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Original Research Paper

Biomarkers Responses of Land Snails *Helix aspersa* Exposed to Chronic Metal Pollution under Field and Laboratory Conditions

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ABSTRACT

The effects of exposure to metals under field and laboratory conditions were investigated in the terrestrial land snail *Helix aspersa*. In this study, terrestrial snails, collected from an uncontaminated site in Guelma city (North east of Algeria) and transplanted at the industrial zone of El hadjar contaminated by several heavy metals. On the other hand groups of *Helix aspersa* were exposed to increasing concentrations of industrial metal dust (100, 300 and 500µg/g of diet) for a total duration of 12 weeks. A battery of non-enzymatic biomarkers malondialdehyde (MDA), glutathione (GSH) and enzymatic biomarkers catalase (CAT), glutathione S-transferase (GST) were applied for the estimation of biochemical effects induced by the chronic exposure of snails to mixture of metal dust. Several responses have been revealed in digestive gland, serum and HLS. The results showed that CAT activity and MDA content were significantly higher in snails from the polluted site of El Hadjar and specimens exposed to high concentration of metal dust. In contrast GST activity and GSH level showed significant decrease in both transplanted and metal dust exposed snails. Therefore, our results showed the importance of *H. aspersa* as a sentinel organism for biomonitoring.

INTRODUCTION

Environmental pollution by metals has become one of the most important problems in the world. Environmental poisoning by heavy metals has increased in the last decades due to extensive use of heavy metals in agriculture, chemical and industrial processes, becoming a threat to living organisms (Mule & Lomte 1994). *Helix aspersa* snails, whose biology and ecology are well known (Cain 1983, Barker 2001), are macro concentrators for several metals (Dallinger 1993) and one of the most commonly used species in ecotoxicological laboratory experiments (Laskowski & Hopkin 1996a, 1996b, Gomot 1997, Gomot-de Vaufleury 2000). *H. aspersa* snails have also been used as MTE bioindicators in polluted areas in passive (Beeby & Richmond 1998, Gomot-deVaufleury & Pihan 2000) as well as active (Gomot-de Vaufleury & Pihan 2000) biomonitoring studies. Cell damage induced by heavy metals is commonly associated with the ROS formation (Leonard et al. 2004), which needs to be counteracted by defence systems. Thus, assessment of parameters related to oxidative stress in specific sentinel organisms could be included in studies of environmental pollution to predict the impact of pollutants present in the environment (Walker et al. 1976, Pellerin-Massicote 1994, Livingstone 2001). The use of a battery of biomarkers is more advantageous than the use of a single biomarker. Lipid peroxidation (LPO) of membrane lipids, or the oxidation of polyunsaturated fatty acids are observed and used as a biomarker to analyse the effect and exposure to metals (Winston & Di Giulio 1991, Romeo et al. 2000, Gravato et al. 2006). Enzymes of the detoxification machinery can serve as important markers of environmental pollution (Filho et al. 2001). One of the most abundant and ubiquitous detoxification enzyme families is the glutathione-S-transferase family. These enzymes play a pivotal role in inhibiting the cellular damage produced by a wide variety of structurally diverse carcinogens and endogenous toxins (Ansher et al. 1986, Rees 1993, Jaiswal. 1994). The utility of antioxidant enzymes as biomarkers of metal pollution was established by several investigators (Geret et al. 2002, 2003, Grara et al. 2012, Boucenna et al. 2015). This includes non-enzymatic parameters such as glutathione (Cossu et al. 1997, Fernandez-Checa 2003), and enzymatic parameters like catalase (CAT). Therefore, CAT is thought as a key enzyme of the antioxidant defence systems which can protect host cells by removing cytotoxic H_2O_2 (Bai et al. 1999, Bai & Cederbaum 2003).

The aim of this study was to assess the effect of the chronic exposure to the industrial metal pollution in land snails under field and laboratory conditions, by using a suite of biomarkers.

MATERIALS AND METHODS

Biological Material

Gastropod terrestrial snails (*Helix aspersa*) (average weight of 8.5 ± 0.15 g) were collected from an uncontaminated, site of Guelma city (Northeast Algeria) considered as reference site.

