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RESEARCH REPORT

Potential toxic effects of titanium dioxide nanoparticles and carbon nanotubes on land snail *Helix aspersa*: use of oxidative stress as a reliable biomarker for ecotoxicology assessment

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Abstract

Oxidative stress represents a main regularly informed mechanism of nanoparticles (NPs) toxicity. The present work was designed to evaluate the oxidative stress in haemolymph and digestive gland of snail *Helix aspersa* after exposure to different dermal doses: 10.8 and 2.17 µg snail^{-1} for titanium dioxide nanoparticles (TiO_2NPs), and 8.0 and 1.6 µg snail^{-1} for Single-Walled Carbon Nanotubes (SWCNTs) during 48 h. The results indicated that, both nanomaterials (NMs) induced significant increases in malondialdehyde (MDA) as biomarker for lipid peroxidation (LPO). However, SWCNTs induced significant decline in reduced glutathione (GSH) content compared with the control. Catalase (CAT) activity significantly increased for the all treatments greater than the control. On the other hand, activities of glutathione-*S*-transferase (GST) and glutathione peroxidase (GPx) slightly decreased for both NMs. Nanoparticles (NPs) of TiO_2 increased activity of glutathione reductase (GR) in both haemolymph and digestive gland homogenates, while SWCNTs treatments exhibited activities did not exceed the value of the control (0.08 U mg^{-1} protein). The present findings indicate that, alterations of antioxidant enzymes activity and levels of MDA and GSH are recognized to oxidative stress. Consequently, the use of snail, *H. aspersa* can offer a respectable sentinel model to assess ecotoxicological effects of NMs on the gastropods.

Key Words*:* nanomaterials; oxidative stress; terrestrial; *Helix aspersa*; dermal toxicity

Introduction

The land snails turn out to be an economic serious pest in Egypt. It origins severe economic damage, particularly in gardening and ornamental plants (Goden, 1983). Land snail, *Helix aspersa* is one of the bio-indicators which used for ecotoxicological assessment (Regoli *et al*., 2006). 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 (Notten *et al*., 2006; Regoli *et al*., 2006; Abdel-Halim *et al*., 2013). As documented in the literature, *H. aspersa* represents a suitable bio-indicator of metal and organic soil contamination (Gimbert *et al*., 2006). Application of synthetic molluscicides stayed the greatest effective method, chiefly above great areas (Heiba *et al*., 2002; Radwan *et al*., 2008). Permitting to current improvements in nanotechnology, exposure to micro-and nano-sized particles or debris has increased (Veranth *et al*., 2007).

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).

The quickly developed field of nanotechnology, which is forming materials with size-dependent properties, is likely to become alternative source of

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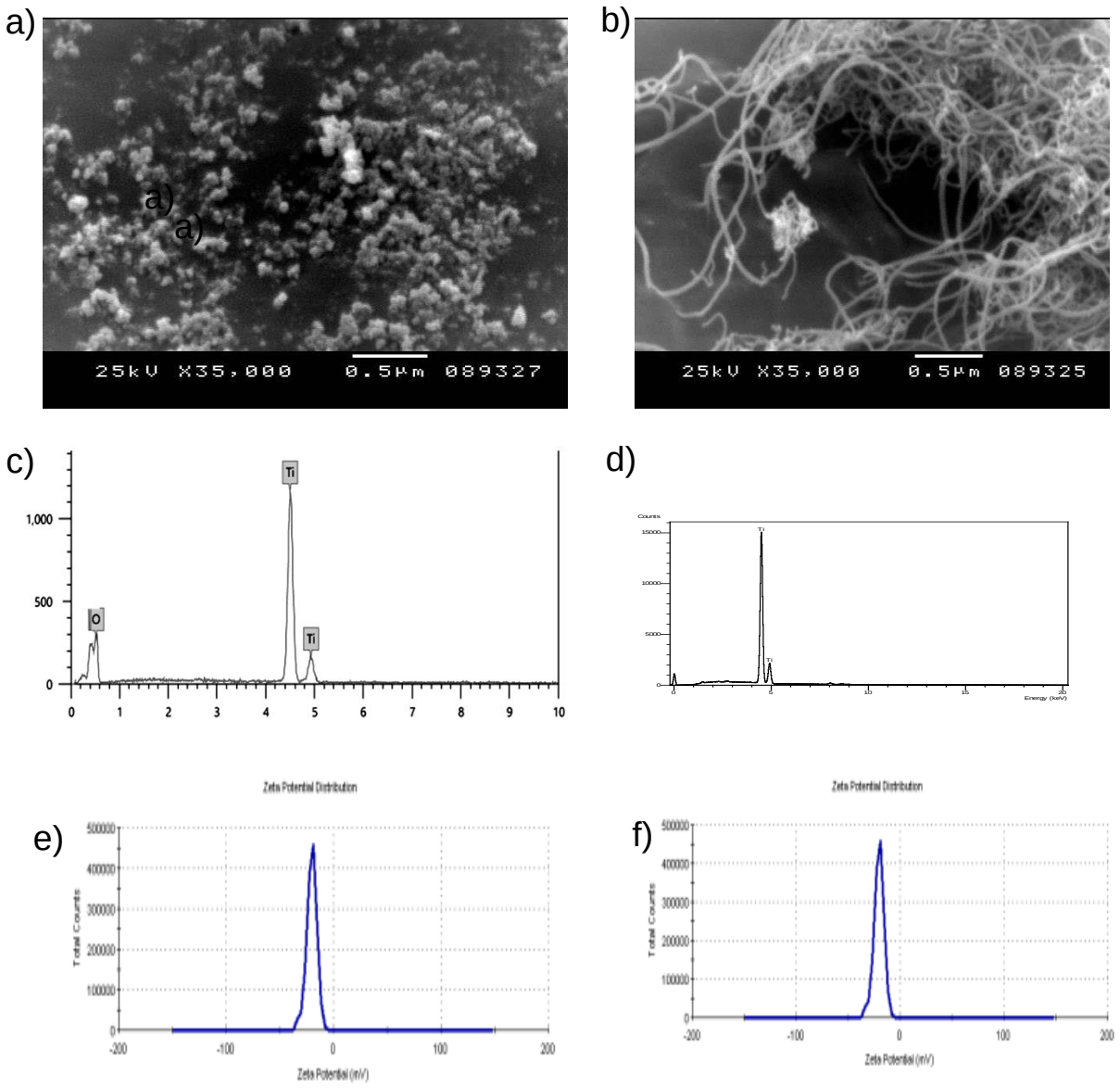

