Original Article

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 (La_2O_3 NPs)-induced toxicity on human livers cells for 48 hrs. Before exposure to La_2O_3 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 La_2O_3 NPs increased. HuH-7 cells were more sensitive than CHANG cells to La_2O_3 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 La_2O_3 NPs. Glutathione was decreased and catalase was increased at 50 μg/ml of La_2O_3 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 La_2O_3 NPs over 48 hrs. Thus, study warrants the application of La_2O_3 NPs in daily life and provides vital information about the toxicity of La_2O_3 NPs.

Keywords

La_2O_3 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. La_2O_3 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 (La_2O_3) 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 La_2O_3 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 La_2O_3 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] Scepanovic et al[6] 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 La_2O_3 NPs. The objective of this study was to investigate the toxic effects of

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La_2O_3 NPs on human normal and cancer cells. Also, our findings will help evaluate the safety of medical device technologies using La_2O_3 NPs as a raw material.

Materials and Methods

Chemical and Reagents

Lanthanum oxide (La_2O_3) nanopowder (La_2O_3, 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 La_2O_3 NPs

The physical properties of La_2O_3NPs 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 La_2O_3NPs in water and culture media were determined by using Zeta-sizer dynamic light scattering instruments (Malvern, UK) as described by Alarifi 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 CO_2 incubator (5%) at 37°C.

La_2O_3NPs Exposure

CHANG and HuH-7 cells were sub-grown for 24 h before exposure to La_2O_3 NPs. Stock suspension of La_2O_3 NPs (1 mg NPs/ml dH_2O) was prepared in dH_2O. 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 La_2O_3 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[8] after exposure to La_2O_3 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 Alarifi 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 nitrotetrazolium violet, phenazine metosulphate, 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) (BioTek 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 La_2O_3 NPs (0, 10, 20, 50, 100, and 300 µg/ml) for 48 h. The cells (3×10^4) were seeded in a black bottom culture plate (96 well) and incubated in a CO_2 incubator at 37°C for 24 h for attachment. After exposure to La_2O_3 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 La_2O_3 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).

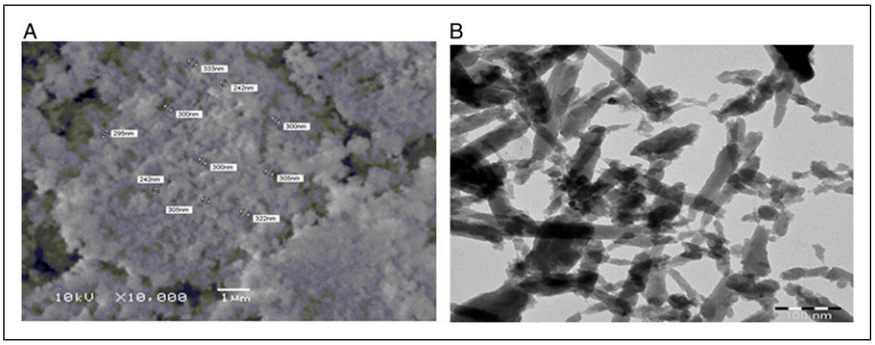

Figure 1. Characterization of La_2O_3NPs. (A) Image of La_2O_3NPs by scanning electron microscope. (B) Image of La_2O_3NPs by transmission electron microscope (JEM 1011).