Metal Releases

Metal dust used in this study was collected at steel complex EL-Hadjar and a chemical analysis by atomic absorption was made to determine the composition of this dust. This analysis identified the presence of 7 heavy metals listed in the Table (1).

Exposure Modalities

Field exposure: Specimens of *Helix aspersa* were handpicked from a relatively unpolluted site of Guelma (Northeast Algeria) and transplanted in the city of El Hadjar in the surroundings of the steel complex EL-Hadjar. This steel complex generates considerable quantities of metal dust released in the atmosphere.

Gastropods *H. aspersa,* divided into 2 groups of 40 specimens, and settled in metallic cages (45×35×25 cm) excluding a direct contact with soil. After 12 weeks of exposure, snails were recovered and sacrificed.

Experiment under laboratory conditions: The experiment was performed over 12 weeks under controlled conditions at room temperature 20 ± 2° and a 18/6-h light : dark cycle, humidity 80 to 95%. Animals were maintained in plastic boxes (40×35×15 cm).

Treatment of animals was performed by adding increasing concentrations of metal dust in the diet (wheat flour). We selected three concentrations and a control medium (100, 300, 500 µg/g of food). Snails were divided into 8 lots (15 snails/lot.)

Collection of haemolymph, isolation of plasma and haemocytes and preparation of haemocyte lysate suspension (HLS): Haemolymph was collected with a glass micropipette after puncturing the shell, and touching the foot with the point of a micro pipette tip, the snail was forced to retract deeply into its shell and haemolymph was extruded (Sminia & Barendsen 1980). In this way about 300 µL of haemolymph was obtained from each snail. The haemolymph was centrifuged at 15000 rpm for 45 min at 4C° to collect serum, and the haemocyte lysate suspension (HLS) was obtained followed the same procedure as that described by Gopalakrishnan et al. (2009, 2011).

Tissue preparation and enzyme determination: At the end of the experimental period the snails were killed by deep freezing. Shells were removed and digestive gland was excised. Digestive gland, serum and HLS were used for determination of responses to oxidative stress by measuring measures of lipid peroxidation and activities of antioxidant enzymes

Lipid peroxidation (LPO) was determined by the measuring of malondi-aldehyde (MDA) equivalents formed by reaction with thiobarbituric acid (Draper & Hadley 1990) and absorbance was measured at 532 nm. Catalase activity was determined according to the method of Regoli & Principato (1995).

Concentrations of GSH were estimated by the method of Weckberker & Cory (1988), by reading the O.D. of the yellow substance formed when 5,5-dithio-2-nitrobenzoic acid is reduced by glutathione at 412 nm.

Glutathione-S-transferase (GST) activity of the fraction obtained with the substrate 1-chloro-2, 4-dinitrobenzene was measured spectrophotometrically by following conjugation of the acceptor substrate with glutathione as described in Habig et al. (1974).

Statistical Data Analysis

The statistical analysis was performed using data analysis software: Minitab (Version 14.0). The mean values obtained in the different groups were compared by unpaired Student's *t*-test, A $P<0.05$ was considered statistically significant. The values of all biochemical parameters were expressed as mean ± SE.

Table 1: Composition in ppm dust rejected by the steelworks 1 and steel works 2 of the steel complex of El Hadjar-Annaba (Tadjine et al. 2008).

Sample	Cu	Zn	Pb	Cr	Ni	Mn	Fe
Dust steelwork 1	3,7	240	24	10	1,2	320	3000
Dust steelwork 2	7	280	62,4	12	1,3	540	3600
Total	10,7	720	88,4	22	2,5	860	6600

