

Effects of Metal Dust on Functional Markers and Histology of Gland Digestive and Kidney of the Land Snails (*Helix aspersa*) in the North East of Algeria

^{1,2}Mounir Boucenna, ²Houria Berrebbah, ²Amira Atailia,
²Nedjoud Grara and ²Mohamed Reda Djebar

¹Biology and Animal Physiology Department,
Faculty of Life and Natural Sciences, Ferhat Abbas Setif 1 University, Setif, Algeria
²Laboratory of Toxicology, Department of Biology,
Faculty of Sciences, Annaba University, BP 23000, Algeria

Abstract: The effect of metal dust on the terrestrial snail *Helix aspersa*, collected from an uncontaminated site in *Guelma city* (North east of Algeria) was investigated. Herein, snail *Helix aspersa* was treated with various doses 100, 500, 1000 and 1500 µg of industrial metal dust/g of diet, for a total duration of two weeks. The obtained results showed that metal dust treatment caused a significant increase in the levels of total protein, carbohydrate and lipids in the digestive gland, along with less effect on the carbohydrates in kidney when compared with their controls. Histological changes which observed in the selected organs following treatment with 1000 and 1500 µg of metal dust/g of diet were atrophy of the connective tissue, membrane destruction, cell necrosis, the appearance of inflammatory infiltrates. All these effects could be attributed in the first biological response of *Helix aspersa* to various pollutants.

Key words: *Helix aspersa* • Metal dust • Digestive gland • Kidney • Histopathology

INTRODUCTION

Trace metals (TMs) are naturally present in the environment but soils can exhibit extremely high levels of these persistent pollutants, often due to anthropogenic activities and such contamination may lead to harmful effects on wildlife [1-2]. World mining activities are known to release significant amounts of toxic metals into the surrounding environment [3]. Having such individual tolerance to metal-induced stress, allows some individuals to be more competitive than non-tolerant ones under polluted environments [4].

Land snails have an ecological role and importance as the most species-rich group of terrestrial mollusks; their ecological and biological attributes closely adhere to the preconditions of serving as suitable bioindicators. These ubiquitous and sinantropic mollusk species (*Helix aspersa*) are abundant in Northeast Algeria [5].

Snails are a good bio-accumulator of metals, may provide main links in transfer of chemicals from vegetation or plant litter to carnivores. Such transfer along food

chain is an important aspect of ecotoxicology. Snails are able to accumulate bioavailable metals in their organs and they present an important organotropism for the digestive gland and the kidney [6,7]. Using snails in toxicity bioassays is an attractive method, since snails are easy to culture in the laboratory and can be fed on artificial diets with the desired amounts of metals and they respond quickly to metal contamination in the range of sublethal doses [8,9]. This is due to their physiological particularities leading to pollutant accumulations through multiple routes of exposure: oral, dermal and respiratory [10].

Several studies have reported the crucial role of the anti-oxidant defense system in attenuating oxidative threat due to various chemicals [11]. Organisms can be tolerant to metal exposure following two main processes: acclimatization, adaptation, or both [12].

The digestive gland (or hepatopancreas) was chosen as a target organ in metal toxicity evaluations; this is due to its ability to uptake and to concentrate the contaminants by 5-10 folds higher than other organs [13].

Additionally, it's involved in accumulation and storage of metals [14-15] and in detoxification processes [16]. Changes in digestive cell structure and function, as a result of metal accumulation, have been well documented [14,17].

Gastropod epithelium consists of three cell types: excretory, calcium and digestive cells, the latter of which is the most abundant cell type [18]. The digestive glands also possess connective tissue, which is composed of pigment cells, rhogocytes (pore cells) and fibroblasts among other tissue types [19].

The kidney is rapidly accumulated Al and Fe but the increase was short-lived. The kidney may therefore be involved in elimination of metal not incorporated into the digestive gland [20]. Matricon-Gondran [21] indicted that the main region of the kidney characterized by epithelial folds of nephrocytes, the very proximal part has a reticular aspect due to the presence of large hemal spaces surrounded by podocyte-like cells.

