Highlights:

- Toxicity of ZnO nanoparticles were for the first time evaluated using human pulmonary adenocarcinoma cells LTEP-a-2;
- Toxic effects of ZnO nanoparticles were examined by detecting the survival and reproduction rates of LTEP-a-2 cells;
- Zn^{2+} ions exerts great toxicity on LTEP-a-2 cells by inducing oxidative stress and apoptosis.
ZnO nanoparticles induced cytotoxicity on human pulmonary adenocarcinoma cell line LTEP-a-2

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Abstract: Novel nanoparticles (NPs) such as zinc oxide (ZnO) NPs are widely produced and applied in our daily lives at a rapid pace. Thus, the toxicity of ZnO NPs should be monitored as an important standard for environmental risk assessment. Here we assessed the in vitro cytotoxicity of ZnO NPs on human pulmonary adenocarcinoma cells LTEP-a-2 by tetrazolium salt colorimetric assay of cell proliferation in the presence or absence of ZnO NPs. ZnO NPs-induced morphological changes in LTEP-a-2 cells were examined by light and scanning electron microscopy. The mechanism by which ZnO NPs impose the cytotoxic effect was investigated by a combination of active oxygen test, lactose dehydrogenase-release assay, and apoptosis detection. Results showed that ZnO NPs significantly inhibited the proliferation and induced evident morphological changes (cell shrinkage and chromosome condensation) in LTEP-a-2 cells. Additionally, ZnO NPs increased the level of intracellular reactive oxygen species and induced the
formation of apoptotic vesicles as well as the lysis of cell nuclei. Zn\textsuperscript{2+} ions released from ZnO NPs into aqueous solution are important components that exert cytotoxic effects on LTEP-a-2 cells. This study provides new insights to the cytotoxicity of ZnO NPs against human health.

**Keywords:** human pulmonary adenocarcinoma cells, ZnO nanoparticles, cytotoxicity, reactive oxygen species, apoptosis

1. Introduction

Nano metal oxides such as zinc oxide (ZnO) nanoparticles (NPs) have been widely used in biological and pharmaceutical applications for their photocatalytic and electrochemical properties [Veranth et al., 2007]. The mass production and wide use of metal NPs will inevitably increase their potential risks to the eco-environment [Lee et al., 2009]. In the processes of production and use, NPs are able to enter human body directly; additionally, NPs can be inhaled through the respiratory tract and accumulated in the food chain [Hanna et al., 2013]. Thus, widespread safety concerns are raised over the toxicity of NPs and their adverse effects on human health.

Toxicological studies have demonstrated that metal NPs such as ZnO NPs exert varying degrees of lethal or inhibitory effects on microalgae *Pseudokirchneriella subcapitata* [Aruoja et al., 2009], bacterium *Escherichia coli* [Nair et al., 2009], and aquatic organisms such as Zebra fish [Zhu et al., 2008; Hu et al., 2011]. Metal NPs show serious adverse effects on human respiratory system, which pass through
alveolar epithelial cells into interstitial lung tissues and trigger severe pulmonary inflammatory response, leading to interstitial pulmonary fibrosis [Hackenberg et al., 2011; Oberdörster et al., 1994; Renwick et al., 2004]. NPs greatly reduce the phagocytic capacity of macrophages and thus decrease their ability to clear foreign matter that seriously affects human health. Moreover, metallic NPs promote the generation of reactive oxygen species (ROS) and cause oxidative stress in human body by interfering with cell metabolism and interactions to impair antioxidant mechanisms [Long et al., 2006; Park et al., 2008; Pujalté et al., 2011].

The toxicity of ZnO NPs has received great attention in toxicological research. It can cause severe anemia and renal damage in mice as well as nephrotoxicity and kidney metabolic alterations in rats. Low dose of ZnO NPs induces various degrees of damage to organs of mice, including stomach, liver, kidney, and spleen [Wang et al., 2008; Yan et al., 2012]. High cytotoxicity of ZnO NPs is demonstrated in in vitro cell viability assessment with a WST-8 cell proliferation assay kit and ROS detection with a reagent kit [Heng et al., 2010]. As for human health, ZnO NPs show potential detrimental effects on human nasal mucosal cells, causing significant DNA damage and inflammatory response [Wiethoff et al., 2003]. In addition, significantly decreased viability of human neuroblastoma cell line (SH-SY5Y) is observed with the addition of ZnO NPs (final concentration ≥0.4 ± 0.02 mM) [Taccola et al., 2011].