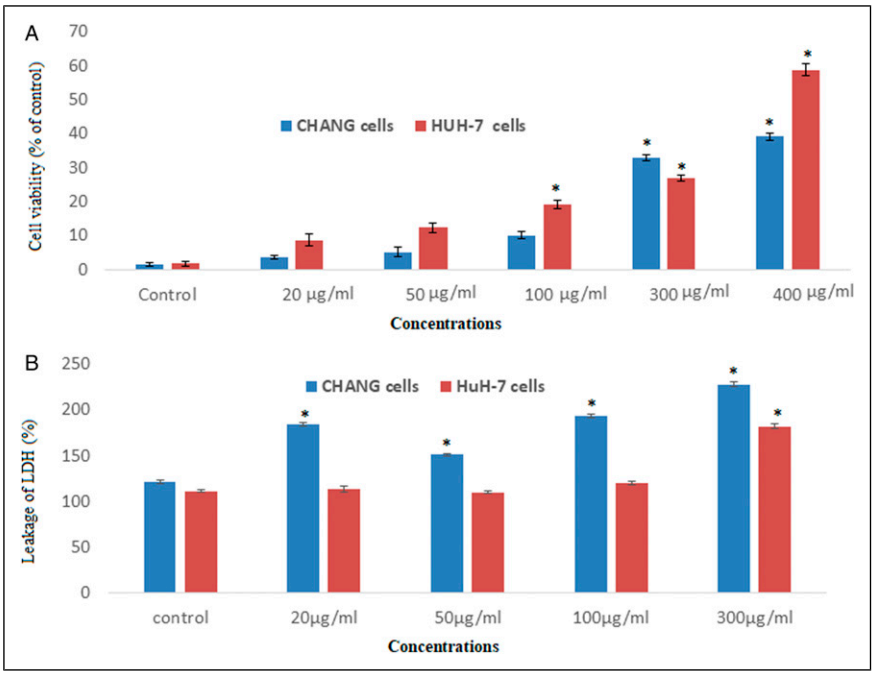

Figure 2. Cytotoxicity of La_2O_3NPs on CHAG and HuH-7 cells for 48 h, as evaluated by (A) MTT and (B) LDH assays. Each value represents the mean ± SE of three experiments. n = 3, *P < .05 vs control.

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 La_2O_3 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.

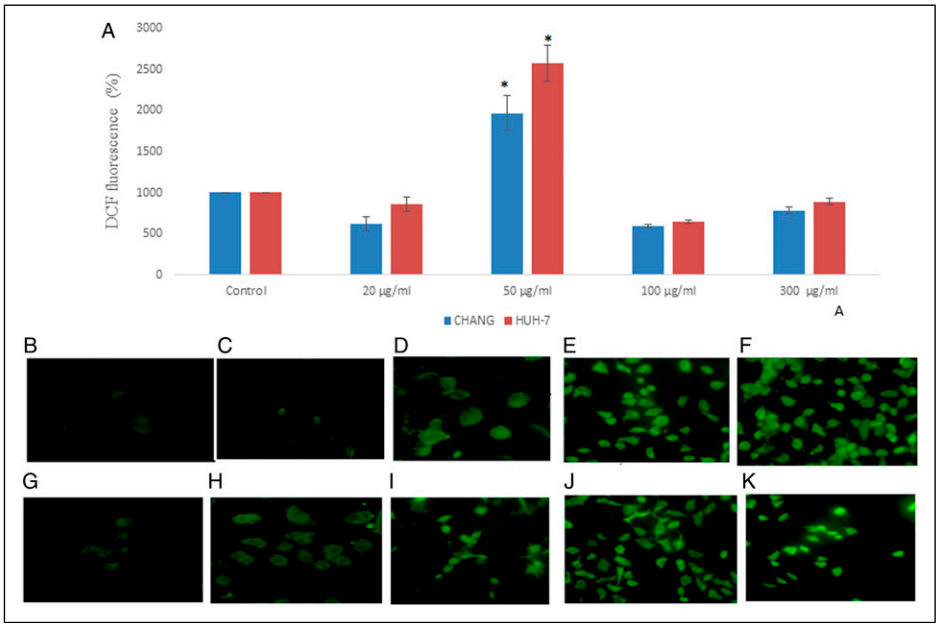

Figure 3. Production of intracellular ROS in CHANG and HuH-7 for 48 h due to La_2O_3NPs exposure. (A) Percent of DCF fluorescence intensity and generation of green fluorescence in CHANG and HuH-7 for 48 h. (B) Control CHANG cells. (C) CHANG cells at 20 µg/mL. (D) CHANG cells at 50 µg/mL. (E) CHAG cells at 100 µg/mL. (F) CHANG cells at 300 µg/mL. (G) Control HuH-7 cells. (H) HuH-7 cells at 20 µg/mL. (I) HuH-7 at 50 µg/mL. (J) HuH-7 cells at 100 µg/mL. (K) HuH-7 cells at 300 µg/mL. Each value represents the mean ± SE of three experiments. *$P < .005$ vs control.