The severity effects cell membranes of the apex and the microvilli, as well as the appearance of the cytoplasm, the nucleus, mitochondria, vacuole, endoplasmic reticulum and the Golgi apparatus could be semi-quantitatively evaluated, allowing to identify three physiological states of the animal state "control" for unexposed animals, compensation status characterized by symptoms of reaction indicates that animals are able to tolerate sublethal poisoning by activating detoxification process and the status of non-compensation with symptoms of destruction that correspond to intracellular structures with visible damage due to toxic effects[22].

The aim of the current study was to evaluate the effects of industrial metal dust on the two target organs (digestive gland and kidney), following some biochemical parameters (metabolic) and histopathology studies of the land snail (*Helix aspersa*) exposed to metal dust in a dose dependent manner.-

MATERIALS AND METHODS

Snail's Collection: Gastropod terrestrial snails (*Helix aspersa*) were collected from an uncontaminated site of *Guelma city* (Northeast Algeria). Snails (10.5±0.26 g) were raised in the following optimal environmental conditions: photoperiod 18h light/24h, temperature (20±2°C), humidity 80 to 95% wheat flour in food. The animals were divided into transparent polystyrene boxes (25 x 15 x 15 cm) with perforated lid; each box contains a wet sponge to retain humidity. During the exposure period food is supplied in petri dishes regularly every day [23].

Chemical Equipment: Metal dust used in this study was collected from steel complex of El-Hajar (13km from the city of Annaba on the road N°. 44 (Northeastern Algeria)). Dust composition was chemically analyzed, using atomic absorption technique, allowing us to determine at least the presence of 07 trace metals (Table 1).

Treatment Mode: Processing snails was made by adding various concentrations of metal dust (100, 500, 1000, 1500 mg /g diet such as wheat flour). Snails were divided into 5 groups of 10 snails in each and they were undergone a daily treatment for two weeks [23].

Dissection and Tissue Preparation: After two weeks of treatment, the snails were sacrificed after freezing (-80°C), without prior fasting which could alter the expression levels of molecules sought. After dissection and removal of the two organs (digestive gland and kidney), from five randomly chosen snails of each experimental group, we evaluated some of biochemical parameters; total proteins, carbohydrates and lipids. From another five snails, we performed the chosen organ histopathological studies, following the usual technique of histology.

Measurement of Biochemical Parameters: The extraction of metabolites in both organs was performed according to the method of Shibko [25] that which total proteins are quantified by the Bradford method [26], the determination of carbohydrates was performed according to the method of Duchateau and Florkin [27]. Lipids were determined by the method of Goldsworthy [28].

Histopathological Study: Sections of the digestive gland and kidney from exposed and control animals were dissected and immediately fixed in 10% neutral buffered formalin for 24 h, processed by using a graded ethanol series and then embedded in paraffin. The paraffin sections were cut into 5µm thick slices and stained with hematoxylin and eosin for light microscopic examination. The sections were viewed and photographed. [29].

Statistical Data Analysis: The results obtained were subjected to analysis by ANOVA one way (treatment Doses), the analysis over treatments was also performed \by using SAS (1999). Significant difference between two means was measured using Fisher's test (LSD_{5%}). The linear regressions were performed with Microsoft excel 2007 (Microsoft, Redmond, WA, USA.) [30].

Table 1: Composition in ppm Dust rejected by the Steelworks 1 and Steelworks 2 of the steel complex of El Hajar-Annaba [24].

Sample	Cu	Zn	Pb	Cr	Ni	Mn	Fe
Dust Steelworks 1	3.70	240	24.00	10	1.20	320	3000
Dust steelworks 2	7.00	480	62.40	12	1.30	540	3600
Total	10.70	720	88.40	22	2.50	860	6600

RESULTS

As shown in Table 2, proteins, carbohydrates and lipid levels were significantly increased ($P < 0.001$) in the digestive glands of treated snails with various concentrations of metal dust, when compared with control group.

In regard to the proportioning effect of total protein in the digestive gland, Table 3 shows that the minimum and maximum values content was registered at the control individuals with 1.62 ($\mu\text{g/g}$ fresh weight) and 3.92 ($\mu\text{g/g}$ fresh weight) as the highest dose respectively (Table 3).

