Evaluation of Cyto - and Genotoxic Influence of Lanthanum Dioxide Nanoparticles on Human Liver Cells

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Abstract

Inorganic nanoparticles are representing an emerging paradigm in molecular imaging probe design. We have determined lanthanum oxide nanoparticles (La₂O₃ NPs)-induced toxicity on human liver cells for 48 hrs. Before exposure to La₂O₃ NPs, the size and shape of NPs were confirmed by transmission electron microscope. It was found at 32 ±1.6 nm with a sheet-like morphological structure. The viability of CHANG and HuH-7 cells was reduced as the concentration of La₂O₃ NPs increased. HuH-7 cells were more sensitive than CHANG cells to La₂O₃ NPs. We observed production of intracellular ROS in HuH-7 cells was more than CHANG cells and the LPO level was more in CHANG cells than in HuH-7 cells at 50 μg/ml of La₂O₃ NPs. Glutathione was decreased and catalase was increased at 50 μg/ml of La₂O₃ NPs. More apoptotic and necrotic cells were observed at 300 μg/ml in HuH-7 cells FACS. DNA damage was observed by the SGCE test and more DNA damage was found in CHANG cells than HuH-7 cells at 300 μg/ml La₂O₃ NPs over 48 hrs. Thus, study warrants the application of La₂O₃ NPs in daily life and provides vital information about the toxicity of La₂O₃ NPs.

Keywords
La₂O₃ NPs, FACS, ROS, human liver cells, genotoxicity

Introduction

Nano-medicine is the science and technology of diagnosing, treating and preventing disease and traumatic injury, relieving pain, and preserving and improving human health, using molecular tools and molecular knowledge of the human body.¹ Asian countries are the richest resource of rare earth elements in the world. La₂O₃ NPs are applied in the manufacturing of various types of sensors for glucose, phosphate, uric acid, and thermal meter. Lanthanum has paramagnetic properties so it is used in drug delivery to target tissue in the human body. Lanthanum trioxide (La₂O₃) is used as an antibacterial, antivirus, overturns Ca channels, and as a selected protein binder.²⁻⁴ However, it is important to describe there is much important use of La₂O₃ NPs in medical science and a little data is available on its toxicity and safety. So in this experiment, we have analyzed cytotoxicity and apoptotic properties of La₂O₃ NPs on two selected liver cells over 48 h. NPs mediated toxicity involves various mechanisms, specifically, the excess generation of ROS in living tissue under stress. The generation of intracellular ROS is within cells by different sources. Mitochondria are the main source of ROS in cells, and the major part of ROS has occurred in the electron transport chain.⁵ Electrons leak from the electron transport chain directly to oxygen, producing short-lived free radicals such as superoxide anion.⁵ Scopanovic et al⁶ had been reported that antioxidant addition with extract of Filipendula ulmaria reduces the toxic effects of nano-sized calcium phosphates in rats. Oxidative stress, apoptotic, and inflammatory response are the main mechanisms of toxicity of La₂O₃ NPs. The objective of this study was to investigate the toxic effects of

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La2O3 NPs on human normal and cancer cells. Also, our findings will help evaluate the safety of medical device technologies using La2O3 NPs as a raw material.

Materials and Methods

Chemical and Reagents
Lanthanum oxide (La2O3) nanopowder (La2O3, 99.99%, 10–100 nm, Stock#: US3265) was purchased from US Research Inc. Houston TX USA. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], H2-DCFH-DA), DMSO, Annexin V-FITC, and PI dye were bought from Sigma-Aldrich (St. Louis, Missouri, United States). DMEM, fetal bovine serum (FBS), and antibiotics were bought from Gibco, USA.

Physical Properties of La2O3 NPs
The physical properties of La2O3 NPs were determined by using a scanning electron microscope (SEM) (JEOL Inc., Tokyo, Japan) and transmission electron microscope (TEM) (JEOL Inc., Tokyo, Japan). The hydrodynamic size and zeta potential of suspension of La2O3 NPs in water and culture media were determined by using Zeta-sizer dynamic light scattering instruments (Malvern, UK) as described by Alari et al.7

Cells
The liver cell lines such as CHANG and HuH-7 were gifted from the Department of Infection and Immunity at King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia. The cells were maintained in DMEM with fetal bovine serum (10%) and antibiotics penicillin and streptomycin (10 000 U/ml) in CO2 incubator (5%) at 37°C.

