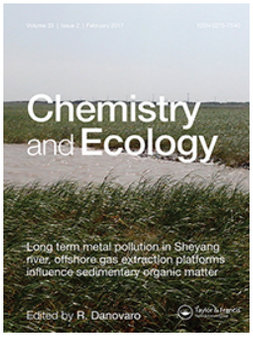

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Genotoxicity in the freshwater gastropod *Lymnaea luteola* L: assessment of cell type sensitivities to lead nitrate

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ABSTRACT

The aquatic ecosystems are converting into the highly contaminated site due to environmental pollutants. The present study explores the oxidative stress and toxic potential of lead nitrate in freshwater snail *Lymnaea luteola* (*L. luteola*) L. The snails were exposed to an environmentally relevant concentration of lead nitrate for 24 and 96 h. Later exposure to lead nitrate (0, 10, 20 and 40 µg/mL) to the freshwater snail, the level of reactive oxygen species, malondialdehyde and nitric oxide (NO) were increased and glutathione, glutathione-S-transferase were decreased. Lead-nitrate-induced haemocyte cell death and it was observed by using Annexin-V FITC/PI through a flow cytometer. DNA damage in haemocyte cells was measured at above doses of lead-nitrate exposure for 24 and 96 h and it was compared to the untreated snail. Average tail DNA (%) and olive tail moment in single-cell gel test were increased dose and duration fashion and maximum DNA damage was measured at 96 h. These results indicate the potential toxicity and genotoxicity of lead nitrate in acute treatment to *L. luteola* and single-cell gel test are the assay for rapid detection of genetic effects.

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KEYWORDS
Snails; comet assay; oxidative stress; apoptosis

1. Introduction

The diverse harmful health effect upon exposure to toxic heavy metals in the environment is a matter of serious concern and a global issue. Various types of metals are used in industry and industrial effluents are directly discharged into aquatic ecosystems (rivers, ponds, etc.). Heavy metals are highly toxic to the aquatic organism and animals. Aquatic pollution due to lead is considered as severe environmental threats to the aquatic organism. Lead does not have any remarkable roles in animals. Lead-induced toxicity on developing a nervous system [1]. Several researchers reported that lead-induced oxidative stress and decreased glutathione [2]. Accidental discharge of harmful chemicals into the environment has potential to disrupt the function and structure of normal ecosystems and

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pose a hazard to living organisms. Fu et al. [3] reported that lead-nitrate damage DNA as single and double strand breaks producing chromosome aberrations.

In vitro and *in vivo* biomarker tests have used to determine mutagenic and genotoxic effects of metal pollutants, but single-cell gel electrophoresis (comet assay) is a rapid, sensitive and widely used parameter under laboratory and field conditions [4]. Gedic et al. [5] and Tice et al. [6] had been reported that comet assay is capable of measuring DNA damage produced by oxidising and alkylating intercalating agents at the small amount (0.1 DNA/10 Da). Fresh water snail is used as sentinel biological model for aquatic pollution and has an important role in determining the harmful risk of contamination in aquatic ecosystems. Several numbers of gastropods snail species have applied for the study of the teratogenic and genotoxic effect of environmental pollution [7]. Due to diverse ecological nature, geographical distribution and availability of *Lymnea luteola* (*L. luteola*), throughout the year, has made it an important test model for aquatic pollution.

However, the availability of environmental pollutants in an aquatic ecosystem is the main concern to the health of the aquatic organism; so toxicity of heavy metals need to be explored prior their release to the aquatic environment. In the current observations, we have determined genotoxicity, apoptosis and oxidative stress due to acute exposure of lead nitrate to *L. luteola*.

2. Materials and methods

2.1. Chemicals and experimental specimens

For the current study, technical-grade lead nitrate ($Pb(NO_3)_2$; 99.9% EC) procured by Hi-Media Laboratories Pvt. Ltd. Mumbai, India. Snail saline buffer (5 mM HEPES, 3.7 M NaOH, 36 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, pH 7.8), ethylene diamine tetra-acetic disodium salt, dimethyl sulphoxide, H_2DCFDA, propidium iodide, Annexin V FITC were purchased from M/s. Sigma (St. Louis, MO).