Fisher's test revealed four homogeneous groups for the carbohydrate parameters. The group (a) includes the D3 and D4 doses with 28.26 and 30.52 ($\mu\text{g/g}$ fresh weight) respectively (Table 3). The group (b) is presented by snails exposed to 500 μg of metal dust/g of diet. The lowest rate of carbohydrates is observed in the control snails with 16.27 $\mu\text{g/g}$ of fresh weight (group (c)). The last group has an overlap between the two latter (b and c) for the 100 $\mu\text{g/g}$ (Table 3).

The average rate of lipids in the digestive gland is estimated at 0.37 ($\mu\text{g/g}$ of fresh weight), 0.16 and 0.81 ($\mu\text{g/g}$ of fresh weight) are the minimum and the maximum values noted at 100 $\mu\text{g/g}$ and 1000 $\mu\text{g/g}$ respectively (Table 3).

The statistical analysis (ANOVA) showed a significant difference in the levels of total proteins and lipids (Table 4), along with a slight significant difference at the 5% threshold ($\text{LSD}_{5\%} = 0.40$) for a dose of 5.13 $\mu\text{g/g}$ fresh weight. The maximum value was noticed in the total protein levels of the snail kidney (*Helix aspersa*). However, 3.20 $\mu\text{g/g}$ fresh weight was noted as a minimum value (Table 4). The lipid rate exhibited a low value (1.58 $\mu\text{g/g}$ fresh weight) in a dose of 1500 $\mu\text{g/g}$, whereas 2.17 $\mu\text{g/g}$ fresh weight is recorded as the maximum value (Table 5).

The analysis of variance showed a high significant dose effect for carbohydrate rates (Table 4). The maximum value 14.85 $\mu\text{g/g}$ fresh weight is obtained in snails treated with 1500 $\mu\text{g/g}$, while the minimum value is 6.26 $\mu\text{g/g}$ fresh weight in the control snails (Table 5).

The Figure 1 illustrates that total protein levels are strongly correlated with doses of metal dust in the digestive gland ($R^2 = 0.83^{**}$). Whilst, the total proteins were less correlated with increasing doses in the kidney of treated snails ($R^2 = 0.0017^{ns}$).

The rate of carbohydrates is positively and significantly correlated with treatment doses where $R^2 = 0.96^{**}$ and $R^2 = 0.78^{**}$ in the digestive gland and kidney, respectively (Figure 2).

Table 2: Statistical analysis of the functional biochemical markers of digestive gland in control and treated land snails (*Helix aspersa*)

Source of variation	Proteins				Carbohydrates			Lipids		
	DF	SS	MS	F	SS	MS	F	SS	MS	F
Doses	4	13.21	3.30	46.93***	672.00	168	40.82***	1.23	0.30	182.38***
Error	15	1.05	0.07		61.73	4.11		0.02	0.001	
Total	19	14.27			733.73			1.25		
C.V %		9.08			8.99			10.92		

df = degrees of freedom, SS = sum of squares, MS = means square, C.V: coefficient of variation, Level of significance: $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$, $p < 0.0001 = ****$.

Table 3: Biochemical parameter averages in the digestive gland of the snail (*Helix aspersa*).

Doses	Proteins	Carbohydrates	Lipids
D0=0 $\mu\text{g/g}$	1.62 (d)	16.27 (c)	0.24 (c)
D1= 100 $\mu\text{g/g}$	2.38 (c)	17.32 (bc)	0.16 (d)
D2=500 $\mu\text{g/g}$	3.29 (b)	20.32 (b)	0.17 (d)
D3=1000 $\mu\text{g/g}$	3.36 (b)	28.26 (a)	0.81 (a)
D4=1500 $\mu\text{g/g}$	3.92 (a)	30.52 (a)	0.49 (b)
Means	2.92	22.54	0.37
Minimum	1.62	16.27	0.16
Maximum	3.92	30.52	0.81
Standard deviation (δ)	0.86	6.21	0.25
$\text{LSD}_{5\%}$	0.40	3.05	0.06

Table 4: Statistical analysis (ANOVA test) of the biochemical markers in kidney of the land snail (*Helix aspersa*) treated with metallic dust

