International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 15-11-2025, Accepted: 14-12-2025, Published: 29-12-2025
Volume 10, Issue 12, 2025, Page No. 145-152

Apoptotic and histopathological biomarkers of gastropods in relation to nano-metallic nanoparticles exposure

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Abstract
Nanoparticles (NPs) will fit proliferation their release into the environment getting up adverse outcomes. The present study aims to evaluate apoptotic and histopathological defects of titanium dioxide nanoparticles (TiO_2NPs) and single walled-carbon nanotubes (SWCNTs) in gastropod, *Helix aspersa* after exposure to sub-lethal dermal doses for 48 h. The obtained data indicated that, response of apoptotic cells to trypan blue dye conjugation with nucleus of the cells showed declines in viability of the haemolymph of treated animals, respect to their control. Treatment 0.1 TiO_2NPs exhibited the greatest decline (71.33%), respect to control (95.60%), followed by 0.1 SWCNTs (74.37%). This finding was in parallel with significant increases in lactate dehydrogenase (LDH) activity and level of carbonyl protein (CP) in haemolymph and digestive gland homogenate of the treated individuals. Ultrastructure investigations in the digestive glands of treated animals showed significant alterations in their components, respect to firm structure of control sections, especially dilated microvilli (MV) and vacuolated lysosomes (Ly) The present findings provide an ideal illustrating potential cytotoxic effect of TiO_2NPs and SWCNTs on the treated snails. Also, the selected snail represents a reliable sentinel model to assess the impacts of these NPs on the gastropods.

Keywords: Nanoparticles, gastropods, apoptosis, TEM, cytotoxicity

Introduction

The land snails turn out to be an economic severe pest in Egypt. It beginning severe economic damage, mainly in the gardening and ornamental plants (Goden 1983) [23]. Land snail, *Helix aspersa* is one of the bio-indicators which used for ecotoxicological assessment (Regoli et al. 2006) [42]. The preferred choice of this species is mainly due to its bioaccumulation capability for many metal pollutants, global distribution, reflecting its ability to adapt to habitats, soil and varied climates and ease rearing (Viard et al. 2004). Land snails have also been widely accomplished as sentinel species for assessing metallic pollution in the global ecosystems (Regoli et al. 2006; Abdel-Halim et al. 2013) [2, 42]. As documented in the literature, *H. aspersa* represents a suitable bioindicator of metal and organic soil contamination (Gimbert et al. 2006) [22]. Application of synthetic molluscicides stayed the greatest effective method, chiefly above great areas (Radwan et al. 2008) [41]. Permitting to current improvements in nanotechnology, exposure to micro-and nano-sized particles or debris has increased (Veranth et al., 2007) [49]. The gastropods have been indicated as suitable invertebrate model-system to assess the toxicity and ecotoxicity of NMs (Amorim et al., 2019; Kaloyianni et al., 2020) [10, 27], especially due to their advantages, such as easy to obtaining and keeping under laboratory conditions, small size, high egg production, short life cycle, adaptation to different environments, some species have transparent embryos and described genome (i.e., *Biomphalaria glabrata*), sensitivity to water and sediment contamination, high susceptibility to anthropogenic factors, high worldwide distribution and the possibility of analyzing multiples biomarkers. Furthermore, snails are an ethically acceptable alternative animal model and regulatory biochemical pathways homologous to vertebrate systems (Oliveira-Filho et al., 2017; OECD, 2016; Ruppert et al., 2017) [38, 45]. They were also used as a model system in the immunology studies (Boisseaux et al., 2017; Boisseaux et al., 2018) [12, 13].

Nanotechnology is not impartial the size of very small things; it is the innovative science and skill employing substance at the microscopic or molecular scale. Nowadays, metal oxide semiconductors e.g. titanium dioxide nanoparticles (TiO_2NPs) and carbon nanotubes (CNTs) are definite critical due to their various practices: environmentally remediators for the contaminants, disinfection and preventive of virus, protecting UV, preserve corrosion way and depigment (Greenwood and Earnshaw, 1997) [24]. The quickly developed field of nanotechnology, which is forming materials with size-dependent properties, is likely to become alternative source of exposure to nanoparticles (NPs). Engineered NPs (ENPs) including, CNTs have an increased surface area greatly enhances the chemical/catalytic reactivity compared with normal-sized form of the same substance (Liu, 2006) [31]. Carbon nanotubes (CNTs) are significant original class of technical materials that have numerous-useful application. It includes single-walled CNTs (SWCNTs) in which a single sheet of graphite is revolved forming a joined tube, and multi-walled CNTs (MWCNTs) in which a number of sheets are revolved forming concentric tubes (Alexander, 2007) [6]. Due to the increasing application of NMs in human health and various segments of society, numerous studies on toxicity and ecotoxicity of NMs have gained scientific distinction, mainly related to human and environmental health (Chaturvedi and Dave, 2018; Fadeel et al., 2018) [14, 20]. The toxicity of NMs to vertebrate and invertebrate models has been reviewed (Wu and Tang, 2018; Pereira et al., 2019; Amaral et al., 2019) [9, 40, 50]. The increased practices of NPs will fit proliferation their release into the environment getting up adverse outcomes. A great ideal displayed that there is strong association between environmental pollutants and stress-related disease

