



Bismuth oxide nanoparticles induce oxidative stress and apoptosis in human breast cancer cells

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Abstract

Metal nanomaterials such as bismuth oxide nanoparticles ($\text{Bi}_2\text{O}_3\text{NPs}$) have been extensively used in cosmetics, dental materials, pulp capping, and biomedical imaging. There is little knowledge about the health risk of $\text{Bi}_2\text{O}_3\text{NPs}$ in humans, which warrants a thorough toxicity investigation of $\text{Bi}_2\text{O}_3\text{NPs}$ at the cellular level. In this experiment, we investigated the cytotoxic effect of $\text{Bi}_2\text{O}_3\text{NPs}$ on human breast cancer (MCF-7) cells over 24 and 48 h. MCF-7 cells were exposed to $\text{Bi}_2\text{O}_3\text{NPs}$ at varying doses (0.1, 0.5, 1.0, 5, 10, 20, 40 $\mu\text{g/mL}$) for 24 and 48 h. We assessed the toxicity of $\text{Bi}_2\text{O}_3\text{NPs}$ by measuring its effect on the viability and oxidative stress biomarkers, e.g., GSH, SOD, and catalase in MCF-7 cells. The pro-apoptotic effects of $\text{Bi}_2\text{O}_3\text{NPs}$ on MCF-7 cells were determined via evaluating dysfunction of mitochondrial membrane potential (MMP), caspase-3 activity, externalization of phosphatidylserine, and chromosome condensation. Furthermore, apoptotic cells were evaluated using 7-AAD fluorescence stain and Annexin V-FITC. $\text{Bi}_2\text{O}_3\text{NPs}$ induced oxidative stress in MCF-7 cells in a time- and dose-dependent manner. $\text{Bi}_2\text{O}_3\text{NPs}$ increased the rate of both necrotic cells and apoptotic cells. In addition, the blue fluorescence of MCF-7 cells with condensed chromatin was increased in a time- and dose-dependent manner. In conclusion, the present study highlights the potential toxic effects of $\text{Bi}_2\text{O}_3\text{NPs}$ at the cellular level and suggests further investigation of $\text{Bi}_2\text{O}_3\text{NPs}$ before any medical purposes.

Keywords $\text{Bi}_2\text{O}_3\text{NPs}$ · MCF-7 cells · Cytotoxicity · Apoptosis

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Introduction

Cancer is the second leading cause of mortality after cardiovascular diseases worldwide (Kooti et al. 2017). In addition to genetic drivers, there are environmental factors that cause cancer (Doubeni et al. 2019). Breast cancer is the most common cancer in women under the age of 60. The rates of treatment success are high in the early stages with high survival rates. However, in advanced or late stages, the treatment is often insufficient, with a 5-year survival rate of less than 25% (Pradeepkumar and Kumar 2018). Breast cancer cell line (MCF-7) has been widely used as an in vitro model for breast cancer (Riaz et al. 2013). One example of MCF-7 contributions in breast cancer research is its utility for the study of the estrogen receptor (ER) alpha, as this cell line expresses substantial levels of ER mimicking the majority of invasive human breast cancers (Novaro et al. 2003; Alberts 2008).

Bismuth nanoparticles have attracted interest in health sciences (bioimaging, biosensing, biomolecular detection, and dental materials) and cosmetic industry owing to their

remarkable physico-chemical properties from nanoelectronics to catalysis (Hyodo et al. 2009; Patil et al. 2020). However, the toxicity of such nanoparticles at the molecular level has not been extensively explored, and it is a crucial step prior to biomedical applications. Some researchers have reported that metal nanomaterials can accumulate in different tissues, leading to toxic effects, such as oxidative stress and apoptosis (Karam et al. 2007; Chen et al. 2015). Bi₂O₃NPs induce toxicity in various mammalian cells (Ding et al. 2011; Abudayyak et al. 2017; Wang et al. 2017) by increasing the production of intracellular reactive oxygen species (ROS) at a rate that overwhelms the cellular defense mechanisms. High levels of ROS in cellular systems can lead to irreversible molecular damages of the genetic materials and organic compounds, resulting in cytotoxicity and genotoxicity (Finkel and Holbrook 2000; Valko et al. 2007).