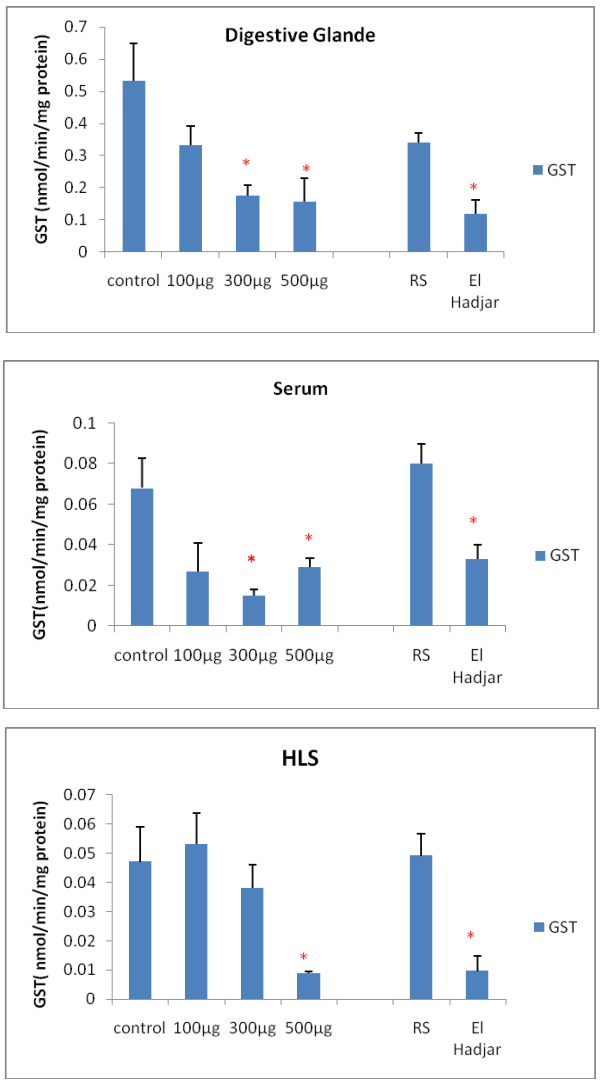

Fig. 1: Glutathione S-transferase activity in digestive gland, serum and HLS of the snails (*Helix aspersa*) exposed to metal dust in the laboratory condition and snails from field (RS: Reference site and El Hadjar). Data are reported as mean ± standard error. Asterisks indicate difference from control values (paired *t* test: *P<0,05, **P<0,01)

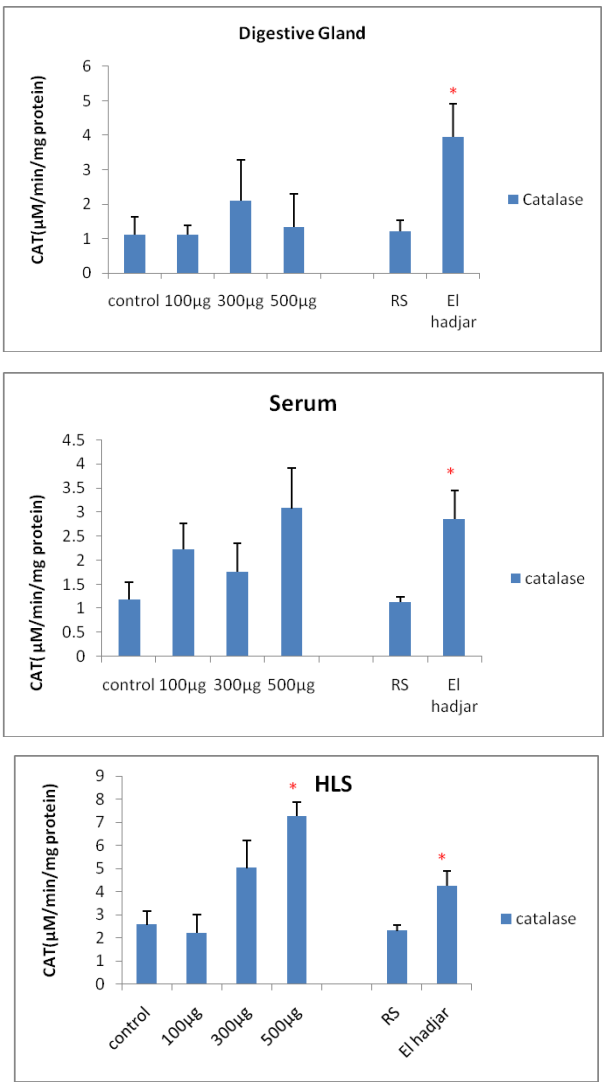

Fig. 2: Catalase activity in digestive gland, serum and HLS of the snails (*Helix aspersa*) exposed to metal dust in the laboratory condition and snails from field (RS: Reference site, and El Hadjar). Data are reported as mean ± standard error. Asterisks indicate difference from control values (paired *t* test: *P<0,05, **P<0,01).

