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Original article:

HIGH DOSES OF ZINC AND COPPER ALTER NEITHER CEREBRAL METAL LEVELS NOR ACETYLCHOLINESTERASE ACTIVITY OF SUCKLING RATS

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ABSTRACT

This research investigated the *in vivo* ($ZnCl_2$ 27 mg/kg; $CuSO_4$ 10.2 mg/kg) and *in vitro* effects of zinc and copper on acetylcholinesterase activity of different cerebral areas, Zn and Cu levels in cerebrum, and body weight gain of young Wistar rats. Three-day-old rats were injected (s.c.) with 5 doses (saline, Zn, Cu or Zn+Cu) for 5 consecutive days and were killed 24 h after the last dose. In the other experiment, 7-day-old rats received only 1 dose (saline, Zn or Cu) and were killed at 1, 6 or 24 h after. For the *in vitro* experiments, the acetylcholinesterase activity from cerebrum of 8-day-old rats was analyzed in presence of Zn or Cu (0.01 to 1 mM). Regarding the *in vivo* experiments, only body weight gain was decreased by 5 simultaneous administrations of Zn and Cu. The acetylcholinesterase activity from cerebrum and cerebellum and cerebral zinc and copper contents were not altered by the treatments. *In vitro*, Cu 0.1 and 1 mM, but not Zn, inhibited the enzyme of both cerebrum and cerebellum. The enzymatic activity from cerebrum and cerebellum homogenate was more sensitive to Cu than the enzymatic activity from S2 and S1 fractions, respectively, since less metal was necessary to inhibit the enzyme.

Keywords: acetylcholinesterase; brain growth; cerebral metal levels; copper; suckling rats; zinc

INTRODUCTION

Zinc and copper trace elements are involved in vital functions in mammalian tissues. Besides being structural components of proteins, they are also co-factors for the activity of many enzymes that are required for growth, development and maintenance of the nervous system. These ions are also involved in the antioxidant defense, since they are part of enzymes, such as zinc/copper-superoxide dismutase (SOD). Furthermore, both ions function as signaling molecules that are released in synaptic terminals and act in membrane proteins playing modulator role in regulating neuronal excitability (Mathie et al., 2006).

It is known that the concentration of zinc in brain is higher than in other organs, around 150 μM (about 10-fold serum zinc levels). As this metal is involved in the be-

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havior and cognitive function, zinc deficiency may lead to alterations in brain activity, motor development, neuropsychological behavior, attention, learning, and memory (Mocchegiani et al., 2005).

Despite the highest concentration of copper being in liver (Gaetke and Chow, 2003), it is also found in the nervous system reaching concentrations around 70 μM in the cerebrospinal fluid and 200 μM in the synaptic cleft (Mathie et al., 2006). Neurological and neuropsychological symptoms of copper toxicity occur during the Wilson's disease development (Strausak et al., 2001). Copper homeostasis alteration in brain is an important possible factor in the etiology of Alzheimer's disease (Camakaris et al., 1999), since the copper concentration may attain 400 μM in this neurodegenerative disease (Mathie et al., 2006).

Acetylcholine, the principal neurotransmitter of the cholinergic neurons, is one of the main neurotransmitters involved in neurodegenerative diseases (Wacker et al., 2005) and is related to cognitive functions involved in the learning and memory process (Blockland, 1995). Cholinergic neurons correspond to 25 % of the brain cells and are represented mainly by cortical and hippocampal neurons (Wacker et al. 2005). Moreover, hypothalamus and cerebellum, among others, are also cerebral areas supplied by cholinergic projection neurons (Das et al., 2001).

The synaptic cholinergic transmission depends on the acetylcholinesterase (AChE) (E.C.3.1.1.7) activity, since this enzyme promotes the hydrolysis of the neurotransmitter acetylcholine in choline and acetic acid, resulting in the terminus of the transmission of the nervous impulse in the synapses (Taylor, 1996). In the mammalian brain, AChE exists in different globular forms, mainly as mono-, di- and tetramers of catalytic subunits (G1, G2, G4). These forms can be classified according to the solubility in salt (G1, cytosolic and water soluble) and detergent (G4, membrane bound) (Nigg and Knaak, 2000; Rieger and Vigny, 1976).