Hence, it is of outmost importance to study the cellular toxicity of Bi₂O₃NPs prior to any translational medical application. Accounting for in vitro genotoxicity and cell apoptosis caused by Bi₂O₃NPs, the present study investigated the involvement of complex cellular responses via diverse biological processes compromised by Bi₂O₃NPs including cell viability, mitochondrial membrane dysfunction, oxidative stress-mediated apoptosis, and genotoxicity on human breast cancer (MCF-7) cell line.

Material and methods

Chemicals

Bismuth oxide nanopowder (Bi₂O₃NPs), < 10 nm particle size, 99% trace metal basis; 3-(4,5-dimethylthiozoly-2)-2,5-diphenyl tetrazolium bromide (MTT); 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA); acridine orange (AO); ethidium bromide (EtBr); phosphate buffer saline (PBS); propidium iodide; and Trypsin-EDTA solution were purchased from Thermo Fisher Life Technologies, USA. The caspase-3 assay kit and fetal bovine serum (FBS), Triton X-100, antibiotics (penicillin–streptomycin), and DMEM medium were purchased from Sigma-Aldrich, USA. The whole consumables and culture wares were procured from Corning, NY, USA.

Cell culture

MCF-7 cells (ATCC® HTB-22™) were seeded in DMEM supplemented with 10% FBS and 1.5% antibiotic solution. Cells were grown in standard conditions. Cells were observed with (Nikon Eclipse TE2000) an inverted microscope.

Treatments of Bi₂O₃NPs

MCF-7 cells were subcultured overnight before exposure to Bi₂O₃NPs (0, 0.1, 0.5, 5, 10, 20, 40, 100 µg/mL). Bi₂O₃NPs was suspended in 1 mg/mL DMEM medium and diluted to 0.1–100 µg/mL. Suspended solution of Bi₂O₃NPs was sonicated by using the Qsonica Q700 sonicator at 40 W at RT for 15 min to prevent aggregation of Bi₂O₃NPs.

Physiochemical characterization of Bi₂O₃NPs

A small amount of nanoparticles was smashed on a slide then inserted into the X-ray diffraction (XRD, Rigaku MiniFlex 600) to characterize crystallinity using CuK α radiation (30 kV, 40 mA, 1.54430 Å) between 5 and 80°. The morphology of Bi₂O₃NPs was measured using a scanning electron microscope (SEM).

Bi₂O₃NPs were suspended in DDW (100 µg/mL), and films of NPs on the transmission electron microscope (TEM) grid were prepared by dipping in NP suspension. The excess solution was removed by using a blotting paper. The size of Bi₂O₃NPs was determined by using TEM (JEM 1011, Japan) with a voltage of 80 kV.

To further measure the hydrodynamic diameters of Bi₂O₃NPs, Bi₂O₃NPs were sonicated using probe sonication. After sonication, Bi₂O₃NP suspension was added into a folded capillary cell (DTS1070), followed by dynamic light scattering (DLS) analysis using Zetasizer (ZEN3600, Malvern, UK) to measure Z-average hydrodynamic diameters, and zeta potential at room temperature.

MTT assay

The cytotoxicity of Bi₂O₃NPs on MCF-7 cells was assessed by using the MTT assay kit (Abcam, ab211091). MCF-7 cells were cultured in 96-wells plate and exposed to Bi₂O₃NPs (0, 0.1, 0.5, 5, 10, 20, 40, 100 µg/mL) for 24 and 48 h. After exposure to nanoparticles, the plate suspension was discarded. New MTT solution (0.5 mg/mL) in DMEM was added and incubated for 4 h. The formazan crystal was formed, and optical density was observed at 570 nm using a microplate reader (Multiskan® Ascent, LabSystems, and Haverhill, MA, USA).

