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# In vitro effect of zinc and cadmium on acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain

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**Abstract**

Zinc and cadmium are environmental contaminants that induce a wide range of effects on CNS. Here we tested the in vitro effect of these metals on acetylcholinesterase (AChE) and ectonucleotidase (NTPDase and ecto-5′-nucleotidase) activities in zebrafish brain. Both zinc and cadmium treatments did not alter significantly the zebrafish brain AChE activity. ATP hydrolysis presented a significant increase at 1 mM zinc (17%) and the AMPase activity had a dose-dependent increase at 0.5 and 1 mM zinc exposure (188% and 199%). After cadmium treatment, ATPase activity was significantly increased (53% and 48%) at 0.5 and 1 mM, respectively. Cadmium, in the range 0.25–1 mM, inhibited ADP hydrolysis in a dose-dependent manner (13.4–69%). Ecto-5′-nucleotidase activity was only inhibited (38%) in the presence of 1 mM cadmium. It is possible to suggest that changes on NTPDase and ecto-5′-nucleotidase activities can be an important mechanism involved in neurotoxic effects promoted by zinc and cadmium.
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## 1. Introduction

Exposure to metals can develop a wide range of deleterious effects on exposed organisms. Zinc and cadmium are neurotoxic metals, known to be important environmental contaminants. Behavioral disorders and biochemical dysfunction have been observed in central nervous system (CNS) of zinc and cadmium-exposed animals (Carageorgiou et al., 2004).

Despite the toxicity observed at higher concentrations, zinc is the second most important trace metal in the body after iron. Total brain zinc concentrations, at micromolar range, are usually associated with zinc metalloenzymes and transcription factors (Barañano et al., 2001; Takeda, 2001). Smart et al. (2004) have shown that low concentrations of zinc are important in neurotransmission. After depolarization induced by electrical stimuli, $K^+$ or kainate, zinc is released at synaptic cleft in a $Ca^{2+}$-dependent manner (Barañano et al., 2001). The importance of zinc on excitatory glutamate receptors and on inhibitory GABA currents has already been described (Smart et al., 2004). Furthermore, the relevance of zinc in cognitive development and CNS homeostasis is well known (Takeda, 2001).

On the other hand, cadmium is a metal with many toxic properties at CNS (Carageorgiou et al., 2004). This metal affects the structure of nucleic acids, the activity of certain enzymes, the uptake of catecholamines and the levels of various neurotransmitters (Cooper and Manalis, 1984).

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Minami et al. (2001) demonstrated the toxicity of this metal in rat brain, relating a possible action of cadmium in the balance of excitatory and inhibitory synaptic neurotransmission.

Acetylcholine is a classical neurotransmitter that plays several roles at CNS. After released, acetylcholine 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. Moreover, studies have been performed in order to elucidate the mechanisms involving the cholinergic and non-cholinergic activities of AChE in both central and peripheral nervous systems (Cousin et al., 2005).

Acetylcholine and ATP are co-released together at nerve endings (Burnstock, 2004). Besides the energetic function, ATP can be an important signaling molecule, acting in metabotropic P2Y receptors and ionotropic P2X receptors (Cunha and Ribeiro, 2000). Studies demonstrated that ATP can control the acetylcholine release through a dual opposite modulation, acting on facilitatory P2X or inhibitory P2Y receptors (Cunha and Ribeiro, 2000).

This nucleotide is converted to adenosine by cell-surface enzymes called ectonucleotidases. These enzymes constitute the pathway responsible for inactivation of the ATP signal controlling the purinergic neurotransmission. ATP is hydrolyzed to AMP by a family of enzymes named NTPDases (nucleoside triphosphate diphosphohydrolases) and an ecto-5′-nucleotidase (EC 3.1.3.5) promotes the AMP hydrolysis to adenosine, an important neuromodulatory messenger (Zimmermann, 2001). Adenosine is able to modulate acetylcholine release through inhibitory adenosine $A_1$ or facilitatory adenosine $A_{2A}$ receptors (Magalhães-Cardoso et al., 2003).

Zebrafish (*Danio rerio*) is a small freshwater teleost widely used as a vertebrate model of developmental, neurobiological and toxicological studies (Hill et al., 2005; Senger et al., 2005). Zebrafish presents a unique situation among vertebrates, because AChE is the only ACh-hydrolyzing enzyme in this organism (Behra et al., 2003). The genome of this teleost does not encode a functional butyrylcholinesterase, another enzyme that can also hydrolyze ACh. AChE gene is already cloned and sequenced and this enzyme activity was detected in zebrafish brain (Bertrand et al., 2001). Furthermore, cholinergic receptors are also expressed in neuronal tissues of this teleost (Zirger et al., 2003).