AChE is used as a biomarker of the cholinergic function, since its activity is inhibited by different toxic agents, such as pesticides (Taylor, 1996) and heavy metals (Najimi et al., 1997; Frasco et al., 2007). However, the effects of zinc and copper on the AChE activity are little known and sometimes contradictory. The brain enzyme of fish is inhibited by zinc (Suresh et al. 1992) and activated by copper (Romani et al., 2003). The brain AChE of rats may be either decreased (Brocardo et al., 2005) or increased by zinc (Carageorgiou et al., 2005) and increased by cadmium (Carageorgiou et al., 2005). Nevertheless, the effects of these metals on AChE from young rats are unknown. This is of interest since animals in development are particularly sensitive to extern insults such as nutritional privation (Rocha et al., 1993; Vendite et al., 1985) and heavy metal (Peixoto et al., 2003; Peixoto and Pereira, 2007; Roza et al., 2005; Franciscato et al., 2009). This high sensitivity is due to the fact that rodents have a high growth and development of brain and organs during the first days of life. Thus, the developmental neurotoxicity with consequent neurodevelopmental disorders and subclinical brain dysfunction may be associated with the increasing risk of neurodegenerative diseases (Grandjean and Landrigan, 2006).

Considering the special sensitivity of young rats, this study aimed to investigate the effects of zinc and copper exposure on the AChE activity of different cerebral areas, brain metal levels and physiological parameters as well as to verify the action of these metals on the AChE activity *in vitro*.

MATERIALS AND METHODS

Chemicals

Acetylthiocholine iodide (ATC), 5-5-dithiobisnitrobenzoic acid (DTNB), Tris (hydroxymethyl-d_3) amino-d_2–methane, Coomassie brilliant blue G and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and zinc chloride ($ZnCl_2$), pentahydrate copper sulfate ($CuSO_4.5H_2O$), potassium phos-

phate dibasic (K_2HPO_4) and monobasic (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sucrose, Triton X-100, and nitric acid were obtained from Merck (Darmstadt, Germany).

Animals

Studies were conducted in accordance with the national and institutional guidelines (University Ethics Committee Guidelines — Process number 23081.013915/2004-06) for experiments with animals. Wistar pregnant rats obtained from the General Animal House of the Federal University of Santa Maria were transferred to the colony room and maintained individually in opaque plastic cages on a 12 h light–dark cycle at a controlled temperature (23 ± 2° C). One day after birth, the number of pups of each litter was reduced to 9. Males and females were used without distinction.

Treatment schedules

1. Five doses of zinc and/or copper:

From the 3rd to 7th day of life, rats received one daily dose of treatment, administered by subcutaneous (s.c.) injections in a constant volume of 10 ml/kg body weight. Animals were weighed daily to adjust the dose.

Copper curve: four litters were used and the rats of each litter were divided into 4 groups of treatment: saline (NaCl 150 mM); 5.1 mg/kg $CuSO_4.H_2O$ (1.3 mg/kg Cu); 10.2 mg/kg $CuSO_4.5H_2O$ (2.6 mg/kg Cu) and 20.4 mg/kg $CuSO_4.5H_2O$ (5.2 mg/kg Cu).

Zinc and copper treatments: five litters were used and the rats were divided into 4 groups: saline (NaCl 150 mM); 27 mg/kg $ZnCl_2$ (12.9 mg/kg Zn); 10.2 mg/kg $CuSO_4.5H_2O$ (2.6 mg/kg Cu) and 27 mg/kg $ZnCl_2$ + 10.2 mg/kg $CuSO_4.5H_2O$ (12.9 mg/kg Zn + 2.6 mg/kg Cu).

Each litter contained two rats for each treatment.

2. One dose of zinc or copper:

Seven-day-old rats received one dose of treatment, administered by subcutaneous (s.c.) injections in a constant volume of 10 ml/kg body weight. Three litters were used and each litter was divided into 3 treatments: saline (NaCl 150 mM); 27 mg/kg $ZnCl_2$ (12.9 mg/kg Zn) and 10.2 mg/kg $CuSO_4.5H_2O$ (2.6 mg/kg Cu).

Tissue preparation

Rats that received five doses of treatments were killed 24 h after the last dose and rats that received one dose were killed at 1, 6 or 24 h after treatment.