When ZnO NPs enter the human body, pulmonary organs would be the first attacked. In vivo airway exposure causes an important hazard. Inhalation or instillation of the NPs results in pulmonary inflammation and systemic toxicity.
Despite previous reports on their biological effects, the toxic effect of ZnO NPs on human pulmonary adenocarcinoma cells is poorly understood. Thus, the present study was conducted to assess the in vitro cytotoxicity of ZnO NPs with human pulmonary adenocarcinoma cell line LTEP-a-2 as target. The major physicochemical properties of ZnO NPs used for toxicity assays were characterized; their cytotoxic effects on LTEP-a-2 cells were investigated by in vitro proliferation assay. Additionally, ZnO NPs-induced morphological changes in LTEP-a-2 cells were examined microscopically. The results were discussed to explore the mechanisms by which ZnO NPs affect LTEP-a-2 cell proliferation.

2. Experimental Design

2.1. Characterization of ZnO NPs

Highly purified (99.9%) ZnO NPs were purchased from Sigma Aldrich (St. Louis, USA). Stock solutions were prepared in Dulbecco’s modified Eagle’s medium (DMEM) containing 50 μg/ml fetal bovine serum (FBS). To avoid particle aggregation, the prepared solutions were sonicated by water bath sonication for three times (20 s/time). ZnO NPs in DMEM were characterized in terms of the morphology, diameter, tendency of aggregation, and intracellular distribution using a scanning electron microscope (SEM, Hitachi S-4800, Japan). Zeta potential analysis of ZnO NPs in DMEM was performed by electrophoretic light scattering (Malvern Zeta sizer ZS9, Worcestershire, UK).

2.2. Cell culture

Human pulmonary adenocarcinoma cells LTEP-a-2 were purchased from China Center for Type Culture Collection (Wuhan, China). Cells were inoculated into
DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and then incubated at 37°C in a humidified 5% CO₂ atmosphere for 3 days before passage. Logarithmically growing LTEP-a-2 cells at the density of 1×10⁵ cells/ml were used for the following experiment. To avoid significant changes in cell characteristics, cells were used between passages 15 and 25.

2.3. *In vitro* cytotoxicity assay of ZnO NPs

*In vitro* cytotoxicity of ZnO NPs on LTEP-a-2 cells were detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) method as described previously [Mosmann, 1983]. Cells were inoculated into 96-well plates in triplicate at approximately 5×10⁴ cells per well. After 24-h incubation at 37°C, cells were exposed to gradient concentrations of ZnO NPs (0.01, 0.25, 0.5, 1.0 and 1.5 μg/ml) for 4, 8, and 12 h. A negative control was prepared by exposing LTEP-a-2 cells to 0 μg/ml ZnO NPs. Then, cells were added with 20 μl of 5 mg/ml MTT solution and incubated at 37°C for another 4 h. The viability of LTEP-a-2 cells was analyzed by measuring the absorbance of cell cultures at 490 nm using a microplate reader (Thermo Varioskan Flash 3001, USA).

2.4. Microscopic examination of cell morphological changes

LTEP-a-2 cells were incubated on coverslips at a density of 1×10⁵ cells/cm² for 24 h and then treated with gradient concentrations of ZnO NPs (0-0.5 μg/ml) for 12 h. Thereafter, cells were washed with phosphate-buffered saline (PBS), fixed with 2.5% glutaraldehyde at 4°C overnight, and post-fixed with 1% osmium tetroxide (pH 7.4) as previously described [Wang *et al*., 2008]. After dehydration in ascending concentrations of ethanol (30-100%), cells were freeze-dried using a lyophilizer (Eyela, Japan). After that, specimens were sputter-coated with gold and cell morphological changes were examined by scanning electron microscopy.
2.5. Detection of intracellular ROS

The intracellular ROS level of LTEP-a-2 cells was detected by active oxygen detection as described previously [Royall and Ischiropoulos, 1993; Park et al., 2013]. Cells were exposed to the non-fluorescent probe 2’,7’-dichlorofluorescein diacetate (DCFH-DA, final concentration 20 μm/l; Sigma, St. Louis, USA) in the presence or absence of 0.1 μg/ml ZnO NPs for 30 min at 37°C [Jiang et al., 2013; Zhang et al., 2012]. Then, cells were washed twice with PBS and dissociated into single cell suspension by trypsin digestion. The 2,7-dichlorofluorescein (DCF) fluorescence intensity of cell cultures at the wavelengths of 488/525 (ex/em) was read using a flow cytometer (BD FACSARia, USA). Results were used to calculate as the relative ROS level to untreated cells.

