

Zinc reverses malathion-induced impairment in antioxidant defenses

Jeferson L. Franco^{a,b}, Thais Posser^b, Jacó J. Mattos^b, Rafael Trevisan^a, Patricia S. Brocardo^b, Ana Lúcia S. Rodrigues^b, Rodrigo B. Leal^b, Marcelo Farina^b, Maria R.F. Marques^b, Afonso C.D. Bainy^b, Alcir L. Dafre^{a,*}

^a Departamento de Ciências Fisiológicas, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil

^b Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil

ARTICLE INFO

Article history:

Received 16 October 2008

Received in revised form 17 February 2009

Accepted 18 February 2009

Available online 4 March 2009

Keywords:

Zinc
Malathion
Antioxidant defenses
Neuroprotection
Heat shock proteins

ABSTRACT

Malathion toxicity has been related to the inhibition of acetylcholinesterase and induction of oxidative stress, while zinc has been shown to possess neuroprotective effects in experimental and clinical studies. In the present study the effect of zinc chloride (zinc) was addressed in adult male Wistar rats following a long-term treatment (30 days, 300 mg/L in tap water *ad libitum*) against an acute insult caused by a single malathion exposure (250 mg/kg, i.p.). Malathion produced a significant decrease in hippocampal acetylcholinesterase, as well as a decrease in the activity of several hippocampal antioxidant enzymes: glutathione reductase, glutathione S-transferase, catalase and superoxide dismutase. The pretreatment with zinc did not completely prevent acetylcholinesterase activity impairment; however, antioxidant activity was completely restored. Zinc administration significantly increased HSP60, but not HSP70, expression. The HSP60 increase suggests a novel zinc-dependent pathway, which may be related to a counteracting mechanism against malathion effects. Based on these results the following hypothesis can be presented: the published “pro-oxidative” effect of malathion may be related, among others, to compromised antioxidant defenses, while the zinc “antioxidant” action may be related to the preservation of antioxidant defenses. In conclusion, our data points to the inhibition of antioxidant enzymes as an important non-cholinergic effect of malathion, which can be rescued by oral zinc treatment.

© 2009 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Zinc occurs in hundreds of enzymes and in even more protein domains, participating in a number of cellular processes, including cell proliferation, differentiation, and apoptosis. Zinc participates in the functioning of the immune system, intermediary metabolism, DNA metabolism and repair, reproduction, among others. About 25% of the human population worldwide is at risk of zinc deficiency, and zinc supplementation can be beneficial (Maret and Sandstead, 2006). In acrodermatitis enteropathica, a genetic disorder of zinc metabolism caused by a mutation in the zinc transporter hZip4, zinc supplementation is necessary to limit zinc deficiency (Mao et al., 2007). In the Wilson's disease, zinc supplementation replaces copper and prevents its excess, limiting oxidative damage (Kitzberger et al., 2005). In diabetes, zincuria is a risk factor for zinc deficiency, while zinc supplementation decreases the chances of deficiency and ameliorates several oxidative parameters (Maret and Sandstead, 2006). Zinc supplementation is also postulated as an adjuvant in the therapy of mood disorders (Nowak et al., 2005).

In preclinical studies, oral zinc supplementation has been shown to have an antidepressant effect (Brocardo et al., 2007). Zinc also protects against oxidative liver damage induced by chronic alcohol ingestion (Zhou et al., 2005), organophosphate treatment (Goel et al., 2005) or by lithium (Chadha et al., 2008). Given the potential for wide therapeutic use of zinc supplementation we wanted to focus on whether zinc has a neuroprotective function.

In the brain, zinc-rich areas display as much as 300–600 μM of available zinc, particularly in the hippocampus and cerebral cortex (Vallee and Falchuk, 1993; Frederickson et al., 2005). Zinc is stored in synaptic vesicles such as in glutamatergic neurons and released simultaneously with glutamate, acting as a neuromodulator (Frederickson et al., 2005). A significant number of reports describe the protective effects of this endogenous metal against excitotoxic insults (Cole et al., 2000; Cohen-Kfir et al., 2005). However, the mechanism whereby zinc displays its protective action remains to be established. One beneficial aspect on zinc action may be the antagonism of NMDA receptors (Chen et al., 1997; Paoletti et al., 1997). Moreover, zinc can modulate GABAergic neurotransmission by inhibiting GABA transporters in the hippocampus, which could reveal a link between excitatory and inhibitory neurotransmission, especially during epileptic seizures (Cohen-Kfir et al., 2005). In addition, zinc is demonstrated to modulate intracellular

* Corresponding author. Tel.: +55 48 3721 9579; fax: +55 48 3721 9672.

E-mail address: aldafre@ccb.ufsc.br (A.L. Dafre).

signaling cascades such as mitogen-activated protein kinases, protein kinase C and Ca^{2+} /calmodulin activated protein kinase II, therefore participating in cell proliferation and differentiation (Beyersmann and Haase, 2001).

Malathion (O,O-dimethyl-S-1,2-bis ethoxy carbonyl ethyl phosphorodithioate) is an organophosphate (OP) pesticide widely employed in agriculture and in domestic pest control. It is considered to be a hazardous compound to human health, pets and wildlife (Flessel et al., 1993). As with other OP agents, malathion is thought to exert its toxic effects by inactivation of serine esterases (Taylor et al., 1995), mainly acetylcholinesterase (AChE; EC 3.1.1.7) as well as butyrylcholinesterase (BuChE; EC 3.1.1.8). The inhibition of AChE leads to the accumulation of acetylcholine in the synaptic terminals of the central and peripheral nervous system with consequent overstimulation of the cholinergic pathways (Kwong, 2002; Bartling et al., 2007). Various studies have reported neurotoxic effects of malathion in both humans (Abdel-Rahman et al., 2004; Rothlein et al., 2006) and animals (Vidair, 2004; Brocardo et al., 2005; da Silva et al., 2006). Malathion is thought to be one of the main agents leading to human OP intoxication in Santa Catarina state in Southern Brazil, according to unpublished data obtained from Toxicological Information Center (Centro de Informações Toxicológicas – CIT) hosted by the Hospital Universitário, Florianópolis, SC, Brazil.

