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Toxicology Letters 169 (2007) 121–128
Toxicology Letters
www.elsevier.com/locate/toxlet

Biomarkers of exposure and effect as indicators of the interference of selenomethionine on methylmercury toxicity

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Received 4 October 2006; accepted 19 December 2006
Available online 3 January 2007

Abstract

The present study was conducted to clarify the interference of selenomethionine (SeMet) on methylmercury (MeHg) toxicity through the evaluation of changes in biomarkers of exposure and effect in rats exposed to MeHg and co-exposed to MeHg and SeMet. Male Wistar rats received two intraperitoneally (i.p.) administrations, either MeHg (1.5 mg/kg body weight), SeMet alone (1 mg/kg body weight) or combined MeHg and SeMet, followed by 3 weeks of rat urine collection and neurobehavioural assays. The effects of different administrations were investigated by the quantification of total mercury in kidney and brain, analysis of urinary porphyrins, determination of hepatic GSH and evaluation of motor activity functions (rearing and ambulation). MeHg exposure resulted in a significant increase of urinary porphyrins during the 3 weeks of rat urine collection, where as it caused a significant decrease in motor activity only at the first day after cessation of rat exposure. Additionally, SeMet co-exposure was able to normalize the porphyrins excretion, and a tendency to restore rat motor activity was observed, on the first day after cessation of exposure. Brain and kidney mercury levels increased significantly in rats exposed to MeHg; however, in co-exposed rats to SeMet no significant changes in Hg levels were found as compared to rats exposed to MeHg alone. Hence, the present study shows that urinary porphyrins are sensitive and persistent indicators of MeHg toxicity and demonstrates for the first time that SeMet reduces its formation. Finally, these results confirm that the mechanism of interaction between SeMet and MeHg cannot be explained by the reduction of Hg levels in target organs and suggestions are made to clarify the interference of SeMet on MeHg toxicity.

Keywords: Methylmercury; Selenomethionine; Biomarkers; Porphyrins; Gluthathione; Behavioural assays

1. Introduction

MeHg, is an hazardous environmental pollutant which bioaccumulates in the marine food chain, being fish and sea food the most important sources of human exposure to this organomercurial compound. Neurotoxic and developmental effects were identified as the major adverse effects resulting from exposure to high doses of MeHg (WHO, 1990; ATSDR, 1999; Clarkson et al., 2003). However, the effects resulting from exposure to low or very low doses of MeHg are not well established and are controversial mainly at prenatal exposure period (Auger et al., 2005; Myers et al., 2003; Aschner, 2002; Grandjean et al., 1997).

Various dietary components are referred as confounding factors and responsible by the modulation of MeHg toxicity (Beyrouty and Chan, 2006; Chapman and Chan, 2000; Egeland and Middaugh, 1997). Given that fish and sea food are also a rich source of Se, its role on MeHg toxicity has been one of the nutritional factors most investigated (Onning and Bergdahl, 1999; Dietz et

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doi:10.1016/j.toxlet.2006.12.007

al., 2000; Storelli and Marcotrigiano, 2002; Beyrouty and Chan, 2006). This micronutrient is essential or toxic depending on its concentration and chemical forms. Several experimental studies have investigated its protective effects against carcinogenic and/or neurotoxic chemicals and various toxicokinetic and toxicodynamic mechanisms have been reported to explain this interaction (Combs Jr., 2005; Pamphlett et al., 2005).

