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RESEARCH ARTICLE

Mechanism of action and toxicological evaluation of engineered layered double hydroxide nanomaterials in *Biomphalaria alexandrina* snails

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Abstract

Layered double hydroxide (LDH) nanomaterials have recently become immense research area as it is used widely in industries. So, it's chance of their release into natural environment and risk assessment to nontarget aquatic invertebrate increasing. So, the present study aimed to synthesize and confirm the crystalline formation of Co-Cd-Fe LDHs and Co-Cd-Fe/PbI_2 (LDH) and then to investigate the toxic impact of the two LDH on the adult freshwater snails (*Biomphalaia alexandrina*). Results showed that Co-Cd-Fe/PbI_2 LDH has more toxic effect to adult *Biomphalaria* than Co-Cd-Fe LDHs (LC_{50} was 56.4 and 147.7 mg/L, 72 h of exposure, respectively). The effect of LC_{25} (117.1 mg/L) of Co-Cd-Fe LDHs exposure on the embryo showed suppression of embryonic development and induced embryo malformation. Also, it showed alterations in the tegmental architectures of the mantle-foot region of *B. alexandrina* snails as declared in scanning electron micrograph. Also, exposure to this sublethal concentration caused abnormalities in hemocyte shapes and upregulated IL-2 level in soft tissue. In addition, it decreased levels of nonenzymatic reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), caspase-3 activity, and total protein content in significant manner. Glutathione S-transferase (GST) activity was not affected by LDH exposure. It caused histopathological damages in both glands of snails and also caused a genotoxic effect in their cells. The results from the present study indicated that LDH has risk assessment on aquatic *B. alexandrina* snails and that it can be used as a biological indicator of water pollution with LDH.

Keywords LDH · Toxicity · *B. alexandrina* · Biomarker · Commet assay

Introduction

Nanomaterials have been applied in many biomedical researches due to their unique optical, electronic, and magnetic characteristics (Tarafdar et al. 2013; Bazrafshan et al. 2017; Corsi et al. 2018). Layered double hydroxide (LDH) is one of two-dimensional layered inorganic nanomaterials. It is one of various cheap nanoparticles bearing positive charge (Malakar et al. 2021). Layered materials have been extensively used in the application of catalysis, polymer nanocomposites, and sensors (Zhao et al. 2007; Manzi-Nshuti et al. 2009; Han et al. 2011), in medicine and pharmacy (Ladewig et al. 2009; Choi and Choy 2011). Additionally, it is used as fertilizers, herbicides, growth regulators, and in removing environmental chemical pollution (Li et al. 2016; Peligro et al. 2016; Benício et al. 2020; Daniel and Thomas 2020). Their unique uses in many applications depend on the host molecule. These have been attributed to their exchange capacity of anionic and capability to accommodate in the interlayer region different types of functional anions/molecules (metals, halocomplexes, polymers, proteins, drugs, etc.). The wide spread utilization of LDHs may lead to increase the chance of their release into the aquatic ecosystem, which has not been investigated. Toxicological evaluation of LDH has been gained environmental and human health care, since it may cause a negative impact to nontarget aquatic fauna.

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Many articles elucidate the toxicological impact of nanomaterials to aquatic organism such as zooplankton, fish, algae, freshwater rotifers, and snails (Zhu et al. 2009; Kim et al. 2012; Long et al. 2012; Myer et al. 2017; Amorim et al. 2019; Martins et al. 2020). However, toxicological impact of inorganic nanomaterial (LDH) to snails does not study until now.

B. alexandrina snails are widely accepted invertebrate models to study the toxicity and toxico-kinetic of inorganic nanomaterial for aquatic ecosystem (Oliveira-Filho et al. 2017; Kaloyianni et al. 2020). It is the intermediate host of *Schistosoma mansoni* that is wildly disseminated throughout tropical and subtropical highly polluted canals and in the Nile River (DeJong et al. 2001). *Biomphalaria* characterized by their availability, easy way for collection, acclimate to laboratory conditions, sensitivity to water, and chemical pollutant. All the previous characters nominate it to use as laboratory monitoring in ecotoxicological studies and for analyzing multiples biomarkers. (Duft et al. 2007; OECD 2016; Oliveira-Filho et al. 2017; Ruppert et al. 2017). Many studies in immunology, reproductive, and developmental biology used *Biomphalaria* as paradigm (Khangarot and Das 2010; Boisseaux et al. 2017; Pirger et al. 2018). Nanomaterials (NMs) such as carbon nanotubes, silver nanoparticles, have potential effects to *B. alexandrina* as conducted in many studies (Moustafa et al. 2018). The toxicities of LDH-NPs depend on their chemical compositions and concentrations used. These LDH-NPs might generate reactive oxygen species (ROS) and so inducing oxidative stress, the expression of antioxidant enzymes (like catalase, glutathione reduced, and super oxide dismutase) and inflammation (Choi et al. 2015). Caixeta et al. (2020) stated that the toxicity of NMs has been attributed to reactive oxygen species (ROS) generated that subsequently by lipid peroxidation, DNA, and protein damage. Also, Choi et al. (2009) stated that nanoparticle induced oxidative stress and might negatively alter the physiological responses, such as carcinogenesis, inflammation, fibrosis, and genotoxicity. These nanomaterials could find their ways to the natural environment of snails by water runoff and drainage canals and caused negative effects on the organisms lived in these environments, and so, the present study aimed to evaluate the toxicity of LDHs NMs on *B. alexandrina* and how it affected their biological processes, and to donate well knowledge about biological behavior and risk assessment of Co-Cd-Fe LDHs in aquatic environments.

Material and method

Preparation of two types of LDHs

Co-Cd-Fe LDH

NaOH (5M) was dissolved in 200 mL of distilled water. Another 200 mL aqueous solution of 1Fe $(NO_3)_3.19H_2O$ (0.1M), Co $(NO_3)_2.16H_2O$ (0.1M), and $Cd(NO_3)_2.4H_2O$ (0.1M) was prepared. This later solution was stirred for 24h. A pH 10 of the reaction is adjusted by using sodium hydroxide solution. At pH 10, the solution was divided into two solutions; one of them is stirred for 24h and the second one is put in the autoclave for 3h. A washing process using DI water is carried out for the resulting precipitate to reduce the pH to 7. Finally, the product is dried at 80°C for 1 day.

T- LDHs/PbI_2 NC

In a general synthesis technique, in situ growth of the metal cations, typically, NaOH (5M) in 200mL of distilled H_2O is prepared. Another solution of $Fe(NO_3)_3.9H_2O$ (0.1M), $Co(NO_3)_{21}.6H_2O$ (0.1M), $Cd(NO_3)_2.4H_2O$ (0.1M), and 2.5g PbI_2 was prepared. This later solution was stirred for 24h. A pH 10 of the reaction is adjusted by using the sodium hydroxide solution. After reaching pH 10, the solution was remained under continuous stirring for 24h. A washing process using DI water is carried out for the resulting precipitate to reduce the pH to 7. After washing, a drying process is carried out at 80°C for 1 day.

Characterization of LDH

The XRD patterns of Co-Cd-Fe LDH and Co-Cd-Fe LDH/PbI_2 NC were obtained by Philips1 X $Pert_1$-MRD1 X-ray diffraction (λ_{CuKa} = 0.15418 nm). Sample morphology is investigated using a 1field-emission1 1scanning electron11 microscope (FESEM, TEM, Zeiss SUPRA/55VP with GEMINI/column). Fourier transform infrared spectroscopy (FTIR) was performed by A Shimadzu1-FTIR-3401-Jasco1 spectrometer to obtain the important functional groups of the samples. Finally, the optical absorbance behaviors of the products are investigated by Lambda 900-UV/Vis/IR Perkin Elmer spectrophotometer up to 1200 nm.

