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Impairment of DNA in a Freshwater Gastropod (*Lymnea luteola* L.) After Exposure to Titanium Dioxide Nanoparticles

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Abstract The apoptotic and genotoxic potential of titanium dioxide nanoparticles (TiO_2NPs) were evaluated in hemocyte cells of freshwater snail *Lymnea luteola* L. Before evaluation of the toxic potential, mean size of the TiO_2NPs was determined using a transmission electron microscopy and dynamic light scattering. In this study, *L. luteola* were exposed to different concentrations of TiO_2NPs (28, 56, and 84 μg/ml) over 96 h. Induction of oxidative stress in hemolymph was observed by a decrease in reduced glutathione and glutathione-*S*-transferase levels at different concentration of TiO_2NPs and, in contrast, an increase in malondialdehyde and reactive oxygen species levels. Catalase activity was decreased at lower concentrations but increased at greater concentration of TiO_2NPs. The extent of DNA fragmentation occurring in *L. luteola* due to ecotoxic impact TiO_2NPs was further substantiated by alkaline single-cell gel electrophoresis assay and expressed in terms of % tail DNA and olive tail moment. The alkaline single-cell gel electrophoresis assay for *L. luteola* clearly shown relatively greater DNA damage at the highest concentration of TiO_2-NPs.The results indicate that the interaction of TiO_2NPs with snail influences toxicity, which is mediated by oxidative stress according dose and in a time-dependent manner. The results of this study showed the importance of a multibiomarker approach for assessing the injurious effects of TiO_2NPs to freshwater snail *L. luteola*, which may be vulnerable due to the continuous discharge of TiO_2NPs into the aquatic ecosystems. The measurement of DNA integrity in *L. luteola* thus provides an early warning signal of contamination of the aquatic ecosystem by TiO_2NPs.

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The rapid increase in productivity and use of nanoparticles (NPs) has raised concerns that releases of engineered nanomaterials may pose a serious environmental threat because aquatic ecosystems likely will serve as terminal sinks for nanomaterials (Oberdorster et al. 2005; Moore 2006; Wallis et al. 2014; Li et al. 2014a, b). Titanium dioxide (TiO2) NPs are used in a variety of consumer products, such as sunscreens, cosmetics, paints, and surface coatings, as well as in the environmental decontamination of air, soil, and water (Kaida et al. 2004; Choi et al. 2006). Such widespread use raises concern that TiO_2NPs could pose a risk to both ecosystems and human beings. Despite the dramatic increase in the use of TiO_2NPs, the little information is available on their potential harmful effects on aquatic organisms (Ma et al. 2014; Li et al. 2014c). In particular, TiO_2NP concentrations in surface water range from 3 to 1.6 μg/l and reach 5 μg/l in effluents from wastewater-treatment plants (Gottschalk et al. 2013). Aquatic pollutants prevalent in the

aquatic ecosystem may likely to cause severe damage to genetic material directly or indirectly. The occurrence of DNA-strand breakage in various species of aquatic organisms exposed to enhanced concentration of genotoxic pollutants is a matter of great concern. Thus, the measurement of DNA integrity in those species of aquatic organisms exposed to pollutants, e.g., TiO_2NPs is indeed of great importance for the biomonitoring of pollution of the aquatic environment.

Freshwater gastropods are common in both lotic and lentic systems and play a vital role in the food chain. Moreover, because of their characteristic of having little mobility, they are useful as sentinel species for the biomonitoring of pollution and ecotoxicological studies (Angeletti et al. 2013). A few data have been observed on the occurrence of DNA damage in gastropods (Benton et al. 2002; Sarkar et al. 2013). Due to their small sizes, NPs are more likely to infiltrate biological systems that larger molecules can not infiltrate (Moore 2006) and diffuse through cell membranes (Lin et al. 2010). Reactive oxygen species (ROS) generation is a parameter that is convenient to measure in ecotoxicity because cells respond to oxidative stress by exerting a number of protective responses that can easily be measured as enzymatic or genetic expression responses (Kovochich et al. 2007). The alkaline single-cell gel electrophoresis assay is a rapid and sensitive method for detecting DNA damage of a variety of toxic agents such as chemical compounds, ionizing radiation, and NPa in cells and aquatic organisms (Ali et al. 2008).

The presence of genotoxic and carcinogenic compounds in aquatic environments is of major concern with respect to the health of aquatic biota; therefore, the genotoxicity of NPa must be identified before their widespread release to the aquatic environment. Therefore, the current study was aimed at evaluating the impact of ecogenotoxicity of TiO_2NPs on the integrity of DNA as a biomarker of aquatic pollution.

Materials and Methods

Chemicals

TiO_2NPs (Product No. 718467 and average particle size $\leq$21 nm), snail saline buffer (SSB) [5 mM of HEPES, 3.7 M of NaOH, 36 mM of NaCl, 2 mM of KCl, 2 mM of $MgCl_2$, and 4 mM of $CaCl_2$ (pH 7.8)], ethylene diamine tetra acetic disodium salt (Na_2EDTA), dimethyl sulphoxide, CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester), 5′,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), propidium iodide, and Annexin V FITC were purchased from M/s Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and were purchased from local markets.