The toxicity induced by OP compounds is also believed to be linked to the pro-oxidative properties of these compounds (Goel et al., 2005; Banerjee et al., 1999; Verma and Srivastava, 2001; Ranjbar et al., 2002). It was described that malathion exposure increases lipid peroxidation in rodent erythrocytes, liver and brain (Hazarika et al., 2003; Akhgari et al., 2003). In a recent report (Brocardo et al., 2007), we showed a neuroprotective action of zinc against malathion which may be related to an up-regulation in neuroprotective effectors (Franco et al., 2008). Among these effectors brain derived neurotrophic factor expression, intracellular signal-regulated protein kinase phosphorylation and GSH synthesis have been postulated (Franco et al., 2008).

Other potential zinc target is the heat shock proteins (HSPs) that work as molecular chaperones, able to protect tissues including brain against cell death (Plumier et al., 1997; Sharp, 1998). It was previously demonstrated that zinc causes increase in heat shock protein 70 kDa (HSP70) expression in a variety of tissues and cell cultures (Lee et al., 2000; Unoshima et al., 2001). The exact role of heat shock proteins in zinc protective/toxic effects is not fully understood. While some authors consider the induction of these molecular chaperones as a beneficial action of zinc (Unoshima et al., 2001; Klosterhalfen et al., 1997; Tons et al., 1997), others, however, believe that such effect is a toxic cellular response (Lee et al., 2000).

In the present study we aimed to investigate whether long-term oral zinc (300 mg/L p.o.) treatment is able to protect the rat brain against toxicity caused by an acute treatment with malathion (250 mg/kg i.p.). Antioxidant activity and the HSPs expression were the endpoints investigated.

2. Materials and methods

2.1. Chemicals and antibodies

Glutathione-disulfide reductase (GR), EC 1.8.1.7, reduced glutathione (GSH), oxidized glutathione (GSSG), *tert*-butylhydroperoxide (*t*-BOOH), 5,5'-dithio-bis(2-nitrobenzoic) acid, cytochrome *c*, xanthine, xanthine oxidase (EC 1.17.3.2), 1-chloro-2,4-dinitrobenzene, acetylthiocholine iodide were purchased from Sigma, São Paulo. NADPH was purchased from Gerbu Biochemicals GmbH, Gaiberg. Zinc chloride was obtained from Merck, Rio de Janeiro and commercial-grade malathion 500 CE (95% purity, CAS 121-75-5) was purchased from BioCarb, Curitiba. The primary antibodies for HSP60 and HSP70 were purchased from StressGen, Ann Arbor, Michigan and secondary antibodies were from Amersham, São Paulo. All other chemicals used in this work were from the highest commercial grade available.

2.2. Animals and treatments

Adult male Wistar rats (3 months old, 250–350 g) were maintained in a room under controlled temperature ($23 \pm 1^\circ\text{C}$). They were subjected to a 12 h light cycle (lights on 7:00 a.m.) with free access to food and water. All procedures used in the present study were approved by the institution ethics committee on the use of animals (CEUA).

Animals were separated to four different groups: (a) the control group was maintained for 30 days and on the 31st day received a saline injection intraperitoneally. (b) In the zinc group, ZnCl_2 (300 mg/L) diluted in tap water was offered *ad libitum* during 30 days. Animals received an i.p. saline injection 24 h after zinc treatment was complete. Based on daily liquid consumption, each animal received between 15 and 18 mg/kg body weight of zinc chloride per day. This protocol was based on previous reports (Goel et al., 2005; Domingo et al., 1988). (c) In the malathion group animals received an i.p. injection of malathion (250 mg/kg) 24 h previous to tissue collection. (d) In the zinc/malathion group, animals received zinc for 30 days in the tap water. In order to avoid the acute effect of zinc, 24 h after oral zinc was interrupted animals received an i.p. injection of malathion (250 mg/kg). Animals were sacrificed after 24 h following the respective saline/malathion i.p. injections, i.e., 48 h after oral zinc was interrupted, and tissues were prepared for biochemical analysis.

2.3. Tissue preparation

The hippocampus was rapidly removed to cooled saline and immediately homogenized in 0.02 M HEPES pH 7.0 and centrifuged at $1000 \times g$. An aliquot of the supernatant (S1) was used for measurements of cholinesterase activity and the remaining S1 was centrifuged at $20,000 \times g$ for 30 min at 4°C . The supernatant (S2) was isolated and utilized for measurements of antioxidant enzyme activity. Blood samples were isolated from rat hepatic portal vein, using heparinized syringes, for measurements of plasma acetylcholinesterase activity and AST/ALT activity, as markers of malathion intoxication.

For western blots, tissues were homogenized at 4°C in a buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Na_3VO_4 , 100 mM sodium fluoride. The homogenates were centrifuged at $1000 \times g$ for 10 min at 4°C and the supernatants (S1) collected. After protein determination, β -mercaptoethanol was added to samples to a final concentration of 8%. Then samples were frozen at -80°C for further determination of HSP60 and HSP70 immunocomponent. Protein levels were quantified according to Bradford (1976) using bovine serum albumin as standard.

2.4. Enzyme assays

The GR activity was determined according to Carlberg and Mannervik (1985). Glutathione peroxidase (GPx), EC 1.11.1.9, activity was measured indirectly by monitoring the consumption of NADPH at 340 nm according to Wendel (1981) using the *t*-BOOH as a substrate. Glutathione transferase (GST), EC 2.5.1.18, activity was assayed by the procedure of Habig and Jakoby (1981) using 1-chloro-2,4-dinitrobenzene as substrate. Catalase (CAT), EC 1.11.1.6, activity was measured according to Aebi (1984). Superoxide dismutase (SOD), EC 1.15.1.1, activity was based on the decrease in cytochrome *c* reduction (Misra and Fridovich, 1977). Acetylcholinesterase activity was measured according to Ellman et al. (1961). Plasma transaminases, aspartate transaminase (AST, EC 2.6.1.1) and alanine transaminase (ALT, EC 2.6.1.2) activity were determined using commercially available kits (Biotécnica Ltda., Varginha) and expressed as percent (%) of controls.

2.5. Western blot

Samples (10 mg of protein) were separated by SDS-PAGE using 10% gels and transferred to nitrocellulose membrane using 400 mA current (3 h at 4°C) (Posser et al., 2007). The membranes were blocked with 5% skim milk (1 h), followed by a second blockage (1 h) with 2.5% gelatin, both solutions in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). All steps were followed by three times washing with TBS-T (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5). For HSP detection, rabbit polyclonal antibodies SPA805 (1:5000) and SPA811 (1:5000) (StressGen), anti-human HSP60 and HSP70, were used as primary antibodies. NA934 (1:1000) goat anti-rabbit IgG peroxidase conjugated (Amersham) was used as the secondary antibody for detecting both isoforms. Loading control was checked by probing for β -actin at dilution of 1:25,000 (A3854, Sigma–Aldrich). Immunoblotting was developed using the enhanced chemiluminescence (ECL) system (Amersham Bioscience). HSP60 and HSP70 expression was quantified by densitometric analysis of the immunoreactive bands using the Scion Image[®] software.

