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Cellular alterations in midgut cells of honey bee workers (*Apis millefera* L.) exposed to sublethal concentrations of CdO or PbO nanoparticles or their binary mixture

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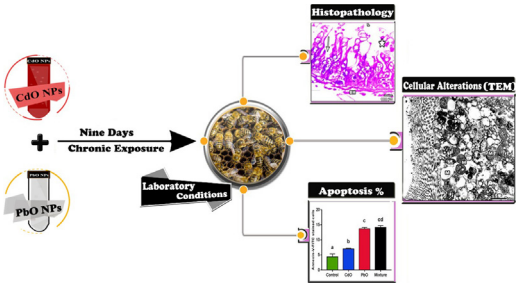
HIGHLIGHTS

- Bee workers have been exposed for 9 days to sublethal conc. of CdO or/and PbO NPs.
- Common histopathological and cellular alterations have been observed in all treated groups.
- The incidence (%) of both apoptosis and necrosis was greater in bees exposed to the binary mixture.
- CdO NPs had greater potential to affect honey bees than did PbO NPs.

GRAPHICAL ABSTRACT



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ABSTRACT

Beside many beneficial applications in industry, agriculture and medicine, nanoparticles (NPs) released into the environment might cause adverse effects. In the present study, effects of exposure to sublethal concentrations of PbO and CdO NPs, either separately or in combination on honey bee (*A. mellifera*) workers were assessed. Honey bee workers were fed sugar syrup contained (20% of LC_{50}) of CdO (0.01 mg ml^{-1}) and PbO (0.65 mg ml^{-1}) NPs either separately or combined for nine days under laboratory conditions. Control bees were fed 1.5 M sucrose syrup without NPs. Effects on histological and cellular structure of mid gut cells were investigated using light and electron microscope. Percentages of incidence of apoptosis or/and necrosis in mid gut cells were also quantified by use of flow cytometry. Rapture of the peritrophic membrane (PM) was among the most observed histopathological alteration in bees fed sugar syrup contained CdO NPs separately or combined with PbO NPs. Common cytological alterations observed in epithelial cells were irregular distribution or/and condensation of nuclear chromatin, mitochondrial swelling and lysis, and rough endoplasmic reticulum (rER) dilation, fragmentation, and vesiculation and were quite similar in all treated groups compared to control. The greatest incidence (%) of necrosis was observed in bees fed the diet that contained CdO NPs alone. The greatest % of both apoptosis and necrosis was observed in bees fed sugar syrup spiked with sublethal concentrations of both metal oxide NPs. Joint action of the binary mixture of Cd and Pb oxide NPs on honey bees was concluded to be antagonistic. Collectively, exposure of honey bees to these metal oxide NPs even at sublethal concentrations will adversely affect viability of the colony and further studies are still required to determine the effects of these metal oxide NPs on behavior and pollination ecology of honeybees.

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1. Introduction

Metals and metalloids are naturally occurring elements that can be toxic to animals. At lesser concentrations some metals and metalloids, including selenium, copper, zinc, manganese and iron, are important trace nutrients, required for the proper function of biochemical processes throughout the body (Fraga, 2005; Torres-Vega et al., 2012; Wright and Baccarelli, 2007). Other metals and metalloids, such as cadmium, lead, mercury, and arsenic, have no known physiological function in animals and are toxic even in small quantities (Neathery and Miller, 1975; Wright and Baccarelli, 2007).

With the rapid development of nanotechnology, metal oxide nanoparticles (MONPs) have been widely used in various fields, such as electronic devices, cosmetics, paints, additives in food, and biological and medical systems (Rana et al., 2011; Wu et al., 2016). Nanoparticles are classified as a material in which at least one dimension is <100 nm in diameter (Auffan et al., 2009). Metal and metal oxide nanoparticles exhibit different physiochemical properties and are different than their native bulk compounds in several respects, which include surface, optical, thermal, and electrical properties (Sanchez-Dominguez et al., 2009). With the world wide utilization of these MONPs, their potential effects on the environment and wildlife have been investigated, but most studies have focused on their toxicity to human health, soil and aquatic organisms (Dorne et al., 2011; Franklin et al., 2007; Limbach et al., 2007). Their inevitable release into the environment might result in harmful effects on honey bees as well (Al Naggar, 2016; Mueller and Nowack, 2008).

The European honey bee *Apis mellifera* is an essential pollinator for agriculture worldwide, and has been widely regarded as a biomonitor of pollutants in the environment as well (Al Naggar et al., 2013; Steen et al., 2015). During their wide-ranging foraging activities, these hymenopterans are exposed to pollutants including metals associated with particulates of various sizes, which are present in the atmosphere, soil, vegetation, and water (Lambert et al., 2013; Al Naggar et al., 2014; Negri et al., 2015). Exposure of honey bees to polluting sources could occur through adhesion of particles to hairs on bodies of insects, inhalation of pollutants via spiracles of the tracheal system or ingestion of contaminated nectar, pollen and water. Contaminants are brought back to the hives and may also be found into the apiary products, such as honey and wax (Satta et al., 2012; Porrini et al., 2014).

Cadmium and lead rank among the priority metals that are of great public health significance due to their high degree of toxicity and carcinogenicity, (Tchounwou et al., 2001; Yedjou and Tchounwou, 2007). Nanoparticles made of cadmium (Cd) and lead (Pb) oxide are now widely used in many industries. Cadmium oxide NPs (CdO) is used in applications, such as solar cells (Mane et al., 2006), transparent electrodes, phototransistors (Ryuji et al., 1971). It is found in air of the industrialized areas, tobacco smoke (Faroon et al., 2012), in the sediments in lakes and streams (Rzętała, 2016), cadmium-containing fertilizers and sewage sludge (Jensen and Bro-Rasmussen, 1992).

Lead oxide NPs (PbO) is an important industrial material due to its unique electronic, mechanical properties and its potential applications in nano devices and functionalized materials (Meshram et al., 2015; Shan Lim et al., 2015). The main sources of lead entering ecosystems

