafish brain. There were no changes in acetylcholinesterase (AChE) activity when fish were exposed to the same concentration of $AlCl_3$ at pH 6.8. In vitro concentrations of $AlCl_3$ varying from 50 to 250 μM increased AChE activity (28% to 33%, respectively). Moreover, we observed that animals exposed to $AlCl_3$ at pH 5.8 presented a significant decrease in locomotor activity, as evaluated by the number of line crossings (25%), distance traveled (14.1%), and maximum speed (24%) besides an increase in the absolute turn angle (12.7%). These results indicate that sublethal levels of aluminum

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might modify behavioral parameters and acetylcholinesterase activity in zebrafish brain.

Keywords Acetylcholinesterase · Aluminum · Behavior · Locomotion · Zebrafish

Abbreviations

ACh Acetylcholine

AChE Acetylcholinesterase

Introduction

Aluminum is a non-essential metal that is extremely common throughout the world, being the third most abundant element in the Earth's crust. Aluminum is innocuous under alkaline or circumneutral conditions whereas in acidic environments, it presents severe risks to the aquatic biota, including fish (Waring et al. 1996). Studies have postulated that chronic water acidification associated with aluminum is involved in the decline in the Atlantic salmon population (Monette and McCormick 2008). Furthermore, aluminum is known to have toxic effects on a variety of organ systems including the brain (Oteiza et al. 1993). The precise molecular mechanisms by which aluminum exerts its neurotoxic effects are still not completely understood. Evidence that aluminum accumulation contributes to Alzheimer's disease (AD) remains contradictory, although some epidemiological studies have indicated a relationship between the concentration of aluminum in potable water and this neurodegenerative condition (Rondeau et al. 2009; Shcherbatykh and Carpenter 2007).

Acetylcholine (ACh) is a classical neurotransmitter secreted from presynaptic nerve terminals. After release, 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 and cleaves acetylcholine into choline and acetate (Soreq and Seidman 2001). Studies have established AChE as a biomarker for several environmental contaminants (Naravaneni and Jamil 2007; Senger et al. 2006), and it has been suggested that aluminum interacts with the cholinergic system in both in vitro and in vivo systems. However, the results of these investigations are conflicting because some authors report decreases in AChE activity (Hetnarski et al. 1980; Kumar 1998) whereas others report an activation of AChE in the presence of aluminum (Peng et al. 1992; Sarkarati et al. 1999; Zatta et al. 1994, 2002).

The zebrafish is a consolidated model system in neuroscience, toxicological, and behavioral studies (Gerlai et al. 2000; Rico et al. 2006; Senger et al. 2005, 2006). Zebrafish have recently become a focus of neurobehavioral studies since their larvae display learning, sleep, drug addiction, and other neurobehavioral phenotypes that are quantifiable and relate to those seen in humans (Guo 2004; Best and Alderton 2008). Furthermore, the organization of the zebrafish genome and genetic pathways controlling signal transduction and development are highly conserved between zebrafish and humans (Postlethwait et al. 2000). This species also holds great potential to improve our understanding of the genetic basis of behavior and associated behavioral disorders (Amsterdam and Hopkins 2006; Krens et al. 2006).

This teleost specie is unique among other vertebrates because AChE is the only ACh-hydrolyzing enzyme in this organism (Behra et al. 2002). It has been demonstrated that butyrylcholinesterase gene is not found in the zebrafish genome and AChE is encoded by a single gene that has already been cloned, sequenced, and functionally detected in zebrafish brain (Bertrand et al. 2001). Furthermore, cholinergic receptors are also expressed in neuronal tissue of this species (Williams and Messer 2004; Zirger et al. 2003).

Considering that aluminum is a pollutant that has been correlated with neurodegenerative disorders and with declining fish populations in soft water acidification and that zebrafish is a relevant model to evaluate behavioral, toxicological, and molecular parameters related to aluminum exposure effects, which can occur in humans, the aim of this work was to investigate the effects of aluminum exposure in two different pH values (pH=5.8 and pH=6.8) on brain acetylcholinesterase activity as well as on the behavior of this species.

Methods

Animals

Adult (around 6–8 month-old) wild-type zebrafish of both sexes was used in this study. The fish were obtained from a commercial supplier (Delphis, RS, Brazil) and acclimatized for at least 2 weeks in a 50-L aquarium. The fish were kept on a 14/10 h light/dark cycle (lights on at 7:00 a.m.) at a temperature of 25± 2°C. Animals were fed and maintained according to Westerfield (2000). All procedures for the use of animals were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Chemicals

Aluminum ($AlCl_3$, CAS number 7784-13-6, 99% min. purity) was purchased from Quimibrás Indústrias Químicas (Brazil). Trizma Base, ethylenedioxy-diethylene-dinitrilo-tetraacetic acid (EDTA), ethylene glycol bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, and 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma (USA). All other reagents used were of analytical grade.