La2O3NPs Exposure
CHANG and HuH-7 cells were sub-grown for 24 h before exposure to La2O3 NPs. Stock suspension of La2O3 NPs (1 mg NPs/ml dH2O) was prepared in dH2O. The stock suspension of NPs was diluted as per experimental concentration. In this experiment, we applied different concentrations of NPs (0, 20, 50, 100, 300, and 400 μg/ml) to determine the toxicity of La2O3 NPs. The unexposed cells were considered as the control set.

MTT Assay
Cell viability of CHANG and Huh-7 cells were determined by using by MTT test after exposure to La2O3 NPs for 48 h. After adding MTT dye (.5 mg/mL) to each well, the plate was incubated for 4 h. Formazan crystal was dissolved in DMSO and optical density was determined at 570 nm by applying a microplate reader (BioTek Instruments, Winooski, VT, USA) with Gen5 software (version 1.09).

LDH Assay
The lactate dehydrogenase (LDH) technique was followed as described by Alari et al (2018). Briefly, 50 μl of culture media was withdrawn from experimental plates and mixed with 50 μl lithium lactate solution of 50 mM, 50 μl tris solution of 200 mM, and 50 μl of NAD solution (a mixture of ni-trotetrazolium violet, phenazine methosulphate, and nicotinamide dinucleotide; Sigma Aldrich, Merck KGaA, Darmstadt, Germany) were added to a new 96 wells plate. After 15 min, the absorbance was taken at 490 nm and the background absorbance was taken at 690 nm using a microplate reader with Gen5 software (version 1.09) (Bio-Tek Instruments, Winooski, VT, USA).

Measurement of ROS Levels
Generation of ROS levels in liver cells was determined by Ali et al,9 due to exposure to La2O3 NPs (0, 10, 20, 50, 100, and 300 μg/ml) for 48 h. The cells (3×104) were seeded in a black bottom culture plate (96 well) and incubated in a CO2 incubator at 37°C for 24 h for attachment. After exposure to La2O3 NPs, the culture plates were incubated for 48 h. After incubation, 10 μM DCFH-DA was added per well for 40 min at 37°C. After incubation, the plate was washed, and fluorescence intensity was measured at 485 nm excitation and 520 nm emissions using by a microplate reader with Gen5 software (version 1.09) (Bio-Tek Instruments, Winooski, VT, USA). Data were represented as a percent of fluorescence intensity in comparison to the control wells. A separate set of experiments were carried out to assess for generation of ROS through the qualitative analysis method.9

Oxidative Stress
Lipid peroxide (LPO) and Glutathione (GSH) assays. The cell lysate was prepared after exposure to La2O3 NPs. The lipid peroxide (LPO) and glutathione (GSH) level were measured as per instructions of lipid hydroperoxide assay kit (item no. 705002) and glutathione (GSH) assay kit (Item no. 703002), respectively, Cayman Chemical, USA.

Superoxide dismutase (SOD) activity. SOD estimation was done in cells collected by centrifugation at 1000×g for 10 min at 4°C. Cell pellets were lysed in cold 20 mM HEPES buffer, pH 7.2, containing 1 mM ethylene glycol tetra-acetic acid, 210 mM mannitol, and 70 mM sucrose. Cells were then centrifuged at 1500×g for 5 min at 4°C. Cell extracts were finally incubated with xanthine oxidase for 20 min according to manufacturer’s protocol and absorbance of the reaction mixture was measured at 450 nm by using a plate reader (Synergy Fluostar, Germany).
Catalase activity. Measurement of catalase activity was based on peroxidatic function of catalase. Briefly, cells were collected and sonicated in buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) followed by centrifugation at 10,000 g for 15 min at 4°C. The supernatant was then assayed for catalase activity using manufacturer’s protocol, and absorbance was monitored at 540 nm by using a plate reader (Synergy Fluostar, Germany). The activity was represented as n mole/(min ml).

AnnexinV-FITC
The apoptotic effect of La₂O₃ NPs (0, 10, 20, 50, 100, and 300 μg/ml) on CHANG and HuH-7 cells was determined by flow cytometry (BD FACS, BD Biosciences) by using Annexin V-FITC and 10 μl PI staining.¹⁰

Comet Assay
DNA damage in CHANG and HuH-7 cells was determined using comet assay as described by Ali et al.⁹,¹¹

Statistical Analysis
The data were analyzed by SPSS 26.0 software (IBM) and expressed as mean ± standard error (SE). *P value < .05 was considered statistically significant.
Figure 3. Production of intracellular ROS in CHANG and HuH-7 for 48 h due to La$_2$O$_3$NPs exposure. (A) Percent of DCF fluorescence intensity and generation of green fluorescence in CHANG and HuH-7 for 48 h. (B) Control CHANG cells. (C) CHANG cells at 20 $\mu$g/mL. (D) CHANG cells at 50 $\mu$g/mL. (E) CHAG cells at 100 $\mu$g/mL. (F) CHANG cells at 300 $\mu$g/mL. (G) Control HuH-7 cells. (H) HuH-7 cells at 20 $\mu$g/mL. (I) HuH-7 at 50 $\mu$g/mL. (J) HuH-7 cells at 100 $\mu$g/mL. (K) HuH-7 cells at 300 $\mu$g/mL. Each value represents the mean ± SE of three experiments. *$P < .005$ vs control.