conditions in animals (Sindermann, 1993) [47]. The present study aims to use of *H. aspersa* as a bio-indicator of TiO_2NPs and SWCNTs adverse impact through quantifying cell viability, cell damage *via* assay of some biochemical defects in the haemolymph and digestive gland as well as ultrastructural investigation of the digestive gland.

Materials and methods

Chemicals

Tested NPs: TiO_2NPs and $SWCNT_S$ were supplied by Nano Lab., Dream Land, 6*th* October city, Egypt. For biochemical analysis, chemicals: Phosphate buffer, sodium phosphate monobasic; dibasic and potassium phosphate monobasic; dibasic were supplied by J.T. BAKER Chem. Co, Phillipsburg, N.J. 08865. Ethanol (C_2H_5OH), 1-Chloro 2, 4-dinitrobenzene (CDNB), reduced glutathione (GSH), 2-amino-2-hydroxy methylpropane-1, 3-diol (Tris-HCl), β-nicotinamide adenine dinucleotide reduced form (β-NADPH), oxidized glutathione (GSSG), and bovine serum albumin (BSA) were obtained from Sigma Chem. Co. P.O. Box 14508 St. Louis MO 63178, USA.

Characterization of NPs

Nanoparticles (NPs) of TiO_2 and SWCNTs were previously subjected for characterization by Abdel-Halim et al. (2021) [1, 4].

Tested animals

Healthy individuals of land snail, *H. aspersa* weighing 4.0±0.7 g was collected from some gardens in Ismailia governorate, Egypt. The individuals were maintained for 14 d in wood aerated cages (40 x 40 x 40 cm; 100 individuals each) under laboratory conditions (25±2˚C; 63±2% relative humidity and 12:12 h light/dark). The animals were fed on lettuce leaves *ad libitum*.

Sub-acute toxicity

Independent on acute LD_{50} values for the tested materials previously published (Abdel-Halim et al., 2021) [1, 4], sub-lethal doses: 1/10 and 1/50 of LD_{50} values representing: 10.8 and 2.17 µg/snail for TiO_2; 8.0 and 1.6 µg/snail for SWCNTs were applied as described above in acute toxicity experiment. Control group was injected with vehicle (as a reference group). Three replicates were maintained for each treatment of the tested dose (each contained 10 individuals). After 48 h of dosage, the live animals were taken for analysis. The haemolymph was carefully collected by inserting under the shell from the hemocoel along the right side of the head. The fluid was withdrawn in anticoagulant's vails and stored at -20˚C until used. Another aliquot was freshly taken for cell viability assay. Then, they were dissected to remove digestive glands and stored as described above.

Biochemical quantifications

One g of each tissue was homogenized for 15 s in potassium phosphate buffer pH 6.5 (1/10 w/v). For ten min, the samples were centrifuged at 5000 rpm. Dilute an aliquot of haemolymph with the same buffer (1/10 v/v). The homogenate was used to measure lactate dehydrogenase (LDH).

Lactate dehydrogenase (LDH)

An aliquot (100 µl) of homogenized tissue was added to 1 ml of the working solution which was prepared by mixing four volumes of Tris buffer pH 7.4 (80 mM), sodium pyruvate 1.6 mM, and NaCl (200 mM) with one volume of α-nicotinamide adenine dinucleotide (NADH) (240 µM). The absorbance change was recorded every min at 340 nm (Mc Queen, 1975) [35].

