

Apoptosis, metallothionein, and bioavailable metals in domestic mice (*Mus musculus* L.) from a human-inhabited volcanic area

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Abstract The influence of extreme environments of volcanic origin over vertebrates and the cellular responses that these may give are almost unknown. The main objectives were to evaluate the exposure of mice to metals in the interior of houses of a small village settled inside a volcanic crater (Furnas, Azores), and the levels of apoptosis and metallothionein in the organs (lung, liver, and kidney) of those animals. Adult mice (*Mus musculus*) were captured in two areas, one with volcanic activity and the other without it over the last three centuries. In the excised organs, analysis of metals (Al, Cd, Pb, Zn), TUNEL assay for apoptosis, and immunohistochemistry for metallothionein were undertaken. Mice from the area with volcanic activity presented higher levels of apoptosis and metallothionein than those from the area without volcanic activity. Such results were in agreement with the differences in metal burdens of the three organs, and interestingly these concentrations were similar to or higher than others found in heavily polluted areas outside the Azores. Thus, there may be a high risk of harmful effects for organisms, including humans, inhabiting areas with volcanism, where hazardous gases and metals in the air are very common during the entire day or even all year round.

Keywords *Mus musculus* · Apoptosis · Metallothionein · Volcanism · Metal

Introduction

Although many metals occur in the environment as a result of anthropogenic activities, their concentration in organisms may also be explained by exposure to natural sources of these elements (Olsvik et al. 2000; Amaral and Rodrigues 2005; Amaral et al. 2006a, b; Zaldibar et al. 2006). One example of natural input of metals in soil, water, and air is volcanic activity, which may manifest through lava emissions, degassing soils, and hydrothermal sources, and is responsible for the presence of metals in those media, as particles or associated with gases (Ferreira and Oskarsson 1999; Delmelle and Stix 2000; Kelepertsis et al. 2001; Durand et al. 2004; Hansell et al. 2006).

Accumulation of trace metals in different tissues and organs of terrestrial invertebrates (Heikens et al. 2001; Lock and Janssen 2001; Amaral and Rodrigues 2005; Amaral et al. 2006b) and vertebrates (Wlostowski et al. 2000; Pereira et al. 2006) exposed to natural and anthropogenic sources of these elements has been reported in many previous studies. For this reason, wild animals have been used to evaluate exposures to inorganic contaminants, and data collected from these animals are considered very useful for human and environmental health risk assessment. Also, it is widely accepted that this type of assessment cannot be based solely on chemical analyses, and that it should include the measurement of biological effects resulting from pollutants (Walker 1998; Kakkar 2005).

Chronic exposure to trace metals and metalloids, resulting in continued bioaccumulation, has been linked to several injurious effects in living organisms. Many metals can be mutagenic (Filipic and Hei 2004; Hei and Filipic 2004), carcinogenic (Waalkes 2003; Waisberg et al. 2003), teratogenic (Calevro et al. 1998), and can also alter the activity of enzymes, transport proteins, cell and tissue

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structure and functions (Wlostowski et al. 2000; Yeh et al. 2000; Damek-Poprawa and Sawicka-Kapusta 2004). Trace metals may also induce apoptosis (Lin et al. 2003; Wätjen and Beyersmann 2004; Banasik et al. 2005; Xu et al. 2006) and metallothionein expression (Bracken and Klaassen 1987; Jeffery et al. 1987; Huang et al. 2007) in several tissues and organs. Apoptosis, under both normal and pathological conditions, controls the size of cell populations in different types of organs and tissues, and directs the morphological restructuring, avoiding modifications to the normal pattern during development. This well-conserved mechanism is involved in cellular turnover in normal adult animals, it occurs spontaneously in malignant tumors, and is also present in embryogenesis, lymphocytic selection within the thymus or involution of the mammary gland after a lactation period (Kerr 2002; Zhang and Xu 2002; Hwang et al. 2004). Metallothioneins (MTs) are low-molecular weight (6–7 kDa) and cysteine-rich proteins that normally bind group 1B and 2B metals, and that are present in all vertebrates and in the majority of eukaryotic organisms (Olsson et al. 1998). These proteins are involved in the homeostasis and storage of copper and zinc, and play a protective role against the toxic effects of heavy metals, making them inactive and promoting their excretion (Kägi and Kojima 1987; Kägi and Schaffer 1988; Roesijadi 1994; Nordberg 1998; Binz and Kägi 1999). Therefore, cellular and tissue injuries, apoptosis and metallothionein expression may eventually be considered important biomarkers of the effects induced by chronic exposure to metals in natural conditions (Wlostowski et al. 2000; Amaral and Rodrigues 2005; Amaral et al. 2006b; Pereira et al. 2006).