ROS measurement

ROS were measured using 2,7-dichlorodihydrofluorescein diacetate (H2-DCFH-DA). H2-DCFH-DA was dissolved in dimethyl sulfoxide to 5 mM as an optimal concentration. A black well plate (96 wells) was used for treatment. The working solutions containing 10 µM H2-DCFH-DA were added to

MCF-7 cells and incubated for 30 min at 37 °C in duplicate. The cells were washed once with PBS and re-suspended in PBS. A spectro-fluorometer was used to measure the relative fluorescence intensity at 480-nm excitation wavelengths and 530-nm emission.

For assessment of the generated ROS, MCF-7 cells were incubated with Bi₂O₃NPs. The cells were incubated with H₂-DCFH-DA (5 mM) at 37 °C for 30 min, then washed with PBS and fixed with paraformaldehyde (4%). The fluorescence of cells was measured by fluorescence microscope (Nikon Eclipse 80i).

Oxidative stress biomarkers

MCF-7 cells (6×10^6) was cultured in a 75-cm² flask and treated with various dose of Bi₂O₃NPs (1, 5, 10, 20, and 40 µg/mL) for 24 and 48 h. MCF-7 cells were washed with PBS and scraped. The scraped cells were incubated in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na₂ EDTA, 1% Triton, and 2.5 mM sodium pyrophosphate). MCF-7 cells were centrifuged at 15,000 rpm at 4 °C for 15 min. The supernatant was kept at – 80 °C in freezer to measure the oxidative stress biomarkers, such as glutathione (GSH), superoxide dismutase (SOD), and catalase. GSH was measured by using a GSH kit (item no. 703002, Cayman chemical). SOD was measured by using a SOD kit (item no. 706002, Cayman chemical), and catalase was measured by using a catalase kit (item no. 707002, Cayman chemical). Protein concentration was measured by using the Bradford method (1976).

JC-1 assay

The JC-1 assay was used to determine mitochondrial membrane potential (MMP) in MCF-7 cells after exposure to nanoparticles using the JC-1 assay kit (Cayman chemical kits), and the test was done according to manufacturer's instruction.

Examination of phosphatidylserine translocation

MCF-7 cells were cultured and incubated with Bi₂O₃NPs (5, 10, and 20 µg/mL) for 24 and 48 h. After incubation, the cells were fixed with paraformaldehyde (4%) for 5 min and then washed with cold PBS. Cells were stained with propidium iodide (10 µL/mL) and Annexin V/FITC conjugate (2 µL) in binding buffer (500 µL). The images were examined by using a confocal microscope with emission 520 nm and excitation 488 nm for Annexin V/ FITC and emission 617 nm and excitation 536 nm for propidium iodide.

Measuring of chromosome condensation and caspase-3 activity

The chromatin condensation was measured in treated MCF-7 cells after exposure to Bi₂O₃NPs (5, 10, and 20 µg/mL) for 24 and 48 h. Chromatin condensation was observed by DAPI staining. The condensed chromatin body in treated cells was detected according to Toné et al.'s (2007) method. In addition,

Fig. 1 **a** TEM image of Bi₂O₃NPs. **b** SEM image of Bi₂O₃NPs. **c** X-ray diffraction spectra of Bi₂O₃NPs