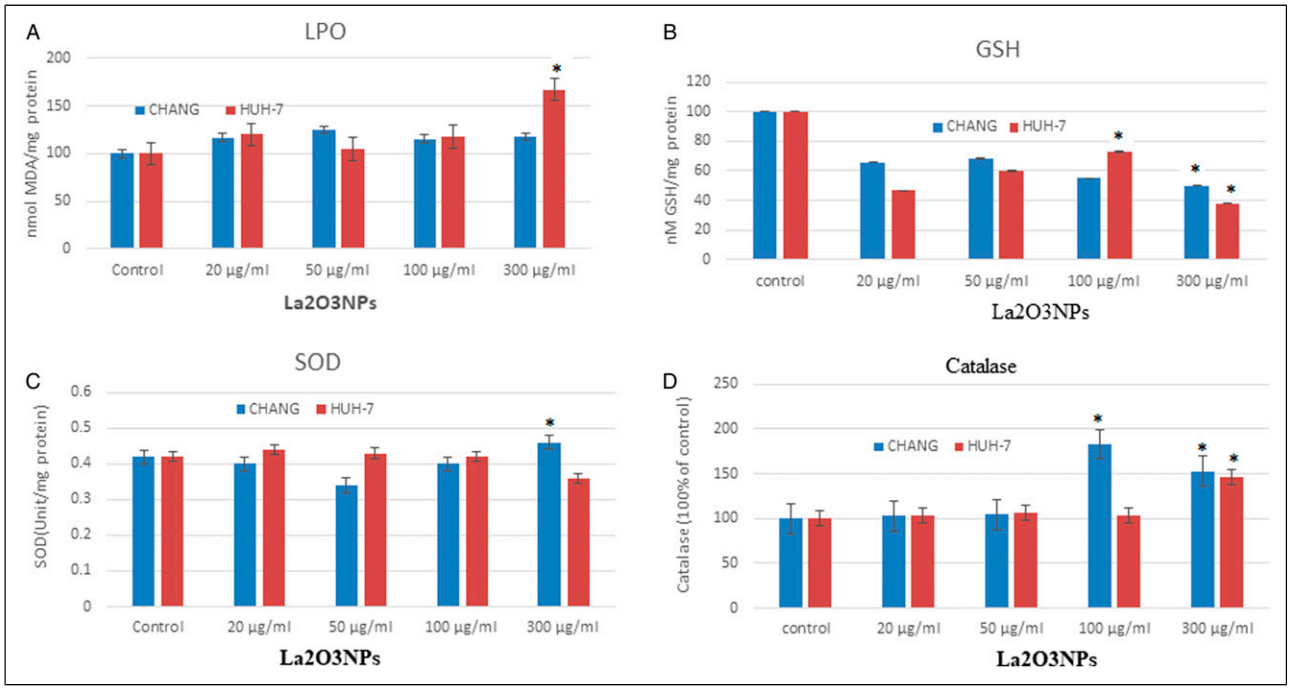

Figure 4. La_2O_3NPs induced (A) Levels of LPO. (B) Glutathione (GSH) cells. (C) Activity of SOD. (D) Activity of catalase in CHANG and HuH-7 for 24 h. Each value represents the mean ± SE of three experiments. *$P < .05$ vs control.