Snail Collection and Culture

Adult *L. luteola* of similar size [average shell length 21.7 mm (range 19.4–26.30)] and weight [average wet weight 494 mg (range 320–650)] were carefully collected from noncontaminated artificial fish culture ponds, transferred to the laboratory, and maintained in glass aquaria. Snails were acclimatized to laboratory conditions for 2 weeks before experimentation at a temperature of 21 ± 1 °C and fed daily ad libitum with thoroughly washed freshwater green aquatic plant leaves (*Marsilia* sp.).

Characterization of TiO_2NPs

TiO_2NPs were suspended in deionized water at a concentration of 1 mg/ml and then sonicated using a sonicator bath at room temperature for 10 min at 40 W to form a homogeneous suspension. Sonicated TiO_2NP stock solution (1 mg/ml) was diluted to 10–200 μg/ml working solutions. Hydrodynamic size and zeta potential of the TiO_2NP suspension in water were measured by dynamic light scattering (DLS) (Zeta Sizer-HT Malvern Instrument, Worcestershire, UK). The size and shape of TiO_2NPs were characterized at an accelerating voltage of 200 kV by transmission electron microscopy (TEM) (FETEM, JEM-2100F; JEOL).

Determination of Sublethal Concentrations

Acute toxicity bioassay to determine the 96-h LC_{50} value of TiO_2NPs was performed in a static-renewal system. The acute bioassay procedure was based on standard methods (Organization for Economic Cooperation and Development (1992)). A stock solution of TiO_2NPs (1 mg/ml) was prepared in deionized water.

A set of 10 acclimatized *L. luteola* was randomly exposed to different TiO_2NP target concentrations (0, 5, 15, 30, 60, 120, and 200 μg/ml) in transparent polystyrene beakers of 1,000 ml test water, and the experiment was repeated twice to obtain the 96-h LC_{50} value of the test TiO_2NPs for the freshwater snail *L. luteola*. Fluorescent lights with two 48-W lamps were used as light source to simulate the natural day-to-light cycle (12 h of darkness to 12 h of light).

The 96-h LC_{50} value (112 μg/ml) of TiO_2NPs for *L. luteola* was determined using the probit analysis method as described by Finney (1971). On the basis of the 96-h LC_{50} value, the three test concentrations of TiO_2NPs, viz., dose 1 (1/4th of LC_{50} = approximately 28 μg/ml), dose 2 (1/2nd of LC_{50} = approximately 56 μg/ml), and dose 3 (3/4th of LC_{50} = approximately 84 μg/ml), were determined.

In Vivo Exposure of TiO_2NPs and Hemolymph Collection

Lymnea luteola L.were exposed to the three aforementioned test concentrations of TiO_2NPs in a static-renewal system for 96 h. The control snails were maintained in tap water. The sampling was performed at intervals of 24 and 96 h at the rate of five snails per duration.

The physicochemical properties of test water, namely, temperature, pH, total conductivity, dissolved oxygen, chloride, and total hardness, were analyzed by standard methods (American Public Health Association, American Water Works Association, Water Pollution Control Federation 2005) during the experimentation period.

For each sampling duration, whole hemolymph was collected through the haemal pore and immediately processed for measurement of ROS generation, apoptosis, and DNA damage, and remaining hemolymph was stored at −80 °C. The stored hemolymph was used for the measurement of CAT, glutathione-*S*-transferase (GST) activities, lipid peroxidation (LPO) level, and reduced glutathione (GSH).

Hemocyte Cell Viability

Gentle prodding of the foot sole forces the snail to retract deeply into its shell, thereby extruding a drop of hemolymph through the hemal pore. A drop of hemolymph (50 µl) was collected using a micropipette and added directly to SSB plus anticoagulant. Hemocyte cell viability was assessed using the trypan blue (0.2 %) exclusion test. Stained (dead) and unstained (viable) hemocytes were counted microscopically. Each hemolymph sample was poured directly into a haemocytometer. The total number of circulating hemocytes was counted using a phase contrast light microscope (Leica). Hemograms for both control and treated snails were expressed as the mean cell number per cubic millimeter of hemolymph.

Detection of ROS

ROS generation was studied by two methods: fluorometric analysis and microscopic fluorescence imaging. In fluorometric analysis, intracellular ROS was measured by oxidation of nonfluorogenic substrate carboxy-H_2DCFDA (5-(-6)-carboxy-2,7-dichloro dihydrofluorescein diacetate), a permanent cationic dye that is readily incorporated in cells and becomes fluorescent after being oxidized in the cytoplasm of cells by ROS. Hemolymph was collected from exposed *L. luteola* and added to 5 µM carboxy-H_2DCFDA and kept in dark for 15 min at room temperature. After incubation of hemocyte cells, the fluorescence was measured at an excitation wavelength (485 nm) and an emission wavelength (535 nm) using a fluorescence microplate reader (Omega Fluostar). The intensity of untreated control well was assumed to be 100 %. A pparallel set of hemolymph was analyzed for intracellular ROS fluorescence using an upright fluorescence microscope equipped with a charge-coupled device cool camera (Nikon Eclipse 80i equipped with Nikon DS-Ri1 12.7 megapixel camera).

LPO Levels

The concentration of malondialdehyde (MDA) as a marker of LPO was determined according to the method of Nair and Turner (1984). Briefly, 0.25 ml of hemolymph was mixed with 2 ml of thiobarbituric acid (TBA) solution that was freshly prepared by mixing a 1:3 volume of TBA (0.8 %) and trichloroacetic acid (20 %). The mixture was incubated for 50 min in a boiling water bath. After cooling, the mixture was centrifuged at 3,000 g for 10 min. The MDA level was measured spectrophotometrically (Varian-Cary 300 Bio) at 532 nm, and the results are expressed as MDA mM/ml.