2.6. Statistical analysis

Data are presented as mean \pm standard deviation (S.D.). Statistical significance was assessed by two-way ANOVA (malathion and zinc as factors) followed by Tukey's test when appropriate. A value of $P < 0.05$ was considered to be statistically significant.

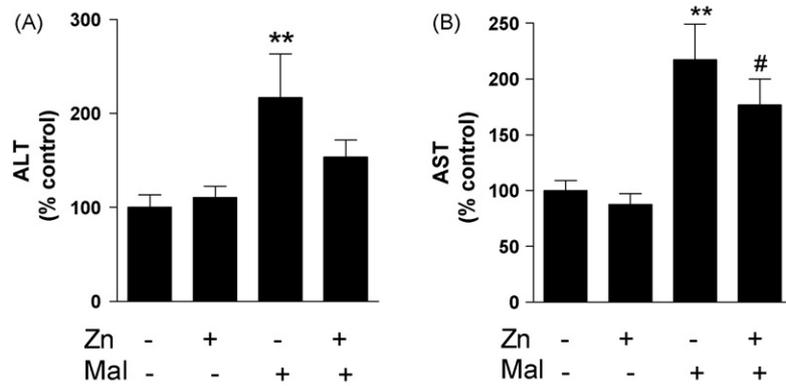


Fig. 1. Plasma AST and ALT activity after zinc/malathion administration. Animals were pre-treated with zinc (300 mg/L p.o.) for 30 days and 24 h later were injected with a single malathion dose (250 mg/kg i.p.) and sacrificed 48 h after zinc treatment closed, as described in Section 2. (A) Plasma ALT levels and (B) plasma AST levels. Data are expressed as % of untreated controls. The values are mean \pm S.E.M. ($n = 4-6$ animals per group). Statistical analysis was performed by two-way ANOVA (malathion and zinc as factors) followed by Tukey's post hoc analysis. ** $P < 0.01$ when compared to control and zinc groups; # $P < 0.05$ when compared to malathion group. Basal AST activity was 63.5 ± 3.8 U/L and ALT activity was 62.2 ± 4.2 U/L.

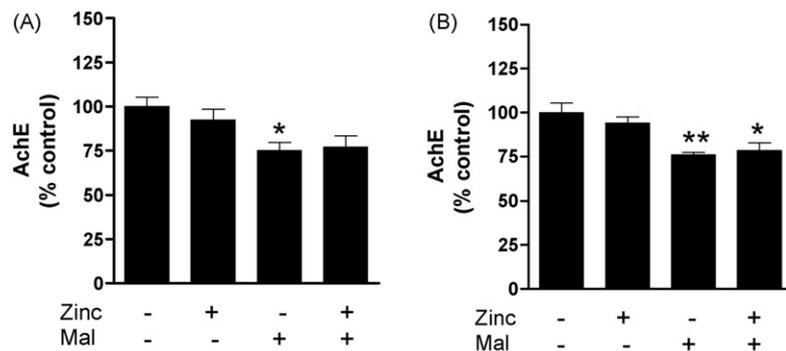


Fig. 2. Hippocampus and plasma AChE activity after zinc/malathion administration. Animals were pre-treated with zinc (300 mg/L p.o.) for 30 days and 24 h later injected with a single malathion dose (250 mg/kg i.p.). Animals were sacrificed 48 h after zinc treatment closed, as described in Section 2. (A) Hippocampus and (B) plasma AChE activity. Data are expressed as % of untreated controls. The values are mean \pm S.E.M. ($n = 4-6$ animals per group). Statistical analysis was performed by two-way ANOVA (malathion and zinc as factors) followed by Tukey's post hoc analysis. * $P < 0.05$ and ** $P < 0.01$ when compared to control and zinc groups. Basal AChE activity was 261.97 ± 35.42 mU/mg protein in the plasma and 23.58 ± 2.82 mU/mg protein in the hippocampus.

3. Results

In order to determine whether the malathion dosing (250 mg/kg, i.p.) produced toxicity to animals, we measured plasma AST and ALT activity. Fig. 1 shows that malathion increased the activity of both enzymes in plasma. This evidence suggests that this malathion dose is able to induce some degree of liver toxicity. Long-term zinc treatment, which did not affect plasma AST or ALT activity, was

able to reverse malathion-induced increase in plasma ALT activity (Fig. 1A). Moreover, zinc produced a slight reduction in malathion-induced AST release, but it was marginally significant ($P = 0.075$; Fig. 1B).

Malathion treatment caused a significant inhibition in both hippocampal and plasma AChE activity (Fig. 2). Zinc alone did not affect AChE activity *per se* neither reverse malathion induced AChE decrease.

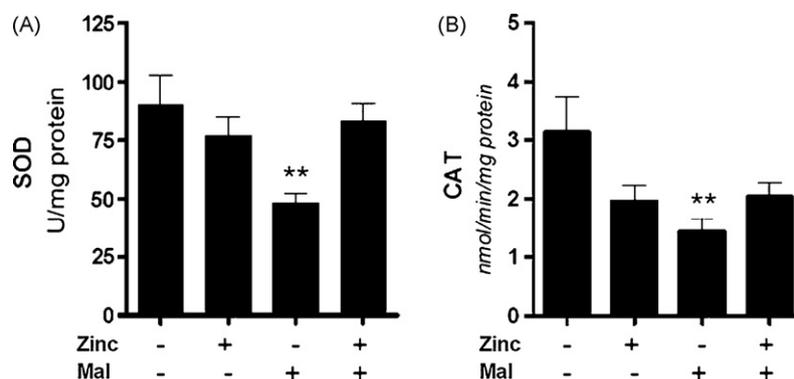


Fig. 3. Activity of SOD and CAT on the hippocampus after zinc/malathion administration. Animals were pre-treated with zinc (300 mg/L p.o.) for 30 days and 24 h later injected with a single malathion dose (250 mg/kg i.p.). Animals were sacrificed 48 h after zinc treatment closed, as described in Section 2. Hippocampus SOD (A) and CAT (B) activity. Data are expressed the rate of hydroperoxide consumption (CAT, nmol/(min mg)) or units of enzyme activity (SOD) normalized to the protein content (mg/mL). One SOD unit corresponds to the amount of protein needed to inhibit the rate of cytochrome *c* reduction by 50%. The values are mean \pm S.E.M. ($n = 5-6$ animals per group). Statistical analysis was performed by two-way ANOVA (malathion and zinc as factors) followed by Tukey's post hoc analysis. ** $P < 0.01$ when compared to all other groups.