Freshwater snail *Lymnaea luteola* were collected from unpolluted ponds with the help of fisherman. The snail had mean shell length 20.5 mm and wet weight 0.51 g. Snails were adapted for two weeks under lab condition at 22°C in static renewal system and everyday nursed *ad libitum* with carefully washed freshwater leaves of *Marsilea* sp. prior to treatments of lead nitrate.

2.2. Exposure to lead nitrate

Snails were treated with three concentrations (0, 10, 20 and 40 µg/mL) for 96 h in a static renewal system. Unexposed snails were used as a control. The sampling was done at intervals of 24 and 96 h at the rate of 5 snails per dose and duration.

The physicochemical properties of test water (e.g. temperature, pH, total conductivity, dissolved oxygen and total hardness) was analysed by standard methods [8]. On each sampling time, the whole haemolymph was collected through the haemal pore and immediately processed for estimation of apoptosis, ROS production, genotoxicity and oxidative stress biomarkers.

2.3. Viability of haemocyte

Haemolymph was collected by gentle prodding of foot forced to snails to retract into their shell, thus extruding haemolymph [9]. Viability of haemocyte was determined by trypan blue exclusion test [10]. Haemograms for both control and exposed snails were expressed as the average number of cell (cells/mm^3) of haemolymph.

2.4. Quantitation of ROS

Determination of reactive oxygen species in isolated haemocytes of snail was done by using CM-DCFH-DA according to Ali et al. [11] method.

Briefly, 10 mM CM-DCFH-DA was diluted in SSB as a working solution (5 mM). Isolated haemocytes cells of treated and untreated snail were incubated with H2-DCFH-DA (5 mM) at room temperature for 30 mn and washed with PBS. Haemocytes cells were fixed on slide and image was observed by upright fluorescence microscope (Nikon Eclipse 80i).

Isolated haemocytes cells (10^3/well) were placed with H2-DCFH-DA (5 mM) in black bottom culture plate (96 well) and incubated for 30 mn. Then it was washed with PBS. The intensity of fluorescence was quantified at the excitation wavelength (495 nm) and emission (525 nm).The results are presented as % of fluorescence intensity relative to control wells. Control represents fluorescence value 100%.

2.5. Oxidative stress markers

2.5.1. Lipid peroxidation (LPO)

LPO was measured by production of malondialdehyde according to Ohkawa et al. [12] method. Haemolymph (330 µL) was added to thiobarbituric acid (3000 µL) reagent and it was freshly made by mixing in 1:3 of thiobarbituric acid (0.8%) and trichloroacetic acid (20%). The mixture was incubated for 65 minutes (mn) in a boiling water bath. After cooling, the mixture was centrifuged at 3000×*g* for 10 mn. The MDA level was measured by a spectrophotometer (Varian-Cary 300 Bio) at 532 nm and the results were expressed as mM MDA per mL.

2.5.2. Glutathione (GSH)

GSH was measured by Owens and Belcher [13] method. The test mixture consists of 0.1 mL of hemolymph, 1.5 mL of 0.5 M phosphate buffer, pH 8.0, followed by 0.4 mL of 3% meta-phosphoric acid and 30 µL DTNB (0.01 M). The amount of reduced GSH present in the hemolymph sample in terms of mg/dL of hemolymph was calculated after calibration against the standard curve of GSH.

2.5.3. Glutathione-S-transferase

It was measured by Vessey and Boyer [14]. The reaction mixture contained 0.2 mL of 4 mM GSH, 20 µL of 0.25 mM CDNB, 20 µL of hemolymph 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 at 340 nm.

Nitric acid was measured by Montgomery and Dymock [15] method and superoxide dismutase was determined according to Misra and Fridovich [16]. The content of protein was measured by Bradford method [17].