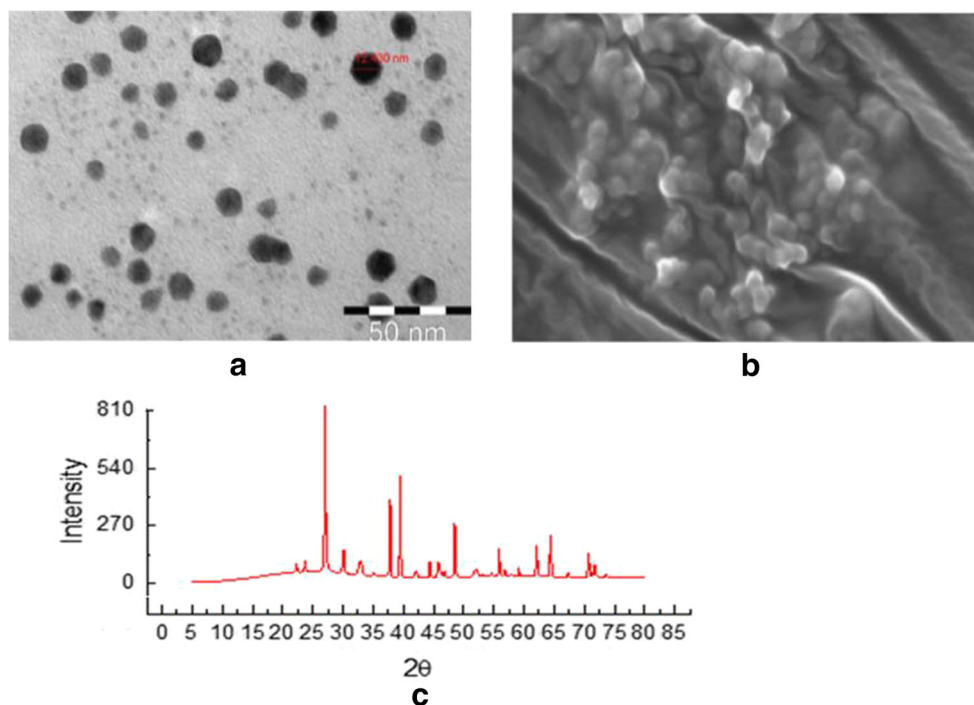


Table 1 The hydrodynamic size and zeta potential of Bi₂O₃NPs

Solvent	Dynamic light scattering
Size in water	470.5667 ± 40.22938
Zeta in water	− 22.6 ± 0.404145
Size in DEME	390.9333 ± 39.6437
Zeta in DEME	− 9.38667 ± 0.491155

the level of caspase-3 enzyme was measured in MCF-7 cells after treating with Bi₂O₃NPs for 24 and 48 h using Cayman Chemical colorimetric assay kits.

Annexin V-FITC/7-ADD staining

The Guava Nexin® assay was used to detect PS on the external membrane of apoptotic cells. The bismuth nanoparticles of different concentrations (5, 10, and 20 µg/mL) and final volume of 200 µL of triton (0.1% v/v) medium were used as positive control to detect PS in MCF-7 cells. Positive control was added to MCF-7 just 15–120 min before running the assay, then incubated for 24 and 48 h (5% CO₂ and 37 °C). The cell line was harvested at 85% confluence (5 × 10⁴) by 0.25% trypsin in 96-well plates. The cells were directly stained in a 96-well microplate with Guava Nexin reagent, a pre-made cocktail containing Annexin V-PE and 7-AAD, in a

