

# Oxidative Stress and Genotoxicity of Zinc Oxide Nanoparticles to *Pseudomonas* Species, Human Promyelocytic Leukemic (HL-60), and Blood Cells

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**Abstract** In the present study, toxicity of commercial zinc oxide nanoparticles (ZnO NPs) was studied on the bacterium *Pseudomonas* sp., human promyelocytic leukemia (HL-60) cells, and peripheral blood mononuclear cells (PBMC). The toxicity was assessed by measuring growth, cell viability, and protein expression in bacterial cell. The bacterial growth and viability decreased with increasing concentrations of ZnO NP. Three major proteins, ribosomal protein L1 and L9 along with alkyl hydroperoxides reductase, were upregulated by 1.5-, 1.7-, and 2.0-fold, respectively, after ZnO NP exposure. The results indicated oxidative stress as the leading cause of toxic effect in bacteria. In HL-60 cells, cytotoxic and genotoxic effects along with antioxidant enzyme activity and reactive oxygen species (ROS) generation were studied upon ZnO NP treatment. ZnO NP exhibited dose-dependent increase in cell death after 24-h exposure. The DNA-damaging potential of ZnO NP in HL-60 cells was maximum at 0.05 mg/L concentration. Comet assay showed 70–80% increase in tail DNA at 0.025 to 0.05 mg/L ZnO NP concentration. A significant increase of 1.6-, 1.4-, and 2.0-fold in ROS level was observed after 12 h. Genotoxic potential of ZnO NPs was also demonstrated in PBMC through DNA fragmentation. Thus, ZnO NP, besides being an essential element having antibacterial activity, also showed toxicity towards human cells (HL-60 and PBMC).

**Keywords** ZnO NP · Cytotoxicity · MTT · Protein expression · ROS · DNA damage

## Introduction

Increasing innovations in nanotechnology is leading to the development of new materials with unusual properties. These materials are collectively termed as engineered nanoparticles (ENPs). In this class, metal oxide nanoparticles are gaining attention due to their increasing applications in commercial products like cosmetics, medicines, food processing, wastewater treatment, and biocides [1–4]. Zinc oxide nanoparticles (ZnO NPs) have excellent UV-adsorbing property, which makes it an important ingredient as UV protector in sunscreens as well as paints [1, 5]. The antibacterial potential of ZnO NP is utilized in food packaging and toothpastes [3]. The increasing production and use of ZnO NP in consumer products have raised the concern for its presence and effects on the environment.

Numerous reports are available on the toxic effects of ZnO NP on different organisms ranging from prokaryotes to mammalian cells [6–9]. In these studies, low concentrations of ZnO NP showed toxic effects in its nano as well as ionic form. The nano dimension of ZnO resulted in lethal effects due to interference with vital processes of the organisms. However, the molecular mechanisms through which ZnO NP elicits toxic effects are yet to be determined.

Different studies have reported the antibacterial effects of ZnO NP. Size-dependent antibacterial effects of ZnO NP on different strains of *Staphylococcus*, *Streptococcus*, and *Bacillus subtilis* indicated that smaller sizes of ZnO NP inhibited growth by 95%, while larger particle sizes reduced growth by only 40–50% [9]. Synergistic antibacterial effect of ZnO NP, along with different antibiotics like ceftazidime,

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tobramycin, and ciprofloxacin, has been reported in *Pseudomonas aeruginosa*. Significant inhibition in bacterial growth was observed at concentrations of 23.4 and 46.8 µg/mL of ZnO NP with other antibiotics in clinical and standard strains of bacteria [10]. Reddy et al. [11] studied ZnO NP toxicity in prokaryotic and eukaryotic cells. Both Gram-negative and Gram-positive bacteria were found to be susceptible to very low ZnO NP concentration, whereas primary human T cells were resistant at similar concentration. Premanathan et al. [12] also reported the antibacterial activity of ZnO NP in both Gram-negative and Gram-positive bacteria. The study revealed higher toxicity of ZnO NP towards Gram-positive bacteria. However, there are no reports on the effects of ZnO NP on protein expression in bacteria.

ZnO NP has been reported to be cytotoxic and genotoxic to different mammalian cells. The toxicity of nanoparticle depends on particle composition, size, and solubility [13]. In human bronchial epithelial cells (BEAS-2B), ZnO NP elicited cytotoxicity and oxidative stress with increase in intracellular calcium ion concentration [14]. ZnO NP resulted in reactive oxygen species (ROS) generation and interleukin 8 (IL-8) release in human colon carcinoma cells (Caco 2) [15]. Premanathan et al. [12] studied toxicity of ZnO NP on human promyelocytic leukemic (HL-60) cells and normal lymphocytes. The study illustrated that cancer cells are more sensitive to ZnO NP as compared to normal cells. ROS generation and apoptosis were suggested as the mechanisms of toxicity in cancer cells.

In the present study, the toxic effect of ZnO NP on soil beneficial bacterium *Pseudomonas* sp. FPC 951 was studied through growth, cell viability, and protein expression analysis. The toxicity of ZnO NP was also studied in the human promyelocytic cells (HL-60) and peripheral blood mononuclear cells (PBMC) to understand the mechanism of ZnO NP toxicity in human cells. ZnO NP cytotoxicity to human cells was observed through cell viability assay, ROS generation, and antioxidant enzyme activity, while the DNA-damaging potential was monitored through DNA fragmentation, comet assay, and cytokinesis block micronucleus (CBMN) assay.