In vivo treatments

For in vivo treatments, animals were divided into four groups: control group (pH 6.8), $AlCl_3$-treated group (pH 6.8), control group (pH 5.8), and $AlCl_3$-treated group (pH 5.8). The control groups were maintained in the 5-L test aquarium water at pH 6.8 or acidified with HCl to reach pH 5.8. The treated fish were maintained in the 5-L test aquarium containing 50 μg/L $AlCl_3$ at pH 5.8 or pH 6.8 for 24 h (acute treatment) or 96 h (subchronic treatment) because there is evidence that soft water acidification associated with aluminum induces changes in swimming activity, higher sensitivity to stress, and the decline in the Atlantic salmon population (Brodeur et al. 2001; Monette and McCormick 2008). For this reason, we evaluated the effect of aluminum on circumneutral pH=6.8 and acid pH=5.8. Immediately after the exposure, the fish were euthanized. A pool of two whole brains of zebrafish was used for each experiment.

In vitro treatments

For in vitro assays, $AlCl_3$, at final concentrations of 50, 100, and 250 μM, was added directly to the reaction medium, pre-incubated for 10 min with the brain homogenate, and maintained throughout the enzyme assay. For the control group, the enzyme assay was performed in the absence of $AlCl_3$. A pool of five whole brains of zebrafish was used for each experiment.

Determination of AChE activity

Zebrafish were euthanized by decapitation, and their brains were removed from the skull by dissection. The brains 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 with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB, using a method previously described (Ellman et al. 1961). Before the addition of substrate, samples containing protein (10 μg) and the reaction medium described above were pre-incubated for 10 min at 25°C. Acetylthiocholine hydrolysis was monitored by the formation of the 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 micromoles of thiocholine (SCh) released per hour per milligram of protein. Four different experiments were performed for each group tested, and the assays were run in triplicate.

Protein determination

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

Behavioral analysis

The behavior of fish was recorded between 10:00 and 12:00 a.m., and all animals were maintained at pH= 5.8. In the behavioral assessment, control and $AlCl_3$-treated groups (50 μg/L of $AlCl_3$ for 96 h) were placed individually into the experimental tank (30× 15×10 cm, length×height×width) and were first habituated to the tank for 30 s, as previously described (Gerlai et al. 2000). Their locomotor activity was videorecorded for 5 min after the habituation period and simultaneously analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, Illinois). The tank was divided into equal sections with four vertical and three horizontal lines, and the following locomotion patterns were measured: distance traveled, maximum speed, number of line crossings (vertical and horizontal lines), and absolute turn angle. For behavioral analysis, a number of ten animals were tested for each group.

Statistical analysis

Data were analyzed using one-way (in vitro treatments) or three-way analysis of variance (ANOVA) using aluminum, time of treatment, and pH as factors, and the results from enzyme assays were expressed as means±SD. A Tukey multiple range test was performed as post hoc considering $P \leq 0.05$ as significant. For behavioral studies, data were expressed as means±SEM and analyzed using an unpaired, two-tailed Student's *t* test, considering $P \leq 0.05$ as significant.

Results

This study examined the effects of aluminum exposure on brain AChE activity and behavioral parameters of zebrafish. The in vivo experiments were performed after 24 and 96 h of exposure to 50 μg/L of $AlCl_3$ at pH 5.8 and pH 6.8. We evaluated the control and $AlCl_3$-treated groups at pH 6.8 in order to confirm the supposed influence of pH on the toxic effects of aluminum. A three-way ANOVA revealed a main effect of Al treatment ($F(1–21)=54,990$, $P<0.01$), time of exposure ($F(1–21)=20,682$; $P<0.01$), and of treatment×time of exposure×pH interaction ($F(1–21)=8,479$; $P<0.01$). Post hoc analyses indicated that this enzyme activity was significantly increased (36%; $P<0.01$) after 96 h of $AlCl_3$ exposure at pH 5.8 when compared to respective control (pH 5.8; Fig. 1). The exposure to $AlCl_3$ at pH 6.8 did not lead to a significant difference in AChE activity in zebrafish brain with either schedule of treatment. There were no significant changes after $AlCl_3$ exposure for 24 h at pH 5.8.