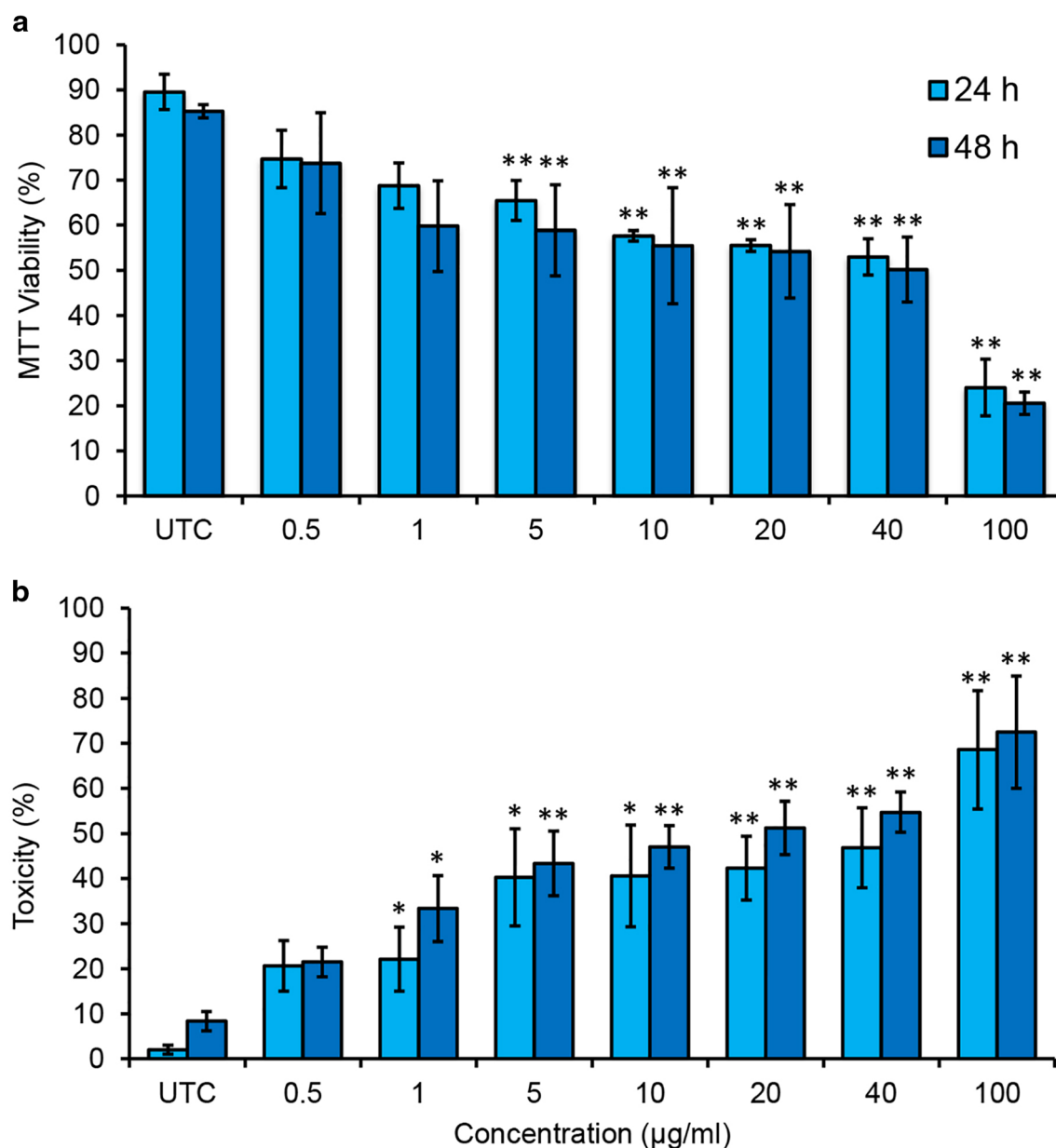


Fig. 2 Cytotoxicity of Bi₂O₃NPs on MCF-7 cells for 24 h and 48 h, as determined by **a** MTT tests and **b** LDH assay. Each value represents the mean ± SE of three experiments. **p* < 0.05 and ***p* < 0.01 vs. control

200- μ L final volume. After a 20-min incubation at room temperature, the samples were acquired on a Guava® System. Apoptosis assay was done according to manufacturer's instructions.

Statistical analysis

Pearson's correlation and one-way analysis of variance (ANOVA) were used to analyze the parametric data using the SPSS software version 26.0 (IBM company, USA). The difference between the results was considered slightly significant at $*p < 0.05$ and highly significant at $**p < 0.01$. Data was expressed as average value of triplicate independent experiment.

