

Metallothionein, essential elements and lipid peroxidation in mercury-exposed suckling rats pretreated with selenium

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Abstract Detoxification of mercury (Hg) with selenium (Se) in the early postnatal period with regard to the expression of metallothionein protein (MT), essential element status, and lipid peroxidation level in tissues has not been studied. Seven-day-old Wistar pups were orally pretreated with Se [6 $\mu\text{mol Na}_2\text{SeO}_3/\text{kg}$ body weight (b.w.)] for 3 days and then cotreated with Hg (6 $\mu\text{mol HgCl}_2/\text{kg}$ b.w.) for the following 4 days. This group (Se + Hg) was compared to the groups treated with Hg, Se, or vehicle (control). Compared to the Hg-group, Se + Hg-group exhibited lower renal MT expression, reduced accumulation of Hg, Cu and Zn, and reduced excretion of Se, Hg and Zn in urine. In the liver, MT was stimulated by Se treatment in both, Se and Se + Hg-group. Hepatic and brain levels of the endogenous essential elements Cu, Fe, Mg, and Zn remained unchanged in all of the studied groups. Brain Hg levels and oxidation of lipids measured as thiobarbituric acid reactive substances were diminished in Se + Hg-group of pups compared

to the Hg-group. This study suggests that Se pretreatment can help reduce Hg in the tissues of suckling rats, simultaneously preventing impairment of essential element levels in the kidneys and their excessive excretion via urine. Also, Se was shown to prevent oxidative damage of lipids in the brain, which is particularly susceptible to Hg during the early postnatal period.

Keywords Selenium supplementation · Mercury exposure · Suckling rat · Metallothionein · Essential element · Lipid peroxidation

Introduction

Mercury (Hg), depending on its chemical form, has shown toxicity in a number of organ systems including the renal, cardiovascular, gastrointestinal, hepatobiliary and neurological system (ATSDR 1999; Clarkson and Magos 2006; Bridges and Zalups 2010). Exposure to Hg in the early postnatal period has been recognized as an important public health issue because of the pronounced sensitivity of this period in life. A newborn organism is much more susceptible to toxic health effects compared to adults. Also, the exposure pathways are different and besides the usual (food and drink) intake, oral exploration and hand-to-mouth activity adds significantly to Hg intake (WHO 1986; U.S. Environmental Protection Agency, EPA 2002; Grandjean et al. 2008). Among the most significant

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channels of exposure to the inorganic form of Hg (I-Hg) for children are ingestion of batteries, homeopathic remedies, skin bleaching creams and soaps although children are most commonly exposed to the organic form of Hg, methylmercury (MeHg), via food (U.S. Environmental Protection Agency, EPA 2007). In industrial China, I-Hg is a more common pollutant in the environment than other forms of Hg (Feng et al. 2004). It is now known that I-Hg can be produced in the human body via slow demethylation of MeHg in the liver and brain, as well as via fast oxidation of elemental Hg in blood and tissues (ATSDR 1999; Clarkson and Magos 2006; Khan and Wang 2009). Both Clarkson (1997) and Khan and Wang (2009) proposed I-Hg as the possible proximate toxic form of Hg upon exposure to elemental Hg or demethylation of MeHg, respectively. Thus, research with the I-Hg is needed to clarify the mechanism of toxic action in the organism (Feng et al. 2004).

One of the toxic effects of Hg is the impairment of essential element [Fe, Cu, Zn, selenium (Se), Ca] homeostasis (Telišman 1995; Peraza et al. 1998). Hg (Cd and Cu, too), as higher affinity metal compared to Zn, was shown to replace Zn from the metal–metallothionein (MT) complex (reviewed in Sabolić et al. 2010). Feng et al. (2004) reported the imbalance of micro-nutritional elements in rats' brain after in utero and lactational exposure to I-Hg. Vice versa, essential elements in excess appear to be protective against Hg toxicity (Chowdhury and Chandra 1987). The most studied interaction of Hg with an essential element is the one with Se (Watanabe 2002). Many studies have shown that supplementation with selenite prevents Hg toxicity (reviewed by Khan and Wang 2009; Rooney 2007; Ralston and Raymond 2010; Yang et al. 2008; Falnoga and Tušek-Žnidarič 2007; Luque-Garcia et al. 2013) but the exact mechanism is still unknown. Generally, Hg, and more rarely Se, content was measured in various tissues of adult (Magos and Webb 1976; Abdulla and Chmielnicka 1990; Nielsen and Andersen 1991; Agarwal and Behari 2007; Su et al. 2008; Agarwal et al. 2010) and young suckling animals (Orct et al. 2009) exposed to Hg and Se, but without exploring the effect on other endogenous essential elements, especially in young animals. Chmielnicka et al. (1986) found in adult rats that Se lowered Hg-induced renal deposition and urinary excretion of Cu and partly Zn in acute single dose experiment. Peixoto et al. (2008) investigated the

effect of Zn-supplementation on Hg-intoxicated suckling rat pups and subsequent essential element changes. In these studies, exposure was parenteral, even though oral treatment would be of greater interest when it comes to human exposure.