Determination of GSH

GSH was measured at 412 nm by the method of Owens and Belcher (1965). The assay mixture consisted of 0.1 ml of hemolymph and 1.5 ml of 0.5 M phosphate buffer (pH 8.0) followed by 0.4 ml of 3 % metaphosphoric acid and 30 µl of DTNB (0.01 M).The amount of GSH present in the hemolymph sample (in mg/dl) was calculated after calibration against the standard curve of GSH.

Determination of Antioxidant Enzyme Activity

CAT activity was measured after the decrease of absorbance at 240 nm due to H_2O_2 consumption (Beers and Sizers 1952). The reaction mixture consisted of 1 ml of 12.5 mM hydrogen peroxide (substrate), 2 ml of 66.7 mM phosphate buffer (pH 7.0), and 0.1 ml of hemolymph. CAT activity was expressed as units per milliliter.

GST activity was assessed by the method of Vessey and Boyer (1984) using 1-chloro-2,4,dinitrobenzene (CDNB) as a substrate. The reaction mixture contained 0.2 ml of 4 mM GSH, 20 µl of 0.25 Mm CDNB, 200 µl of hemolymph, and 2.76 ml of 0.1 M phosphate buffer (pH 7.0). The formation of the CDNB-GSH conjugate was evaluated by monitoring the increase in absorbance at 340 nm.

Apoptosis Detection by Annexin V-FITC

For analysis of apoptotic hemocyte cells, hemolymph was collected from treated and untreated snails during a 96-h

exposure in glutaraldehyde and resuspended in binding buffer (100 µl, 1×), transferred into a 5-ml fluorescent antibody cell separation tube and added with 5 µl of Annexin V-FITC (conjugated with fluorescein isothiocyanate) and 10 µl of propidium iodide. After incubation for 30 min at room temperature in dark, 400 µl of 1× binding buffer was added to each tube, and flow cytometry analysis was performed immediately. Data acquisition and analysis were performed by BD LSRII analyzer (Becton Dickinson, USA) using CELL Quest software. Cells that were Annexin V (−) and PI (−) were considered viable cells. Cells that were Annexin V (+) and PI (−) were considered early stage apoptotic cells. Cells that were Annexin V (+) and PI (+) were considered late-stage apoptotic cells.

Determination of DNA-Strand Breakage

Alkaline single-cell gel electrophoresis assay was performed as a three-layer procedure (Singh et al. 1988) with slight modification, in which conventional microscopic slides were used (Ali et al. 2008). The hemolymph of the exposed snails was quickly collected in glutaraldehyde–added ice–cold SSB. Viability of cells was evaluated by the trypan blue exclusion method (Anderson et al. 1994). In brief, approximately 15 µl of cell suspension was mixed with 85 µl of 0.5 % low melting–point agarose and layered on one end of a frosted plain glass slide that had been precoated with a layer of 200 µl of normal agarose (1 %). Thereafter, it was covered with a third layer of 100 µl of low melting–point agarose. After solidification of the gel, the slides were immersed in lysing solution (2.5 M of NaCl, 100 mM of Na_2EDTA, 10 mM of Tris (pH 10) with 10 % DMSO and 1 % Triton X-100 added fresh) overnight at 4 °C. For positive control, the hemolymph cells were treated ex vivo with 100 µM of H_2O_2 for 10 min at 4 °C. The slides were then placed in a horizontal gel electrophoresis unit. Fresh cold alkaline electrophoresis buffer [300 mM of NaOH, 1 mM of Na_2EDTA, and 0.2 % DMSO (pH 13.5)] was poured into the chamber and left for 20 min at 4 °C for DNA unwinding and conversion of alkali-labile sites to single-strand breaks. Electrophoresis was performed using the same solution at 4 °C for 20 min using 15 V (0.8 V/cm) and 300 mA. The slides were neutralized gently with 0.4 M of Tris buffer at pH 7.5 and stained with 75 µl of ethidium bromide (20 µg/ml). Two slides were prepared from each specimen, and 50 cells/slide (100 cells/concentration) were scored randomly and analyzed using an image analysis system (Komet-5.0; Kinetic Imaging, Liverpool, UK) attached to a fluorescent microscope (DMLB; Leica, Germany) equipped with appropriate filters. The parameters selected for quantification of DNA damage in the hemocyte cells were percentage of DNA in the tail (*i.e.*, % tail DNA = 100 % head DNA) and olive tail moment [OTM (arbitrary units, the products of the distance of DNA migration from the body of the nuclear core and the total fraction of DNA in the tail)] as determined by the software.

Estimation of Protein

The total protein content was measured by the Bradford (1976) method using Bradford reagent (Sigma-Aldrich, USA) and bovine serum albumin as the standard.

Statistical Analysis

At least three independent experiments were performed each evaluation. Data were expressed as mean (±SE) and analysed by one-way analysis of variance). The p value of <0.01 was considered statistically significant.

Results

TiO_2NP Characterization

The TEM image (Fig. 1a) shows the shape and size of the TiO_2NPs. The average size ± SE of the TiO_2NPs measured by TEM was 34.10 ± 2.70 nm (Fig. 1b). The mean hydrodynamic diameter and zeta potential of the TiO_2NPs in water as determined by the DLS measurement were 190.5 ± 3.4 nm and −13.9 mV, respectively.