Brain was removed and cerebrum and cerebellum were dissected on ice. For metal content determination, cerebrum and cerebellum were weighed, placed in vials, and frozen at -20° C until analysis. For the determination of the AChE activity, cerebrum and cerebellum were homogenized (1:10, w/v) in 10 mM Tris-HCl buffer, pH 7.2 with 160 mM sucrose. Homogenates were frozen at -20° C until analysis.

Enzyme assay

The AChE activity was determined by the method of Ellman et al. (1961), modified as described in Pereira et al. (2004). The mixture assay contained DTNB 1.04 mM, potassium phosphate buffer 24 mM pH 7.2 and 25 μL of enzymatic material. It was pre-incubated for 2 min at 30º C and the reaction was started with the addition of acetylthiocholine (ATC) 0.83 mM. The product of thiocholine reaction with DTNB was determined at 412 nm every 30 sec during 2 min. The specific activity was expressed as μmol ATC hydrolyzed/h/mg protein. All samples were run in triplicate.

Protein determination

Protein concentrations in the samples were determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard. All samples were run in triplicate.

Metal content determination

The determination of cerebral zinc and copper contents was made by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) as described in detail in Peixoto et al. (2008). Samples were di-

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gested using a Model Multiwave 3000 microwave oven equipped with high-pressure quartz vessels (80 bar, 250 °C, Anton Paar, Graz, Austria). Zinc and copper contents are expressed as μg of metal/g wet tissue.

In vitro zinc and copper assays

Brain of 8-day-old rats was removed and cerebrum, cerebellum hippocampus and hypothalamus were dissected, weighed and homogenized. For the determination of the AChE activity in fractions of cerebrum and cerebellum, homogenate aliquots (H) were separated and the remaining homogenates were centrifuged at 14,500 *g* for 30 min at 4^{o} C for the separation of the salt soluble fraction present in the supernatant (S1). The pellet was suspended in a mixture of 1 % Triton X-100 and 50 mM sodium phosphate buffer, pH 7.2. The suspension was agitated for 10 min at 10^{o} C in ice bath. Afterwards, it was centrifuged at 23,400 *g* for 30 min at 4^{o} C for the separation of the detergent soluble fraction present in the supernatant (S2) (Das et al., 2001).

Zinc and copper were tested varying final concentrations from 0.01 to 1 mM. The metals were pre-incubated with the enzyme for 2 min, and the enzymatic reaction was conduced similarly to *in vivo* enzymatic assays. The AChE activity is expressed as delta extinction/min (ΔE/min). The concentration of the metal required to induce 50 % inhibition of the AChE activity (IC_{50}) was calculated according to Dixon method (1/V versus [I]) (Dixon and Webb, 1964).

Statistical analysis

Results were analyzed by one- or two-way ANOVA followed by Duncan's multiple range test or paired Student *t*-test when appropriate.

RESULTS

Copper curve

Results demonstrated that the treatments with the different doses of copper (1.3, 2.6 or 5.2 mg/kg) did not alter the body weight. All groups presented significant increase of weight from the initial to the end of the treatment [two-way ANOVA (4 treatments x 2 days): $F(1,14)=729.24$, $p<0.001$]. The cerebrum weight was not modified by the treatments (one-way ANOVA) (data not shown). However, the highest dose caused lesions in the skin at the site of the injection. Thus, the intermediate dose of the copper was chosen for the experiments with zinc and copper.

Treatments with five doses of zinc and/or copper

Effects of the treatments with saline, 12.9 mg/kg Zn, 2.6 mg/kg Cu or both on body weight gain are showed in Figure 1. Two-way ANOVA (4 treatments x 6 days) revealed a significant effect of days [$F(5,80)=421.04$, $p<0.001$] and treatments x days interaction [$F(15,80)=9.61$, $p<0.001$]. The interaction was significant since Zn+Cu animals had lower weight gain than the other groups. In fact, on day 8 (24 h after the last dose) the Zn+Cu group presented smaller body weight than the others (one-way ANOVA, $F(3,16)=3.35$ $p<0.05$) (see Table 1).