Knowing that repeated exposure of rodents to low doses of MeHg induces the accumulation of Hg in target organs (Friberg and Mottet, 1989; Berntssen et al., 2004; Harry et al., 2004), considerable attention was focused on brain, kidney and liver Hg levels after co-exposure of animals (fish, aquatic birds, rodents and primates) to different Se compounds; however, a number of inconsistencies related to the changes of Hg levels in target organs were found (Cuvin-Aralar and Furness, 1991; Heinz and Hoffman, 1998; Bjerregaard et al., 1999). Additionaly, no epidemiological studies have shown a correlation between Se intake and the occurrence, or absence, of symptoms for MeHg toxicity (Watanabe, 2002). The implementation of measures to monitor the Hg exposure in high fish consumption populations is an important task and will contribute to elucidate the risk/benefit binomium resulting from ingestion of contaminated fish. In this context, it is imperative to select appropriate biomarkers to validate the MeHg exposure and to detect and/or predict the protective effects resulting from Se dietary content in the populations under study. The selection of biomarkers to apply in epidemiological studies must be based on its early specific biological responses and on the possibility to be measured in accessible biological matrices (Costa, 1996; Manzo et al., 1995, 2001). One of the most used biomarkers of exposure are the urinary porphyrins, which are sensitive and persistent indicators of exposure to various inorganic and organic agents. Hence, porphyrinurias are not specific of mercury exposure, and different metals can inhibit the same biosynthetic heme pathways at different steps (Fowler, 2001; Wang et al., 2002; Wu et al., 2004). MeHg-induced porphyrinuria of renal origin was described in rats and humans at dose levels that did not produce signs of neurological dysfunction. The characterization of urinary porphyrins permitted the identification of a new keto-isocoproporphyrin defined as a specific indicator of Hg exposure and recently this porphyrin has been proposed as a biomarker of susceptibility to Hg (Woods, 1995, 1996; Pingree et al., 2001; Woods et al., 2005; Heyer et al., 2006).

On the other hand, liver is also a target organ which accumulates MeHg and causes oxidative stress associated to thiol hepatic depletion. In fact, it has been shown that MeHg is one of the strongest thiol-binding agents forming complexes with critical endogeneous molecules, particularly intracellular GSH. Besides, one of the major mechanisms of MeHg toxicity is through generation of ROS (Yee and Choi, 1996; Shanker et al., 2005; Chen et al., 2006) and the quantification of GSH levels has been proposed as a biomarker of oxidative stress and as an indicator of Hg toxicity (Thompson et al., 2000; Arteel and Sies, 2001). In this study, the interference of SeMet on GSH hepatic levels is justified by its capacity to promote the synthesis of several selenoenzymes, namely, glutathione-transferase and glutathione-peroxidase, which catalyze the reaction of MeHg liver detoxication and act as scavengers of ROS, respectively (El-Sayed et al., 2006; Arteel and Sies, 2001).

Finally, neurobehavioural functions can be used as a tool for the detection of early changes due to neurotoxic agents and the high sensitivity of several biomarkers of effect has been applied to predict the neurotoxicity induced by MeHg (Manzo et al., 1995; Vezer et al., 2005). In primates, exposure to MeHg mimics the neurological sensory and motor dysfunctions observed in humans. Neurological effects were also observed in rodents, being the motor deficits generally the most evident effects following developmental exposure (Dietrich et al., 2005; Goulet et al., 2003; Kim et al., 2000; Rice, 1996).

The aim of this study is to clarify the interference of Se on MeHg neurotoxicity, through the determination of changes in biomarkers of exposure and effect in rats exposed to MeHg and co-exposed to MeHg and SeMet.

2. Material and methods

2.1. Chemicals

Methylmercury hydroxide (Alfa Aesar), stannous chloride (Fluka), mercury chloride (Riedel), selenomethionine (Sigma), sodium bicarbonate, disodium EDTA, monobasic sodium phosphate, HPLC-grade methanol, hydrochloridric acid, acetic acid, nitric acid, sulphosalicylic acid, 5,5′-dithiobis-(2-nitrobenzoic acid), porphyrin chromatographic markers: uroporphyrin I, heptacarboxyl porphyrin I, hexacarboxyl porphyrin I, pentacarboxyl porphyrin I, coproporphyrin I, and mesoporphyrin IX (Porphyrin Products). All chemicals were reagent grade. Distilled deionised water was used for aqueous solutions.