Physicochemical Analysis of the Test Water

The water temperature varied from 21.8 to 22.2 °C, and pH values ranged from 7.50 to 8.06. The dissolved oxygen concentration was normal, varying from 6.20 to 8.05 mg/l, during experimental period. The conductivity of the water ranged from 251.8 to 272 µM/cm and chloride ion from 46.5 to 54 mg/l. The total hardness ranged from 178.0 to 183 mg/l, and total alkalinity ranged from 267 to 282.0 mg/l as $CaCO_3$.

Hemocyte Cell Viability and Induction of ROS in Hemocyte Cells

Cell viability of hemocyte was 98 % in the control, and it was decreased as concentration and in a time-dependent manner (Fig. 2). TiO_2NPs induce intracellular ROS generation in hemocyte cells, and this was observed by measuring DCF fluorescence as a reporter of ROS generation. Compared with the control, a significant ($p < 0.01$) increase in DCF intensity was observed in all snail hemocyte cells in concentration and in a time-dependent manner (Fig. 3).

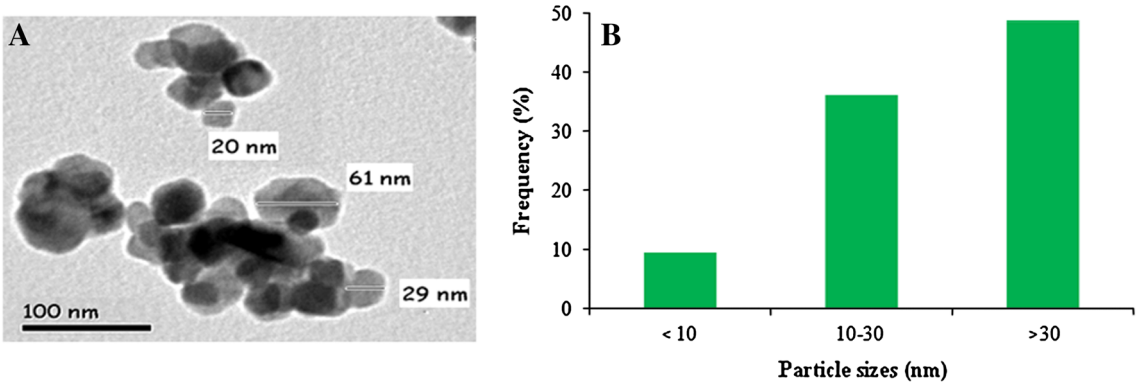

Fig. 1 Characterization of TiO_2NPs. **a** The TEM image. **b** Size distribution histogram generated using the TEM image

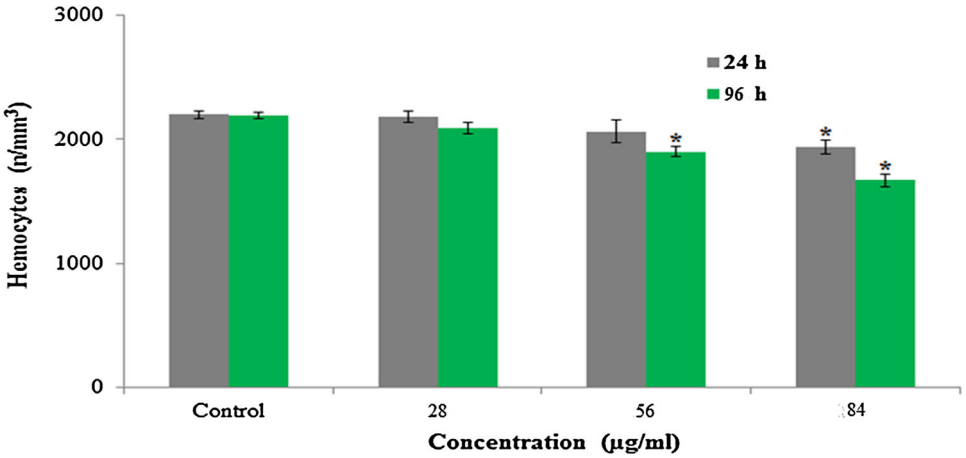

Fig. 2 Alterations in the total number of circulating hemocyte/mm^3 after exposure to various concentrations of TiO_2NPs in *L. luteola*. for 24 and 96 h. Each value represents the mean ± SEM of three experiments. *$p < 0.01$ versus the control

TiO_2NP-Induced Oxidative Stress

The MDA level, GSH and CAT, and GST activities in the hemolymph of TiO_2NP-exposed snails were investigated and illustrated (Fig. 4). At sublethal concentration of TiO_2NP exposure to the snails exhibited significant increase ($p < 0.01$) in MDA content compared with the control, which indicates increased LPO level (Fig. 4a).

GSH level was decreased significantly ($p < 0.01$) as concentration and in a time-dependent manner (Fig. 4b). All concentrations of TiO_2NP caused a significant ($p < 0.01$) decrease in GST activities in the hemolymph of snails (Fig. 4c). CAT activity in hemolymph of snail was increased significantly ($p < 0.01$) at greater doses of TiO_2NPs (Fig. 4d).

Apoptosis

Annexin V-FITC and propidium iodide staining were applied to detect apoptotic and necrotic stimulation in hemocyte cells by TiO_2NP exposure. TiO_2NPs showed a significant increase in Annexin V (+) cells (13.10 %) and late necrotic cells (17.59 %) compared with the untreated snail hemocyte cells (Fig. 5).