Carbonyl protein (CP)

Oxidative damage to protein was quantified as a carbonyl protein (Stadman and Levine, 2000) [48]. Frozen samples of tissues were weighed, homogenized (1: 20 w/v) in ice-cold 5% w/v sulfosalicylic acid and then centrifuged at 15000xg in an Eppendorf micro centrifuge for 15 min. The supernatant was removed and 0.5 ml of 2, 4-dinitrophenyl hydrazine (10 mM/L in 2 mM/L of HCl) solution was added to the pellet. The samples were kept at room temperature for 1h with vigorous vortex every 15 min, then 0.5 ml of 20% w/v TCA was added and the tubes were re-centrifuged for 3 min at 15000xg. The supernatant was again discarded and the excess of 2, 4- dinitrophenyl hydrazine was removed by washing the pellet three times with 1 ml of ethanol: ethyl acetate (1:1 v/v), followed by vigorous vortex and re-centrifuging as described before. Finally, the pellet was dissolved in 6 M/L of guanidine chloride and incubated for 15 min at 37 ˚C. The maximum absorbance in the range of 360-370 nm was recorded and the find carbonyl protein values expressed by using the extinction coefficient of 22 mM/L. Blank was prepared by replacing 2, 4-dinitrophenyl hydrazine with 2 mM/L HCl.

Cell viability

Fifty µl of freshly haemolymph sample were mixed with equal parts of 0.4% trypan blue dye to obtain a 1–2 dilution by pipetting up and down in a cryo-vial. The mixture was incubated for up to 3 min at room temperature, and examined on a haemocytometer counter under the light microscope. Percentages of viable cells were calculated according to the formula: -

$$\% \text{ viable cells} = 1 - \text{No of blue cells/No of total cells} \times 100$$

Ultrastructural investigation

The selected organs were dissected. Small bits of were fixed as quickly as possible with 2 ml of 2.5% glutaraldehyde diluted in 0.1 M phosphate buffer, pH 7.2, and kept at 4 ˚C until needed. The fixed tissues were rinsed with 0.1 M phosphate buffer pH 7.2. They were then immersed in 1 % osmium tetraoxide (OsO_4) for 1-2 h at 4 ˚C and washed for 2 min with buffer. For 5 min, the samples were dehydrated using an ascending sequence of acetone concentrations: 25, 50, 75, and 100%. Following dehydration in 100% acetone, propylene oxide was used to enter the tissues. Epon araldite was used to embed the specimen for 48 h at 48˚C. capsulated samples were sectioned at a thickness of 20–30 nm using an ultratome machine. Then, the sections were collected on metal mesh (grids) and dyed with toluidine blue. After staining the grids with 4% uranyl acetate for 5 min, they were washed in a series of four beakers of clear water. The grids were stained for 5 min with 1% of lead acetate, washed with water, and kept in a grid box until inspected (Reynold, 1963) [43]. The grids were viewed using a Transmission Electron Microscope (TEM) (JOEL 1400 Plus, Japan).

Statistical analysis

The ANOVA was used to compare the significant means of treatments using the Student-Newman-Keuls test. The means were assessed for significance using the least significant difference (LSD) technique at a probability of 0.05 (Cohort Software Inc, 1985) [16].

Results

Biochemical responses

Cytotoxic impacts of TiO_2NPs and SWCNTs on haemolymph and digestive gland of gastropod, *H. aspersa* after 48 h exposure were investigated. Activity of LDH and level of CP in the above samples were altered in the treated individuals, respect to untreated group (control). Also, similar patterns were noticed for cell viability of haemolymph samples in association with that observed in ultrastructural investigation in sections of the digestive gland.

LDH

Enzyme activity in both haemolymph and digestive gland of the treated individuals was greater than control (Table 1). Treatment 0.1 TiO_2NPs exhibited the greatest activity (177.50 U/L) in the digestive gland homogenate, respect to control (73.81 U/L, 2.41-folds), followed by 0.1 SWCNTs (142.71 U/L, 1.93-folds). Slight increases in the enzyme activity were recorded for 0.02 SWCNTs (1.44-folds) and 0.02 TiO_2NPs (1.52-folds). In case of haemolymph, slight increases were noticed for treatments, with respect to control which did not exceed 34.29 U/L.

Table 1: Activity of lactate dehydrogenase (LDH) (U/L) in haemolymph and digestive gland homogenate of gastropod, *Helix aspersa* treated with TiO_2NPs and SWCNTs

Treatment (µg/snail)	Mean±SD	95% CL	Folds
Haemolymph			
SWCNTs			
8.00	50.42±1.94	(48.26-52.59)	1.47
1.60	45.18±1.97	(43.02-47.34)	1.31
TiO_2NPs			
10.80	53.77±1.378	(51.61-55.94)	1.56
2.17	52.15±1.82	(49.99-54.31)	1.52
Control	34.39±1.395	(32.22-36.55)	-
Digestive gland			
SWCNTs			
8.00	142.71±2.17	(128.78-156.64)	1.93
1.60	106.99±5.64	(93.06-120.92)	1.44
TiO_2NPs			
10.80	177.50±19.8	(163.5-191.4)	2.41
2.17	112.55±6.92	(98.62-126.48)	1.52
Control	73.81±10.56	(59.88-87.74)	-