Source of variation	Proteins				Carbohydrates			Lipids		
	DF	SS	MS	F	SS	MS	F	SS	MS	F
Doses	4	8.22	2.05	3.60*	179.55	44.88	28.12****	0.93	0.23	2.38*
Error	15	8.56	0.57		23.94	1.60		1.48	0.099	
Total	19	16.78			203.50			2.42		
C.V %		18.70			11.00			17.86		

df = degrees of freedom, SS = sum of squares, MS = means square, C.V: coefficient of variation, Level of significance: p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****

Table 5: Statistical analysis (Average comparison) of the biochemical markers in kidney of the land snail (*Helix aspersa*) treated with metallic dust.

Doses	Proteins	Carbohydrates	Lipids
D0=0 µg/g	3.20 (b)	6.26 (c)	1.61 (b)
D1= 100 µg/g	5.13 (a)	10.75 (b)	1.66 (b)
D2=500 µg/g	3.64 (b)	11.65 (b)	2.17 (a)
D3=1000 µg/g	4.07 (ab)	13.86 (a)	1.74 (ab)
D4=1500 µg/g	4.12 (ab)	14.85 (a)	1.58 (b)
Minimum	3.20	6.26	1.58
Maximum	5.13	14.85	2.17
Means	4.03	11.48	1.75
Standard deviation (δ)	0.94	3.27	0.35
LSD _{5%}	1.13	1.90	0.47

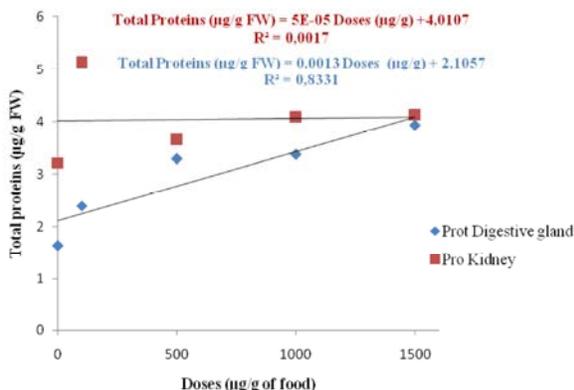


Fig. 1: Variation of total protein rate in the digestive gland and kidney of the snails (*Helix aspersa*) exposed to various doses of metal dust

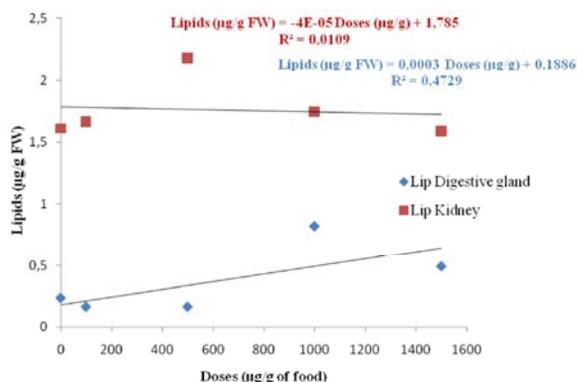


Fig. 3: Variation of lipids in the digestive gland and kidney of the snails (*Helix aspersa*) exposed to various doses of metal dust

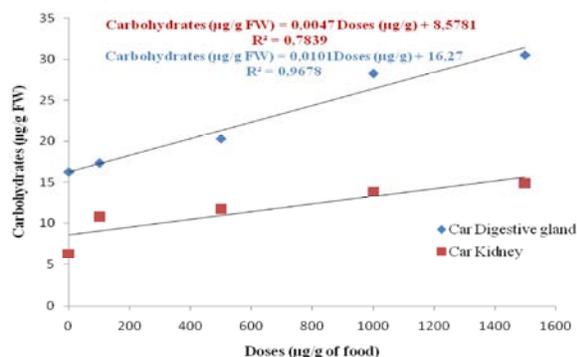


Fig. 2 : Variation of carbohydrate rates in the digestive gland and kidney of the snails (*Helix aspersa*) exposed to various doses of metal dust

The lipids in the digestive gland have low correlations with treatment doses which $R^2 = 0.47$, on the other hand, the change in lipids is not very linked to different treatment doses in the kidney ($R^2 = 0.0109$) (Figure 3).