## Materials and Methods

### Chemicals

Highly purified water-dispersed ZnO NPs (50 wt%) were purchased from Sigma-Aldrich (USA). A 50 mg/L ZnO NP working stock solution was made in double-distilled water (Millipore, USA) and analyzed by inductively coupled plasma-optical emission spectroscopy (iCAP 6000, Thermo, USA). Human promyelocytic lymphoma (HL-60) cells were purchased from the National Center for Cell Sciences (NCCS), Pune, India. Normal-melting-point agarose, low-

melting agarose, Trizma base, ethylene diamine tetraacetic acid (EDTA), ethidium bromide, cytochalasin B, and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). Fetal bovine serum (FBS), phytohemagglutinin (PHA), and antibiotics were purchased from Gibco (USA). ROS/reactive nitrogen species (RNS) assay kit (Cell Biolabs, USA) and microtiter plates (Corning, Costar, USA) were purchased for biochemical assays. Triton X-100 and dimethyl sulfoxide (DMSO) were purchased from Fischer Scientific (USA).

### Bacterial Strain and Cell Viability

A *Pseudomonas* strain was isolated from soil and used as a representative soil beneficial bacterium for studying the toxicity of silver nanoparticles (AgNPs) in our previous article [16]. The same isolate was used as the model bacterial system for the current study. In brief, the bacterial culture was grown overnight in minimal medium with 0.1% glucose at 37 °C and 150 rpm as reported earlier [17]. It was inoculated to optical density of 0.1 at 600 nm ( $OD_{600}$ ) in minimal medium and exposed to increasing ZnO NP concentrations (0, 0.05, 5, 10, 15, and 20 mg/L). The flasks were incubated for 24 h in a shaker incubator at 37 °C. The growth was monitored after every 3 h in a spectrophotometer at 600 nm. During exponential growth, the specific growth rate was determined using the following equation [18]:

$$x = x_0 e^{\mu_{\max} t}$$

where

$x_0$	Cell dry weight (g/L) at time 0
$X$	Cell dry weight (g/L) at time $t$
$\mu_{\max}$	Maximum specific growth rate ( $\text{h}^{-1}$ )
$t$	Time (h)

MTT assay was used to determine the viability of bacteria. The cells were treated with increasing ZnO NP concentration as used for the growth assay. Samples were harvested at 3-h interval, and MTT (1.28 mg/mL) was added and incubated at 37 °C for 1 h. The cells were then harvested, and formazan crystals formed by MTT reduction were dissolved in DMSO, and color development was monitored at 550 nm in a microplate reader (Infinite 200 PRO, Tecan).

### Protein Expression Study

Bacterial cells were exposed to different concentrations of ZnO NP (0, 5, 10, 15, 20 mg/L) for 24 h and collected by centrifugation in three biological replicates as reported in our earlier study [16]. In brief, cells were suspended in lysis buffer (8 M urea, 4% [(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.03 M Tris, pH 8.0) and disrupted by

sonication in ice (Vibra-Cell, Sonics). Cell extract was collected after centrifugation, and the protein concentration was measured using the Bradford reagent [19].

Of protein samples, 50  $\mu\text{g}$  was applied to 12% SDS-PAGE as reported earlier [16]. The protein bands were separated through electrophoresis for 12 h at 60 V in stacking gel and 80–100 V in resolving gel. The gel was stained with silver stain and photographed using the Gel Doc system (Bio-Rad, USA). The protein bands with consistent differential expression between control and ZnO NP-exposed samples were cut from the gel. These bands were tryptic digested for matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. The MALDI-TOF MS spectra were recorded at AIRF facility at Jawaharlal Nehru University, India.

### Cell Culture

Peripheral blood mononuclear cells (PBMC) were obtained from healthy human donor (nonsmoker, nonalcoholic, and under no medication) following the ethical approval from the research ethics committee and standards laid down in 1964 with latest amendments of Declaration of Helsinki [20]. Cells were seeded in cell culture tubes with RPMI 1640 medium supplemented with 20% FBS, 100 U/mL of penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, 250 ng/mL amphotericin B, and 1% PHA. HL-60 cells were seeded into cell culture flasks with the same medium composition excluding PHA. The cells were incubated in 5%  $\text{CO}_2$  atmosphere at 37 °C for 24 h prior to treatment with ZnO NP. HL-60 cells were used for cytotoxicity and genotoxicity assays, while PBMC cells were used for genotoxicity testing through micronucleus assay. Deionized water was used as a control in all the assays. All experiments were executed in triplicate.