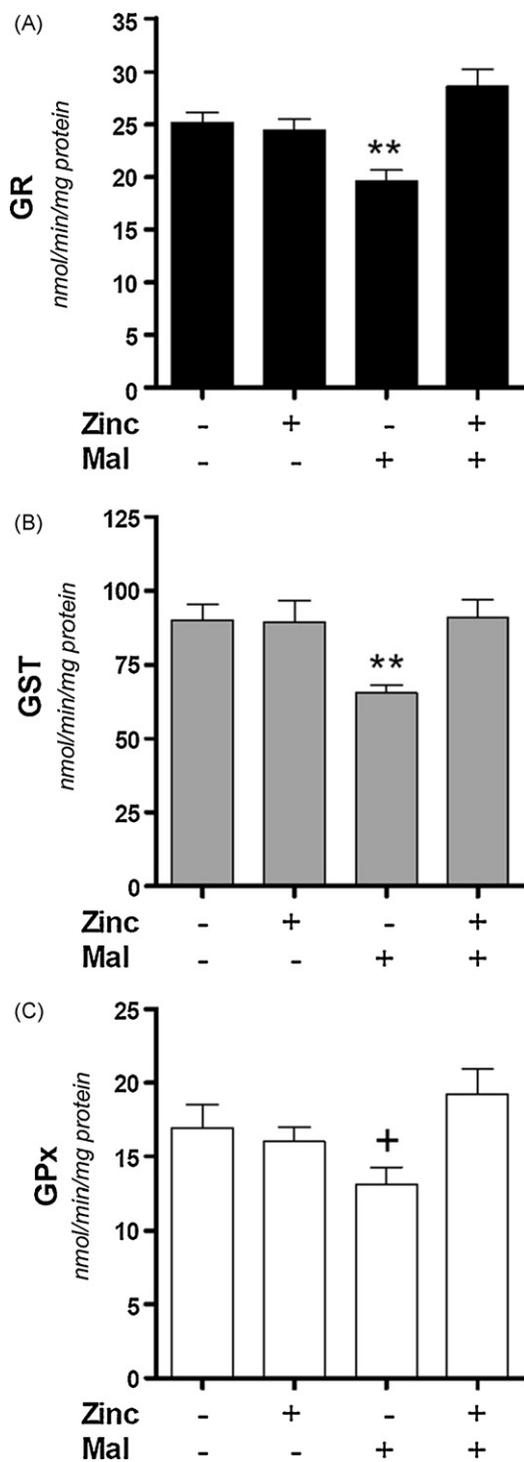


Fig. 4. Activity of GPx, GR and GST in the rat hippocampus after zinc/malathion administration. Animals were pre-treated with zinc (300 mg/L p.o.) for 30 days and 24 h later injected with a single malathion dose (250 mg/kg i.p.). Animals were sacrificed 48 h after zinc treatment closed, as described in Section 2. Hippocampus GPx (A), GR (B) and GST (C) activity. Data are expressed as the rate of NADPH consumption (GR and GPx) or the rate of the CDNB-GSH conjugation normalized to the protein content (nmol/(min mg) protein). The values are mean \pm S.E.M. ($n=10$ animals per group). Statistical analysis was performed by two-way ANOVA (malathion and zinc as factors) followed by Tukey's post hoc analysis. ** $P<0.01$ when compared to all other groups; + $P<0.05$ when compared to malathion plus zinc groups.

Malathion caused a significant decrease in the activity of the antioxidant enzymes SOD and CAT (Fig. 3), as well as GR and GST (Fig. 4). Pretreatment of animals with a zinc (300 mg/L in tap water) for 30 days caused a complete blockade of the malathion-induced

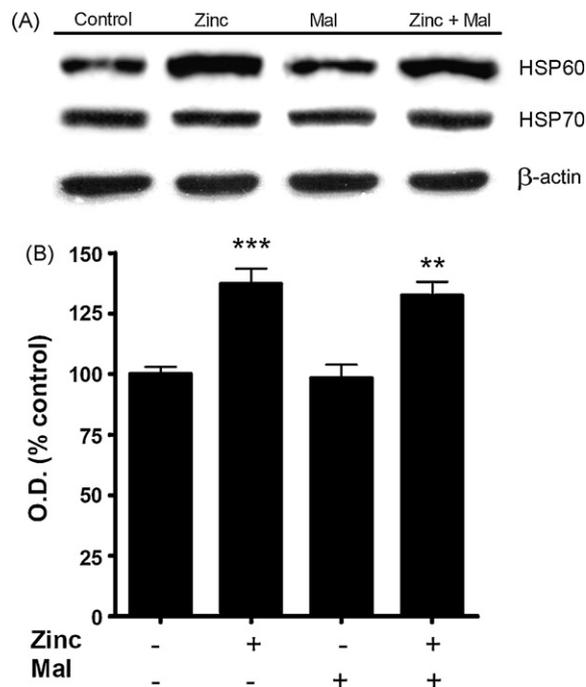


Fig. 5. Hippocampus HSP60 and HSP70 expression after zinc/malathion administration. Animals were pre-treated with zinc (300 mg/L p.o.) for 30 days and 24 h later injected with a single malathion dose (250 mg/kg i.p.). Animals were sacrificed 48 h after zinc treatment closed. Hippocampal homogenates were prepared and analyzed as described in Section 2. (A) Representative western blot for HSP60 and HSP70 content and (B) quantitative analyses for HSP60 immunoreactive bands, expressed as % of untreated controls. The values are mean \pm S.E.M. ($n=4-6$ animals per group). Statistical analysis was performed by two-way ANOVA (malathion and zinc as factors) followed by Tukey's post hoc analysis. ** $P<0.01$ and *** $P<0.001$ when compared to control and malathion groups.

decrease in antioxidant enzymes activity in the hippocampus (Figs. 3 and 4). GPx activity was not significantly decreased as compared to control animals, however, the mean value was lower than in the malathion/zinc-treated animals.

