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Research Article
Histological and Ultrastructure Alterations in the Midgut of *Blaps polycresta* and *Trachyderma hispida* (Coleoptera: Tenebrionidae) Induced by Heavy Metals Pollution

Dalia Abdel Moneim Kheirallah and Lamia Mostafa El-Samad

Department of Zoology, Faculty of Science, Alexandria University, 21511 Alexandria, Egypt

Abstract

Background and Objective: The present research focused on using two coleopteran insects *Blaps polycresta* and *Trachyderma hispida* as bioindicators for heavy metals pollution that resulted from ceramic industry in an urban area, Khorshed district, Alexandria, Egypt. **Materials and Methods:** Bioaccumulation of the heavy metals was quantified in the soil and midgut tissues of the two insects by using x-ray microprobe analysis. Histological and ultrastructure preparations were performed at the Electron Microscope Unit at Faculty of Science, Alexandria University, Egypt. **Results:** The x-ray analysis revealed high metal percentage in the soil and midgut tissues of the insects collected from the polluted site compared to the reference site (the garden of Faculty of Science, Alexandria University, Egypt). Histological and ultrastructure alterations were observed in the midgut cells of the two insects collected from the polluted site compared to the reference site. The most observed histological alteration was the distortion of the brush borders of the microvilli. Ultrastructure alterations included: Nuclear distortion of regenerative and digestive cells, lysis of the mitochondrial matrices, the appearance of electron-dense vesicles, the presence of myelin figures, vacuolated cytoplasm and dilation of the rough and smooth endoplasmic reticulum. **Conclusion:** The study validates that heavy metals pollution leads to cellular and sub-cellular alterations in insects inhabiting industrial districts.

Key words: Industrial pollution, heavy metals, beetles, histological and ultrastructure aberrations, x-ray analysis

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Corresponding Author: Dalia Abdel Moneim Kheirallah, Department of Zoology, Faculty of Science, Alexandria University, 21511 Alexandria, Egypt
Tel: 002-01221775286 Fax: 002-034264455

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Industrialization poses serious risks to human and ecosystems' health. Populations in developing countries are especially at risk because of the lacking of appropriate regulations and enforcement as well as barriers to accessing "cleaner" technologies. The undesirable outcomes from industrial plants such as the emission of toxic chemicals in the atmosphere resulted in environmental pollution[1-3]. Ceramic industry is an important source of pollutants to the environment[4]. Many relevant aspects such as air pollution, human toxicity, global warming, acidic rain and ozone depletion from ceramic tile processes have been discussed by several authors[2,3,5-11]. Residential plants and animals were found to be affected in ceramic industrial areas[12]. Because of the usage of many chemicals and high energy which is essential in this industry soil pollution occurs[13].

The chemicals that have been used in manufacturing ceramic contains heavy metals which are the primary cause of ecotoxicity[11]. Variable fractions of the total concentration of the heavy metals are bioavailable to the soil animals[14]. The more accurate information is the measurements of the heavy metals concentrations within the inhabitants[15]. Heavy metals reside in the organisms' tissues and may pose health problems. Xenobiotic undergo a series of biotransformation reactions catalyzed by different enzymes, their activation provides evidence for pollution exposure[16].

Insects are good monitoring models to evaluate environmental alterations and heavy metals pollution[17-22]. The midgut in insects is directly subjected to pollutants as it acts as a physical and chemical barrier against the ingested toxic matters while feeding[23-25]. Histological and ultrastructure biomarkers deliver good surveillance for environmental health. Hence, the focal point of this survey is reporting structural abnormalities at the cellular and subcellular levels in the midgut of *B. polycresta* and *T. hispida* as biomarkers of ceramic industrial pollution and heavy metals toxicity. These anomalies will serve in the identification of different health risks that resulted from exposure to environmental stresses.

MATERIALS AND METHODS

Study sites: The two selected sites in the study were a rustic area, the garden of Faculty of Science Moharam Bek, Alexandria University, Alexandria, Egypt which considered as reference site (site A)[18] and a densely populated area, Khorshed district which located in the suburbs of the eastern edge of Alexandria, Egypt (Latitude: 31.2018066, Longitude: 30.0300721), the polluted site (site B) (Fig. 1). Ceramic factories allocated through Khorshed district (Lecico and Khorshed ceramic factories).

Fig. 1: Map of Alexandria Governorate showing Khorshed district. Arrow pointed at ceramic factories. https://www.google.com/maps/Alexandria, Egypt, Khorshed (31.2018066, 30.0300721)

Specimens identification: The coleopteran species *Blaps polycresta* and *Trachyderma hispida* were the superior beetles populated in the nominated sites. Beetles' identification was performed at Entomology Department, Faculty of Agriculture, Alexandria University. They belong to Coleoptera: Tenebrionidae.