### Cell Viability Assay

HL-60 cell viability was measured using MTT assay. MTT forms purple-colored formazan crystals after interacting with the living cells [21]. HL-60 cells were plated in 96-well plate at a density of  $1 \times 10^4$  cells in 200- $\mu\text{L}$  medium per well. The cells were treated with increasing ZnO NP concentrations (0.008, 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1, and 2 mg/L) for a period of 24 h. At the end of exposure, 20  $\mu\text{L}$  of MTT solution (0.5 mg/mL) was added and cells were incubated at 37 °C for 3 h. The cells were then settled by centrifugation at  $1000 \times g$  for 5 min, supernatant was discarded, and 200  $\mu\text{L}$  of DMSO was added to solubilize the formazan crystals. Absorbance was recorded at 550 nm using a microplate reader (Infinite 200 PRO). The percent cell viability was measured with respect to control cells that were considered to be 100% viable. The experiment was carried out in triplicate.

### DNA Fragmentation

HL-60 cells were seeded in 25-cm<sup>2</sup> flask at a cell density of  $2 \times 10^5$  cells/mL medium and treated with different concentrations of ZnO NP (0, 0.01, 0.025, and 0.05 mg/L) for 24 h. After exposure period, the cells were harvested and washed twice with phosphate-buffered saline. The cell pellet was lysed in 950  $\mu\text{L}$  lysis buffer (Tris EDTA buffer, pH 8.0, 10% SDS, and 20 mg/mL proteinase K) for 2 h at 55 °C. RNA was removed by incubating the sample with 10 mg/mL of RNase A enzyme at 37 °C for 1 h. The resulting lysate was extracted using phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). DNA was precipitated from the aqueous phase with ethanol and resuspended in Tris EDTA buffer (pH 8.0). DNA fragmentation was observed by agarose gel electrophoresis in 1.2% agarose gel at 80 V.

### Genotoxicity Assays

#### *Alkaline Single-Cell Gel Electrophoresis (Comet Assay)*

The DNA-damaging potential of ZnO NP was studied in HL-60 cells using comet assay as described by Singh et al. [22]. HL-60 cells were seeded at a density of  $2 \times 10^5$  cells/mL in the RPMI-1640 medium in 24-well plate. The cells were treated with different concentrations of ZnO NP (0, 0.01, 0.025, and 0.05 mg/L) for 24 h. Deionized water was added as negative control. The cells were harvested by centrifugation and washed with phosphate-buffered saline. The cells were then resuspended in 100  $\mu\text{L}$  of low-melting agarose and spread on base slides coated with 1% normal-melting agar. Then, the cells were lysed with lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10) for 1 h followed by treatment with alkaline buffer (0.3 M NaCl, 1 mM EDTA) for 40 min in the dark. The slides were electrophoresed at 20 V and 300 mA for 30 min and then neutralized with neutralization buffer. The slides were stained with ethidium bromide and analyzed on a fluorescence microscope. One hundred comets were analyzed from each sample, including control, in triplicate. The percent tail DNA was determined using the Komet 5.5 software.

#### **CBMN Assay**

CBMN assay was carried out using the protocol described by Fenech [23]. In brief, PBMC cells were incubated in RPMI-1640 medium supplemented with 20% FBS and 1% PHA (v/v) for 24 h to stimulate cell division. This was followed by treatment with different concentrations of ZnO NP (0, 0.01, 0.025, and 0.05 mg/L) and deionized water as control. At 44 h, cytochalasin B was added to arrest the cell division. The cells were further incubated for a period of 24 h and then harvested for micronucleus study. The cells were kept in hypotonic

solution for 5 min, washed, and fixed in methanol/acetic acid fixative (3:1) for 20 min at 4 °C. The cells were further incubated with fixative overnight. Finally, the cells were harvested and stained with Giemsa for 10 min. The slides were dried and then scored for number of micronuclei per thousand binucleated cells.

## Oxidative Stress Assays

### Intracellular ROS Measurement

Free radicals induced in HL-60 cells after 24-h ZnO NP treatment (0, 0.01, 0.025, and 0.05 mg/L) were quantified with the fluorescent indicator 2',7'-dichlorofluorescein diacetate (DCFHDA) using OxiSelect In Vitro ROS/RNS Assay Kit. In brief, lysate and assay standard were added to wells with catalyst to accelerate the oxidation reaction. After 5-min incubation, the DCFHDA probe was added to all wells and incubated further for a period of 15–45 min. The fluorescence was then read in a microplate reader at 480-nm excitation/530-nm emission. The free radical content in the cell was represented as fold change with respect to control.

### Antioxidant Enzyme Activity

HL-60 cells were treated with increasing ZnO NP concentrations (0, 0.01, 0.025, and 0.05 mg/L) for 24 h. After harvesting, the cells were washed with phosphate buffer and lysed using a sonicator (Sonics Vibra Cell) for 2 min (5-s on, 5-s off). The lysate was centrifuged at 12,000 rpm for 10 min at 4 °C, and protein concentration was measured in the supernatant using the Bradford method [19]. Supernatant was used to measure the antioxidant enzyme activities.

Superoxide dismutase (SOD) activity was measured as percent inhibition of nitroblue tetrazolium (NBT) reduction using the method described by Janknegt et al. [24]. Fifty micrograms

of protein was used for measuring the SOD activity. The reaction mix containing 0.05 M phosphate buffer,  $5.9 \times 10^{-5}$  M NBT,  $9.9 \times 10^{-3}$  M methionine,  $1.17 \times 10^{-6}$  M riboflavin, and 0.025% Triton X 100 was mixed with the cell protein. The reaction mix was exposed for 10–15 min in bright light. The absorbance of resulting blue color was read at 560 nm.