Results

Physical characterization of Bi₂O₃NPs

The shape, surface, size, and area of investigated nanoparticles were examined by TEM and SEM. The average size of

Bi₂O₃NPs was 14 ± 11 nm by TEM (Fig. 1a). The SEM image shows that the powder of Bi₂O₃NPs is strongly agglomerated. The surface charge density of Bi₂O₃NPs in culture media suspension was determined by DLS with a zeta potential of -22.6 ± 0.4 mV (Table 1). XRD analysis showed the high crystallinity of Bi₂O₃NPs (Fig. 1c). The estimated size of NPs as measured by XRD was 14 ± 7 nm, which is comparable to the TEM results in Fig. 1a.

Cytotoxicity

The viability of MCF-7 cells was evaluated after treatment with different doses (0.1, 0.5, 1, 5, 10, 20, 40, and 100 μ g/mL) of Bi₂O₃NPs for 24- and 48-h incubation time using two methods (MTT and LDH assays). Pronounced dose-dependent response of cells was observed as suggested by the loss of cell survival as a result of elevation in the concentration of nanoparticles (Fig. 2a). The data of LDH assay were accorded with the MTT test (Fig. 2b). Thus, it is clear that Bi₂O₃NPs induced cytotoxic effects in a concentration- and time-dependent manner (Fig. 2). The cytotoxicity was more pronounced when exposing cells to 5 μ g/mL of Bi₂O₃NPs and