To determine whether aluminum had a direct effect on the enzyme activity, we tested the in vitro effect of $AlCl_3$ on AChE activity in zebrafish brain. The $AlCl_3$ concentrations tested (50–250 μM) caused a significant increase in AChE activity ranging from 28% to 33% (Fig. 2).

The swimming activity of the control group (pH 5.8) and the $AlCl_3$-treated group (pH 5.8) was evaluated in the open field. The $AlCl_3$-treated group presented impaired locomotor activity (25%), as evidenced by a decrease in the number of crossings, when compared to the control group (Fig. 3). The results show that the $AlCl_3$-treated group significantly decreased the distance traveled (14.1%) during the 5 min of the task. When maximum speed was analyzed, the $AlCl_3$-treated group showed a decrease of 24% when compared to its respective control group (Fig. 3). The measurement of absolute turn angle

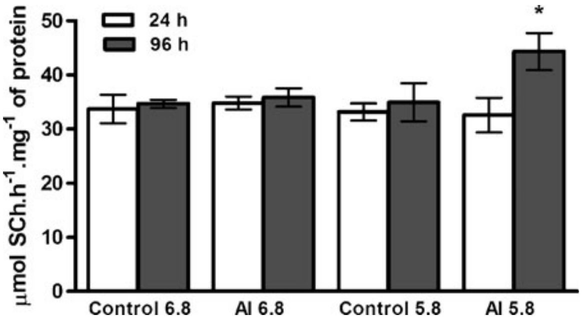

Fig. 1 In vivo effect of $AlCl_3$ on acetylcholinesterase activity in zebrafish whole brain. Data represent means±SD of four different experiments ($n=4$, each "*n*" containing a pool of two zebrafish brains), performed in triplicate. *Asterisk* indicates a difference when compared to the control group. Data were analyzed statistically by three-way ANOVA followed by Tukey's post hoc test, considering $P<0.05$ as significant

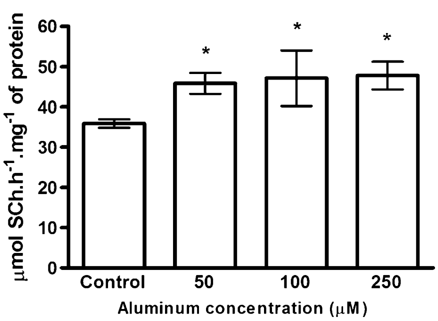

Fig. 2 In vitro effect of different concentrations of aluminum on acetylcholinesterase activity in zebrafish whole brain. Data represent means±SD of four different experiments ($n=4$, each "*n*" containing a pool of five zebrafish brains) performed in duplicate. *Asterisk* indicates a difference when compared to the control group. Data were analyzed statistically by one-way ANOVA followed by Tukey's post hoc test, considering $P<0.05$ as significant

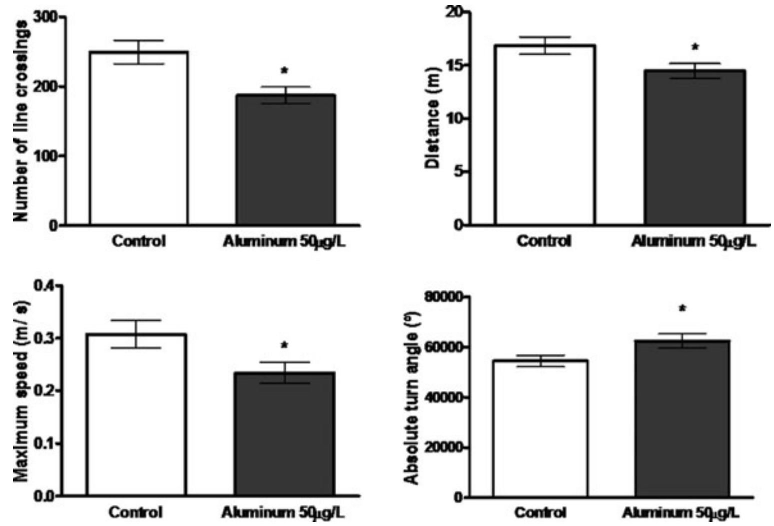

Fig. 3 Effect of aluminum acid exposure (50 μg/L, for 96 h at pH 5.8) on the swimming behavior during 5 min of videorecording. The parameters evaluated were the number of line crossings, total distance traveled, maximum speed, and the absolute turn angle values, as described in "Methods" section. Data are expressed as mean±SEM of ten different animals per group (n=10), considering P<0.05 as significant

behavior reflects changes in the zebrafish swimming direction, and this parameter showed a significant increase in the absolute turn angle values for the $AlCl_3$-treated group (12.7%; Fig. 3).