Snail source and maintenance

Adult *B. alexandrina,* snails (810 mm in diameter; 0.26g weight) have been obtained from Theodor Bilharz Research Institute (TBRI), (Giza, Egypt). Snails were transferred to Medical Malacology Lab and kept in plastic tank with dechlorinated aerated tap water (10 snails/L) with a photoperiodicity of 12h light/12 h dark cycle, a temperature of 25 ±3 °C, pH: 7± 0.2 and fed on ovendry lettuce leaves (1gm/10 snails) and Tetramin. The tank water was changed every 3 days. For collecting egg masses, pieces of polyethylene sheets (5 × 10 cm) were used (OECD 2016).

Toxicity study

Acute toxicity test in adult *B. alexandrina*

The toxicity of the two layered materials Co-Fe-Cd and Co-Fe-Cd/PbI_2 LDH against adult mature snails (10–12 mm; 150 snails) was determined. Stock solution of two layered materials was prepared using dechlorinated tap water (1000 mg/L). A series of concentrations of Co-Fe-Cd LDH (100, 75, 50, 25, and 20) and of Co-Fe-Cd/PbI_2 LDH (200, 150, 100, 75, and 50) were prepared to calculate LC_{50} and LC_{90} at laboratory temperature (22–25°C). Three replicates were conducted for each concentration and the control group (30 snails per experimental group): 72 h after, the snails were transferred from the exposure concentrations and maintained in dechlorinated tap water for another 24 h of recovery. Mortality percent of snails was recorded and lethal concentration and slope values were analyzed by Probit analysis (WHO 1965).

Bioassay

After calculation of the sublethal concentration, snails were exposed to LC_{25} of LDH for 24h followed by a recovery period of another 24 h in dechlorinated water. Then, the following experiments were done:

Embryotoxicity test

According to Rapado et al. (2011), pieces of polyethylene sheets (5 × 10 cm) containing egg clutches (100 eggs) were collected for the embryotoxicity assay. The egg masses were transferred to Petri dishes contains LC_{25} of LDH for 24h, subsequently washed with filtered and dechlorinated water (pH 7.0). Seven days after exposure, the embryos were examined for unviability (malformed embryos or dead) by stereomicroscope. Another egg clutches was transferred to dechlorinated aerated tap water as a control. Assays were performed in triplicate.

Scanning electron microscope of the mantle-foot region

The mantle-foot regions of snails were separated under a stereomicroscope. Then, the specimens were fixed, dehydrated, critically dried, and coated as recommended by Ibrahim and Abdel-Tawab (2020). Finally, they were analyzed by JSM-6510 LA.

Immunocytotoxicity

Cytotoxicity assay in hemocytes of *B. alexandrina* According to Nduku and Harrison (1980), hemolymph of ten snails was collected from the snail heart by insertion a capillary tube into the snail shell that is directly over the heart: 10 µl of hemolymph was spared on a glass slide to prepare hemocytes monolayers and leave to air-dry for 15 min at laboratory temperature. Hemocytes were fixed with 100% methanol for 5 min and then stained with 10% Giemsa stain (Aldrich) for 20 min, then examined under the light microscope. This assay was done in triplicate for each group to study the outer morphological changes in the hemocytes.

Measurement of IL-2 level and Caspase-3 activity IL-2 in tissue homogenate (1gm/10 mL phosphate buffer) of five snails was measured by enzyme-linked immunosorbant assay (ELISA). Cytokine levels were determined by commercially available ELISA kits for IL-2 (OptEIA™ Kits; BD Biosciences). The depth of the color can then be measured spectrophotometrically at appropriate wave length. The intensity of colored end product provided a measure of the cytokine concentration (Hemdan et al. 2007). Caspase-3 activity was determined according to Bonomini et al. (2004). The released p-nitroaniline (pNA) moiety concentration was measured colorimetrically at 405 nm.

Tissue preparation for oxidant/antioxidant biomarker and biochemical studies

The soft tissues of five snails were removed from the exposure group and the control one, weighted (1gm/10 mL phosphate buffer), and then homogenized in ice cold, twice-distilled water using a glass Dounce homogenizer. The supernatants were separated using high speed centrifuged (3000 rpm for 10 min) and stored at − 80 °C until used; then experiments were done according to the pamphlet of each kit.

Oxidant/antioxidant defense biomarker These biomarkers have been measured in the supernatant of the tissue homogenate of five snails for LDH exposure group and control one. The enzymatic responses SOD, CAT, and GST were measured according to Aebi 1984 (Mannervik and Guthenberg 1981), and nonenzymatic responses GSH was determined according to the method of Ellman (1959). For biochemical studies, the snails' total protein was done according to the method of Gornall et al. (1949). All parameters determined using biodiagnostic kits (Biodiagnostic Dokki, Giza, Egypt).

Genotoxicity evaluation was done by detecting of DNA single-strand breaks (comet assay)

DNA single-strand damage of snails exposed to LC_{25} of LDH for 48 h was detected by single-cell gel assay as previously described by Singh et al. (1988) and Grazeffe et al. (2008). The hemolymphs of twenty snails were collected by inserting a capillary tube into the heart of each snail. The DNA fragment migration patterns of 100 cells were evaluated with a fluorescence microscope at 510 nm. The comet tail lengths

were measured from the middle of the nucleus to the end of the tail with 40× increase for the count and measure the size of the comet. Visualization of DNA damage was observed by Ethidium Bromide Staining using a 40× objective. Slides were scored blindly.

Histological evolution of the digestive and hermaphrodite glands

Ten adult *B. alexandrina* snails (8–10 mm) were exposed to LC_{25} of LDH for 24h followed by another 24 h of recovery for 2 weeks. The digestive and hermaphrodite glands of the surviving *B. alexandrina* snails were removed and fixed in Bouin's solution. The glands dehydrated, embedded in paraffin wax. Then, they both sectioned and stained with hematoxylin and eosin (Mohamed and Saad 1990). Five slides/each gland/snail were examined by light microscopy for any alterations in the histological architecture compared to the control snails and photographed by using a microscopic camera.

Statistical analysis

Data analysis were performed by *t*-test to determine the significant difference between exposure and control group and expressed as mean ± SME of mean (Graph Pad Prism 6.04 software). The lethal concentration (LC_{10}, LC_{25}, LC_{50}, and LC_{90}) values, slope, and respective 95% confidence limit (CL) of LC_{50} was calculated by Probit analysis (Finney 1971).

Result

Characterization of Co-Cd-Fe LDH, and T-LDH/PbI_2 NC

Function groups identification

The FTIR charts of (Co-Cd-Fe) LDH and its composite are displayed in Fig. 1A (A, B) and Table 1. After combination of PbI_2, there are red shifts in absorption bands and some peaks changed in intensity and other broads Fig. 1A (B).

Structural properties

The structure and crystalinity of (Co-Cd-Fe) LDH was confirmed by XRD diffract gram. Its chart displays highly matching of hydrotalcite LDH with hexagonal phase (Fig. 1B). XRD peaks referred to diffractions (003), (006), (101), (009), (107), (018), (110), and (113). It is noticed that these peaks have high intensity which was reflected the high crystallinity of the studied LDH.

Their crystal sizes were calculated using Scherrer's relation (R). The mean size was ~23.5 nm. In addition to their average microstrain value was ~ 0.7% and its dislocations density was 0.0018 that evaluates the density of defects and the quality of the crystal. This result gives a reflection to high quality of the synthesized LDH crystal.