2.6. Lactose dehydrogenase (LDH)-release assay

The LDH activity in LTEP-a-2 cells was measured according to the method of Saffar Mansoor [Mansoor et al., 2013]. Cells were inoculated into 96-well plates in accordance with the procedure mentioned above. After being treated with gradient concentrations of ZnO NPs (0, 0.01, 0.1, 0.2 μg/ml), cells were precipitated by centrifuging the plates at 1000xg for 10 min. The supernatants were collected to quantify LDH activity using a LDH-cytotoxicity assay kit (Nanjing Jiansheng Bioengineering Institute, Nanjing, China). The absorption of cell cultures at 440 nm was measured using a Tecan microplate reader (Infinite M200) [Yang et al., 2012; Liu et al., 2013]. All treatments were performed in triplicate.

2.7. Detection of cell apoptosis

The morphological features of LTEP-a-2 cells undergoing apoptosis and necrosis were examined by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Cells were seeded in 6-well plates and pretreated with different concentrations of ZnO
NPs (0, 0.05, 0.2 μg/ml) for 12 h. Then, cells were fixed with paraformaldehyde solution (final concentration 4%) and stained with DAPI solution (final concentration 2 μg/mL) (Sigma, St. Louis, USA) for 30 min at room temperature [Li et al., 2012]. After being washed twice with PBS, changes in nuclear morphology were captured directly by fluorescence microscopy (Leica DM5000, Germany).

2.8. Cytotoxic effects of Zn\textsuperscript{2+} ions

To test whether the cytotoxicity of ZnO NPs was imposed by Zn\textsuperscript{2+} ions released into solution, LTEP-a-2 cells were incubated with the supernatant of ZnO NPs solution (0-1.5 μg/ml) that were filtered with 0.22μm membrane previously in complete DMEM for 4, 8, 12, and 24 h. Thereafter, Zn\textsuperscript{2+} ions in the supernatant fraction were quantified by inductively coupled plasma mass spectrometry (ICP-MS). Cell viability was evaluated by MTT assay as described above.

2.9. Statistical analysis

All experimental data are presented as means ± standard error of the mean from at least three independent experiments. Data comparison between treatments was accomplished by one-way analysis of variance followed by Student’s t-test (P < 0.05 considered statistically significant). Statistical analysis was performed in SPSS16.0 and Origin 6.0.

3. Results and discussion

This study evaluated the in vitro cytotoxicity of ZnO NPs and associated morphological changes in LTEP-a-2 cells. Results indicated that ZnO NPs caused a range of cytotoxic responses in the human pulmonary adenocarcinoma cells tested.

3.1. Characteristics of ZnO NPs
Characterizing the morphological and physicochemical properties of NPs has long been recognized as an important issue in comparative studies of cytotoxicity [Johnston et al., 2009; Gonzalez et al., 2008]. In this study, SEM image shows that ZnO NPs are mainly axiolitic shaped and partially rhombic (Fig. 1A). The estimated mean grain diameter of ZnO NPs is 30±5 nm by SEM, which matches the supplier's declaration. Zeta potential data indicate that ZnO NPs have a positive surface charge, 18.6 mV at pH 7.4 in DMEM (Fig. 1B). This level of surface charge is inadequate to stabilize the suspension via repulsive force, thus may cause NPs aggregation in DMEM [Mahmoudi et al., 2012]. The size distribution of ZnO NPs in DMEM, as determined by dynamic light scattering, shows great variations (Fig. 1C).