Catalase activity was measured using the method of Jing et al. [25]. Fifty micrograms of protein in 50 mM phosphate buffer (pH 7.0) was used for the assay with 10% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The decrease in absorbance of  $\text{H}_2\text{O}_2$  was recorded at 240 nm. The activity of catalase was expressed after normalization to cell count.

Peroxidase activity was measured using 0.05 M pyrogallol in 0.1 M phosphate buffer (pH 6.5) with 1%  $\text{H}_2\text{O}_2$  as described by Luna et al. [26]. The assay mixture consisted of 0.5 mL of 1%  $\text{H}_2\text{O}_2$  in 3 mL pyrogallol-phosphate buffer and 0.02 mL of enzyme extract. The absorbance was read at interval of 30 s for 3 min at 430 nm.

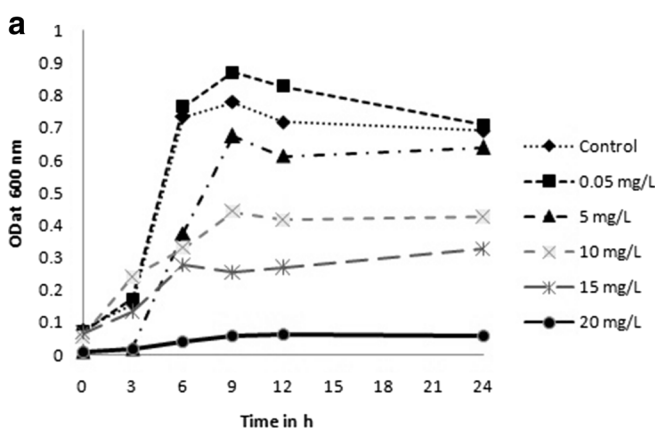
### Statistical Analysis

The results obtained were expressed as mean  $\pm$  standard error. The effect of ZnO NP was estimated by comparison to negative control in each assay at the statistical significance level of 0.05.

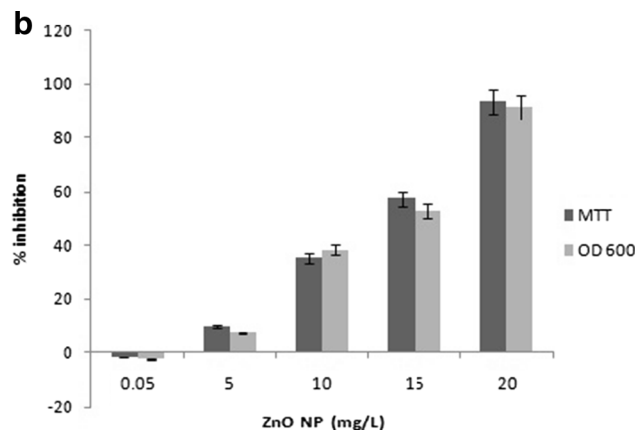
## Results

### ZnO NP Antibacterial Action

The growth of bacterial cells was monitored at increasing concentrations of ZnO NP (0, 0.05, 5, 10, 15, 20 mg/L) through  $\text{OD}_{600}$  measurement. No significant change in growth of bacteria was observed at 0.05 and 5 mg/L of ZnO NP, but growth declined with increasing ZnO NP concentrations (Fig. 1). The specific growth rate for control was  $0.5 \text{ h}^{-1}$ ,



**Fig. 1** **a** Antibacterial activity of ZnO NP at different concentrations (0, 0.05, 5, 10, 15, 20 mg/L) on *Pseudomonas* sp. **b** Percent inhibition in growth of *Pseudomonas* sp. at different concentrations of ZnO NP (0,



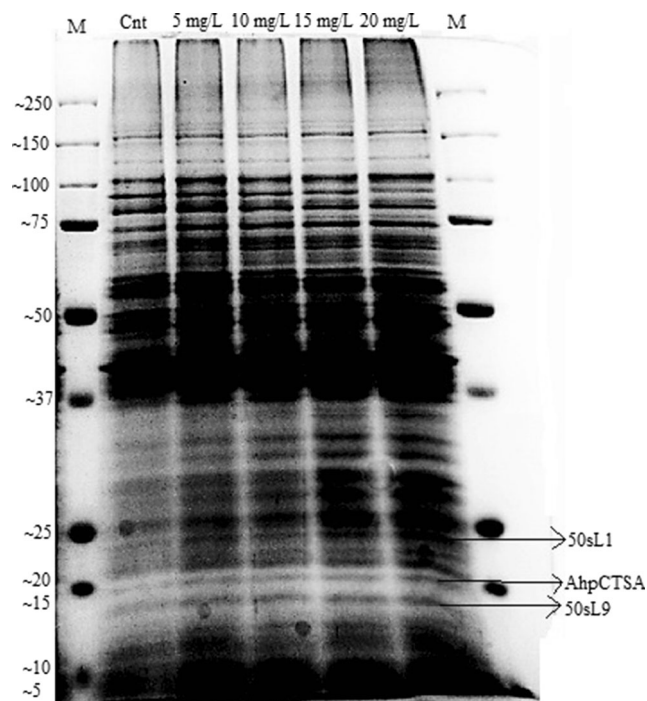
0.05, 5, 10, 15, 20 mg/L) using  $\text{OD}_{600}$  and MTT measurements. Error bar shows mean  $\pm$  standard error



which decreased to  $0.1 \text{ h}^{-1}$  at  $20 \text{ mg/L}$  ZnO NP concentrations. ZnO NP effect on bacterial cell viability was also determined after 24-h exposure using the MTT assay. Toxic effects were prominent above  $10 \text{ mg/L}$  concentration. Cell growth and viability results were comparable from both  $\text{OD}_{600}$  and MTT assays (Fig. 1).

