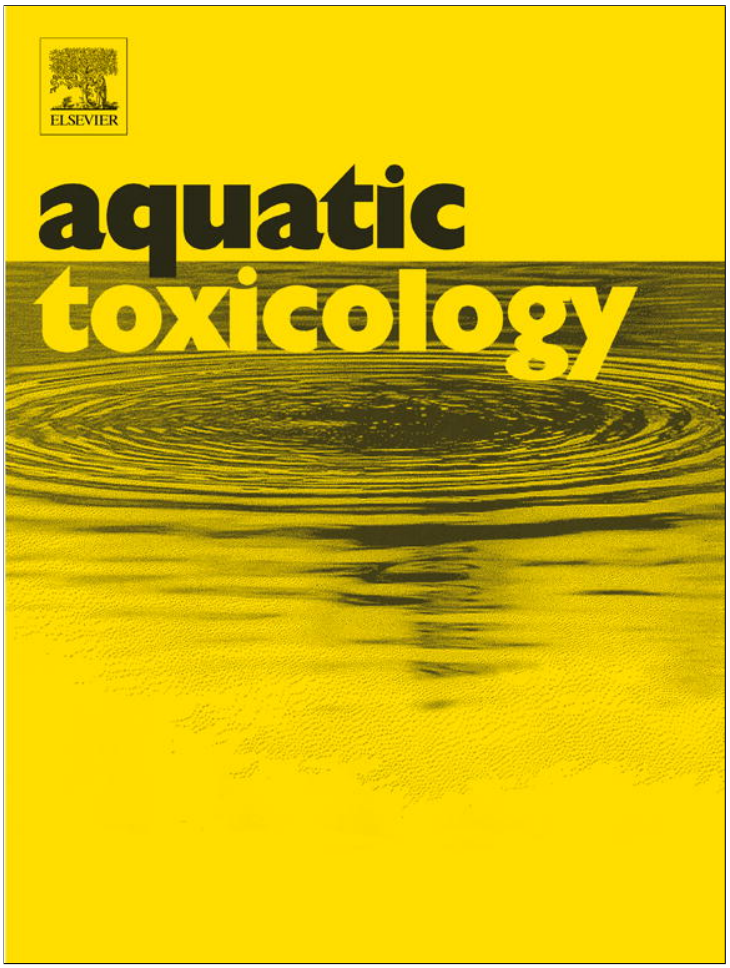

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Oxidative stress and genotoxic effect of zinc oxide nanoparticles in freshwater snail *Lymnaea luteola* L.

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ABSTRACT

Understanding the toxic effects of nanoparticles on aquatic organism is the biggest obstacle to the safe development of nanotechnology. However, little is known about the toxic mechanisms of zinc oxide nanoparticles (ZnONPs) in freshwater snail *Lymnaea luteola* (*L. luteola*). This study was designed to investigate the possible mechanisms of genotoxicity induced by ZnONPs in freshwater snail *L. luteola*. ZnONPs (32 μg/ml) elicited a significant ($p < 0.01$) reduction in glutathione (42.10% and 61.40%), glutathione-S-transferase (25.60% and 40.24%) and glutathione peroxidase (21.73% and 39.13%) with a concomitant increase in malondialdehyde level (54.50% and 57.14%; $p < 0.01$) and catalase (34.88% and 52.56%; $p < 0.01$) in digestive gland of *L. luteola* after 24 and 96 h exposure, respectively. However, a statistically significant ($p < 0.01$) induction in DNA damage was observed by the comet assay in digestive gland cells treated with ZnONPs for 24 and 96 h. Thus, the results demonstrate that ZnONPs induce genotoxicity in digestive gland cells through oxidative stress. Freshwater snail *L. luteola* may be used as suitable test model for nanoecotoxicological studies in future.

1. Introduction

The rapid expansion of nanotechnology has led to a large increase in the number of products containing nano-sized materials. However, the novel physicochemical properties of nanoparticles emphasize the need for proper assessment of its potential effects on aquatic organisms as well as human health. Recently, metal nanoparticles production volumes are highest among metal oxide production (Kumar, 2006). One of the reasons, metal nanoparticles are in high demand is because it is used in different types of hazardous materials such as chlorinated solvents and pesticides (Kumar, 2006). Kumar (2006) reported that ZnONPs efficiently transform hydrogen sulfide (H_2S) to ZnS and dehalogenate chlorinated solvents. Zinc has been reported to be frequently occurring in freshwater habitats at concentrations of approximately 4.50–20 μg/l (Adhikari et al., 2009). ZnONPs have antimicrobial properties and are used in sunscreens, cosmetics, coatings, caulks and adhesive (Australian Academy of Science, 2008). Antimicrobial wallpaper was made by researchers from National Tsing Hua University in Taiwan by coating paper with ZnONPs (Richards, 2006). ZnONPs reach to the aquatic environment through different commercial and consumer applications. So, it is imperative to investigate possible ecological effects of ZnONPs.