2.2. Animals

Male Wistar rats (197.3 ± 25.5 g) were obtained from Charles River Lab (Barcelona, Spain). They had free access to tap water and to a daily dose of food and were acclimatized

to the environment for 1 week before dosing. After this period, all the animals were placed in metabolic cages, and identified individually by tail tattoo. Four groups of five rats were intraperitoneally (i.p.) administrated for 2 days (days 1 and 3) with: G1—methylmercury hydroxide (1.5 mg/kg); G2—methylmercury hydroxide + selenomethionine (1.5 mg/kg + 1.0 mg/kg); G3—selenomethionine (1.0 mg/kg); G0—physiologic serum (vehicle), as control group. The choice of doses was based on a preliminary experiment showing that few toxic end points were affected at 1.0 mg/kg of MeHg and no toxicity was observed at 1.0 mg/kg of SeMet.

2.3. Collection of samples

After the period of adaptation to the new controlled environmental conditions, 24-h urine samples were collected for baseline porphyrin measurements. The urine samples were collected once a week, over ice, out of light and in presence of sodium bicarbonate and EDTA. At the end of the experiment, the brain, kidney and liver were removed, after the sacrifice of the animals. Samples were kept at −80 °C until analysis.

2.4. Analytical and behavioural assays

2.4.1. Urinary porphyrin analysis

Urine samples of exposed and unexposed rats were analysed for the determination of total porphyrins using a spectrophotometric method (Soulsby and Smith, 1974). This method is basically an extraction of porphyrin and porphyrinogen into ether from an acidified urine. The ether is then shaken with a solution of iodine in hydrochloric acid to oxidize any porphyrinogen to porphyrin and to extract the porphyrins. Finally, the solution is analysed spectrophotometrically at the peak of the Soret band and at wavelengths on either side to correct any present impurities.

2.4.2. Total mercury determination

Analysis of total mercury in kidney and brain samples was carried out by Cold Vapour-Atomic Fluorescence Spectrometry (CV-AFS) (Merlin, PSA 10.023; P.S. Analytical Ltd.). The Hg concentrations of the samples were determined after acid microwave digestion (Parr Bomb 4781, 23 mL). The solubilized samples were then added to the gas–liquid separator (PSA Analytical) by a valve (Omnifit), and the formed Hg^0 is transported by argon gas passing through a membrane (Permapur), to the detector. The inorganic Hg is reduced to Hg^0 by addition of stannous chloride and chloridric acid to the separator. All utensils used were acid washed to reduce the risk of contamination. All samples were analysed in duplicate.

2.4.3. Hepatic glutathione determination

To quantify hepatic glutathione (GSH) concentrations, the rats of the different groups were sacrificed at the end of the experiment by cervical dislocation. The livers were removed immediately and rinsed in saline at low temperature. The livers were homogenized with 0.1 M sodium phosphate (pH 7.4) and the same volume of 3% sulphosalicylic acid, on ice, followed by centrifugation (2500 rpm, for 10 min at 4 °C). The resultant supernatant was mixed with 0.1 M sodium phosphate (pH 8.0) containing 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid). The absorbance at 412 nm was quantified. Determinations were performed in triplicate and GSH levels were expressed as μmol GSH/g of liver.

2.4.4. Behavioural assays

The first behavioural experiment was started immediately after the end of the exposure and completed within 3 weeks. Two behavioural parameters were studied once a week: ambulation (number of crossings within 5 min in an open field) and rearing (number of times within 5 min where both forelegs were risen from the floor in the same open field). The floor of the open field measured 60 cm × 90 cm and was divided into six equal squares. The floor was surrounded by a 30-cm-high opaque wall. The rats were tested individually, and after each session, the open field was cleaned with 70% alcohol followed by wet cotton. These assays were always performed at a fixed hour of the day with controlled light and sound. At the beginning of each session the animals were placed at the centre of the open field, and the behaviour of each rat was observed for 5 min.

2.4.5. Statistical analysis

Data are presented as mean values ± standard deviations. Statistical comparisons between two groups were made using the Student's *t*-test. With several groups, one-way analysis of variance (ANOVA) was used and when significant *F* values were obtained, group differences were evaluated by the Tukey's post hoc test. A minimum of five animals were included for statistical analysis in each group, and the significance level was $p < 0.05$ unless otherwise indicated.