Fig. 1 Characterization of NPs, where (a) SEM image of TiO_2 visualized at 35.000X, (b) image of SWCNTs visualized at 35.000X, EDA pattern of (c) Ti and (d) purity in prepared particles, (e) Zeta potential distribution of TiO_2 and (f) Zeta potential distribution of SWCNTs determined by using DLS

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).

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).

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). Oxidative stress displays the main regularly described mechanisms of NPs toxicity (Mocan *et al*., 2010). Particles with metal atoms or ions can provide free radicals on their surface in the attendance of atmospheric oxygen or ozone (Nel *et al*., 2006). Reactive oxygen species (ROS) created through exposure to NPs can disrupt cellular key components and interact with the normal metabolism (Nel *et al*., 2006; Choi and Hu, 2008), may bind with macro-molecules interpreting them dysfunctional (Gogoi *et al*., 2006), may offer a basis of soluble metal ions, leading to disrupt the antioxidant system and damage of lipids, protein,

and DNA (Kelly *et al*., 1998). Numerous studies verified the potential toxic effects of NPs on bacteria, human cells, rodents, aquatic organisms and others (Lin and Xing, 2007). For example, Khene *et al*. (2017) observed the oxidative stress responses of TiO_2NPs in a single exposure on the gastropod, *H. aspersa*, where some biomarkers have been described in homogenates of digestive gland and kidney. The present study aims to assess the effectiveness of *H. aspersa* in the environmental monitoring as a bio-indicator of TiO_2NPs and SWCNTs contamination through quantifying certain biomarkers (oxidative stress parameters) in haemolymph and digestive gland.

Materials and methods

Chemicals

Tested NPs: TiO_2NPs and SWCNTs were supplied by Nano Lab., Dream Land, 6th October city, Egypt. For biochemical analysis, chemicals: thiobarbituric acid (TBA) and sodium azide (NaN_3) were supplied by LOBA CHEMIE PVT. Ltd, Mumbai-400005, India. Phosphate buffer, sodium phosphate monobasic; dibasic and potassium phosphate monobasic; dibasic were supplied by J.T. BAKER Chem. Co, Phillipsburg, N.J. 08865. Trichloroacetic acid (TCA; Cl_3C_3COOH), hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) were obtained from Research Lab. Fine Chem. Indust., Mumbai 400002, India. Ethylene diamine tetra acetic acid disodium salt (EDTA), ethanol (C_2H_5OH), 1-Chloro 2, 4-dinitrobenzene (CDNB), reduced glutathione (GSH), 2-amino-2-hydroxy methyl-propane-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 employed for Scanning Electron Microscope (SEM) observation (JOEL, model, JSM 5300, Japan) with great perseverance filament gun of 80 Kev. An aliquot of each material was layered on copper grid and visualized for its shape and dimension. On the other hand, TiO_2NPs and SWCNTs were subjected to X-ray Electron Dispersive Analysis (EDA) using an X-ray Oxford detector unit (model 6697, England) equipped with SEM instrument to achieve the purity of NPs. Moreover, the prepared solutions of NPs were subjected to Dynamic Light Scattering (DLS) (DTS Nano v 5.2; Malvern Zeta sizer Nano ZS, Malvern Instruments, UK) to obtain the charge for each one.

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*.

Acute toxicity

The contact toxicity of the examined NPs against *H. aspersa* snails was evaluated by using topical application method (Hussein *et al*., 1994; Radwan *et al*., 2008), to determine the median lethal and the sublethal doses. The tested doses of TiO_2NPs were 10, 20, 40, 80, 160, 320, and 640 µg snail^{-1} and 10, 20, 40, 80, 160, 320, and 640 µg snail^{-1} for SWCNTs. For each treatment, three replicates (10 animals for each) were used in plastic boxes. The tested doses were gently applied once on the surface of the snail body inside the shell using micropipette containing 10 µl of vehicle (phosphate buffered saline pH 7.0). All boxes were sprayed with water to provide suitable humidity for snail activity. LD_{50} values were determined after 48 h of exposure by using Probit analysis program (Finney, 1971).