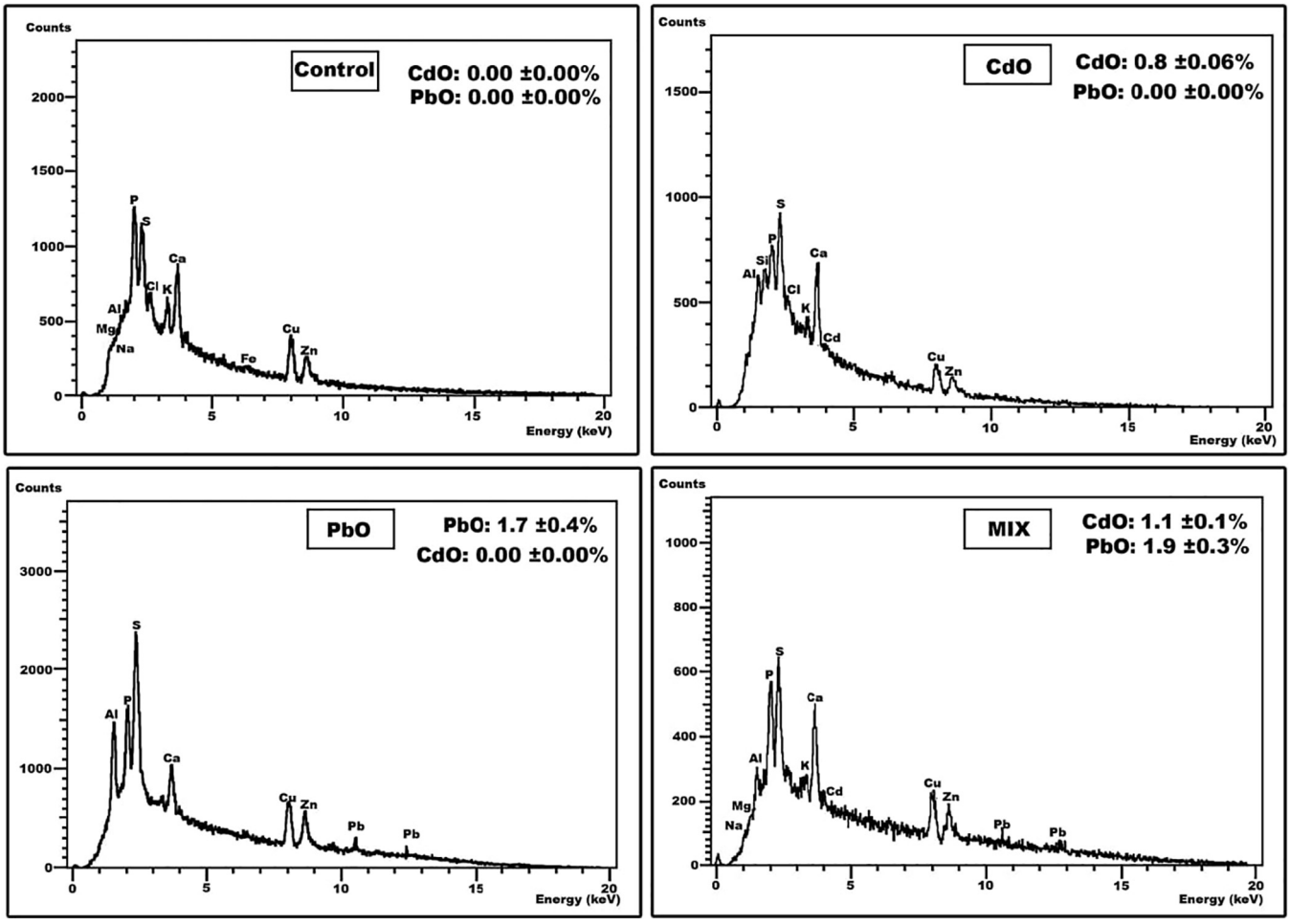


Fig. 1. SEM-EDX spectra of air-dried mid gut tissues of honey bee workers, chronically exposed to; a suspension of CdO NPs (0.01 mg ml^{-1}), suspension of PbO NPs (0.65 mg ml^{-1}) and their binary mixture in 1.5 M sucrose as compared to control (1.5 M sucrose). Semi-quantitative detection (% of height peak) of metal oxides NPs (mean ± SD; $n = 3$).

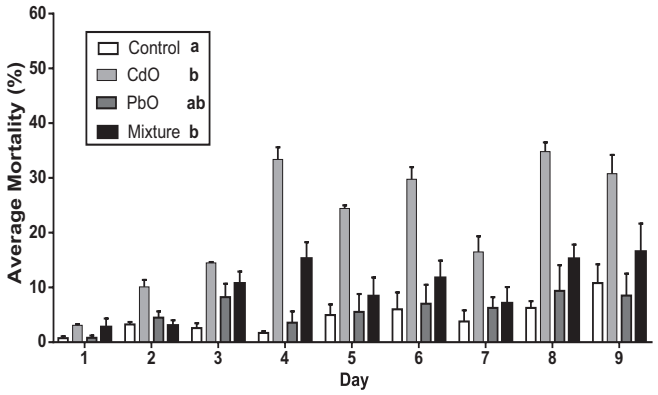


Fig. 2. Cumulative mortalities (%) (mean ± SD) of honey bee workers exposed for nine days to a control or sucrose solution diet containing sublethal concentrations of CdO NPs (0.01 mg ml^{-1}) or PbO NPs (0.65 mg ml^{-1}) or their binary mixture. Letters denote significant differences compared to control (One-way repeated measure (RM) ANOVA followed by Tukey's post hoc test ($P < 0.05$).

are atmospheric, primarily from automobile emissions, paint chips, used ammunition, fertilizers or pesticides. Other sources of Pb and PbO NPs include power plants and high temperature processes such as those used in sintering plants (Oravisjärvi et al., 2003), lead smelters (Sobanska et al., 1999), electric steel plants (Sammut et al., 2010; Oszlánczi et al., 2011) or welding of lead painted metal and in battery plants (Järup, 2003).

Although adverse effect of MONPs on *A. mellifera* such as nano Boron particles (Dağlıoğlu et al., 2015a), Poly (Vinylferrocene) supported platinum nanoparticles (Dağlıoğlu et al., 2016), TiO_2, Ag-TiO_2 and ZnO-TiO_2 nanoparticles (Dağlıoğlu et al., 2015b), Cerium(IV) oxide nanoparticles (Kos et al., 2017) and ZnO nanomaterials (Glavan et al., 2017) have been investigated, CdO and PbO nanoparticles had not yet been tested on honey bees. Considering their wide applications and their relative great toxic potencies, there was a need to investigate their adverse effects on honey bees.

The digestive system is a critical organ for honey bee health because it is the site of contact with pathogens and xenobiotics (Han et al., 2012; Johnson et al., 2009). The midgut epithelium is responsible for detoxification of ingested xenobiotics (Higes et al., 2013). It is the only tissue of adult honey bees that exhibits widespread cell proliferation (Ward et al., 2008). Thus, one important organ for toxicity analysis is the midgut, since it is responsible for digestion and absorption of ingested food. Moreover, it is one of the primary interfaces where bees come in contact with injected metals (Fernanda Catae et al., 2014). Several authors have reported that the alimentary canal (midgut) of insects is the major tissue through which metals are accumulated (Maroni et al., 1986; Suzuki et al., 1984; Schmidt and Ibrahim, 1994; Zhang et al., 2001).

Alterations of ultrastructure in organisms exposed to either Pb or Cd have been reported to include swollen mitochondria, which are a well-documented response to intoxication with Pb, by both vertebrate and invertebrate cells (Cheville, 2009; Vandenbulcke et al., 1998) and Cd (Hemelraad et al., 1990; Hirano et al., 1991). Diplopods exhibited disconnected epithelial cells, electron-dense cytoplasm and elongated liver cells after exposure to Cd or Pb (Köhler and Alberti, 1992). Mitochondrial swelling and lysis, dilation of rough endoplasmic reticulum (rER) and shortened-disorganized microvilli observations were also reported in for *Boettcherisca peregrina* larvae after exposure to Cd (Wu et al., 2009).

Considering the importance of honey bees, the reported toxicity of Cd and Pb as metals and as metal oxide NPs and the possibility of exposure of honey bees to these environmental pollutants, the present study was carried out to assess whether chronic exposure to sublethal concentrations of PbO and CdO NPs either separately or combined exerts adverse effects on honey bee workers. Effects on mortality, histological and cellular structure, and death of midgut cells of honey bees were investigated. It was hypothesized that exposure of bees to Cd or/and Pb oxide NPs could exert adverse effects on endpoints examined in individual bees.

2. Materials and methods

2.1. Preparation of metal oxide NPs

Cadmium oxide (CdO) NPs were prepared according to previously published methods (DurgaVijaykarthik et al., 2014), while Lead Oxide (PbO) NPs were prepared according to alternative published methods (Alagar et al., 2012).

2.2. Characterization of synthetized CdO and PbO NPs

Crystalline phases of CdO and PbO NPs were characterized by use of X-ray diffraction (XRD), by use of a Siemens D500 X-ray diffractometer (Table S1). The size and morphology of both CdO and PbO NPs were also characterized by use of a HR-TEM (High Resolution Transmission Electron Microscopy) using a JEOL 100CX (Jeol Inc., Peabody, MA, USA) operated at 80 kV (Fig. S1). Particle sizes were analyzed using Image J software.

2.3. Test animals

Experiments were conducted during July–October 2017 with hybrid *A. mellifera* workers of the subspecies *carnica*, obtained from hives maintained in the apiary of the experimental farm of the city of Scientific Research and Technological Applications (SRTA-City) (New Borg El Arab, Alex., Egypt). Nurse bees collected from brood frames of three colonies were taken to the department of plant protection and molecular diagnosis laboratory and placed in a refrigerator at 4 °C for approximately 10 min to slow movement.

Five serially diluted concentrations of synthetized NPs (CdO: 0.002, 0.02, 0.2, 2, 20 mg ml^{-1}; PbO: 0.6, 1.2, 2.5, 5, 10 mg ml^{-1}) were tested against honey bee workers to determine the median lethal concentration (LC_{50}), which is the concentration required to cause 50% of a group of test organisms within a specified period to be killed (OECD, 1998). Access to sucrose solution was through a 50 mm diameter Petri dish placed at the bottom of each cage. The Petri dish was covered with a plastic net to prevent drowning of bees. During the experiment, caged bees ($n = 25$ per cage) were kept in an incubator at 27 °C and 65% relative humidity. Experiments were conducted in triplicate for each concentration and the bioassay was done on three separate occasions (i.e. blocks in time) during July 2017. Preliminary results of the LC_{50} bioassay for both CdO and PbO NPs indicated that the data obtained after exposure for 24 h or 48 h failed to fulfill the requirements for determination of LC_{50}. Therefore, mortality was recorded daily for 4 days and then cumulative mortality was calculated for each concentration tested. LC_{50} for each metal oxide NPs was estimated by the log-probit model using the LdP Line^{R} software (Ehabsoft (http://www.ehabsoft.com/ldpline) (Table S2).