3.2. In vitro cytotoxicity of ZnO NPs

Cytotoxicity is the primary biological endpoint in determining the toxicity of an environmental contaminant. Previous studies have indicated the ability that NPs interact with cells can be determined by colorimetric assays with fluorometric dyes or reaction products in MTT, neutral red, monosodium salt (WST-1), and Coomassie blue assays [Jaeger et al., 2012; Doak et al., 2009; Hanley et al., 2009; Heng et al., 2011]. In the present study, the percentage of cell mortality relative to the control was calculated to reflect the cytotoxicity of ZnO NPs in LTEP-a-2 cells. Significant inhibition of cell proliferation was observed in a concentration- and time-dependent manner (Fig. 2A). With increasing concentration of ZnO NPs, cell morphology exhibited great changes while the number of cells underwent significant declines (Fig. 2B). This effect was more evident with higher concentrations of ZnO NPs and longer exposure time.

3.3. Morphological changes in LTEP-a-2 cells
The cytotoxic effect of ZnO NPs in LTEP-a-2 cells was confirmed by microscopic examination of morphological changes in LTEP-a-2 cell. Electronic microscopic images show that after 12-h exposure to ZnO NPs, LTEP-a-2 cells underwent great morphological changes (Fig. 3). In the control group, LTEP-a-2 cells maintained a regular round shape, whereas in the experimental groups, LTEP-a-2 cells turned to a blackberry-like shape due to the formation of apoptotic vesicles after NPs exposure, with some cells weakly adhering to the plate wall. SEM image confirms that ZnO NPs induced obvious changes in the morphology of LTEP-a-2 cells (Fig. 3). In addition, the number of experimental cells decreased dramatically after NPs exposure (Fig. 3). The above changes were more significant in cells exposed to higher concentrations of ZnO NPs.

3.4. ROS generation stimulated by ZnO NPs

ROS, including reactive superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH·), are important signal molecules with comprehensive effects on cell signal transduction [Yin and Casey 2010; Zheng et al., 2011]. Under normal conditions, cells commonly present low levels of ROS and balance redox reactions; such balance can be broken in the presence of any injurants or hazardous substances, leading to stimulated ROS generation and induction of oxidative stress [Xiao et al., 2003]. In the present study, potential effect of ZnO NPs on the generation of ROS in LTEP-a-2 cells was examined by fluorescence assay with hydrophobic molecules DCFH-DA, which can penetrate cellular membranes, then hydrolyzed by intracellular esterases to non-fluorescent dichlorofluoroscin (DCFH) and oxidized by ROS to fluorescent dichlorofluorescein (DCF).

The results showed that LTEP-a-2 cells treated with 0.1 μg/ml ZnO NPs had significantly higher fluorescence intensity of DCF than the control treatment (Fig. 4).
This observation implied that the generation of ROS in LTEP-a-2 cells was greatly stimulated by ZnO NPs exposure. That is, ZnO NPs induced a significant increase in the ROS level and thus caused a strong oxidative stress in LTEP-a-2 cells. High intracellular ROS level and oxidative stress have been acknowledged as the main causes for cellular damage induced by metal oxide NPs such as ZnO NPs [Karlsson et al., 2008; Xia et al., 2006]. The mechanism by which ZnO NPs damage LTEP-a-2 cells is explored in the following sections.

3.5. LDH leakage in LTEP-a-2 cells exposed to ZnO NPs

LDH is a cytoplasmic enzyme presents in all cells. It can be released into the extracellular medium when the cell membrane is damaged. The experimental results showed that large amounts of LDH were released from LTEP-a-2 cells after 24-h exposure to ZnO NPs (>0.2 μg/ml), leading to LDH accumulation in apical and basolateral fluids; much less LDH was accumulated in the control treatment (Fig. 5). More LDH was released from cells when they were exposed to higher concentrations of ZnO NPs. That is, LDH leakage in LTEP-a-2 cells exposed to different levels of ZnO NPs was induced in a concentration-dependent manner.

3.6. Induction of chromosome condensation

The results of DAPI staining and fluorescence microscopy showed that LTEP-a-2 cells underwent significant chromosome condensation after 12-h exposure to ZnO NPs (0.05-0.2 μg/ml); the degree of condensations in cells was enhanced with increasing exposure concentration of ZnO NPs (Fig. 6). These observations suggested that ZnO NPs exposure caused LTEP-a-2 cell damage by inducing the apoptotic process.