2.6. Assessment of apoptosis by Annexin V FITC

The apoptotic producing capacity of lead nitrate was analysed by annexin V FITC test kit (ab14085, Abcam® discover more UK). Haemolymph of control and exposed snail were taken in glutaraldehyde and made a suspension in binding buffer (1×, 150 µL) and mixed 5 µL annexin-V and 10 µL PI. After 30 mn 350 µL binding buffer (1×) added and shifted into Fluorescence-activated cell sorting (FACS) tube for analysis. Acquisitions of results were done in a flow cytometer (Becton Dickinson, USA) using 'CELL Quest' software. For each concentration examined, 10,000 events were assessed in the triplicate test.

2.7. DNA damage test

Test for DNA damage in haemocyte of *L. luteola* was performed by method proposed by Singh et al. [18].

2.8. Statistical analysis

Results were outfitted using excel and presented as average ± SE. ANOVA (one-way analysis of variance) was applied to compare significance. A p value of less than .01 was considered statistically significant.

3. Results

3.1. Test water quality

The physiochemical characteristics of test water are listed in Table 1. Temperature ranged from 25°C to 27°C during experimentation. The pH of the water ranged from 7.6 to 7.8, which was slightly higher than neutral. Dissolved oxygen ranged from 6.8 to 7.9 mg/L.

3.2. Cytotoxicity of haemocyte cells and production of ROS

The viability of haemocyte cells are determined by Trypan blue by using haemocytometer and it observed that 97.5% cells are live. Percent of live cells are more decreased at 40 µg/mL of lead nitrate in 96 h (Figure 1).

Table 1. Average and standard deviation values of physiochemical properties of the test water.

Parameters	Value
Temperature (°C)	26.48 ± 0.37
pH	7.6–7.8 ± 0.11
Dissolved oxygen (mg/L)	6.8–7.9 mg/L ± 0.4
Total hardness (as $CaCO_3$)	145–160 mg/L± 8.20

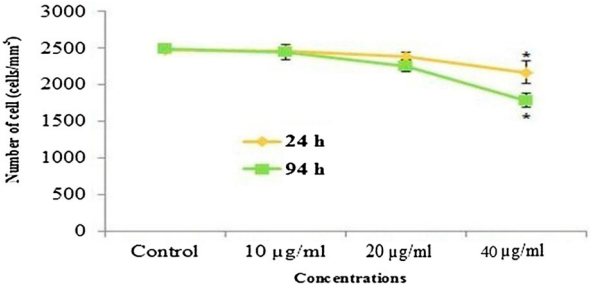

Figure 1. Changes circulating hemocyte cells in *L. luteola* due to lead nitrate. *$p < .01$ vs. control. Number of experimental observation (n) = 3.

Later exposure to lead nitrate generates reactive oxygen species in haemocyte cells was determined by using DCFDA fluorescent reagent. The ROS generation was significantly increased in haemocyte cells of exposed snails (Figure 2A–C). Figure 2D showed a significant correlation between ROS generation and DNA damage at different doses of lead nitrate within exposure time.

3.3. Oxidative stress

Haemocyte concentration of malondialdehyde points out lipid peroxidation (Figure 3A).It was hiked concentration and time basis. Alterations in the activities of GSH, GST, NO and SOD in lead-nitrate-exposed snail were illustrated in Figure 3B–E.