In Furnas (Azores, Portugal), plants and animals, including a population of approximately 1,500 humans, live on top of geothermal ground where soil degassing, hydrothermal vents and gas emissions occur, and as a consequence of this they are chronically exposed to volcanic gases and metals. Besides the high bioavailability of cadmium and zinc found in soil invertebrates from this area (Amaral et al. 2006b), high incidence rates of some cancer types and chronic bronchitis has also been registered in local humans (Amaral et al. 2006c; Amaral and Rodrigues 2007). In order to assess the exposure and effects to a mixture of trace metals in this area, domestic mice (*Mus musculus* L.) from several households were collected for the present study. This species was chosen because it is considered a good sentinel, as (i) it is ubiquitous and can be found in both active and non-active volcanic areas; (ii) it shares the same houses as humans and eat their food; (iii) it occupies a middle position in many food chains; (iv) it contacts with soil, not only by living in holes made in the soil but also by ingesting the soil; (v) it has small ranges of action, usually an area of 64 m² (Timm 1994); and (vi) its populations are usually

large. The aims of this study were (i) to evaluate the exposure of *M. musculus* to four metals (Al, Cd, Pb, Zn) occurring naturally in the interior of several houses settled inside the crater of Furnas volcano, and outside of it; and (ii) to investigate the influence of an extreme environment of volcanic origin, with hazardous gases and metal emissions, over apoptosis and metallothionein synthesis in some organs of the same mice.

Materials and methods

Samples collection

Mice were collected in human-inhabited houses at Furnas and Rabo de Peixe (São Miguel Island, Azores, Portugal). The former is a rural locality built upon actively degassing ground inside a volcanic crater, where fumarolic fields and hydrothermal vents are current manifestations of volcanism and are the cause for ongoing natural exposure to high levels of metals and gases (Baxter et al. 1999; Cruz 2003; Ferreira et al. 2005). The latter is approximately 20 km apart from Furnas, and does not present any type of volcanic manifestations since the seventeenth century (Carvalho 1999). During spring and summer, two groups of male mice (*M. musculus*) were collected using mechanical traps that kept them alive and without wounds. One group corresponded to 11 adult mice from several houses in Furnas, and the other (control) corresponded to 8 mice, also adult, collected in Rabo de Peixe. Mice were transferred to the laboratory, where they were anesthetized and sacrificed. For this study, only males were used, because females present important and exclusive ways of detoxification associated with menstruation, pregnancy, parturition and suckling. These phenomena grant them a much higher variability, since they have 5 to 10 liters of 5 to 6 pups each (Timm 1994), and through blood and milk they eliminate many organic and inorganic toxicants (Messiha 1989; Manfroi et al. 2004).

Chemical analyses and histological processing

From each mouse, both kidneys, both lungs, and the liver were excised. One kidney, one lung and a piece of the liver were dried (130°C), digested in concentrated nitric acid (HNO₃), and finally dissolved in 0.1 N HNO₃, prior to analysis of Al, Cd, Pb, and Zn by flame atomic absorption spectrophotometry. The other kidney, lung and rest of the liver were fixed, for 5 h, in neutral buffered formaldehyde (Hopwood 1996), dehydrated in alcohol, cleared in xylene, embedded in paraffin, and sectioned. Three sets of sections were obtained from each organ, one of 7 μm thick was for routine histological staining with haematoxylin-eosin, and

the other two of 4 μm thick were for in situ detection of apoptosis and metallothionein.