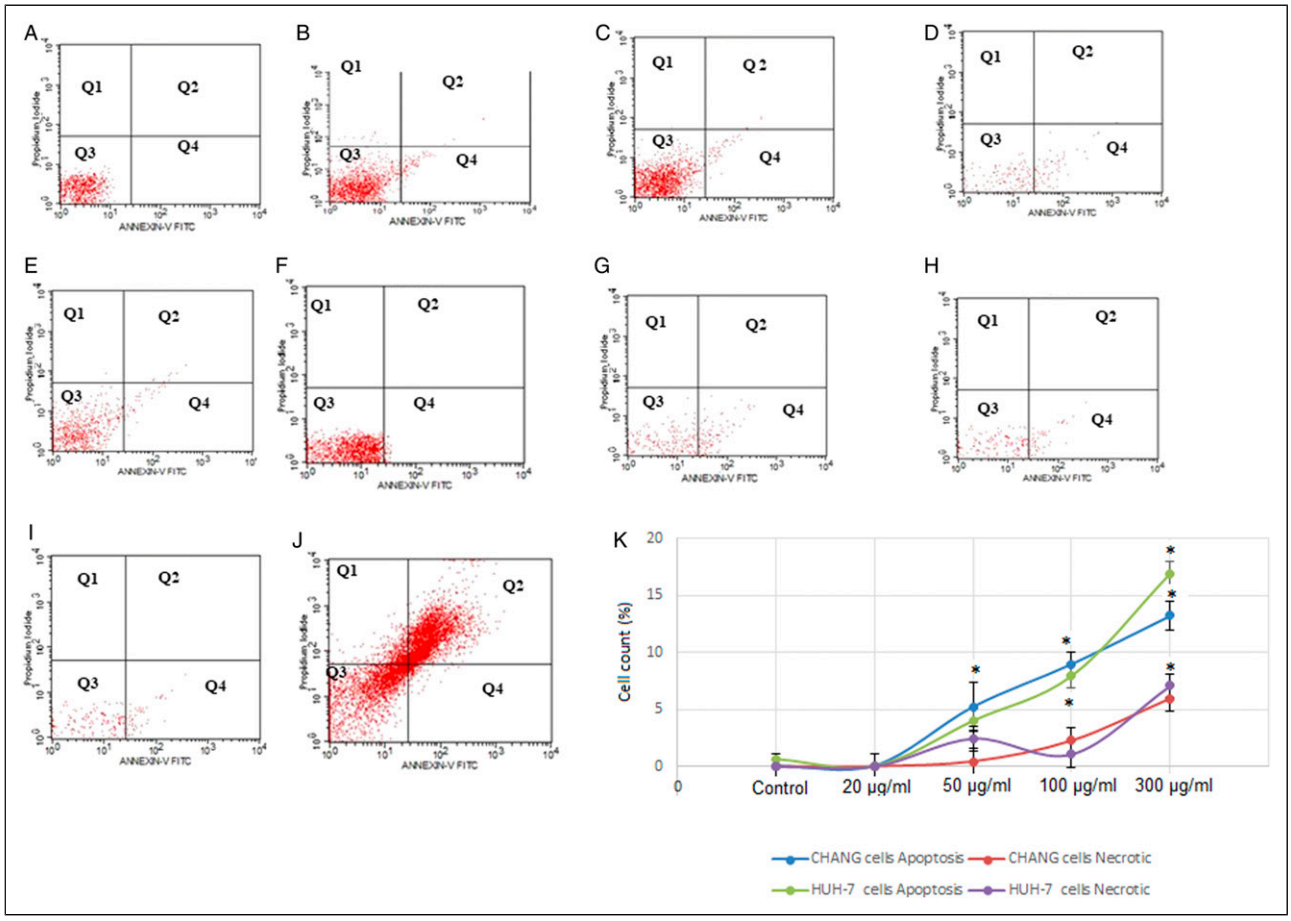

Figure 5. After exposure of La_2O_3NPs, scatter diagram of apptotic and necrotic CHANG and HuH-7 for 48 h. (A) Control CHANG cells. (B) CHANG cells at 20 µg/mL. (C) CHANG cells at 50 µg/mL. (D) CHANG cells at 100 µg/mL. (E) CHANG cells at 300 µg/mL. (F) Control HuH-7 cells (G) HuH-7 cells at 20 µg/mL. (H) HuH-7 cells at 50 µg/mL. (I) HuH-7 cells at 100 µg/mL. (J) HuH-7 cells at 300 µg/mL. (K) Percent of apoptotic and necrotic CHANG and HuH-7 for 4 h after exposure of La_2O_3NPs. Each value represents the mean ± SE of three experiments. *$P < .05$ vs control.

Results

Characterization of La_2O_3 NPs

Physical characterization of La_2O_3 NPs was done by TEM and DLS methods. Results showed that La_2O_3 NPs. Nanoparticles were in irregular form, sheet-like structure, and agglomeration of La_2O_3 NPs as determined by SEM and TEM. Figures 1A and 1B.

The shape of La_2O_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_2O_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_2O_3 NPs on liver cells was determined MTT test. La_2O_3 NPs induced toxicity in a dose-dependent manner. La_2O_3 NPs induce more cell death in HuH-7 cells than CHANG cells. A more toxic effect of La_2O_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_2O_3 NPs exposure increased. Thus, it was confirmed that La_2O_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_2O_3 NPs exposed cells (Figures 3A and 3B).