Sub-acute toxicity

Independent on acute LD_{50} values for tested materials, sublethal doses: 1/10 and 1/50 of LD_{50} values (10.8 and 2.17 µg snail^{-1} for TiO_2; 8.0 and 1.6 µg snail^{-1} 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. Then, they were dissected to remove digestive glands and stored as described above.

Table 1 The relative toxicities of the examined NPs on snail *H. aspersa*

NPs	No.	LD_{50} (µg snail^{-1})	Lower Limit	Upper limit	1	2	index	folds	Slope
SWCNTs	1	79.6	66.39	96.54	*	*	100	1	1.41
TiO_2NPs	2	108.2	91.29	129.28	*	*	73.5	1.36	1.42

Biochemical quantifications

Sample preparation

One g of digestive gland tissue was homogenized in 0.05 M potassium phosphate buffer pH 6.5 (1/10 w/v) using a polytron for 15 s. The samples were centrifuged at 5000 rpm for 10 min at 4 °C. The homogenate was used for lipid peroxidation (LPO) and reduced glutathione content (GSH) assays, while supernatant was used for catalase (CAT), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST) and glutathione reductase (GR) assays. An aliquot of haemolymph was diluted with the above buffer (1/10 v/v) before used for all the above assays.

LPO

The thiobarbituric acid reactive substances (TBARS) were used as a technique according to Rice-Evans *et al*., (1991). Spectrophotometric quantification of malondialdehyde (MDA) content in tissue homogenate was done. An aliquot (250 µl) of homogenate was mixed with 1ml of 15% (w/v) trichloroacetic acid (TCA) in 25 mM HCl. The mixture was boiled for 10 min, quickly cooled, and immediately centrifuged at 5000 rpm for 5 min. The developed colour was determined at 535 nm. MDA level was estimated using an extinction coefficient of 156 mM^{-1} and expressed as nM g^{-1} tissue.

GSH

The method was designed on the reduction of 5, 5´-dithiobis 2-nitrobenzoic acid (DTNB) with GSH to give a yellow composite which is measured at 405 nm (Beutler *et al*., 1963). An aliquot (500 µl) of enzyme source, was mixed with same volume of 500 mM TCA, followed by centrifugation at 3000 rpm for 15 min. Half ml of the supernatant was well mixed with 1 ml of each 100 mM of phosphate buffered saline (PBS), pH 7.4 and 1 mM DTNB. After 10 min, the absorbance was measured at 405 nm against blank. GSH level was expressed as nM mg^{-1} protein.

CAT

Catalase (CAT) activity was measured independence on decrease of absorbance at 240 nm associated with hydrogen peroxide (H_2O_2) consumption (Beers and Sizer, 1952). The reaction mixture consisted of 1 ml of 12.5 mM H_2O_2 (substrate), 2 ml of 66.7 mM phosphate buffer, pH 7.0 and an aliquot of enzyme source. The activity was expressed as U mg^{-1} protein, where the unit of CAT is the amount of enzyme which liberates half the peroxide oxygen from hydrogen peroxide solution of any concentration in 100 at 25 °C.

GPx

The enzyme activity was measured according to method of Flohe and Gunzler (1984). In cuvette, phosphate buffer solution (100 mM, pH 7.0), EDTA (50 mM), sodium azide (250 mM), H_2O_2 (10 mM) and enzyme were well mixed. The change in absorbance was recorded every 3 s for 40 s at 340 nm. The activity was expressed as mU GP_x mg^{-1} protein, where one unit of GP_x is defined as the amount of enzyme necessary to oxidize 1 µM of NADPH per min.

GST

Spectrophotometric method of Habig and Jakoby (1981) by using 1-Chloro, 2-4 dinitrobenzene (CDNB) was used. An aliquot (250 µl) of enzyme source was mixed with 500 µl of potassium phosphate buffer (50 mM; pH 6.5). Then, the mixture was incubated at 25 °C for 5 min, followed by adding of 100 µl of 0.2 M CDNB and 150 µl of 10 mM GSH. After 1 min, the change of absorbance was recorded every 30 s for 6 min at 340 nm. The enzyme activity was expressed as µM mg^{-1} min^{-1}.

GR

The activity of GR was measured independent on the decrease in the absorbance during NADPH oxidation (Goldberg and Spooner, 1987). In each cuvette, 0.1 M potassium phosphate buffer, 3.4 mM EDTA, pH 7.6, 30 mM oxidized glutathione (GSSG), 0.8 mM β-NADPH and 1.0% of bovine serum albumin (BSA) was mixed by inversion. Then, 100 µl of enzyme was added. The absorbance was recorded at 340 nm for approximately 5 min. The activity was expressed as U mg^{-1} protein**.** One unit will reduce 1.0 µM of GSSG per min at pH 7.6 and 25 °C.

Protein content

Protein level was determined according to the method of Lowry *et al*., (1951). Intensity of the developed blue colour was measured a 750 nm against the blank. Bovine serum albumin (BSA) was used as a standard.