Morphological properties

The morphological properties were examined through FESEM and TEM, at Fig. 1C (A, B). The morphology of Co-Cd-Fe LDH was characterized with the agglomeration of the particles which have plate-like morphology. This behavior was similar for all hydrotalcites prepared by coprecipitation method. TEM clarified the plate like of LDH layers and proved the morphology of the LDH.

Zeta potential and particle size distribution

The value of zeta potential of the fabricated LDH after dilution is depicted from Fig. 1D to be −8.46 mV. Also, the value the conductivity was 2.47ms/cm. In addition, LDH had a large particle size distribution (1911 nm), as calculated by DLS measurements. This value is larger than that reported using SEM or TEM images and this could be attributed to the aggregate LDH in aqueous solution through DLS technique in contrast to SEM or XRD techniques which do not allow for aggregation.

Toxic impact of LDH on adult *B. alexandrina*

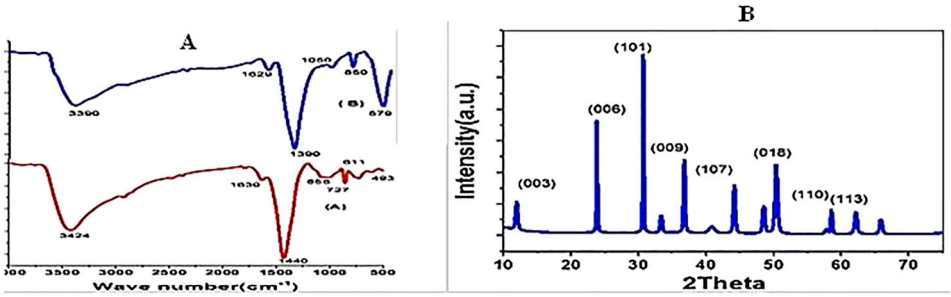
In the present study, Co-Cd-Fe LDHs and Co-Cd-Fe LDHs/PbI_2(LDH) were tested for its toxic effect against *B. alexandrina*. Snails were exposed to different concentrations of Co-Cd-Fe LDHs and Co-Cd-Fe LDHs/PbI_2 (LDH) for 72 h of exposure followed by another 24 h for recovery. Probit analysis showed that the LC_{50} of Co-Cd-Fe LDHs was 147.7 with confidence limits 110.99–188 mg/L. While Co-Cd-Fe /PbI_2 LDH showed more toxic effect, LC_{50} was 56.4 with confidence limits 21.52–85.07 mg/L (Table 2).

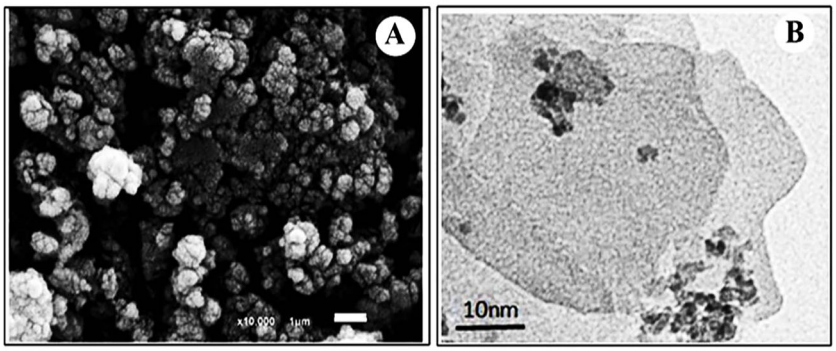
Embryotoxicity

Exposure of embryos of *Biomphalaria alexandrina* snails to Co-Cd-Fe LDHs caused delay of embryonic development (30%), embryo malformation (20%), and accumulation of LDH NPs in the egg clutches leading to death of some embryos (50%) (Fig. 2B).

Effect of LDH on *B. alexandrina* ultrasturacture

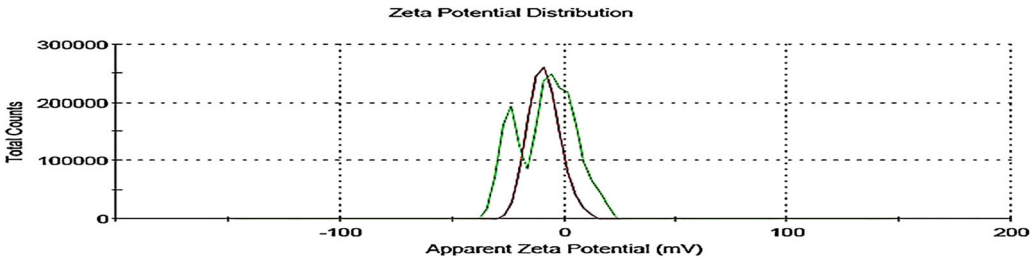
The scanning electron micrographs of the soft part of *B. alexandrina* snails showing the normal foot plantaris with notable surface fold and covered with fine and smooth cilia (Fig. 3A), and tegmental surface of mantle with microvilli and fine spines (Fig. 3D). Following the exposure to LC_{25}, the foot





D

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-8.46	Peak 1:	-2.28	70.4	8.58
Zeta Deviation (mV):	12.7	Peak 2:	-24.2	29.6	4.62
nductivity (mS/cm):	2.47	Peak 3:	0.00	0.0	0.00
Result quality	See result quality report				



			Size (d.nm...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	1911	Peak 1:	679.0	91.9	131.2
PdI:	1.000	Peak 2:	103.2	8.1	13.96
Intercept:	0.506	Peak 3:	0.000	0.0	0.000
Result quality	Refer to quality report				

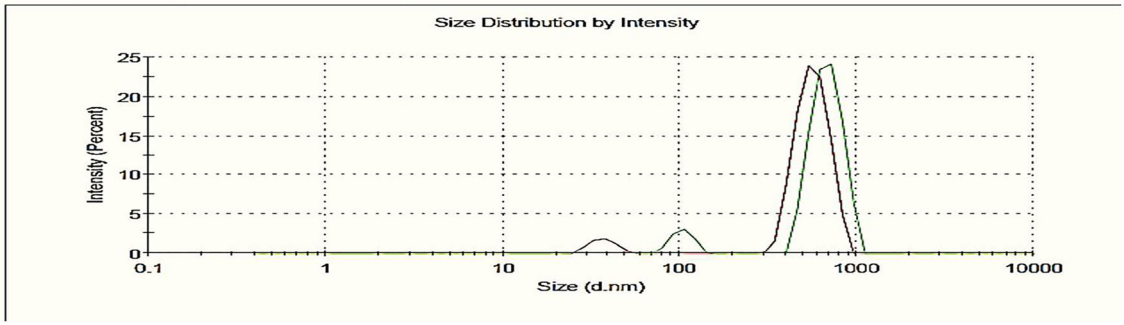


Fig. 1 Characterization of LDH. (**1A)** FTIR spectra of Co-Cd-Fe LDH (A) and Co-Cd-Fe LDH composite with PbI_2 (B), (**1B**) XRD patterns of Co-Cd-Fe LDH, (**1C**) FESEM of fabricated Co-Cd-Fe LDH (A) TEM of Co-Cd-Fe LDH (B). (**1D**) Zeta potential for the size particle of Co CdFe LDH

Table 1 FTIR peaks of Co-Cd-Fe LDH and its composite with PbI_2

Function group	Co-Cd-Fe LDH	Composite
H stretching	3424	3390 broaing of peak which is attributed to O–H stretching and symmetric mode of Pb-I cluster
The O–H bending	1630	1629 cm^{-1}
Bending of H2O molecule	1350	1390 cm^{-1}
NO3−-stretching mode26		1430 cm^{-1}
M–O vibrations of LDH	Below 1000 cm^{-1}	580

cilia became tangled, adherent, and ultimately peeled off (Fig. 3A and 3B). Also, the tegmental surface of mantle became rough, most microvilli completely destroyed, nipples and erosion (Fig. 3E and 3F).