### ZnO NP Effect on Protein Expression in *Pseudomonas* sp.

Bacterial protein expression was studied by the SDS-PAGE and MALDI-TOF analysis. The bacterial cells were grown in minimal medium with 0, 5, 10, 15, and  $20 \text{ mg/L}$  ZnO NP for 24 h. Cellular protein was extracted, electrophoresed on SDS-PAGE, and visualized by silver stain (Fig. 2). The differentially expressed proteins were tryptic digested and identified using MALDI-TOF. The major upregulated proteins were 50S ribosomal proteins L1, L9, and alkyl hydroperoxide reductase-thiol-specific antioxidant (AhpC/TSA) family protein (Table 1). AhpC/TSA and ribosomal proteins L1 and L9 showed increased expression by 2.0-, 1.5-, and 1.7-fold, respectively, in ZnO NP-exposed cells ( $p < 0.05$ ) in comparison to control, as analyzed by protein density measurement.



**Fig. 2** SDS-PAGE gel depicting upregulation of protein bands in *Pseudomonas* sp. on exposure to 5, 10, 15, and  $20 \text{ mg/L}$  of ZnO NP with respect to control after 24 h. 50S ribosomal proteins L1 and L9 and AhpC/TSA-alkyl hydroperoxide reductase C/thiol-specific antioxidant were overexpressed. M 250-kDa protein marker

### Cytotoxicity of ZnO NP in HL-60 Cells

HL-60 cells were used for the determination of cytotoxic potential of ZnO NP. Increasing concentration of ZnO NP ( $0.008\text{--}2 \text{ mg/L}$ ) was used to generate dose-response curve. The cytotoxicity of ZnO NP was compared against control, and percent viability was calculated. The  $\text{LC}_{50}$  was found to be  $0.05 \text{ mg/L}$  (Fig. 3).

### DNA Fragmentation

The genotoxic effect of ZnO NP was studied through DNA fragmentation, which is a hallmark of apoptosis. DNA fragmentation was evaluated after 24-h treatment with different concentrations of ZnO NP (0, 0.01, 0.025, and  $0.05 \text{ mg/L}$ ). Increase in DNA fragmentation with increasing concentration of ZnO NP was observed through agarose gel electrophoresis (Fig. 4).

### Genotoxicity of ZnO NP

The percentage of DNA damage was assessed by comparing % tail DNA in treated cells with that of control. The % tail DNA showed an increase of 70–80% with increasing concentration of ZnO NP as compared to control (Fig. 5a). Chromosomal damage was further substantiated by the CBMN assay. The average number of micronuclei was found to be  $0.5 \pm 0.01$  per thousand binucleated cells in control cells, while the exposed cells showed  $2.75 \pm 0.02$  micronuclei per thousand binucleated cells at  $0.05 \text{ mg/L}$  concentration of ZnO NP (Fig. 5b).

### Oxidative Stress Measurement

To measure ZnO NP-induced oxidative stress, time-dependent experiment was performed at different concentrations of ZnO NP (0, 0.01, 0.025, and  $0.05 \text{ mg/L}$ ). ROS was found to be higher at 12 h as compared to 24 h of ZnO NP exposure (Fig. 6). It was observed that ZnO NP induced significant increase of 1.4-, 1.6, and 2.0-fold in ROS level after 12-h exposure to 0.01, 0.025, and  $0.05 \text{ mg/L}$  of ZnO NP, respectively. At 24 h, the ROS level showed an increase of 1.2-, 1.3-, and 1.6-fold at above ZnO NP concentrations, respectively. This could be due to increase in cell death with time. ROS generation in cells normally leads to alteration in the level of antioxidant enzymes like SOD, catalase, and peroxidase. Hence, antioxidant enzyme activity was also evaluated after ZnO NP exposure. SOD showed no significant difference with respect to control. Peroxidase and catalase enzymes showed decrease in activity with increasing concentration of ZnO NP as compared to control. Peroxidase enzyme showed a decrease of 0.83- to 0.4-fold at 0.025 and  $0.05 \text{ mg/L}$  ZnO NP, respectively (Fig. 7a). Similarly, catalase enzyme showed a

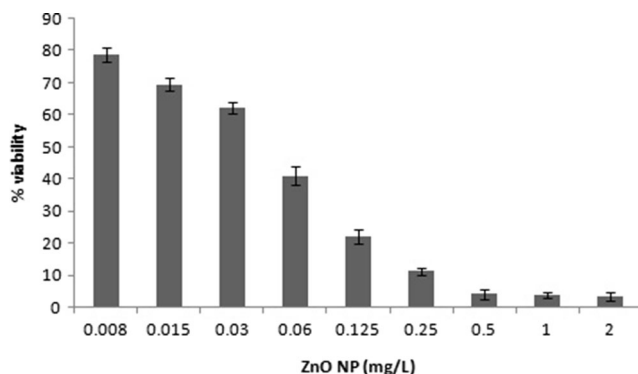
**Table 1** Protein spots overexpressed in *Pseudomonas* sp. after ZnO NP exposure as identified by MALDI-TOF