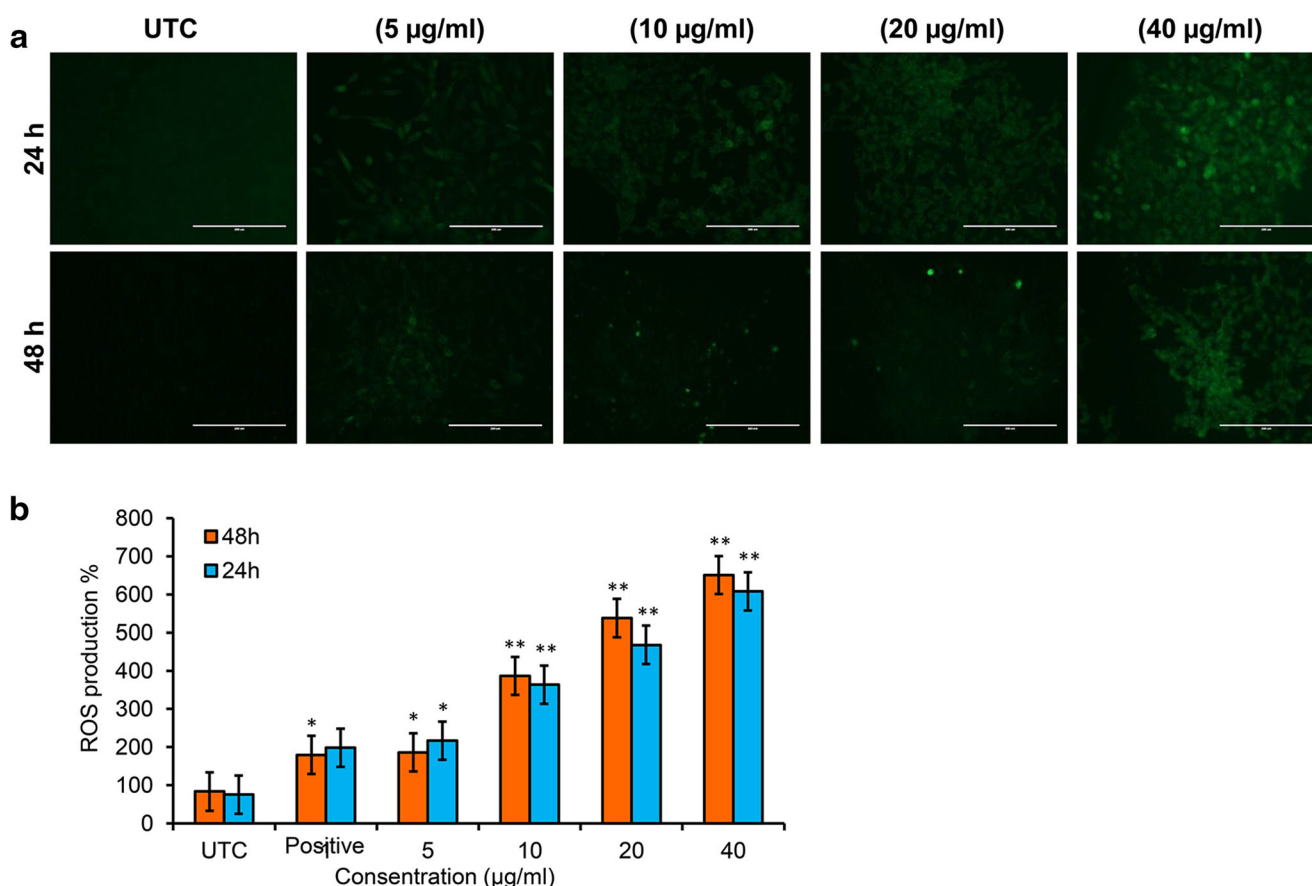


Fig. 3 Generation of ROS due to Bi₂O₃NPs. **a** The fluorescence image of MCF-7 cells treated for 24 h and 48 h and stained with DCFH-DA. **b** % ROS production due to Bi₂O₃NPs in MCF-7 cells. Each value represents the mean \pm SE of three experiments. $*p < 0.05$ and $**p < 0.01$ vs. control

beyond; therefore, we limited our further toxicity studies to the high concentrations of Bi₂O₃NPs.

Oxidative stress

Intracellular ROS were measured using DCFDA fluorescent dye after incubating MCF-7 cells with Bi₂O₃NPs for 24 and 48 h. ROS generation was significantly increased (Fig. 3 a and b). The higher the concentration of Bi₂O₃NPs, the more significant the stress was on MCF-7 cells at the two time points. Glutathione reductase was increased up to 43%, 84%, and 96% in 24 h (Fig. 4a), and 41%, 80%, and 83% in 48 h

(Fig. 4b) post-exposure to 5, 10, and 20 µg/mL of Bi₂O₃NPs, respectively.

The activity of SOD enzyme decreased significantly from 100 down to 49% and 38% in 24 h and down to 61% and 9% in 48 h (Fig. 4d) at 10 and 20 µg/mL Bi₂O₃NPs exposure, respectively (Fig. 4 c, d). Furthermore, there was a significant increase in the catalase concentrations in MCF-7 cells following Bi₂O₃NPs exposure. The results showed that the catalase activity increased significantly ($p < 0.05$) up to 35%, 40.1%, 40%, and 56% in 24 h (Fig. 4e), and up to 63%, 70%, 82%, and 83% in 48 h (Fig. 4f) in cells incubated with 5, 10, 20, and 40 µg/mL Bi₂O₃NPs, respectively.