Impact of LDH on hemocytes of *B. alexandrina*

In control group, microscopical examinations of *B. alexandrina* hemocytes showed three types of cell that differentiated morphologically. The first type is hyalinocytes; the second is granulocytes (spreading hemocytes), and the third is round small (undifferentiated) (Fig. 4A, 4B, 4C). After exposure to the LDH at sublethal concentration (LC_{25}), hyalinocytes nucleus showed shrinkage and others had two separate nuclei; also, aggregations of hyalinocytes were more evident after exposure to LC_{25}. While granulocytes having irregular cell membrane aggregate and form either pseudopodia or filopodia (Fig. 4D, 4E).

Influence of LDH on IL-2 level and caspase-3 activity

In the present study, there are a marked increase in expression of IL-2 in LDH exposure group ($p< 0.001$) in compared to non-exposure one (152.14±0.1 and 72.04±0.21 Pg/mL, respectively) (Fig. 5B). Also, caspase-3 activity was slightly increased ($p < 0.05$) in exposed snails compared to control one (60.95±0.11 and 54.8±1.8 nmol pNa min^{-1} mg^{-1} protein, respectively) (Fig. 5A).

Impact of LDH on oxidant/antioxidant defense biomarker and biochemical studies

In the present study, exposing of snails to LC_{25} of LDH induced significant decreased ($p<0.001$) in SOD and CAT ($p<0.01$) activity compared to the non-exposer group (control), (Fig. 6A and 6B) with no change in GST activity ($p>0.05$), (Fig. 6C). Concomitantly, a significant decrease of GSH levels and total protein content ($p<0.001$) in tissue homogenate was observed in LDH exposure group compared with their time-matched controls (Fig. 6D and 6E).

Influence of LDH on DNA

The present results showed that the olive tail moment (OTM) of snails subjected to sublethal concentrations of LDH was highly increased ($p < 0.01$) than control snails (5.63±0.1 and 3.25±0.12 μm, respectively) (Fig. 7A, 7B, and 7C**).**

Impact of LDH on digestive and hermaphrodite gland of *B. alexandrina*

Examination of the histological sections through digestive gland showed many tubular glands with single layer of secretory cells (SC) and digestive cells (DC) (Fig. 8A). Treatment these snails, with LC_{25} of LDH, showed rupturing, vacuolation, and a significant increase in the number of SC. Also, the lumen (L) increased; most of the DC and SC were degenerated and ruptured while the tubular glands lose their confirmed shape (Fig. 8B). Meanwhile, the histological sections of *B. alexandrina* snails of the control group through the hermaphrodite gland revealed female oogenic cells with normal oocytes and mature ova and male reproductive cells with normal spermatocytes, and sperms (Fig. 8C). The treatment of snails with a dose of LC_{25} caused degenerations and destruction of some oocytes, mature ova, spermatocytes, and sperms (Fig. 8D).

Table 2 Shows molluscicidal activity of Co-Fe-Cd and Co-Cd-Fe/PbI_2 for adult *B. alexandrina*, snails after 72h of exposure followed by 24 h for recovery

	LC_{10} (mg/L)	LC_{25} (mg/L	LC_{50} (mg/L)	Confidence limits of LC_{50} (mg/L)	LC_{90} (mg/L)	Slope
Co-Cd-Fe	89.5	117.1	147.7	110.99–188.07	205.9	1.1
Co-Cd-Fe/PbI_2	17.4	35.91	56.4	21.52–85.07	95.3	1.2

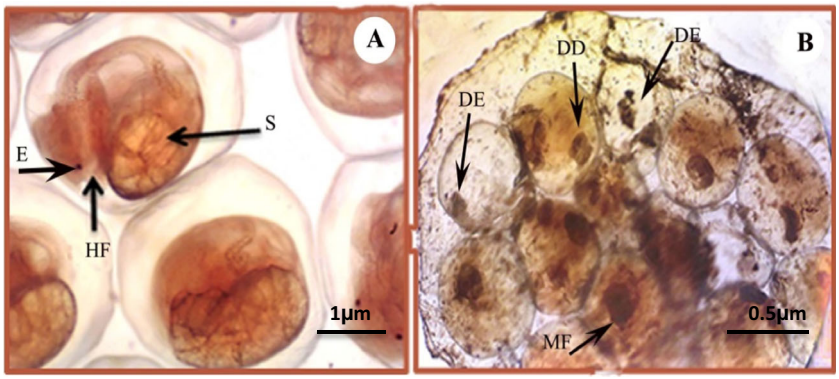


Fig. 2 Morphological abnormalities in *Biomphalaria* embryos after exposure to Co-Fe-Cd LDH. (**2A**) Normal control embryos of 7-days aged where the snails completely formed (*E* eye; *HF* head foot; *S* shell). (**2B**) After exposure of the egg mass to LC_{25} Co-Fe-Cd LDH (*DE* dead embryo, 50%; *MF* malformed embryo, 20%; *DD* development delay, 30%)

Discussion

Layered double hydroxide (LDH) gains significant attention in life science applications due to their extremely governable synthesis and high biocompatibility. But few studies highlight toxicity and toxico-kinetic of LDH. In the present study, we engineered Co-Cd-Fe LDH and T-LDH/PbI_2 NC, and its toxicological impact was evaluated. The results of Co-Cd-Fe LDH characterization by XRD were matched with a usual LDH with crystallinity mean size ~23.5 nm (Lu et al. 2015; Mohamed et al. 2018). Also, their FTIR spectra were similar to that previously recorded (Shaban et al. 2018; Mohamed