2.4. Exposure protocol and effects on survival

Bee workers were chronically exposed to sublethal concentrations (i.e concentration of a potentially lethal substance that is not large enough to cause death) (20% of LC_{50}) of CdO (0.01 mg ml^{-1}) or PbO (0.65 mg ml^{-1}) NPs separately or combined for 9 days according to standard guidelines that recommended a ten-day trial for chronic toxicity evaluation on adults (OECD, 2014). These concentrations are comparable to concentrations of their metals that found in contaminated environments and measured in floral parts of plants grown in contaminated soils (Al Naggar et al., 2014; Hladun et al., 2015).

A mixture of sublethal concentrations of both metal oxide NPs was prepared according to LC_{50} of each metal oxide NP (i…e 0.4 mg of CdO NP + 26 mg of PbO NP were added to 40 ml of sugar syrup to get 0.01 mg ml^{-1} and 0.65 mg ml^{-1} for CdO and PbO NPs, respectively).

Numbers of dead bees were recorded daily. Each bioassay consisted of three replicate cages per treatment, and the bioassay was done on three separate occasions (i.e. blocks in time). On the ninth day, numbers of surviving bees were lesser in CdO NPs treated group. Therefore, subsamples of surviving bees from different replicates were collected, dissected and alimentary canals were removed for histopathology, ultrastructural and cell death investigations. Some alimentary canals were also used to monitor possible accumulation of both metal oxide NPs in mid gut tissues employing proton-induced X-ray emission (PIXE) spectroscopy.

õFor histopathological investigations using hematoxylin and eosin (H&E), alimentary canals were fixed in 10% formalin while for ultrastructure investigations using transmission electron microscopy, a fixation solution (4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) was used Additionally, some survived

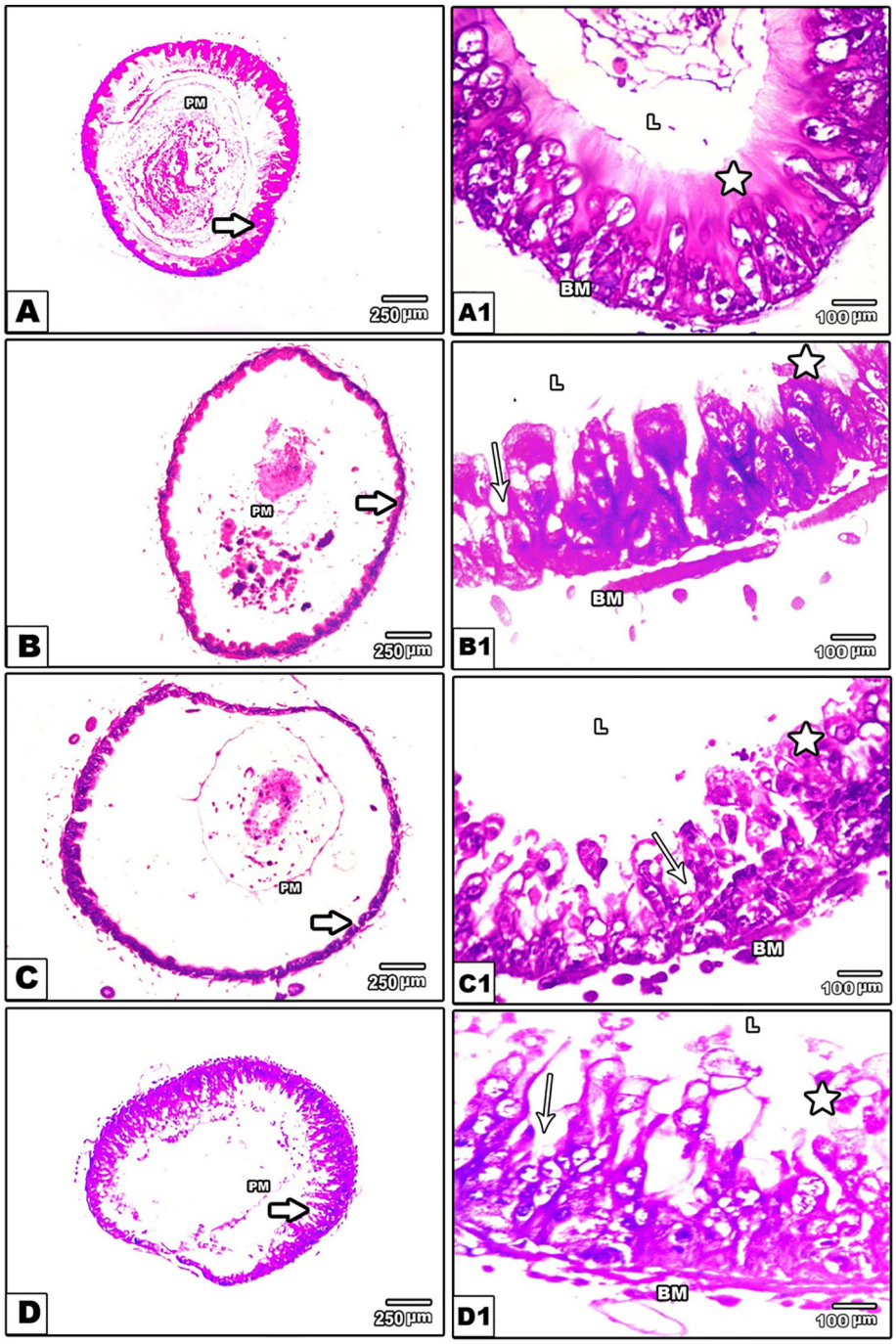
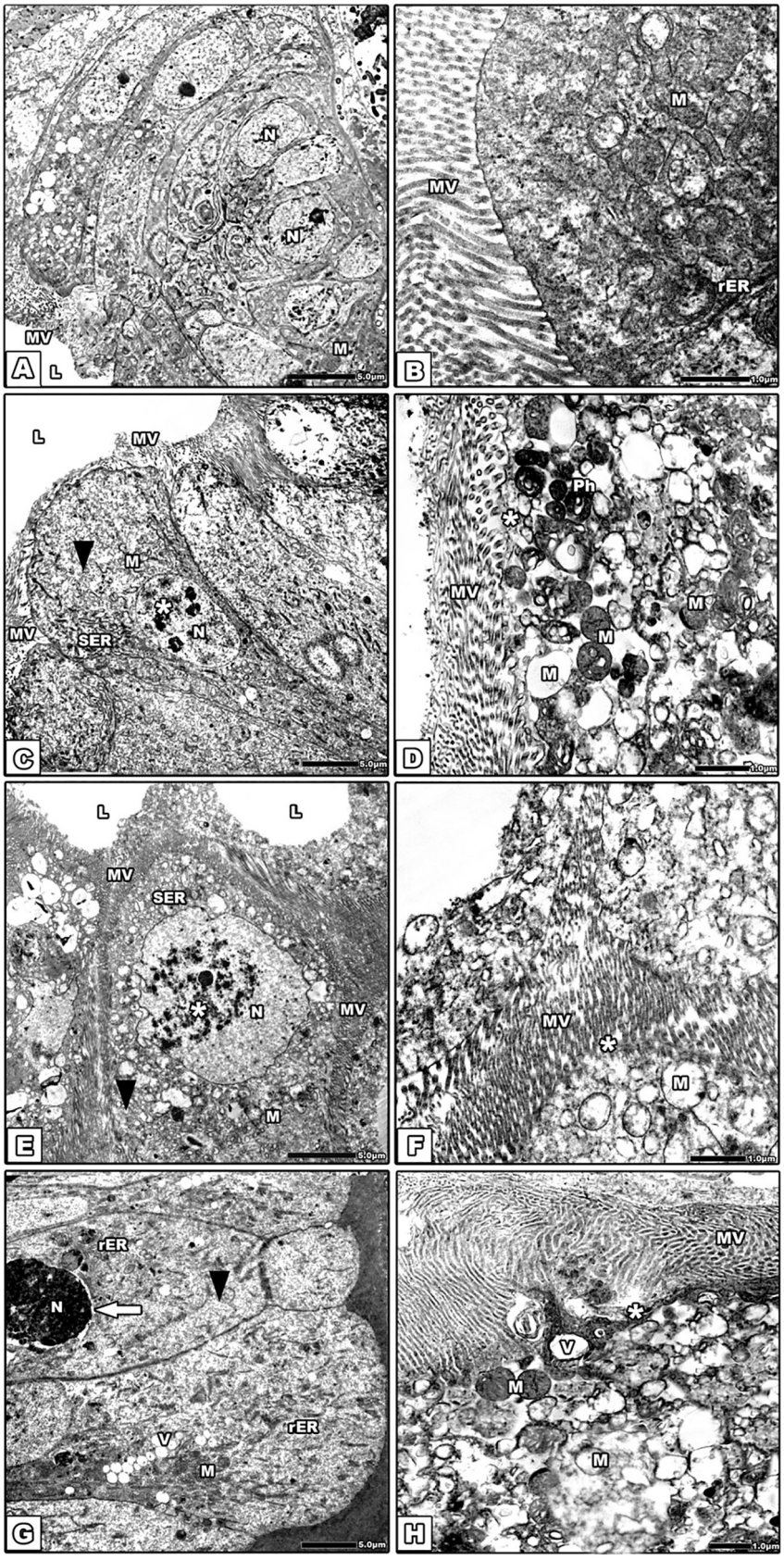