Statistical analysis:

LD_{50} value was expressed as µg $snail^{-1}$ with confidence limit (CL) and slope for TiO_2NPs and SWCNTS which computed using Probit analysis. All data and means were compared to significance by Student-Newman Keuls at the probability of 0.05 (Cohort Software Inc., 1985).

Results

Nanoparticles characterization

Titanium dioxide nanoparticles (TiO_2NPs) exhibited characteristic spherical shape with size ranged from 16.00 to 55.00 nm as made-up in SEM image (Figure 1a). However, SWCNTs exhibited single tubes with size ranged from 11.0 to 62.0 nm (Figure 1b). In addition, EDA pattern for elemental analysis is plotted in Figure 1c displaying the dominance of TiO_2 (100.0 %) of the total content and SWCNTs exhibited carbon percent over 97.0 % (Figure 1d). The average of zeta potential of TiO_2NPs and SWCNTs in their vehicle solutions as determined by DLS were -20.3 and 5.2 mv, respectively (Figure 1e and f).

Acute toxicity of NPs

The examined NPs represented LD_{50} values on the investigated snail *H. aspersa* after 48 h to be 108.3 and 79.6 µg $snail^{-1}$ for TiO_2NPs and SWCNTs, respectively (Table 1). The percent mortality in sublethal dose experiments did not exceed 10 % of the total treated individuals against control group.

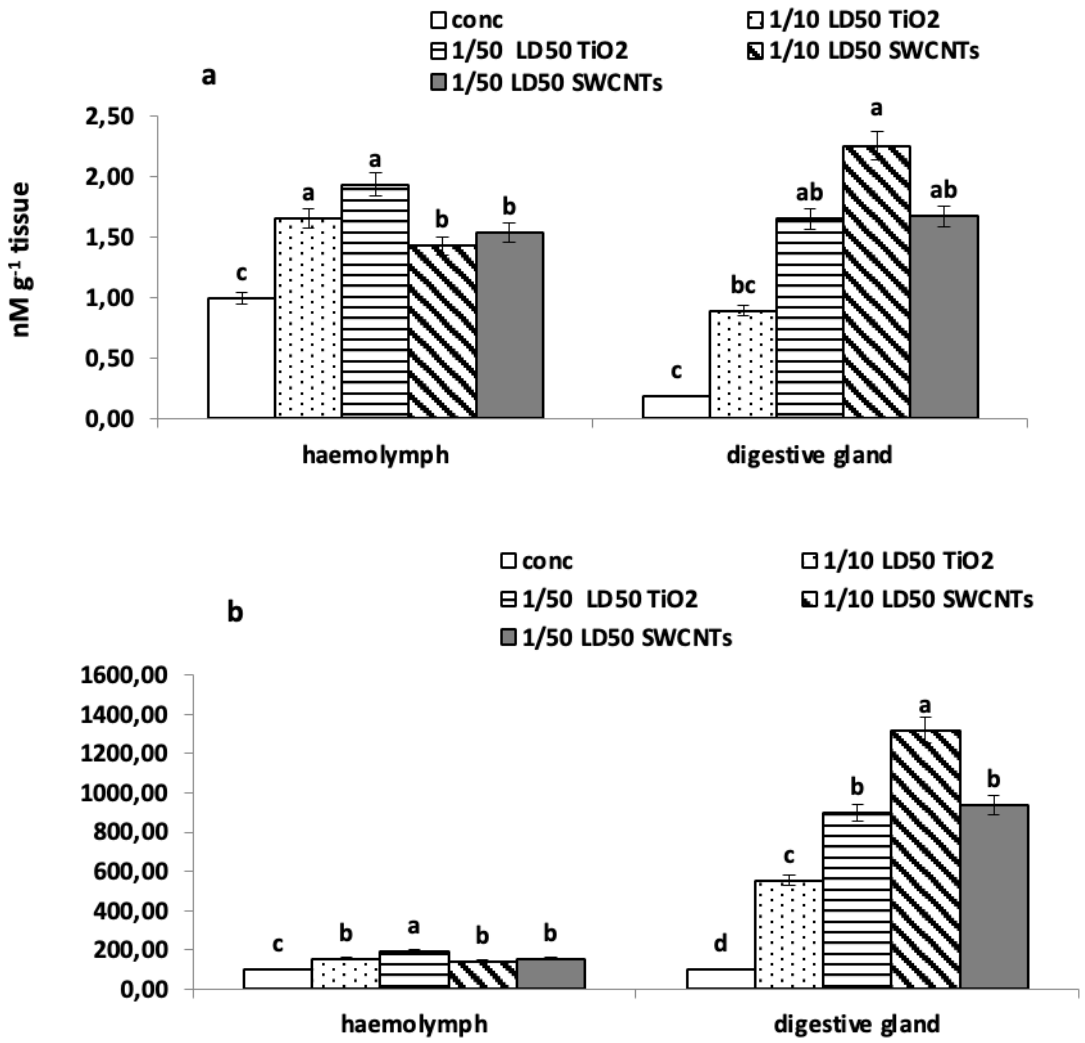

Fig. 2 a) MDA level (nM g^{-1} tissue) and b) % of control in the digestive gland and haemolymph of *H. aspersa* treated with NPs for 48 h. Each value represents mean of three replicates ± SE. The same letters indicate no significant difference at 0.05 levels

Oxidative stress

The effect of 1/10 and 1/50 of LD_{50} treatments for TiO_2NPs and SWCNTs after 48 h were investigated on antioxidant stress biomarkers in haemolymph and digestive gland homogenate samples. The MDA and GSH levels and CAT, GPx, GST and GR activities in the above samples were altered in treated snails, respect to untreated individuals (control).