Toxicity of metal oxide nanoparticles has been reported in fresh water crustaceans, *Daphnia magna* and Zebra fish. In vivo inhibition or induction of oxidative stress biomarkers is a good ecotoxicological tool to assess the effects of xenobiotics in organisms (McLoughlin et al., 2000). In snails, xenobiotics are transferred by haemocytes to the digestive gland. Gomot (1998) reported the toxicity of metals such as cadmium, mercury, silver, zinc and chromium in *L. stagnalis*. Antioxidant enzymes have been used as a biomarker for metals and organic compounds pollution, which induced oxidative stress in marine and freshwater organisms (Regoli et al., 1997; Doyotte et al., 1997). Zhu et al. (2008) reported that ZnONPs significantly increased mortality and decreased hatchability of Zebra fish embryos. Moreover, most of the ecotoxicity studies on snails have been done by using organism level end-points, such as mortality and growth (Crane et al., 2002). Oxidative stress in terms of reactive oxygen species (ROS) generation is a convenient parameter to measure the toxicity and ecotoxicity, because cells respond to oxidative stress by mounting a number of protective responses that can be easily measured as enzymatic or genetic expression (Regoli et al., 2002; Kovochich et al., 2007). Oxidative stress may manifest as damage to tissue macromolecules, including proteins and DNA (Di Giulio et al., 1989). DNA may in cases show base-pair damages,

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strand cross links and strand breaks as a result of free radical generation, which may be produced directly by chemicals, such as H_2O_2 or other ROS. Comet assay is a promising technique that has been applied to the study of DNA single strand breaks inducing a variety of toxic agents such as chemical compounds, ionizing radiation and NPs in cells and aquatic organisms (Fairbairn et al., 1995; Tice et al., 2000; Collins, 2004; Ali et al., 2008).

Therefore, in the present study, the oxidative stress and genotoxicity of ZnONPs were investigated in freshwater snail *L. Luteola*, which is an important aquatic organism of river, ponds and lakes of South East Asian freshwater ecosystems.

2. Materials and methods

2.1. Chemicals

Zinc oxide nanoparticles (ZnONPs) dry powder (Product No. 677450, APS: 50 nm and SSA: >10.8 m^2/g) was purchased from M/s. Sigma–Aldrich, St. Louis, MO, USA.

Ethylene diamine tetra acetic disodium salt, dimethyl sulfoxide, CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester), thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were procured from M/s. Sigma (St. Louis, MO). All other chemicals used were of the highest purity available from commercial sources.

2.2. Snail collection and culture

Individuals of adult snail (*L. luteola*) of similar size and weight were carefully collected from non-contaminated artificial fish culture ponds situated at Indian Institute of Toxicology Research (IITR), Gheru Campus, Lucknow, and transferred to the laboratory. They were maintained in glass aquaria. Snails were acclimatized to laboratory conditions for 2 weeks before experimentation, at temperature 21 ± 1 °C and fed daily ad libitum with thoroughly washed freshwater green aquatic plant (*Marsilia* sp.) leaves. They had an average wet weight of 500 mg (range 350–650 mg) and shell length 22 mm (range 19–25 mm).

2.3. Characterization of ZnONPs

The optical absorption of the ZnONPs suspension was measured using a double beam UV–vis spectrophotometer (Varian-Cary 300 Bio) in the wavelength range of 200–800 nm at room temperature. The crystalline nature of ZnONPs was carried out by taking X-ray diffraction (XRD) pattern. The XRD pattern of ZnO nanopowder was acquired at room temperature with the help of PANalytical X'Pert X-ray diffractometer equipped with a Ni filtered using Cu K_α (λ = 1.54056 Å) radiations as X-ray source. Structural studies of ZnONPs were done by field emission transmission electron microscopy (FETEM, JEM-2100F, JEOL Inc., Japan) at an accelerating voltage of 200 kV respectively.

The average hydrodynamic size of ZnONPs in water was determined by dynamic light scattering (DLS) (Nano-ZetaSizer-HT, Malvern Instrument, UK). The ZnONPs suspension was sonicated using a sonicator bath at room temperature for 15 min at 40 W and performed the DLS experiments as described by Murdock et al. (2008).

2.4. Determination of Zn^{2+} in test water

Zn^{2+} concentration was determined in dosing solutions with flame atomic absorption spectroscopy (GBC Avanta Ver 2.01). Dosing solution (10, 21 and 32 μg/ml) was acidified, to pH 2, by adding 150 μl concentrated nitric acid to 20 ml of sample. The Zn^{2+} concentration analysis was conducted within 96 h of sample preparation.

2.5. Determination of sub lethal concentrations

The acute toxicity bioassay to determine the LC_{50}-96 h value of ZnONPs was conducted in the semi-static system. The acute bioassay procedure was based on standard methods (APHA et al., 2005). A stock solution of ZnONPs (1 mg/ml) was prepared in deionized water.