Fig. 3. Photomicrographs of mid gut cells of honey bee workers, control groups and fed for nine days sublethal concentrations (20% of LC_{50}) of CdO (0.01 mg ml^{-1}) or/and PbO NPs (0.65 mg ml^{-1}) stained with hematoxylin–eosin. **A, A1** normal epithelial cells of control group presenting a typical morphology, with normal columnar cells resting on basement membrane (BM), thick gelatinous layer and multilayer peritrophic membrane intact (pm). **B, B1** honey bee workers fed a sublethal concentration of CdO NPs. Note, PM was completely ruptured, basement membrane ruptured and slight vacuolization. The gelatinous layer and the digestive vesicles were absent. **C, C1** honey bee workers fed a sublethal concentration of PbO NPs. Note, PM was not affected and appeared normal and transparent. Columnar cells suffered loss of matrix with numerous small vacuoles, degenerated basement membrane and gelatinous layer was absent. **D, D1** honey bee workers fed a binary mixture of sublethal concentration of both CdO and PbO NPs. Note, PM was partially ruptured, numerous large vacuoles between columnar cells and digestive vesicles. The BM and the muscles were intact. *Thick arrow*, epithelial cells: *PM* Peritrophic membrane; *BM* basement membrane; *L* Lumen of the gut; *Star* columnar cells; *Thin arrow* vacuoles.

bees were also dissected on ice and alimentary canals were removed and stored at −80 °C to quantify the potential cell death by annexin V/PI assay using flowcytometric analysis.

2.4.1. Concentrations of Cd and Pb in mid gut tissues

Preparations of honey bee tissues were carried out as described previously by Kuterbach and Walcott (1986). Specimens were investigated



using field emission scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM–EDX) (FE-SEM; JEOL JSM-5300 LV EDX; EDS/WDS Oxford Instruments INCA).

2.4.2. Light microscopy

Midguts (ventriculus) of three composite samples ($n = 15$) per treatment; one randomly selected from each set of treatments from each of the three bioassays (blocks), were passed through various concentrations of alcohol for dehydration. Processed tissues were then blocked in suitable moulds with molten wax and allowed to cool on a cool plate. Blocks were trimmed and serial sections 6 μm thick were cut on a Leitz–Wetzlar microtome using Richert Jung disposable microtome blades. Sections were collected and arranged in rows, putting the slide on a moderately hot stretching table and the tissue was allowed to dry. The slides were then treated in xylene for 5 min to remove wax, followed by treatment with a descending grade of alcohol. The slides were then dipped in hematoxylin, and washed in water for 30 min, followed by eosin staining for 3 min. Stained tissue sections were observed under an Olympus compound microscope (×1000) and photomicrographs from representative histological sections were taken on a Leitz–Wetzlar photomicroscope. Three slides containing serial histological sections of midgut per treatment were examined and compared.

2.4.3. Transmission Electron Microscopy (TEM)

For transmission electron microscopy studies, fragments of midgut from three composite samples ($n = 15$) per treatment; one randomly selected from each set of treatments from each of the three bioassays (blocks), were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.3) for 24 h at room temperature, post-fixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature. After washing in distilled water, the material was stained with 0.5% uranyl acetate in water solution for 2 h at room temperature. Afterwards, fragments were dehydrated in graded acetone series (50%, 70%, 90% and 100%), and embedded in Araldite® resin. Selected semi-thin sections were stained with 1% toluidine blue, while ultrathin sections were stained with 2% aqueous uranyl acetate and lead citrate (Reynolds, 1963), and were examined and photographed using a JEM-1200EX transmission electron microscope (JEOL, Japan) at an accelerating voltage of 60 kV.

2.4.4. Annexin V/PI assay

Cells were assessed for apoptosis and necrosis by use of the annexin V/propidium iodide (PI) assay (van Engeland et al., 1996). Flowcytometric analysis was performed on FAC Scan (Becton Dickinson) using standard settings: fluorescence 1 (FL1), 4 decades (logarithmic), detector 496 V, log amplifier, compensation 22.8%. Data analysis was performed using lysis software (Becton Dickinson) (Neubourg et al., 1996). Biopsies from alimentary canal tissues of surviving bees were taken, and a suspension of cells was prepared in Tris-EDTA buffer (PH 74) (Sigma-Aldrich Co.). The cell suspension was fixed in ice-cold 96–100% ethanol (Sigma) at 4 °C overnight, centrifuged at 1500 rpm for 10 min, and then re-suspended in PBS containing 50 μg/ml propidium iodide (PI) (Sigma-Aldrich Co.).

Apoptosis was assessed with flow cytometry using an annexin V FITC/PI staining kit (Pharmingen, Becton Dickinson Co., San Diego, CA, USA) (Peng et al., 2002; Yedjou et al., 2010). After 48 h of transfection, alimentary canal tissues were harvested, washed twice in PBS (sodium chloride NaCl 40.0 g, potassium chloride KCl 1.0 g, potassium dihydrogen phosphate anhydrous Na_2HPO_4 4.6 g, and distilled water to make up to 51 ml; 4 °C), resuspended in binding buffer (10 mm HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), stained with fluorescein isothiocyanate-conjugated annexin V (annexin V-FITC), mixed gently and incubated for 15 min at room temperature in the dark, and then washed with binding buffer and analyzed with flowcytometry (FACS Calibar; Becton-Dickinson) using CellQuest software (Becton-Dickinson, San Jose, CA, USA).

2.5. Data analyses

Data were analyzed by use of GraphPad Prism version 5.00 for Windows, (GraphPad Software, La Jolla California USA, www.graphpad.com). Normality of data was assessed by use of the Kolomogrov-Smirnov test, and homogeneity of variance was assessed by use of Levene's test. If necessary, data was log10-transformed to better approximate normality and homogeneity of variance. Mortality was analyzed by one-way repeated measure (RM) ANOVA. Incidence (%) of apoptosis and necrosis were assessed using one-way ANOVA followed by Tukey's post hoc test ($P < 0.05$). An alpha level of 0.05 was used for all tests.

3. Results

3.1. Content of CdO and PbO NPs in midgut tissues

Energy dispersive X-ray (SEM-EDX) spectra of air-dried midgut tissues of bee workers that consumed sugar syrup contained sublethal concentrations of CdO or PbO NPs showed small peaks for CdO (0.8 ± 0.06%) and PbO (1.7 ± 0.4%) NPs, respectively. Midgut tissues of bee workers fed sugar syrup containing a mixture of sublethal concentrations of both metal oxide nanoparticles contained traces of both CdO (1.1 ± 0.1%) and PbO (1.9 ± 0.3%) NPs while mid gut tissues of bee workers fed on sugar syrup only (control) contained no traces for any of these metal oxide NPs (Fig. 1).

3.2. Survival of honey bees

Metal oxide NPs tested either separately or combined had a significant effect on cumulative nine days mortality (%) of honey bee workers (Repeated measure ANOVA: $P < 0.01$) compared to control. Cumulative mortality (%) was significantly greater in bees that consumed diets containing sublethal concentrations of CdO NPs alone or in combination with sublethal concentrations of PbO NPs ($P < 0.05$) compared to survival of controls. There was no significant difference ($P > 0.05$) in mortality (%) of bees fed sugar syrup contained sublethal concentrations of PbO NPs compared to control (Fig. 2.).