Microscopic Examination of Histological Sections of the Digestive Gland: The digestive gland of control group of snails (Fig. 4A) consists of a digestive epithelium composed of lobules of acinus forming a coherent maintained by connective tissue. The epithelium contain three types of cells and each cell has the essential components (membrane and nucleus) digestive cells DC

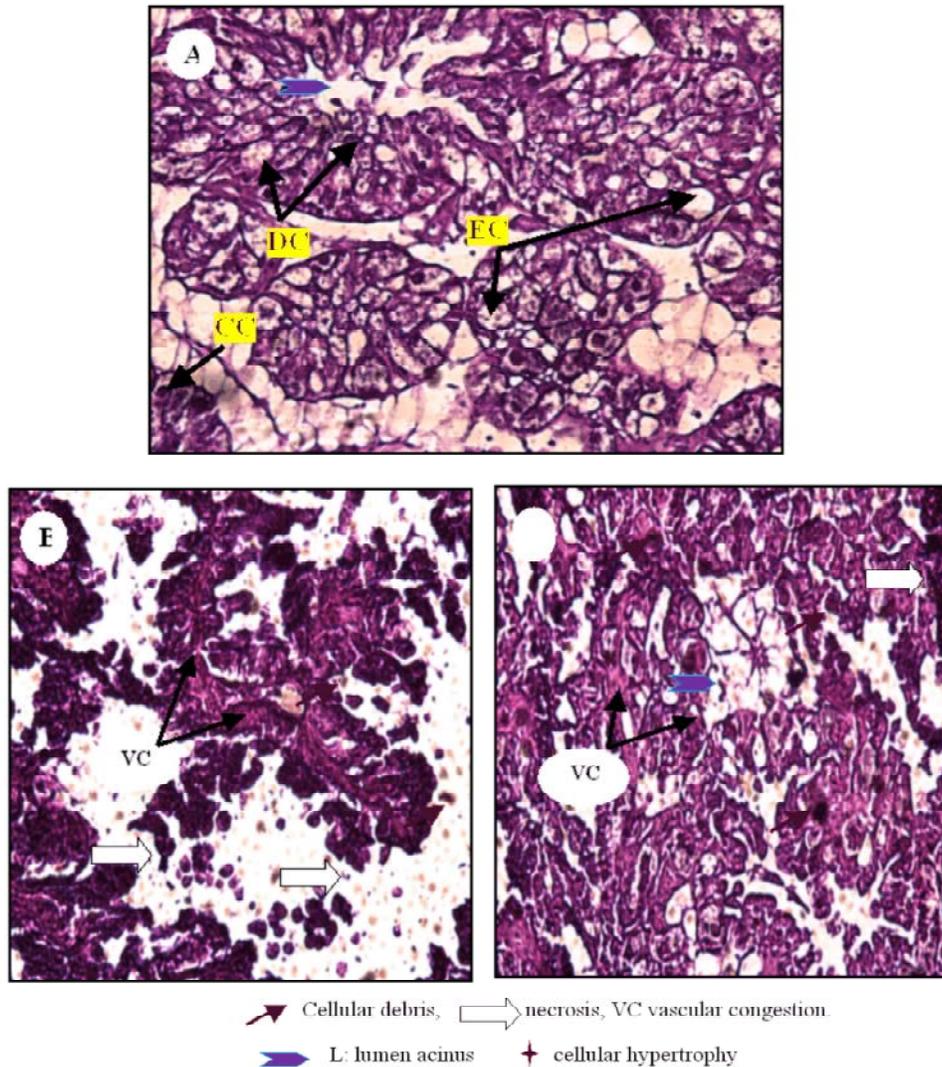


Fig. 4: Histological sections of digestive gland of *Helix aspersa* control group (A) and treated with doses 1000 ($\mu\text{g/g}$ of food) (B), and 1500 ($\mu\text{g/g}$ of food) (C). (DC: digestive cell, EC: excretory cell, CC: calcium cell, L: lumen acinus)

(most abundant), EC excretory cells and the calcium cells (CC). However, among treated groups of this cellular structure present visible alterations manifested by vascular congestion (VC) of acinus with lumen expanded and necrosis cell structures especially in snails treated with dose 1000 ($\mu\text{g/g}$ of food) (Fig. 4B). The microscopic observation of organ histology of treated animals with the dose of 1500 $\mu\text{g/g}$ of food (Fig. 4C) illustrates the destruction of the parenchyma of the acinus, irregular lumen with cellular debris, the disappearance of the digestive cells and a decrease in the thickness of the epithelium confirmed the necrosis phenomenon.