Figure 4. La$_2$O$_3$NPs induced (A) Levels of LPO. (B) Glutathione (GSH) cells. (C) Activity of SOD. (D) Activity of catalase in CHANG and HuH-7 for 24 h. Each value represents the mean ± SE of three experiments. *$P < .05$ vs control.
Results

Characterization of La\textsubscript{2}O\textsubscript{3} NPs

Physical characterization of La\textsubscript{2}O\textsubscript{3} NPs was done by TEM and DLS methods. Results showed that La\textsubscript{2}O\textsubscript{3} NPs. Nanoparticles were in irregular form, sheet-like structure, and agglomeration of La\textsubscript{2}O\textsubscript{3} NPs as determined by SEM and TEM. Figures 1A and 1B.

The shape of La\textsubscript{2}O\textsubscript{3} NPs was sheet-like structure (Figures 1A and 1B). Average size of NPs was 32 ±1.6 nm (Figure 1C). After suspension of La\textsubscript{2}O\textsubscript{3} NPs in Mille Q water and DMEM, their size was determined using Zeta-sizer and size and zeta potential were 296±6.0 nm and ∼10.7 ±3.7 mV and 161±9.9 nm and ∼11.5 ±4.9 mV.

Cytotoxicity

Cytotoxicity of La\textsubscript{2}O\textsubscript{3} NPs on liver cells was determined MTT test. La\textsubscript{2}O\textsubscript{3} NPs induced toxicity in a dose-dependent manner. La\textsubscript{2}O\textsubscript{3} NPs induce more cell death in HuH-7 cells than CHANG cells. A more toxic effect of La\textsubscript{2}O\textsubscript{3} NPs was observed in CHANG cells than in HuH-7 cells at a higher dose of 300 μg/ml (Figure 2A).

Leakage of lactate dehydrogenase enzymes was measured to confirm the early apoptotic and to the plasma membrane. The level of LDH was increased as the concentration of La\textsubscript{2}O\textsubscript{3} NPs exposure increased. Thus, it was confirmed that La\textsubscript{2}O\textsubscript{3} NPs deteriorated the membrane of cells and as a result apoptosis and cytotoxicity occurred (Figure 3B). The pattern of cytotoxicity in both cells showed an irregular pattern (Figures 2A and 2B).

Oxidative Stress

ROS was increased at 50 μg/ml and it decreased at higher concentrations (Figure 3A). In this study, we have observed the production of intracellular ROS by measuring DCF intensities in La\textsubscript{2}O\textsubscript{3} NPs exposed cells (Figures 3A and 3B).
DCF fluorescence (green color) was observed at 100 $\mu$g/ml $\text{La}_2\text{O}_3$ NPs in CHANG and HuH-7 cells (Figures 3 E and 3F) than in HuH-7 cells (Figures 3J and 3K).

The LPO level was increased after exposing $\text{La}_2\text{O}_3$ NPs, and maximum induction was found in HuH-7 cells (Figure 4A) at 300 $\mu$g/ml $\text{La}_2\text{O}_3$ NPs. Reduction of GSH was observed in NPs exposed NPs exposed to CHANG and HuH-7 cells (Figure 4B). The level of SOD and catalase was decreased and increased irregularly in both cells (Figures 4 C and 4D).

**Apoptosis**

Apoptotic and necrotic effects of NPs on CHANG and HuH-7 were evaluated by using FACS. We observed 17.5% apoptotic cells in HuH-7 cells and 12.8% apoptotic cells in CHANG cells at 300 $\mu$g/ml $\text{La}_2\text{O}_3$ NPs (Figure 5K). We have presented cell scatter diagram of apoptotic and necrotic cells in Figure 5 A-J.

**DNA Damage**

Damage to DNA in liver cells due to $\text{La}_2\text{O}_3$ NPs exposure was measured by comet assay and results were presented in Figure 6 A-K. We observed dose-dependent DNA damage in both cells (Figure 6 A-K). Maximum fragmentation of DNA was observed in HuH-7 cells treated at 300 $\mu$g/ml $\text{La}_2\text{O}_3$ NPs (Figures 6J and 6K). The fragmentation of DNA was found higher in HuH-7 cells than in CHANG cells (Figures 6J and 6K).