3. Results

3.1. Mercury in target organs

The Hg quantification in brain and kidney was determined after the sacrifice of the animals 3 weeks after cessation of exposure. As shown in Table 1, the levels of Hg in these organs were found to be significantly

Table 1
Mercury concentration, expressed in μg/kg of tissue, in brain and kidney of unexposed G0 and exposed rats to: G1—MeHgOH (1.5 mg/kg), G2—MeHgOH + SeMet (1.5 mg/kg + 1.0 mg/kg) and G3—SeMet (1.0 mg/kg)

	Brain (μg/g)	Kidney (μg/g)
G1 (MeHg)	0.43 ± 0.21	11.94 ± 1.62
G2 (MeHg + SeMet)	0.33 ± 0.10	12.78 ± 2.96
G3 (SeMet)	nd	nd
G0 control group	nd	nd

Values are means ± S.D. for $n = 5$ (nd: not detectable).

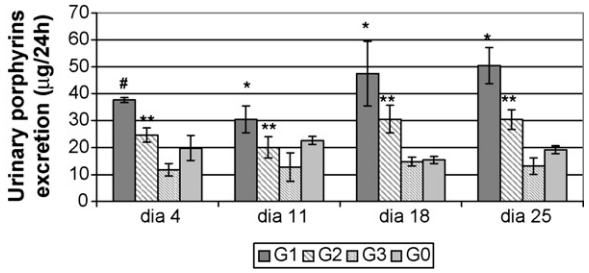

Fig. 1. Urinary porphyrins excretion at days 4, 11, 18 and 25 after the first administration, expressed in μg/24 h, of unexposed G0 and exposed rats to: G1—MeHgOH (1.5 mg/kg), G2—MeHgOH + SeMet (1.5 mg/kg + 1.0 mg/kg) and G3—SeMet (1.0 mg/kg). The intraperitoneally administrations were made at days 1 and 3. Data are means ± S.D. ($n = 5$). $^{\#}p < 0.01$ statistical difference compared with control group. $^{*}p < 0.05$ statistical difference compared with control group. $^{**}p < 0.05$ statistical difference compared with MeHg group.

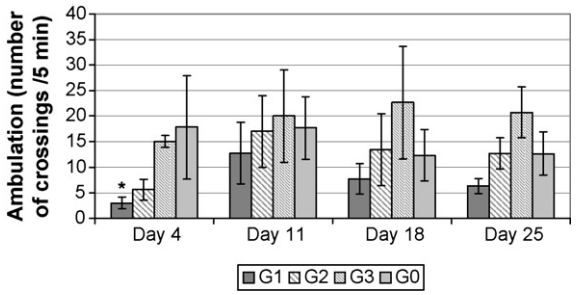

Fig. 2. Ambulation (mean number of crossings within 5 min in an open field) of i.p. administered rats with: G0—physiologic serum (vehicle), G1—MeHgOH (1.5 mg/kg), G2—MeHgOH + SeMet (1.5 mg/kg + 1.0 mg/kg) and G3—SeMet (1.0 mg/kg). $^{*}p < 0.05$ statistical difference compared with control group (day 4).

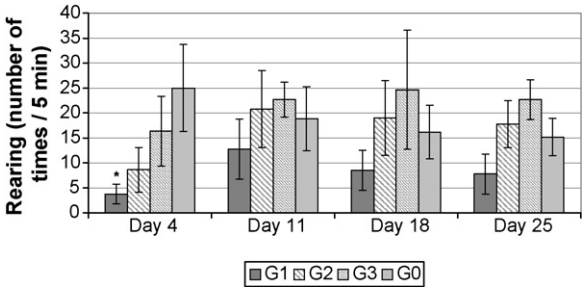

Fig. 3. Rearing (mean number of times within 5 min where both forelegs are raised from the floor in an open field) of i.p. administered rats with: G0—physiologic serum (vehicle), G1—MeHgOH (1.5 mg/kg), G2—MeHgOH + SeMet (1.5 mg/kg + 1.0 mg/kg) and G3—SeMet (1.0 mg/kg). $^{*}p < 0.01$ statistical difference compared with control group (day 4).

increased ($p < 0.001$) in MeHg-treated animals (G1) when compared to control animals (G0). However, no significant changes in kidney and brain Hg levels were observed in SeMet + MeHg-treated animals (G2).