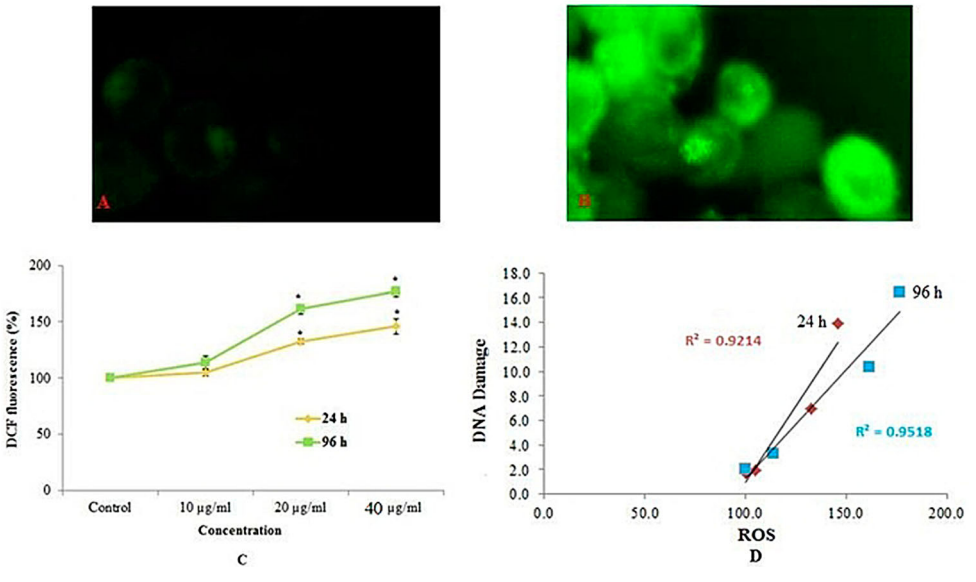

Figure 2. Photographs showing lead-nitrate-induced ROS generation in hemolymph of *L. luteola*. **A.** Control **B.** 40 µg/mL of lead nitrate **C.** ROS generation (%). **D.** Significant correlation between ROS generation and DNA damage. *$p < .01$ vs. control. Number of experimental observation (n) = 3.

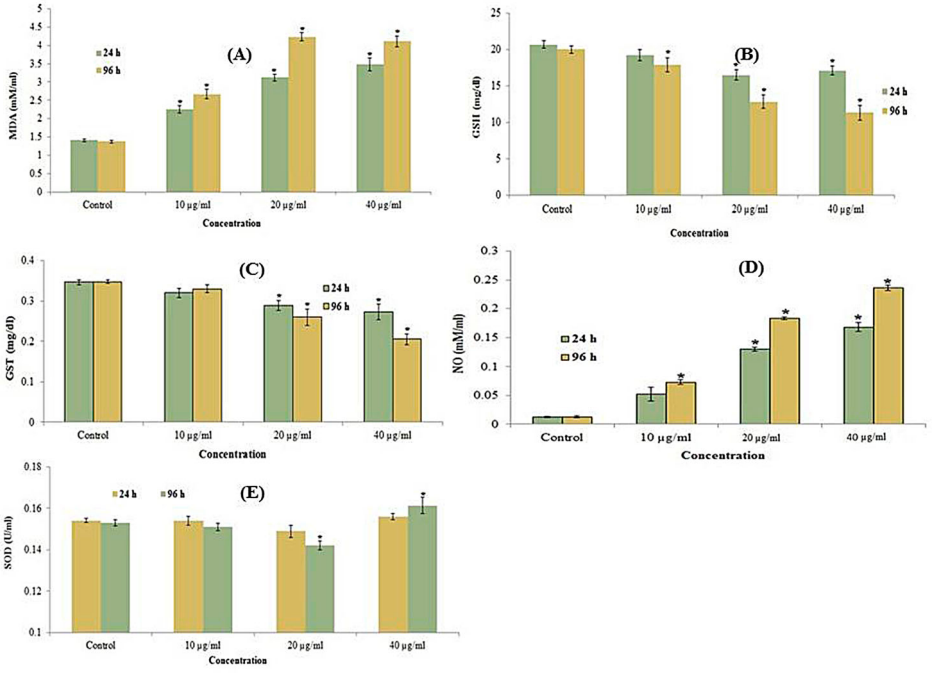

Figure 3. A. LPO **B.** GSH **C.** GST **D.** Nitric oxide (NO) **E**. SOD activity in hemolymph after exposure of lead nitrate to *L. luteola*. *$p < .01$ vs. control. Number of experimental observation (n) = 3.