Sampling procedure: The beetles, *B. polycresta* and *T. hispida* were collected randomly from ten sampling areas (1 m^2 each) at each site in August, 2018. Synchronously with the beetle collection, soil samples at a depth of 30 cm below the surface were gathered from the specific sites. The ten areas in Khorshed district were selected around the ceramic factories (five sites around each factory). Air temperature in July ranged from 27-35°C and the mean relative humidity was 76% with nearly no differences between the two sites. About 100 beetles from each species were collected from each site. The specimens were sustained alive in local soil and plants in glass containers until processing. Beetles were anesthetized with absolute ethanol (95%), then dissected under a dissecting microscope in a drop of Ringer's physiological solution. The abdominal cavity was opened and the midgut was taken out. Maintenance of the insects was done in compliance with ethical guidelines for the protection and use of laboratory animals. The methodology was approved by the Ethics Committee of Alexandria University (protocol approval number is 0302440).

Bioaccumulation of metals in the soil and in the midgut tissues: Metals accumulation in sieved soil and in un-coated specimens of the midgut were detected using Jeol scanning electron microscope-5300 equipped with a Link-Isis energy dispersive X-ray micro-analyzer. A stationary spot (X500) was analyzed at random for 110 sec. Due to the divergent distribution of trace metals, four points were measured to check for the variability of trace metal composition.

Histological and ultrastructure investigations: The histological preparation followed Anderson and Gordon[26] methods of dehydration, clearing and paraffin embedding. Xylene was the clearing agent. Midgut was fixed in paraffin wax (65-60°C) and 5 µm thick sections were stained with hematoxylin and eosin.

In ultrastructure preparation, midgut was fixed in $_4F_1G$ in phosphate buffer solution (pH 7.2) at 4°C for 3 h and post-fixed in 2% OsO_4 in the same buffer for 2 h. Samples were washed in the buffer and dehydrated at 4°C through a series of ethanol. Specimens were immersed in Epon-Araldite mixture in labeled beam capsules. For semithin sections, LKB ultramicrotome was used (1 µm thick). Sections were mounted on a glass slide, stained with toluidine blue and examined with the light microscope to determine the orientation and the structural features. Ultrathin sections (0.06-0.07 µm thick)[27] were cut for TEM and picked upon 200 mesh naked copper grids. Grids were stained with uranyl for half an hour and lead citrate for 20-30 min.

Statistical analysis: Data analysis was performed using the program SPSS[28]. Data were analyzed statistically by using t-test[29] to determine the difference between the two studied sites.

RESULTS

X-ray microanalysis: Twelve elements were represented in the x-ray spectra including Na, Al, P, S, K, Pb, Cd, Ca, Fe, Ni, Cu and Zn. The soil samples and the midgut tissues of the two beetles collected from the polluted site showed a significant elevation in the percentages of Al, Cu and Zn with detection of Cd and Pb compared to the reference site (Table 1, 2). Proportions of Na, P, S, K and Fe were higher in the reference site than the polluted site.

Histological and ultrastructure archetypes observed in the midgut of *B. polycresta* and *T. hispida* collected from the reference site (site A)

Histological observations: The epithelial sheath is covered by two muscle layers, external longitudinal and internal circular (Fig. 2a, b, 3a). The epithelium composed of two types of cells: (1) The columnar digestive cells with a brush border of microvilli on the apical membrane, facing the peritrophic membrane and the gut lumen and (2) The regenerative cells occur singly or in small groups called "nidi", forming crypts, in contact with the basal lamina (Fig. 2a-c, 3a, b).

Table 1: Metal percentages in the soil collected from either reference or polluted site using energy dispersive X-ray micro-analysis (EDX)

Metals	Site A	Site B	p-vale
Na	9.5±0.06	7.4±0.06	<0.001*
Al	2.0±0.06	8.5±0.06	<0.001*
P	37.1±0.06	41.2±0.06	<0.001*
S	35.7±0.06	11.6±0.06	<0.001*
K	1.2±0.06	ND	-
Pb	ND	7.3±0.06	-
Cd	ND	11.6±0.06	-
Ca	2.7±0.06	1.4±0.06	<0.001*
Fe	ND	1.2±0.06	-
Cu	1.9±0.06	16.9±0.06	<0.001*
Zn	1.1±0.06	13.8±0.06	<0.001*

For each metal, the percentage expressed by using minimum-maximum values and mean (n = 3) using Student t-test, p: p-value for comparing between site A and site B, *: Statistically significant at $p \leq 0.05$, ND: Not detected