Histological Examination of the Kidney: Kidney of control snails (Fig. 5A) showed an epithelium-lined

excretory kidney sections, connective acinus incorporated columnar cells with a brush border. The kidney epithelium contains only one type of cells named excretory cells, which have a nucleus and a granular membrane, as well as they are subdivided into two other cell types known as rod and ciliate cells.

The histological weathering induced by the presence of metal particles (Fig. 5B) result following change as a response in renal tissue: hypertrophy epithelia, necrosis with irregular lumen obstructed by cell debris. Similarly, histological examination of snails treated with dose 1500 ($\mu\text{g/g}$ of food) (Fig. 5C) shows the disappearance of the excretory cell membranes, following their destruction and proliferation of connective tissue.

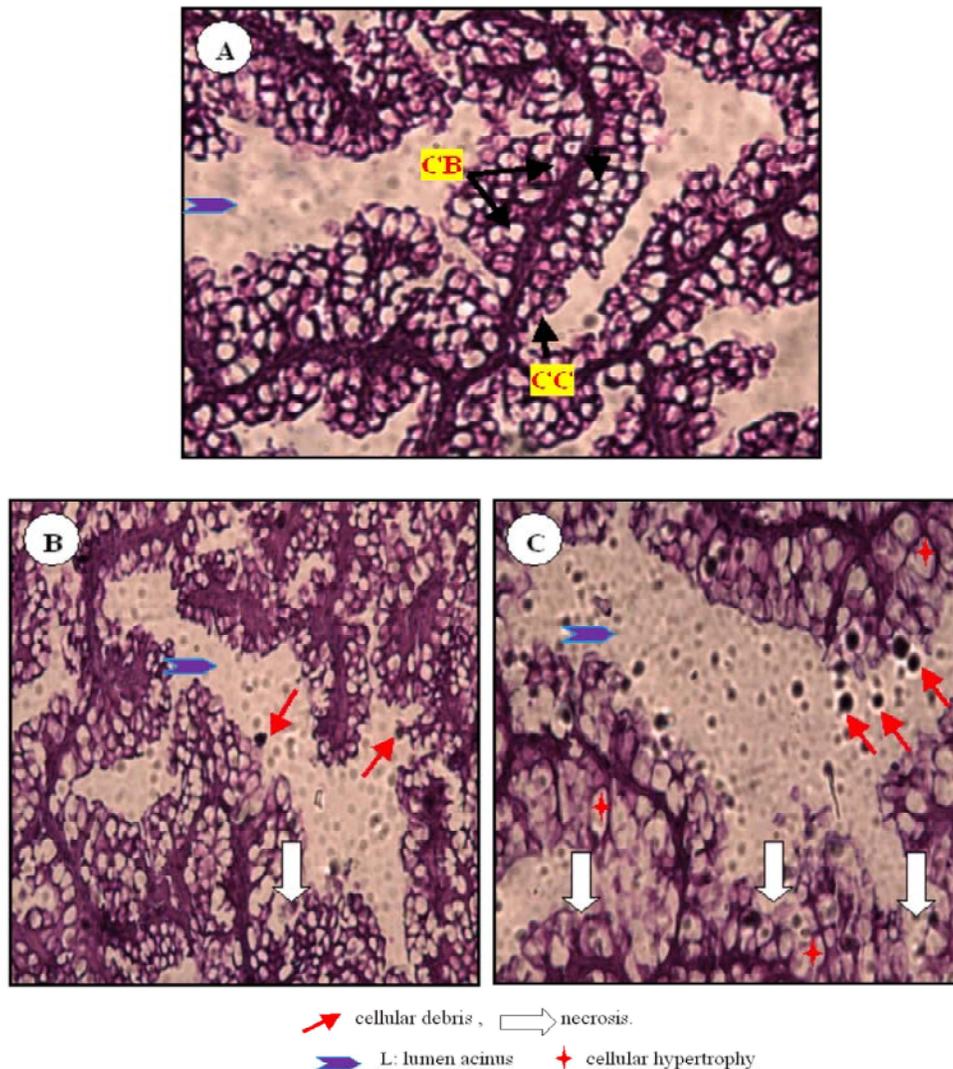


Fig. 5: Histological sections of the kidney of *Helix aspersa* control group (A) and treated with doses 1000 ($\mu\text{g/g}$ of food) (B), and 1500 ($\mu\text{g/g}$ of food) (C).

DISCUSSION

Several studies have shown that most heavy metal toxicities are strongly associated to over production of intracellular reactive oxygen species (ROS) in biological systems [31]. Oxidative stress defines the potential of ROS to damage cellular components such as biomembranes, proteins, DNA and RNA [32]. In molluscs heavy metals can induce a state of general stress, resulting in the reduction of their ability to adapt to hypoxia.

Our study showed that the protein levels in both organs increased in metal dust exposure following dose-dependent manner, which is agreed with those of Grara *et al.* [33]. Here, *Helix aspersa* were exposed for a

period of 28 days to a various metals (Cu, Zn, Ni and Pb). The results showed a significant increase in total protein content. Alike, Radwan and Mohamed [34] observed a significant increase in protein rate in the same species treated with 0.6 LD₅₀ of imidacloprid. While El Gohary and Genena, [35] was found to decrease the level of total protein. Heavy metal storage in molluscs is often due to the increase of low molecular weight proteins belonging to the metallothionin family (MTs), which play a major role in metal homeostasis. Three isoforms of MTs are known [36] and one of them (CdMT) is induced by Cd.