A set of 10 acclimatized *L. luteola* specimens was randomly exposed to each of the seven ZnONPs target concentrations (0, 5, 10, 20, 40, 60, 80 and 100 μg/ml) in transparent polystyrene beakers of 200 ml test water and the experiment was repeated twice to obtain the LC_{50}-96 h value of the test ZnONPs for the freshwater snail *L. luteola*. Photoperiod was controlled to simulate the natural day:light cycle (12 h:12 h). Fluorescent light with two 48 W lamps was used as light source.

The LC_{50}-96 h value (42.67 μg/ml) of ZnONPs for *L. luteola* was determined by using the probit analysis method as described by Finney (1971). On the basis of LC_{50}-96 h value, the three test concentrations of ZnONPs viz., concentration I (1/4th of LC_{50} = ∼10 μg/ml), concentration II (1/2nd of LC_{50} = ∼21.33 μg/ml) and concentration III (3/4th of LC_{50} = ∼32.0 μg/ml) were determined.

2.6. In vivo exposure of ZnONPs and isolation of digestive gland cells

The *L. luteola* specimens were exposed to the three aforementioned test concentrations of ZnONPs in a semi-static system. The exposure was continued up to 96 h and isolation of digestive gland cells was done at intervals of 24 and 96 h at the rate of five snails per duration. The snails maintained in tap water were considered as negative control.

The physicochemical properties of test water, namely temperature, pH, total conductivity, dissolved oxygen and total hardness were analyzed by standard methods (APHA et al., 2005).

At each sampling duration, the digestive glands of the exposed snails were quickly removed, washed with ice-cold saline (0.9%), and cleaned from accessory connective adipose tissues. Digestive gland tissues from each group were weighed and homogenized in 10 vol. ice-cold saline solution (w/v ratio) using a polytron homogenizer for 1 min. The homogenates were centrifuged at 5000 rpm for 30 min at 4 °C. The supernatants were used for the measurement of catalase, glutathione peroxidase (GPx), glutathione-S-transferase (GST) activities, lipid peroxidation (LPO) level and glutathione (GSH) content.

2.7. LPO levels

The concentration of malondialdehyde (MDA) as a marker of LPO was determined according to the method of Nair and Turner (1984). Briefly, 0.33 ml of digestive gland homogenate was mixed well with 3 ml of thiobarbituric acid (TBA) reagent that was freshly prepared by mixing 1 vol. of 0.8% TBA to 3 vol. of 20% trichloroacetic acid. The mixture was incubated for 50 min in a boiling water bath. After cooling, the mixture was centrifuged at 3000 × *g* for 10 min. The MDA level was measured spectrophotometrically (Varian-Cary 300 Bio) at 532 nm and the results are expressed as *n* moles of MDA per milligram of wet tissue.

2.8. Reduced GSH contents

Glutathione content as a nonenzymatic antioxidant was measured according to Owens and Belcher (1965) at 412 nm. The assay

mixture consisted of 0.1 ml of the homogenate, 1.5 ml of 0.5 M phosphate buffer, pH 8.0, followed by 0.4 ml of 3% metaphosphoric acid and 30 μl 5′,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) (0.01 M). The amount of reduced GSH present in the digestive glands sample in terms of μg/g of wet tissue was calculated after calibration against the standard curve of GSH.

2.9. Catalase activity determination

Catalase activity was measured following the decrease of absorbance at 240 nm due to H_2O_2 consumption (Beers and Sizer, 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 an aliquot amount of the supernatant. Catalase activity was expressed as units per gram of wet tissue. The unit of catalase is the amount of enzyme that liberates half of the peroxide oxygen from the hydrogen peroxide solution of any concentration in 100 s at 25 °C.

2.10. GPx activity determination

Glutathione peroxidase activity was assayed according to the method described by Chiu et al. (1976). The assay mixture consisted of 2.6 ml of 0.4 M Tris–HCl buffer, pH 8.9, 100 μl of the supernatant, 100 μl of 1 mM GSH, 100 μl of 0.05% cumene hydroperoxide, and 100 μl of 0.01 M Ellman's reagent (DTNB). The mixture was vortexed and incubated at 25 °C for 5 min. GPx activity was monitored at 412 nm and expressed as optical density (O.D.) per gram of protein per minute.

2.11. GST activity determination

Glutathione-S-transferase activity was assayed 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, 20 μl of supernatant, and 2.76 ml of 0.1 M phosphate buffer, pH 7.0, in a final volume of 3.0 ml. The formation of the CDNB–GSH conjugate was evaluated by monitoring the increase in absorbance at 340 nm. Results are expressed as O.D. per gram of protein per minute.