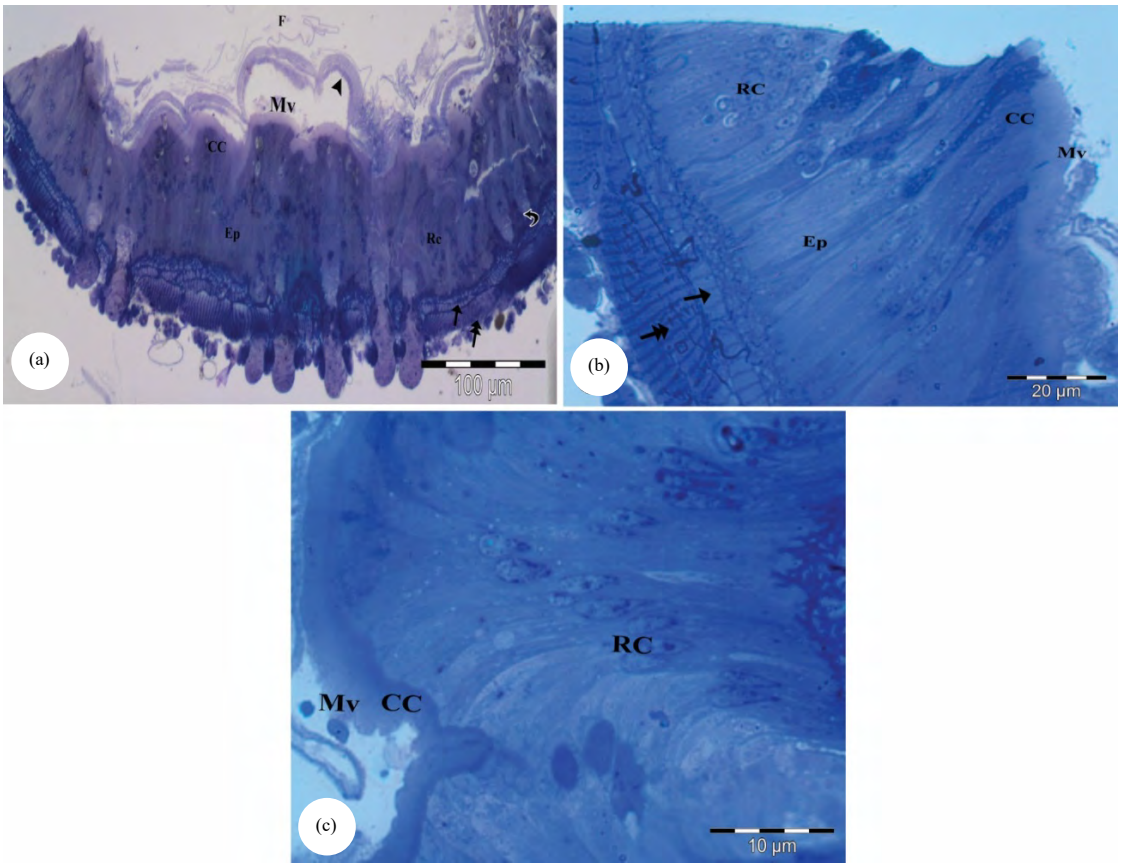

Fig. 2(a-c): Semithin sections of the midgut tissues of *B. polycresta* collected from the reference site (site A) (a) Epithelial sheath (Ep), longitudinal muscle (double head arrow), circular muscle (arrow), regenerative cells (RC) forming crypts (curved arrow), columnar digestive cells (CC), microvilli (MV), peritrophic membrane (arrow head) evolving food particles (F). (b, c) Magnified parts of Fig. 2a

Table 2: Metal percentages in midgut tissues of *B. polycresta* and *T. hispida* collected from either reference or polluted sites using energy dispersive X-ray micro-analysis (EDX)

	B. polycresta			*T. hispida*		
Metals	Site A	Site B	p-value	Site A	Site B	p-value
Na	11.3±0.06	7.1±0.06	<0.001*	14.6±0.06	7.4±0.06	<0.001*
Al	5.8±0.06	11.8±0.06	<0.001*	6.9±0.06	16.5±0.06	<0.001*
P	26.2±0.06	43.4±0.06	<0.001*	26.5±0.06	41.1±0.06	<0.001*
S	34.2±0.06	26.5±0.06	<0.001*	31.4±0.06	20.4±0.06	<0.001*
K	8.0±0.06	ND	-	ND	ND	-
Pb	ND	4.3±0.06	-	ND	4.1±0.06	-
Cd	ND	7.6±0.06	-	ND	12.3±0.06	-
Ca	ND	0.3±0.06	-	4.9±0.06	ND	-
Fe	0.5±0.06	0.8±0.06	0.021*	0.3±0.06	0.2±0.06	0.288
Cu	2.9±0.06	7.9±0.06	<0.001*	3.4±0.06	6.7±0.06	<0.001*
Zn	2.1±0.06	4.2±0.06	<0.001*	2.6±0.06	5.47±0.03	<0.001*

For each metal, the percentage expressed by using minimum-maximum values and mean (n = 3) using Student t-test, p: p-value for comparing between site A and site B, *Statistically significant at $p \leq 0.05$, ND: Not detected

Ultrastructure observations: The regenerative cells appeared with an oval nucleus, patches of heterochromatin and regular nuclear envelope (Fig. 4a, 5a, b). In the cytoplasm, uniformly distributed rounded mitochondria, frequent cisterns of rough endoplasmic reticulum (RER) and free ribosomes were noticed (Fig. 4a, 5a, b). Smooth septate junctions were found between adjacent epithelial cells (desmosomes) (Fig. 4a, b, 5a, b). The columnar digestive cells appeared with an oval nucleus and regular nuclear envelope (Fig. 5c). The cytoplasm possesses numerous mitochondria, cisterns of the rough and smooth endoplasmic reticulum, glycogen granules and free ribosomes (Fig. 4b, 5c). The luminal border has a uniformly distributed microvilli (Fig. 4b, 5c).