Each value represents the mean of 3 replicates±SD

CP

Levels of CP in haemolymph and digestive gland homogenate of the treated individuals were significantly increased, with respect to their untreated group (control) (Table 2). Treatment 0.025 SWCNTs exhibited the greatest level (23.18 nM/g tissue; 21.82-folds) in the haemolymph, with respect to control (1.063 nM/g tissue), followed by 0.1 dosage of SWCNTs (12.97-folds), 0.025 TiO_2NPs (8.69-folds), and 0.1 TiO_2NPs (8.08-folds). In case of digestive gland, 0.1 SWCNTs exhibited the greatest value (600.60 nM/g tissue), compared to control (40.30 nM/g tissue; 14.90-folds). No significant differences in CP level values were obtained between 0.02 SWCNTs and 0.1 TiO_2NPs treatments (5.63; 5.38-folds). While, 0.025 dosage of TiO_2NPs exhibited the lowest value (161.10 nM/g tissue; 3.99-folds).

Table 2: The levels of carbonyl protein (CP) (nM/g tissue) in haemolymph and digestive gland homogenate of gastropod, *Helix aspersa* treated with TiO_2NPs and SWCNTs

Treatment (µg/snail)	Mean±SD	95% CL	Folds
Haemolymph			
SWCNTs			
8.00	13.79±3.41	(8.81-18.77)	12.97
1.60	23.18±6.79	(18.20-28.17)	21.82
TiO_2NPs			
10.80	8.59±2.28	(3.61-13.58)	8.08
2.17	9.24±3.41	(4.26-14.23)	8.69
Control	1.063±0.693	(-3.919-6.045)	-
Digestive gland			
SWCNTs			
8.00	600.6±59.7	(558.6-642.7)	14.90
1.60	227.33±4.85	(185.28-269.38)	5.64
TiO_2NPs			
10.80	216.8±26.4	(174.8-258.9)	5.38
2.17	161.1±31.1	(119.0-203.1)	3.99
Control	40.30±9.60	(-1.75-82.35)	-

Each value represents the mean of 3 replicates±SD

Cell viability

The response of apoptotic cells to trypan blue dye conjugation with nucleus of the cells showed declines in the haemolymph of treated animals, respect to their control (Figure 1).

Treatment 0.1 TiO_2NPs exhibited the greatest decline (71.33%), respect to control (95.60%), followed by 0.1 SWCNTs (74.37%). Slight declines in the cell viability were noticed in 0.02 TiO_2NPs (90.63%), and 0.02 SWCNTs (91.63%).

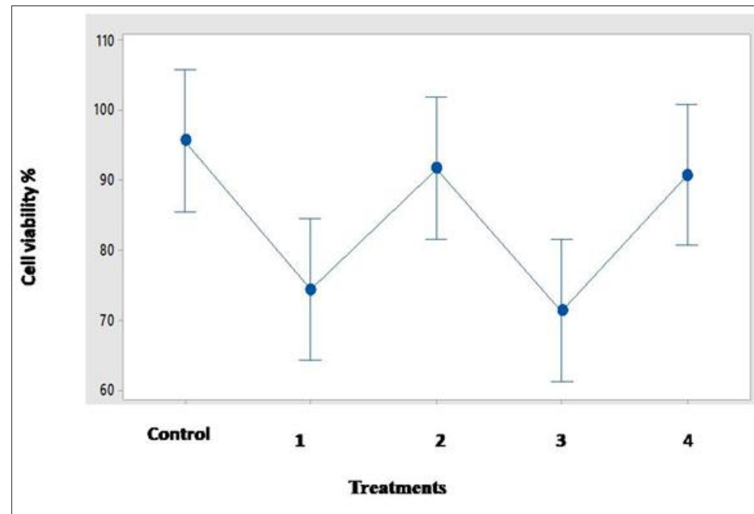

Figure 1: Cell viability % of haemolymph of gastropod, *Helix aspersa* treated with (1) 1/10 LD_{50} SWCNTs, (2) 1/50 LD_{50} SWCNTs, (3) 1/10 LD_{50} TiO_2NPs, and (4) 1/50 LD_{50} TiO_2NPs, respect to their control. Each value represents mean of 3replicates±SD. F-value=5.97, P-value=0.010 at confidence limit (CL) of 95%.