Long-term zinc treatment induced a significant increase in the expression of HSP60 in the rat hippocampus, while HSP70 immunocent content remained unaltered (Fig. 5A). Malathion treatment did not change HSP60 or HSP70 expression. Animals receiving zinc/malathion showed similar results regarding HSP60 expression when compared to the zinc group. This increased HSP60 expression appears to be a result of zinc treatment, independent on malathion administration (Fig. 5A), as confirmed by two-way ANOVA that identified zinc as a significant factor ($P<0.0001$). Fig. 5B depicts the densitometric quantification of the immunoreactive bands for HSP60.

4. Discussion

It has been previously reported that zinc is able to protect against alcohol- (Zhou et al., 2005) and OP-induced (Goel et al., 2005) increases in oxidative markers of rat liver. In line with these reports, the present study demonstrates the beneficial effect of a long-term zinc treatment (300 mg/L in tap water) against malathion-induced impairment in the rat hippocampal antioxidant defenses. In a previous study, we showed that *in vivo* acute treatment with zinc causes signs of toxicity (Franco et al., 2008), confirming *in vitro* studies (Walther et al., 2003; Bossy-Wetzels et al., 2004). As the period of treatment increases, protective effects could be disclosed. Short-term treatment with zinc protects against chromatin condensation and recovers from depressant-like behavior induced by malathion (Brocardo et al., 2007; Franco et al., 2008). The long-term

oral treatment utilized in this study displayed no signs of toxicity, on the contrary, neuroprotective pathways were activated by the zinc treatment protocol, as demonstrated in our previous report (Franco et al., 2008). In the present study we demonstrate that long-term oral zinc administration leads to a reversion of OP effects by preventing malathion-induced decreases in antioxidant defenses and liver damage. Since OP intoxication represents an important human health problem (Flessel et al., 1993) and one of the major targets for OP toxicity is the CNS, that besides cholinergic effects there are evidences of oxidative stress. Our study constitutes an important contribution regarding a preventive treatment against the OP-induced impairment in antioxidant defense enzymes.

The malathion dose used in the present study caused an inhibition of plasma AChE. In parallel, it was possible to observe an increase in plasma AST and ALT activity, which are markers of hepatic toxicity. Altogether, these data point to a significant level of systemic toxicity for the malathion dosage used. Zinc treatment was unable to completely reverse malathion-induced AChE inhibition; however it caused some degree of hepatic protection by recovering plasma ALT activity, as shown in Fig. 1A.

In addition to the systemic effects of malathion on rats, we also demonstrated the inhibition of AChE in rat hippocampus (Fig. 2A). Inhibition of AChE by OP leads to an accumulation of acetylcholine and subsequent impairment of numerous body functions (Bartling et al., 2007). Clinically, the standard treatment of OP poisoning includes the combined administration of atropine (an anti-muscarinic compound) and pralidoxime (an AChE re-activator) (Peter et al., 2008). However, the counteracting actions of these compounds against non-cholinergic effects of OP are not understood. In our study, the pretreatment of rats with zinc did not strongly protect against malathion-induced inhibition of AChE in the hippocampus (as demonstrated in Fig. 2A), suggesting that the preventive actions of this metal against malathion-induced decrease in antioxidant defenses are not entirely related to the reactivation of brain acetylcholinesterase. However, a growing number of authors point to the toxicological relevance of non-cholinergic targets OP-induced damage (Masoud et al., 2003; Saleh et al., 2003). In a previous work we showed that chromatin condensation, lipid peroxidation and behavioral alterations were produced without a perceptible decrease in the AChE activity (Brocardo et al., 2007), indicating that non-cholinergic actions of malathion are toxicologically relevant.

There is evidence for a role of oxidative stress in the toxicity of OP agents. In recent studies from our group we reported increased lipid peroxidation levels in mouse and rat brain (Brocardo et al., 2007; da Silva et al., 2006), as well as by others (Fortunato et al., 2006a,b; Delgado et al., 2006). Published data depict increased lipid peroxidation as a general finding after acute or chronic treatment with malathion or other OP (Banerjee et al., 1999; Verma and Srivastava, 2001; Ranjbar et al., 2002; Goel et al., 2005; Fortunato et al., 2006a,b; Delgado et al., 2006; Brocardo et al., 2007; da Silva et al., 2006). Other evidences of oxidative stress have been presented. A recent experiment demonstrated DNA damage in brain structures after short-term (Brocardo et al., 2007) and chronic treatment with malathion (Reus et al., 2008).

In the present study we showed that malathion caused a significant decrease of the activity of several important antioxidant defenses (GR, GST, CAT and SOD). Impaired antioxidant defenses would reduce the CNS protection against an oxidative challenge (Dringen et al., 2005). Thus, our data supports a pro-oxidative action as a mechanism for malathion neurotoxicity, in addition to its inhibitory action toward brain cholinesterases. As demonstrated in other studies, the activities of antioxidant enzymes can be altered in a variety of animal tissues poisoned with malathion (Hazarika et al., 2003; Akhgari et al., 2003; Ahmed et al., 2000; John et al., 2001). The marked reduction on the activity of glutathione-

related antioxidant enzymes, catalase and SOD indicates that such an effect may be a characteristic feature for rodent hippocampus under acute malathion exposure. Taken the extensive literature data showing increased lipid peroxidation as general finding after malathion exposure, it is clear that the antioxidant system are not coping with the oxidative challenge. One possible reason for this increased lipid peroxidation would be the parallel impairment in antioxidant defenses, as presented in the present work.

Previous works demonstrated that oral zinc treatment is able to prevent alcohol-induced (Zhou et al., 2005) or OP-induced oxidative damage to the liver (Goel et al., 2005). In the present work we show that pretreatment of male rats with oral zinc (300 mg/L in tap water) completely reversed the malathion-induced impairment on rat hippocampus antioxidant defenses, corroborating data obtained for rodent liver. As the maintenance of normal levels of antioxidants in the brain is crucial in protecting neural cells against pro-oxidative conditions (Dringen et al., 2005), our data represent an important finding showing the neuroprotective potential of oral zinc treatment in animal models of oxidative stress. The exact mechanism whereby zinc counteracts malathion effects on antioxidant enzymes needs further investigation. Zinc can modulate glutathione synthesis (Andrews, 2001) and defense enzymes such as glucose-6-phosphate dehydrogenase and glutathione S-transferase (Chung et al., 2006) via activation of metal-responsive transcription factor 1 (MTF1), but, since we did not find any zinc-dependent alteration in the antioxidant defenses studied, and due to the homeostatic regulation of zinc (Andrews, 2001), the involvement of MTF1 is not likely (Chung et al., 2006).