Histological and ultrastructure archetypes observed in the midgut of *B. polycresta* and *T. hispida* collected from the polluted site (site B)

Histological observations: Incisions between adjacent cells (Fig. 6a), vacuolation (Fig. 6a-c, 7a-c) and destruction of the brush border of the microvilli (Fig. 6c, 7c) were observed.

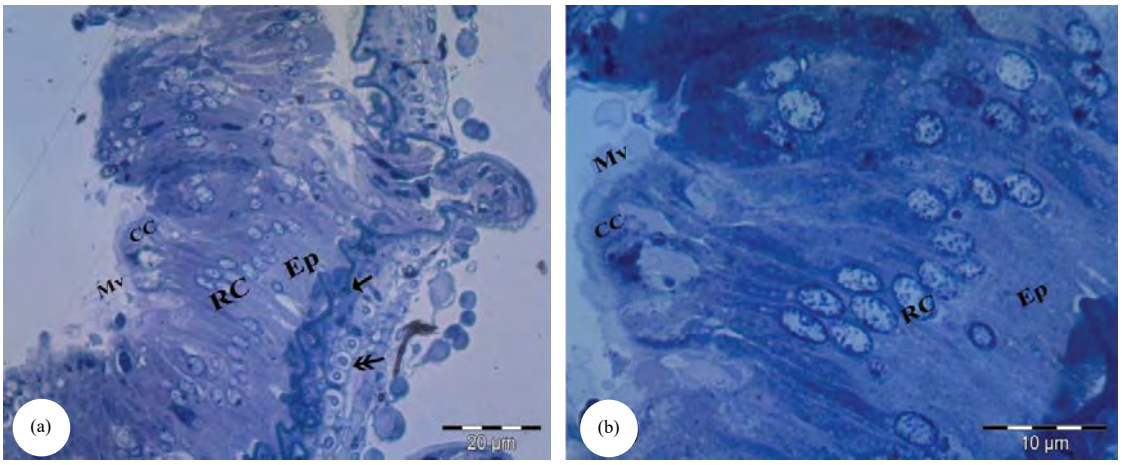

Fig. 3(a-b): Semithin sections of the midgut tissues of *T. hispida* collected from the reference site (site A) (a) Epithelial sheath (Ep), muscle layers: Longitudinal (double head arrow) and circular (arrow), regenerative cells (RC), columnar digestive cells (CC), microvilli (MV) and (b) Magnified part of Fig. 3a

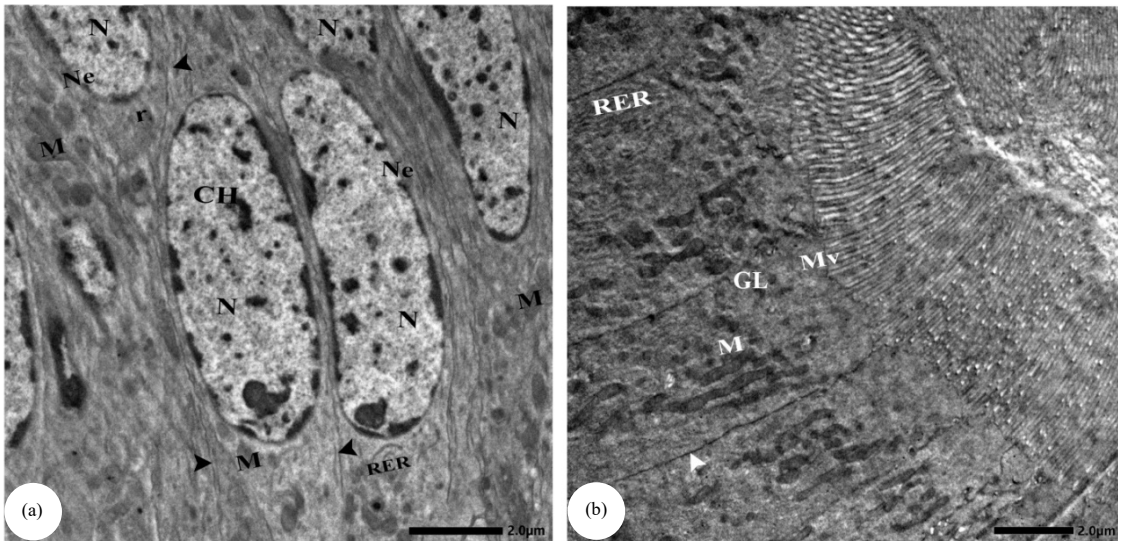

Fig. 4(a-b): Electron micrographs midgut cells of *B. polycresta* collected from the reference site (site A) (a) Nucleus (N), nuclear envelope (Ne), chromatin (CH), mitochondria (M), rough endoplasmic reticulum (RER), free ribosomes (r), desmosomes (arrow head) and (b) Basal region of the digestive cells with numerous mitochondria (M), glycogen granules (GL) and rough endoplasmic reticulum (RER), luminal border with microvilli (MV), desmosomes (arrow head)