MT, a protein of low molecular weight ($M_r \sim 6$ kDa), is inducible by various metals (Zn, Cu, Cd, Hg, Ag, Au, As) and reactive oxygen species (ROS), among other stimuli. Following binding to MT, otherwise reactive metal ion is stored inactive. Although it is a clearly confirmed fact in adult animals, induction of MT by metals in young animals has not been well studied (Zalups 2000; Sabolić et al. 2010), and the thus far published results are conflicting. Peixoto et al. (2007) confirmed induction of MT by $ZnCl_2$, but not by $HgCl_2$ in the kidney of 13-day-old rats. Also Brambila et al. (2002) failed to see induction of MT in the kidney of 1, 7, 14 and 21-day-old rats exposed to Hg^0 vapor in utero. Cherian et al. (1987) noted induction of MT by $ZnSO_4$, but not by $CdCl_2$ in newborn rats' liver, while Fernandez et al. (2007) measured an up-regulation of MT-1 gene expression in the mice embryo after single $CdCl_2$ injection to mice mothers.

Essential elements are needed in the active sites of some endogenous antioxidant enzymes that cleave ROS and protect cells from oxidative damage. Ercal et al. (2001) pointed out a few indirect mechanisms of Hg-induced oxidative damage: depletion of glutathione (GSH) which results with the accumulation of ROS in the cell; inhibition of mitochondrial oxidative phosphorylation suggested in renal tubular damage; alteration of Ca (activates hydrolytic enzymes) homeostasis; and effect on the activity of catalase, Cu–Zn superoxide dismutase, GSH peroxidase, and xanthine oxidase. It was reported that exposure to Hg results in oxidative stress followed by lipid peroxidation (reviewed in Telišman 1995; Peraza et al. 1998; Zalups 2000; Ercal et al. 2001; Ralston and Raymond 2010; Romero et al. 2014; Agha et al. 2014). The impact of Se supplementation on Hg-induced lipid peroxidation has been studied only in adult animals (Farina et al. 2003; Perottoni et al. 2004a, b; Agarwal and Behari 2007; Su et al. 2008; Brandão et al. 2009; Agarwal et al. 2010; Agha et al. 2014).

The present work was designed to study the effect of oral Se supplementation of Hg-intoxicated suckling rats on MT, endogenous essential elements and the

level of lipid peroxidation in kidney, liver, brain, and urine of rats in the sensitive early postnatal period.

Materials and methods

Animals

Before the beginning of this experiment, 20 female Wistar rats bred at the Laboratory Animal Unit of the Institute for Medical Research and Occupational Health, Zagreb, Croatia were mated with males in a 3:1 ratio. After birth, the number of pups was reduced to eight in the respective litter. Four females with deliveries on the same day and 40 pups of both sexes were used in this experiment. The animals were maintained in a 12 h light/dark cycle at room temperature of 21 ± 1 °C and constant humidity of 40 %. Each litter was kept in an individual polycarbonate cage (26.5 × 20.7 × 14.0 cm) with a stainless steel lid. The cages were cleaned and pine shaving bedding was changed daily. Mother rats were given a normal rat diet (Mucedola, Italy) and deionised water ad libitum throughout the experiment. All research procedures were carried out in accordance with the National Animal Welfare Act, and approved by the Institute's Ethics Committee and the Croatian Ministry of Agriculture, Forestry, and Water Management.

Experimental design

The experiment started at 7 days of age, as it was proposed that rats at this age may be more comparable to humans at birth (Miller 1983). Eight pups (four male and four female) were randomly assigned to each of the four litters (with one mother rat in each) on postnatal day 2 (PND 2; day of birth = PND 0). Two pups were taken from each litter in order to form one of the four experimental groups with eight animals per group:

- (1) *Control group* distilled water orally for 7 days,
- (2) *Se-group* oral doses of 6 μmol Na₂SeO₃/kg body weight (b.w.)/day for 7 days,
- (3) *Hg-group* oral doses of 6 μmol HgCl₂/kg b.w./day for 4 days,
- (4) *Se + Hg-group* oral doses of 6 μmol Na₂SeO₃/kg b.w./day for 7 days + 6 μmol of HgCl₂/kg b.w./day for 4 days.

Water, Se, and/or Hg solutions were administered to pups using an artificial feeding method introduced by Kostial et al. (1971). Every morning before the first administration, each pup was weighed. The daily dose was freshly prepared and administered in two portions (at 9:00 a.m. and 2:00 p.m.) with an automatic pipette (25 μl), four drops a day in total. In-between administrations, all pups were returned to their lactating mother rats and allowed to suckle. Pups always received Se 15 min before Hg (Se + Hg-group). Animals pretreated with Se (Se- and Se + Hg-group) received sodium selenite (p.a., Sigma-Aldrich, USA) at a daily dose of 0.5 mg Se/kg b.w. for seven consecutive days (PNDs 7–13). This procedure is called pretreatment because the Se + Hg pups had been receiving Se for 4 days before they were cotreated with Hg for the following 4 days. The daily dose of Se was calculated to achieve an equimolar ratio to the daily dose of Hg (Hg:Se, 1:1). Hg was administered as HgCl₂ (p.a., Kemika, Croatia) at a daily dose of 1.3 mg Hg/kg b.w. for four consecutive days (PND 10–13). The same Hg dose was given to the Hg alone and the Se + Hg-group. The daily dose of Hg administered to each animal was 5 % of the acute LD₅₀ oral dose found previously for HgCl₂ (35 mg/kg b.w.) in suckling rats (Kostial et al. 1978). The daily dose of Hg applied here caused no apparent adverse health effects in suckling rats (Orct et al. 2009) and was lower than the one from the study of Nielsen and Andersen (1991) where no renal necrosis was found (oral dose 25 μmol HgCl₂/kg b.w.) in mice.

On PND 14, 24 h after the last treatment, pups were anaesthetised (Narketan 0.8 ml/kg b.w. + Xylapan 0.6 ml/kg b.w., i.p., Vetoquinol AG, Switzerland), and dissected in the same sequence for each animal.