RESULTS

Our results revealed significant differences in the oxidative stress and antioxidant defence system of the snails. GST activity (Fig. 1) was significantly less ($P < 0.05$) in HLS, serum and digestive gland of both caged snails in the contaminated site of El Hadjar and organisms exposed to the highest concentration of metal dust (300µg/g) and (500µg/g) compared respectively to samples from the reference site of Guelma and control snails under laboratory conditions.

Catalase activity reported in (Fig. 2) revealed a significant increase in catalase activity ($P<0.05$) in HLS, serum and digestive gland in the snails from polluted site of El Hadjar compared to samples from the reference site. In contrast no significant differences were observed between control and exposed snails to metal under laboratory conditions , except in HLS of snails exposed to the highest metal concentration (500µg/g), which showed significant increase in CAT activity.

The level of glutathione in snails from field and laboratory conditions are reported in (Fig. 3). GSH content was significantly lower in digestive gland tissue and

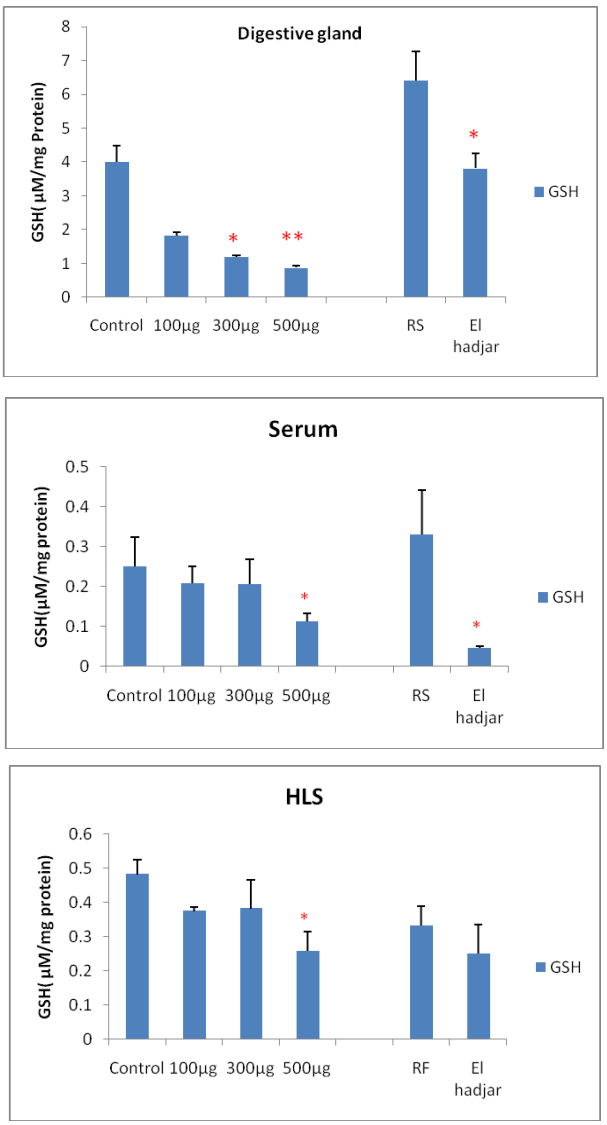

Fig. 3: Glutathione content in digestive gland, serum and HLS of the snails (*Helix aspersa*) exposed to metal dust in the laboratory condition and snails from field (RS: Reference site and El Hadjar). Data are reported as mean ± standard error. Asterisks indicate difference from control values (paired *t* test: *P<0,05, **P<0,01).