Spot no.	Protein name	Accession no.	Mascot score (MALDI)	Fold change
1	50S ribosomal protein L1	Gi/26987185	110	1.5
2	Antioxidant AhpC/TSA family protein	Gi/26987820	94	2.0
3	50S ribosomal protein L9	Gi/270265017	86	1.7

decrease of 0.6- to 0.3-fold at 0.025 and 0.05 mg/L ZnO NP, respectively (Fig. 7b). The decreasing enzyme activity is an indication of the inability of cells to protect themselves against ZnO NP-mediated ROS.

## Discussion

Increasing use and environmental discharge of nanoparticles like ZnO NP have raised concerns about their impact on humans and other living organisms. In humans, the nanoparticles have been reported to enter the circulatory system and spread throughout the body. Therefore, toxicity of nanoparticles to blood cells is currently a very important topic of research [27]. ZnO NPs have also been proposed to be effective in cancer treatment. Hence, several studies are being undertaken to assess the effect of ZnO NP on different cancer cells in comparison to primary human cells [28]. The current study used human promyelocytic lymphoma cells (HL-60) as the cancer cell line and PBMC as primary cells to study the effect of commercially available ZnO NP. Further, the toxicity of ZnO NP towards ecologically important native soil microorganisms is understudied. The current study attempted to determine the toxicity of ZnO NP against natural soil microflora. *Pseudomonas* sp. constitutes predominant fraction of soil microflora. Many organic pollutants are reported to be degraded by *Pseudomonas* sp., and they also protect plants from pathogens [29–31]. Hence, a soil isolate, *Pseudomonas* sp. FPC 951, was selected as the model organism in this study as reported earlier [16].



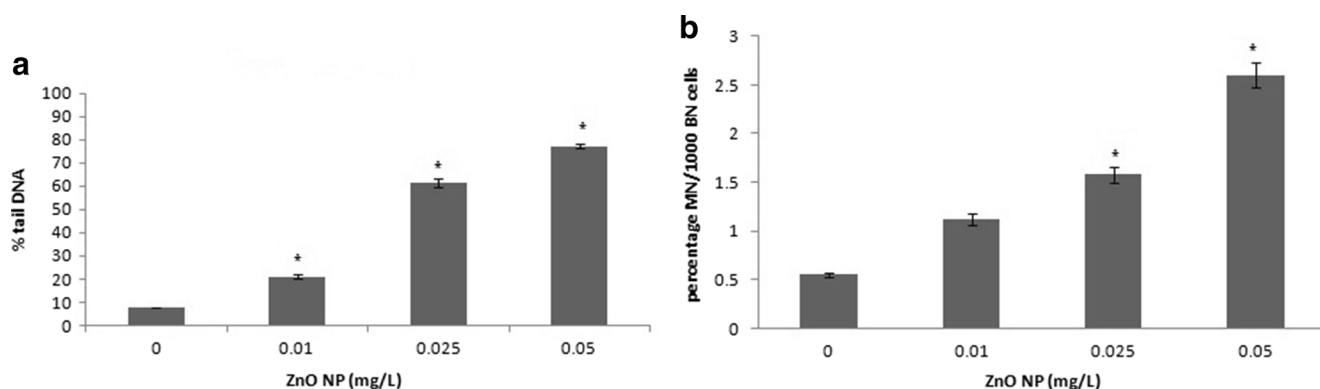
**Fig. 3** Viability of HL-60 cells exposed to different concentrations of ZnO NP (0.008 to 2 mg/L) for 24 h using MTT assay. Data represents mean  $\pm$  standard error

The growth and cytotoxicity study revealed that ZnO NPs are toxic to *Pseudomonas* sp. However, the toxicity was lower than the earlier reported AgNP toxicity to the same isolate. The difference in toxicity of these two nanoparticles may be due to chemical composition or other parameters such as nanoparticle size, charge, and morphology, which were different for both the nanoparticle preparations [16].

Three proteins namely 50S ribosomal proteins L1 and L9 and AhpC/TSA showed upregulation in the presence of ZnO NP. As reported earlier, ribosomal proteins are involved in structural and stress regulatory functions with translational regulation [16]. 50S ribosomal protein L1 is one of the largest proteins located opposite the L7/L12 stalk of ribosomal subunit. It regulates its own expression in *Escherichia coli* and *Methanococcus* sp. by binding to its messenger RNA (mRNA) [32]. 50S ribosomal protein L9 is also a structural protein associated closely with the same L7/L12 complex just beneath the L1 protein [33]. This protein interacts with amino acid addition p-site of tRNA and mRNA [34]. L1 and L9 protein expressions upon ZnO NP exposure may indicate the toxic effects of ZnO NP on translation process.