3.4. Determination of apoptosis through FACS

After exposure of lead nitrate, the apoptotic and necrotic cell death were determined by Annexin V FITC and PI through the flow cytometer and results are illustrated in Figure 4. After treatment of lead nitrate (40 µg/mL) for 24 and 96 h, it was found that Annexin V positive (apoptotic) cells were high at 24 h; on the other hand propidium iodide positive (necrotic) cells populations were high at 96 h (Figure 4A–D).

3.5. DNA fragmentation

Genotoxic effect of lead nitrate was quantified by comet test and the DNA fragmentation was measured in the unit % tail DNA and OTM. We found that fragmentation of DNA occurred in a dose- and time-dependent manner when compared to control (Figure 5).

4. Discussion

Industrial waste and urban sewage water are discharged into rivers, lakes and coastal waterways; thus, it is unavoidable that industrial metals and byproducts enter into aquatic systems. One of the main reasons of aquatic pollution is the discharge of heavy metals, while its toxic effects were explored some decade years ago. Osman [19] first reported alkaline single-cell gel test and this test have been accepted as a suitable test to measure genotoxic effects in invertebrates. Low DNA damage at 10 µg/mL of lead nitrate occurred in snail but slightly increased with the extension of treatment

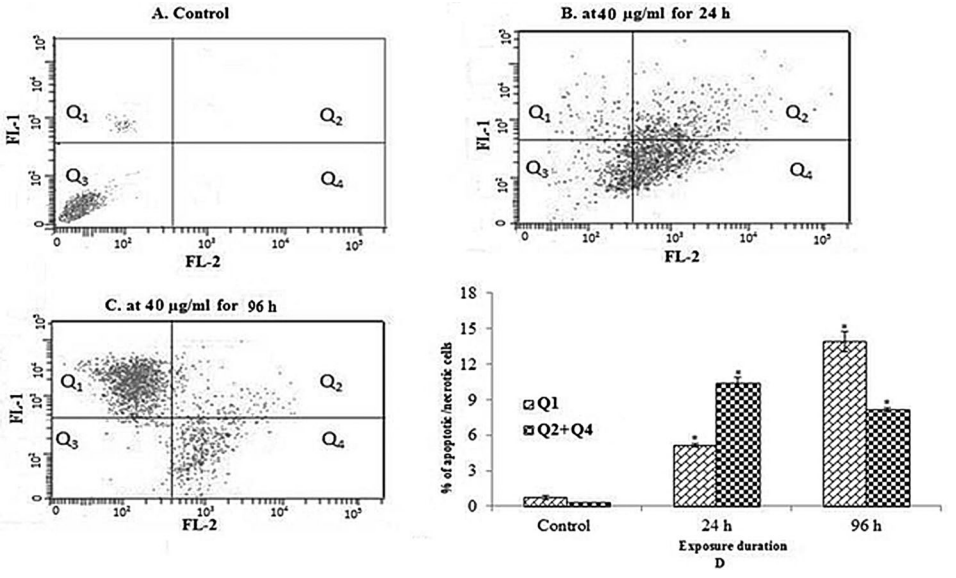

Figure 4. Lead nitrate (40 µg/mL) induced apoptosis in hemocyte cells of *L. luteola*. **A–D**, flow cytometric analysis of annexin V-FITC/PI stained cells. *$p < .01$ vs. control. Number of experimental observation (n) = 3.

duration. This occurrence might have activated antioxidant system against ROS or activation of DNA repair in *L. luteola*. DNA repair system could be involved in the accumulation of toxicant above the threshold level in invertebrates [20]. Russo et al. [21] reported controversy in the genotoxic mechanism of heavy metals. Genotoxic effects