Carbohydrates are the primary and immediate source of energy. Under stress conditions, carbohydrate reserves are depleted to meet increased an energy demand [37]. Our results show a significant elevation of carbohydrates

rates at two organs tested. In the study of Barky *et al.* [38-39] toxic effect of Atrazine caused increase in glucose concentration in *Biomphalaria alexandrina* snails. Unlike, the work of Radwan *et al.* [40] indicated a significant decrease in carbohydrate rate.

Lipids are also a main source of metabolic energy and essential component for the formation of cells and tissue membrane [41]. The total lipid was significantly increased in the digestive gland of *Eobania vermiculata* snails that was collected from polluted areas [42], unlike the work of Grara *et al.* [33] which recorded a significant decrease in lipid concentration.

The kidney lipid levels did not show any significant difference, similar results were noted by Boshoff *et al.* [7] indicating that the biomarker concentrations (lipids, glycogen, proteins), were not related to the metal concentrations in digestive gland of *Cepaea nemoralis* snails.

The midgut gland or digestive gland or hepatopancreas, similar in its function than the pancreas and liver for humans, is a major organ involved in metal uptake and storage in molluscs. This gland is responsible for digestive enzyme production, nutrient absorption, endocytosis of certain food ingredients and also food storage and excretion [43]. The digestive glands are the most important gastropod organs involved in pollutant detoxification [44]. The histological and histochemical changes are expected to be useful biomarkers of copper exposure [14]. The histopathological responses of several organs (digestive gland and kidney) in *Helix aspersa* exposed to increasing concentrations of Cd in food occur by reactions involving epithelial hyperplasia at the lowest doses tested cell destruction and important is accompanied by a proliferation of connective tissue and necrosis at high concentrations [45].