DNA Damage

NA damage was measured as % tail DNA and OTM in control as well as in the TiO_2NP-exposed snail hemocyte cells. The cells exposed to different concentrations of TiO_2NPs exhibited significantly ($p < 0.01$) greater DNA damage in cells than those of the control groups. A gradual nonlinear increase in DNA damage was observed in cells as concentrations of TiO_2NP and duration increased. The highest DNA damage was recorded at the highest TiO_2NP concentration at 96 h (Fig. 6).

Discussion

Industrial waste and urban water sewage are released into rivers, lakes, and coastal waterways; thus, it is inevitable that industrial nanoscale products and byproducts enter into these aquatic systems (Daughton 2004). Freshwater snails

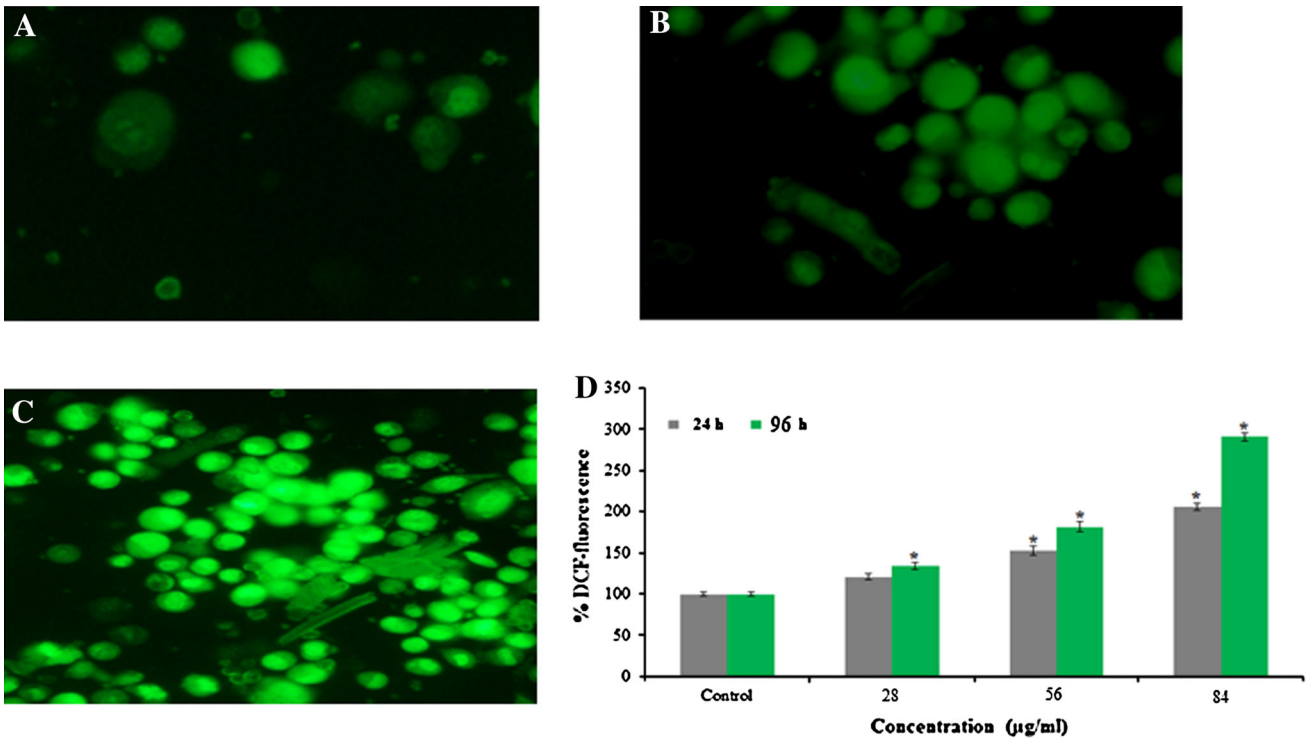

Fig. 3 Photographs showing TiO_2NP-induced ROS generation in hemolymph of *L. luteola*. **a** Control. **b** 28 µg/ml of TiO_2NPs. **c** 84 µg/ml of TiO_2NPs. **d** Percentage of ROS generation. Each value represents the mean ± SEM of three experiments. $^*p < 0.01$ versus control