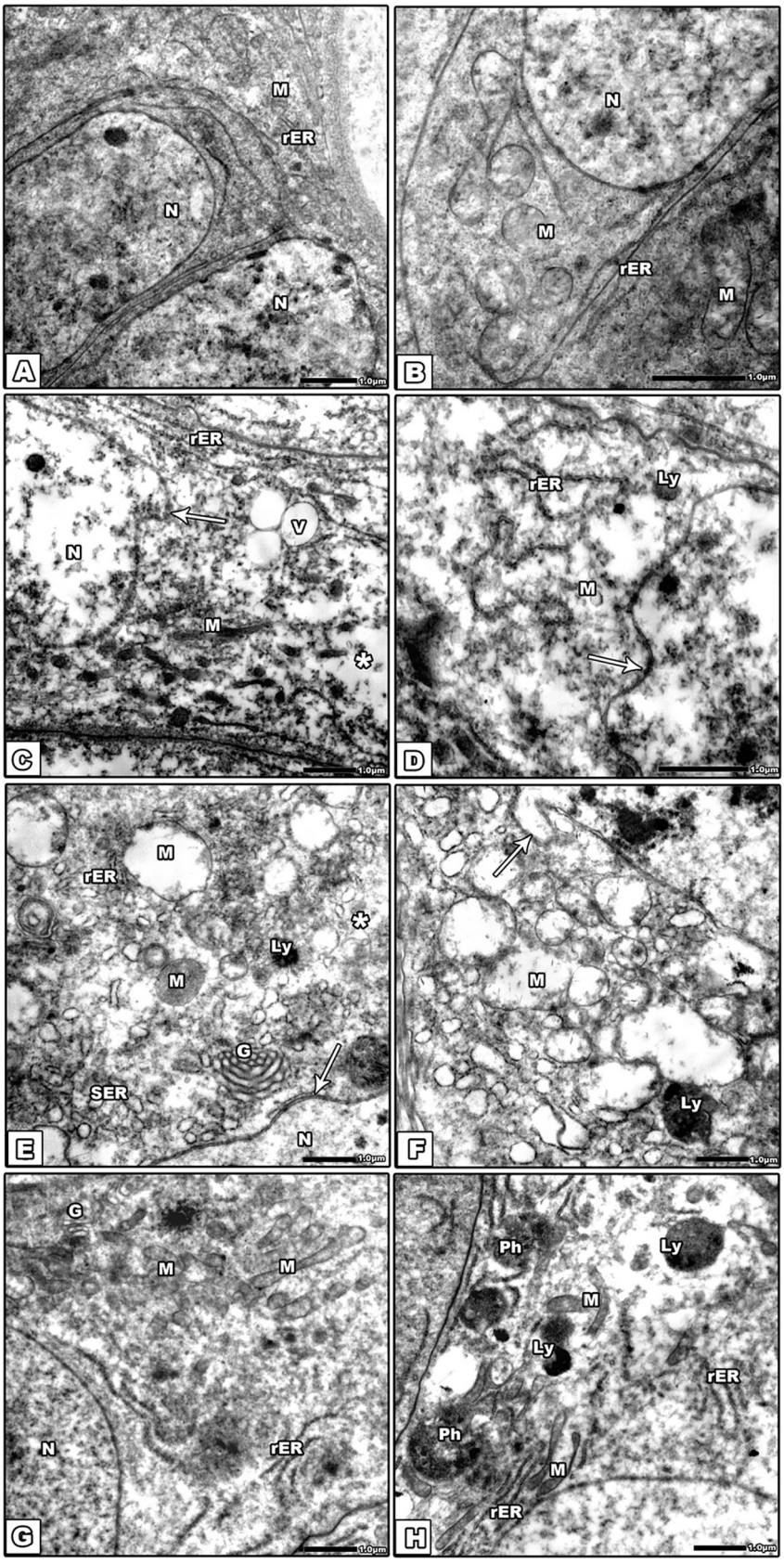
4. Morphological assessment of Mid gut tissues by light microscopy

The mid gut epithelial cells of bees fed the sugar syrup free from any metal oxide NPs (control) showed normal appearance, the peritrophic membrane (PM) appeared visible, multi-layered, intact with normal thickness, contained some food debris and separated from the epithelial cells (Fig. 3-A). Epithelial cells possess normal nuclei with high stainability, homogenous cytoplasm inclusion with intact and regular cell

Fig. 4. Transmission electron microscopy photomicrographs of mid gut cells of honey bee workers, control groups and fed nine days a sublethal concentration (20% of LC_{50}) of CdO or/and PbO NPs. **A, B** Control group, exhibiting typical morphology of columnar cells laterally joined by long septate junctions and the apical border was straight bearing numerous, long filament like microvilli. **C, D** honey bee workers fed a sublethal concentration of CdO NPs (0.01 mg ml^{-1}). Note, cytoplasmic proteolysis (black arrow head), swollen nuclei and abnormal distribution of heterochromatin into patches (star), microvilli appeared withered, detached, and fragmented and some mitochondria were found swollen and showed lysis of matrix and breakage of mitochondrial cristae. **E, F** honey bee workers fed a sublethal concentration of PbO NPs (0.65 mg ml^{-1}). Note, cytoplasmic proteolysis, lysis of smooth endoplasmic reticulum (sER), swollen-irregularly shaped nuclei, abnormally distributed heterochromatin and some vacuoles were found. Microvilli looked detached and fragmented. **G, H** honey bee workers treated with a binary mixture of sublethal concentration of both CdO and PbO NPs. Note, nuclear pyknosis, dilated nuclear periphery, lysis of rER and small vacuoles. Irregular elongation of microvilli and some mitochondria showed lysis of matrix. *N* Nuclei; sER Smooth endoplasmic reticulum; arrow head cytoplasmic proteolysis; * distributed heterochromatin; arrow nuclear pyknosis; MV microvilli; M mitochondria.

boundary. The most abundant type of epithelial cells was columnar cells that were arranged in one layer and settled on the basement membrane (Fig. 3-A1).

In honey bee workers fed sugar syrup contained sublethal concentrations of CdO NPs, adverse effects were severe where the PM was completely raptured, which led to dispersal of midgut contents in the



lumen of alimentary canal. Also, the thickness of epithelial cells was less compared to those of controls (Fig. 3-B). Raptures of basement membrane, nuclei were not visible, but slight vacuolization and lysis of cytoplasm was observed. The gelatinous layer and the digestive vesicles were absent as well (Fig. 3-B1).

In honey bee workers chronically, fed sugar syrup containing sublethal concentrations of PbO NPs, adverse effects were less severe, and the PM was not affected and appeared normal and transparent, however it was only one layer thick. Thicknesses of epithelial cells were less than that of controls (Fig. 3C). Columnar cells exhibited a loss of matrix with numerous small vacuoles, degenerated basement membrane and the gelatinous layer was absent (Fig. 3-C1).

In bees fed the diet containing the binary mixture of sublethal concentrations of both CdO and PbO NPs, the PM was partially ruptured, however the thickness of epithelial cells was comparable to that observed in controls (Fig. 3D). Numerous large vacuoles were observed between columnar cells and digestive vesicles, however; the basement membrane and the muscles remained intact (Fig. 3-D1).

4.1. Ultrastructure observations by TEM

The midgut epithelium of the control group that were fed only sugar syrup, was characterized by oval nuclei and cells were laterally joined by long septate junctions, which were tight and intact. Moreover, the apical border was straight bearing numerous, long filament like microvilli extending into the midgut lumen (Fig. 4 A&B). Abundant and dense mitochondria, rER and an oval cell nucleus were evident (Fig. 5 A&B).

Cytological observations of the CdO NPs-treated bees revealed a dramatic, cytotoxicity of midgut epithelial cells. Cytoplasmic proteolysis, swollen nuclei and abnormal distribution of heterochromatin into patches of varying densities were observed. Microvilli appeared withered, detached, and fragmented and some mitochondria were swollen and exhibited lysis of matrix and breakage of mitochondrial cristae. Moreover, CdO NPs were located in phagosomes or formed clusters within cytoplasmic vesicles (Fig. 4 C&D). Indentation in the nuclear membrane, lysis of nuclear content, cytoplasmic proteolysis and elongation of mitochondria were observed. rER membranes were less, damaged, but occasionally dilated; thus, their layered structure was lost (Fig. 5 C&D).