Recently, the characterization of NTPDase and ecto-5′-nucleotidase activities in zebrafish brain membranes have been described (Rico et al., 2003; Senger et al., 2004). In the literature, both P2X and P2Y receptors have already been identified in this specie (Kucenas et al., 2003).

Considering that both cholinergic and purinergic systems are present in zebrafish brain and that zinc and cadmium are important toxic substances, the aim of this study was to test the in vitro effect of zinc and cadmium on AChE and ectonucleotidase activities in zebrafish brain.

## 2. Materials and methods

### 2.1. Animals

Adult male and female zebrafish were obtained from commercial supplier and housed for 2 weeks in a 50-L aquarium containing continuously aerated distilled water. The fish were kept between 23 and 26 °C under a natural light–dark photoperiod. All procedures for the use of animals were according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

### 2.2. Chemicals

Zinc chloride ($ZnCl_2$; CAS number 7648-85-7) and cadmium acetate [$Cd(CH_3COO)_2$; CAS number 543-90-8] were purchased from QM (Brazil) and Nuclear (Brazil), respectively. Trizma Base, malachite green, ammonium molybdate, polyvinyl alcohol, nucleotides, EDTA, EGTA, sodium citrate, Coomassie Blue G, bovine serum albumin, calcium and magnesium chloride, acetylthiocholine, 5,5′-dithiobis-2-nitrobenzoic acid (DNTB) were purchased from Sigma (USA). All other reagents used were of analytical grade.

### 2.3. In vitro treatments

The metals were added to reaction medium before the pre-incubation with the enzyme and maintained throughout the enzyme assays. Zinc and cadmium were tested in a final concentration of 0.05–1 mM.

### 2.4. Biochemical measurement of AChE activity

Zebrafish brains were gently homogenized on ice in 60 vol (v/w) of 0.05 M Tris–HCl, pH 8.0, using a Teflon–glass homogenizer. AChE activity was measured according to the method of Ellman et al. (1961). AChE activity in the homogenate was measured by determining the rate of hydrolysis of acetylthiocholine (ACSCh, 0.8 mM) in 2 mL assay solutions with 100 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB. Before starting the reaction with addition of substrate, samples containing 10 μg of protein and the reaction medium described previously were pre-incubated at 25 °C for 10 min. The hydrolysis was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Protein concentration and linearity of absorbance towards time were also investigated. Controls without zebrafish brain homogenate were performed in order to determinate the non-enzymatic hydrolysis of ACSCh. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. We performed at least three different replicate experiments.

### 2.5. Determination of ectonucleotidase activities

Brain membranes were prepared as described previously (Barnes et al., 1993). Whole zebrafish brains were

homogenized in 60 vol (v/w) of chilled 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. This preparation was centrifuged at 1000*g* for 10 min and the pellet was discarded in order to remove the nuclear and cell debris. The supernatant was centrifuged for 25 min at 40,000*g*. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris–citrate buffer, and recentrifuged for 20 min at 40,000*g*. The final pellet was resuspended and used in the enzyme assays. The material was maintained at 2–4 °C throughout preparation.

NTPDase and 5′-nucleotidase assays were performed according Rico et al. (2003) and Senger et al. (2004), respectively. Brain membranes of zebrafish (3–10 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the NTPDase activity) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the ecto-5′-nucleotidase activity) in a final volume of 200 μL. The samples were pre-incubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The assays were stopped by the addition of 200 μL 10% trichloroacetic acid and the samples were chilled on ice for 10 min before the measurement of inorganic phosphate (Pi) (Chan et al., 1986). Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid were used to correct non-enzymatic hydrolysis of the substrates. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity is expressed as nanomole of Pi released per minute per milligram of protein. We performed at least three different replicate experiments.

### *2.6. Protein determination*

Protein was measured by the Coomassie Blue method (Bradford, 1976), using bovine serum albumin as a standard.

### *2.7. Statistical analysis*

Data were expressed as means ± SD and analyzed by one-way analysis of variance (ANOVA). A Duncan multiple range test considering $P \leqslant 0.05$ as significant followed the analysis.

## 3. Results

The effect of zinc and cadmium (varying from 0.05 to 1 mM) was demonstrated on AChE, NTPDase and ecto-5′-nucleotidase activities in zebrafish brain. Both zinc (Fig. 1A) and cadmium (Fig. 1B) treatments were not able to promote any significant changes on zebrafish brain AChE activity.

Zinc, when added to the reaction medium, affected ATP and AMP hydrolysis. Whereas ATP hydrolysis presented a significant increase only at 1 mM zinc (17%), the AMP hydrolysis promoted by 5′-nucleotidase significantly increased at 0.5 and 1 mM zinc exposure (188% and 199%) (Fig. 2A). However, there were no significant changes on ADP hydrolysis in all zinc concentrations tested (Fig. 2A).