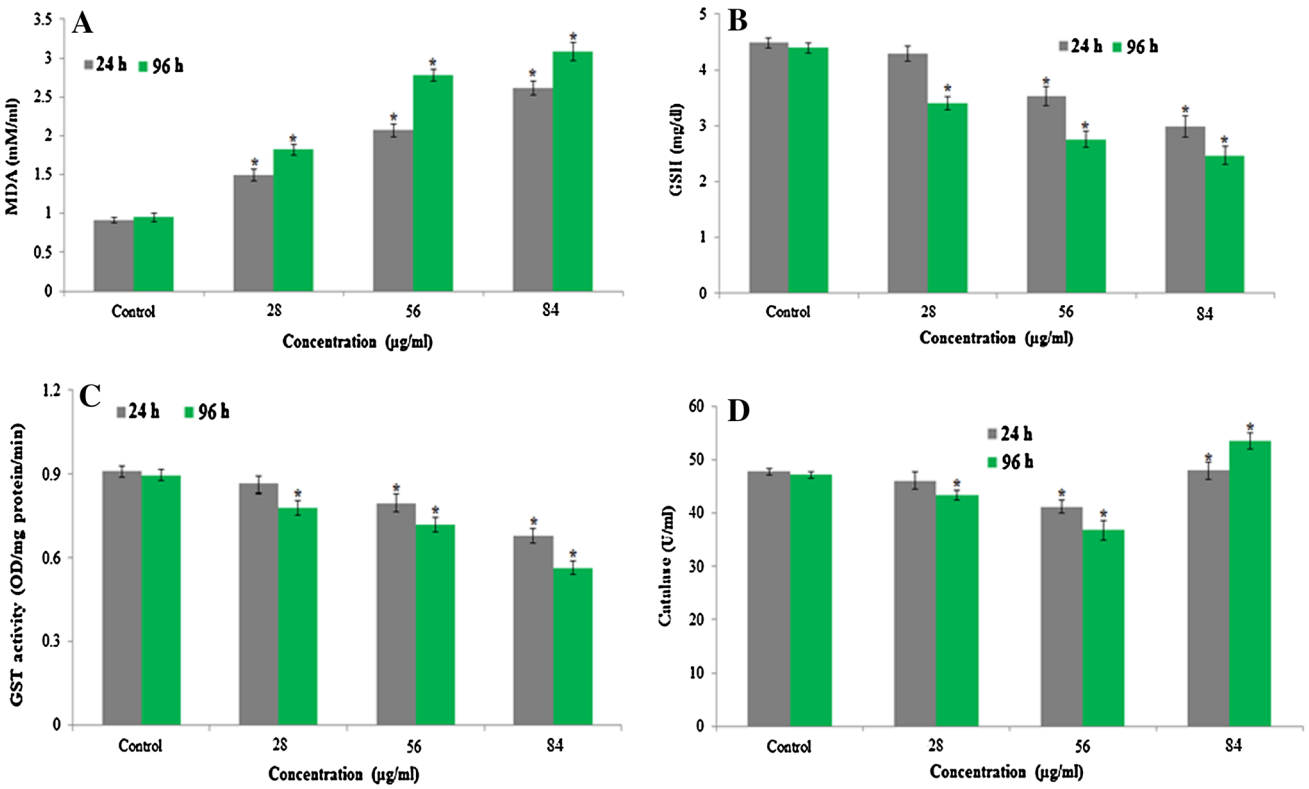

Fig. 4 **a** LPO, **b** GSH, **c** GST, and **d** CAT activity in hemolymph after exposure of *L. luteola* to TiO_2NPs. Each value represents the mean ± SEM of three experiments. $^*p < 0.01$ versus control