3.2. Urinary porphyrins

Using a sensitive and rapid colorimetric method, the total porphyrins were quantified in urine of G0, G1, G2 and G3 groups, 1 day after and 1, 2, and 3 weeks following the last exposure. The results presented in Fig. 1 show a significant increase ($p < 0.05$ and 0.01) in the concentration of total porphyrins following MeHg treatment (G1) when compared to G0, which persists along the 3 weeks after the last exposure. In addition, the quantification of total porphyrins in the SeMet + MeHg (G2)-treated animals, shows a significant decrease in porphyrins excretion ($p < 0.05$) as compared to G1, and these changes persisted over the following 3 weeks. Finally, no significant differences were observed in the porphyrin levels among the SeMet + MeHg (G2), the SeMet (G3)-treated animals and the control animals (G0).

3.3. Behavioural assays

The neurological alterations were tested the day after and 1, 2, and 3 weeks after exposure cease. As is shown in Figs. 2 and 3, the locomotor activities were evaluated with two commonly used assays in open field (rearing and ambulation) and at the first day after cessation of exposure a significant reduction in the frequency of rearings ($p < 0.01$) and ambulations ($p < 0.05$) was observed in G1 (exposed) when compared to G0. However, the behavioural assays performed in G1, 1, 2, and 3 weeks after exposure, show a tendency to attenuation on motor activity dysfunctions. In addition, in the co-exposed group (G2), no significant increase on motor activity was found, and the data obtained along the 3 weeks of observations, show no significant differences among G0, G2 and G3 groups.

3.4. Liver GSH

The hepatic GSH levels were determined at the sacrifice of the animals, 3 weeks after the last exposure. As shown in Fig. 5, there is a marked decrease in GSH levels in G1 (exposed) when compared to G0 (not exposed). Additionally, no significant decrease of GSH levels was observed in G2 (co-exposed), although when comparing to G1 values a slight enhancement of GSH contents was found.

4. Discussion

In this study, changes on biomarkers of exposure and effect were used to investigate the protective effects of

SeMe against MeHg toxicity. In order to clarify the mechanism of this interaction, analysis of four different biomarkers, namely, (i) Hg contents in kidney and brain; (ii) urinary porphyrins; (iii) hepatic GSH; (iv) motor activity functions, were determined in groups of rats not exposed (G0) and exposed to MeHg (G1), MeHg + SeMet (G2), and SeMet (G3). Among the endogeneous selenium species, selenoaminoacids play an essential role and SeMet serve for selenium storage in proteins and also incorporates selenium-specific enzymes (El-Sayed et al., 2006). On the other hand, the majority of fish species is rich in several selenium compounds, namely SeMet and SeCyst (Onning and Bergdahl, 1999) and in our previous work (Cabanero et al., 2005) SeMet was the only selenium compound identified in the analysed fish species. Thus, we selected SeMet to study the interference of Se on MeHg toxicity. As referred above, it is well known that repeated exposure of rodents to low doses of MeHg induces the accumulation of mercury in target organs and Hg quantification in those organs has been proposed as an indicator of target tissue dose. Neverthless, the changes on Hg levels found in brain and kidney after co-exposure of animals to different Se compounds were contradictory. In this study, the interference of SeMet on MeHg toxicokinetics was firstly investigated through the analysis of Hg in the kidney and brain of all the rats under study. The results (Table 1) show a significant increase of Hg in the kidney and brain of G1 when compared to G0; however, no significant changes of Hg concentration were observed in rats co-exposed (G2) as compared to G1. Our findings suggest that for the tested experimental conditions, Se does not interfere on the redistribution of MeHg in the referred organs. Even so, a different range of doses of MeHg and SeMet, different time periods of treatment and the oral route must be tested.