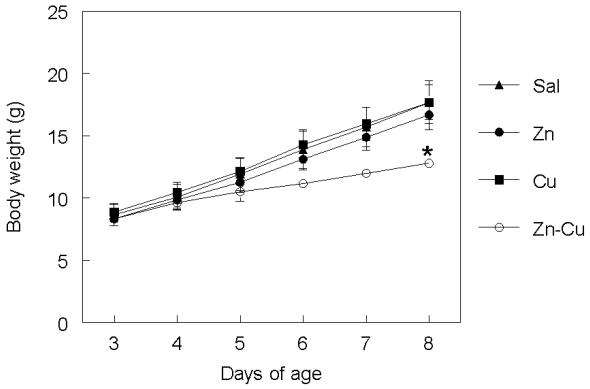

Figure 1: Body weight of rats (n=5) treated for five consecutive days (from the 3rd to 7th day of age) with saline, 12.9 mg/kg Zn, 2.6 mg/kg Cu or 12.9 mg/kg Zn + 2.6 mg/kg Cu. Results are presented as mean ± SEM.
(*) Significant difference from the other groups: $p<0.05$ (Duncan's multiple range test)

Cerebrum and cerebellum weight, and cerebrum/body and cerebellum/body weight ratios are shown in Table 1. These parameters were not significantly altered by the treatments (one-way ANOVA).

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Table 1: Body, cerebrum and cerebellum weights at 24 h after the last dose (8 days old), and relation between weights of brain/body and cerebellum/body of rats treated (s.c.) for five consecutive days with saline, 12.9 mg/kg Zn, 2.6 mg/kg Cu or 12.9 mg/kg Zn + 2.6 mg/kg Cu.

	Weight (g)			Relative weight (%)	
Treatment	**Body**	**Cerebrum**	**Cerebellum**	**Cerebrum/Body**	**Cerebellum/Body**
Saline	17.68 ± 1.74	0.53 ± 0.04	0.13 ± 0.004	2.99 ± 0.07	0.76 ± 0.06
Zn	16.66 ± 1.17	0.48 ± 0.03	0.11 ± 0.01	2.95 ± 0.01	0.64 ± 0.07
Cu	17.68 ± 1.38	0.49 ± 0.03	0.12 ± 0.01	2.81 ± 0.06	0.66 ± 0.04
Zn + Cu	12.78 ± 0.43*	0.42 ± 0.03	0.09 ± 0.01	3.19 ± 0.02	0.74 ± 0.06

Results are presented as mean ± SEM, n=5.
(*) Significant difference from the other groups in the same column: $p<0.05$ (Duncan's multiple range test)

Cerebrum and cerebellum AChE activities and cerebral zinc and copper contents are showed in Table 2. The treatments with zinc and/or copper modified neither the AChE activity from cerebrum and cerebellum homogenate nor the zinc and copper cerebral contents (one-way ANOVA).

Treatments with one dose of zinc or copper

Cerebrum and cerebellum AChE activities are showed in Table 3. Zinc and copper did not modify the enzymatic activity when animals were killed 1, 6 or 24 h after the injection of the metal (one-way ANOVA).

Table 2: Cerebrum and cerebellum AChE activities and zinc and copper concentrations in cerebrum of rats treated (s.c.) for five consecutive days with saline, 12.9 mg/kg Zn, 2.6 mg/kg Cu or 12.9 mg/kg Zn + 2.6 mg/kg Cu.

	AChE activity		Metal contents (μg/g)	
Treatment	**Cerebrum**	**Cerebellum**	**Cu**	**Zn**
Saline	2.36 ± 0.09	2.72 ± 0.13	0.99 ± 0.02	9.60 ± 0.15
Zn	2.61 ± 0.29	3.63 ± 0.25	0.92 ± 0.02	9.38 ± 0.27
Cu	2.67 ± 0.27	3.41 ± 0.29	1.07 ± 0.05	9.50 ± 0.40
Zn + Cu	2.09 ± 0.18	3.13 ± 0.23	0.96 ± 0.03	9.56 ± 0.20

Specific enzyme activity is expressed as μmol ATC hydrolyzed/h/mg protein (n=5). Metal content is expressed as μg/g wet tissue (n=3). Results are presented as mean ± SEM.