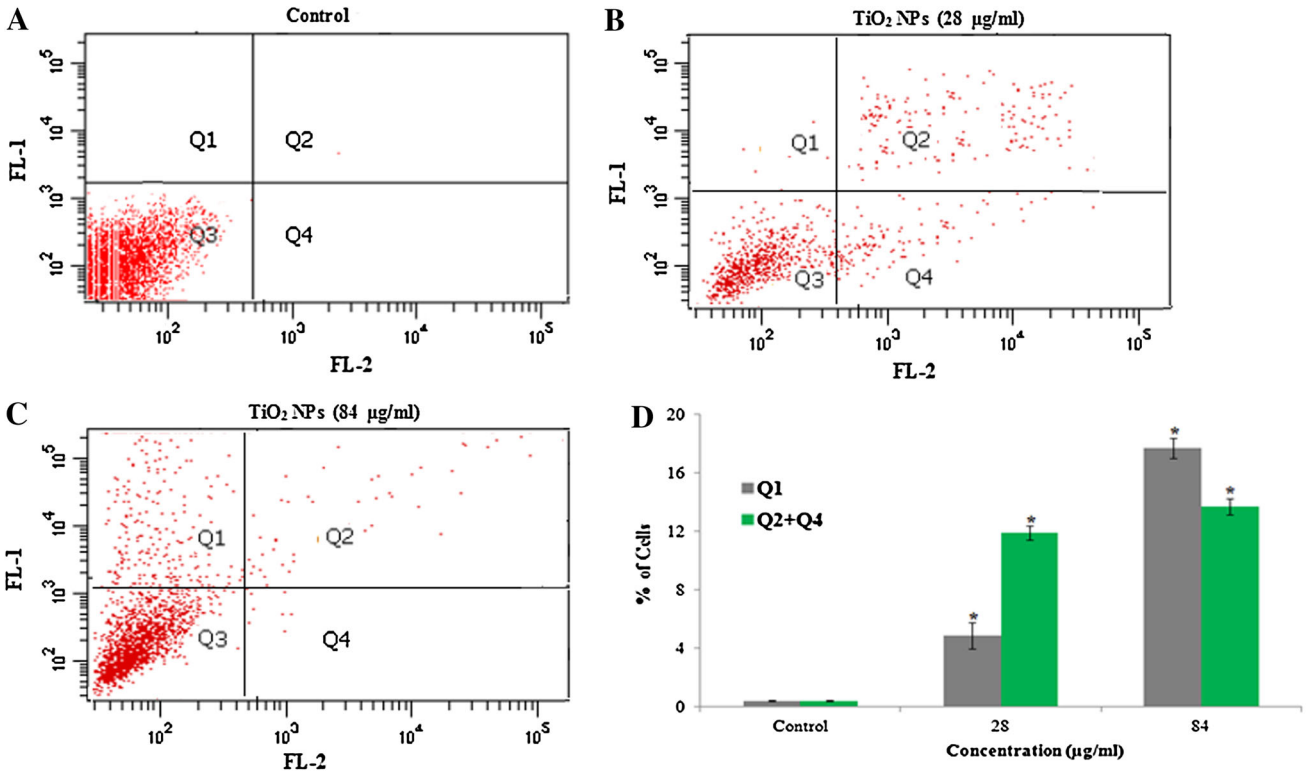

Fig. 5 TiO_2NP-induced apoptosis in hemocyte cells of *L. luteola*. **a–d** Flow cytometric analysis of Annexin V-FITC/PI—stained cells. Data represent mean ± SEM of three experiments. Representative *dot plots* of three independent experiments are presented. *$p < 0.01$ versus control

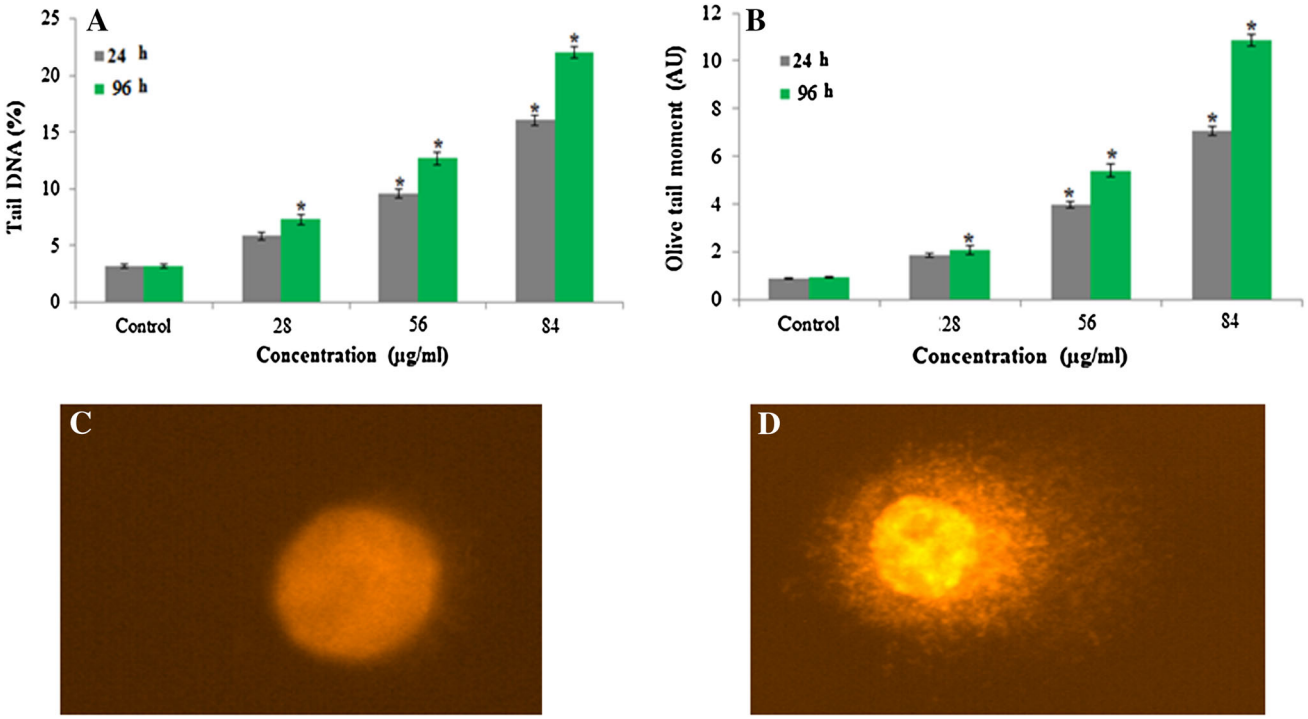

Fig. 6 DNA damage in hemocyte cells of *L. luteola* after exposure of TiO_2NPs. **a** % Tail DNA. **b** OTM. **c** Control. **d** Exposed *L. luteola*. Data are presented as the mean ± SEM of three experiments. *$p < 0.01$ versus control

are often used to monitor aquatic pollution (Ali et al. 2012), so the present study was designed to evaluate the ecogenotoxicological mechanisms of TiO_2NPs in the freshwater snail *L. luteola*.

Nigel and Mitchell (2007) reported that smaller particle increased toxicity due to the chemical reactivity that accompanies greater surface area-to-volume ratio. Before studying the genotoxicity and apoptotic potential of TiO2NPs, we had characterized its zeta potential and size by DLS as well as TEM. However, the size obtained from DLS was greater than the size measured by TEM. The difference in size is due to the fact that different size-determination methods give different results based on the principles used; First, DLS measures Brownian motion and subsequent size distribution of an ensemble collection of particles in solution and gives mean hydrodynamic diameter, which is usually larger than TEM diameter because it includes a few solvent layers; second, during DLS measurement, there is a tendency of particles to agglomerate in the aqueous state, thereby giving the size of clustered particles rather than individual particles; third, DLS reports an intensity-weighted average hydrodynamic diameter of a collection of particles, so any polydispersity of the sample will skew the average diameter toward larger particle sizes.