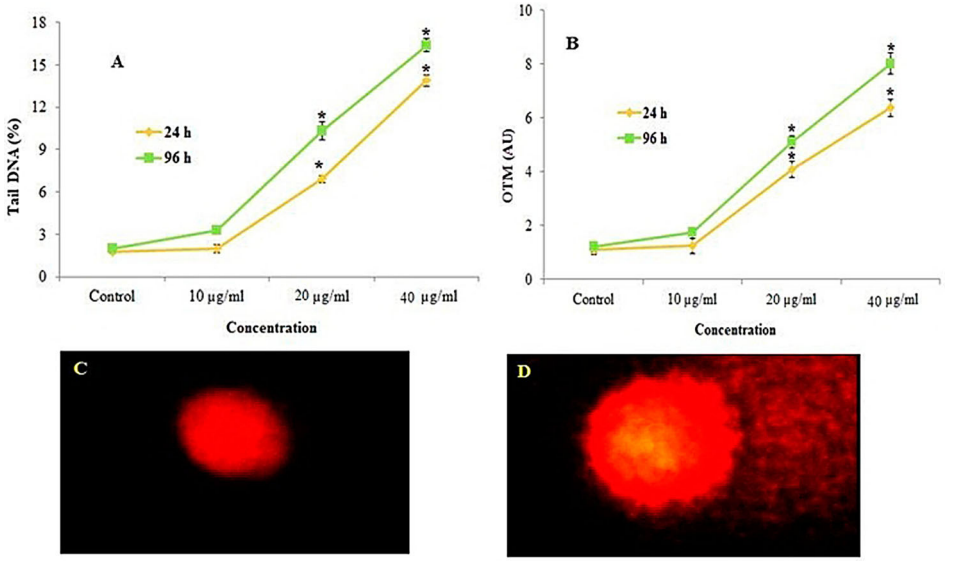

Figure 5. DNA damage in *L. luteola* after exposure of lead nitrate. **A.** % tail DNA **B.** OTM **C.** Control **D.** Exposed. *$p < .01$ vs. control. Number of experimental observation (n) = 3.

of metals may be an accumulation of nucleic-acid-damaging-free radicals or clastogenic and aneugenic action in aquatic organisms [22]. We have observed abnormal behaviour in exposed snail in accordance with the finding of Gomot [23] in freshwater snail *Lymnaea stagnalis* due to cadmium toxicity. Toxicity of lead may depend on many factors such as pH of test water, snail species, size, dissolved oxygen and total hardness. In the present study, lead-nitrate-induced reactive oxygen species is increased with the increase of sampling duration, and concentration of lead nitrate. Overall results from this observation demonstrate that lead-nitrate-induced oxidative stress. Our results indicated inhibition in SOD activity at a lower dose of lead nitrate, whereas a significant increase was observed at a higher dose of lead nitrate in the haemolymph of snails. SOD activity was increased at a higher concentration; it may be due to a result of GSH declination or induction of enzyme. The inhibition of the SOD activity after exposure to low concentration of lead nitrate indicates oxidative stress, which may arise due to an imbalance in ROS formation and the antioxidant defence system of the cells [24]. Increased activity of SOD removed radicals' anion in snails. NO and SOD are very strong biomarkers of stress and may be used as indicators for early challenges of aquatic pollution. These results validate with the findings of earlier researchers report that described heavy metals have the possibility to produce oxidative stress. DNA damage in haemocyte of snail proved that lead nitrate not only induces cell damage through the generation of ROS but induced apoptosis and genetic instability. Thus, lead-nitrate-induced apoptosis and DNA fragmentation (Figures 4 and 5). Thus our study indicated that Annexin V staining and comet assay are sensitive in finding apoptosis and genotoxicity in lead-nitrate-treated snail, and effects were found to be dependent on concentrations and duration.

This study supports the view that *L. luteola* is sentinel bioindicators for sub-tropical and tropical ecosystems. Oxidative stress and DNA damage were sensitive biomarkers of lead nitrate toxicity in aquatic organisms and require complete investigation with other industrial pollutants, organic contaminants and heavy metals.

Disclosure statement

No potential conflict of interest was reported by the authors.

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