TUNEL assay

The detection of apoptotic nuclei in the tissues of kidney, lung and liver was performed using a DeadEndTM kit (Promega). Briefly, tissue sections were dewaxed and rehydrated. Sections were then washed in phosphate buffered saline (PBS) and treated with 20 $\mu\text{g}/\text{ml}$ proteinase K for 20 min at room temperature. DNA of the tissue sections was labeled at 3' ends with biotin-dUTP by incubation with the reaction buffer containing recombinant terminal deoxynucleotidyl transferase, for 60 min at 37°C. Tissues were then treated with 3% H_2O_2 for 5 min to inactivate endogenous peroxidase, and incubated with streptavidin horseradish peroxidase conjugate to detect biotinylated nucleotides for 30 min at room temperature. Diaminobenzidine reacted with the labeled samples to generate an insoluble colored substrate at the site of DNA fragmentation. Finally, sections were counterstained with methyl green or haematoxylin to aid in the morphological evaluation and characterization of normal and apoptotic cells. The percentage of apoptotic nuclei was graded as follows: 0 (no staining); 1 (>0 a 50%); 2 (>50%).

MT quantification

Sections were dewaxed in xylene, hydrated in acetone, brought to distilled water and washed in PBS. Endogenous peroxidase activity was quenched by shortly incubating the sections in 3% hydrogen peroxide. Sections were then washed in PBS and incubated at room temperature, inside a moist chamber, for 30 min with a blocking solution, which was made of 5% normal goat serum diluted in PBS. After a brief rinse in PBS, sections were incubated overnight, inside a moist chamber, at 4°C with the antibody Mouse Anti-Metallothionein, clone E9, (Zymed) diluted (1:100) in PBS. After several baths in PBS, sections were incubated during 1 h at 4°C, inside a moist chamber, with the antibody Goat Anti-Mouse IgG (Sigma) diluted (1:20) in PBS. Then rinsed in PBS, incubated with ExtrAvidinTM (Sigma) (1:20) in PBS, for 30 min. Following several rinses in PBS, the visualization of peroxidase activity was achieved using 3-amino-9-ethylcarbazole (AEC) (Sigma). Finally, after a brief rinse in PBS, sections were counterstained with haematoxylin (5–10 s), washed in water and mounted in glycerol gelatine. In control sections PBS was used instead of the primary antibody solution. The semi-quantitative assessment of the MT immunohistochemical levels found on tissue sections was performed on a consensus basis by four observers (A.A., C.C., C.G. and A.R.) using a Sony

CCD-Iris camera coupled to a Laborlux S (Leitz) light microscope. After establishing the criteria to state the consensus basis, a previous trial was done with no significant differences among the results obtained by the four observers. According to a method previously described (Tuccari et al. 2000; Amaral et al. 2002), the percentage of stained cells (staining score) was graded as follows: 0 (no staining); 1 (>0 to 5%); 2 (>5 to 50%); 3 (>50%). Additionally, an intensity-distribution index (IDI) was calculated by multiplying, for each case, the staining score by the staining intensity (weak = 1; moderate = 2; strong = 3).

Statistical analyses

Differences in metal concentrations of the three organs were examined by a one-way ANOVA and considered significant when $P \leq 0.05$. Differences between the levels of apoptotic nuclei and in situ metallothionein were examined by a Mann–Whitney U test. Pearson's correlations between the metal burdens in the three organs and apoptotic nuclei and metallothionein were calculated using SPSS 13.0 (SPSS Inc.).

Results

In general, the concentrations of Al, Cd, Pb and Zn in mice from Furnas were higher than those from Rabo de Peixe. The kidney of mice from Furnas presented significantly higher levels of all the above metals than those from Rabo de Peixe. The same tendency was observed for the liver. With a mixed behavior, the lung of mice from Furnas presented Al and Cd concentrations significantly higher than those from Rabo de Peixe, but the opposite occurred in the case of Pb. Among the three studied organs the lung was the one presenting higher levels for all of the above elements (Fig. 1).