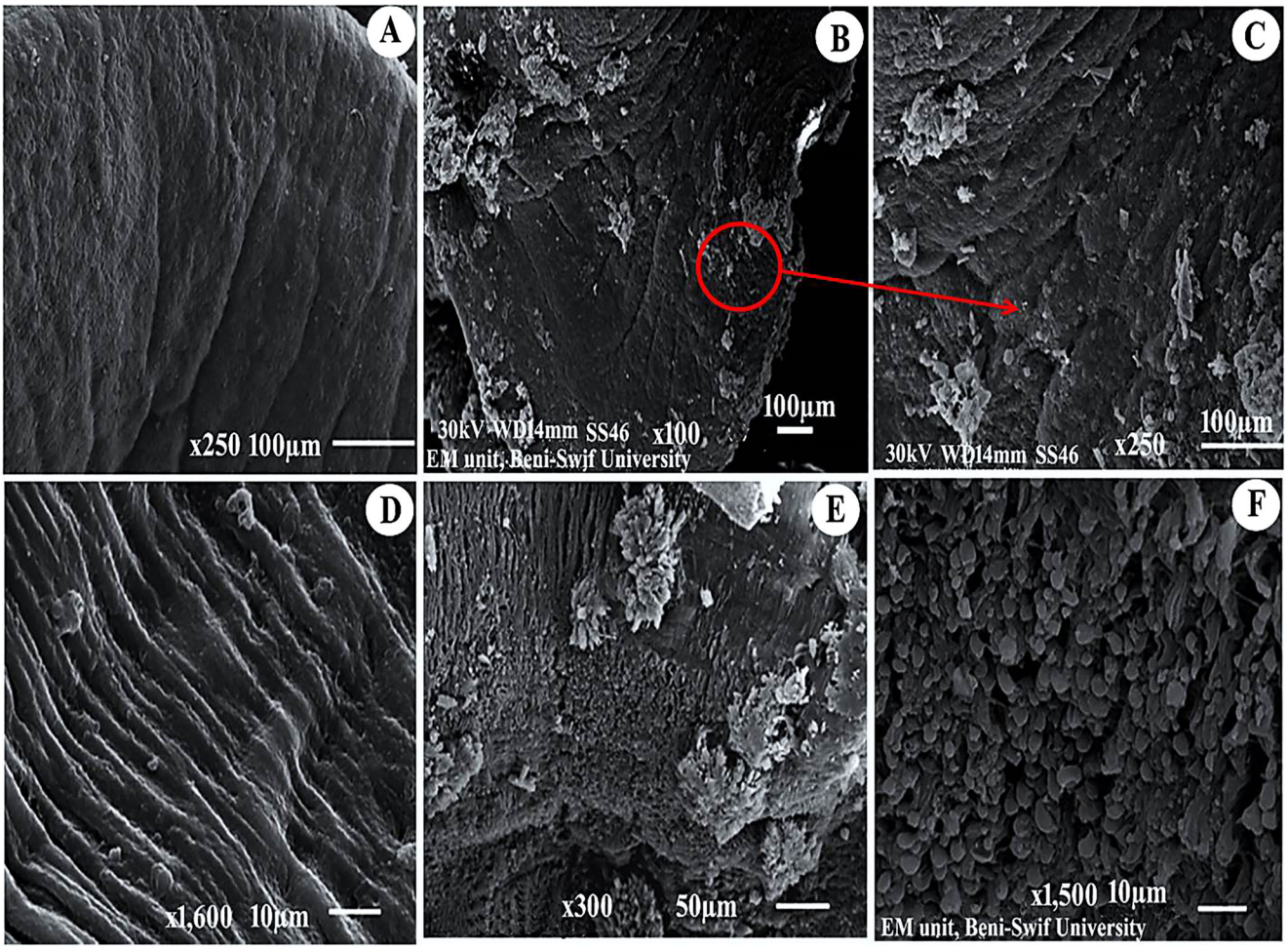


Fig. 3 Scanning electron micrographs (SEM) of *B. alexandrina* snails (soft part), (**3A**) Normal ultrastructure of foot with smooth and regular cilia, (**3B**) foot plantaris after exposure to Co-Fe-Cd LDH, the cilia became tangled and adherent, (**3C**) higher magnification of 3B, (**3D**) normal mantle showing smooth tegmental surface and microvilli, (**3E**) mantle after exposed to Co-Fe-Cd LDH showing tortuosity, nipples, erosion, and accumulation of LDH NMs in tegmental surface, (**3F**) higher magnification of 3E

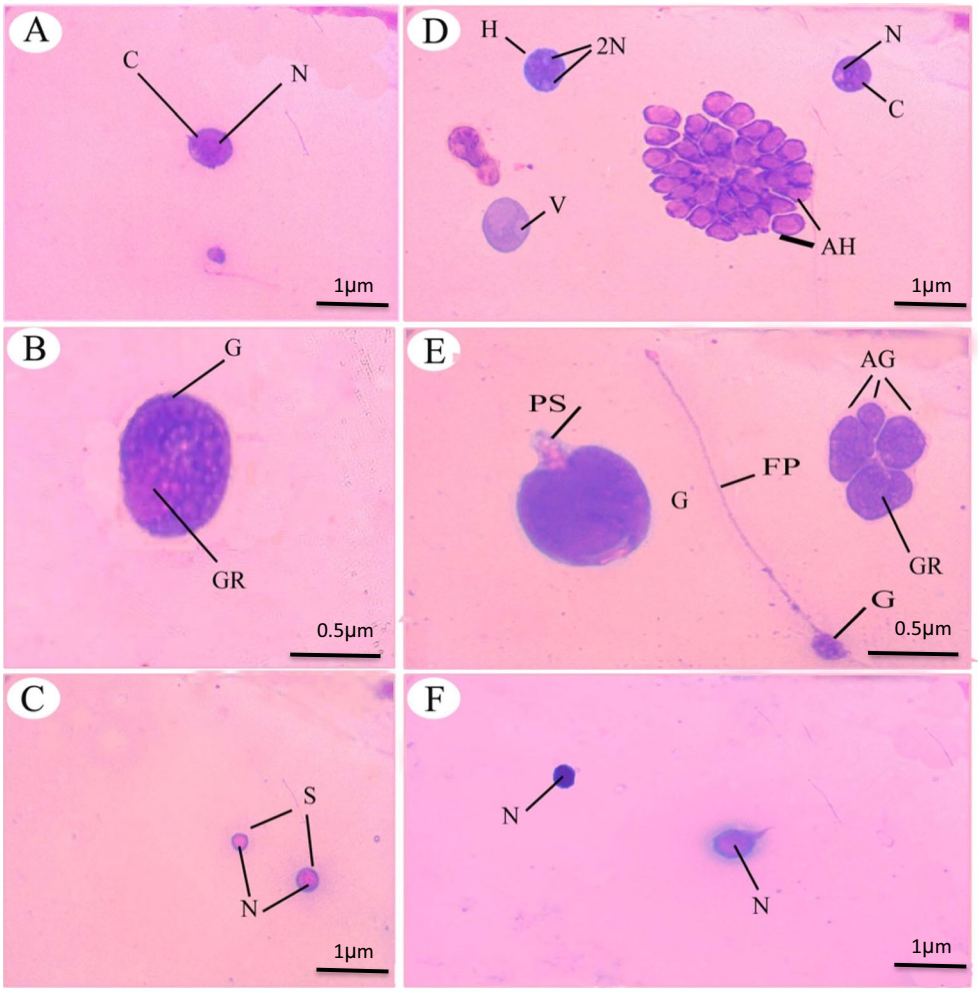


Fig. 4 Light micrographs show hemocytes of adult *Biomphalaria alexandrina* snails. **4A** Hyalinocyte (22.32%); **4B** granulocyte (37.5%); **4C** small (40.17%) (×40); **4D**, **4E**, and **4F** show the abnormalities following exposure to LC_{25} of Co-Fe-Cd LDH for 48h, 4D hyalinocytes increased in number (43.58%), some forming aggregations, some have two separate nuclei (2N) and vacuoles (V). 4E some granulocytes (35.9%) forming either pseudopodia (PP) or filopodia (FP) and aggregation (AG), 4F some hyalinocytes forming pseudopodia (PP), while small cells formed 20.51%. *C* cytoplasm, *PS* pseudopodia, *G* granulocyte, *GR* granules, *H* hyalinocyte, *N* nucleus, *S* round small

et al. 2018; Parida and Mohapatra 2012), while FSEM and TEM clarified the plate like of LDH layers (Tedim et al. 2011; Chen et al. 2017). The zeta potential reflected the stability of nanoparticles in suspension and is also the major factor in the initial adsorption of nanoparticles onto the cell membrane or the uptake inside these cells. The zeta potential and size could affect nanoparticle toxicity (Mohamed et al. 2021).

The present study confirmed that Co-Cd-Fe LDH and T-LDH/PbI_2 NMs showed toxic effect against *B. alexandrina* and that T-LDH/PbI_2 NM more toxic to adult *Biomphalaria* than Co-Cd-Fe LDHs. This result is in good accordance with Cardinale et al. (2012) who stated that TiO_2 nanoparticles showed more inhibition in the growth rate in *Scenedesmus quadricauda* than Al_2O_3nano-powder and concluded that the toxicity of LDH on algae was time- and concentration-dependent. Also, it was proven that LDH has toxic impact against human cell line (Choi et al. 2007) and green algae *Scenedesmus quadricauda*(Ding et al. 2018).