Ultrastructural profiles

Electron micrograph (Figure 2a) illustrates firm structure of the digestive gland tissues of control snail, *H. aspersa* showing regular microvilli (MV), smooth endoplasmic reticulum (SER), mitochondria (M), nucleus (N), and lysosomes (Ly) at magnification of 2500X. Section of TiO_2NPs-treated snails showed enlargement nucleus (N) with multi-nuclei (Nu), and few of mitochondria (M) and lysosomes (Ly). Also, it was observed irregular and diluted nuclear membrane (arrow), digestive gland (DG), and intracellular space between the cells (head arrow) (Figure 2b). On the other hand, section of SWCNTs-treated snails showed lack of microvilli (MV), intracellular space, fibers (Fi), vacuolated lysosomes (Ly), electron dense of mitochondria (M), and nucleus (N) with multi-nuclei (Nu) (Figure 2c).

At magnified field (4000X), section of the digestive gland of control snails stated regular nuclear membrane (arrow), microvilli (MV), mitochondria (M), nucleus (N) with heterochromatin with dark dense matrix, and extensive distribution of Golgi bodies (G) (Figure 3a). While, section of TiO_2NPs-treated snails showed disrupted microvilli (MV) and extensive food fibers (Fi) (Figure 3b). On the other hand, section of SWCNTs-treated snails showed intracellular space (head arrow), fibers (Fi), extensive distribution of mitochondria (M) with dark dense cristae, extensive distribution of vacuoles (V), vacuolated lysosomes (Ly), Golgi bodies (G) (arrow), and NPs-vacuolated digestive gland (VDG). It was documented formation of nucleus (N) with light dense matrix and multi-nuclei (Nu) (Figure 3c). The above observations were documented at other magnified fields at 6000X, where section of control tissue did not confirm any alterations in the cellular components (Figure 4a). While, section of TiO_2NPs-treated snails documented precipitation of NPs in mitochondria (M) (Notched Right Arrow), extensive vacuoles (V), light dense of digestive gland (DG), hetero distribution and shape of mitochondria (M). The nucleus (N) contains aggregated chromatin and some dilated parts of the membrane (Figure 4b). On the other hand, section of SWCNTs-treated snails showed distracted nucleus (N) with loss its components. Few mitochondria (M) filled with NPs and large vacuoles (V) were noticed (Figure 4c).

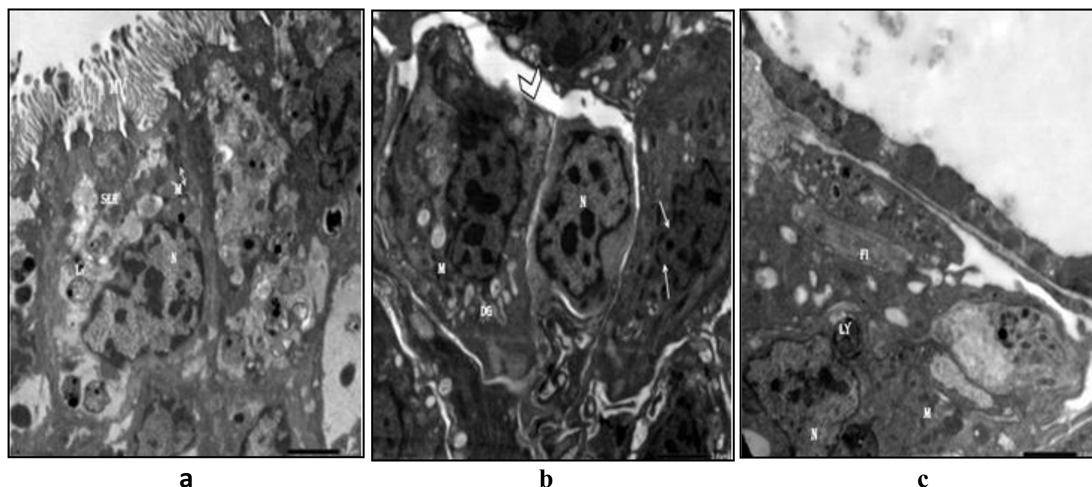

Fig 2: Electron micrographs illustrate (a) firm structure of the digestive gland section of control snail, *Helix aspersa* showing regular microvilli (MV), smooth endoplasmic reticulum (SER), mitochondria (M), nucleus (N), and lysosomes (Ly); (b) section of TiO_2NPs-treated snails showing enlargement nucleus (N) with multi-nuclei (Nu), and few of mitochondria (M) and lysosomes (Ly). Also, it was observed irregular and diluted nuclear membrane (arrow), digestive gland (DG), and intracellular space between cells (head arrow); and (c) section of SWCNTs-treated snails showing lack of microvilli (MV), intracellular space, fibers (Fi), vacuolated lysosomes (Ly), electron dense of mitochondria (M), and nucleus (N) with multi-nuclei (Nu) [Glutaraldehyde-OsO_4 fixed uranyl acetate lead citrate stained preparation 2500X]

