Ecotoxicology and Environmental Safety 74 (2011) 480–486

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Delayed biochemical changes induced by mercury intoxication are prevented by zinc pre-exposure

C. Franciscato [a], L. Moraes-Silva [a], F.A. Duarte [b], C.S. Oliveira [b], R.P. Ineu [a], E.M.M. Flores [b], V.L. Dressler [b], N.C. Peixoto [a,b], M.E. Pereira [a,b,*]

[a] Programa de Pós-Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil
[b] Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil

ARTICLE INFO

Article history:
Received 25 August 2009
Received in revised form
19 October 2010
Accepted 28 November 2010
Available online 22 December 2010

Keywords:
Mercury
Zinc
Porphobilinogen-synthase
Renal insufficiency
Young rats
Body weight
Mercury-induced delayed effects
More sensitive rats to mercury
Less sensitive rats to mercury

ABSTRACT

This work evaluated the delayed effects of mercury and the effectiveness of zinc in preventing such effects. Pups were pre-treated with 1 daily dose of $ZnCl_2$ (27 mg/kg/day, by subcutaneous injections) from 3rd to 7th postnatal day and received 1 daily dose of 5 mg/kg of $HgCl_2$, for 5 subsequent days (8–12 days old). Animals were euthanized 21 days after the end of Hg-exposure. Porphobilinogen-synthase activity as well as zinc and mercury contents was determined in the liver and kidneys. Alanine aminotransferase, aspartate aminotransferase and lactic dehydrogenase activities as well as urea, creatinine and glucose levels were analyzed in plasma or serum. Some animals were considered more sensitive to mercury, since they did not recover the body weight gain and presented an increase of renal and hepatic mercury content, urea and creatinine levels; a decrease in renal porphobilinogen-synthase and alanine aminotransferase activities, as well as a decrease in the liver and an increase in kidney weights. Some animals were considered less sensitive to mercury because they recovered the body weight and presented no biochemical alterations in spite of mercury in the tissues. Zinc prevents partially or totally the alterations caused by mercury even those that persisted for a long time after the end of exposure. These findings suggest that there is difference among the animals regarding the sensitivity to mercury.

1. Introduction

Mercury is a hazardous pollutant and can be found in three different chemical forms: elemental mercury vapor, inorganic mercury and organic mercury. The toxicity of this metal depends on its chemical form (Clarkson, 1997). The exposure to inorganic mercury, which is the object of this study, is mainly occupational-like, occurring in industrial activities and mining (Nevado et al., 2003). In fact, the exposure to this form of mercury causes serious risks to gold miners' health (Barregard, 2008). It is also known that inorganic mercury can be alkylated to methylmercury by anaerobic bacterium in water or in sediments (Bisinoti and Jardim, 2004; Wasserman et al., 2003). Organic mercury accumulates in the aquatic food chain through the process of biomagnification, i.e., the metal concentration increases in living organisms when passing from herbivores to carnivorous animals (Boening, 2000).

Although the inorganic form, mercury chloride, is known as a nephrotoxic agent (Clarkson, 1997; Emanuelli et al., 1996; Goyer, 1995), it causes renal insufficiency, produces changes in body and organ weights and decreases the serum alanine aminotransferase activity of rats exposed to metal during the precocious phase of development (8–12th day of age) (Peixoto et al., 2003, 2007a; Peixoto and Pereira, 2007).

Other researchers have demonstrated that inorganic mercury causes hepatotoxicity, since adult mice treated with one dose of $HgCl_2$ (5 mg/kg) showed significant elevation in serum alanine (ALT) and aspartate (AST) aminotransferase activities (Kumar et al., 2005). In fact, these enzymes are known as important markers of hepatocellular damage (Meyer et al., 1992).

The large affinity of mercury by sulfhydryl groups is an important contributor to its toxicity (Clarkson, 1997). Therefore, porphobilinogen-synthase [PBG-synthase (E.C.:4.2.1.24)], an enzyme involved in the heme biosynthesis with high content of sulfhydryl residues (Sassa, 1982), has its activity inhibited by toxic metals in different tissues from mice (Emanuelli et al., 1996), rats (Peixoto et al., 2003, 2007a; Rocha et al., 1993, 1995, 2001), and humans (Calderón et al., 2003). This enzyme requires zinc for its maximum activity, although the precise function of this metal is not clear (Bevan et al., 1980; Hasnain et al., 1985). However, it is known that PBG-synthase contains two sites binding to zinc with different affinities (Dent et al., 1990). The B site of the enzyme binds Zn^{2+} with relatively low affinity, and the maintenance of their sulfhydryl groups in a reduced state is fundamental for the enzyme activity (Jaffe, 1995, Barbosa et al., 1998).

Zinc is an essential metal involved in several metabolic functions (Fang et al., 2002). It is a catalytic and structural element of

* Corresponding author at: UFSM, CCNE, Departamento de Química, Campus Universitario, Camobi, 97105-900 Santa Maria, RS, Brazil. Fax: +55 55 32208799.
E-mail address: pereirame@yahoo.com.br (M.E. Pereira).

0147-6513/
doi:10.1016/j.ecoenv.2010.11.011

several proteins (Mathie et al., 2006) and plays an important role in the metallothionein synthesis (Pedersen et al., 1998; Peixoto et al., 2003, 2007b). However, an excessive intracellular increase of zinc can be cytotoxic and causes, for instance, neuronal degeneration (Choi and Koh, 1998). Our previous results suggest zinc as a protector agent against several mercury toxic effects in rats. In fact, zinc pre-treatment avoids the hepatic and renal PBG-synthase inhibition, the decrease in body weight gain (Peixoto et al., 2003, 2007b) and the renal insufficiency (Peixoto and Pereira, 2007) induced by mercury in rats exposed to it during the second phase of development (8–12th day of age). This developmental period is characterized by rapid protein, DNA, and RNA synthesis (Gottileb et al., 1977).

In fact, rodents have three principal postnatal phases (0–6, 8–12 and 17–23 days old) characterized by remarkable development and growth of organs and body (Gottlieb et al., 1977; Winick and Noble, 1965). Thus, insults, even those considered mild, may cause pronounced damages when applied to developing organisms (Smart and Dobbing, 1971). Therefore, animals in their early life stage are particularly sensitive to insults such as heavy metal exposure (Franciscato et al., 2009; Peixoto et al., 2003, 2007c; Rocha et al., 1993).