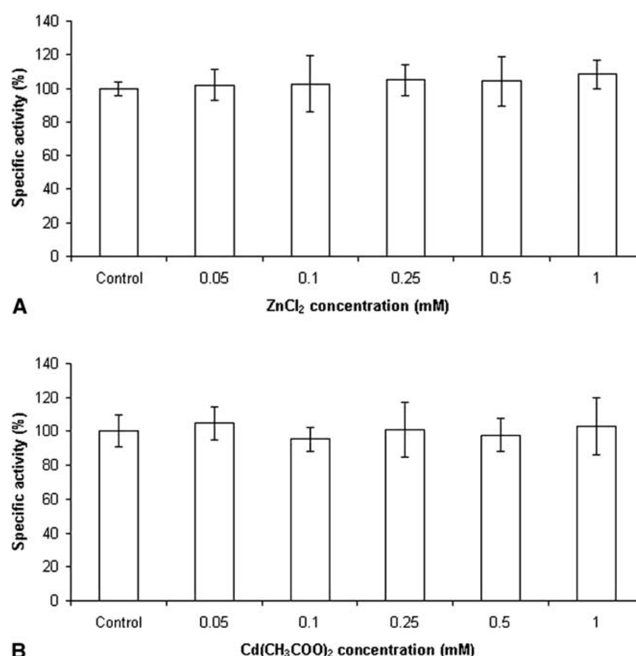


Fig. 1. In vitro effects of varying concentrations of zinc (A) and cadmium (B) on AChE activity in zebrafish brain. Bars represent the mean ± of at least three different experiments. The AChE control activity (without metal) was 37.97 ± 3.59 and 35.21 ± 1.45 μmol of thiocholine released per hour per milligram of protein for cadmium and zinc, respectively (ANOVA followed by a Duncan multiple range test, considering $P \leqslant 0.05$ as significant).

Cadmium, at 0.5 and 1 mM, promoted a significant increase in ATPase activity (53% and 48%, respectively) (Fig. 2B). ADP hydrolysis was inhibited in a dose-dependent manner ranging from 0.25 to 1 mM cadmium (13.4–69%). The ecto-5′-nucleotidase activity was only inhibited (38%) in the presence of 1 mM cadmium (Fig. 2B).

## 4. Discussion

Here, we have tested the in vitro effect of zinc and cadmium on AChE and ectonucleotidase activities in zebrafish brain. The AChE activity was not altered, but nucleotide hydrolysis were affected in the presence of zinc and cadmium. Zinc increased ATPase and AMPase activities, but there were no changes on ADPase activity. However, cadmium strongly increased ATP hydrolysis, but promoted a significant inhibition of ADP and AMP hydrolysis in zebrafish brain membranes.

Several studies have shown the influence of zinc on many cellular mechanisms. This divalent cation is an important molecule involved at neurotransmission (Barañano et al., 2001; Smart et al., 2004) and in activation of different metalloenzymes, such as zinc hydrolases (Hernick and Fierke, 2005). In addition, zinc is also necessary to

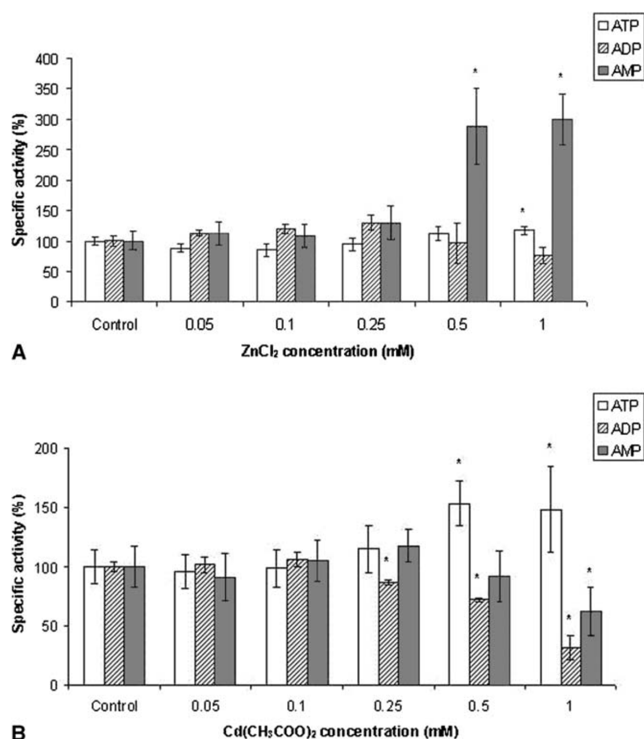


Fig. 2. Effects of varying concentrations of zinc (A) and cadmium (B) on nucleotide hydrolysis in zebrafish brain membranes. Bars represent the mean ± of at least three different experiments. The control ATPase, ADPase and AMPase activities for cadmium were 617.1 ± 88.3; 147.9 ± 5.5 and 30.5 ± 5.4 nmol Pi $min^{-1}$ $mg^{-1}$ of protein, respectively. The control ATPase, ADPase and AMPase activities for zinc were 564.4 ± 58.2; 147.9 ± 28.21 and 24.2 ± 5.2 nmol Pi $min^{-1}$ $mg^{-1}$ of protein, respectively. *Significantly different from control group ($P \leqslant 0.05$) using ANOVA followed by a Duncan multiple range test.