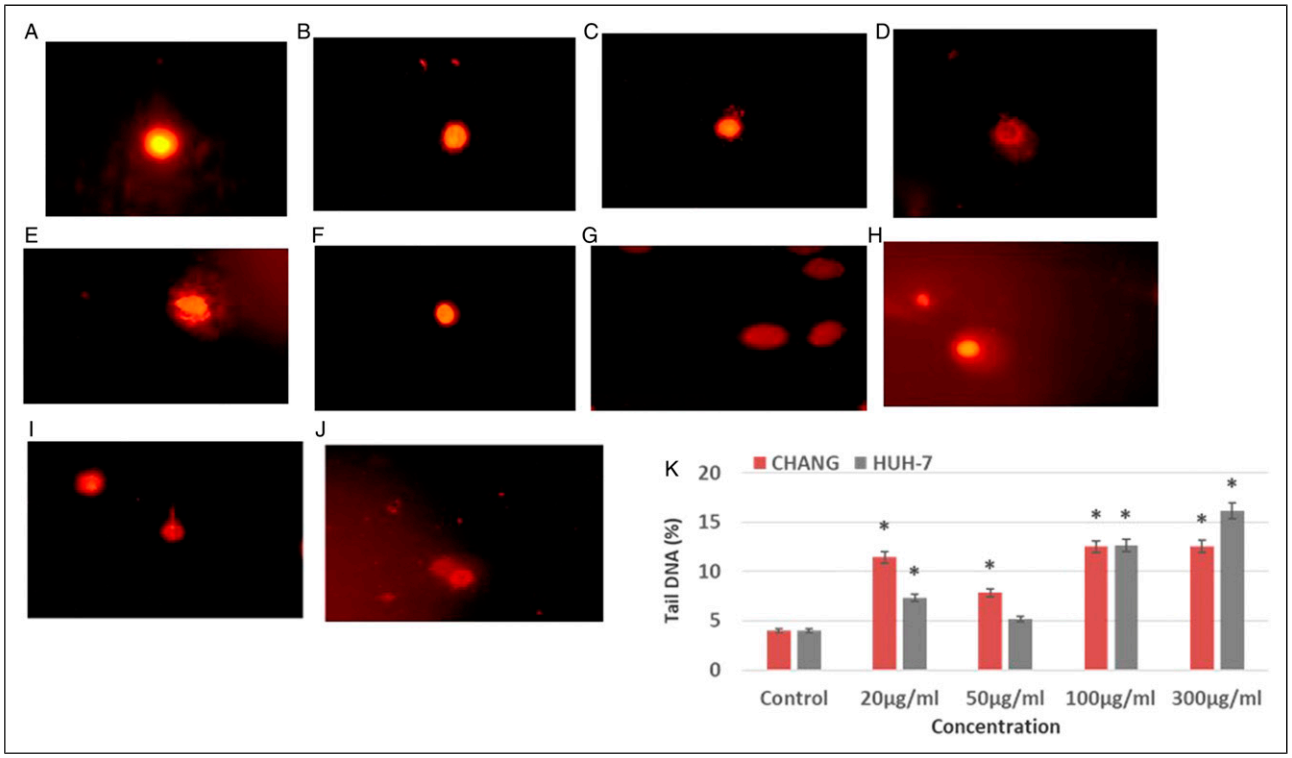

Figure 6. DNA damage in CHANG and HuH-7 over 48 h after treatment of La_2O_3NPs. (A) Control CHANG cells. (B) CHANG cells at 20 µg/mL. (C) CHANG cells at 50 µg/mL. (D) CHANG cells at 100 µg/mL. (E) CHANG cells at 300 µg/mL. (F) Control HuH-7 cells. (G) HuH-7 cells at 20 µg/mL. (H). HuH-7 cells at 50 µg/mL. (I) HuH-7 cells at 100. (J) HuH-7 cells at 300 µg/mL. (K) Percent of tail DNA damage in CHANG and HuH-7 for 48 h after exposure of La_2O_3NPs. Each value represents the mean ± SE of three experiments. *$P < .05$ vs control.

DCF fluorescence (green color) was observed at 100 µg/ml La_2O_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 La_2O_3 NPs, and maximum induction was found in HuH-7 cells (Figure 4A) at 300 µg/ml La_2O_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 µg/ml La_2O_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 La_2O_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 µg/ml La_2O_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.[12] The La_2O_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 La_2O_3 NPs to CHANG and HuH-7 cells induced toxicity and reactive oxygen species. In this experiment, we have observed the toxic potential of La_2O_3 NPs on CHANG and HuH-7 cells. Before exposure of La_2O_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 La_2O_3 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 La_2O_3 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,[13] regarding the toxicity of silver nanoparticles in mammalian cell lines. Hence, La_2O_3 NPs exposure outcompeted in a reduction viability and leakage of lactate dehydrogenase in CHANG and HuH-7 cells. The cellular level La_2O_3 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.[14] Oxidative stress is also critical for La_2O_3 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 La_2O_3 NPs exposure.[15] Yuan et al[15] 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.[16] Some researchers reported the phytotoxicity of La_2O_3 NPs on *Zea mays* L.[17] Eftekhari et al[18] 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.[19] Interestingly, among these two cell types, HuH-7 cells were more sensitive than CHANG cells to La_2O_3 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 La_2O_3 NPs.

It is more important finding that HuH-7 cells more susceptible to La_2O_3 NPs than CHANG cells in 48 h. In the future, we will do investigations about the mechanism of toxicity due to La_2O_3NPs 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

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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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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