Enzymatic (GST, SOD, and CAT) and nonenzymatic (GSH) antioxidant markers play a vital role in protection of

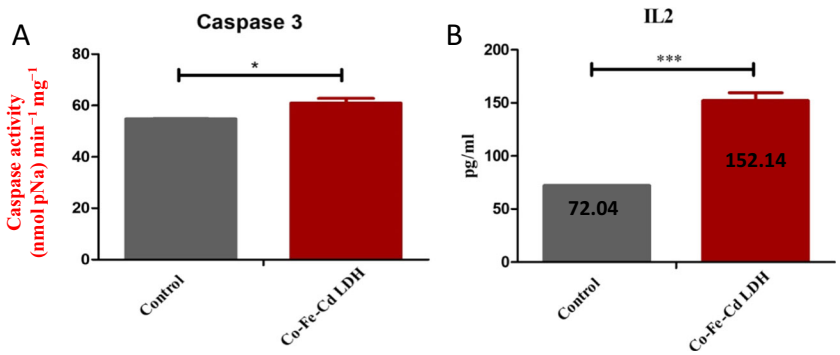


Fig. 5 Effect of Co-Fe-Cd LDH on the expression of Caspase 3 and IL-2. All values presented as mean ± SE. *, *** Significant difference as compared to control (p <0.05, p < 0.001)

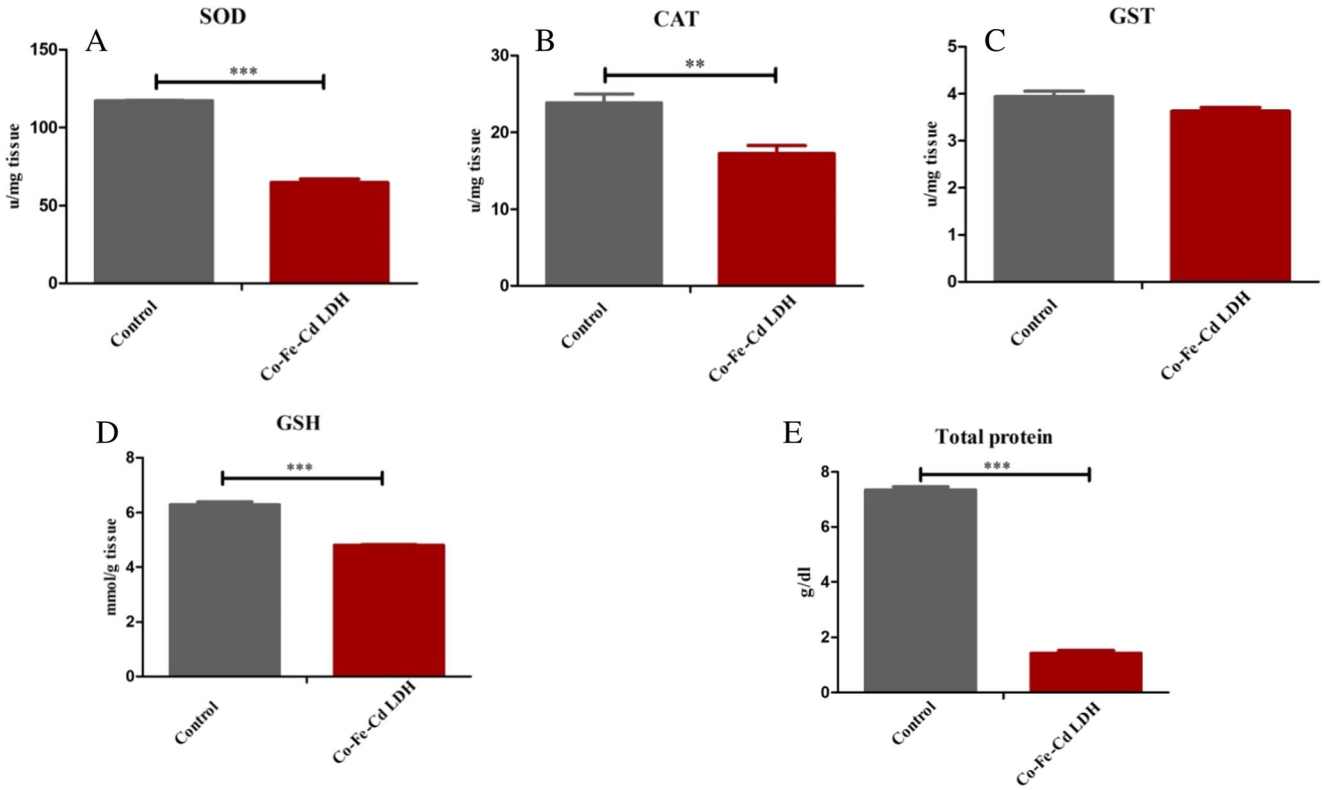


Fig. 6 Effect of Co-Fe-Cd LDH on the levels of enzymatic and nonenzymatic parameters and total protein in soft tissue of *Biomphalaria alexandrina* snail. All values presented as mean ± SE. ******* Significant difference as compared to control ($p < 0.05$, $p < 0.01$, $p < 0.001$)

the organisms from oxidative stress and suppression of its cellular damage as it reduce and converted H_2O_2 and superoxide anion radical. While GSH act as a reducing agent in conjugation with xenobiotics (Pena-Llopis et al. 2001). Disturbance of oxidant/antioxidant system has been the main toxic impact induced by NMs in snails. LDH increased the ROS production and subsequently altered the enzymatic and nonenzymatic antioxidant enzyme, such as SOD, CAT, GSH (Ali 2014; Ali et al. 2012; Bao et al. 2018). In addition, this reduction can be elucidated to the direct combination of metal with active site of enzyme and its biotransformation. The present data showed significant decrease in SOD, CAT, GSH and this in agreement with Gnatyshyna et al. (2020) who reported that the nonenzymatic marker activity decreased in *Lymnaea stagnalis* after exposure to Cu (10 μg L^{-1}), Zn (130 μg L^{-1}), Cd (15 μg L^{-1}), and thiocarbamate fungicide (91 μg L^{-1}) for 14 days.

Also, a decrease of catalase activity was seen previously in snail exposed to herbicides (Bhagat et al. 2016). In addition, exposure of *B. alexanderina* snails to ZnONPs showed significant inhibition of GSH and CAT ((Fahmy et al. 2014). In contrast with the present result, Atli and Grosell (2016) reported that exposing *L. stagnalis* to only the highest concentrations of Cu caused an increase in antioxidant enzyme.

Exposed snails showed no significant variation in GST activity compared to control. This finding agreed with those obtained by Sánchez-Marín et al. (2020) as they indicated that this enzyme is not activated in response to organophosphate flame retardants, tris (1,3-dichloro-2-propyl) phosphate in mussels.

Also, the present study declared a marked decrease in the total protein content ($p< 0.001$) compared with controls. Fahmy et al. (2014) recorded a significant decrease in *B. alexanderina* snail protein content after exposure to ZnONPs and related the differences in the activity of the antioxidant markers with the tissue type and the metal concentrations. SOD and CAT activities in *Daphnia magna* exposed to Cd and Cu varied according to metal concentration. Also, *L. natalensis* snails collected from polluted dams in Zimbabwe showed variation in SOD and CAT activities (Siwela et al. 2010). In addition, Achatina fulica showed reduction in CAT and SOD activities after exposure to Cd and Zn. This variation could be attributed to the excess production of ROS (Chandran et al. 2005). Similarly, such enzyme reductions were also observed in the present study in response to LDH exposure.