2.12. Determination of DNA strand breakage

The alkaline single cell gel electrophoresis (SCGE) 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 digestive glands of the exposed snails were quickly removed, washed with ice-cold saline (Ca^{2+}, Mg^{2+} free), and cleaned from accessory connective adipose tissues. The tissue was cut into small pieces using scissors and finally homogenized to obtain single-cell suspension. The cell suspension was centrifuged at 3000 rpm at 4 °C for 5 min and the cell pellet was finally suspended in chilled phosphate buffer saline for comet assay. Viability of cells was evaluated by trypan blue exclusion method (Anderson et al., 1994). The samples showing cell viability higher than 84% were further processed for comet assay. In brief, about 15 μl of cell suspension (approx. 20,000 cells) was mixed with 85 μl of 0.5% low melting point agarose and layered on one end of afrosted plain glass slide, pre-coated with a layer of 200 μl normal agarose (1%). There after, it was covered with a third layer of 100 μl low melting-point agarose. After solidification of the gel, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10 with 10% DMSO and 1% Triton X-100 added fresh) overnight at 4 °C. For positive control, the digestive gland cells were treated ex vivo with 100 μM 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 NaOH, 1 mM 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 carried out 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 Tris buffer at pH 7.5 and stained with 75 μl ethidium bromide (20 μg/ml). Two slides were prepared from each specimens and 50 cells per slide (100 cells per concentration) were scored randomly and analyzed using an image analysis system (Komet-5.0, Kinetic Imaging, Liverpool, U.K.) attached to fluorescent microscope (DMLB, Leica, Germany) equipped with appropriate filters. The parameters selected for quantification of DNA damage in the digestive gland cells were percent tail DNA (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.

2.13. 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.

2.14. Statistical analysis

At least three independent experiments were carried out each experiment. Data were expressed as mean (±SE) and analyzed by one-way analysis of variance (ANOVA). p-Value less than 0.01 was considered statistically significant.

3. Results

3.1. Physicochemical characterization of ZnONPs

The UV–vis spectrophotometer showed a sharp absorption band at 390 nm (Fig. 1A). The crystal structure of ZnONPs was characterized by X-ray diffraction (PANalytical X'Pert Pro X-ray diffractometer) with Cu Kα radiation (λ = 0.15418 nm). Fig. 1B shows X-ray diffraction patterns of ZnONPs. The peaks at 2θ = 31.67°, 34.31°, 36.14°, 47.40°, 56.52°, 62.73°, 66.28°, 67.91°, 69.03°, 72.48° and 78.64° were assigned to (1 0 0), (0 0 2), (1 0 1), (1 0 2), (1 1 0), (1 0 3), (2 0 0), (1 1 2), (2 0 1), (0 0 4) and (2 0 2) of ZnONPs, indicating that the samples were polycrystalline wurtzite structure (Zincite, JCPDS 5-0664). No characteristic peaks of any impurities were detected, suggesting that high-quality ZnONPs were synthesized. The average crystallite size (d) of ZnONPs was estimated by Scherrer's formula (Patterson, 1939).

$$d = \frac{K\lambda}{\beta \cos\theta}$$

where K = 0.9 is the shape factor, λ is the X-ray wavelength of Cu Kα radiation (1.54 Å), θ is the Bragg diffraction angle and β is the FWHM of the respective diffraction peak.

The average crystallite size of ZnONPs was found to be around 22 nm. Fig. 1C shows the typical TEM image of ZnONPs. This picture exhibits that the majority of the particles were in polygonal shape with smooth surfaces. TEM average diameter was calculated from measuring over 100 particles in random fields of TEM view. The average TEM diameter of ZnONPs was around 22 nm supporting the XRD data. Fig. 1D represents the frequency of size (nm) distribution of ZnONPs.

The average hydrodynamic size and zeta potential of ZnONPs in water determined by DLS were 264.8 nm and −15.3 mV, respectively (Fig. 1E).