Another hypothesis is that pretreatment of animals with zinc may modulate the expression of protective cell stress proteins, such as heat shock proteins, which consists in cellular chaperones that protect brain cells against death induced by several stress conditions (Plumier et al., 1997; Sharp, 1998). The constitutive forms of HSPs are thought to be involved in the regulation of the correct folding of newly formed proteins, being crucial to maintenance of cellular homeostasis. The inducible HSPs work to repair damaged proteins, thus preventing their aggregation and degradation (Kiang and Tsokos, 1998). Recent data support the idea that increased levels of HSPs may be useful cellular tools to afford neuroprotection against cell stress events (Escobedo et al., 2007; Perrin et al., 2007). In order to investigate this hypothesis we measured HSP60 and HSP70 expression in the hippocampus of animals treated with zinc and/or malathion.

Our data demonstrate that the administration of a long-term zinc dose (30 days p.o.) caused a significant increase in the expression of HSP60 in the rat hippocampus, while HSP70 immuncontent remained unaltered. Malathion *per se* did not change either HSP60 or HSP70 levels in the rat hippocampus. The induction of HSP60 by zinc was retained when animals were subsequently treated with malathion. It is established that zinc is able to induce HSP70 expression in a variety of tissue models and cell culture conditions (Lee et al., 2000; Unoshima et al., 2001), which was not observed with the present oral zinc treatment. However, at least to our knowledge, our study is the first report showing the modulation of HSP60 in rat hippocampus during *in vivo* zinc exposure. In a previous report, we have demonstrated that exposure of mussels to zinc for 48 h caused a significant increase in HSP60 in the gills, while HSP70 remained unchanged (Franco et al., 2006). A recent study reported increased levels of HSP60 in the hippocampus of gerbils submitted to an ischemic insult, correlating with a neuroprotective action of this chaperone (Hwang et al., 2007). In addition, a correlation between brain ischemia and synaptic release of zinc in the hippocampus has been extensively reported (Lee et al., 2000; Koh et al., 1996), thus suggesting a possible correlation between zinc release and induction of HSP60 during ischemia. Mitochondrial HSP60 is an essential element for functional apoptosis (Arya

et al., 2007) and chronic malathion treatment has been shown to induce oxidative stress and mitochondrial dysfunction (Delgado et al., 2006). In this regard, HSP60 expression may present a counteracting action against mitochondrial dysfunction, which needs to be further addressed.

Both, literature evidences and the present data point to HSP60 as an important molecular target for zinc in mammalian and non-mammalian tissues. Moreover, the fact that zinc treatment completely reversed the deleterious effect of malathion toward antioxidant enzymes, concomitantly with increased HSP60 expression in rat hippocampus, supports the idea that this cellular chaperone may afford neuroprotection by counteracting the malathion effects. Nevertheless, such hypothesis needs to be further investigated.

In conclusion, our results indicate that zinc was able to recover malathion-induced impairment in several antioxidant enzymes, showing to be a potential agent to be used against the pro-oxidative effects of OP compounds such as malathion. In addition, the data showed an increased expression of HSP60 after long-term oral zinc administration, which may be a possible mechanism in counteracting deleterious malathion effects.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported in part by a grant (W/3636) from the International Foundation for Science to ALD. The Brazilian agencies Capes (JLF and TP) and CNPq (ALD; ACDB; MF; RBL and RT) fellowships are acknowledged. The authors are in debt with Mr. Péricles A. Mitozo for laboratory technical support.