It is known that postnatal exposure to mercury causes behavioral (Peixoto et al., 2007c) and biochemical alterations in rats (Peixoto et al., 2003; Peixoto and Pereira, 2007) even with an elapsed time after exposure (Franciscato et al., 2009; Rocha et al., 2001; Stringari et al., 2006). Recently, we verified that there are rats more and less sensitive to mercury when exposed to it during developmental period. These subgroups of animals were classified according to body development after the end of mercury exposure. Mercury exposed rats that recovered the body weight gain did not show behavioral alterations (Franciscato et al., 2009).

The aim of this study was to evaluate the toxic effects of mercury in 33-day-old rats exposed to metal during one of the developmental postnatal phases (8–12 days old), as well as to verify the effectiveness of zinc as preventive treatment. We investigated if animals present different sensitivities to mercury regarding biochemical parameters. For this, we evaluated body and organ weights, mercury and zinc levels, and PBG-synthase activity. We also investigated ALT, AST, and lactic dehydrogenase (LDH) activities as hepatic damage markers (Devlin, 1997), and urea and creatinine levels as renal function markers (Meyer et al., 1992). The blood glucose level was evaluated since at 24 h after mercury exposure rats presented hypoglycemia (Peixoto and Pereira, 2007).

2. Materials and methods

2.1. Chemicals

Zinc ($ZnCl_2$), mercury ($HgCl_2$) , sodium chloride (NaCl), glacial acetic acid, *ortho*-phosphoric acid, nitric acid (HNO_3), perchloric acid, absolute ethanol as well as ethyl ether were obtained from Merck (Rio de Janeiro, RJ, Brazil); δ-aminolevulinic acid (δ-ALA), Coomassie brilliant blue G, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA); trichloroacetic acid was obtained from Reagen (Colombo, PR, Brazil) and *para*-dimethylaminobenzaldehyde was obtained from Riedel (Seelze, Han, Germany). The kits for the determination of creatinine, urea, glucose, ALT, AST, and LDH were obtained from Labtest (Lagoa Santa, MG, Brazil).

2.2. 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 at room temperature (23 ± 2 °C). One day after the birth, the number of pups of each litter was reduced to 8 to avoid undernutrition effect due to the number of teats. Studies were conducted in accordance with the national and institutional guidelines (University Ethics Committee Guidelines—Process number 23081.014805/2007–68) for experiments with animals.

2.3. Treatments

Pups were pre-treated from postnatal days 3–7 of life with one daily dose of saline (NaCl 90 mg/kg/day) or $ZnCl_2$ (27 mg/kg/day). After the pre-treatment, the animals received saline or $HgCl_2$ (5 mg/kg/day) for 5 consecutive days (from 8 to 12 days old). These doses were chosen according to Peixoto et al. (2003), who showed that 5 mg/kg/day of $HgCl_2$ caused toxic effects that was prevented by zinc (27 mg/kg/day) dose in rats killed 24 h after the end of mercury exposure. In the present study, the same model of treatment was used; however, the animals were killed 21 days after mercury exposure. Treatments were administered by subcutaneous (s.c.) injections in a constant volume of 10 mL/kg body weight. Animals were weighed daily to adjust the dose. A total of 34 litters were exposed to treatments. Twenty-seven litters were used for biochemical analysis and metal quantification, since from other seven litters, two animals of the same treatment died before the end of the experimental period. Each litter contributed with only one *n* to each experimental group. Two rats per litter were assigned to each of the four experimental groups. At the end of experimental period (33 days old) the litters were divided in two groups according to their sensitivities to mercury. Thus, the experiment was performed with rats that were 13 days old but divided into two groups: one group containing rats that were more sensitive to mercury (MSR) and the second group containing rats that were less sensitive to mercury (LSR). The groups were confirmed by correlations between body weight and mercury contents in cerebrum and cerebellum (Franciscato et al., 2009).

2.4. Tissue preparation

Twenty-one days after the end of mercury exposure (33 days old), the animals were weighed, anesthetized and euthanized by decapitation. Liver and kidneys were removed, weighed and minced. Portions of each organ were used for the determination of the enzymatic activity and for the analysis of metal contents.

For the PBG-synthase activity determination, the liver and kidneys were homogenized in 7 and 5 volumes of 150 mM of NaCl, respectively. Homogenates were centrifuged at 8000*g* for 30 min to obtain the supernatant with the enzymatic material (Peixoto et al., 2003, 2004; Roza et al., 2005). For metal content a portion of each tissue was frozen at −20 °C until analysis.

For other biochemical analysis the plasma was obtained from heparinized blood and the serum was obtained from blood without anticoagulant. Both plasma and serum were separated by centrifugation at 3000*g* for 10 min, and were frozen until analysis (up to 5 days). Another portion of blood (around 0.5 mL) was frozen for the analysis of metal contents.

2.5. Biochemical determinations

2.5.1. PBG-synthase activity

PBG-synthase activity was determined according to the method of Sassa (1982) by measuring the rate of formation of the product (porphobilinogen), except that 76 mM potassium phosphate buffer (pH 6.8) and 2.2 mM δ-ALA were used (Peixoto et al., 2003; 2004; Roza et al., 2005). The reaction product was determined using the modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×10^4 for the Ehrlich-porphobilinogen salt. The incubation was initiated by adding 100 μL of tissue preparation and was carried out for 40 and 90 min for liver and kidney, respectively, at 39 °C. The specific activity was expressed as nmol of PBG formed per hour per mg of protein. Protein concentrations in the samples were determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as standard. All samples were run in triplicate.

2.5.2. ALT and AST activity

The ALT activity was determined in a medium containing buffer 55.8 mmol/L, α-ketoglutaric acid 1.67 mmol/L, L-alanine 83.3 mmol/L, sodium azide 12.8 mmol/L and 25 μL of serum incubated at 37 °C for 30 min. The reaction was stopped by adding HCl 0.45 mmol/L. The color reactive (2.4-dinitrophenylhydrazine 0.45 mmol/L) was added and the medium was incubated for 20 min at room temperature. The color was intensified by NaOH 0.33 mmol/L and the absorbance was determined at 505 nm. The activity (in U/mL) was calculated by comparing with a calibration curve utilizing sodium pyruvate as standard. The AST activity was determined similarly to ALT enzyme, except that L-aspartic acid 83.3 mmol/L was used as substrate and that the medium was incubated at 37 °C for 60 min.