Table 3: Cerebrum and cerebellum AChE activities of rats treated (s.c.) with 1 dose of saline, 12.9 mg/kg Zn or 2.6 mg/kg Cu and killed 1, 6 or 24 h after injection

		AChE activity Intervals after injection		
Tissue	**Treatment**	**1 h**	**6 h**	**24 h**
Cerebrum	Saline	2.17 ± 0.15	1.91 ± 0.14	1.85 ± 0.32
	Zn	1.92 ± 0.11	2.41 ± 0.66	2.45 ± 0.58
	Cu	2.41 ± 0.62	1.67 ± 0.66	1.86 ± 0.29
Cerebellum	Saline	2.21 ± 0.24	2.59 ± 0.49	2.79 ± 0.56
	Zn	2.69 ± 0.13	1.67 ± 0.34	1.67 ± 0.34
	Cu	2.45 ± 0.64	2.29 ± 0.32	2.07 ± 0.17

The enzyme activity is expressed as μmol ATC hydrolyzed/h/mg protein. Results are presented as mean ± SEM (n=3).

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Zinc and copper in vitro effects

In vitro effects of zinc and copper (0.01 to 1 mM) on the AChE activity of cerebrum and cerebellum are presented in Figure 2A and B. Two-way ANOVA (tissues x metal concentrations) revealed a significant effect of copper [$F(4,24)=29.69$, $p<0.001$] but not of zinc. Copper at 0.1 and 1 mM inhibited the AChE activity of both cerebrum [0.1 mM, $t(3)=4.90$, $p<0.02$; 1 mM, $t(3)=8.19$, $p<0.04$] and cerebellum [0.1 mM, $t(3)=3.52$, $p<0.04$; 1 mM, $t(3)=6.16$, $p<0.009$] homogenates. However, no difference between the tissues was found.

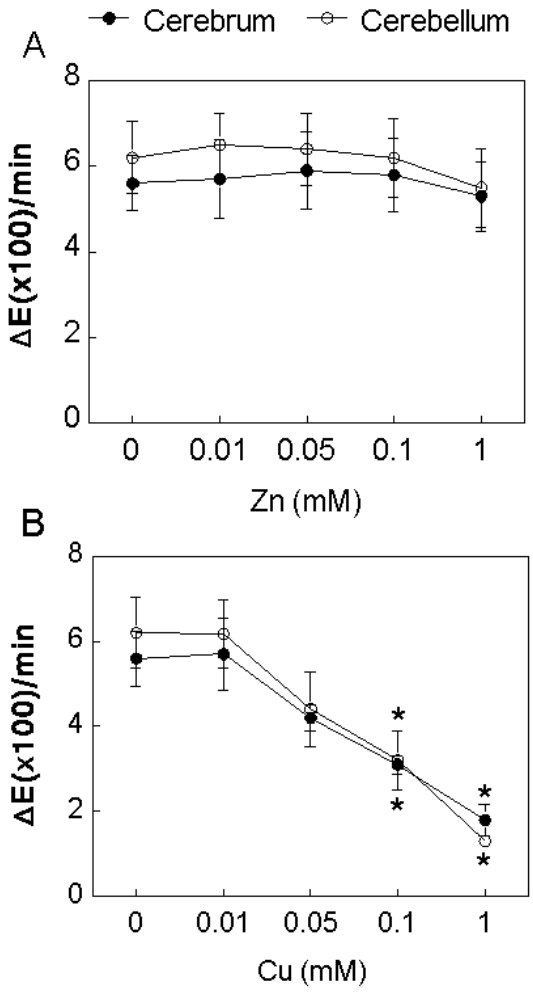

Figure 2: *In vitro* effects of different concentrations of zinc (A) and copper (B) on the AChE activity of rat cerebrum and cerebellum homogenates. Results are presented as mean ± SEM, n=4.
(*) Significant difference from 0 mM: $p<0.05$ (paired *t*-test).

IC_{50} of copper for AChE from cerebrum, cerebellum, hippocampus and hypothalamus homogenates were similar (one-way ANOVA): 0.30 ± 0.05, 0.37 ± 0.05, 0.35 ± 0.07 and 0.24 ± 0.05 mM, respectively.