LPO

Levels of MDA in both haemolymph or digestive gland of NP-treated snails were significantly greater than control (at $p < 0.05$) (Figure 2a and b). MDA levels for 1/10 LD_{50} of TiO_2 treatment were 1.66 and 0.89 nM g^{-1} tissue in haemolymph and digestive gland displaying % of control 154.42 and 553.68 %. However, 1/50 LD_{50} of TiO_2 treatment exhibited levels: 1.94 and 1.60 nM g^{-1} tissue in the above samples displaying % of control 194.41 and 898.28 %, respectively. Regarding SWCNTs, 1/10 LD_{50} treatment exhibited levels 1.43 and 2.26 nM g^{-1} tissue with % of control 143.12 and 1319.78 % in haemolymph and digestive gland samples. However, 1/50 LD_{50} treatment exhibited 1.54 and 1.68 nM g^{-1} tissue with % of control 155.32 and 938.49 % for the above samples.

GSH

The reduced GSH content in haemolymph and digestive gland of NP-treated snail is illustrated in Figure 3. In haemolymph, no significant difference ($p < 0.05$) was observed in GSH levels for 1/10 and 1/50 LD_{50} of TiO_2 treatments (27.76 and 27.90 nM mg^{-1} protein) against control group (27.90 nM mg^{-1} protein). However, SWCNTs treatments at the same manner decreased GSH content (24.80 and 26.03 nM mg^{-1} protein). Regarding digestive gland, all treatments decreased GSH content than the control (37.81 nM mg^{-1} protein). Treatments of 1/10 and 1/50 LD_{50} for TiO_2 exhibited levels: 22.41 and 13.84 nM mg^{-1} protein, while SWCNTs treatments

exhibited the levels: 23.22 and 29.12 nM mg^{-1} protein.

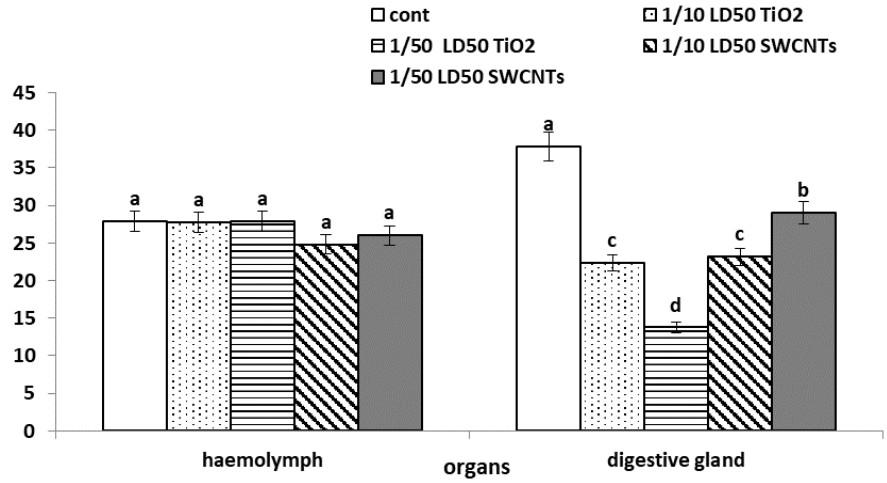

Fig. 3 GSH content (nM mg^{-1} protein) in the digestive gland and haemolymph of snail *H. aspersa* treated with NPs for 48 h. Each value represents mean of three replicates ± SE. The same letters indicate no significant difference at 0.05 levels

CAT

The activity of CAT enzyme increased in both haemolymph and digestive gland in all treated snails compared with untreated group. However, its activity was greater in digestive gland than haemolymph (Figure 4). In haemolymph, 1/10 LD_{50} of TiO_2 treatment exhibited the greatest activity (19.83 U mg^{-1} protein), followed by 1/50 LD_{50} treatment (16.23 U mg^{-1} protein). However, SWCNTs treatments exhibited the activities: 9.81 and 10.02 U mg^{-1} protein, respectively, against control (5.23 U mg^{-1} protein). Regarding digestive gland, the treatments were in the following order: 1/10 TiO_2, 1/50 SWCNTs, 1/10 SWCNTs, and 1/50 TiO_2 with mean values: 50.91, 39.00, 22.93 and 16.33 U mg^{-1} protein, respectively.