Sampling and analysis of elements

Blood was collected from the heart in BD Vacutainer® tubes with lithium heparin anticoagulant. Plasma was obtained by centrifugation of the collected blood samples (3000 rpm, for 15 min). Urine was collected directly from urinary bladder by a syringe. Animals were killed by bleeding from the abdominal aorta. Their liver, kidneys, and brain were removed, weighed, and rinsed with cold deionised water. Liver and brain were cut in half. The first part of the liver, brain, the right kidney, whole blood, and plasma were stored at –20 °C until element analysis. The other half

of the liver and brain tissues, and the left kidney were homogenised on ice in 1.15 % KCl buffer in a Potter–Elvehjem homogeniser (Cole and Palmer, USA) with a Teflon pestle, and used immediately to measure lipid peroxidation. Aliquots of the liver and kidney homogenates were stored at $-20\text{ }^{\circ}\text{C}$ until used for MT immunoblotting. Hg and essential elements (Cu, Fe, Mg, Se, Zn) mass fractions were analysed from the same thawed tissue samples (right kidney, part of liver, part of brain) digested in an UltraCLAVE IV microwave digestion system (Milestone, Sorisole, Italy) after adding 2 ml of concentrated HNO_3 , 65 % s.p. grade (Merck, Germany) in quartz vessels with Teflon caps. Whole blood, plasma and urine analyses were carried out without sample digestion. Total Hg was analyzed by total decomposition, amalgamation, atomic absorption spectrometry (TDA-AAS) in an Hg analyzer (AMA 254, Leco, USA). Se and Cu were analyzed by electrothermal AAS with Mg and Pd as a matrix modifier and Zeeman background correction (AAAnalyst 600, Perkin Elmer Instruments, USA). For Fe, Mg and Zn quantification, the digested samples were aspirated into the air-acetylene flame of a Varian AA-375 atomic absorption spectrometer (Mulgrave, Victoria, Australia). The reliability of analytical methods was evaluated using standard reference materials: bovine liver 1577b (NIST, USA), horse kidney H8 (IAEA, Austria), SeronormTM Trace Elements Whole Blood L-2 (Sero AS, Norway), SeronormTM Trace Elements Serum L-2 (Sero AS, Norway), and SeronormTM Trace Elements Urine (Sero AS, Norway). The results of our analyses were within $\pm 10\%$ of the certified values.

The results of element mass fractions in organs are expressed as micrograms per gram of wet tissue weight ($\mu\text{g/g w.w.}$). Hg and Se in whole blood and plasma are expressed as $\mu\text{g/l}$ and in urine as $\mu\text{g/mg creatinine}$.

Metallothionein determination

Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and Western blotting was used for MT quantification in rat pups' liver and kidney tissues. Crude tissue homogenates (20 % w/v) were rehomogenized through 25 gauge needle, diluted 1:1 in buffer (300 mM mannitol, 5 mM ethylene glycol tetraacetic acid, 12 mM Tris/HCl, pH 7.4) and centrifuged (Eppendorf Centrifuge 5417 R, Eppendorf AG, Hamburg, Germany) at $5000\times g$ at $4\text{ }^{\circ}\text{C}$ for

15 min. The pellet was discarded. Proteins were measured in the supernatant by the dye-binding assay (Bradford 1976). Before SDS-PAGE, the supernatant was mixed with sample buffer (1 % SDS, 12 % v/v glycerol, 30 mM Tris/HCl, pH 6.8) containing reducing agent (5 % 2-mercaptoethanol), and denatured by heating at $95\text{ }^{\circ}\text{C}$ for 5 min. The heat-treated proteins (60 μg protein/lane) were then separated through 18 % SDS-PAGE MiniProtean III system and wet-transferred using mini trans-blot electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA, USA) to Immobilon membrane (PVDF, Millipore, Bedford, MA, USA). Following transfer, the Immobilon membrane was incubated in 1 % glutaraldehyde for 60 min to enhance MT retention in the membrane (Mizzen et al. 1996), washed several times with water and preincubated with the blotting buffer (0.15 M NaCl, 1 % Triton-X-100, 20 mM Tris/HCl, pH 7.4) with 5 % nonfat dry milk, at room temperature for 60 min to block nonspecific binding. The membrane was then incubated overnight at $4\text{ }^{\circ}\text{C}$ in the blotting buffer (0.15 M NaCl, 1 % Triton-X-100, 20 mM Tris/HCl, pH 7.4) with a monoclonal mouse antibody against the horse MT (clone E9, DAKO, Carpinteria, CA, USA; 1:500), which recognizes a highly conserved domain common to MT-I and MT-II of many species, including rats. The membrane was then washed with several changes of blotting buffer, incubated for 60 min in the same buffer that contained secondary antibody (0.1 $\mu\text{g/ml}$, alkaline phosphatase-labeled goat anti-mouse IgG; GAMAP; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), washed again, and stained for alkaline phosphatase activity using the 5-bromo-4-chloro-3-indolyl phosphate in conjunction with nitro blue tetrazolium as indicators of the protein bands.

Relative density of each protein band was evaluated by densitometry using the Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Inc.). An area of the MT-related strongest protein band in the control protein band of the respective blot was marked, and an equal area was applied to all other relevant bands in the same blot. The area density of each band was expressed in arbitrary units, relative to the strongest band density (=1 arbitrary unit) in the corresponding control samples. The immunoblotting figures are representative from findings in six control and six treated rats per experimental group. The numeric data are expressed as mean \pm SEM.