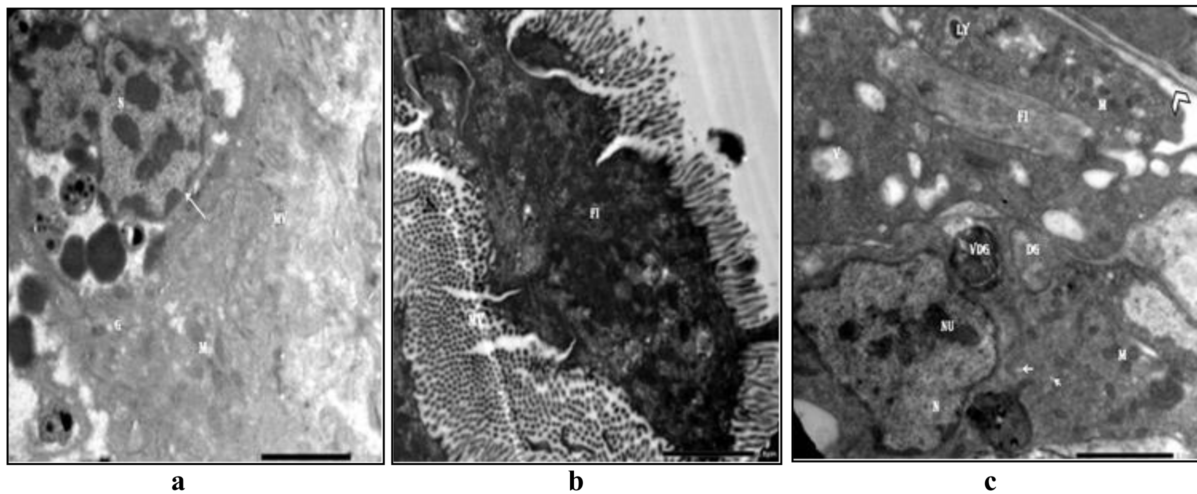

Fig 3: Electron micrographs illustrate (a) section of the digestive gland of control snails states regular nuclear membrane (arrow), microvilli (MV), mitochondria (M), nucleus (N) with heterochromatin with dark dense matrix, and extensive distribution of Golgi bodies (G); (b) section of TiO_2NPs-treated snails showing disrupted microvilli (MV) and extensive food fibers (Fi), and (c) section of SWCNTs-treated snails showing intracellular space (head arrow), fibers (Fi), extensive distribution of mitochondria (M) with dark dense cristae, extensive distribution of vacuoles (V), vacuolated lysosome (Ly), Golgi bodies (G) (arrow), and NPs-vacuolated digestive gland (VDG). It was documented formation of nucleus (N) with light dense matrix and multi-nuclei (Nu) [Glutaraldehyde-OsO_4 fixed uranyl acetate lead citrate stained preparation 4000X]