**Fig. 4** DNA fragmentation pattern in HL-60 cells exposed to increasing concentration of ZnO NP for 24 h. C control, T1 0.01 mg/L, T2 0.025 mg/L, T3 0.05 mg/L of ZnO NP, and M 100-bp DNA marker



**Fig. 5** **a** Comet assay in HL-60 cells treated with ZnO NP (0, 0.01, 0.025, and 0.05 mg/L) for 24 h. **b** Cytokinesis block micronucleus assay in peripheral blood mononuclear cells (PBMC) treated with

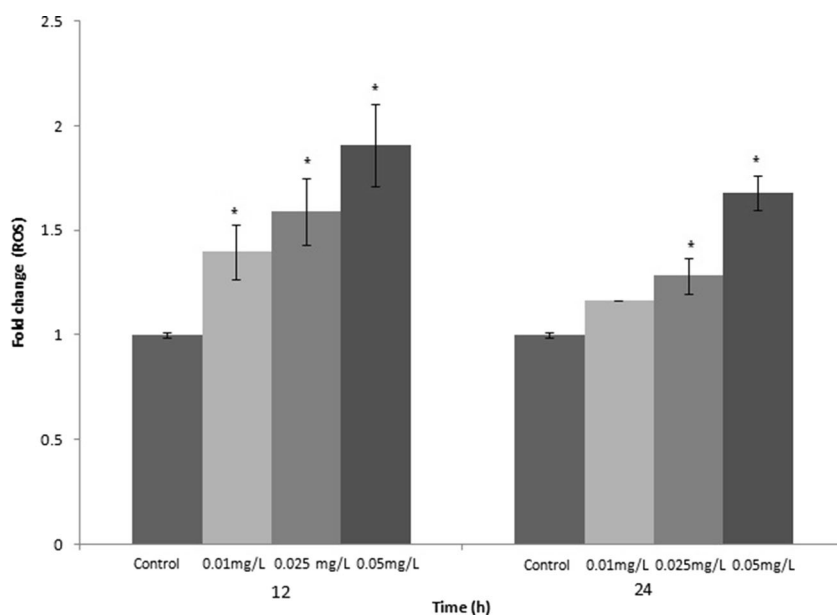
different concentrations of ZnO NP (0, 0.01, 0.025, 0.05 mg/L) for 24 h. Data represents mean  $\pm$  standard error. Asterisks indicate statistically significant difference from control at  $p < 0.05$

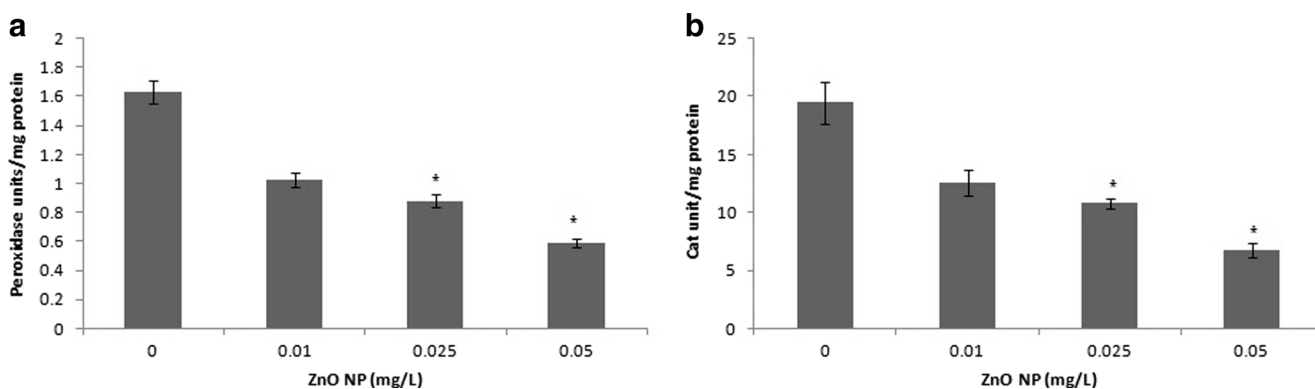
Increased expression of antioxidant family protein AhpC/TSA indicates generation of ROS due to ZnO NP as reported in our previous report [16]. AhpC/TSA protects cell against reactive sulfur species (RSS) and peroxides [35]. It also protects cell from intracellular oxidative stress and prevents DNA damage [15]. The inhibitory effects of ZnO NP have earlier been reported in both Gram-positive and Gram-negative bacteria. ZnO NP enhances antibacterial activity of conventional antibiotics and induces lipid peroxidation and ROS generation in bacterial cell as reported earlier [10, 12]. The antibacterial mechanism of ZnO NP has been postulated from cell interaction, cellular internalization, and generation of ROS like  $H_2O_2$  [36]. Thus, ZnO NP-induced oxidative stress might be responsible for the upregulation of antioxidant protein AhpC/TSA in the current study.