Lipid peroxidation measurement

Lipid peroxidation was measured in tissue homogenate aliquots as the amount of malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acid hydroperoxides, formed in the thiobarbituric acid reaction as described by Ohkawa et al. (1979). Thiobarbituric acid reactive substances (TBARSs) were quantified by comparing absorption on a spectrophotometer Cecil 9000 series (Cecil Instruments, Cambridge, UK) at 532 nm to the standard curve of MDA equivalent generated by hydrolysis of 1,1,3,3-tetramethoxypropane. TBARS data were expressed as nmol/g w.w.

Statistical analysis

Results are presented as mean \pm SEM. Statistical analysis was performed using Statistica for Windows software, version 12.0 (StatSoft, Inc., Tulsa, USA) after log-transformation of elementary data. When data showed equal variance (Bartlett) and followed a normal distribution (Shapiro–Wilk), one-way analysis of variance with post hoc analysis (Tukey's HSD test) was used to determine significant differences between the groups. In other cases, when the criteria for parametric methods were not fulfilled, the effect of treatment was assessed using the Kruskal–Wallis test with different subsets identified by the Mann–Whitney U-test. Differences were considered significant at $P \leq 0.05$.

Results

Whole b.w. gain during the experimental period (PND 7–14) and organ weight at the last day of experiment (PND 14) did not differ significantly between the four studied groups (Table 1). Exposure to Hg elevated Hg content in all of the studied compartments ($P < 0.001$), most pronouncedly in the kidney (Table 2). Se supplementation before and during exposure to Hg reduced Hg in whole blood, plasma, brain, kidney, and urine ($P < 0.001$). Supplementation with Se (Se-group) significantly elevated Se content in plasma, urine ($P < 0.001$) and tissues ($P < 0.001$) of suckling rats (Table 3). However, when pups were supplemented with Se and exposed to Hg, except in urine where Se level decreased, the level of Se remained unchanged in

plasma, liver and kidney of suckling pups compared to the ones administered Se alone. Unlike in the kidney and urine, exposure to Se or/and Hg did not cause disturbances in the brain and hepatic levels of essential elements (Cu, Fe, Mg and Zn; Fig. 1). Hg exposure caused an increase in renal Cu ($P < 0.001$) and Zn ($P < 0.001$) compared to control pups, while in the group administered both Se and Hg, this effect was diminished. We also noticed higher excretion of Cu ($P < 0.01$), Mg ($P < 0.01$) and Zn ($P < 0.01$) in the urine of pups exposed to Hg compared to control pups. This Hg-induced impairment in excretion of Cu, Mg and Zn was diminished in Se + Hg-group, but the observed decrease in comparison to the Hg group was statistically significant only for Zn ($P < 0.05$). Pups exposed to Se and Hg had higher renal Mg than the control ($P < 0.01$) and Hg exposed animals ($P < 0.05$).

The immunoblotting studies with anti-MT antibody exhibited in the kidney and liver homogenates two major protein bands with Mr of ~ 12 kDa (possibly MT dimer) and ~ 18 kDa (possibly MT trimer; Fig. 2). Since the monomeric protein has Mr of 6–7 kDa we assume that these higher Mr bands are a consequence of protein aggregation due to well-known self oligomerization property of MT (Haase and Maret 2008) in spite of the presence of reducing agent (2-mercaptoethanol). Generally, higher MT expression was observed in the liver compared to kidney (data not shown), which is in accordance with previous findings by Mehra and Bremner (1984). The density data of combined (12 + 18 kDa) protein bands revealed that exposure to Hg strongly enhanced MT expression in the kidney ($P < 0.001$), but not in the liver of treated pups compared to control rats. Supplementation with Se (Se + Hg-group) strongly reduced the Hg-stimulated renal MT expression ($P < 0.001$), but not entirely to the level of control animals (control vs. Se + Hg: $P < 0.01$). Immunoblotting of the liver samples showed a significant heterogeneity in the protein band density in all experimental groups, but there was an obvious trend of MT upregulation related to Se supplementation (Se-group vs. Se + Hg-group), while HgCl₂ alone had no effect on MT expression compared to control pups.

Lipid peroxidation was enhanced in the brain ($P < 0.01$) and liver ($P < 0.01$) of Hg-exposed rats and supplementation with Se managed to significantly diminish this effect only in the brain ($P < 0.01$), although the same trend was obvious also in the liver

Table 1 Body weight gain during the 8-day experimental period and organ weight on the last day of the experiment (g)

Groups	n	Body weight (g)		Body weight gain (PND 7–14)	Organ weight (PND 14)		
		PND 7	PND 14	% of initial weight	Brain	Liver	Kidney
Control	10	16.5 ± 0.5	32.9 ± 0.8	100 (69–127)	1.25 ± 0.02	0.854 ± 0.029	0.349 ± 0.012
Se	10	16.6 ± 0.4	33.3 ± 0.9	102 (73–122)	1.24 ± 0.01	0.955 ± 0.032	0.345 ± 0.009
Hg	10	16.6 ± 0.4	32.9 ± 0.7	99 (76–126)	1.28 ± 0.02	0.829 ± 0.026	0.367 ± 0.008
Se + Hg	10	16.5 ± 0.4	33.0 ± 0.9	101 (71–141)	1.25 ± 0.02	0.875 ± 0.041	0.352 ± 0.011

The results are presented as mean ± SEM or mean (range in parenthesis). The percent of the initial body weight was calculated as (body weight on PND 14 – body weight on PND 7)/(body weight on PND 7) × 100