3.7. In vitro cytotoxicity of Zn$^{2+}$ ions released from NPs
The results of ICP-MS showed a significant dose-dependent linear increase of Zn$^{2+}$ ions in DMEM medium (Fig. 7A). Experimental data of MTT assay showed that with increasing Zn$^{2+}$ concentration, the viability of LTEP-a-2 cells decreased significantly in a dose-dependent manner (Figs. 7B, C). Clearly, Zn$^{2+}$ ions released from ZnO NPs into solution are important components that exert toxic effect on LTEP-a-2 cells.

4. Conclusions

With rapid scientific and technological progress, the safety of NPs should be taken up on priority in nanotechnology industry, which is continuing its march toward a $1 trillion enterprise by 2015 [Nel et al., 2006]. Evaluation of the toxicity and the health risks of novel NPs to human beings are of great importance. Our study indicated that ZnO NPs have significant dose-dependent cytotoxic effect on human pulmonary adenocarcinoma cells LTEP-a-2. The shape of LTEP-a-2 cells underwent dramatic changes after exposure to ZnO NPs, and the degree of change was enhanced with increasing concentration of ZnO NPs. Additionally, ZnO NPs caused a variety of morphological changes in LTEP-a-2 cells, including cell shrinkage, connection disappearance, cytoplasmic density increase, and apoptotic body appearance. The cytotoxicity of ZnO NPs had been reported to stimulate the generation of ROS, induce oxidative injury, and excite inflammation [Xia et al., 2008; Yang et al., 2009]. Our results showed that ZnO NPs could induce the accumulation of ROS with the release of LDH in LTEP-a-2 cells. Considering the positive relationship between the levels of Zn$^{2+}$ ions and ZnO NPs as well as the negative relationship between Zn$^{2+}$ ion concentration and cell viability, we proposed that Zn$^{2+}$ ions are important components that contribute to the cytotoxic effects of ZnO NPs on LTEP-a-2 cells. This study provides valuable data for assessing the toxicity of ZnO NPs in vitro. Further study is
needed to investigate the molecular mechanisms regarding the dose-response relationship of ZnO NPs with cells targeted.

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References


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Figure captions:

**Fig.1.** Major physical and chemical parameters of ZnO nanoparticles. A, scanning electron micrograph of ZnO nanoparticles; B, physical properties of ZnO nanoparticles; and C, size distribution of ZnO nanoparticles.

**Fig.2.** A, Viability of LTEP-a-2 cells exposed to different concentrations of ZnO nanoparticles (0-1.5 μg/ml) for 4, 8, and 12 h; B, microphotographs showing the morphology of LTEP-a-2 cells after exposed to ZnO nanoparticles for 12 h, examined by normal contrast microscopy (×40 magnification).

**Fig.3.** Scanning electron microscope images showing the morphology of LTEP-a-2 cells after exposed to different concentrations of ZnO nanoparticles (1, control, 0 μg/ml; 2, 0.01 μg/ml; 3, 0.05 μg/ml; 4, 0.1 μg/ml; 5, 0.2 μg/ml; and 6, 0.5 μg/ml).

**Fig.4.** Detection of intracellular reactive oxygen species of LTEP-a-2 cells by flow cytometry (Control 001, cells treated with 0 μg/ml ZnO nanoparticles and incubated without DCFH-DA probe; 002, cells treated with 0 μg/ml ZnO nanoparticles and incubated with a DCFH-DA probe; and 003, cells treated with 0.1 μg/ml ZnO nanoparticles and incubated with a DCFH-DA probe.)

**Fig.5.** Effects of ZnO nanoparticles on lactate dehydrogenase leakage in LTEP-a-2 cells. Cells treated with 0-0.2 μg/ml of ZnO nanoparticles for 24 h. Data expressed as the percentages relative to the control (means ± standard error of the mean). *P < 0.05, **P < 0.01.

**Fig.6.** Increase of chromosome condensation in LTEP-a-2 cells after exposed to different concentrations of ZnO nanoparticles for 12 h, monitored by DAPI staining (a DAPI solution was applied to cultured cells [untreated control and ZnO-treated cells] in chamber slides, followed by incubation for 10 min in the dark at 37°C) and fluorescent microscopy (×40 magnification).

**Fig.7.** A, Concentrations of Zn²⁺ ions in the cell culture medium released from ZnO nanoparticles; B, C: viability of LTEP-a-2 cells at 4, 8, 12, and 24 h.
### A.

![SEM Image](image.png)

### B.

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C. Figure 1.
Figure 2.
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