2.5.3. LDH activity

The activity of this enzyme (in U/L) was determined by the formation of NADH. The medium containing buffer 200 mmol/L pH 8.2, lactic acid 260 mmol/L, sodium azide 7.7 mmol/L and 25 μL of plasma was incubated at 37 °C for 2 min. After this period, the color reactive (INT 0.64 mmol/L, NAD+ 1.2 mmol/L, phenazine 0.26 mmol/L and sodium azide 1.23 mmol/L) was added and the medium was incubated for another 5 min at 37 °C. The reaction was stopped by adding HCl 200 mmol/L and the tubes remained at room temperature for 5 min until the reading of the absorbance at 500 nm.

2.5.4. Creatinine

The determination of creatinine (mg/dL) was carried out by measuring the quantity of creatinine picrate formed at 510 nm. The medium containing picric acid 7.79 mmol/L, NaOH 145.9 mmol/L and 100 μL of serum was incubated at 37 °C for 10 min. After this period of incubation, the first absorbance was determined. The acetic acid 0.4 mmol/L was added and the medium was incubated for another 5 min at room temperature before determining the second absorbance.

2.5.5. Urea

Urea (mg/dL) was determined by the quantity of indophenol blue formed at 600 nm. The medium containing phosphate buffer 19.34 mmol/L, sodium salicylate 58.84 mmol/L, sodium nitroprusside 3.17 mmol/L, urease (≥ 12.63 UK/L) and 10 μL of serum was incubated at 37 °C for 5 min.

2.5.6. Glucose

The reaction for the determination of the glucose concentration was conducted in a medium containing phosphate buffer 9.9 mmol/L, phenol 9.9 mmol/L, glucose oxidase (≥ 12,000 U/L), peroxidase (≥ 1000 U/L), 4-amino-antipyrine 0.4 mmol/L and 10 μL of serum that was incubated at 37 °C for 10 min. The product formed from the complete oxidation of the glucose is a red-violet quinoneimine, whose absorbance was measured at 500 nm.

2.6. Metal content determination

The samples of wet tissue (0.5 g of kidney and 2 g of liver) and blood (0.5 mL) were placed in vials and frozen at −20 °C until analysis.

The digestion of samples and the determination of Zn and Hg contents were conducted as described in detail by Peixoto et al. (2008). Samples were digested with HNO_3 using a Model Multiwave 3000 microwave oven equipped with high-pressure quartz vessels (max 80 bar, 280 °C, Anton Paar, Graz, Austria). After digestion, samples were diluted with water to 25 mL and transferred to graduated polypropylene vials. Colorless and clear solutions were obtained after the digestion step. Spike recovery tests and biological certified reference material (SRM NIST 1577, bovine liver) were carried out to validate the results. Blanks were run and analyzed after each 10 measurements in order to check eventual memory effects for all elements.

Metal analyzes were carried out using a Model AAS EA 5 atomic absorption spectrometer (Analytik Jena, Jena, Germany) equipped with a transversely heated graphite tube atomizer with pyrolytic coated tubes for zinc. A batch-operated chemical vapor generation system, HS 5 (Analytik Jena, Jena, Germany), was adapted to this equipment for mercury determinations. A deuterium background corrector was used for all determinations. Hollow cathode lamps were operated at 4 mA. Wavelength was set at 213.9 and 253.7 nm for Zn and Hg, respectively, and the spectral band passes at 0.5 nm. Integrated absorbance (peak area) was used for all measurements. Heating program for Zn was carried out according to the recommendations of the manufacturer. For Hg determination, 3 M HCl and 0.25% m/v $NaBH_4$ solutions were used as acid medium and reductant, respectively. Argon was used as purge gas. Results for all elements determined were periodically evaluated by measurements of analytical standards (each 10 measurements) as well as by the digests analysis of certified reference material SRM NIST 1577 (each 3 measurements). When the result for standard checking presented a bias higher than 5%, a recalibration procedure was performed.

2.7. Statistical analysis

Results were analyzed by the non-parametric method of the Kruskal–Wallis test with Dunn's post-hoc test when appropriate. Effects were considered significant when $p \leq 0.05$.

3. Results

The body weight gain was used as a parameter to classify the animals in more or less sensitive to Hg. Thus, animals (27 litters) were divided into two groups. The first group ($n=15$), MSR litters, comprised the litters whose mercury exposed rats did not recover the impairment in body weight until 33 days old (Kruskal–Wallis test, $H(3)=27.6$, $p<0.0001$). The second group ($n=12$), LSR litters, corresponded to litters whose mercury exposed rats recuperated the body weight gain and presented body weight similar to control group starting at 14 days old (Table 1). The presence of more and less sensitive rats to mercury was previously demonstrated in a recent study and these definitions were confirmed by correlations between body weight and mercury contents in cerebrum and cerebellum, where for MSR litters, the body weight decreased significantly with the increase in mercury levels in both cerebrum ($r=0.73$, $p<0.001$) and cerebellum ($r=0.76$, $p<0.001$). On the other hand, there was no relationship between these two parameters for LSR litters (Franciscato et al., 2009).

3.1. Liver and kidney weights

The Kruskal–Wallis test revealed a significant effect of treatment on liver [$H(3)=31.71$, $p<0.0001$] and kidney [$H(3)=26.06$, $p<0.0001$] weights of MSR litters (Table 1). Sal-Hg group presented liver weight significantly lower and kidney weight significantly higher than the other groups (Dunn's post-hoc test: $p<0.05$). Regarding LSR litters, the mercury treatment did not alter these parameters. The presence of more and less sensitive rats to mercury was also observed when considered the relationship (Pearson correlations) between body and renal weights. For MSR litters, there was a negative correlation between body and renal weights, i.e., the body weight decreases significantly with the increase of the renal weight ($r=0.46$, $p<0.05$). However, for LSR litters, there was a positive correlation between body and renal weight ($r=0.71$, $p<0.05$). These different correlations confirm that the MSR litters contain animals that presented impairment in body weight gain and renal damage, whereas the LSR litters contain the animals that do not present alterations induced by mercury at 33 days old.

Table 1
Body, liver and kidney weights of rats treated (s.c.) with saline or $ZnCl_2$ (27 mg/kg/day), from 3rd to 7th, and with saline or $HgCl_2$ (5 mg/kg/day), from 8th to 12th day old.