A slightly increase of caspase-3 activity was detected as unspecific response to LDH stress. Its elevation was observed previously in apoptotic cells (Elmore 2007; Florentin and

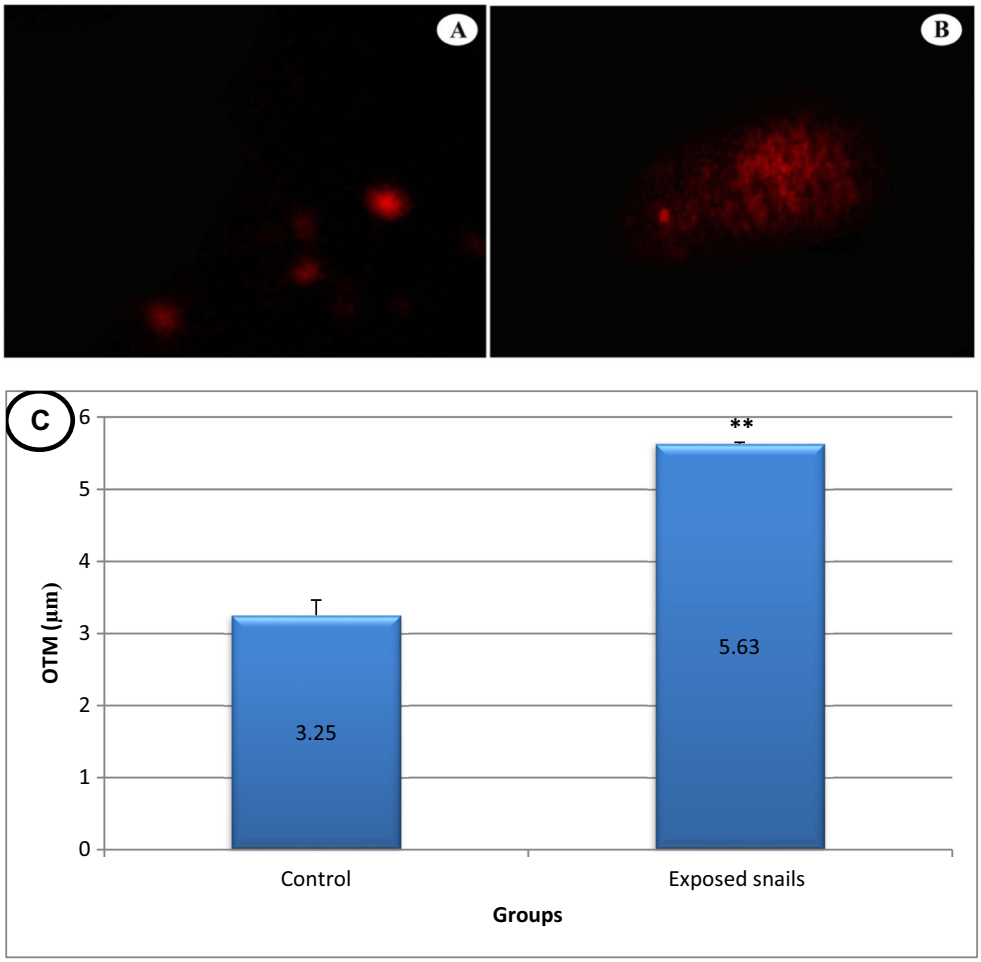


Fig. 7 Light micrograph shows the extent of DNA migration by comet assay. (**7A**) Control *B. alexandrina*; (**7B**) snails exposed to sublethal concentration of Co-Fe-Cd LDH for 48 h with high DNA migration ($p < 0.01$) than control snails; (**7C**) showed the increase in OTM in exposed snails than control one (5.63±0.1 and 3.25±0.12 µm, respectively).

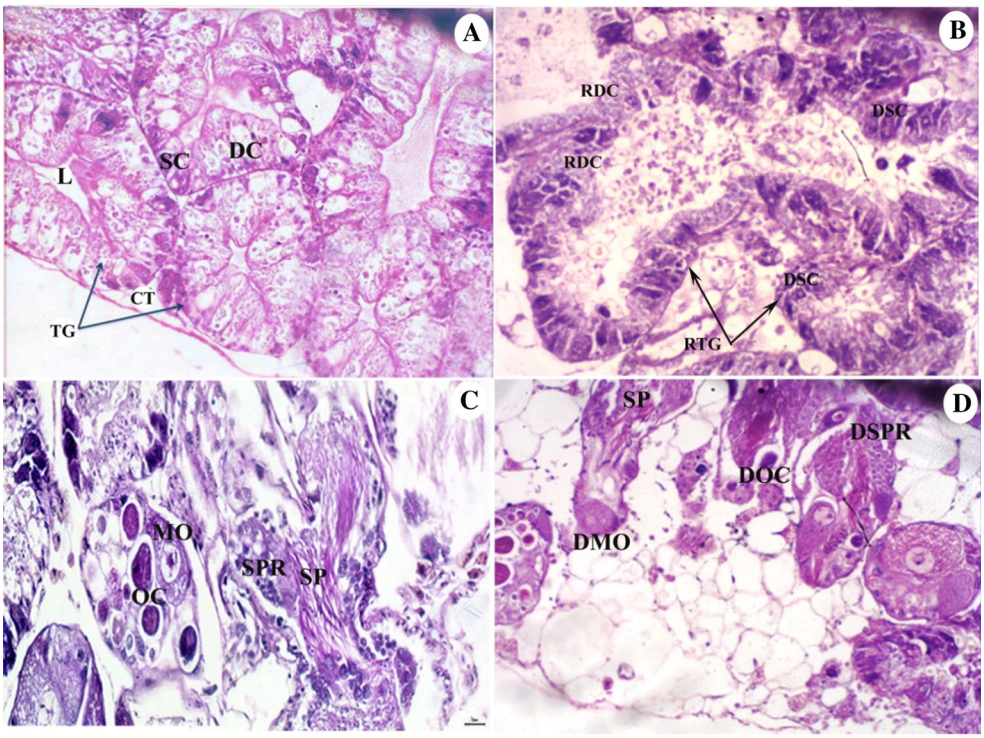


Fig. 8 Light micrographs of the hermaphrodite and the digestive glands of *B. alexandrina* snails (H&E) (×40): (**8A**) normal digestive gland of *B. alexandrina* snails (**8B**) snails exposed to LC_{25} of Co-Fe-Cd LDH (**8C**) normal hermaphrodite gland of *B. alexandrina* snails (**8D**) snails exposed to LC_{25} of LDH. *MO* mature ovum, *OC* oocytes, *SP* sperms, *SPR* spermatocytes, *OC* oocyte, *DOC* degenerated oocytes, *DSPR* degenerated spermatocytes, *DC* digestive cells, *SC* secretory cells, *L* lumen, *TG* tubular gland, *CT* connective tissue, *RDC* ruptured digestive cells, *DDC* degenerated digestive cells, *RTG* ruptured tubular gland, *RSC* ruptured secretory cells

Arama 2012), and this elevation may be due to cytological changes in the digestive gland of LDH-stressed snails (Zaldibar et al. 2007a, 2007b; Hödl et al. 2010; Benito et al. 2017). Previously, increasing in caspase-3 activity has been detected in *L. stagnalis* in response to pollutant stress (Gnatyshyna et al. 2020). Also, caspases-3 levels increased in *Helix aspersa* snails after exposure to iron oxides nanoparticles (Sidiropoulou et al. 2018).

In the declared data, LDH at sublethal concentration (LC_{25}), caused abnormalities in hyalinocytes and granulocytes shapes as nucleus shrinkage, divided to two separate nuclei, aggregate or formed pseudopodia. The immuno-cell responses and molecular aspects in *B. alexandrina* snails considered as important biomarkers of exposure to environmental pollutants (Mohamed 2011). *Biomphalaria* snails immunology can be attributed to hemocyte which are the critical line of cellular defense (Larson et al. 2014), where they contributed in many defense mechanisms against several pathogens as it is responsible for the phagocytosis, cytotoxic reactions (Fried 2016), and release soluble compounds including agglutinins and antimicrobial peptides (Ottaviani 2006; Mitta et al. 2000).