While the toxicity of ZnO NP to *Pseudomonas* was studied at much higher concentration (20 mg/L), very low concentrations of ZnO NP (0.008–2 mg/L) were used to investigate the

cytotoxicity and genotoxicity towards HL-60 and human PBMC cells. However, in order to compare the toxicity of ZnO NP in *Pseudomonas* and human cells, the toxicity of ZnO NP to *Pseudomonas* was studied at a representative low concentration of 0.05 mg/L, which was the  $LC_{50}$  for HL-60 cells. As expected, the bacterial cells did not show any change in growth at this concentration of ZnO NP. Further, earlier studies have reported ZnO NP toxicity in human cells at relatively higher concentrations [12, 37]. However, the predicted environmental concentrations of ZnO NP are much lower, 0.8–115 ng/L in surface water and 1.58 g/L in treated wastewater [38]. Hence, it is necessary to study the toxicity at lower ZnO NP concentrations to get relevant ecotoxicological information. It has also been reported that the mechanism of nanoparticle toxicity varies with its concentration. Xiao et al. showed that ZnO NP exerted toxicity to *Daphnia magna* predominantly through dissolved ions at low concentration, while particles played a major role in

**Fig. 6** ROS assay in HL-60 cells at different time intervals (12 and 24 h) in the presence of different concentrations of ZnO NP (0, 0.01, 0.025, 0.050 mg/L). Asterisks indicate statistically significant difference from control at  $p < 0.05$





**Fig. 7** Activity of **a** peroxidase enzyme and **b** catalase enzyme in HL-60 cells after ZnO NP exposure (0, 0.01, 0.025, 0.05 mg/L) for 24 h. Asterisks indicate statistically significant difference from control at  $p < 0.05$

toxicity at high nanoparticle concentration [39]. Therefore, the current study investigated ZnO NP toxicity to HL-60 and human PBMC at much lower concentrations.

In HL-60 cells, an increase in cell death was observed with increasing concentration of ZnO NP. The cytotoxic effects were observed in a dose-dependent manner through MTT assay. An 85% reduction in cell viability was observed at 0.25 mg/L concentration, while 4 mg/L concentration showed complete cell death after 24-h exposure (not shown in the figure). Significant toxicity at lower dose may be attributed to the smaller size of the ZnO NP, and release of  $Zn^{2+}$  ions may be the other major reason for the induction of toxic effects [8]. Cytotoxicity of ZnO NP has earlier been reported in other cells, such as A549, primary and immortalized human epithelial cells (BEAS-2B), and Neuro-2A cells [13, 37, 40, 41]. ZnO NPs were also found to induce DNA damage through the comet assay. The DNA-damaging potential increased with increasing concentrations of ZnO NP. Seventy- to eighty-percent DNA damage was observed at the  $LC_{50}$  concentration of ZnO NP (0.05 mg/L). The tail DNA observed in the comet assay could be a consequence of apoptosis, which has been reported to lead to DNA fragmentation. DNA damage was further studied through CBMN assay on human PBMC cells. Cells showed a significant increase in the frequency of micronuclei with increasing concentrations of ZnO NP ( $p < 0.05$ ).

Most metal oxide nanoparticles exert their toxic effects through ROS generation [12, 21, 37, 42]. ROS can interact with the biomolecules (proteins, enzymes, membrane lipids, DNA) resulting in their inactivation and ultimately cell death [40, 41]. Premanathan et al. [12] related the toxic effects of ZnO NP on HL-60 and normal lymphocytes to ROS generation and apoptosis. However, the study was carried out at concentrations much higher than those reported here. In the current study, generation and accumulation of ROS (superoxide radicals  $O_2^{\cdot-}$ , hydroxyl radical  $OH^{\cdot}$ , hydrogen peroxide  $H_2O_2$ ) in cells resulted in depletion of antioxidant enzymes.

Different studies have reported a variety of mechanisms for ZnO NP-mediated toxic effects. ZnO NP has been shown to induce ROS generation leading to apoptosis through activation of caspase cascade and higher expression level of bax/bcl-2 proteins. Intracellular ROS generation leads to chromosome condensation, mitochondrial membrane potential (MMP) depolarization, and induction of apoptosis mediated by the c-jun N-terminal kinase (JNK) signaling pathway. This involves phosphorylation of JNK, extracellular signal-related kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK), and poly(ADP-ribose) polymerase 1 (PARP) cleavage [42]. The up-regulation of protein p53 and downregulation of antiapoptotic protein survivin were also observed in ZnO NP-induced toxicity [21]. In the present study, ZnO NP-induced ROS generation was followed by DNA damage, apoptosis, and cell death.

ZnO NPs are being targeted as chemotherapeutic drug due to their ability to preferentially kill cancer cells with minimal toxicity to normal human cells [27]. However, the current study demonstrated toxicity of ZnO NP to normal PBMC even at low concentrations. This may be due to the physicochemical properties of nanoparticle preparation or the microenvironment used for cell growth and treatment. Thus, the results from the current study emphasize the need of more studies to establish the safety of ZnO NP towards normal cells and its subsequent clinical application as an anticancer drug. In conclusion, ZnO NP truly represents an ecotoxicological threat and their application needs to be closely monitored in the future.

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**Compliance with Ethical Standards** Peripheral blood mononuclear cells (PBMC) were obtained from healthy human donor (nonsmoker, nonalcoholic, and under no medication) following the ethical approval from the research ethics committee and standards laid down in 1964 with latest amendments of Declaration of Helsinki [20].



**Conflict of Interest** The authors declare that they have no conflict of interest.

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