Feeding bees with sugar syrup containing a sublethal concentration of PbO NPs for nine days resulted in more injury to cells. Cytoplasmic proteolysis, lysis of smooth endoplasmic reticulum (sER), swollen-irregularly shaped nuclei, abnormally distributed heterochromatin and some vacuoles were also observed. Moreover, micovilli were detached and fragmented (Fig. 4 E&F). Mitochondria exhibited visible damage; appeared swollen and their cristae were fractured and dissolved. Indentation of nuclear membrane, tortuous-bulbous Golgi bodies and swollen lysosomes were observed as well (Fig. 5 E&F).

For honey bees fed sugar syrup contained sublethal concentrations of both CdO and PbO NPs, various cytological alterations were also observed. Nuclear pyknosis, dilated nuclear periphery, lysis of rER and small vacuoles were observed. Irregular elongation of microvilli and some mitochondria exhibited lysis of the matrix (Fig. 4 G&H). Additionally, arc-like mitochondria, swollen lysosomes, enlarged phagosomes and a dilatation of cisternae with less cytoplasmic matrix of rER were observed in midguts of worker bees fed metal oxide NPs (Fig. 5 G&H).

4.2. Flow cytometry analysis

Incidence (%) of apoptosis (summation of UR+ LR) and necrosis (UL) of midgut epithelia of honey bee workers after exposure for nine days to CdO or/and PbO NPs were evaluated by flow cytometry analysis in combination with FITC-Annexin V/PI staining and illustrated (Fig. 6). Compared to control, there were significant differences in incidences of both apoptosis and necrosis in honey bee workers fed sugar syrup contained sublethal concentrations of CdO or/and PbO NPs. The greatest incidence (%) of both apoptosis and necrosis was observed in bees fed sugar syrup spiked with sublethal concentrations of both metal oxide NPs (Fig. S2).

5. Discussion

Metals and metalloids are persistent environmental pollutants and that can cause adverse effects on wildlife. Even at sublethal concentrations, these metals have toxic effects (Hasiang and Díaz, 2011; Xu et al., 2009). However, for the honey bee, an important insect pollinator, little is known about the sublethal effects of metal and metal oxide NPs, even though bees can be exposed to these toxins chronically in some environments (Al Naggar, 2016; Burden, 2016). Therefore, this study is first of its kind to investigate and report the adverse cellular effects on midgut epithelial tissues of honey bee workers after chronic exposure to sublethal concentrations of CdO and PbO NPs either separately or combined under laboratory conditions.

In the current study, bees were allowed to feed freely, so the metal accumulation data indicate the balance between the ingested metal and the physiologically excreted metal, but do not indicate the amount of metal neutralized by cellular detoxification processes. It is well known that arthropods can detoxify metals, either by binding to specific proteins, such as metallothionines, or by a compartmentation process within membrane-limited vesicles (Bouquegneau et al., 1985; Köhler and Alberti, 1992). Accumulation of Cd and Pb MONPs in mid gut tissues of bee workers might be attributed to unique properties of NPs that enable them to enter organisms and be transported into tissues, cells and even into organelles, in ways that larger particles might not (Kovochich et al., 2007).

In the present study, the greatest cumulative mortality (%) was observed in bees chronically fed sugar syrup containing sublethal concentrations of CdO NPs alone or in combination with sublethal concentrations of PbO NPs compared to that of the untreated control. Similar results have been reported previously (Di et al., 2016; Hladun et al., 2016), in which exposure to soluble salts of $CdCl_2$ caused adverse effects on development and survival of honey bees under field and laboratory conditions. In contrast, the rate of survival was greater in bees that consumed sugar syrup spiked with sublethal concentration of PbO NPs. This result is also consistent with those of previous studies (Di et al., 2016; Nikolic et al., 2016), in which Pb showed lesser effect on honey bees than did Cd. Additionally, Zhang et al. (2001) reported that; the rate of survival of German cockroaches treated with Pb was greater than that of those treated with Cd, Hg, or Cr. It was hypothesized that mortality would be greater in bees fed the combined mixture of both metal oxide NPs. However, exposure to both types of NPs were antagonistic. These results are consistent with those of a previous study where antagonistic effects on luminosity between Cd and Pb on the luminescent bacterium Photobacterium phosphoreum T3S were observed (Zeb et al., 2017).

Fig. 5. Transmission electron microscopy photomicrographs of mid gut cells of honey bee workers, control groups and fed nine days a sublethal concentrations (20% of LC_{50}) of CdO or/and PbO NPs. **A, B** Control group, presenting a typical morphology of columnar cells with oval nucleus, abundant and dense mitochondria, rough endoplasmic reticulum (rER). **C, D** honey bee workers fed a sublethal concentration of CdO NPs (0.01 mg ml^{-1}). Note, indentation in the nuclear membrane, lysis of nuclear content, cytoplasmic proteolysis and elongation of mitochondria were observed. rER membranes were reduced, destroyed, and occasionally dilated. **E, F** honey bee workers fed a sublethal concentration of PbO NPs (0.65 mg ml^{-1}). Note, mitochondria exhibited visible damage; appeared swollen and their cristae were fractured and dissolved. Indentation of nuclear membrane, tortuous-bulbous Golgi bodies and swollen lysosomes were observed as well. **G, H** honey bee workers fed a binary mixture of sublethal concentration of both CdO and PbO NPs. Note, arc-like mitochondria, swollen lysosomes, enlarged phagosomes and a dilatation in cisternae with less cytoplasmic matrix of rER. N Nuclei; sER Smooth endoplasmic reticulum; rER rough endoplasmic reticulum; arrow nuclear membrane; * cytoplasmic proteolysis; M mitochondria; G Golgi complex; Ly Lysosomes; pH Phagosomes; V vacuoles.