Animals	Treatment groups	Weight (g)			
		Body 13 days old ($n=15$)	Body 33 days old ($n=15$)	Liver ($n=15$)	Kidney ($n=15$)
MSR	Sal–Sal	25.8[a] (23.4/27.2)	93.9[a] (86.1/99.2)	4.43[a] (4.00/5.30)	0.86[a] (0.70/0.96)
	Sal–Hg	21.0[b] (19.2/23.0)	60.0[b] (35.5/74.9)	3.06[b] (1.80/3.70)	1.50[b] (1.20/2.10)
	Zn–Sal	25.1[a] (22.3/27.4)	95.7[a] (87.2/98.9)	4.60[a] (4.40/5.20)	0.90[a] (0.80/1.00)
	Zn–Hg	24.0[a,b] (21.0/26.0)	91.0[a] (83.5/96.2)	4.40[a] (4.00/4.80)	0.98[a] (0.89/1.04)
		($n=12$)	($n=12$)	($n=12$)	($n=12$)
LSR	Sal–Sal	25.35[a] (24.4/25.9)	90.7 (86.9/101.1)	4.70 (4.10/5.40)	0.90 (0.83/1.10)
	Sal–Hg	22.2[b] (20.9/24.8)	85.4 (78.1/97.7)	4.60 (4.20/4.70)	1.05 (0.89/1.20)
	Zn–Sal	24.2[a,b] (23.5/25.5)	91.4 (84.4/98.6)	4.80 (3.80/5.30)	0.97 (0.80/1.03)
	Zn–Hg	23.9[a,b] (22.4/24.7)	90.4 (79.7/97.3)	4.80 (4.50/5.10)	0.99 (0.95/1.10)

The results are presented as median (interquartile intervals). Dunn's post-hoc test: different letters confer significant statistical difference among groups ($p<0.05$).

3.2. Renal and hepatic PBG-synthase activity

Renal and hepatic PBG-synthase activities are shown in Fig. 1. For MSR litters, the Kruskal–Wallis test revealed a significant effect of treatment on renal [$H(3)=12.36$, $p<0.01$] but not on hepatic PBG-synthase activity. Duncan's multiple range test showed that the Sal–Hg rats presented a significant inhibition in renal PBG-synthase activity when compared to Sal–Sal and Zn–Hg groups ($p<0.05$). For LSR litters, renal and hepatic enzyme activities were similar between the groups.

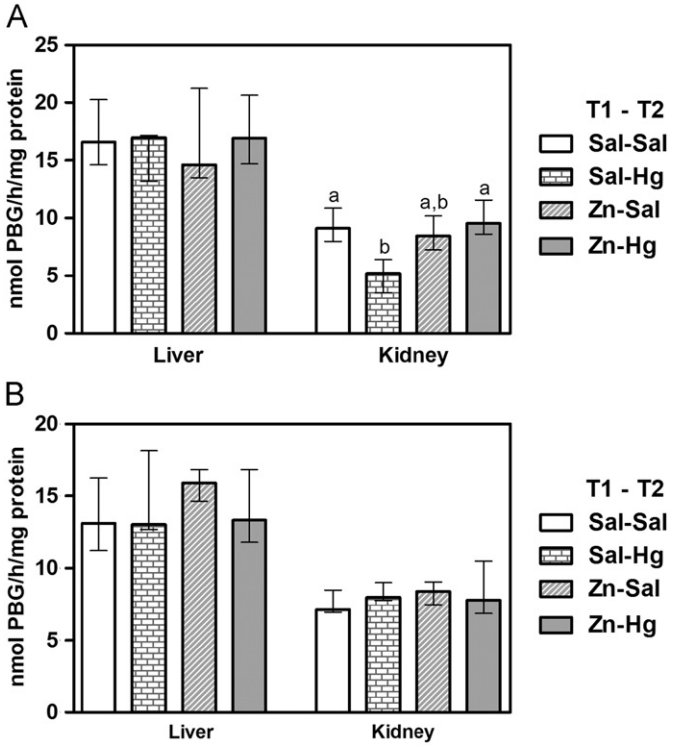

Fig. 1. Hepatic and renal PBG-synthase activity of more sensitive (MSR litters, $n=5$) (A) and less sensitive (LSR litters, $n=4$) (B) rats treated as described in Table 1. The results are presented as median (interquartile intervals). Dunn's post-hoc test: different letters confer significant statistical difference among groups ($p<0.05$).

3.3. Hepatic toxicity

Parameters of hepatic toxicity (ALT, AST and LDH activities) are shown in Table 2. For MSR litters, the Kruskal–Wallis test revealed a significant effect of the treatment on ALT activity [$H(3)=10.83$, $p<0.05$]. Sal–Hg rats presented ALT activity significantly lower than the control group. Zinc pre-exposure partially prevented this decrease (Dunn's post-hoc test: $p<0.05$). AST and LDH activities were not altered by treatments. For LSR litters, enzyme activities were similar between the groups.

3.4. Renal toxicity

Parameters of renal function (creatinine and urea levels) are shown in Table 3. For MSR litters, Kruskal–Wallis test revealed a significant effect of treatment on creatinine and urea levels [$H(3)=13.18$, $p<0.001$] and [$H(3)=17.17$, $p<0.001$, respectively]. Sal–Hg rats showed creatinine and urea levels significantly higher than Sal–Sal and Zn–Sal rats. Zinc pre-exposure partially prevented this increase (Dunn's post-hoc test: $p<0.05$). For LSR litters, statistical analysis showed absence of mercury effect on these metabolite levels.

3.5. Glucose

Treatments altered serum glucose levels neither for MSR nor LSR litters (Kruskal–Wallis test) (Table 3).

Table 2
Serum ALT and AST and plasma LDH activity of rats treated as described in Table 1.

Animals	Treatment	ALT (U/mL)	AST (U/mL)	LDH (U/L)
MSR ($n=5$–6)	Sal–Sal	71.0[a] (61.5/74.5)	126.0 (120.0/131.5)	248.6 (163.7/299.9)
	Sal–Hg	24.0[b] (19.0/32.0)	122.0 (115.5/124.0)	170.2 (108.0/258.0)
	Zn–Sal	62.0[a,b] (53.5/83.5)	121.0 (112.0/138.0)	242.6 (142.8/326.0)
	Zn–Hg	58.0[a,b] (55.0/81.0)	131.0 (124.5/138.0)	315.5 (259.4/336.7)
LSR ($n=3$–4)	Sal–Sal	54.0 (39.75/64.0)	108.5 (103.5/121.0)	158.2 (153.1/418.5)
	Sal–Hg	56.5 (39.75/62.0)	120.0 (105.3/122.8)	174.2 (155.5/325.4)
	Zn–Sal	51.0 (34.25/69.25)	109.5 (100.0/119.8)	239.0 (178.9/333.9)
	Zn–Hg	52.0 (45.5/62.25)	120.0 (110.3/125.3)	213.3 (187.5/463.1)

The results are presented as median (interquartile intervals). Dunn's post-hoc test: different letters confer significant statistical difference among groups ($p<0.05$).