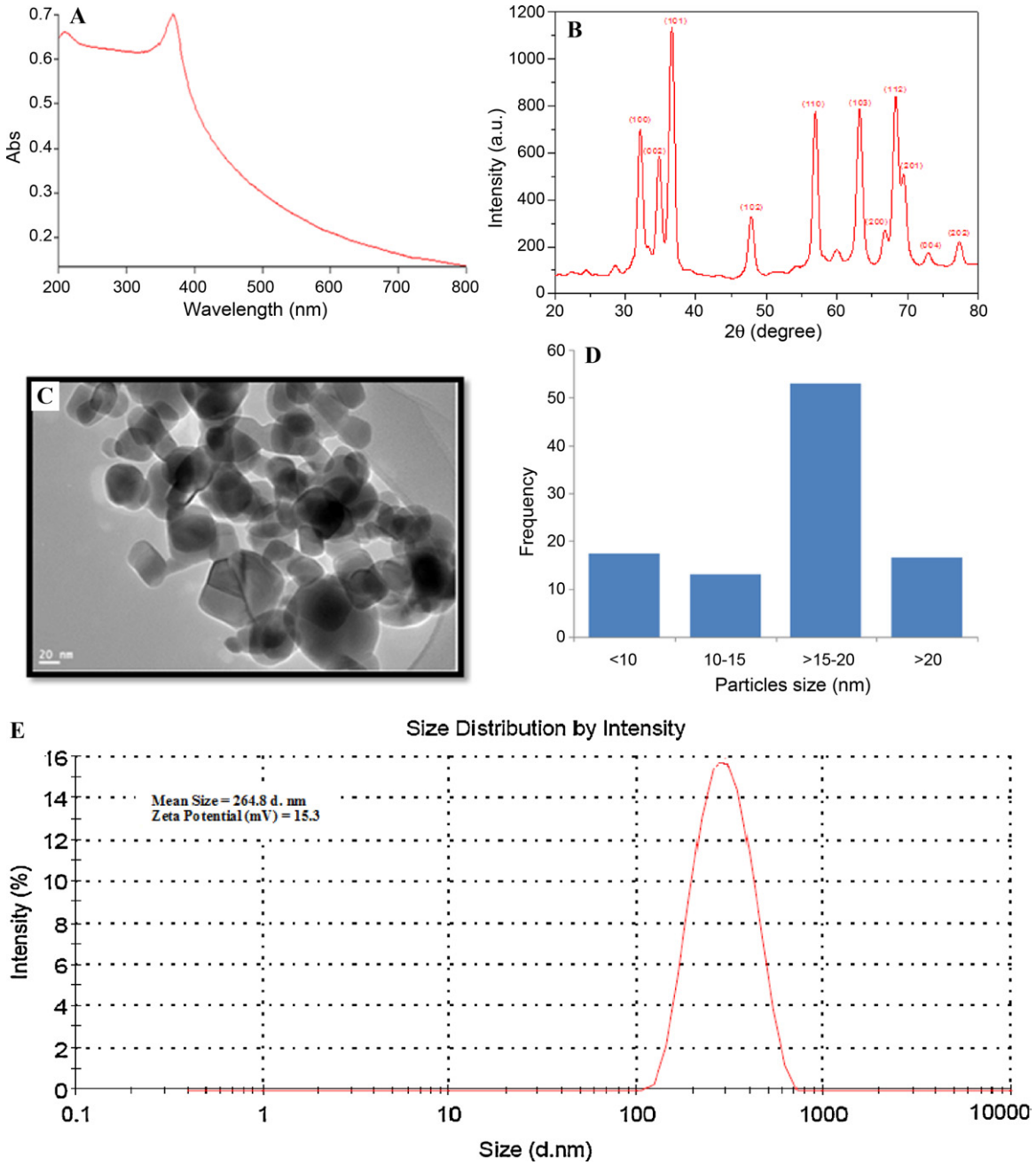

Fig. 1. Characterization of ZnONPs: (A) UV–visible spectrum of ZnONPs and absorbance maxima at 390 nm and narrow peak indicate small size of the particles, (B) XRD pattern of ZnONPs, (C) TEM image, (D) the size distribution histogram generated by using TEM image, and (E) size distribution and zeta potential of ZnONPs were determined using dynamic light scattering (DLS). Analysis was performed from the stock solution.

3.2. Physicochemical analysis of the test water

The water temperature varied from 25.8 to 27.4 °C and pH values ranged from 7.05 to 8.10. The dissolved oxygen concentration was normal, varying from 6.0 to 8.2 mg/l, during experimental period. The conductivity of the water ranged from 244 to 298 μM/cm and chloride from 46 to 56 mg/l. The total hardness ranged from 160 to 180 mg/l and total alkalinity from 259 to 292 mg/l as $CaCO_3$.

3.3. Release of Zn^{2+} concentration

The released Zn^{2+} concentrations in test solution of ZnONPs were measured. Zn^{2+} concentrations in test water were found to be 1.098 μg/ml at lowest and 2.040 μg/ml at highest exposure solution (Table 1).

3.4. ZnONPs induced oxidative stress

The MDA level, GSH content, CAT, GPx and GST activities in the digestive gland of ZnONPs exposed snails were investigated and illustrated in Figs. 2–6.

Sub lethal in vivo exposure of different concentration of ZnONPs to the snails exhibited significant elevation ($p < 0.01$) in MDA level

Table 1
Concentration of Zn^{2+} in ZnONPs dosing solutions.

ZnONPs dose (μg/ml)	Calculated Zn (μg/ml)
Control	>0.001
10	1.098
21	1.552
32	2.040

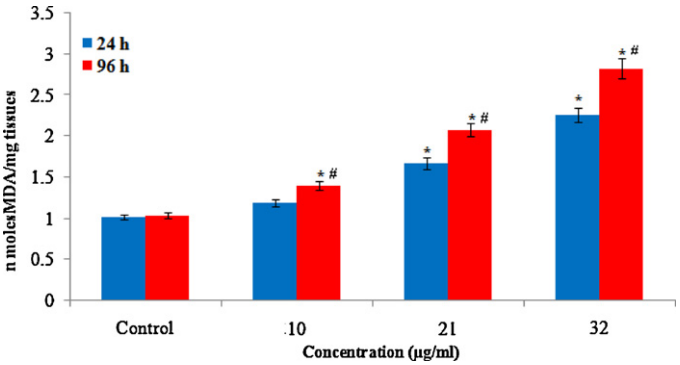

Fig. 2. LPO level in the digestive gland of *L. luteola* snails after 24 h and 96 h of exposure to different concentrations of ZnONPs. Each value represents the mean ± S.E. of three experiments, performed in duplicate. $^{*}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. between durations within concentration.