Discussion

In the present study, we observed significant changes in brain AChE activity and behavioral parameters in zebrafish exposed to sublethal levels of aluminum. The precise mechanisms by which aluminum exerts its neurotoxic effects are still not completely understood; however, the literature suggests that aluminum interacts with the cholinergic system, acting as a cholinotoxin (Gulya et al. 1990).

In vivo experiments involving exposure for 96 h to 50 μg/L $AlCl_3$ at pH 5.8 showed a significant increase of AChE activity in zebrafish brain. However, this metal with the same concentration and exposure time did not have any effect on zebrafish brain AChE at the circumneutral pH 6.8. Studies have reported that exposure to aluminum chloride at low pH values results in more severe physiological consequences in fish when compared to circumneutral exposure. Brodeur et al. (2001) have shown that Atlantic salmon (*Salmo salar*) exposed for 36 days to acidified water (pH 5.2) containing 50 μg/L $AlCl_3$ present altered bioenergetics when compared to circumneutral aluminum-exposed fish. Furthermore, Monette and McCormick (2008) demonstrated that gill ionoregulatory responses of the same fish species were more prominent with a 6-day exposure time with 43–68 μg/L of aluminum at acid pH. In addition, the solubility of aluminum increases as a direct result of decreased pH, leading to the increased presence of inorganic aluminum, the form of aluminum most toxic to fish (Finn 2007; Gensemer and Playle 1999). Therefore, our results are in agreement with the above mentioned studies since we observed significant changes in AChE only in the $AlCl_3$-treated group at pH 5.8.

Our in vitro experiments showed that $AlCl_3$ was able to increase AChE activity. This finding could be related to the fact that in vitro experiments evaluate the direct effect of the metal on the enzyme, without the influence of other biological systems such as cell signaling pathways. Alterations of AChE by divalent cations mediated through allosteric anionic sites are well-known (Roufogalis and Wickson 1973). In addition, Gulya et al. (1990) suggested that an increase in AChE activity after aluminum exposure may be due to allosteric interaction between the cation and the peripheral anionic site of the enzyme. Furthermore, previous studies have suggested that modifications to the lipid membrane could be responsible for a change in the conformational state of the AChE molecule, which could be responsible for the induction of AChE activity observed after long-term exposure to aluminum (Kaizer et al. 2005).

The involvement of cholinergic systems in locomotor activity, operant tasks, responses to novel stimuli, and the performance of spatial memory tasks is well established (Pepeu and Giovannini 2004). Our results evaluating zebrafish behavior after $AlCl_3$ exposure are in accordance with those of Allin and Wilson (1999), who observed reduced swimming activity in juvenile rainbow trout when exposed to aluminum in acidic water. However, Brodeur et al. (2001), using Atlantic salmon, observed a contradictory result, reporting that the animals presented increased swimming activity when exposed to acidic water and aluminum. These contrasting findings indicate that fish can react differently to sublethal levels of aluminum in acidic waters. Many factors might be responsible for these differences, such as the relative level of toxicity of the acidic water or species-specific variations. Nevertheless, the possibility of such alterations in fish behavior is important because it can limit fish survival in the wild (Brodeur et al. 2001), where reduced swimming activity can affect the ability of the fish to forage, avoid predation, migrate, and successfully reproduce (Allin and Wilson 1999).

Reliable animal models are required to facilitate the understanding of neurodegenerative pathways in Alzheimer's disease. Models should allow for the testing of compounds at various points of the pathogenic cascade in order to search for disease-modifying drugs. Furthermore, they remain a valuable tool for identifying molecular, cellular, and pathological changes that trigger the onset of cognitive decline in AD. The zebrafish is an effective and simple model organism for studies of development and disease processes in the nervous system (Newman et al. 2007). Considering that studies have linked aluminum-induced neurotoxicity with neurodegenerative disorders such as AD (Flaten 2001), the evaluation of neurochemical and behavioral changes induced by aluminum in zebrafish can contribute to a better understanding of the mechanisms related to the neurodegeneration induced by this metal.

In summary, this study showed that aluminum treatment, at acid pH, causes changes in brain AChE activity and behavioral parameters in zebrafish. The induction of brain AChE activity could be involved in the behavioral and neurotoxic effects of aluminum on the central nervous system. The use of zebrafish as model to evaluate the biochemical and behavioral changes induced by aluminum exposure might represent a relevant contribution to the understanding of its toxic effects on human health.

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Conflict of interest statement The authors declare that there is no conflict of interest.

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