References

- Abdel-Rahman, A., Abou-Donia, S., El-Masry, E., Shetty, A., Abou-Donia, M., 2004. Stress and combined exposure to low doses of pyridostigmine bromide, DEET, and permethrin produce neurochemical and neuropathological alterations in cerebral cortex, hippocampus, and cerebellum. *Journal of Toxicology and Environmental Health* 67, 163–192.
- Aebi, H., 1984. Catalase in vitro. *Methods in Enzymology* 105, 121–126.
- Ahmed, R.S., Seth, V., Pasha, S.T., Banerjee, B.D., 2000. Influence of dietary ginger (*Zingiber officinale* Rosc) on oxidative stress induced by malathion in rats. *Food and Chemical Toxicology* 38, 443–450.
- Akhgari, M., Abdollahi, M., Kebryaezadeh, A., Hosseini, R., Sabzevari, O., 2003. Biochemical evidence for free radical-induced lipid peroxidation as a mechanism for subchronic toxicity of malathion in blood and liver of rats. *Human & Experimental Toxicology* 22, 205–211.
- Andrews, G.K., 2001. Cellular zinc sensors: MTF-1 regulation of gene expression. *Biometals* 14, 223–237.
- Arya, R., Mallik, M., Lakhotia, S.C., 2007. Heat shock genes—integrating cell survival and death. *Journal of Biosciences* 32, 595–610.
- Banerjee, B.D., Seth, V., Bhattacharya, A., Pasha, S.T., Chakraborty, A.K., 1999. Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. *Toxicology Letters* 107, 33–47.
- Bartling, A., Worek, F., Szinicz, L., Thiermann, H., 2007. Enzyme-kinetic investigation of different sarin analogues reacting with human acetylcholinesterase and butyrylcholinesterase. *Toxicology* 233, 166–172.
- Beyersmann, D., Haase, H., 2001. Functions of zinc in signaling, proliferation and differentiation of mammalian cells. *Biometals* 14, 331–341.
- Bossy-Wetzel, E., Talantova, M.V., Lee, W.D., Scholzke, M.N., Harrop, A., Mathews, E., Gotz, T., Han, J., Ellisman, M.H., Perkins, G.A., Lipton, S.A., 2004. Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K⁺ channels. *Neuron* 41, 351–365.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248–254.
- Brocardo, P.S., Assini, F., Franco, J.L., Pandolfo, P., Muller, Y.M., Takahashi, R.N., Dafre, A.L., Rodrigues, A.L., 2007. Zinc attenuates malathion-induced depressant-like behavior and confers neuroprotection in the rat brain. *Toxicological Science* 97, 140–148.
- Brocardo, P.S., Pandolfo, P., Takahashi, R.N., Rodrigues, A.L., Dafre, A.L., 2005. Antioxidant defenses and lipid peroxidation in the cerebral cortex and hippocampus following acute exposure to malathion and/or zinc chloride. *Toxicology* 207, 283–291.
- Carlberg, I., Mannervik, B., 1985. Glutathione reductase. *Methods in Enzymology* 113, 484–490.
- Chadha, V.D., Bhalla, P., Dhawan, D.K., 2008. Zinc modulates lithium-induced hepatotoxicity in rats. *Liver International* 28, 558–565.
- Chen, N., Moshaver, A., Raymond, L.A., 1997. Differential sensitivity of recombinant N-methyl-D-aspartate receptor subtypes to zinc inhibition. *Molecular Pharmacology* 51, 1015–1023.
- Chung, M.J., Hogstrand, C., Lee, S.J., 2006. Cytotoxicity of nitric oxide is alleviated by zinc-mediated expression of antioxidant genes. *Experimental Biology and Medicine* (Maywood, NJ) 231, 1555–1563.
- Cohen-Kfir, E., Lee, W., Eskandari, S., Nelson, N., 2005. Zinc inhibition of gamma-aminobutyric acid transporter 4 (GAT4) reveals a link between excitatory and inhibitory neurotransmission. *Proceedings of the National Academy of Sciences of the United States of America* 102, 6154–6159.
- Cole, T.B., Robbins, C.A., Wenzel, H.J., Schwartzkroin, P.A., Palmiter, R.D., 2000. Seizures and neuronal damage in mice lacking vesicular zinc. *Epilepsy Research* 39, 153–169.
- da Silva, A.P., Meotti, F.C., Santos, A.R., Farina, M., 2006. Lactational exposure to malathion inhibits brain acetylcholinesterase in mice. *Neurotoxicology* 27, 1101–1105.
- Delgado, E.H., Streck, E.L., Quevedo, J.L., Dal-Pizzol, F., 2006. Mitochondrial respiratory dysfunction and oxidative stress after chronic malathion exposure. *Neurochemical Research* 31, 1021–1025.
- Domingo, J.L., Llobet, J.M., Paternain, J.L., Corbella, J., 1988. Acute zinc intoxication: comparison of the antidotal efficacy of several chelating agents. *Veterinary and Human Toxicology* 30, 224–228.
- Dringen, R., Pawlowski, P.G., Hirrlinger, J., 2005. Peroxide detoxification by brain cells. *Journal of Neuroscience Research* 79, 157–165.
- Ellman, G.L., Courtney, K.D., Andres Jr., V., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* 7, 88–95.
- Escobedo, I., Peraile, I., Orío, L., Colado, M.I., O'Shea, E., 2007. Evidence for a role of Hsp70 in the neuroprotection induced by heat shock pre-treatment against 3,4-methylenedioxymethamphetamine toxicity in rat brain. *Journal of Neurochemistry* 101, 1272–1283.
- Flessel, P., Quintana, P.J., Hooper, K., 1993. Genetic toxicity of malathion: a review. *Environmental and Molecular Mutagenesis* 22, 7–17.
- Fortunato, J.J., Agostinho, F.R., Reus, G.Z., Petronilho, F.C., Dal-Pizzol, F., Quevedo, J., 2006a. Lipid peroxidative damage on malathion exposure in rats. *Neurotoxicology Research* 9, 23–28.
- Fortunato, J.J., Feier, G., Vitali, A.M., Petronilho, F.C., Dal-Pizzol, F., Quevedo, J., 2006b. Malathion-induced oxidative stress in rat brain regions. *Neurochemical Research* 31, 671–678.
- Franco, J.L., Posser, T., Brocardo, P.S., Trevisan, R., Uliano-Silva, M., Gabilan, N.H., Santos, A.R., Leal, R.B., Rodrigues, A.L., Farina, M., Dafre, A.L., 2008. Involvement of glutathione, ERK1/2 phosphorylation and BDNF expression in the antidepressant-like effect of zinc in rats. *Behavioural Brain Research* 188, 316–323.
- Franco, J.L., Trivella, D.B., Trevisan, R., Dinslaken, D.F., Marques, M.R., Bainy, A.C., Dafre, A.L., 2006. Antioxidant status and stress proteins in the gills of the brown mussel *Perna perna* exposed to zinc. *Chemico-biological Interactions* 160, 232–240.
- Frederickson, C.J., Koh, J.Y., Bush, A.I., 2005. The neurobiology of zinc in health and disease. *Nature Reviews* 6, 449–462.
- Goel, A., Dani, V., Dhawan, D.K., 2005. Protective effects of zinc on lipid peroxidation, antioxidant enzymes and hepatic histoarchitecture in chlorpyrifos-induced toxicity. *Chemico-biological Interactions* 156, 131–140.
- Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-transferases. *Methods in Enzymology* 77, 398–405.
- Hazarika, A., Sarkar, S.N., Hajare, S., Kataria, M., Malik, J.K., 2003. Influence of malathion pretreatment on the toxicity of anilofos in male rats: a biochemical interaction study. *Toxicology* 185, 1–8.
- Hwang, I.K., Ahn, H.C., Yoo, K.Y., Lee, J.Y., Suh, H.W., Kwon, Y.G., Cho, J.H., Won, M.H., 2007. Changes in immunoreactivity of HSP60 and its neuroprotective effects in the gerbil hippocampal CA1 region induced by transient ischemia. *Experimental Neurology* 208, 247–256.
- John, S., Kale, M., Rathore, N., Bhatnagar, D., 2001. Protective effect of vitamin E in dimethoate and malathion induced oxidative stress in rat erythrocytes. *The Journal of Nutritional Biochemistry* 12, 500–504.
- Kiang, J.G., Tsokos, G.C., 1998. Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacology & Therapeutics* 80, 183–201.
- Kitzberger, R., Madl, C., Ferenci, P., 2005. Wilson disease. *Metabolic Brain Disease* 20, 295–302.
- Klosterhalfen, B., Hauptmann, S., Offner, F.A., Amo-Takyi, B., Tons, C., Winkeltau, G., Affify, M., Kupper, W., Kirkpatrick, C.J., Mittermayer, C., 1997. Induction of heat shock protein 70 by zinc-bis-(DL-hydrogenaspartate) reduces cytokine liberation, apoptosis, and mortality rate in a rat model of LD100 endotoxemia. *Shock* (Augusta, GA) 7, 254–262.
- Koh, J.Y., Suh, S.W., Gwag, B.J., He, Y.Y., Hsu, C.Y., Choi, D.W., 1996. The role of zinc in selective neuronal death after transient global cerebral ischemia. *Science* (New York, NY) 272, 1013–1016.
- Kwong, T.C., 2002. Organophosphate pesticides: biochemistry and clinical toxicology. *Therapeutic Drug Monitoring* 24, 144–149.