Table 3
Serum creatinine, urea and glucose of rats treated as described in Table 1.

Animals	Treatment	Creatinine (mg/dL)	Urea (mg/dL)	Glucose (mg/dL)
MSR ($n=6$)	Sal–Sal	0.46[a] (0.38/1.00)	45.9[a] (43.1/52.8)	147.9 (135.0/177.9)
	Sal–Hg	1.74[b] (1.39/2.10)	156.2[b] (99.7/173.2)	125.4 (95.2/155.6)
	Zn–Sal	0.65[a] (0.59/0.76)	46.5[a] (40.6/52.0)	145.5 (117.4/164.5)
	Zn–Hg	0.72[a,b] (0.59/1.03)	61.5[a,b] (52.1/83.3)	137.6 (124.7/150.6)
LSR ($n=4$)	Sal–Sal	0.44 (0.36/0.52)	49.4 (40.2/57.4)	135.4 (120.0/171.7)
	Sal–Hg	0.53 (0.34/0.64)	56.8 (44.8/70.8)	168.5 (112.9/216.1)
	Zn–Sal	0.74 (0.43/1.10)	50.9 (46.5/52.6)	153.6 (144.9/180.7)
	Zn–Hg	0.60 (0.47/0.83)	61.1 (56.4/74.4)	142.8 (125.0/159.0)

The results are presented as median (interquartile intervals). Dunn's post-hoc test: different letters confer significant statistical difference among groups ($p<0.05$).

3.6. Metal content

3.6.1. Mercury levels

Hepatic and renal mercury contents are shown in Fig. 2. For MSR litters (Fig. 2A), the Kruskal–Wallis test showed significant effect of treatment on hepatic [$H(3)=20.34$, $p<0.0001$] and renal [$H(3)=18.89$, $p<0.0001$] Hg contents. Sal-Hg rats presented hepatic Hg levels significantly higher than Sal–Sal and Zn–Sal rats. Zinc pre-exposure partially prevented this increase (Dunn's post-hoc test: $p<0.05$). Regarding renal mercury levels, the Sal–Hg group presented a partial increase of renal mercury content when compared to Sal–Sal and Zn–Sal rats. However, zinc pre-treatment induced an additional increase in renal mercury levels when compared to the control group (Dunn's post-hoc test: $p<0.05$).

For LSR litters (Fig. 2B), the Kruskal–Wallis test showed absence of mercury effect on hepatic and renal mercury content.

Treatments altered the blood mercury levels neither for MSR nor for LSR litter (data not shown).

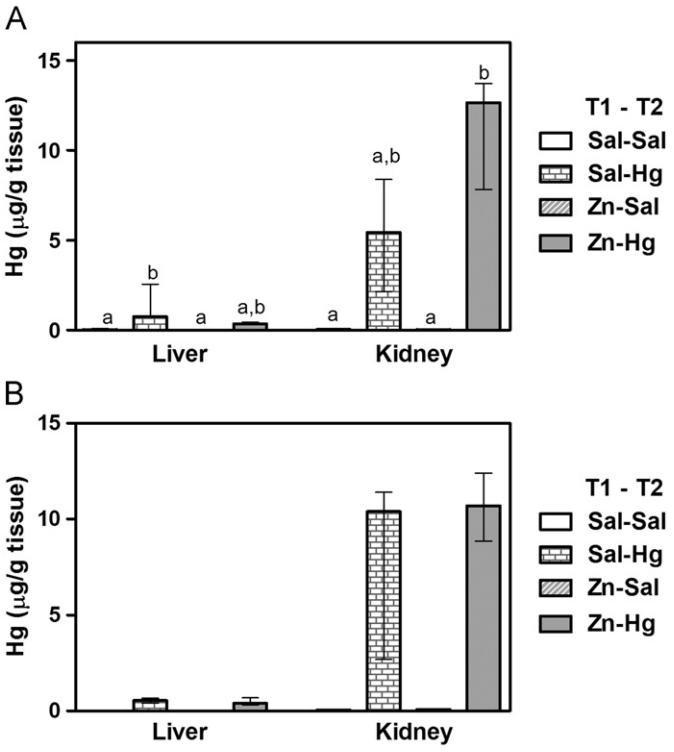

Fig. 2. Hepatic and renal mercury content of more sensitive (MSR litters, $n=6$) (A) and less sensitive (LSR litters, $n=3$) (B) rats treated as described in Table 1. The results are presented as median (interquartile intervals). Dunn's post-hoc test: different letters confer significant statistical difference among groups ($p<0.05$).

3.6.2. Zinc content

Treatments neither altered blood, hepatic and renal zinc contents neither for MSR nor for LSR litters (Kruskal–Wallis test) (Table 4).

4. Discussion

This research investigated the delayed effect of mercury in rats exposed to this metal during the second phase of postnatal development (8–12th day of age), evaluating body weight gain, organ weight, PBG-synthase activity, mercury and zinc content, as well as hepatic and renal damage. Moreover, we verified the effectiveness of zinc in protecting against toxic effects caused by mercury in MSR litters.

This study confirms that there are rats more and less sensitive to mercury for both biochemical and physiological parameters. Previously, using the same protocol of treatment, we observed that young rats exposed to mercury presented different sensitivity regarding behavioral alterations (Franciscato et al., 2009). At 12 and 13 days old, i.e., after the 4th and 5th days of exposure, all Hg-exposed rats presented body weight significantly lower than other treatment groups (data not shown). However, 33 days old, it was observed that there are litters whose Hg-animals recovered the body weight [called litter containing less sensitive rats (LSR) to mercury] and litters whose Hg-animals did not recover the body weight gain [called litter containing more sensitive rats (MSR) to mercury] (Franciscato et al., 2009).