TiO_2NPs have been widely used in industries, such as electronics, optics, and material sciences, as well as in architecture, medicine, and pharmacology (Liu et al. 2013). In the present study, the ecogenotoxicity of TiO_2NPs was investigated in the freshwater snail *L. luteola*. We found that TiO_2NPs were in sediment at the bottom of beaker. The results of 96-h lethality test showed that TiO_2NPs are toxic to *L. luteola*. However, the long-lasting environmental impact of TiO_2NPs must be investigated because these are continuously introduced to the aquatic environment due to their use in several products.

Production of ROS could cause DNA oxidation and strand breaks leading to a great amount of cell death. Free radicals produced in mitochondria also partially account for these ROS (Valencia and Kochevar 2006). The secondary ROS are detected because singlet oxygen is too short-lived to be scavenged by the probe used for the ROS measurements. The DCF fluorescence in hemocyte cells was increased as concentration and in a time-dependent manner, thus indicating the generation of ROS induced by TiO_2NPs, and this could induce oxidative stress in hemocyte cells. The level of ROS generated by TiO_2NPs was of biological significance as shown by the enhancement of hemocyte cell death. When CAT activity is impaired, H_2O_2 accumulates in the cell and damages several structures including the enzyme itself.

TiO_2NPs activate the apoptotic mechanism in hemocytes. The data indicate that ROS induced by TiO_2NPs might directly account for the decrease in apoptotic mitochondrial membrane potential and that cells undergoing apoptosis and necrosis can facilitate cell renewal. In the current study, the ecotoxic response of TiO_2NPs is accordance with the findings of Lee et al. (2007) in the aquatic sentinel species *Daphnia* and *Chironomus* for CeONPs, SiO_2NPs, and TiO_2NPs. Livingstone et al. (1992) reported that mitochondrial membrane perturbations resulted from interaction with oxygen species in *Mytilus edulis*. Zhao et al. (2003) suggested that even nonoxidant agonists of apoptosis interact through oxidant mechanisms, thus inducing mitochondrial ROS production by breaking lysosomes. Released lysosomal enzymes attack intact lysosomes causing further ruptures; finally, they induce the release of mitochondrial proteins, including cytochrome c, and promote the apoptotic cascade that occurs long after the removal of the oxidant stress (Zhao et al. 2003).

In this study, we observed a decrease in GSH accompanied by the increase of MDA levels. GSH is one of the most important factors protecting from oxidative attacks by ROS, such as LPO, because GSH acts as a reducing agent and free-radical trapper and is known to be a cofactor substrate and/or GSH-related enzyme (Verma et al. 2007). CAT is an extremely important component of intracellular and antioxidant defenses of organisms (Jamil 2001). It reduces H_2O_2 into water and oxygen to prevent oxidative stress and for maintaing cell homeostasis. Many other studies have found varying responses of CAT to increased metal exposure with some organisms exhibiting increased activity, others exhibiting decreased activity, and still others showing no CAT response at all (Regoli et al. 1998). We observed that CAT activity was significantly increased; these data suggest that the increase in antioxidant defenses would be due to enhanced oxygen free radical production, which could stimulate antioxidant activities (Torres et al. 2002) to cope with this increased oxidative stress and protect the cells from damage. The results are in accordance with the findings of Almeida et al. (2004), who found that CAT activity was increased in mussels after exposure to lead. The role of GST is to conjugate tripeptide glutathione with electrophilic and other xenobiotics. Inhibition of GST activity could have occurred either through direct action of the metal on the enzyme or indirectly by way of the production of ROS, which interact directly with the enzyme, deplete its substrate (GSH), and/or downregulate GST genes through different mechanisms (Roling and Baldwin 2006). This might be the reason for the GST activity decrease that was caused in the present study in snails exposed to TiO_2NPs.

Genotoxicity is considered one of the most important end points in most chemical toxicity testing and risk assessment. The migration of DNA observed by alkaline single-cell gel electrophoresis assay is a function of the number of breaks and the increases in tail length based on damage (Singh et al. 1988). The results of this assay, at a concentration of TiO_2NPs of 28 μg/l, show that the genotoxic action of TiO_2NPs causes

less significant damage: Maximum damage was at the highest concentrations at 96 h of exposure. Apoptotic cells have a nucleus of decreased dimensions with DNA projections, the extension of which is proportional to the genetic damage (Rocco et al. 2012). Alkaline single-cell gel electrophoresis assay has been previously used to investigate levels of DNA damage in marine and freshwater bivalves exposed to waterborne pollutants (Pavlica et al. 2001) and silver NPs to freshwater snails (Ali et al. 2014). Many studies have applied alkaline single-cell gel electrophoresis assay to assess DNA damage in cells from a single type of tissue after the exposure of bivalves to a variety of contaminants (Mitchelmore and Chipman 1998). Others have used alkaline single-cell gel electrophoresis assay to measure DNA damage in hemolymph and gill cells in mussels (Rank 1999). Recently a few studies have been performed in snails using alkaline single-cell gel electrophoresis assay (Mohamed 2011). 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). 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. The results obtained from alkaline single-cell gel electrophoresis assay have proved that TiO_2NPs are capable of inducing DNA damage. As a consequence of generation of ROS and DNA damage, TiO_2NPs were mainly responsible for cytotoxicity in hemocyte cells.

Finally it can be concluded that TiO_2NPs induce apoptosis and DNA damage in hemocyte cells of *L. leuteola* by producing ROS in response to NP exposure. Thus, the present work addresses 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.

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