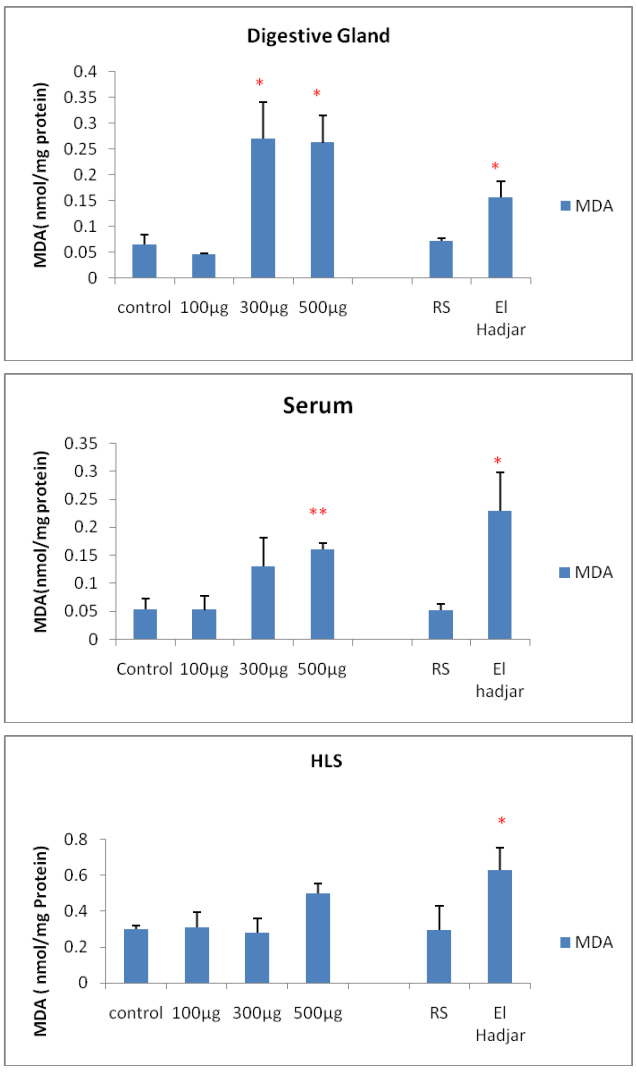

Fig. 4: Lipid peroxidation level content in digestive gland , serum and HLS of the snails (*Helix aspersa*) exposed to metal dust in the laboratory condition and snails from field (RS: Reference site and El Hadjar). Data are reported as mean ± standard error. Asterisks indicate difference from control values (paired *t* test : *P<0,05, **P<0,01).

serum of snails from polluted site than the GSH level in animals from the reference site. However, concentration of GSH in HLS was not significantly different from that of snails from the reference site. On the other hand, our results showed that the concentration of GSH in snails exposed to the elevated concentrations of metal dust was significantly less than in the controls, the lowest level of GSH was found in digestive gland ($p<0.01$).

Oxidative damage in lipids (LPO) are presented in (Fig. 4). MDA levels in digestive gland, HLS and serum were significantly higher in snails from the contaminated site of El Hadjar than in those from reference site. This parameter increased significantly in digestive gland and serum of the high-concentration treated groups compared with control group. However, in HLS of snails exposed to metal dusts which was not significant.

DISCUSSION

The aim of this study was to examine effects of chronic exposure to metal dust released by the steel complex of El Hadjar. Our results can be used to explain the impact of

heavy metal toxicity on organisms. The oxidative effect of the metal dust on the snails *Helix aspersa* was investigated by the analyses of oxidative stress biomarkers (CAT, GSH, GST, MDA).

In our study the results demonstrate that GST activity decreased both in land snails treated with higher metal dust concentrations and in the specimens from the El Hadjar site. GST is known to play a major role in cell protection against the effects of toxic compounds and reactive metabolites produced during oxidative processes (Kamel et al. 2012). GST is considered as sensitive biomarker of exposure to a broad range of contaminants usually used in laboratory and field studies (Faria et al. 2009). The inhibition of GST during this exposure period may suggest failure of detoxification process and development of oxidative stress. This result agrees with data provided by (Borkovic-Mitic et al. 2013) who observed decreased GST in freshwater bivalve *Uniotumidus* exposed to metal pollution.

Borkovic-Mitic et al. (2013) reported that GST activity inhibition could occur either through direct action of the metal on the enzyme or indirectly, via the production of ROS that interact directly with the enzyme, deplete its substrate GSH, and/or through downregulation of GST genes. Thus after exposure to metals such as Cr, Cd, Hg, Zn and As the GST downregulation observed has been attributed to the inhibition of nuclear transcription factors (NF-κB, AP-1) binding to the gene promoter region, either directly or through indirect mechanisms that involve ROS generation (Roling & Baldwin 2006).