Table 2 Mercury retention in blood, plasma and tissues, and excretion after selenium supplementation in suckling rats exposed to mercury

Groups	Hg (% dose) ^a					
	Whole blood (µg/l)	Plasma (µg/l)	Brain (µg/g wet weight)	Liver (µg/g wet weight)	Kidney (µg/g wet weight)	Urine (µg/mg creatinine)
Control	6.15 ± 0.37	4.19 ± 0.36	0.027 ± 0.003	0.130 ± 0.007	0.245 ± 0.015	0.0014 ± 0.0002
Se	5.97 ± 0.30 [#]	2.71 ± 0.19 ^{*#}	0.023 ± 0.002 [#]	0.054 ± 0.003 ^{*#}	0.172 ± 0.013 ^{*#}	0.0006 ± 0.0000 [#]
Hg	273 ± 13 [*]	283 ± 13 [*]	0.150 ± 0.003 [*] (0.136)	7.38 ± 0.39 [*] (4.31)	35.5 ± 1.5 [*] (9.22)	0.557 ± 0.103 [*]
Se + Hg	643 ± 36 ^{*#}	843 ± 46 ^{*#}	0.121 ± 0.002 ^{*#} (0.107)	6.44 ± 0.33 [*] (3.94)	2.68 ± 0.12 ^{*#} (0.669)	0.034 ± 0.009 ^{*#}

Suckling rats received deionised water (control group) or Se or Se and Hg orally in a water solution at 9:00 a.m. and 2:00 p.m. using an artificial feeding method. Selenium supplement was given as sodium selenite and mercury as mercury chloride. Rats were treated from postnatal day (PND) 7 to 13 as follows: control (H₂O, PND 7–13), Se (PND 7–13), Hg (PND 10–13), and Se + Hg (Se PND 7–13 + Hg PND 10–13). Mercury and selenium daily doses were equimolar (6 µmol/kg b.w./day)

* Values significantly different from control group (P < 0.05), # values significantly different from Hg-group (P < 0.05)

^a The results are presented as mean ± SEM, n = 10. The percent of the initial Hg dose in parentheses was calculated from (total mercury in tissue)/(total mercury administered) × 100

of pups (Fig. 3). Significant changes in lipid peroxidation were noticed in the liver and kidney of Se-treated pups compared to control group (P < 0.01).

Discussion

A number of studies on experimental animals have confirmed that Se supplementation decreases I-Hg toxicity, but knowledge on the effects on MT, endogenous essential elements, their excretion and lipid peroxidation, especially in young animals during oral exposure, is lacking. Young animals, especially those on a milk diet (suckling animals) are due to age specificities and enhanced absorption of toxic metals a good model for studying toxic metal exposure in this sensitive life period.

The doses of inorganic Hg and Se to which pups were exposed in this study did not cause adverse health effects in view of decreased b.w. or organ weight. However, Hg and Se administered to pups did cause significant redistribution of Hg, whose levels are usually highest in the kidneys and largely bound to MT. Western blot of renal samples confirmed stimulated expression of MT that correlated with elevated Hg concentration in Hg-group. Also, following Hg redistribution in Se + Hg-group, immunoblots revealed lower MT expression in the renal tissue. According to the available literature, this is the first report of Hg-stimulated renal MT expression in neonatal rat. Peixoto et al. (2007) did not find enhanced MT expression in the liver or kidneys of rat pups of similar age receiving HgCl₂ (s.c.) in even higher dose than in our study. Also Brambila et al. (2002) failed to see an

Table 3 Selenium retention in plasma and tissues, and excretion after selenium supplementation in suckling rats exposed to mercury

Groups	Se (% dose) ^a			
	Plasma (µg/l)	Liver (µg/g wet weight)	Kidney (µg/g wet weight)	Urine (µg/mg creatinine)
Control	111 ± 4	0.392 ± 0.036	0.571 ± 0.096	0.063 ± 0.008
Se	850 ± 38*	12.5 ± 0.6* (14.1)	3.61 ± 0.24* (1.47)	4.30 ± 0.34*
Hg	76.1 ± 3.1 ^s	0.349 ± 0.033 ^s	0.600 ± 0.090 ^s	0.161 ± 0.017* ^s
Se + Hg	748 ± 38*	14.7 ± 1.6* (14.9)	3.30 ± 0.24* (1.37)	2.76 ± 0.12* ^s

Suckling rats received deionised water (control group) or Se or Se and Hg orally in a water solution at 9:00 a.m. and 2:00 p.m. using an artificial feeding method. Selenium supplement was given as sodium selenite and mercury as mercury chloride. Rats were treated from postnatal day (PND) 7 to 13 as follows: control (H₂O, PND 7–13), Se (PND 7–13), Hg (PND 10–13), and Se + Hg (Se PND 7–13 + Hg PND 10–13). Mercury and selenium daily doses were equimolar (6 µmol/kg b.w./day)

* Values significantly different from control group ($P < 0.05$), ^s values significantly different from Se-group ($P < 0.05$)

^a The results are presented as mean ± SEM, $n = 10$. The percent of the initial Se dose in parentheses was calculated from (total selenium in tissue)/(selenium administered) × 100

induction of MT in renal tissue of neonatal rats exposed in utero to Hg^o vapor.