In the kidney and liver, the four metals presented significantly strong and positive correlations between each other, while in the lung this behavior was not that clear (Table 1).

After application of the TUNEL assay and the protocol for in situ detection of MT, one found that the levels of apoptotic nuclei (Fig. 2) and MT (Fig. 3) in the kidney, liver, and lung of mice from Furnas were higher than in the organs of those from Rabo de Peixe (Fig. 4). In the kidney and liver, the levels of apoptotic nuclei were found to be significantly correlated to the amount of MT expressed in situ, but this was not the case in the lung. However, the same levels of apoptotic nuclei in the three organs presented significant correlations to Cd and Pb. The

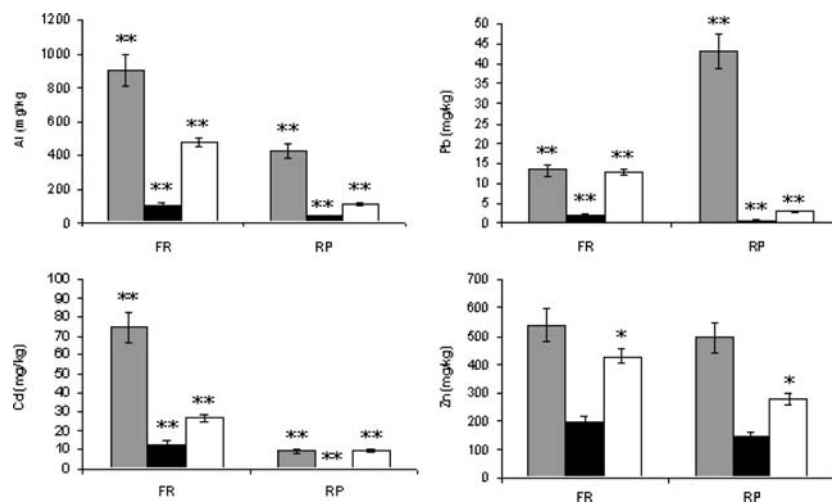


Fig. 1 Concentrations of metals (mg/kg dry weight) [$X \pm se$] in the lung (■), liver (▀) and kidney (□) of *M. musculus* from Furnas (FR; with volcanic activity) and Rabo de Peixe (RP; without volcanic

activity). Asterisk and double asterisk over the bars indicate significant differences at $P \leq 0.05$ e a $P \leq 0.01$, respectively

Table 1 Significant Pearson's correlations between apoptotic nuclei (AN), metallothionein (ID_{MT}), aluminum (Al), cadmium (Cd), lead (Pb), and zinc (Zn) found in the kidney, liver, and lung of *M. musculus* from Furnas and Rabo de Peixe

| | Kidney | Liver | Lung |
|-------|---------|-------|---------|
| AN/MT | 0.645** | AN/MT | 0.557* |
| AN/Cd | 0.551* | AN/Zn | 0.538* |
| AN/Pb | 0.551* | AN/Cd | 0.618** |
| AN/Al | 0.551* | AN/Pb | 0.614** |
| MT/Zn | 0.517* | AN/Al | 0.611** |
| MT/Cd | 0.597* | MT/Zn | 0.746** |
| MT/Pb | 0.606* | MT/Cd | 0.759** |
| MT/Al | 0.606* | MT/Pb | 0.780** |
| Zn/Cd | 0.932** | MT/Al | 0.782** |
| Zn/Pb | 0.884** | Zn/Cd | 0.834** |
| Zn/Al | 0.887** | Zn/Pb | 0.914** |
| Cd/Pb | 0.993** | Zn/Al | 0.932** |
| Cd/Al | 0.994** | Cd/Pb | 0.986** |
| Pb/Al | 1.000** | Cd/Al | 0.977** |
| | | Cd/Pb | 0.999** |

*Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level

expressions of MT in the kidney and in the liver were found to be correlated to the concentrations of all metals, and again the lung presented a different behavior (Table 1).