GSH, plays an important role in maintaining cellular redox status and protecting cells from oxidative injury (Dickinson & Forman 2002). It is the cofactor of many enzymes catalyzing the detoxification and excretion of several toxic compounds (Regoli & Principato 1995).

The decrease in GSH concentrations observed in HLS, serum and the digestive gland may be due to the high affinity of metals for this molecule. Gopalakrishnan et al. (2013) reported that the lesser concentration of GSH is consistent with greater utilization of GSH by GPx to catalyze the reduction of H_2O_2 to H_2O and O_2. On the other hand the depletion of glutathione may be related to the formation of complexes GSH/metals or to the oxidation of GSH by metals (Regoli & Principato 1995). Both of these reactions could explain the observed decrease of glutathione content in snails exposed to metals under field microcosms and laboratory conditions. Quig (1998) and Hultberg et al. (2001) reported that GSH depletion is due to chronic metal exposure. These results agreed with the findings of Abdel-Halim et al. (2013) who showed that GSH depletion in the land snails *Helix aspersa* exposed to urban metal pollution.

Quantification of MDA as product of lipid peroxidation is a direct indicator of oxidative stress (Tao et al. 2013). In the present study, the level of LPO in digestive gland tissue, HLS and serum were significantly higher in the land snails from polluted site of EL Hadjar than other snails in the reference zone. On the other hand significant increased level of malondialdehyde was observed in land snails treated with higher metal dust concentrations, both in digestive gland and serum, greater concentration of MDA observed in this study indicate oxidative injury caused by metal bioaccumulation. It has been reported that, the high level of LPO indicating that the antioxidant enzymes system could not wholly eliminate O_2 (Tao et al. 2013), also Giarratano et al. (2014) reported that the high lipid radical formation rate is related to an elevated content of Fe in tissues. Fe initiates formation of highly toxic radicals from H_2O_2 via Fenton-type reactions and, moreover, exacerbates lipid peroxidation (Punta rulo & Cederbaum 1988), therefore the high increase of MDA content showed in our results may be due to the high concentration of Fe in the metal dust.

These results are in agreement with the findings of Radwan et al. (2010) who indicated that metals showed positive significant correlations with LPO, due to the presence of ionic metals in the digestive gland of snails which catalyze the Fenton reaction and increase the risk of cell damage.

The enzymatic antioxidant system SOD/CAT provides a first line of defence against ROS (Saïdi et al. 2013). However, CAT is better than SOD as a sensitive biomarker of oxidative stress (Wang et al. 2012). Catalase is an enzyme involved in antioxidant defence that eliminates hydrogen peroxide ($2H_2O_2 = 2H_2O+O_2$).

Our results highlight a significant induction of catalase in digestive gland, serum and HLS of snails from field contaminated site. This rise in CAT activity can be an adaptive mechanism to prevent the accumulation of toxic reactive oxygen intermediates. It has been reported that heavy metal exposure induces antioxidant enzymes like CAT to neutralize the impact of the ROS generation (Wu & Yi 2015). Our results are in agreement with those of Radwan et al. (2010) and Wang et al. (2012) who found increase in CAT activity in different species of molluscs exposed to metal pollution.

On the other hand, the current study did not show any significant increase of CAT activity both in digestive gland and serum of snails exposed to metal contamination under laboratory conditions, but in contrast we observed a high level of CAT activity in HLS. Gopalakrishnan et al. (2013) reported that the antioxidants produced by haemocytes are likely involved with the immune response and might be com-

pensated for by other defence mechanisms.

CONCLUSIONS

The obtained results from this study would provide baseline data for future impact assessments concerning industrial heavy metal pollution. The present study demonstrates that the chronic exposure of snails to mixture of metal dust cause changes in the non-enzymatic and enzymatic biomarkers, and also responsible for the development of oxidative stress, as shown by increased activity of catalase and lipid peroxidation, and the decrease of GST activity and GSH level. In addition, the same response was exhibited by snails from the contaminated site of El Hadjar and individuals exposed to the highest metal concentrations under laboratory conditions. This, suggests that the site of El Hadjar is heavily polluted by metals. *Helix aspersa* is a good tool for biomonitoring ecotoxicological effects. More studies must be done in El Hadjar region to know the bioaccumulation of metals to complete the information about the deleterious risk.

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