Ultrastructure observations: Signs of necrosis were observed in the nuclei of some regenerative cells which included: Formation of blebs (Fig. 8a, b, 9a) and pseudo-inclusions at the nuclear envelopes (Fig. 8c, 9a), globular inclusions in the nucleoplasm (Fig. 8a, b, 9a) and Karyorrhexis (Fig. 8a, 9a). Abnormal chromatin clumps were also observed in the nucleoplasm (Fig. 8a, b). Apoptotic regenerative and digestive cells were noticed (Fig. 8c, e). Mitochondria appeared with lysed matrices (Fig. 8a, b). Lysosomes (Fig. 8b, c, e, 9a, b), electron-dense vesicles (Fig. 8d, e, 9b), myelin figures (Fig. 8c, e, 9b) and cytoplasmic vacuolation (Fig. 8a-e, 9a, b) were frequently observed. In addition, dilated rough and smooth endoplasmic reticulum (Fig. 8c) were remarked. Microvilli appeared with distorted brush borders (Fig. 8d, e, 9b).

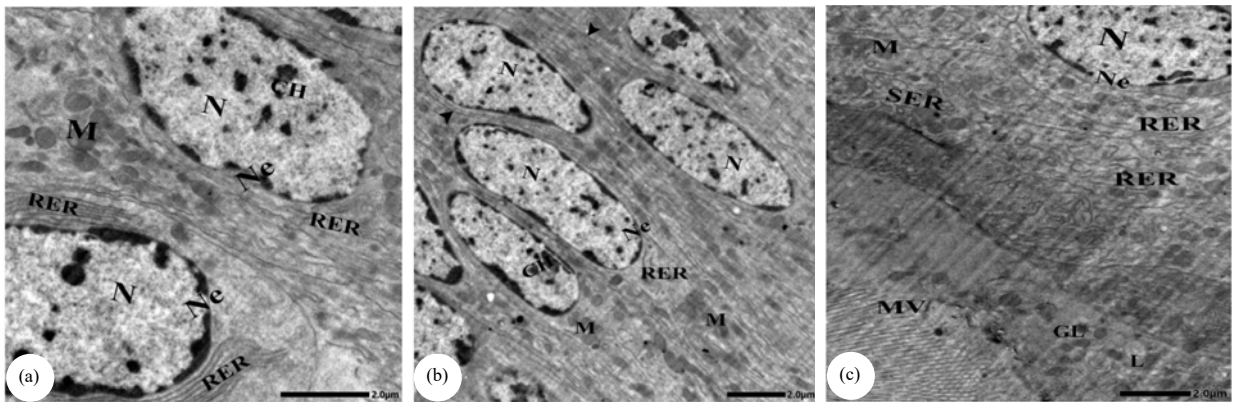

Fig. 5(a-c): Electron micrographs of midgut cells of *T. hispida* collected from the reference site (site A) (a-b) Nucleus of regenerative cells (N), regular nuclear envelope (Ne), chromatin (CH), mitochondria (M), rough endoplasmic reticulum (RER), free ribosomes (r), desmosomes (arrow head). (c) Digestive cells with nucleus (N) regular nuclear envelope (Ne), mitochondria (M), rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), microvilli (MV), glycogen granules (GL), lysosomes (L)