Our findings of delayed transport of Hg from the blood of Se + Hg-group and higher affinity for storage in the liver than in the kidneys of suckling rats compared to Hg-group are in agreement with previous findings in adult animals (Chmielnicka et al. 1986; reviewed in Yang et al. 2008; Khan and Wang 2009) and with our earlier experiments with suckling pups (Orct et al. 2009). Fast formation of water soluble mercuric selenide–selenoprotein P complex in the blood markedly reduces transport of Hg to renal cells but is proven to appear in the hepatocytes after 24 h where it might undergo proteolysis and form inert Hg–Se complex with a very slow elimination time (reviewed in Yang et al. 2008). Unlike the situation in brain and kidneys, where Se supplementation lowered Hg content, Se did not change Hg accumulation in the liver of our pups compared to the Hg-group, as also found in our earlier study with Se:Hg 1:1 ratio (Orct et al. 2009). In that study, Se supplementation in higher Se:Hg ratios decreased the hepatic Hg content by 2.8 (ratio 2:1) and 3.9 times (ratio 3:1) compared to Hg-treated pups, but also increased the liver weight. Because of the dual nature of Se and elevated liver weight in Se + Hg-group (ratio 3:1), indicating possible toxic effects of Se (Orct et al. 2009), we further investigated only the equimolar ratio following the “primum non nocere” demand for supplements/remedies. On the contrary, Su et al. (2008) in their chronic exposure study reported an increase in hepatic and blood Hg and no change in renal Hg in the Se + Hg-group of orally treated adult

rats. In both the chronic (Agarwal and Behari 2007) and acute parenterally exposed rats (Agarwal et al. 2010), Hg was reported to increase in liver and kidneys when Se was coadministered. In the latter studies, the authors exposed rats to lower Se doses compared to the Hg dose, unlike in our experiment where the doses were equimolar, which could partly explain the discrepancy between the results. The phenomena that increased Se intake changes I-Hg distribution between the kidney and liver was confirmed earlier by Nielsen and Andersen (1991). Unlike in the kidneys, Western blot of the hepatic tissues indicated Se, not Hg as a stimulator of MT synthesis. Similar results were found by Iwai et al. (1988) in adult mice injected with sodium selenite. In the Se-treated animals they also noted elevated Zn concentrations and suggested perturbation of Zn homeostasis as a mechanism for stimulation of MT synthesis. Latter findings, reviewed by Maret (2000), confirmed Se compounds as oxidants of MT in vivo, which may act to release Zn from MT, and possibly stimulate thionein synthesis via the Zn-mediated mechanism.

The fact that Se supplementation of Hg-exposed pups causes reduction in excretion of Hg was published in our previous study (Orct et al. 2009), and confirmed here. Magos and Webb (1976) and Chmielnicka et al. (1986) reported the same findings in adult rats—reduction of urinary and faecal Hg mass fraction in the Se + Hg-group. A detail left unexplored in our previous study (Orct et al. 2009) concerned the final Se concentrations in the urine of Se + Hg-group compared to the Se-group. Here we revealed that urinary Se dropped when Se-