IC_{50} of copper for AChE from cerebrum and cerebellum cellular fractions are shown in Figure 3. Two-way ANOVA (2 tissues x 3 fractions) revealed a significant effect of fractions [$F(2,12)=6.22$, $p<0.05$]. To cerebrum, the IC_{50} for AChE S2 fraction was significantly [$t(3)=-4.28$ $p<0.05$] higher than IC_{50} for AChE homogenate, while to cerebellum IC_{50} for AChE S1 fraction was significantly [$t(3)=-4.14$ $p<0.05$] higher than IC_{50} for AChE homogenate (paired *t*-test).

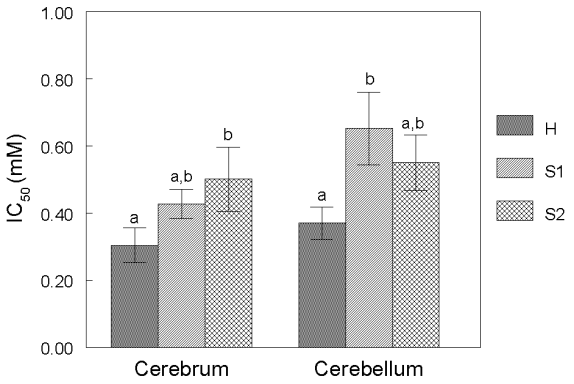

Figure 3: IC_{50} of $CuSO_4.5H_2O$ for *in vitro* inhibition of the AChE activity of brain and cerebellum homogenates, S1 and S2 fractions. Results are presented as mean ± SEM of metal concentration required to induce 50% inhibition of the AChE activity, n=4. Different letters confer significant statistical difference with $p<0.05$ (paired-*t* test).

DISCUSSION

Zinc and copper are essential trace elements for the development and maintenance of the cerebral function; nevertheless, when in excess both may be neurotoxic (Mathie et al., 2006). The equilibrium between the two metal levels seems to be fundamental to several neurological functions (Barnham et al., 2004). However, there is a lack of studies on the effects of essential metals on young animals. Thus, in this study we investigated if the cholinergic systems

through the AChE activity of lactating rats were sensitive to zinc and copper exposure.

Before testing the relationship between the two metals, it was necessary to determine the higher dose of copper inert in inducing any general physiologic parameter damage inspected by both visual observation and corporal development. This determination is important since the zinc dose chosen has no damaging effect and still prevents several toxic effects of mercury (Peixoto et al., 2003; Peixoto and Pereira, 2007; Franciscato et al., 2009). Thus, the animals were submitted to copper dose curve to search the highest dose inert in inducing any toxic effect that could be used in further studies, as zinc is used in preventive treatments (Peixoto et al., 2003; Peixoto and Pereira, 2007; Franciscato et al., 2009). We could observe that although the highest dose tested did not alter the body and brain weight, it caused lesions in the skin at the site of the injection. Thus, based on the findings we have chosen the intermediate dose of the copper curve to carry out the experiments with zinc and copper.

Whereas zinc or copper treatments modified neither the body weight gain nor cerebrum and cerebellum weights, the simultaneous administration of the two metals for five consecutive days induced a decrease in the body weight gain (Figure 1) and a slight, but not significant, reduction of cerebrum and cerebellum weight (Table 1). Our results agree with those obtained by Smith et al. (1997) that found a decrease in the growth of pigs fed on a diet with zinc and copper addition, but not supplemented only with zinc. According to these authors, the decrease in the development may be due to an inhibition of nutrient metabolism. In fact, an imbalance between these two metals may lead to peripheral metabolic toxic effects (Klevay, 1973). Furthermore, the effects of Zn+Cu administration on body, cerebrum and cerebellum weights were simultaneous since the ratios between two parameters were also similar (Table 1).