GPx

The activity of GPx in digestive gland was significantly greater than haemolymph (at $p < 0.05$) (Figure 5). In haemolymph, the all treatments decreased enzyme activity lower than control (1.92 mU mg^{-1} protein) in the following order: 1.12, 1.84, 1.32 and 1.54 mU mg^{-1} protein for 1/10 TiO_2, 1/50 TiO_2, 1/10 SWCNTs, and 1/50 SWCNTs, respectively. Regarding digestive gland, 1/10 LD_{50} SWCNTs treatment exhibited the greatest activity (48.43 mU mg^{-1} protein), followed by 1/50 LD_{50} of TiO_2 treatment (27.12 mU mg^{-1} protein) and 1/10 LD_{50} of TiO_2 treatment (14.74 mU mg^{-1} protein), respectively. However, 1/50 LD_{50} of SWCNTs treatment exhibited enzyme activity equal that of control group (12.53 mU mg^{-1} protein).

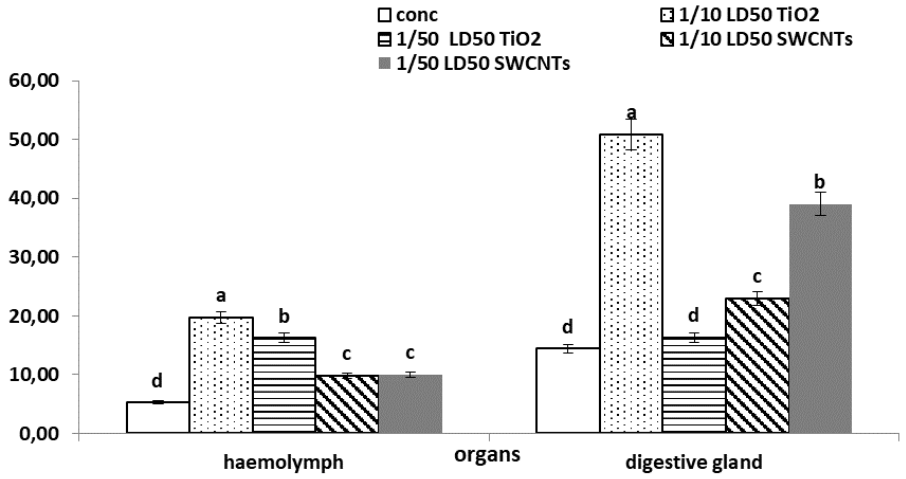

Fig. 4 CAT activity (U mg^{-1} protein) in the digestive gland and haemolymph of snails *H. aspersa* exposed to sublethal doses of TiO_2NPs and SWCNTs. Each value represents mean of three replicates ± SE. The same letters indicate no significant difference at 0.05 levels

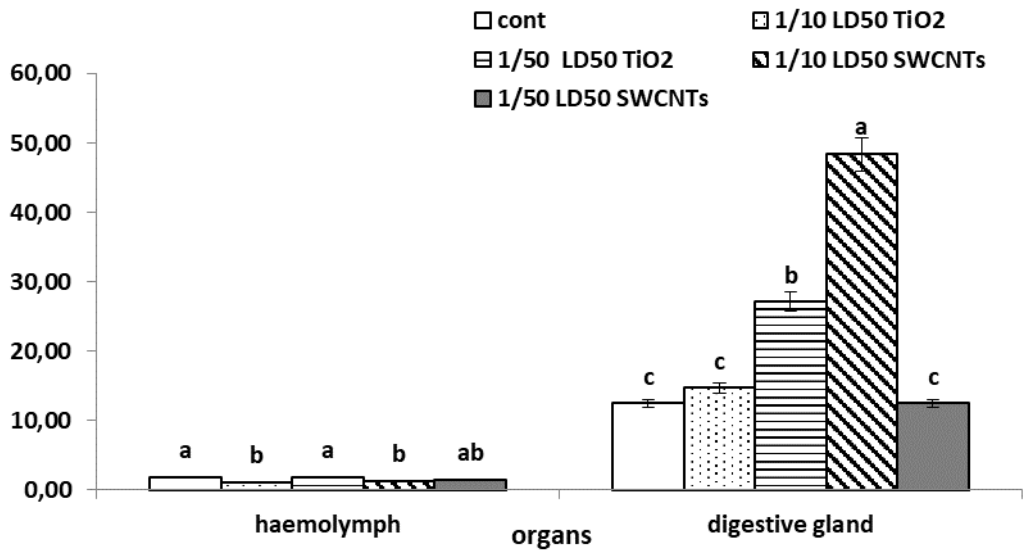

Fig. 5 GPx activity (mU mg^{-1} protein) in the digestive gland and haemolymph of snail *H. aspersa* treated with NPs for 48 h. Each value represents mean of three replicates ± SE. The same letters indicate no significant difference at 0.05 levels

GST

The activities of GST in haemolymph and digestive gland of treated animals and control (untreated) are illustrated in Figure 6. In haemolymph, 1/10 and 1/50 LD_{50} of TiO_2 treatments exhibited activities: 0.14, and 0.21 mU mg^{-1} protein min^{-1} against control (0.22 µM mg-1 min^{-1}). However, 1/10 SWCNTs treatment exhibited the greatest activity (0.26 µM mg^{-1} protein min^{-1}), followed by 1/50 LD_{50} of SWCNTs treatment (0.21 µM mg^{-1} protein min^{-1}). Regarding digestive gland, all treatments exhibited activities lower than control as obtained in the following order: 19.92, 4.01, 8.13, 27.32 µM mg^{-1} protein min^{-1} for 1/0 TiO_2, 1/50 TiO_2, 1/10 SWCNTs, and 1/50 SWCNTs, respectively.