In humans, the different responses to toxic metals have been attributed to a genetic predisposition. In fact, researchers have described a polymorphism within the human gene encoding coproporphyrinogen oxidase (an enzyme of the heme biosynthetic pathway), which significantly modifies the effects of mercury on urinary porphyrin excretion (Woods et al., 2005) and also increases the vulnerability to mercury-induced neurotoxicity (Echeverria et al., 2006). Still, data suggest that a genetic predisposition may increase vulnerability leading to toxicity during critical windows of prenatal and postnatal neurodevelopment (Rose et al., 2008). Therefore, the different sensitivity to mercury presented by the animals studied here is likely to be due to some genetic modifications.

Body weight alterations in MSR litters occurred in parallel to a decrease in liver weight and an increase in kidney weight. These effects had already been found 24 h after Hg-exposure (Peixoto et al., 2003). We can observe that these effects persist even after a long time elapsed from mercury intoxication. Interestingly, these mercury effects are totally prevented by previous exposure to zinc, when the parameters are evaluated at 33 and at 13 day (Peixoto et al., 2003) (21 days or 24 h after mercury exposure, respectively).

In this study the PBG-synthase activity was inhibited for a long time after the end of exposure. This is an important aspect, since in

Table 4
Blood, hepatic and renal zinc content (μg of Zn/g of wet tissue) of rats treated as described in Table 1.

Animals	Treatment	Blood (μg/g)	Hepatic (μg/g)	Renal (μg/g)
MSR ($n=5$–6)	Sal–Sal	4.78 (4.19/5.54)	25.25 (23.75/25.73)	22.05 (15.75/23.43)
	Sal–Hg	4.79 (4.13/4.89)	26.70 (26.00/34.93)	17.80 (10.63/22.33)
	Zn–Sal	4.70 (4.27/4.96)	24.80 (24.15/27.28)	22.95 (15.3/24.93)
	Zn–Hg	4.85 (4.27/4.92)	25.75 (24.10/26.88)	27.90 (18.10/29.13)
LSR ($n=3$)	Sal–Sal	4.35 (4.30/4.53)	25.80 (22.30/27.90)	24.40 (23.40/25.50)
	Sal–Hg	3.31 (2.33/4.03)	25.40 (24.00/26.00)	26.20 (13.70/27.80)
	Zn–Sal	4.33 (4.15/4.49)	26.10 (24.50/30.90)	25.60 (24.60/25.90)
	Zn–Hg	4.35 (4.16/4.40)	25.00 (26.10/26.30)	26.50 (24.20/27.90)

The results are presented as median (interquartile intervals).

most of the studies the animals are euthanized 24 h after exposure (Peixoto et al., 2003, 2007a). Results showed that only renal PBG-synthase activity from MSR litters, but not from LSR litters, presented inhibition by mercury and that this inhibitory effect was prevented by zinc pre-exposure. For liver, the absence of PBG-synthase inhibition may be due to the fact that this organ presented small mercury content (see below). However, mercury content does not explain the absence of Hg effect on renal enzyme activity from LSR litters (Hg exposed rats presented high Hg content) as well as of Zn–Hg rats from MSR litters (Hg content higher than Sal–Hg rats).

Amino transferases and LDH enzymes are sensitive indicators of hepatocellular damage therefore an increase in these serum activities can represent liver lesion (Devlin, 1997; Meyer et al., 1992). Regarding MSR litters, results revealed that AST and LDH activities were not altered by mercury exposure. However, we observed a reduction of 66.2% in ALT activity 21 days after the mercury intoxication, since this effect was partially prevented when the animals were pre-exposed to zinc. Previously, Peixoto and Pereira (2007) showed a reduction of 40% in this enzyme activity 24 h after Hg-exposure, which was prevented by zinc exposure. Thus, we can conclude that the mercury effect continues for a long time after metal intoxication and that this alteration does not indicate hepatic damage. Our results differ from those reported by Kumar et al. (2005) who found elevation in serum ALT and AST activities of adult mice 1, 3, 7, 15 and 30 days after the exposure to one dose of $HgCl_2$ (5 mg/kg) intraperitonially, revealing hepatotoxicity. Still, we observed that these parameters were not modified in LSR litters that also presented low Hg-hepatic content (see below).

Different from hepatic parameters, the renal function of MSR litters, but not of LSR litters, was significantly altered by mercury exposure, reinforcing the previous finding indicating inorganic mercury as a potent nephrotoxic agent (Clarkson, 1997; Emanuelli et al., 1996; Goyer, 1995; Peixoto and Pereira, 2007). Twenty-one days after the end of mercury exposure, rats presented an increase in serum creatinine and urea levels of 278% and 240%, respectively. When animals were pre-exposed to zinc, this effect was partially prevented, indicating zinc pre-exposure as an important preventive treatment against mercury-induced nephrotoxicity. Similar results about renal toxicity and zinc preventive action were also obtained when animals were euthanized 24 h after the end of mercury exposure (Peixoto and Pereira, 2007). Even 21 days after the end of exposure, MSR litters, presented an increase of mercury content in both liver and kidney tissues. However, it is interesting to observe that the level of mercury found in kidney was around 7.3-fold higher than in liver. This result differs from those obtained when animals were euthanized 24 h after exposure, which presented liver mercury levels higher than renal mercury levels (Peixoto et al., 2003, 2007b). Still, we can observe that the content of mercury in both tissues was reduced as a function of the long interval after exposure (at 24 h, ~40 and ~70 μg/g, kidney and liver, respectively (Peixoto et al., 2003); at 21 days, ~5 and ~0.7 μg/g, kidney and liver, respectively). This fact may be related to an attempt of the organism to eliminate the metal. In fact, several studies have demonstrated the formation of complexes such as metallothionein–Hg (Goering, Klaassen, 1983; Peixoto et al., 2007b), glutathione–Hg and cysteine–Hg–cysteine. Furthermore, mercury bound to these small-molecular-weight thiols is easily taken up by kidney, causing accumulation of the metal in this organ (Zalups, 1998).

Blood mercury contents were similar among all groups, showing that the high blood Hg level presented 24 h after exposure (Peixoto et al., 2007b) is reduced after 21 days. This reduction is probably due to the elimination of this metal from the bloodstream by renal filtration and tissue uptake, which occurs during this long interval (21 days) between intoxication and euthanasia. In fact, mercury is eliminated from the body in the urine and feces, and its half-life is around 20 days (Timbrell, 2008).