Our histological study establishes the tissue damage in the digestive gland and kidney of *Helix aspersa* in response to the toxicity of the metal particles which could result in visible structural changes in snails treated with high doses. Further histological examination showed revealed dilation hemolymphatic spaces between the tubules, cellular degeneration, with a more expanded lumen, cell necrosis, with atrophy of the connective tissue of the digestive gland and desquamation of epithelial cells, accompanied by hypertrophy in calcium cells. Furthermore, TMs deteriorate the dynamics of cells and damage their membranes. Therefore, the intercellular exchange and fluidity are disrupted. Accordingly, the diffusion of heavy metals increases in cells causing cellular necrosis [46]. According to Osterauer *et al.* [47],

the changes in digestive gland of ramshorn snails (*Marisa cornuarietis*) were characterized by large hemolymphic spaces between the tubules, enlarged tubule lumen, flattened epithelia, irregular shape of cells, cytoplasmatic protuberances of digestive cells, increased amount of vacuoles in digestive cells and rarely, necrosis of digestive and basophilic cells at 50 and 100 g/l of PtCl₂. The same results were observed in *Planorbarius corneus* exposure to 0.4 mg/l of endosulfan [48]. Thus in adult of *Helix aspersa* fed a copper-contaminated diet exhibited significant effects of copper dose on the height of hepatopancreatic epithelium cells and digestive glandular epithelium area [14].

Gust *et al.* [49] reported histological lesions of the digestive gland which were observed with hypertrophy of calcium cells and vacuolization of digestive cells. In the mud snail *Potamopyrgus antipodarum* exposed to complex field poly-metallic pollution. Contrary, Bacchetta *et al.* [50], where found no histological damage on the hepatopancreas of the snail *Physa fontinalis* even after 14 days of exposure to 0.500 mg/l of Paraquat. No histopathological change (necrosis) was observed and evidence of programmed cell death was very rarely observed in the midgut gland of *Helix pomatia* [51].

The kidney of gastropods is a blind sac located along the dorsal region of the mantle cavity. The wall of the kidney has a uniform structure with epithelial infoldings, the cells of which, called nephrocytes, are characterized by apical microvilli forming a brush border, a large apical vacuole containing a spherocrystal and a basal labyrinth consisting of intricated cytoplasmic processes associated with mitochondria [21].

As reported previously, the kidney is defined as a sac of some gastropods [52]; they may play a part in the regulating of the paracellular pathway followed by some molecules and ions.

In the kidney of *Helix aspersa* treated with increasing doses of metal dust, the histopathological examinations revealed the following changes like adaptive responses: structural integrity of the kidney epithelium is not preserved and cellular debris accumulates in the enlarged lumen, cellular hypertrophy, cell necrosis and widespread proliferation of excretory cells. These results are in agreement with those of Russell [53] observed in Cd-treated *Helix aspersa*. Histopathological analysis revealed significantly enhanced metallic concretion in nephrocyte of snail's kidney *Bellamya bengalensis* exposed to copper sulfate (3CuSO₄·5H₂O) and tubule enlargement, destructed epithelial lining of tubule with luminal spaces filled by metallic concretion as well as cellular debris [54].

Unlike, Chabicoovsky *et al.* [55] indicated that feeding snails on Cu-enriched diet resulted in a decrease of Cd-MT gene expression in the snail kidney, whilst, a very low Cd concentrations were detected. While snail's kidney does not play a major role in the accumulation and excretion of Cd and the specific function in metal metabolism however remains unknown.

CONCLUSION

The present study provided a convenient experimental design which can be useful for easy and quick assessment of metal exposure, under laboratory conditions. We are interested in the changes of some biochemical parameters and in the second part to the histopathological study of the metal dust toxicities. The results of this study showed a significant increase of the rate of total protein, carbohydrate and lipids in the digestive gland, unlike the kidney that does not respond to increasing doses of different metal dusts, except carbohydrates which recorded a slight increase compared to the control group.

Moreover, toxic effects of metal dust induced histopathological alterations of snail target organs were evidenced by dilated hemolymphatic spaces between the tubules, cellular degeneration, with an enlarged lumen, cell necrosis, with atrophy of the connective tissue of the digestive gland and desquamation of epithelial cells, accompanied by hypertrophy in calcium cells in the tissues of the land snails tested. Cellular debris accumulates in the enlarged lumen, cellular hypertrophy; cell necrosis characterized the kidney epithelium after two week of expose to dust metal. Finally, the usefulness of the terrestrial snail *Helix aspersa* as a potential bioindicator species of environmental contamination becomes a topic of great interest.

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