GR

The activities of GR enzyme in digestive gland samples were greater than in haemolymph (p < 0.05) (Figure 7). In haemolymph, 1/50 LD_{50} of TiO_2 treatment exhibited the greatest activity (0.19 U mg^{-1} protein), followed by 1/10 LD_{50} of TiO_2 treatment (0.13 U mg^{-1} protein) against control which did not exceed 0.08 U mg^{-1} protein. However, SWCNTs treatments exhibited activities did not exceed the value of control (0.08 U mg^{-1} protein).

Discussion

The present findings provide an ideal illustrating potential toxic effects 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 (Gomot de-Vaufleury and Pihan, 2000; Beeby and Richmond, 2003). Also, haemolymph was chosen, because it non-bold represents the main compartment for nutrients and xenobiotic distribution in the body's compartments.

Oxidative stress can arise liberated to an imbalance in the biological oxidant-to antioxidant ratio. This explanation obtains the damage which may be caused to cell components e.g. lipids, proteins, enzymes, and nucleic acids. This damage to cell organization provides a critical signal of organ dysfunction (El-Demrdash, 2007). The mechanism refers to the mode of action of metallic NPs is in employment to generation of ROS resulting in direct interaction with the biological targets (Ma and Diamond, 2013). As documented by Pamplona (2008), 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 is 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) or in depletion of antioxidant species with the cell (Ahmad *et al*., 2012). CNT-induced oxidative stress mediates important cellular developments including inflammation, cell injury, apoptosis, and activation of cellular pathways (Bonner, 2002; Iavicoli *et al*., 2012).

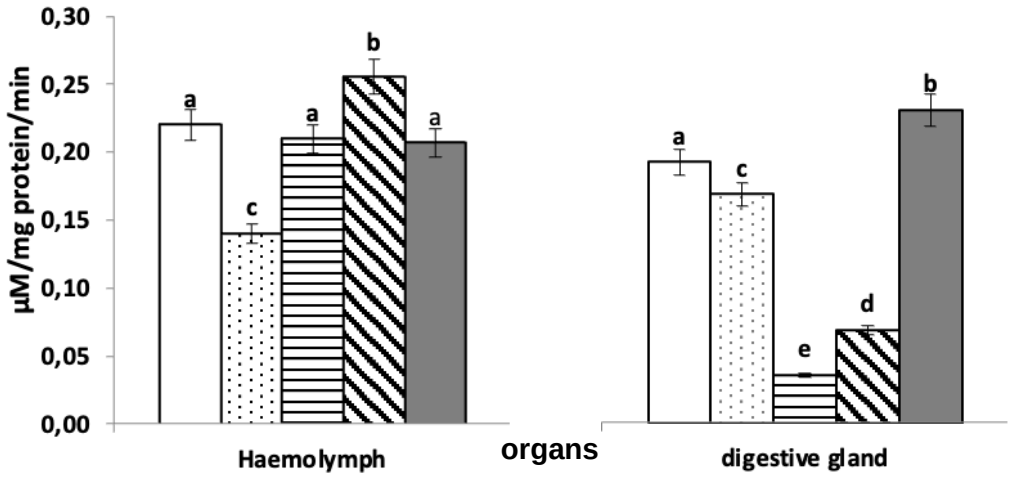

Fig. 6 GST activity (µM mg^{-1} protein min^{-1}) in the digestive gland and haemolymph of snail *H. aspersa* treated with NPs for 48 h. Each value represents mean of three replicates ± SE. The same letters indicate no significant difference at 0.05 levels

The obtained data also disclosed a correlation between the heightening of LPO and reduction of GSH content. In fact, GSH depletion after NPs exposure may refer to its increased consumption *via* free radicals scavenging. Decreased GSH content may be induced by free radicals produced by NPs and/or by the direct binding of glutathione to the metal as described by Barillet (2007). Moreover, ENMs and ultrafine particles are categorized by their binding with thiol group, resulting complexes and/or macro-molecules (Xiong *et al*., 2011; Fahmy *et al*., 2014). For example, some gastropods were exposed to different concentrations of Cu resulting in significant declines in GSH contents (Regoli *et al*., 1997; Canesi *et al*., 1999). Also, Chandran *et al*. (2005) stated declines in GSH content of gastropod, *Achatina fulica* treated with Cd and Zn. A finding was conducted by Ramsden *et al*. (2013) in some organ of zebrafish exposed to TiO_2 demonstrating the same concept. Also, the same depletion in GSH content was noted by Khene *et al*. (2017) on snail, *H. aspersa* exposed to TiO_2 microparticles. Another finding was demonstrated by Ali *et al*. (2015), where freshwater snail, *Lymnea luteola* was exposed to different concentrations of TiO_2NPs for 96 h. After that, significant depletion in GSH content and GST activity as well as increases in MDA level and CAT activity were induced. In addition, 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 (Ali *et al*., 2015). On the other hand, increased activity of CAT in the present study is in accordance with the findings of Almeida *et al*. (2004), who found that CAT activity was increased in mussels after exposure to lead (Pb). In another study, SWCNTs exhibited a significant reduction in GSH, GST, and GPx in hepatopancreas of *L. luteola* after exposure to different concentrations for 96 h (Ali *et al*., 2013).