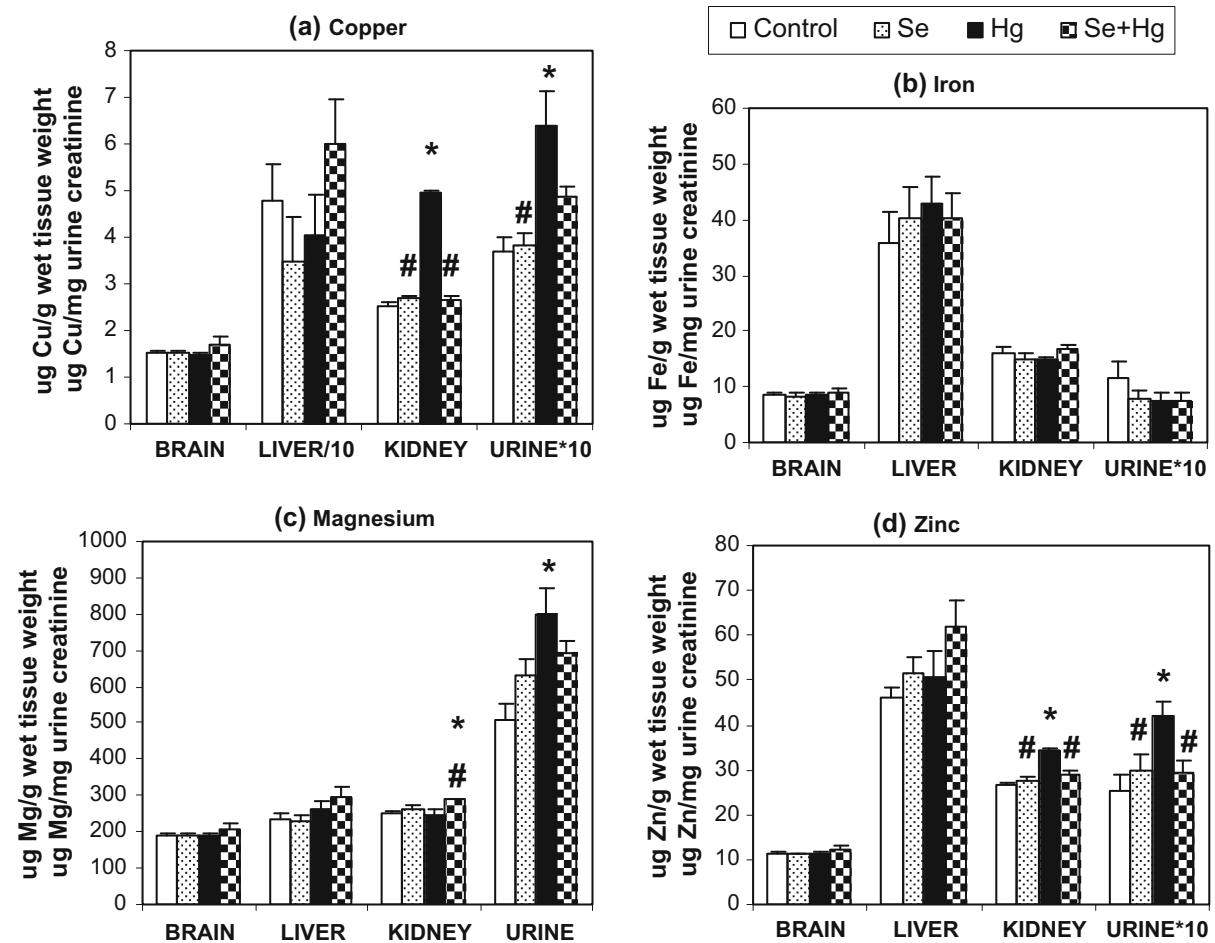


Fig. 1 The effect of Hg or/and Se oral exposure on Cu (a), Fe (b), Mg (c) and Zn (d) levels in brain, liver, kidneys and urine of suckling rats (mean + SEM). Exposures are described in

“Materials and methods” section. * $P < 0.05$ versus control group, # $P < 0.05$ versus Hg-group

supplemented pups were exposed to Hg, as also reported by Magos and Webb (1976) for adults, thereby sharing the faith of Hg due to the formation of the aforementioned mercuric selenide–selenoprotein P complex. Similar results in young animals are not available in other studies. Our study showed that Se supplementation of suckling rats before and during exposure to Hg can reduce Hg accumulation in the kidney and brain and MT expression in the kidney, but also reduces the excretion of both Se and Hg. At the same time, supplemented Se is deposited to tissues irrespective of the presence of Hg and can benefit the respective cells with its antioxidative potential.

The most prominent changes in essential elements in our study were found in the kidneys of Hg- and/or

Se-exposed pups. Significant increase of renal Cu and Zn after oral HgCl_2 exposure is in agreement with reports in adult animals (Bogden et al. 1980; Chmielnicka et al. 1986; Liu et al. 1992) and suckling pups (only renal Cu, not Zn; Peixoto et al. 2008). Our report of Hg-induced excretion of Cu and Zn in urine of suckling rats is unique, and correlates with findings in adult animals (Chmielnicka et al. 1986; Liu et al. 1992). It is known from the literature that HgCl_2 stimulates MT synthesis in kidneys, and not in the liver of adult animals (Zalups 2000). The same was confirmed in this study with suckling rats. Due to the strong binding and sequestration affinity of MT for Cu, Zn, Cd and Hg, increase of Hg-stimulated MT content might result with more binding sites for Cu and Zn

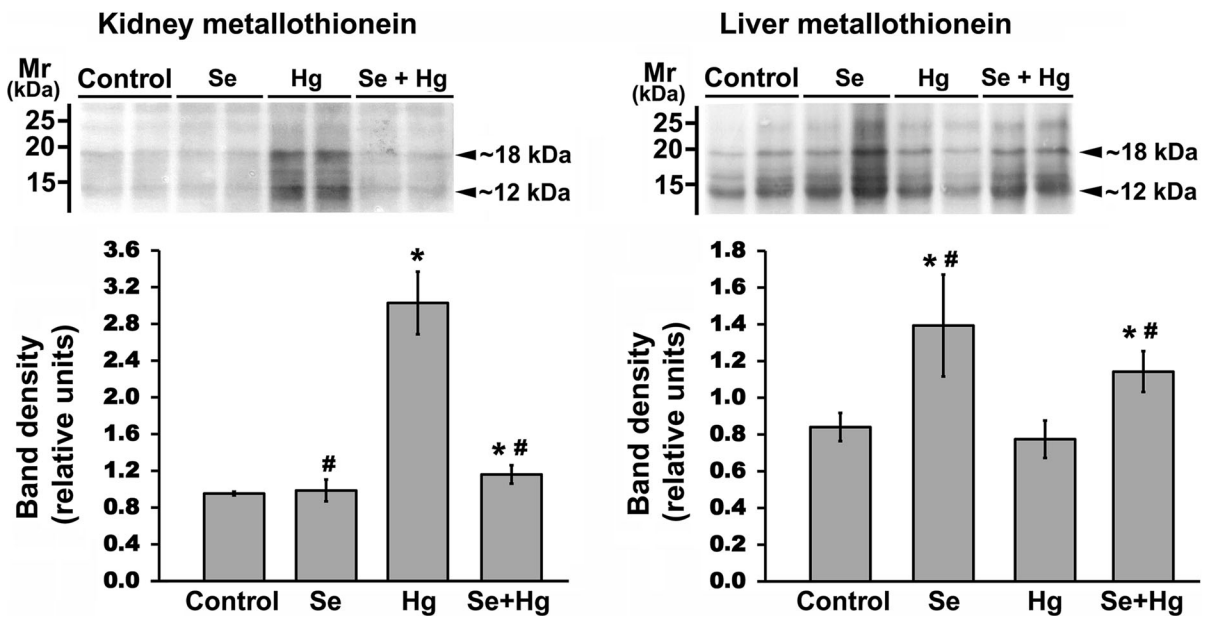


Fig. 2 The effect of Hg or/and Se oral exposure on metallothionein levels in kidneys and liver of suckling rats, as shown in Western blots of tissue homogenates. Exposures and experimental details are described in “Materials and methods” section. Each blot is representative for the findings in six control and six

treated rats per experimental group. Bars represent mean ± SEM (n = 6 in each bar) of the combined density of 12 and 18 kDa protein bands. *P < 0.05 versus control group, #P < 0.05 versus Hg-group