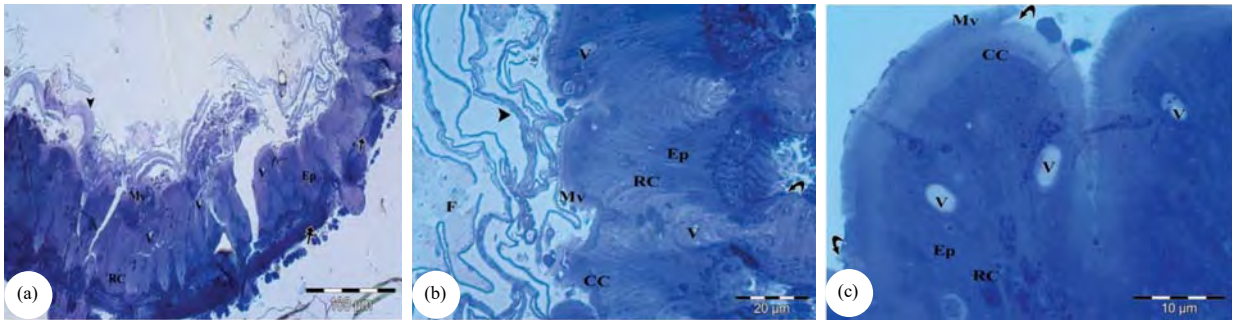

Fig. 6(a-c): Semithin sections of the midgut tissues of *B. polycresta* collected from the polluted site (site B) (a-b) Lacerated (*) and vacuolated (V) epithelial sheath (Ep), (c) Vacuolated (V) epithelial sheath (Ep), distorted microvilli (MV) (curved arrow). Muscle layers: Longitudinal (double head arrow) and circular (arrow), regenerative cells (RC), columnar digestive cells (CC), peritrophic membrane (arrow head), food particles (F)

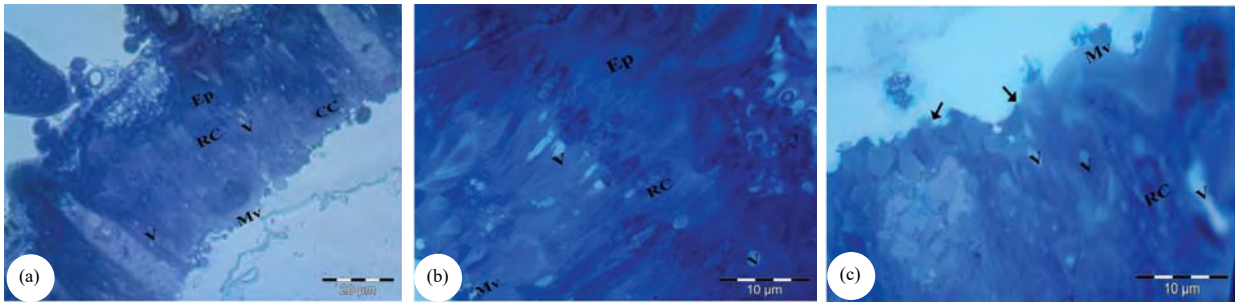

Fig. 7(a-c): Semithin sections of the midgut tissues of *T. hispida* collected from the polluted site (site B) (a) Vacuolated (V) epithelial sheath (Ep), (b-c) Vacuolated (V) epithelial sheath (Ep), distorted microvilli (MV) (arrow), regenerative cells (RC), columnar digestive cells (CC), peritrophic membrane (arrow head)