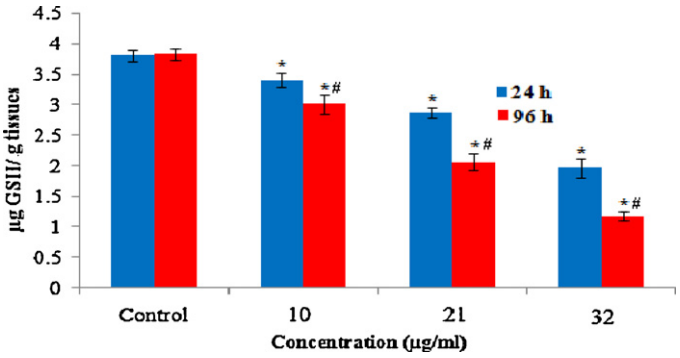

Fig. 3. GSH level in the digestive gland of *L. luteola* snails after 24 h and 96 h of exposure to different concentrations of ZnONPs. Each value represents the mean ± S.E. of three experiments, performed in duplicate. $^{*}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. between durations within concentration.

as compared to the control value, which indicates increased LPO. It was observed that MDA level was elevated as exposure concentration and time increased (Fig. 2).

As shown in Fig. 3, GSH content was significantly ($p < 0.01$) decreased due to ZnONPs exposure in time and concentration dependent manner. The highest reduction in GSH level (68.94%) was observed in snail exposed to 32 µg/ml of ZnONPs at 96 h. Catalase activity in digestive gland of snail was found to be increased significantly ($p < 0.01$) at different treatment concentrations of ZnONPs when compared with control group (Fig. 4). The maximum increased catalase activity was also observed at 32 µg/ml ZnONPs treatment.

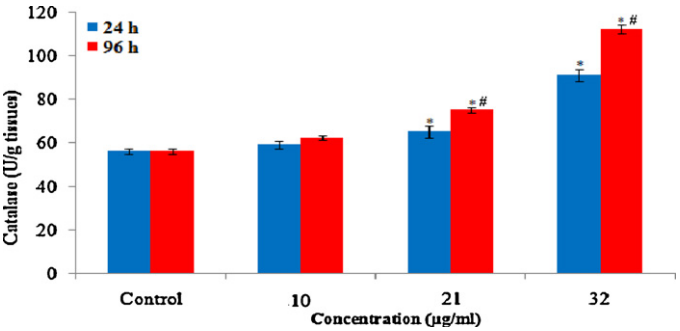

Fig. 4. Catalase activity in the digestive gland of *L. luteola* snails after 24 h and 96 h of exposure to different concentrations of ZnONPs. Each value represents the mean ± S.E. of three experiments, performed in duplicate. $^{*}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. between durations within concentration.

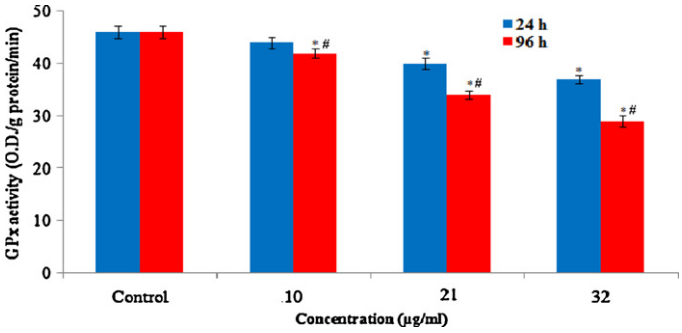

Fig. 5. GPx activity in the digestive gland of *L. luteola* snails after 24 h and 96 h of exposure to different concentrations of ZnONPs. Each value represents the mean ± S.E. of three experiments, performed in duplicate. $^{*}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. between durations within concentration.

Treatment with different concentrations of ZnONPs caused significant ($p < 0.01$) reduction in GPx and GST activities in the digestive gland of snails. GPx activity was highly reduced by ZnONPs at the highest concentration (Fig. 5). The highest reduction in GST activity (42.16%) was also observed at the highest concentration of ZnONPs at 96 h (Fig. 6).

3.5. DNA damage

The DNA damage was measured as % tail DNA and olive tail moment in the control as well as exposed cells. During electrophoresis, the cell DNA was observed to migrate more rapidly toward the anode at the highest concentration than the lowest concentration. The cells exposed to different concentrations of ZnONPs, exhibited significantly ($p > 0.01$) higher DNA damage in cells than those of the control groups (Fig. 7). The gradual nonlinear increase in DNA damage was observed in cells as concentration and time exposure of ZnONPs increased and the highest DNA damage was recorded at 32 µg/ml ZnONPs treated snails for 96 h (Fig. 7a, b and d).