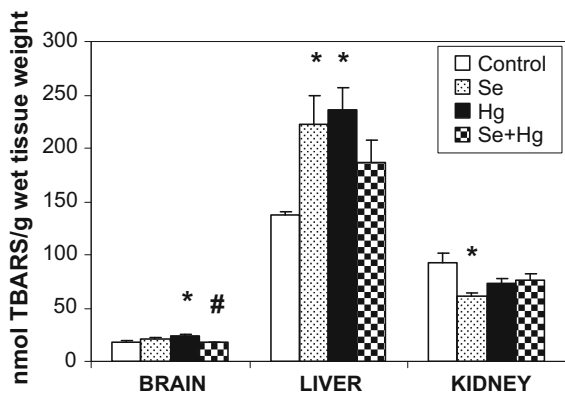


Fig. 3 The effect of Hg or/and Se oral exposure on the level of lipid peroxidation (TBARS) in the brain, liver and kidneys of suckling rats (mean + SEM). Exposures are described in “Materials and methods” section. *P < 0.05 versus control group, #P < 0.05 versus Hg-group

and consequently with their higher retention in the kidney, as proposed earlier (Bogden et al. 1980; Chmielnicka et al. 1986; Liu et al. 1992; Peixoto et al. 2008). Abdulla and Chmielnicka (1990) reported that MT induced by metals contains Zn and/or Cu together with toxic metals. The more prominent increase in

renal Cu than Zn concentrations observed in our pups after exposure to HgCl₂ could be the consequence of physiological differences in Cu/Zn domination in MT, while Cu is more abundant in renal MT and Zn in hepatic MT (Abdulla and Chmielnicka 1990). One of the most original findings of this study is the effect of Se on the above reported Hg-induced changes in essential metals of suckling rats; we found that Se pretreatment modified HgCl₂-induced increase in renal Zn and Cu by lowering them to the levels of the control group. Although reduced, the renal MT levels in Se + Hg- versus Hg-group did not drop to control levels as seen for the content of essential elements Cu and Zn, but rather followed the trend of Hg levels. This might be a consequence of well known higher affinity of Hg to bind MT compared to Zn/Cu (Zalups 2000). In addition, excretion of Zn (and Cu, but not significantly) in urine completely followed the pattern of renal Zn and Cu levels in the experimental groups. Exogenous Se influences the shift of Hg, otherwise bound to MT, to form a high molecular weight complex with Se and selenoprotein P, thought to be a precursor of inert HgSe (s) (Khan and Wang 2009). Once bound to an inert complex, Hg would not

be able to exert biological effects as a free ion (e.g., induction of MT) and influence the essential elements in tissue. Opposite to our Mg quantification data and results from Bogden et al. (1980), Peixoto et al. (2008) reported a Hg-induced decrease in renal, but not hepatic Mg concentrations in suckling rats. We noticed an impairment of Mg metabolism only when we supplemented Hg-exposed pups with Se. Although Mg was elevated in the Se + Hg-group in all three tissues compared to other experimental groups, the difference was significant only in the kidney and we cannot propose the underlying mechanism. On the other hand, this study is the first to report that Hg exposure to suckling pups induced magnesuria, also seen before in adult animals (Liu et al. 1991) as a result of the nephrotoxic effect of HgCl₂. Iron was the only element that showed no impairment of metabolism in any tissue, and excretion in any experimental group of pups. Neither Hg nor Se influenced endogenous essential elements in the liver and brain tissue of our test animals. Similar findings were reported by Bogden et al. (1980) and Liu et al. (1992), whereas Peixoto et al. (2008) found elevated liver Zn and Fe concentrations after exposure to I-Hg. Our study showed that Se supplementation was able to prevent renal accumulation of endogenous Cu and Zn mediated by increased MT levels, and to prevent excretion of Zn and, less distinctly, of Cu and Mg, via urine in Hg-exposed suckling rats.

This study is also the first to report the effect of Se on the TBARS level, as a marker of lipid peroxidation in Hg-exposed suckling rats. The protective effect of Se was confirmed only in the brain tissue of Se + Hg-group compared to the Hg-only group. The proposed mechanisms of protection imply that Se, as a constitutive part of selenoproteins, either scavenges ROS (which induce lipid oxidation in cells and tissues) issued by Hg-exposure, or prevents creation of ROS by complexing Hg ions into inert compounds as HgSe (s). Results from the available literature are numerous, but varying. Organic Se prevented hepatic and renal I-Hg-induced lipid peroxidation in chronic (Su et al. 2008), and subchronic (Brandão et al. 2009) experiments in vivo. Perottoni et al. (2004b) reported that organic Se in vitro prevented I-Hg-induced lipid peroxidation, while in acute in vivo experiment there was no such effect. Inorganic Se was reported to diminish I-Hg-induced lipid peroxidation in serum of subchronically (Agha et al. 2014) and in kidneys of

acutely exposed rats, but failed to exert the same effects in vitro (Perottoni et al. 2004a), in acutely exposed mice (Farina et al. 2003), or in subchronically exposed rats (Agarwal and Behari 2007). In the acute study performed by Agarwal et al. (2010), pretreatment and posttreatment with inorganic Se restored lipid peroxidation in the kidneys and brain of adult rats exposed to I-Hg. Regarding the level of TBARS, we can conclude that Se supplementation before and during exposure to Hg can prevent the oxidation of lipids in the brain, but not liver and kidneys of suckling rats.

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