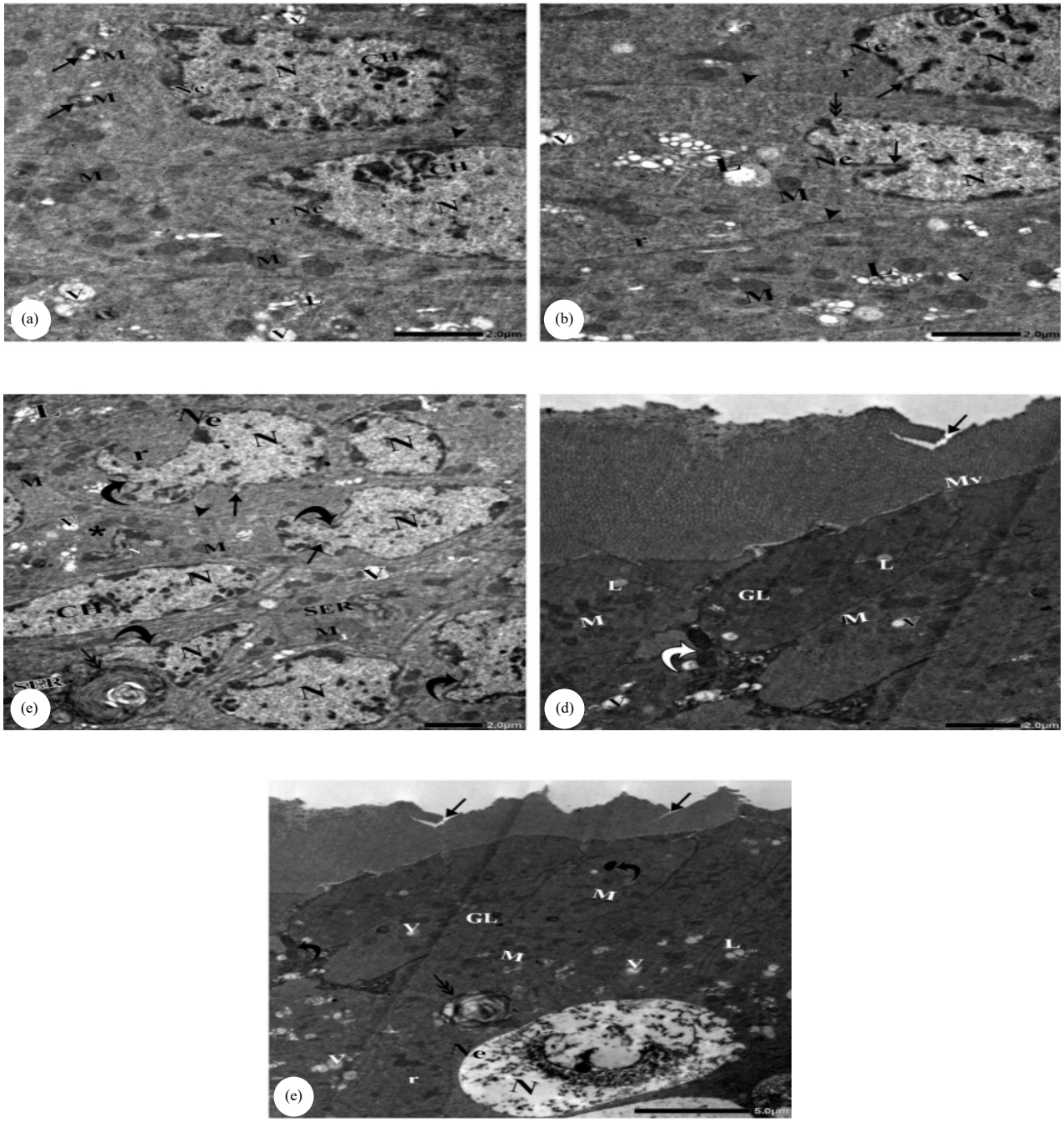

Fig. 8(a-e): Electron micrographs of abnormal midgut cells of *B. polycresta* collected from the polluted site (site B) (a) Nucleus (N), abnormal chromatin clumps (CH), intended nuclear envelope (Ne), lysis of mitochondrial matrices (arrow), vacuoles (V), (b) Globular inclusions (arrow). Karyorrhexis (double head arrow), vacuoles (V), lysosomal vesicles (L), (c) Pseudo-inclusions (curved arrow), globular inclusions (arrow), lysed mitochondria (M), vacuoles (V), lysosomes (L), myelin figure (double head arrow), dilated rough (RER) and smooth endoplasmic reticulum (SER), apoptotic cell (*), (d) Distorted microvilli (arrow), vacuoles (V), electron dense vesicles (curved arrow), glycogen granules (GL) and (e) Karyolysed nucleus (N), myelin figure (double head arrow), vacuoles (V), lysosomes (L), glycogen granules (GL), free ribosomes®

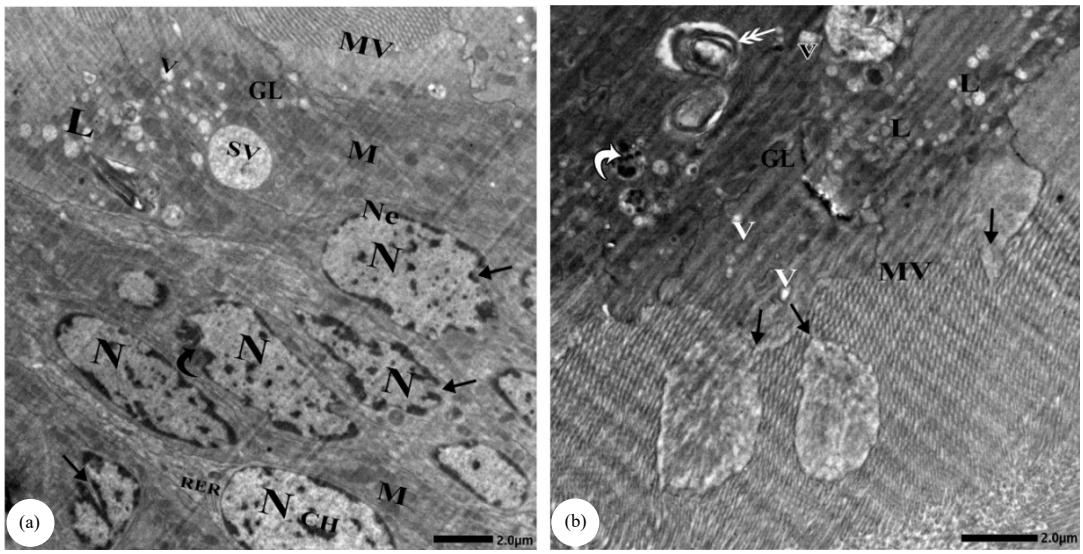

Fig. 9(a-b): Electron micrographs of abnormal midgut cells of *T. hispida* collected from the polluted site (site B) (a) Nucleus (N), chromatin (CH), irregular nuclear envelope (Ne), pseudo-inclusions and globular inclusions (arrow), Karyorrhexis (curved arrow), secretory vesicle (SV), lysosomes (L), rough endoplasmic reticulum (RER), microvilli (MV) and (b) Distorted microvilli (arrow), myelin figure (double head arrow), vacuoles (V), lysosomes (L), dense vesicles (curved arrow), mitochondria (M), glycogen granules (GL)