Regarding the use of porphyrins as biomarkers of MeHg exposure, studies from Woods et al. (1991), refer that MeHg accumulated in kidney is slowly demethylated to Hg^{2+}, and in this form, can inhibit specific enzymes of kidney heme biosynthetic pathway, increasing the excretion of several porphyrins, namely copro-, penta- and keto-isocoproporphyrins. In the present study, a routine and sensitive colorimetric assay was selected to quantify precisely the changes in total porphyrins levels, which were identified (Mateus et al., 2005) as representing mainly coproporphyrins (97%). The data obtained show, a significant increase in total porphyrins excretion in G1 (exposed), when compared to G0. Moreover, it was observed a significant depletion of porphyrins excretion in G2 (co-exposed), when compared to G1. On the other hand, previous work of Woods (1995), reported the characterization of urinary porphyrins in humans and animals, followed by the identification of a new keto-isocoproporphyrin proposed as a specific indicator of Hg exposure. In this study, the urinary porphyrins profile was also performed using HPLC/FLD and the results validated the data obtained for total porphyrins and also confirm coproporphyrins and pentaporphyrins as the most sensitive indicators of Hg exposure (Batoréu et al., 2005). Keto-isocoproporphyrin was also quantified in rat urine; however, due to the trace levels detected it was not considered an useful tool to be used as an indicator of changes in Hg kidney toxicity.

Taking into account the settled experimental conditions, these preliminary results suggest that the interference of SeMet on MeHg porphyrin metabolism is not due to the decrease of Hg accumulation in kidney, but confirm other experimental assays which consider Se responsible for the lower Hg^{2+} availability in the referred organ. In fact, several studies, associate the lower availability of Hg with the formation of Se–Hg biological inert complexes, present in the form of nanocrystals which can be visualised by histochemical techniques (Cuvin-Aralar and Furness, 1991; Frisk, 2001; Loumbourdis and Danscher, 2004). Concerning the neurotoxic effects induced by methylmercury, some clinical signs in motor impairment have been observed in adult humans after environmental exposure to MeHg (Auger et al., 2005; Zahir et al., 2005). Moreover, the inhibitory effects of MeHg in rodent locomotion have been extensively reported (Dietrich et al., 2005). Thus, two functional tests related to the locomotor activity in open field (rearing and ambulation) were selected to assess the neurological changes, and apply as potential predictive biomarkers of Hg neurotoxic effects. Regarding the effects of MeHg treatment, in the first neurobehavioural assay performed in G1, a significant reduction on the frequency of rearings and ambulations was observed when compared to G0 (Figs. 2 and 3); however, in the same group, the assays performed 1, 2, and 3 weeks after cessation of exposure, show a less evident decrease in motor activity, when compared to G0. We also observed a marked attenuation in the motor activity depression in G2 (co-exposed), being the mean value of frequencies on motor activity tests, higher than in G1, and their values along the 3 weeks period came close to the G0 group. These results may be interpreted as a result of a progressive normalization of neurological changes over the post-exposure period. Nevertheless, one limitation of the behavioural assays is their reliability, as they are susceptible to several interferences due to the slightest individual and environmental changes. In fact, the high standard deviation (R.S.D. ~35%) obtained either

with the control or the treated animals groups strongly limits the interpretation of the results.

When comparing the results of Table 1 with the data of Figs. 2 and 3, we may conclude that Se does not interfere on brain mercury levels, whereas its interference on MeHg toxicity is evidenced by a temporary decrease in neurobehavioural motor dysfunctions. Again, the protector effects of SeMet against MeHg neurotoxicity may be explained by its capacity to form insoluble Hg complexes, which lower Hg^{2+} brain availability (Watanabe, 2002).

When comparing the data of Fig. 1 with Figs. 2 and 3 results, a positive correlation was found between the increase of porphyrins excretion and the increase of motor activity dysfunctions in rats exposed to MeHg. Our results also suggest a positive correlation between the decrease of motor activity dysfunctions and the decrease of porphyrins excretion in rats co-exposed to SeMet (Fig. 4a and b).