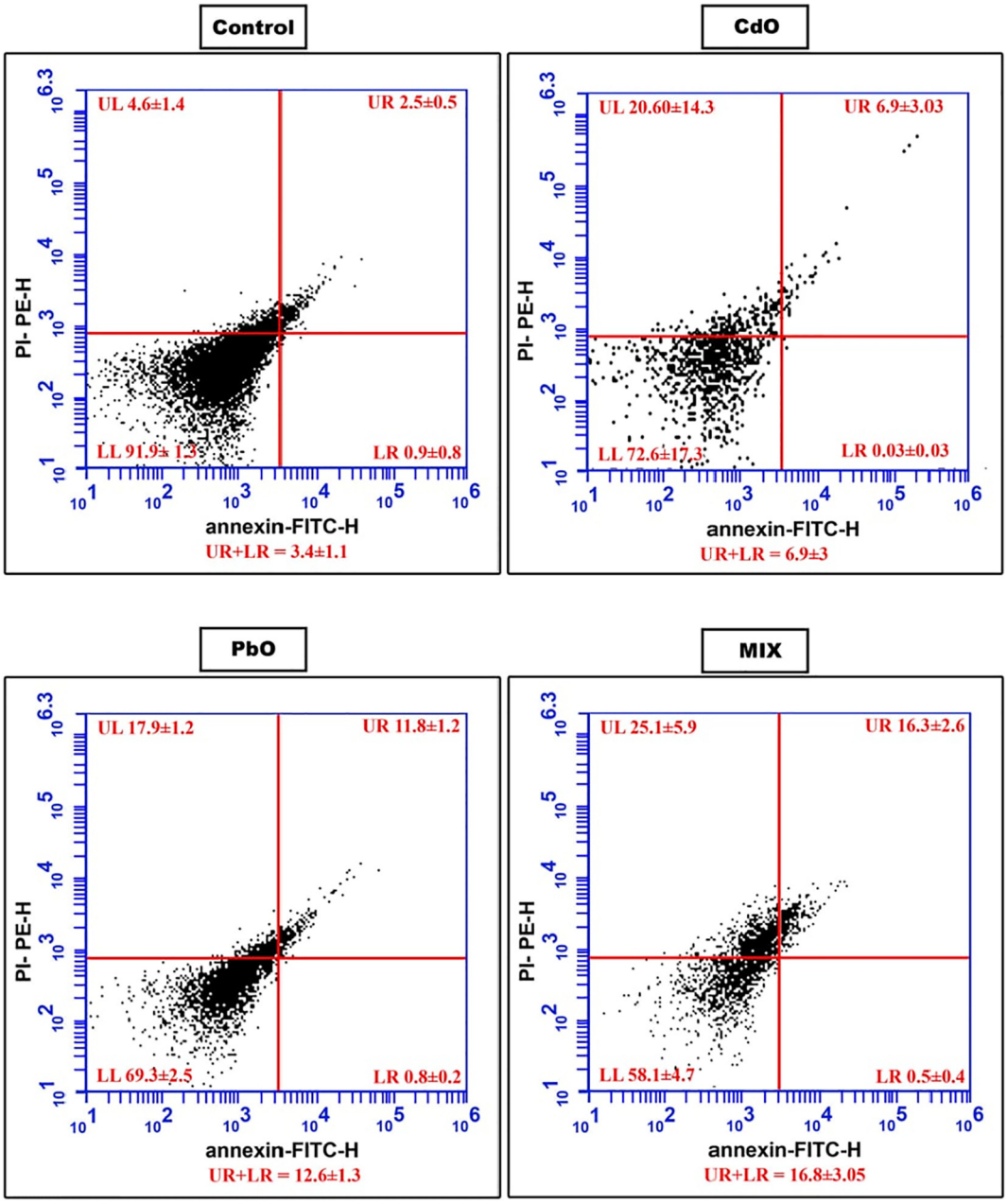


Fig. 6. Flow cytometry analysis of annexin-V-FITC and propidium iodide staining of midgut cells of honey bee workers *(A. mellifera)* exposed for nine days to a control (A) or sucrose solution diet containing sublethal concentrations of (B) CdO NPs (0.01 mg ml^{-1}) or (C) PbO NPs (0.65 mg ml^{-1}) or (D) their binary mixture. The upper left (UL) quadrant ($PI^{+/}$ Annexin V^{-}) represents necrotic cells, the left lower (LL) quadrant (PI^{-}/Annexin V^{-}) represents healthy cells, the upper right (UR) quadrant ($PI^{+/}$Annexin V^{+}) represents early apoptotic cells and the lower right (LR) quadrant (PI^{-}/Annexin V^{+}) represents late apoptotic cells. Apoptosis (summation of UR+ LR). FITC, fluorescein isothiocyanate; PI, propidium iodide. Values represent average percentage (± SEM) of three samples ($n = 3$).

Cadmium and Pb are non-degradable metals, they can be accumulated in animal tissues particularly if present in nanosized materials that can disturb physiological functions causing severe damage to internal tissues (Suganya et al., 2016). In the present study, both CdO and PbO NPs fed to bees at sublethal concentrations either individually or in combination were potent to honey bee workers and demonstrated marked histological anomalies compared to controls. Similar findings have been reported in tissues of German cockroaches exposed to Hg, Pb, Cr and Cd (Zhang et al., 2001) and also in midgut epithelial cells of honey bees exposed to pesticides and pathogens (da Silva Cruz et al., 2010; Dussaubat et al., 2012; Oliveira et al., 2014).

In insects, most digestion and absorption of nutrients and chemicals occurs in the midgut. In this region in most insects, food is enveloped by a semipermeable cellular layer known as peritrophic membrane (PM) that has several functions (Cruz-Landim, 1985). Among the most observed histopathological alterations in the midgut tissues of bees fed sugar syrup containing CdO NPs either alone or in combination with PbO NPs in the current study, is the rapture of PM. These results are consistent with those reported by Zhang et al. (2001) who observed disintegration or/and complete destruction of PM after chronic exposure of German cockroaches to Hg or Cd for eight days. The PM acts as a protective barrier against various chemical, physical and microbial food components (Terra et al., 1988; Terra, 1996). Therefore, any alteration in its structure can lead to dramatic adverse effects on epithelial cells of midgut tissues of insects. Alterations in midgut cells and PM have been shown to be a suitable indicator of prior exposure to environmental stressors (Pawert et al., 1996; Zhong et al., 2012).

In contrast, the PM was intact in bees that consumed sugar syrup contained sublethal concentrations of PbO NPs. These findings are consistent with its lesser effect on survival of honey bees in the current study and in agreement with results of previous studies (Di et al., 2016; Nikolic et al., 2016; Zhang et al., 2001) who reported lesser effects of Pb on midgut tissues and PMs of honey bees and cockroaches. CdO NPs were more potent (more toxic) to honey bee workers than were PbO NPs, a result which is consistent with those of previous studies (Gauthier et al., 2016; Di et al., 2016; Nikolic et al., 2016), in which cadmium salts were more potent to bee workers than were lead salts. The greater potency of Cd salts or its oxide NPs might be attributed to its ability to inhibit the activity of Na^+/H^+ exchange, which alters the passive H^+ permeability that can inhibit the transporter and impair overall osmoregulatory processes (Vilella et al., 1991).

In the current study, common cytological alterations observed in epithelial cells were irregular distribution or/and condensation of nuclear chromatin, mitochondrial swelling and lysis, and rER dilation, fragmentation, and vesiculation. These ultrastructure changes represent the major characteristics of both cell necrosis and apoptosis (Elmore, 2007; Proskuryakov et al., 2003; de Moraes and Bowen, 2000). Picnotic nuclei found in bees fed the mixture of both MONPs indicate a reduction of rates of transcription, which is subsequently responsible for a decrease in metabolic activity, which suggested that cells are in an advanced cell death process (Häcker, 2000; Silva-Zacarin et al., 2008). The results of flow cytometry analysis also confirmed that where, the greatest % of both apoptosis and necrosis incidence was detected in bees fed sugar syrup spiked with sublethal concentrations of both metal oxide NPs. Moreover, the picnotic nuclei observed are often described in organs of Hymenoptera undergoing degeneration (da Cruz-Landim and Cavalcante, 2003; Silva-Zacarin et al., 2007).

Ultrastructural alterations reported due to exposure to CdO or PbO NPs either individually or combined are comparable to those found in the midgut of the fruit fly treated with Cd (Lauverjat et al., 1989) and in midgut and Malpighian tubules of *Boettcherisca peregrina* larvae exposed to Cd or Cu (Wu et al., 2009). Several studies have confirmed the relationship between exposure to metals and incidence of apoptosis (Risso-de Faverney et al., 2004; Luzio et al., 2013). In rainbow trout cell lines, apoptosis was shown to be the most prominent mechanisms of death of cells after exposure to Cd, although other effects, such as necroptosis were also strongly suggested (Krumschnabel et al., 2010).

Nanoparticles are frequently detected in lysosomes upon internalization, and a variety of nanomaterials have been associated with lysosomal dysfunction (Stern et al., 2012). Unique properties of nanoparticles (NPs), enable them to enter organisms and be transported into tissues, cells and even into organelles, in ways that larger particles might not (Kovochich et al., 2007). In present study, precipitates for both metal oxide NPs were observed in lysosomes and phagosomes and therefore, lysosomes appeared swelling and phagosomes were enlarged. It has been established that lysosomal destabilization triggers the mitochondrial pathway of apoptosis (Česen et al., 2012; Repnik et al., 2012). Moreover, mitochondrial swelling and lysis was obvious in bees fed both metal oxide NPs either individually or combined in the current study. Mitochondria behave as osmometers, and the swelling that developed reflects the entry of solutes and water into the mitochondrial matrix (Cheville, 2009). It has been concluded that mitochondria are an important target for toxic effects of metals and its oxide NPs (Belyaeva et al., 2008, 2012).

Cadmium and Pb oxide NPs are increasingly discharged into the environment due to their wide production and application (Mane et al., 2006; Meshram et al., 2015) and can potentially interact with each other and with other contaminants, leading to biological effects (bioaccumulation and/or toxicity) that are poorly understood (Deng et al., 2017). Based on current findings and previously reported results (Al Naggar et al., 2018, in press), the joint action between Cd and Pb oxide NPs tested on honey bees was determined to be antagonistic. These results are in agreement with previous studies (Weltje, 1998; Fulladosa et al., 2005: Zeb et al., 2017) where it was reported antagonistic effects between Cd and Pb on earthworms (Oligochaeta), *Vibrio fischeri* bacteria and on luminosity of *Photobacterium phosphoreum* T3S bacteria, respectively. However, future studies are required to unravel the mechanistic aspect underlining the antagonistic effect observed between Cd and Pb as metals and MONPs.