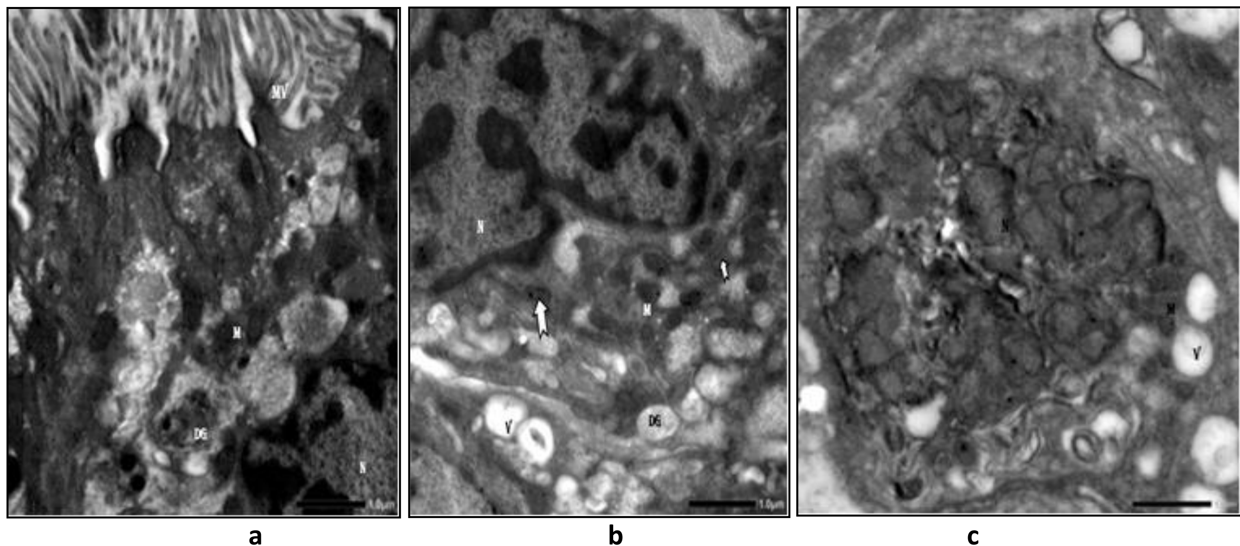

Fig 4: Electron micrographs illustrate (a) section of digestive gland of control snails confirming firm shape of microvilli (MV), and distribution of mitochondria (M), and some digestive gland (DG), (b) section of TiO_2NPs-treated snails documents precipitation of NPs in mitochondria (M) (Notched Right Arrow), extensive vacuoles (V), light dense of digestive gland (DG), hetero distribution and shape of mitochondria (M). The nucleus (N) contains aggregated chromatin and some dilated parts of the membrane, and (c) section of SWCNTs-treated snails showing distracted nucleus (N) with loss its components. Few mitochondria (M) filled with NPs and large vacuoles (V) were noticed [Glutaraldehyde-OsO_4 fixed uranyl acetate lead citrate stained preparation 6000X]

Discussion

The present findings provide an ideal illustrating potential cytotoxic effect of TiO_2NPs and SWCNTs on the treated snails. So, the selected snail represents a reliable sentinel model to assess the impacts of these NPs on the gastropods. Considering of LD_{50} on this species is of credible importance, because it offers appropriate evidence independent on getting total amount of dosage to the whole body of animal. The obtained LD_{50} values of TiO_2NPs and SWCNTs in the present work mean that, these particles are very toxic on the gastropods. The digestive gland was chosen as an object tissue for biochemical assays independent on its ability to absorb and accumulate the pollutants with 5-10 folds greater than other sites (Beeby and Richmond, 2003) [11]. The results seem to confirm that the digestive gland is the most affected organ, as expected, since it is the primary site of biotransformation of xenobiotic and oxy-radical generating enzymes in the snails (Livingstone et al., 1992) [32]. Also, haemolymph was chosen, because it non-bold represents the main compartment for nutrients and xenobiotic distribution in the body's compartments.

The examined NPs showed significant decline in cell viability in the haemolymph samples of treated individuals. Such this profile is in accordance with that obtained by Ali et al. (2015a) [7, 8], where TiO_2NPs are capable to activate the apoptotic mechanism in hemocytes of some snails as well as genotoxic effects which were documented by Comet assay. Also, CNT-induced oxidative stress mediated important cellular developments including inflammation, cell injury,

apoptosis, and activation of cellular pathways (Iavicoli et al., 2012) [26].

As documented by Pamplona (2008) [39], the shifts in function and physical integrity modified bio-molecules through oxidative stress result in broad spectrum of downstream functional magnitudes and explain the cause of some cellular dysfunctions and tissue damage. One the most frequently reported toxicity endpoints for CNTs are the formation of ROS. Oxidative stress may be caused directly by CNT-induced ROS in the vicinity or inside the cell or could arise more indirectly due to the effects of internalized CNT on mitochondrial respiration (Xia et al., 2008) [51] or in depletion of antioxidant species with the cell (Ahmad et al., 2012) [5]. It was stated that, oxidative stress is the primary mechanism of action for NPs and ultrafine particles. The findings herein showed damage in cell components: organelles and nucleic acids ending in organ dysfunction and/or apoptosis (Ma and Diamond, 2013). Such modifications in the structural and functional integrity of adjusted bio-molecules across the process of oxidative stress of NMs might initiate a range of downstream functional consequences associated with cellular damage and organ dysfunction (Pamplona, 2008) [39].