Moreover, GSH as non-enzymatic defense is being a necessary cofactor for GPx and GST activities which play a principal role in conserve cellular redox status and protective cells from oxidative damage (Dickinson and Forman, 2002). Furthermore, part of the system is GST which enhances the conjugation of GSH to nucleophilic xenobiotics or cellular components broken by oxyradicals award causing of their detoxication (Halliwell and Gutteridge, 1989; Singal *et al*., 1992). GR catalyzes the NADPH dependent regeneration of GSH from the oxidized form (GSSG) (Kehrer and Lund, 1994). Decline of GSH levels in analyzed samples of the examined snail proved to be a good bio-indicator for NPs impacts. This explains the high affinities of NPs to GSH molecule.

CAT detoxifies H_2O_2 in the biological systems. Hermis-Lima (2004) indicated that, under oxidative stress, CAT activity often increases due to the regulation by ROS. In the present findings, GPx activity in NP-treated snails increased in comparison with control. This concept is considered a result of the oxidative stress from NPs conjugation and accumulation in the selected samples of haemolymph and digestive gland. On the other hand, SWCNTs exhibited an increase of GST activity, which may be due to the activation of the natural antioxidant defense system by these particles, however, the detoxication process versus the pro-oxidation forces was mediated by this

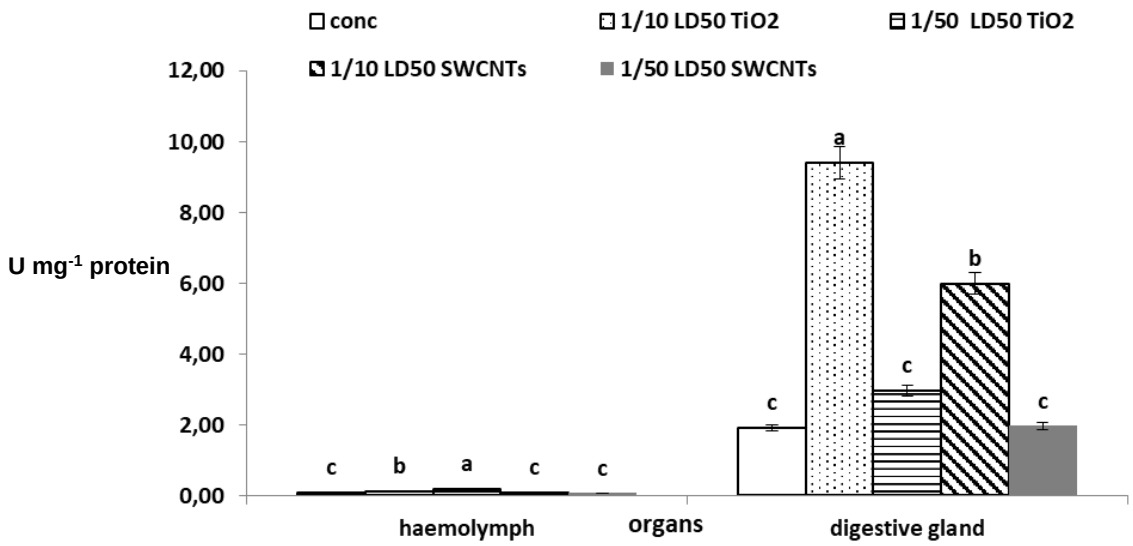

Fig. 7 GR activity (U mg^{-1} protein) in the digestive gland and haemolymph of snail *H. aspersa* treated with NPs for 48 h. Each value represents mean of three replicates ± SE. The same letters indicate no significant difference at 0.05 levels

enzyme (Elia *et al*., 2007). This concept was documented by Canesi *et al*. (1999), where copper treatment increased GST activity in reflecting to increased utilization of GSH conjugation in lipid hydro peroxides and carbonyl compounds metabolism after peroxidation of cellular membranes by the metal. Similarly, the present data are in accordance with that obtained by Radwan *et al*. (2010) and Abdel-Halim *et al*. (2013), where GST activity increased in snails: *Teba pisana* and *H. aspersa* exposed to heavy metals contamination in some urban regions of Egypt. The increase in GR activity in the treated individuals of *H. aspersa* is in accordance with that obtained by Regoli *et al*. (2006), who suggested that the increase of GR activity was reflected in more integrated unbalance of oxyradical metabolism.

Conclusion

The results of this work provide potential toxic effects of TiO_2NPs and SWCNTs on snail *H. aspersa* and show variations of both NMs in relationship to their properties and toxicities on the snail. These findings could explain the ability of NPs to penetrate into the cells and induce damage resulting in dysfunctions and generation of ROS that initiated actions referring to oxidative stress. The use of oxidative stress parameters could be established as a reliable tool to assess the impacts of NPs on terrestrial ecosystems. So, further investigations are required to ensure the safe use of such materials without any risks.

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Compliance with ethical standards

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

Declaration of Conflicting of Interest

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

Supplementary data

No supplementary data are provided.

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