DISCUSSION

The current study illustrated the cellular anomalies that resulted from ceramic industrial pollution in two bioindicator beetles, *B. polycresta* and *T. hispida*. Ceramic industry has been retreated from most of the countries and progressed in the developing countries. The problem is the management of the pollution control planet. Factories are required to eliminate the operational defects[10]. The chemicals that are contributed to the manufacturing process contain heavy metals that are released in the environment and threat the biota[13]. Many authors have reported the prominent act of insects as bioindicators[18,20-22,25,30,31]. The x-ray microanalysis in the present study proclaimed significant high percentages of heavy metals in the soil and midgut tissues of beetles assembled from the polluted site. The midgut in insects is the main site of absorption and plays an essential role in ionic regulation and mineral[32].

Ingestion of heavy metals by insects leads to histological and ultrastructure anomalies[18,20-22,30,31,33].

The observed alterations in the midgut tissues of the two beetles collected from the polluted site could be ascribed to metals accumulation in these tissues as reported previously by Xie *et al.*[34]. Several studies recorded structural alteration in the midgut of insects exposed to pollutants[24,25,35,36].

A normal architecture of the midgut epithelium cells was noticed in the beetles collected from the reference site. An outer longitudinal and inner circular muscle layers covering the midgut epithelium, designed for slow and super contractions and an absorption surface, the microvilli[37].

Ultrastructurally, the nuclei, the rough endoplasmic reticulum and the mitochondria appeared in a healthy manner. Regenerative cells lack lysosomes, glycogen granules and secretory vesicles, they are found only in the digestive cells [38]. The digestive cells have numerous mitochondria in the apical and basal thirds of the cells to support active transport role[38]. The rough endoplasmic reticulum was arranged in spirals or vesicles throughout the cytoplasm, contrary to Silva-Olivares *et al.*[37]. The abundance of the rough endoplasmic reticulum denotes that a large amount of proteins is synthesized in these cells[39].

Contrarily, the midgut of the beetles collected from the polluted site exhibited several histological and ultrastructure deformations. The earliest changes involve chromatin clumping suggesting a less efficient transcription[40]. The formation of blebs at the nuclear envelopes, globular inclusions in the nucleoplasm and Karyorrhexis were signs of cell death pathway[41,42]. Loeb *et al.*[43] found that Cd and Pb produce apoptosis in the midgut of lepidopteran larvae *Heliothis virescens*. They observed cellular shrinkage,

chromatin condensation, blebbing of the cytoplasm and DNA fragmentation. Several authors attributed apoptosis to metal accumulation[44-47] which are in agreement with the current results. The vacuolated areas in the cytoplasm may be ascribed to the breakage of the mitochondria[48] or sometimes may be due to the enhanced endocytotic activity[49]. The presence of electron-dense vesicles in the midgut tissues may be attributed to metal accumulations as a detoxification mechanism[50]. The present results are in accordance with Polidori *et al.*[48] who detected metal precipitation in spherites in the midgut of paper wasps (*Polistes dominula*) collected from Urban environments. Also, a similar observation was noticed by Pigino *et al.*[51], who reported that a large number of electron-dense granules, composed of a variety of heavy metals were accumulated in the epithelium of the midgut ventriculus of the mite *Xenillus tegeocranus* from a deserted mining and smelting area. Disruption of microvilli is one of the major findings in this study since it is the first site facing and interacting with the pollutants[48,52]. Some changes in the cytoplasmic organelles were distinguished in the electron micrographs such as lysis of mitochondrial matrices, dilated rough and smooth endoplasmic reticulum and presence of myelin figures. It was found that heavy metals distort cytoplasmic membranes[20,22,30,53]. Mitochondrial alteration is a reflection of the deregulation of mitochondrial membrane transport[54]. Also, the interference of heavy metals with proper processing in the ER causes its dilation and activates the ER stress response[55]. The proliferation of myelin figures in the current preparations has been interpreted as a symptom of intoxication trigged by xenobiotics which implies an adaptive mechanism in response to the high degradation of cellular organelles[56]. Eventually, these results form a pathway of cell injury resulted from industrial pollution and diagnose the toxicological pathology accompanied manufacturing ceramic. Precautions against manufacturing ceramic should be taken to limit the operational defects that may result in a polluted environment.

CONCLUSION

The study validates that the beetles are sensible bioindicators for industrial pollution and reveals the ultrastructural damage to the midgut cells since they are the first sites of the action of heavy metals through ingestion. It also elucidates the threat resulted from ceramic industries in urban areas that may lead to a serious risk to human health.

SIGNIFICANCE STATEMENT

This study discovered the heavy metals toxicity that resulted from ceramic industrial planets by using ground beetles as bioindicators that can be beneficial for the detection of health problems. This study will help the researcher to uncover the critical areas of cell injuries that many researchers were not able to explore. Thus a new theory on "Hazards of ceramic industry" may be arrived at.

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