mobilize defense against reactive oxygen species (ROS) and $H_2O_2$-induced apoptosis (Chung et al., 2005).

Considering the toxicological effects, zinc is a metal tested in many distinct organisms with variable biochemical and physiological responses. For example, Brocardo et al. (2005) showed an inhibitory effect of zinc on AChE activity from cerebral cortex and hippocampus of rats. Another report demonstrated different results: the ChE activity of the *Adamussium colbecki* scallop is not altered by the in vivo zinc exposure (Corsi et al., 2004). The results presented herein demonstrated that zebrafish brain AChE is not affected by in vitro zinc treatment.

There was no evidence about a possible toxicity of zinc on NTPDase activity at the present moment. On the other hand, studies demonstrate that zinc can modulate ATPase activity in brain. This metal significantly increases the copper-transporting ATPase (DiDonato et al., 2002), $Na^+$–$K^+$–ATPase (Lovell et al., 1999) and $Mg^{2+}$-independent $Ca^{2+}$ activity (Gandhi and Ross, 1988). The results obtained are consistent with these studies, demonstrating that the ATP hydrolysis in zebrafish brain membranes is also modulated by zinc. Furthermore, ecto-5′-nucleotidase contains catalytically important zinc at active site (Senger et al., 2004). Based on these findings, it is possible to suggest the importance of this zinc ligand-site on the activation of AMPase activity in zebrafish brain.

The effect of cadmium exposure on AChE was also tested in another animal species. Carageorgiou et al. (2004) verified the in vitro and in vivo effect of cadmium on this enzyme activity on adult rat brain. After in vitro treatment, concentrations higher than 0.1 mM decreased the acetylcholine hydrolysis. However, after in vivo short (8 h) and long-term (4 months) exposure, cadmium promoted different effects on rat brain AChE. The short-term exposure caused a dose-dependent reduction, while a long-term cadmium administration activated the brain AChE activity. Studies also have shown the in vivo effect of cadmium on brain AChE of different fishes. In *Barbus conchonious*, an exposure to cadmium concentration of 12.6 mg/L during 48 h stimulated the brain AChE activity (Gill et al., 1991). Nevertheless, De La Torre et al. (2000) demonstrated that *Cyprinus carpio* AChE presented no changes after transferred to water contaminated cadmium solution (1.6 mg/L). The similar response to cadmium found to *D. rerio* and *C. carpio* could be attributed to the relatively low phylogenetic distance showed between these species. An inhibition of the NTPDase activity by cadmium acetate has been already described on rat cerebral cortex synaptosomes (Barcellos et al., 1994). This metal inhibited both ATP and ADP hydrolysis in a concentration-dependent manner. The concentration tested ranged to 0.05–1 mM, the same used in our experiments.

A plausible explanation to the contrasting cadmium effect on ATP and ADP hydrolysis is that it causes activation of one member of the NTPDase family and a concomitant inhibition on another member in brain membranes of zebrafish. From the eight-well characterized enzymes of mammals NTPDase family, four members (NTPDase 1–3 and 8) are tightly bound to plasma membrane with active site facing the extracellular milieu. According to the literature, mammalian NTPDase1 hydrolyzes both ATP and ADP at the same rate, whereas NTPDase2 substantially prefers triphosphonucleosides as substrates (Zimmermann, 2001). Orthologous NTPDase1 (GenBank accession number AAH78240) and NTPDase2 (GenBank accession numbers CAE49096, NP_001004643 and XP_697600) genes are present in zebrafish genome. Hence, the decreased ADP hydrolysis and increased ATP hydrolysis observed in the presence of cadmium may be due to NTPDase1 inhibition and NTPDase2 activation.

This study has shown that the purinergic, but not cholinergic system, is affected by in vitro zinc and cadmium treatments in zebrafish brain. Our findings are important to better understand the toxic effect of both metals on CNS. E-NTPDases that are being expressed and contributing for these metals responses are still unknown. Based on the complexity of zinc and cadmium effect in this teleost, further molecular analysis and in vivo assays will be important to elucidate the influence of both contaminants in neurotransmission.

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