Blood and tissue zinc levels were not altered after a long time (26 days) elapsed from zinc exposure. However, literature data have shown that exposure to zinc chloride induces increase of zinc content in the liver (around 400%) and kidney (around 130%), but not in the blood from rats euthanized 6 days after exposure to zinc (Peixoto et al., 2008). This suggests that zinc was redistributed by tissues, since it is an essential metal (Fang et al., 2002).

Despite the lower zinc levels found in 33-day-old rats, zinc-exposure caused a reduction of two-fold in hepatic mercury levels and an increase of two-fold in renal mercury levels. Similar results were found by Peixoto et al. (2003), (2007a), (2008) in rats euthanized 24 h after mercury exposure. Literature data suggest that zinc is a preventive agent against mercury-induced toxicity and this effect can be attributed to metallothionein synthesis induced by zinc pre-exposure (Peixoto et al., 2003, 2007a). In fact, an increase in liver and kidney metallothionein content was found in rats exposed to zinc (Peixoto et al., 2007a), since the liver is the main organ involved in the synthesis of this metalloprotein (Peixoto et al., 2003; Tandon et al., 2001). Thus, the mercury bound to metallothionein synthesized in the liver can be transported to kidney where it continues to be deposited. Although rats pre-exposed to zinc presented larger kidney mercury levels, this toxic metal seems to be unavailable to cause effects, probably due to its binding to metallothionein, explaining the protective effect of zinc.

Regarding LSR litters, despite that Hg-exposed rats presented high mercury content in renal tissues, these Hg levels were not significantly different from the control group, probably due to reduced n and to interquartile intervals of medians.

This study shows that there is difference among the animals regarding the sensitivity to mercury. In MSR litters, mercury accumulates in the tissues and its toxic effects persist for a long time after the end of exposure, causing renal insufficiency and inhibition of the PBG-synthase activity. In addition, zinc is an effective protector against mercury toxicity in MSR litters, even when these analyzes are conducted a long time after exposure. Further studies are necessary to discover the reason why animals present different sensitivity to mercury.

Acknowledgments

Financial support-provided by FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" # 01.06.0842-00. M.E.P is recipient of CNPq fellowships (307814/2008–4); C.F., L.M-S and R.P.I. are recipients of fellowships from CAPES. C.S.O. is recipient of CNPq (PIBIC) fellowship.

References

Barbosa, N.B.V., Rocha, J.B.T., Zeni, G., Emanuelli, T., Beque, M.C., Braga, A.L., 1998. Effect of organic forms of selenium on D-aminolevulinate dehydratase from liver, kidney, and brain of adult rats. Toxicol. Appl. Pharmacol. 149, 243–253.

Barregard, L., 2008. Exposure to inorganic mercury: from dental amalgam to artisanal gold mining. Environ. Res. 107, 4–5.

Bevan, D.R., Bodlaender, P., Shemin, D., 1980. Mechanism of porphobilinogen synthase. Requirement of Zn2+ for enzyme activity. J. Biol. Chem. 255, 2030–2035.

Bisinoti, M.C., Jardim, W.F., 2004. O comportamento do metilmercúrio (MetilHg) no ambiente Quim. Nova 27, 593–600.

Boening, D.W., 2000. Ecological effects, transport, and fate of mercury: a general review. Chemosphere 40, 1335–1351.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Calderón, J., Ortiz-Pérez, D., Yáñez, L., Díaz-Barriga, F., 2003. Human exposure to metals. Pathways of exposure, biomarkers of effect, and host factors. Ecotoxicol. Environ. Saf. 56, 93–103.