- Lee, J.Y., Park, J., Kim, Y.H., Kim, D.H., Kim, C.G., Koh, J.Y., 2000. Induction by synaptic zinc of heat shock protein-70 in hippocampus after kainate seizures. *Experimental Neurology* 161, 433–441.
- Mao, X., Kim, B.E., Wang, F., Eide, D.J., Petris, M.J., 2007. A histidine-rich cluster mediates the ubiquitination and degradation of the human zinc transporter, hZIP4, and protects against zinc cytotoxicity. *The Journal of Biological Chemistry* 282, 6992–7000.
- Maret, W., Sandstead, H.H., 2006. Zinc requirements and the risks and benefits of zinc supplementation. *Journal of Trace Elements in Medicine and Biology* 20, 3–18.
- Masoud, L., Vijayarathay, C., Fernandez-Cabezudo, M., Petroianu, G., Saleh, A.M., 2003. Effect of malathion on apoptosis of murine L929 fibroblasts: a possible mechanism for toxicity in low dose exposure. *Toxicology* 185, 89–102.
- Misra, H.P., Fridovich, I., 1977. Superoxide dismutase: "positive" spectrophotometric assays. *Analytical Biochemistry* 79, 553–560.
- Nowak, G., Szweczyk, B., Pilc, A., 2005. Zinc and depression. An update. *Pharmacological Reports* 57, 713–718.
- Paoletti, P., Ascher, P., Neyton, J., 1997. High-affinity zinc inhibition of NMDA NR1-NR2A receptors. *Journal of Neuroscience* 17, 5711–5725.
- Perrin, V., Regulier, E., Abbas-Terki, T., Hassig, R., Brouillet, E., Aebischer, P., Luthi-Carter, R., Deglon, N., 2007. Neuroprotection by Hsp104 and Hsp27 in lentiviral-based rat models of Huntington's disease. *Molecular Therapy* 15, 903–911.
- Peter, J.V., Moran, J.L., Pichamuthu, K., Chacko, B., 2008. Adjuncts and alternatives to oxime therapy in organophosphate poisoning—is there evidence of benefit in human poisoning? A review. *Anaesthesia and Intensive Care* 36, 339–350.
- Plumier, J.C., Krueger, A.M., Currie, R.W., Kontoyiannis, D., Kollias, G., Pagoulatos, G.N., 1997. Transgenic mice expressing the human inducible Hsp70 have hippocampal neurons resistant to ischemic injury. *Cell Stress & Chaperones* 2, 162–167.
- Posser, T., de Aguiar, C.B., Garcez, R.C., Rossi, F.M., Oliveira, C.S., Trentin, A.G., Neto, V.M., Leal, R.B., 2007. Exposure of C6 glioma cells to Pb(II) increases the phosphorylation of p38(MAPK) and JNK1/2 but not of ERK1/2. *Archives of Toxicology* 81, 407–414.
- Ranjbar, A., Pasalar, P., Abdollahi, M., 2002. Induction of oxidative stress and acetylcholinesterase inhibition in organophosphorous pesticide manufacturing workers. *Human & Experimental Toxicology* 21, 179–182.
- Reus, G.Z., Valvassori, S.S., Nuernberg, H., Comim, C.M., Stringari, R.B., Padilha, P.T., Leffa, D.D., Tavares, P., Dagostim, G., Paula, M.M., Andrade, V.M., Quevedo, J., 2008. DNA damage after acute and chronic treatment with malathion in rats. *Journal of Agricultural and Food Chemistry* 56, 7560–7565.
- Rothlein, J., Rohlman, D., Lasarev, M., Phillips, J., Muniz, J., McCauley, L., 2006. Organophosphate pesticide exposure and neurobehavioral performance in agricultural and non-agricultural Hispanic workers. *Environmental Health Perspectives* 114, 691–696.
- Saleh, A.M., Vijayarathay, C., Masoud, L., Kumar, L., Shahin, A., Kambal, A., 2003. Paraoxon induces apoptosis in EL4 cells via activation of mitochondrial pathways. *Toxicology and Applied Pharmacology* 190, 47–57.
- Sharp, F.R., 1998. Stress genes protect brain. *Annals of Neurology* 44, 581–583.
- Taylor, P., Radic, Z., Hosea, N.A., Camp, S., Marchot, P., Berman, H.A., 1995. Structural bases for the specificity of cholinesterase catalysis and inhibition. *Toxicology Letters* 82–83, 453–458.
- Tons, C., Klosterhalfen, B., Klein, H.M., Rau, H.M., Anurov, M., Oettinger, A., Schumpelick, V., 1997. Induction of heat shock protein 70 (HSP70) by zinc bis (DL-hydrogen aspartate) reduces ischemic small-bowel tissue damage in rats. *Langenbecks Archiv fur Chirurgie* 382, 43–48.
- Unoshima, M., Nishizono, A., Takita-Sonoda, Y., Iwasaka, H., Noguchi, T., 2001. Effects of zinc acetate on splenocytes of endotoxemic mice: enhanced immune response, reduced apoptosis, and increased expression of heat shock protein 70. *The Journal of Laboratory and Clinical Medicine* 137, 28–37.
- Vallee, B.L., Falchuk, K.H., 1993. The biochemical basis of zinc physiology. *Physiological Reviews* 73, 79–118.
- Verma, R.S., Srivastava, N., 2001. Chlorpyrifos induced alterations in levels of thio-barbituric acid reactive substances and glutathione in rat brain. *Indian Journal of Experimental Biology* 39, 174–177.
- Vidair, C.A., 2004. Age dependence of organophosphate and carbamate neurotoxicity in the postnatal rat: extrapolation to the human. *Toxicology and Applied Pharmacology* 196, 287–302.
- Walther, U.J., Czermak, A., Muckter, H., Walther, S.C., Fichtl, B., 2003. Decreased GSSG reductase activity enhances cellular zinc toxicity in three human lung cell lines. *Archives of Toxicology* 77, 131–137.
- Wendel, A., 1981. Glutathione peroxidase. *Methods in Enzymology* 77, 325–333.
- Zhou, Z., Wang, L., Song, Z., Saari, J.T., McClain, C.J., Kang, Y.J., 2005. Zinc supplementation prevents alcoholic liver injury in mice through attenuation of oxidative stress. *The American Journal of Pathology* 166, 1681–1690.