4. Discussion

The fresh water snails are often used to monitor aquatic pollution (Dallinger, 1994), so it is imperative to study the toxic mechanisms of ZnONPs in freshwater snail *L. luteola*. In the present study we observed that ZnONPs induce oxidative stress and DNA damage in digestive gland of *L. luteola* in dose and time dependent manner. The digestive gland (hepatopancreas) of gastropod molluscs is the key organ of metabolism and it is concerned with the production of digestive enzymes, absorption of nutrients,

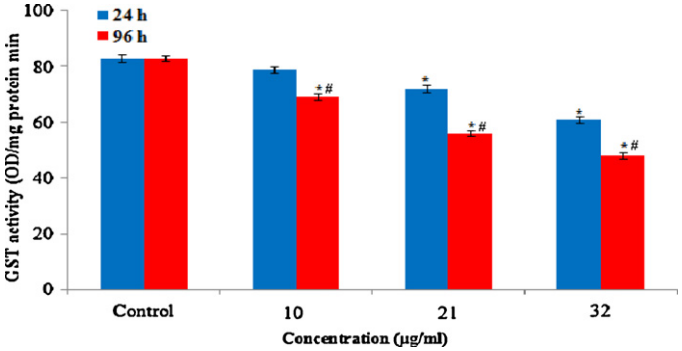

Fig. 6. GST activity in the digestive gland of *L. luteola* snails after 24 h and 96 h of exposure to different concentrations of ZnONPs. Each value represents the mean ± S.E. of three experiments, performed in duplicate. $^{*}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. between durations within concentration.

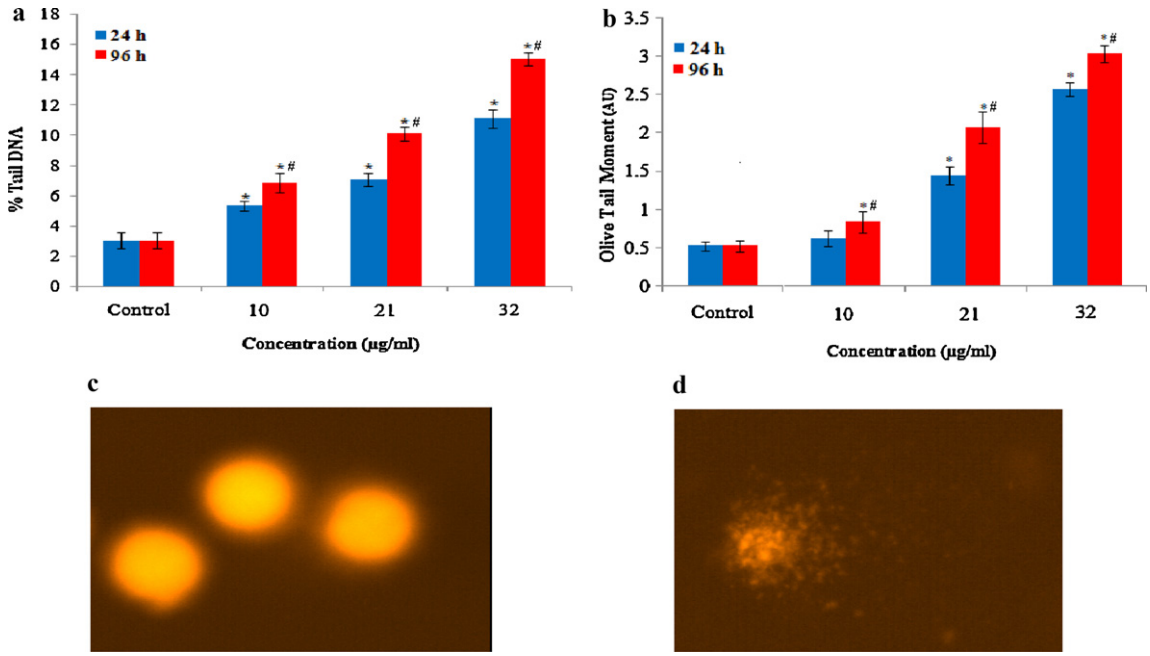

Fig. 7. DNA damage in the digestive gland of *L. luteola* snails after 24 h and 96 h of exposure to different concentrations of ZnONPs. (a) % Tail DNA, (b) olive tail moment (arbitrary unit), (c) control cell and (d) exposed cell. Each value represents the mean ± S.E. of three experiments, performed in duplicate. $^{*}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. between durations within concentration.