Zinc and copper are important ions involved in the neurotransmission process (Smart et al. 2004). Both may act as neuromodulators in the excitatory synapses (Mathie et al., 2006; Mocchegiani et al., 2005). Alterations in zinc and copper homeostasis have been reported in neurodegenerative diseases due to the interaction of metals with key proteins and subsequent induction of oxidative stress leading to neurodegeneration (Barnham et al., 2004). In this study, the exposure to zinc and/or copper produced no changes on the AChE activity of cerebrum and cerebellum (Table 2). These results differ from those reported by Brocardo et al. (2005) that found a decrease in the AChE activity of cerebral cortex and hippocampus of adult rats treated with one dose of $ZnCl_2$ (5 mg/kg), as well as from those by Romani et al. (2003) that related an increase of the AChE activity of brain and muscle of fish exposed to sublethal concentrations of $CuSO_4$. Differences among the results may be related to the age of the animals treated with zinc and to the species of animals exposed to copper, respectively. Furthermore, it was possible to observe that the simultaneous exposure to the two metals did not alter the enzyme activity. The absence of effects on the AChE activity was accompanied by the absence in the variation of zinc and copper contents in cerebrum and cerebellum. Similar levels of these metals among groups may be due to the rapid metal redistributions (Komsta-Szumska and Chmielnicka, 1983). In fact, in a recent study we observed that zinc treatment (same schedule of treatment) causes a high accumulation of this metal in liver and in kidney, without alteration in blood levels (Peixoto et al., 2008).

With the objective to investigate if the absence of the effect of zinc and/or copper could be due to the interval in which the animals were killed after the treatment (24 h after the last dose), other animals were sacrificed 1, 6 or 24 h after treatment. The results of this experiment showed that the brain enzyme activity was not altered in any of the intervals (Table 3), confirming that these metals, in the doses used, did not modify the AChE activity.

Results obtained with *in vivo* experiments have shown that, although the young animals are very sensitive to different extern insults (Peixoto et al., 2003; Rocha et al., 1993; Roza et al., 2005; Vendite et al., 1985) due to accelerated growth and development of brain (Gottlieb et al., 1977), the doses of zinc and copper used in this study were not sufficient to cause accumulation of these metals in the central nervous system nor to produce damage to cholinergic system when considered the AChE activity as biomarker in suckling rats.

We also studied the *in vitro* effects of zinc and copper on the AChE activity of cerebrum and cerebellum of rats of the same age used in the *in vivo* study. Copper 0.1 and 1 mM inhibited the AChE activity of both cerebrum (45 % and 68 %) and cerebellum (48 % and 79 %) (Figure 2A and 2B). The absence of zinc *in vitro* effects on brain AChE activity is in accordance with the results obtained by Senger et al. (2006) who reported that zinc does not affect this enzyme activity from brain of zebrafish. The AChE copper inhibition may be due to an action of Cu^{2+} on the catalytic site of the enzyme by an electrostatic interaction involving specific aminoacidic residues in the active site (Berman and Leonard, 1990). However, studies with enzymatic kinetic are necessary to sustain this hypothesis.

Considering that only copper was effective in inhibiting the cerebral AChE activity, the concentration of copper necessary to inhibit 50 % (IC_{50}) of the enzyme activity of cerebrum, cerebellum, hypothalamus and hippocampus was evaluated and we found a similar IC_{50} for AChE of these structures. Analyzing the sensitivity of AChE of subcellular fractions enriched with G4 globular form (membrane bound form; S2 fraction) or with G1 globular form (cytosolic form, S1 fraction) (Nigg and Knaak, 2000; Rieger and Vigny, 1976) and comparing these activities with the activity from total homogenate, we observed that S2 fraction from cerebrum and S1 fraction from cerebellum were less sensitive to the inhibitor than the mixture of these forms contained in the homogenate, since a higher Cu concentration to inhibit 50 % of enzyme activity (Figure 3) was necessary. These results suggest that the residual activity (pellet 2) in the fraction not evaluated but present in the total homogenate seems to be the most vulnerable to inhibitory effects of copper. Specific experiments are necessary to fundament this hypothesis. However, this supposition does not interfere in the absence of the metal effects *in vivo* since the enzyme activity was analyzed in total homogenate.

In conclusion, although the simultaneous administration of zinc and copper caused a decrease in the body weight gain, these metals separately did not alter the cerebral and corporal development. Zinc and copper treatment did not change the cerebral AChE activity and zinc and copper contents; whereas *in vitro* high concentrations of copper decreased the cerebrum and cerebellum AChE activity. Despite the *in vitro* results, the absence of *in vivo* effects permits to suggest that copper may be investigated as a possible preventive essential metal to toxic effects of the heavy metals as observed to zinc (Peixoto et al. 2003, 2008; Peixoto and Pereira 2007; Franciscato et al., 2009).

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