Discussion

The concentrations of metals in mice from Furnas and also from Rabo de Peixe were very high and unusual for regions considered unpolluted and marked by the lack of heavy industry, such as the Azores. However, these high concentrations are in agreement to previous studies performed with marine and terrestrial invertebrates from this volcanic archipelago, where barnacles presented the highest concentrations of Cd ever measured in this type of organism

(Weeks et al. 1995), and where amphipods and annelids exhibited Zn levels only comparable to those found in industrialized areas (Moore et al. 1995; Amaral et al. 2006b).

In mice from Furnas, the concentrations of Al, Cd, Pb, and Zn were, in general, significantly higher than those from Rabo de Peixe. In the liver and kidney of mice from the volcanic active area, the levels of those elements were so high that in the case of Pb they were very close to the ones found in bank voles from an abandoned lead mine in UK, and in mice and rats from a sulfur mine in Portugal mainland. In the case of Cd and Zn, levels found in the liver and kidney were 10–30 times and 2–6 times, respectively, higher to those of rodents from the abandoned mines mentioned before (Milton et al. 2003; Viegas-Crespo et al.

Fig. 2 Sections of the kidney (A), liver (B) and lung (C) of *M. musculus* from Furnas with many apoptotic nuclei. Sections of kidney (D), liver (E) and lung (F) of *M. musculus* from Rabo de Peixe almost without apoptotic nuclei. Scale bars = 10 μ m

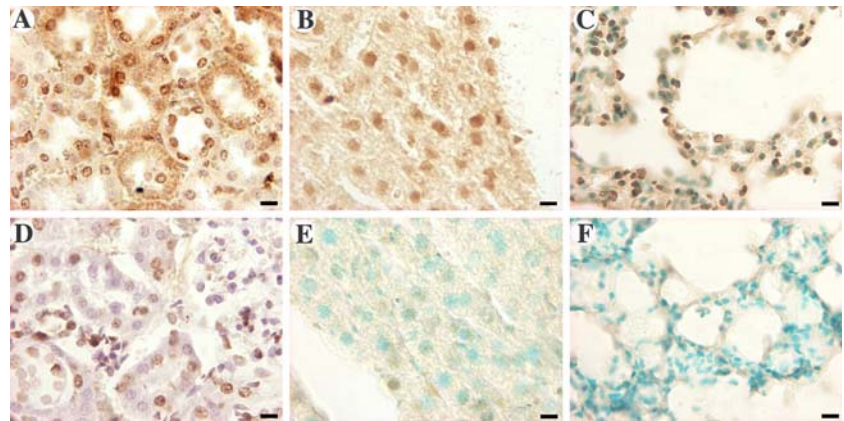
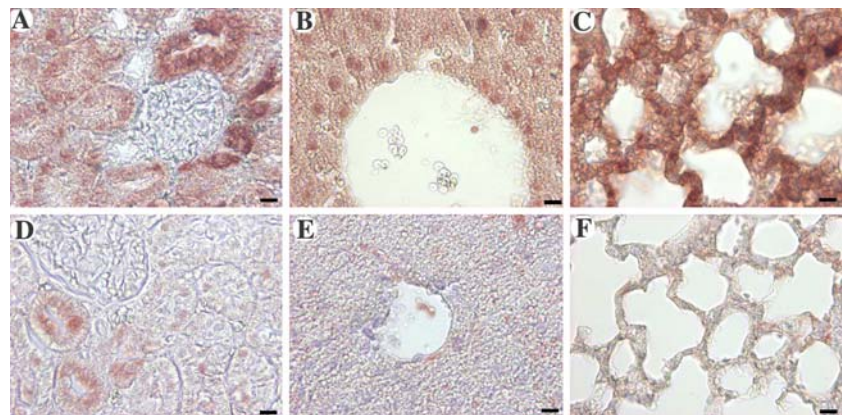