endocytosis of food substances, food storage and excretion (Morton, 1983; Dallinger et al., 2002). Livingstone et al. (1992) reported that digestive gland is major site of biotransformation of xenobiotic and oxy-radical-generating enzymes in snail. Pollutants accumulation through different routes are transported by haemocytes to the digestive gland (Beeby and Richmond, 2002; Regoli et al., 2005). So in the present study, the digestive gland was used to investigate the biochemical responses of ZnONPs. A general pathway of toxicity of many environmental pollutants is mediated by enhancement of intracellular ROS, which induce cell damage (Regoli et al., 2002). LPO is a complex process in which polyunsaturated fatty acids of biological membrane system undergo changes by chain reactions and form lipid hydroperoxides, which decompose double bonds of unsaturated fatty acids and disrupt membrane lipid (Gutteridge, 1995). The measurement of MDA content (an index of LPO) provides a relative potential effect of ZnONPs to cause oxidative injury. Nusetti et al. (2001) reported that LPO levels increased during exposure to metals in several organisms. In the present study, significantly elevated levels of LPO in the digestive gland of *L. luteola* snails indicate that some cell have damaged. Our result coincides with results of Viarengo et al. (1990) study in which LPO level increased in mussel tissues due to copper metal exposure. Glutathione is a tripeptide non enzymatic antioxidant with a single cysteine residue and constitutes an important pathway of the antioxidant and detoxification defence. Chemical compounds, such as trace metals, are biotransformed to a conjugate of GSH. It is also a cofactor of many enzymes catalyzing the detoxification and excretion of several toxicants, which will be destroyed in the cytosolic and mitochondrial compartments by GPx in the presence of GSH (Doyotte et al., 1997). The decrease in GSH content in the digestive gland appears to be a common response of molluscs to metal exposure, partly explained by the high affinity of zinc metal for the GSH molecule (Regoli and Principato, 1995). Chandran et al. (2005) reported that GSH level decreased in the digestive gland and kidney in zinc-treated *Achatina fulica* snails. In this study, we observed decline of GSH content accompanied by elevation of LPO levels. GSH is one of the most important factor protecting from oxidative attacks by reactive oxygen species, because GSH acts as a reducing agent and free-radical trapper and is known to be a cofactor substrate and/or GSH-related enzymes (Verma et al., 2007).

The antioxidant catalase is an important component of intracellular and antioxidant defences of organisms (Jamil, 2001). It reduces the H_2O_2 into water and oxygen to prevent oxidative stress and for maintaining cell homeostasis. Many studies have found varying responses of catalase to increased metal concentrations, with some organisms exhibiting increased activity, others exhibiting depressed activity, and still others showing no catalase response at all (Regoli et al., 1998). In the present study, we observed that catalase activity was significantly increased; this data suggests that the increase in antioxidant defence would be due to enhanced oxygen free radicals production, which could stimulate antioxidant activities (Torres et al., 2002) to cope with increased oxidative stress and protect the cells from damage. The obtained results are in accordance with the findings of Almeida et al. (2004), who found that catalase activity was increased in mussels after exposure to lead.

Glutathione peroxidase (GPx) is the most important peroxidase for the detoxification of hydroperoxides (Orbea et al., 2000). However, the decreased activities of GPx might be due to over production of ROS, especially O_2^-, by the ZnONPs and depletion of its substrate level (GSH). GPx activity in the present study is in accordance with the findings of Chandran et al. (2005) in the digestive gland and kidney of Zn-treated *A. fulica* snails. It is also reported that under high rates of free radicals input, enzyme inactivation prevails and the enzymatic activities are reduced, leading to autocatalysis of oxidative damage process (Escobar et al., 1996).

The role of GST is to conjugate tripeptide glutathione with electrophilic and other xenobiotics. Inhibition of GST activity has occurred either through direct action of metal on the enzyme or indirectly via the production of ROS that interact directly with the enzyme, depletion of its substrate (GSH) and/or down regulation of GST genes through different mechanisms (Roling and Baldwin, 2006). This explanation might be the reason for the GST activity decrease that was caused in the present study in the case of snails exposed to ZnONPs. Similarly, Regoli et al. (1997) reported that GST activity significantly reduced in aquatic organisms due to

copper. Changes in the level of antioxidants have been proposed as biomarker of a contaminant-mediated prooxidant challenge in a variety of invertebrates (Regoli et al., 2002). Genotoxicity is considered one of the most important toxic endpoints in chemical toxicity testing and risk assessment; however, little is known about the genotoxicity of ZnONPs, especially toward aquatic organisms *L. luteola*. The results of the comet assay suggested that ZnONPs may provoke DNA damage in *L. luteola*. It is also observed that oxidative stress may be one of the probable cause in DNA damage. 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.

In conclusion, present data demonstrate that ZnONPs induces genotoxic effects in a fresh water snail *L. luteola* which may be mediated through lipid peroxidation and oxidative stress. This investigation point out ecological implications of ZnONPs release in aquatic ecosystems and warranted to regulatory agencies and industry for the need of monitoring and regulation regarding ZnONPs.

Conflict of interest

There is no conflict of interest.

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