Choi, D.W., Koh, J.Y., 1998. Zinc and brain injury. Annu. Rev. Neurosci. 21, 347–375.
Clarkson, T.W., 1997. The toxicology of mercury. Crit. Rev. Clin. Lab. Sci. 34, 369–403.
Dent, A.J., Beyersmann, C.B., Hasnain, S.S., 1990. Two different zinc sites in bovine 5-aminolevulinate dehydratase distinguished by extended X-ray absorption fine structure. Biochemistry 29, 7822–7828.
Devlin, T.M., 1997. Textbook of Biochemistry with Clinical Correlations 4th ed. Wiley, New York 1007p.
Emanuelli, T., Rocha, J.B.T., Pereira, M.E., Porciúncula, L.O., Morsch, V.M., Martins, A.F., Souza, D.O., 1996. Effect of mercuric chloride intoxication and dimercaprol treatment on δ-aminolevulinate dehydratase from brain, liver and kidney of adult mice. Pharmacol. Toxicol. 79, 139–143.
Echeverria, D., Woods, J.S., Heyer, N.J., Rohlman, D., Farin, F.M., Li, T., Garabedian, C.E., 2006. The association between a genetic polymorphism of coproporphyrinogen oxidase, dental mercury exposure and neurobehavioral response in humans. Neurotoxicol. Teratol. 28, 39–48.
Fang, Y.Z., Yang, S., Wu, G., 2002. Free radicals, antioxidants, and nutrition. Nutrition 18, 872–879.
Franciscato, C., Goulart, F.R., Lovatto, N.M., Duarte, F.A., Flores, E.M.M., Dressler, V.L., Peixoto, N.C., Pereira, M.E., 2009. $ZnCl_2$ exposure protects against behavioral and acetylcholinesterase changes induced by $HgCl_2$. Int. J. Dev. Neurosci. 27, 459–468.
Goering, P.L., Klaassen, C.D., 1983. Altered subcellular distribution of cadmium following cadmium pretreatment: possible mechanism of tolerance to cadmium-induced lethality. Toxicol. Appl. Pharmacol. 70, 195–203.
Gottlieb, A., Keydar, I., Epstein, H.T., 1977. Rodent brain growth stages: an analytical review. Biol. Neonate 32, 166–176.
Goyer, R.A., 1995. Toxic effects of metal. In: Klaassen, C.D. (Ed.), Casarett & Doull's Toxicology—The Basic Science of Poisons Fifth ed. McGraw-Hill Companies, New York, pp. 691–736.
Hasnain, S.S., Wardell, E.M., Garner, C.D., Schlösser, M., Beyersmann, D., 1985. Extended-X-ray-absorption-fine-structure investigations of zinc in 5-amino-laevulinate dehydratase. Biochem. J. 230, 625–633.
Jaffe, E.K., 1995. Porphobilinogen synthase, the first source of heme's asymmetry. J. Bioenerg. Biomembr. 27, 169–179.
Kumar, M., Sharma, M.K., Kumar, A., 2005. *Spirulina fusiformis*: a food supplement against mercury induced hepatic toxicity. J. Health Sci. 51, 424–430.
Mathie, A., Sutton, G.L., Clark, C.E., Veale, E.L., 2006. Zinc and copper: pharmacological probes and endogenous modulators of neuronal excitability. Pharmacol. Ther. 111, 567–583.
Meyer, D.J., Coles, E.H., Rich, L.J., 1992. Veterinary laboratory medicine, interpretation and diagnosis. W. B. Saunders, Philadelphia 350 pp.
Nevado, J.J.B., Bermejo, L.F.G., Martn-Doimeadios, R.C.R., 2003. Distribution of mercury in the aquatic environment at Almaden, Spain. **Environ. Pollut**. 122, 261–271.
Pedersen, S.O., Pedersen, K.L., Hojrup, P., Knudsen, J., Depledge, M.H., 1998. Induction and identification of cadmium-, zinc- and copper-metallothioneins in the shore crab *Carcinus maenas* (L.). Comparat. Biochem. Physiol. Part C 120, 251–259.
Peixoto, N.C., Roza, T., Flores, E.M.M., Pereira, M.E., 2003. Effects of zinc and cadmium on $HgCl_2$-δ-ALA-D inhibition and Hg levels in tissues of suckling rats. Toxicol. Lett. 146, 17–25.
Peixoto, N.C., Roza, T., Flores, Pereira, M.E., 2004. Sensitivity of Ó-ALA-D (E.C. 4.2.1.24) of rats to metals in vitro depends on the stage of postnatal growth and tissue. Toxicol. in Vitro 18, 805–809.
Peixoto, N.C., Pereira, M.E., 2007. Effectiveness of $ZnCl_2$ in protecting against nephrotoxicity induced by $HgCl_2$ in newborn rats. Ecotoxicol. Environ. Saf. 66, 441–446.
Peixoto, N.C., Kratz, C.P., Roza, T., Morsch, V.M., Pereira, M.E., 2007a. Effects of $HgCl_2$ on porphobilinogen-synthase (E.C. 1.2.1.24) activity and on mercury levels in rats exposed during different precocious periods of postnatal life. Cell Biol. Int. 31, 1057–1062.
Peixoto, N.C., Serafim, M.A., Flores, E.M.M., Bebianno, M.J., Pereira, M.E., 2007b. Metallothionein, zinc, and mercury levels in tissues of young rats exposed to zinc and subsequently to mercury. Life Sci. 81, 1264–1271.
Peixoto, N.C., Roza, T., Morsch, V.M., Pereira, M.E., 2007c. Behavioral alterations induced by $HgCl_2$ depend on the postnatal period of exposure. Int. J. Dev. Neurosci. 25, 39–46.
Peixoto, N.C., Rocha, L.C., Moraes, D.P., Bebianno, M.J., Dressler, V.L., Flores, E.M.M., Pereira, M.E., 2008. Changes in levels of essential elements in suckling rats exposed to zinc and mercury. Chemosphere 72, 1327–1332.
Rocha, J.B.T., Freitas, A.J., Marques, M.B., Pereira, M.E., Emanuelli, T., Souza, D.O., 1993. Effects of methylmercury exposure during the second stage of rapid postnatal brain growth on negative geotaxis and on delta-aluminolevulinate dehydratase of suckling rats. Braz. J. Med. Biol. Res. 26, 1077–1083.
Rocha, J.B.T., Pereira, M.E., Emanuelli, T., Christofari, R.S., Souza, D.O., 1995. Effects of treatment with mercury chloride and lead acetate during the second stage of rapid postnatal brain growth on δ-aminolevulinic acid dehydratase (ALA-D) activity in brain, liver, kidney and blood of suckling rats. Toxicology 100, 27–37.
Rocha, J.B.T., Rocha, L.K., Emanuelli, T., Pereira, M.E., 2001. Effect of mercuric chloride and lead acetate treatment during the second stage of rapid post-natal brain growth on the behavioral response to chlorpromazine and on δ-ALA-D activity in weaning rats. Toxicol. Lett. 125, 143–150.
Rose, S., Melnyk, S., Savenka, A., Hubanks, A., Jernigan, S., Cleves, M., James, S.J., 2008. The frequency of polymorphisms affecting lead and mercury toxicity among children with autis. Am. J. Biochem. Biotechnol. 4, 85–94.
Roza, T., Peixoto, N.C., Welter, A., Flores, E.M.M., Pereira, M.E., 2005. 2,3-Dimercapto-1-propanol does not alter the porphobilinogen synthase inhibition but decreases the mercury content in liver and kidney of suckling rats exposed to $HgCl_2$. Basic Clin. Pharmacol. Toxicol. 96, 302–308.
Stringari, J., Meotti, F.C., Souza, D.O., Santos, A.R.S., Farina, M., 2006. Postnatal methylmercury exposure induces hyperlocomotor activity and cerebellar oxidative stress in mice: dependence on the neurodevelopmental period. Neurochem. Res. 31, 563–569.
Sassa, S., 1982. Delta-aminolevulinic acid dehydratase assay. Enzyme 28, 133–145.
Smart, J.L., Dobbing, J., 1971. Vulnerability of developing brain. II. Effects of early nutritional deprivation on reflex ontogeny and developmental of behaviour in the rat. Brain Res. 28, 85–95.
Tandon, S.K., Singh, S., Prasad, S., Mathur, N., 2001. Hepatic and renal metallothionein induction by an oral equimolar dose of zinc, cadmium or mercury in mice. Food Chem. Toxicol. 39, 571–577.
Timbrell, J.A., 2008. Principles of Biochemical Toxicology, 4th ed. Informa Healthcare, USA 583p.
Wasserman, J.C., Hacon, S., Wasserman, M.A., 2003. Biogeochemistry of mercury in the Amazonian environment. Ambio 32, 336–342.
Winick, M., Noble, A., 1965. Quantitative changes in DNA, RNA and protein during prenatal and postnatal growth in the rat. Dev. Biol. 12, 451–466.
Woods, J.S., Echeverria, D., Heyer, N.J., Simmonds, P.L., Wilkerson, J., Farin, F.M., 2005. The association between genetic polymorphisms of coproporphyrinogen oxidase and an atypical porphyrinogenic response to mercury exposure in humans. Toxicol. Appl. Pharmacol. 206, 13–120.
Zalups, R.K., 1998. Basolateral uptake of inorganic mercury in the kidney. Toxicol. Appl. Pharmacol. 151, 192–199.