Fig. 3 Sections of kidney (A), liver (B) and lung (C) of *M. musculus* from Furnas with high IDI_{MT}. Sections of kidney (D), liver (E) and lung (F) of *M. musculus* from Rabo de Peixe almost without stain for MT, thus with low IDI_{MT}. Scale bars = 10 μ m



2003; Pereira et al. 2006). The very high levels of Al, Cd, Pb, and Zn in mice from Furnas may result from the ingestion of food or soil rich in those metals but also from the inhalation of volcanic gases and aerosols that transport those elements (Durand and Grattan 1999; Ferreira and Oskarsson 1999; Durand et al. 2004), as confirmed by the high levels found in the lung. In mice from Rabo de Peixe, the lung presented levels of Pb higher than those from Furnas, that may be justified by the proximity of that sampling area to a site where extraction of basaltic rock and aggregate production occurs, this way contributing with dusts produced during the separation and transformation of basalt rich in Pb (Drever 1997) that can be retained, in part, in the respiratory tract but also in the lung. Cd and Pb, on their own or in mixtures, besides being able to cause the diminishment of ciliated bronchial cells, which are responsible by the secretion of pulmonary surfactants necessary to protect the bronchi (Fortoul et al. 2004, 2005), can also cause either apoptosis or lung cancer (Singh et al. 1999; Kwon et al. 2003). In spite of the few known harmful effects of Al over biologic systems, it is recognized its preferential accumulation in the lung, where it can originate fibrosis and granulomatosis when in high concentrations (Agency for Toxic Substances and Disease Registry 1999).

According to several studies, high concentrations of Al and Zn may be associated to the formation of β -amyloid in the brain, which are implicated in Alzheimer's disease (Bush et al. 1994; Zatta et al. 2002; Kaizer et al. 2005; Mekmouche et al. 2005); therefore it should not be excluded the hypothesis of these metals being mobilized from the lung, liver or kidney to the brain where they act as neurotoxins.

The levels of apoptotic nuclei, in all organs, were higher in mice from Furnas than in those from Rabo de Peixe. However, significant differences were only seen in the case of the lung, which can be attributable to the high concentrations of metals, especially Cd, found in this organ. The action of metals, such as Cd and Pb, can deteriorate DNA and originate cancer, yet the same metals can also initiate the process of apoptosis capable of eliminating cells with damaged DNA and this way prevent cancer (Singh et al. 1999). From the four metals taken into account, Cd is one of the most toxic and the one that recognizably induces apoptosis in cells from different organs (Hamada et al. 1997; Habeebu et al. 1998; Wätjen and Beyersmann 2004), and is capable of causing this type of cellular death in the lung (Lag et al. 2002) even at 2.5 mg/kg (Kwon et al. 2003), i.e. at levels well below the ones found in the present study. The other three metals are also known to