**Discussion**

Now nanotechnology has rapidly been growing worldwide for the past decades showing tremendous applicability in a variety of different sectors for the betterment of human life. However, a major and simultaneous outcome of these increasing nano-based applications is that humans no ware at a greater risk of exposure to nanomaterials which can enter the biological systems through different routes. The $\text{La}_2\text{O}_3$ NPs are one of the major rare earth metals used in the area of electronics, fuel cells, optics, magnetic data storage, ceramics, catalysis, automobiles, biosensor, water treatment, and biomedicine. In this study, cellular responses to the treatment in human liver cells (CHANG and HuH-7 cells) were investigated. We observed that treatment of $\text{La}_2\text{O}_3$ NPs to CHANG and HuH-7 cells induced toxicity and reactive oxygen species. In this experiment, we have observed the toxic potential of $\text{La}_2\text{O}_3$ NPs on CHANG and HuH-7 cells. Before exposure of $\text{La}_2\text{O}_3$ NPs to cells, we
have characterized the nature and size of nanoparticles using TEM, XRD, and Zeta-sizers. TEM analysis explored that the average size of NPs was 32 nm with a sheet-like structure shape. Lim (2015) reported toxic potential of La2O3 NPs has increased as the surface area of NPs decrease and increase, respectively, because it indicates a greater surface area per unit deposited mass. We have observed the toxic effect of La2O3 NPs at all concentrations in both cells and increase, respectively, because it indicates a greater surface area per unit deposited mass. We have observed the toxic effect of La2O3 NPs at all concentrations in both cells but maximum toxicity exerted in CHANG cells than HuH-7 cells at 300 μg/ml for 48 h. The current finding was corroborated by the finding of Sambale et al. regarding the toxicity of silver nanoparticles in mammalian cell lines. Hence, La2O3 NPs exposure outcompeted in a reduction viability and leakage of lactate dehydrogenase in CHANG and HuH-7 cells. The cellular level La2O3 NPs act in a pH-dependent transformation process; the sequestration of a phosphate group from membranes and phosphate deposition on the NPs surface can induce lysosomal membrane biotransformation. Oxidative stress is also critical for La2O3 NPs induced toxicity on cells. The increased malondialdehyde and 8-OHdG activity and decreased SOD activity indicate that oxidative DNA damage may occur early after La2O3 NPs exposure. Yuan et al has reported oxidative stress-mediated inflammation in toxicity of La NPs on the testicular cells. Angiotensin II has a detrimental role in the pathogenesis of chronic liver disease through possessing pro-oxidant, fibro genic, and pro-inflammatory impact in the liver. Some researchers reported the phytotoxicity of La2O3 NPs on Zea mays L. Eftekhari et al reported that cimetidine, N-acetylcysteine, and taurine on thioridazine metabolic activate and induce oxidative stress in isolated rat hepatocytes.

In this experiment, we investigated generation of intracellular reactive oxygen species and its generation was increased in a dose-dependent manner and subsequently, the production of ROS was higher in CHANG cell than HuH-7 cells. In this experiment, apoptosis was observed more in HuH-7 than in CHANG cells. ROS has been linked to different mechanisms viz damaging of nuclear components (DNA and RNA), protein, interference of cellular signaling pathways, and alteration in gene expression and ultimately the mechanism of cell growth was stopped. Interestingly, among these two cell types, HuH-7 cells were more sensitive than CHANG cells to La2O3 NPs in 48 h. To confirm whether the proliferation of cells was inhibited by the apoptotic response, we have determined apoptotic cells by using Annexin V-FITC and PI staining after exposure to La2O3 NPs.

It is more important finding that HuH-7 cells more susceptible to La2O3 NPs than CHANG cells in 48 h. In the future, we will do investigations about the mechanism of toxicity due to La2O3 NPs in vivo experiments. The biological activity of lanthanum nanoparticles might depend upon the size and pH of nanoparticle suspension is worthy of further analysis.

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Author Contributions

Conceptualization: DA and SA; data curation: DA; formal analysis: DA and BA; funding acquisition: SA; investigation: DA, BA, SMA, SA, and RA; methodology: SMA, DA, BA, and RA; project administration: SA and DA; software: DA and BA; supervision: DA and SA; validation: DA and SA; writing-original draft: DA; and writing-review and editing: DA, SA, BA, and RA.

Declaration of Conflicting Interests

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Data Availability Statement

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

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