In this work the GSH levels were used as a biomarker of oxidative stress (Carvalho et al., 2006) and in Fig. 5a lower hepatic GSH in G1 was observed when compared to G0. However, this decrease was not significant, probably due to the known capacity of liver to resynthesize GSH, which could occur during the period preceding the sacrifice of the animals. In addition, in co-exposed

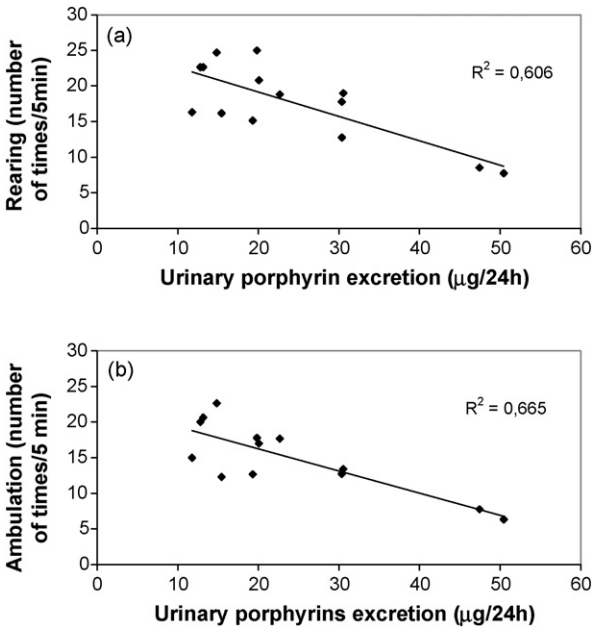

Fig. 4. Correlation between the urinary porphyrin excretion (μg/24 h) and two motor activity functions: (a) rearing (number of times within 5 min where both forelegs were raised from the floor in an open field) $R^2 = 0.60$; (b) ambulation (number of crossings within 5 min in an open field) $R^2 = 0.66$.

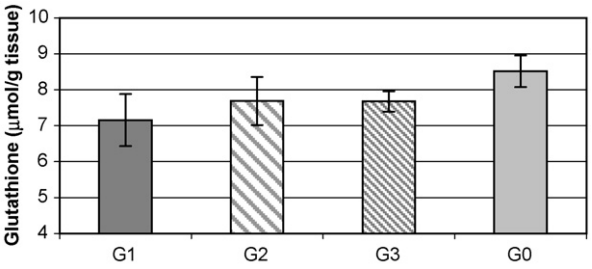

Fig. 5. Liver concentrations of GSH (μmol/g of tissue) in animals exposed to: G1—MeHgOH (1.5 mg/kg), G2—MeHgOH + SeMet (1.5 mg/kg + 1.0 mg/kg) and G3—SeMet (1.0 mg/kg). Unexposed rats (G0) were used for basal values. Data are means ± S.D. $n = 5$.

group (G2) a slight increase in GSH levels was detected when compared to G1. Although these results are not conclusive, they may be an indicator of the capacity of Se to bind Hg^{2+}, leaving GSH available to its detoxification and antioxidant endogeneous functions. Moreover, it is possible that the administrated SeMet induces the synthesis of seleno-enzymes involved in the catalysis of MeHg detoxification process and/or acting as scavengers of ROS, reducing the pro-oxidant MeHg toxic effects (El-Sayed et al., 2006; Arteel and Sies, 2001).

In conclusion, the analysis of biomarkers of exposure and effect in rats exposed to MeHg and co-exposed to SeMet, shows that Se interferes on MeHg toxicity demonstrated by the significant decrease of porphyrins excretion and by a temporary attenuation of motor activity dysfunctions. However, these results also confirm that the mechanism of this interaction cannot be explained by the reduction of Hg levels in kidney and brain. Finally, among all the selected biomarkers, the most sensitive and persistent indicators of MeHg toxicity were the urinary porphyrins.

These results provide a baseline for future works dealing with the role of SeMet on MeHg redistribution and/or its bioavailability in target organs, in order to clarify how Se may contribute to the detoxication of MeHg and the delay or protection against its toxic effects.

Acknowledgement

Project POCTI/41741/ESP/2001—Risk assessment of methylmercury exposure: integrated actions through the food chain. Eixo 2, medida 2.3 POCTI do QCA III (components FEDER e OE).

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