6. Conclusions

Nanoparticles of various chemistries and sizes are becoming a reality in many industrial applications. As a result, there is an increasing need for understanding the adverse effects that nanoparticles may have on human and animal health. To this point, the results of this study revealed for the first time that chronic exposure to sublethal concentrations (20% of LC_{50}) of either CdO or PbO NPs separately or in combination, caused histological and cellular anomalies to midgut epithelial cells of honey bee workers. Common cytological alterations observed in epithelial cells were irregular distribution or/and condensation of nuclear chromatin, mitochondrial swelling and lysis, and rER dilation, fragmentation, and vesiculation were almost similar to those described in other insects or invertebrates exposed to these metals. Collectively, exposure of honey bees to these metal oxide NPs even at sublethal concentrations will adversely affect the colony health and further studies are also required to determine the behavioral effects of these metal oxide NPs on behavior and pollination ecology of honeybees.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.09.311.

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Cellular Alterations in Midgut Cells of Honey Bee workers (*Apis millefera* L.) exposed to Sublethal Concentrations of CdO or PbO Nanoparticles or Their Binary Mixture

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Supplementary material

Table S1. XRD data of CdO and PbO nanoparticles prepared at 0.5 M and 0.02 M, respectively.

Metal	d-spacing (Å) Observed	Intensity (cps)	FWHM β (deg)	(2Theta)	Grain size (nm)
CdO	2.71	100.00	0.17	33.03	50.93
	2.34	91.25	0.17	38.34	51.70
	1.66	55.26	0.10	55.32	95.64
	1.41	34.97	0.12	65.94	81.11
PbO	4.01	100.00	0.12	22.11	67.12
	3.29	99.24	0.15	27.03	56.91
	2.93	79.61	0.23	30.39	36.17
	2.52	47.41	0.17	35.52	50.38

Table S2. Susceptibility of honey bee workers *(Apis mellifera*) to CdO and PbO NPs after 96 h exposure.

Metal	N[(a)]	LC25, (95 % CLd[(b)]) (mg/ml)	LC50, (95 % CLd[(b)]) (mg/ml)	LC90, (95 % CLd[(b)]) (mg/ml)	Slope± SE[(c)]
CdO	**300**	**0.01 (0.006-0.02)**	**0.06 (0.04-0.09)**	**1.46 (0.78-3.51)**	**0.94 ± 0.09**
PbO	**300**	**1.18 (0.65- 1.70)**	**3.27 (2.35-4.81)**	**22.50 (12.10-75.55)**	**1.53 ± 0.27**

(a) N is the number of bee workers tested.

(b) CL is the 95 % confidence limits.

(c) Slope ± standard error.

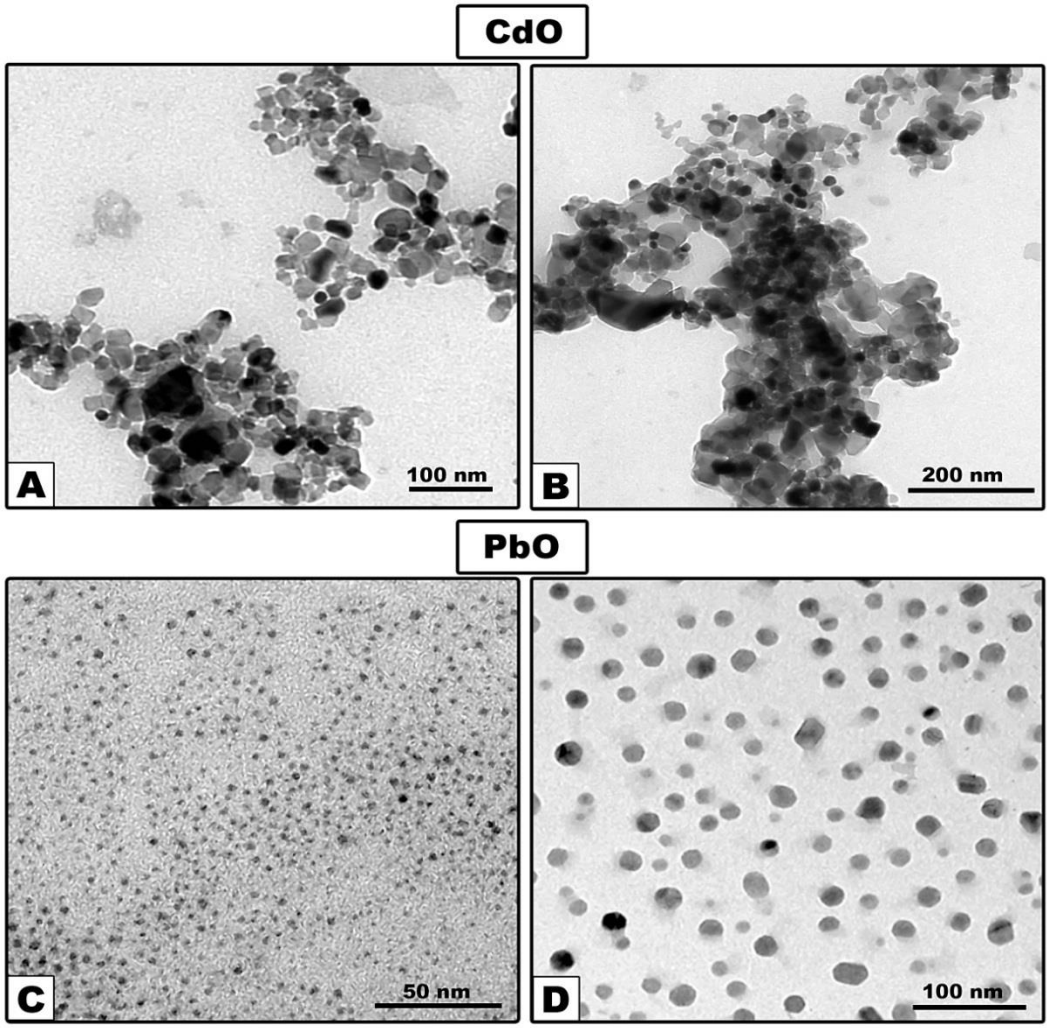

Fig. S1. HRTEM micrograph of synthesized CdO NPs scale bar: (A) 100 nm (B) 200 nm and PbO NPs scale bar (C) 50 nm and (D) 100 nm.

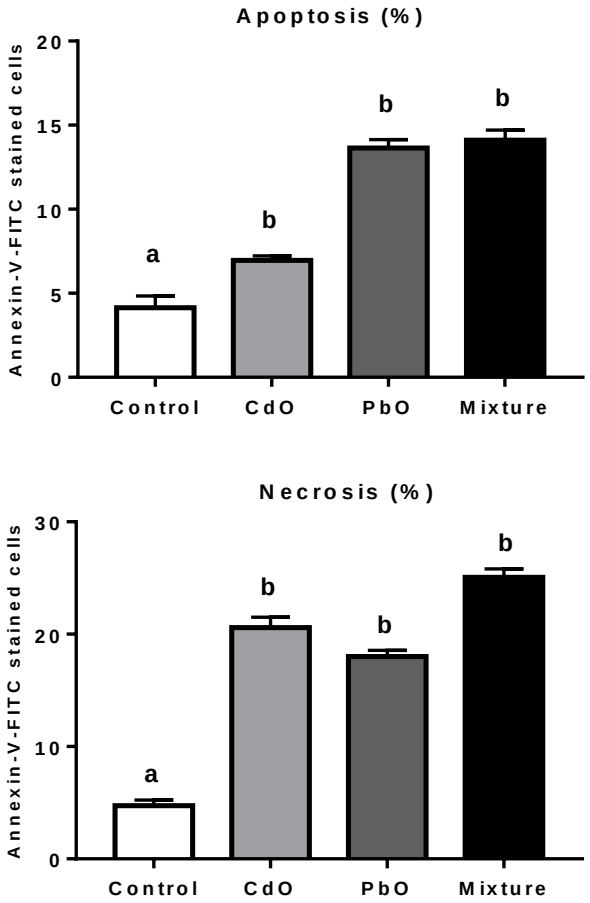

Figure S2. Proportions (%) of cell death (apoptosis or necrosis) (%) quantified by flow cytometry of annexin-V-FITC and propidium iodide staining of midgut epithelia of honey bee workers fed nine days a control or sucrose solution diet containing sublethal concentrations of CdO NPs (0.01 mg ml^{-1}) or PbO NPs (0.6 mg ml^{-1}) or their binary mixture. Bars represent mean percentage (± SEM) of three samples. Letters denote significant differences compared to control (one-way ANOVA with Tukey post-hoc test, $P < 0.05$).