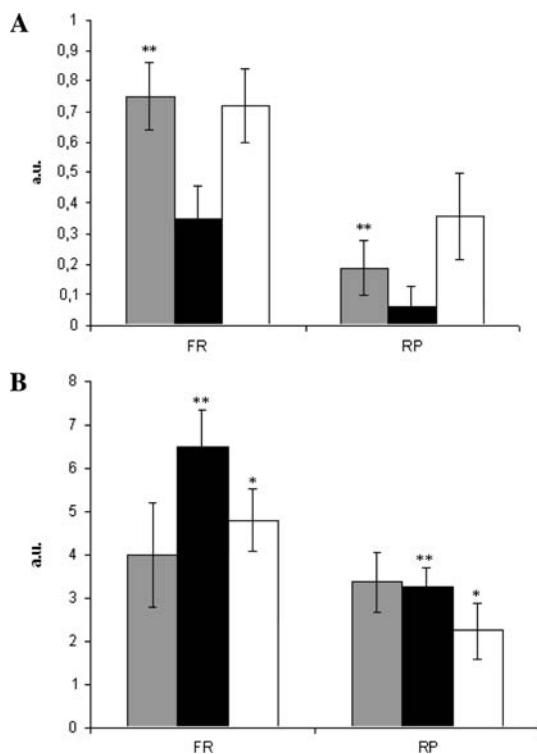


Fig. 4 (A) (Semi)quantification of apoptotic nuclei [$X \pm se$] in the lung (■), liver (■) and kidney (□) of *M. musculus* from Furnas (FR; with volcanic activity) and Rabo de Peixe (RP; without volcanic activity). (B) (Semi)quantification of IDI_{MT} [$X \pm se$] in the lung (■), liver (■) and kidney (□) of *M. musculus* from Furnas (FR; with volcanic activity) and Rabo de Peixe (RP; without volcanic activity). a.u. = arbitrary units. Asterisk and double asterisk over the bars indicate significant differences at $P \leq 0.05$ and $P \leq 0.01$, respectively

cause apoptosis in several organs (Sensi et al. 1999; Singh et al. 1999; Jiang et al. 2001; Lin et al. 2003; Toimela and Tähti 2004; Banasik et al. 2005; Xu et al. 2006). Besides metals, gases like hydrogen sulfide (H₂S) and sulfur dioxide (SO₂), as well as sulfuric acid aerosols, present in the air of Furnas should be pondered since an association between H₂S and SO₂ and apoptosis in lung and aorta cells has been found elsewhere (Yang et al. 2004; Bai and Meng 2005).

The levels of MT (IDI_{MT}) were higher in mice from Furnas than in those from Rabo de Peixe. This is coincident with the levels of apoptotic nuclei and with the concentrations of metals in the three analyzed organs. Besides that, differences between MT levels in mice from the two areas, especially in the kidney and liver, suggest that the four metals may all be partly responsible for the observed results. All four metals have been shown to induce MT expression, either directly or indirectly by altering the concentrations of Cu and/or Zn in the cytosol of cells from different organs (Bracken and Klaassen 1987; Jeffery et al. 1987; Bobillier-Chaumont et al. 2006; Huang et al. 2007). The synthesis of MT is efficiently induced in several

organs to cope with the excessive exposure to metals, acting as a way of neutralizing and eliminating those elements (Kägi and Kojima 1987; Mullins and Fuentealba 1998; Amaral et al. 2002; Bobillier-Chaumont et al. 2006). Without MT synthesis, damages in the affected organ produced by the excess of metals could be larger than those occurring in the presence of MT, since detoxification would be delayed (Jia et al. 2004).

In volcanic environments, soils, vegetables grown in these, and gases constitute natural sources of metals, such as Al, Cd, Hg, Pb, and Zn, to living organisms (Ferreira and Oskarsson 1999; Kelepertsis et al. 2001; Turkdogan et al. 2002) that can accumulate those in different cellular or organ compartments. Previous studies, in areas with volcanic activity, showed (i) the existence of high accessibility and bioavailability of Zn and Cd in soils and terrestrial invertebrates living in these soils; (ii) that high levels of apoptosis in these organisms were associated to morphologic changes in the chloragogenous tissue and intestinal epithelium caused by high levels of Zn and Cd (Amaral and Rodrigues 2005; Amaral et al. 2006a, b); and (iii) that volcanic gases and metals, as well as vegetables grown in volcanic soils are associated to high incidence of gastrointestinal cancer as was found in Turkey (Turkdogan et al. 2002).

Taking from this study, the level of exposure of *M. musculus* to metals (Al, Cd, Pb, and Zn) in the area with volcanic activity is extremely high and preoccupying if one takes into account that these small rodents share the same houses as humans in that area. Although more research is needed to improve the knowledge of risks associated to living in volcanic environments, where hazardous gases and metals are present in a daily basis, it seems realistic to suggest the existence of an increased risk of noxious effects for those inhabiting areas with volcanic activity. Thus, volcanic environment acts as a stress factor to organisms leading their organs and tissues to adapt, e.g. altering their rates of cellular turnover and detoxification, as a way of preventing severe damages to their health.

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