Chronic exposure of the *T. pisana* to Ag NPs caused alterations in hemocytes, such as micronuclei, binucleated cell, and kidney-like nuclei (Radwan et al. 2019). Also, *B. glabrata* exposed to CdTe quantum dot showed altered hemocytes binucleates, micronuclei, and apoptosis (de Vasconcelos et al. 2019). Cell–cell aggregation was considered as an immunological response for host defense. Cellular aggregation of the invertebrates' hemocytes prevented the accidental blood loss by the formation of a biological plug at the site of the wound and resisted the entry of pathogenic microorganism (Guria et al. 2016).

Hughes et al. (1990) and Ottaviani et al. (1993) were detected cytokine-like molecules in marine and freshwater mollusks. IL-2 was one of the cytokines assayed. It is responsible for phagocytosis and provokes the strongest response in the synthesis of biogenic amines, nitric oxide (NO), or oxygen radicals (Ottaviani et al. 1995a, 1995b). In the present study, there are marked increase in expression of IL-2 in LDH exposure group ($p< 0.001$) in compared to non-exposure one. (IL)-2–like peptide was also detected in sea mussel which may be involved in the regulation of responses to different types of stress (Cao 1998; Barcia et al. 1999).

On the level of DNA damage, as an important biomarker of NM toxicity in snails, comet assay is a sensitive tool to detect DNA damages like DNA single-strand breaks (SSBs)(Ibrahim et al. 2018). The present results showed that the olive tail moment (OTM) of snails exposed to sublethal concentrations was increased than control snails. This in agreement with Ibrahim and Ghoname (2018) who demonstrated that the OTM of snails exposed to LC_{10} (27.5 mg L^{-1}) or LC_{25} (32.4 mg L^{-1}) of the aqueous leaves extract of *Anagalis arvensis* was significantly higher than the control group. Such genotoxic effects might be due to either oxidation of DNA bases or covalent binding to DNA resulting in strand breaks. Some studies link DNA SSBs in aquatic animals to effects on the immune system, reproduction, growth, and population dynamics (Lee and Steinert 2003). Exposure to inorganic nanomaterial as Ag NPs, CuO NPs, IONPs, MgO NPs, TiO2 NPs, and ZnO NPs induced genotoxic effects in snails (Caixeta et al. 2020). Ye et al. (2012) stated that the amount of DNA strand breaks were higher after exposure to DNA damaging chemicals compared with controls.

The embryotoxicity observed after exposure has been attributed to ROS production, oxidative stress, and damage. Also, penetration of LDH NPs to gelatinous capsule and cross the egg membrane reduces essential growth metabolism aspects, changes in its permeability, consuming energy for the development, and finally interrupting the mechanics of hatching (de Chavez and de Lara 2003; de Vasconcelos et al. 2019).

Our result is in agreement with Besnaci et al. (2016), who state morphological changes and precipitation of Fe_2O_3 NPs in the egg mass. Also, morphological abnormality and hatchability hinder was seen in *B. pfeifferi* embryos following exposure to curcumin-nisin polylactic acid NPs for 96 h. In addition, hydrophilic nanosilica induced embryotoxic effects in *B. alexandrina* snail at concentration 590 ppm for 6 h and 980 ppm for 48 h (Attia et al. 2017). Similarly, the growth and hatching rate reduction was seen in *B. glabrata* embryos exposed to CdTe NPs for 24 h (de Vasconcelos et al. 2019). In contrast, dimer captosuccinic acid (DMSA)-functionalized Fe_2O_3 NPs did not induce embryo mortality, morphological alterations, and hatching inhibition due to their physical properties and limited internalization in the egg clutches (Oliveira-Filho et al. 2017). LDH posed a significant suppression in the growth of *S. quadricauda* algae after 72 h of incubation and a complete growth inhibition (100%) at higher LDH concentration. LDH had a higher inhibitory effect to growth than the other NPs (Ding et al. 2018).

In the present study, the foot and mantle of *B. alexandrina* snails showed bioaccumulation of LDH in its surface and some morphological disturbances after the exposure to LDH for 24 h followed by 24-h recovery as was detected by scanning electron microscope. LDH can interact, accumulated in foot and digestive gland of snails, and distributed to the mantle. Both Ag NPs and CuO NPs accumulation in mantle, foot and digestive gland of *B. aeruginosa*(Bao et al. 2018; Oliver et al. 2014; Croteau et al. 2014; Ma et al. 2017). Also, NMs possessed a highly adhesive property to a cell membrane; therefore, it could affect the membrane structures and its macromolecules (Rasel et al. 2019). In addition this damage in ultrastructure could lead to snail death (Ibrahim and Abdel-Tawab2020).

The deformation declared in the hermaphrodite gland of *B. alexandrina* histological sections after exposure to LC_{25} of LDH was accompanied with a great damage in the gonadal

cells where degenerations of some mature ova, spermatocytes, oocytes, and sperms. Also, the connective tissue was dissolved and replaced by vacuoles. Saad et al. (2019) reported similar histological alterations in the hermaphrodite glands of *B. alexandrina* snails treated with copper oxide nanocomposite (CuO NC), where the ova and sperms degenerated and there were loss in the connective tissues between acini (Saad et al. 2019). The exposure of the snail to LDH may be lead to metabolic changes, destruction of gametogenic cells and damage of hermaphrodite glands which possibly resulting from a decrease in tissue proteins, apoptosis, or degeneration of cells of these vital organs (Omobhude et al. 2017).

The digestive gland was the main organ analyzed in studies concerning oxidative stress induced by NM due to its higher accumulation capacity and role in the metal detoxification. Exposing of the digestive gland of *B. alexandrina* snails to LC_{25} of the LDH showed significant increase in the number and degeneration of the SC. The DC ruptured and vacuolated in addition, the tubular glands lose their confirmed shape. In like manner, Saad et al. reported histological alterations in the digestive gland of *Coelatura aegyptiaca* following treatment with ZnONPs for 6 consecutive days, where there were gradual hypertrophy and hyperplasia in the glandular cells (Fahmy and Sayed 2017).

Conclusion

The data of the current study consider the first toxicological evaluation of LDH nanomaterial on freshwater snail *B. alexandrina*. In light of the above, LDH induces disturbance in both enzymatic and nonenzymatic antioxidant marker in the tissues of *Biomphalaria* following exposure to sublethal concentration, suppression the embryonic development. It caused alteration in mantle foot ultrastructure, immune response, histopathology of gland, and finally genotoxic effect. This result reflects the possible ecological implications of LDH release in aquatic ecosystems and its risk assessment to aquatic invertebrate.

Code availability Not applicable

Author contribution Conceived and designed experiments; Heba Abdel-Tawab, Amina M. Ibrahim
Data curation; Taghreed Hussein, Fatma Mohamed
Formal analysis; Taghreed Hussein, Fatma Mohamed
Methodology; Heba Abdel-Tawab, Amina M. Ibrahim
Software; Taghreed Hussein, Fatma Mohamed
Supervision; Heba Abdel-Tawab, Amina M. Ibrahim
Validation; Heba Abdel-Tawab, Amina M. Ibrahim
Visualization; Heba Abdel-Tawab, Amina M. Ibrahim
Roles/writing-original draft; Taghreed Hussein, Fatma Mohamed
Writing—review & editing; Heba Abdel-Tawab, Amina M. Ibrahim

Data availability The data that supports the findings of this study are available in the material of this article.

Declarations

Ethics approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

Conflict of interest/competing interests The authors declare no competing interests.

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