Snail, *H. aspersa* exposed to different concentrations of ZnONPs had altered digestive glands through increased secretory cells, vacuolation, and hemocytic infiltration (Abdel-Azeem and Osman, 2021) [1, 4]. In ultrastructural investigation, Abdel-Halim et al. (2022) [4] showed that, gastropod, *Monacha cartusiana* exposed to ZnONPs (74 µg/L) in its diet (Lettuce) for 14 d showed significant decline in genomic template stability (GTS%) of haemolymph and digestive glands. Also, significant defects in the cellular components of the digestive glands of treated individuals were observed, respect to their controls indicating cytotoxicity of such NPs on the gastropods. In other investigations, ZnONPs initiated histopathological alterations in bivalves' DG and gills of *C. aegptiaca* (Hu et al., 2019) [25]. The pathological reactions to ZnONPs were cell hypertrophy and hyperplasia. The organs demonstrated gradual alterations in glandular activity. In addition, the lamellae of the gills were uneven, and the filaments were swollen (Fahmy et al., 2017) [21]. In fact, the measurement of mitochondrial swelling can be explained by the activation of membrane permeability in the amended mitochondria. This discovery involves necrosis or death of apoptotic cells as a function of the involved mitochondria number (Salmi et al., 2017). Also, Ali et al. (2015b) [7, 8] documented an extensive formation in the excretory cells and flattened digestive glands of AgNPs-treated snail, *Eobania vermiculata*. Sawasdec et al. (2021) showed damage to tubule, vacuoles, enlarged tubule lumen, and destructiveness of digestive glands in copper and lithium-treated snail, *Marisa cornuarietis*. Moreover, dilation and vacuolation in the crayfish hepatopancreas were induced after exposure to CuONPs (El-Atti et al., 2019) [19]. However, TiO_2NPs caused histopathological defects in the red swamp crayfish organs, where concentration, 25 mg/L triggered minor changes in hepatopancreatic tubules, such as the emergence of colored pyknotic nuclei and epithelial cell lysis in certain tubules. However, at high concentration (250 mg/L) epithelial cells lysed, cellular atrophy and significant vacuolation occurred. Also, TiO_2NPs caused significant deterioration of the cellular organelles, including ruptured microvilli (mv), distorted mitochondria (M), vacuolated cytoplasm, pyknotic nuclei, and fragmented endoplasmic reticulum (Roncero et al., 1992) [44]. Therefore, the interaction of NMs with enzymes might cause oxidative stress and the production of ROS, which could lead to cell necrosis (Chio et al., 2012) [15]. Previous findings indicated that, cellular changes such as reduced lysosomal membrane stability, autophagic swelling, lipofusion accumulation and other lysosomally related assays, are good indicators of cell injury; and in a situation where exposure to environmental stressors is likely to be sustained, they can be used to predict that further pathological changes will occur (Marigömez and Baybay-Villacorta, 2003; Moore et al., 2004; Moore et al., 2006) [36, 37].

Therefore, DNA damage resulting from contaminant exposure is a key factor when assessing the general health of an organism, as is the need to recognize the cause, the seriousness, and the consequences of genotoxicity on populations and communities (Depledge et al. 1995) [17]. Li et al (2014a, b, c) reported that TiO_2NPs induced phototoxicty in freshwater benthic amphipod (*Hyalella azteca*). ROS is known to react with DNA molecule causing damage to purine and pyrimidine bases as well as DNA backbone. Another important outcome of ROS production, DNA damage resulting from any of these probable mechanisms may trigger signal-transduction pathways leading to apoptosis or cause interferences with normal cellular processes, thereby causing cell death. On the other hand, alkaline single-cell gel electrophoresis assay have proved that TiO_2NPs are capable to induce DNA damage. As a consequence of generation of ROS and DNA damage, TiO_2NPs were mainly responsible for cytotoxicity in hemocyte cells. It was obtained that, TiO_2NPs induced apoptosis and DNA damage in hemocyte cells of *L. leuteola* by producing ROS in response to NP exposure. Such these investigations address the deleterious effects caused by TiO_2NPs in freshwater aquatic organism. Many ways exist by which hemocyte injuries caused by toxicants can be detected, but the apoptosis and genotoxic pathways may provide a sensitive indicator of environmental pollution (Ali et al., 2015a) [7, 8].

Conclusion

This was clear in the reported patterns of cytotoxic and histopathological defects data. Such changes may represent the outcome of NMs' stress in organisms. Moreover, the present study showed the utility of land snail *H. aspersa* as a bio-indicator for NPs toxicity. Furthermore, prolonged exposure of the snails to NMs may enhance the adverse effects. This finding highlights the importance of taking long-term applications of NPs into account to better understand the ecological risks of them in terrestrial ecosystems.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicting of Interest

The authors declare that no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Ethical approval

The experiments have been carried out in accordance with the European Ethical Guidelines (Directive 2010/63/EU, 2010) [18]

Author contribution
All authors contributed to the study conception and design. All authors read and approved the final manuscript.

Data availability statement
The data are available within the article or its supplementary materials. The authors confirm that the data supporting the findings of this study are available within the article.

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