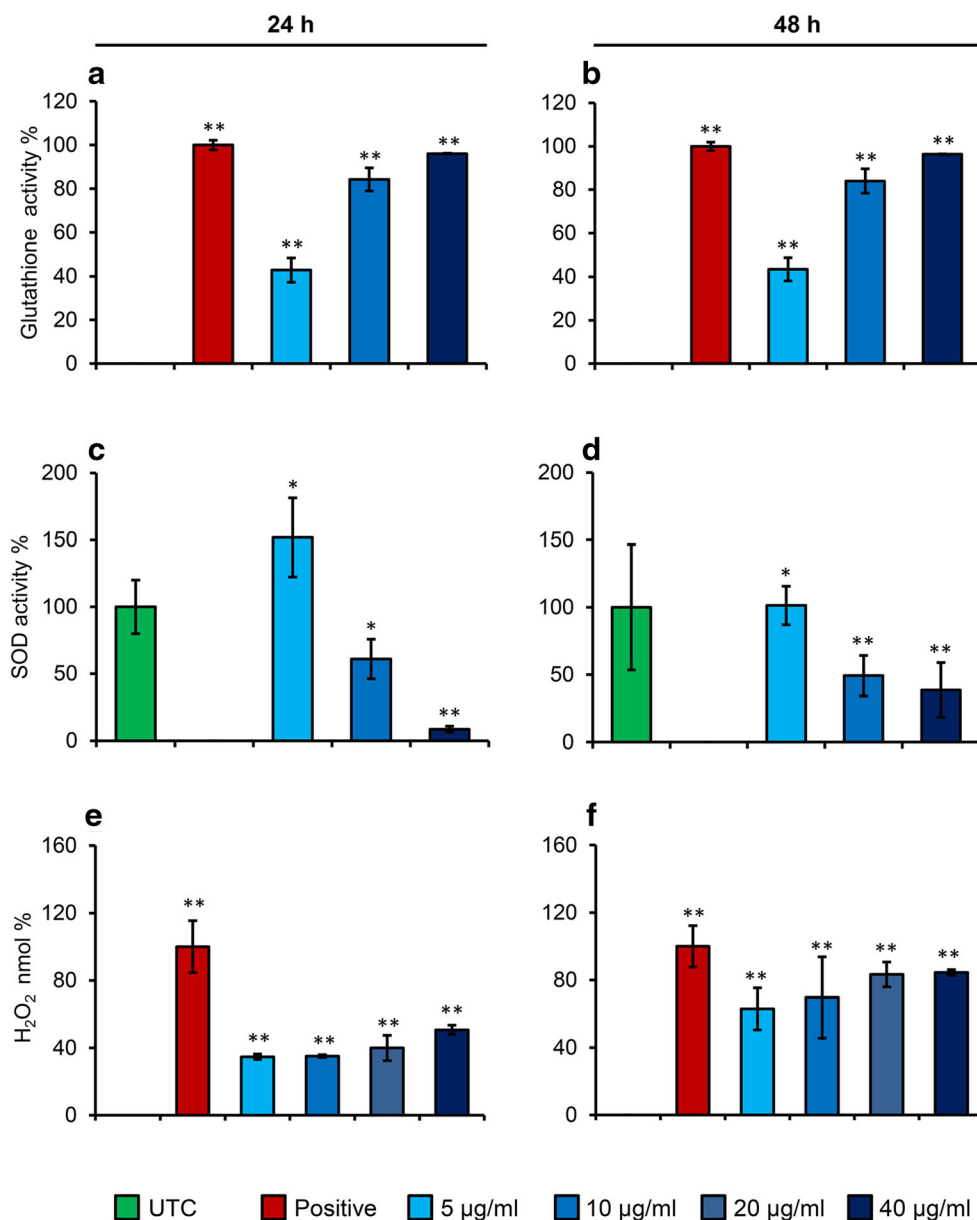


Fig. 4 a Levels of GSH in 24 h. b GSH in 48 h. c SOD in 24 h. d SOD in 48 h. e Catalase in 24 h. f Catalase in 48 h in MCF-7 cells after exposure to Bi₂O₃NPs for 24 and 48 h. Each value represents the mean \pm SE of three experiments. * $p < 0.05$ and ** $p < 0.01$ vs. control

Mitochondrial membrane potential

After exposing MCF-7 cells to $\text{Bi}_2\text{O}_3\text{NPs}$, the lost MMP was evaluated by using the JC-1 fluorescent dye staining method. The strong MMP was observed in untreated cell as the JC-1 dye enters to cells and causes J aggregates with deep red fluorescence. On the other hand, MMP was compromised when cells were incubated with $\text{Bi}_2\text{O}_3\text{NPs}$, especially at higher concentrations and longer durations due to inability of the cells to incorporate JC-1 stain (Fig. 5a). Pearson correlation for JC-1 24 h (Fig. 5b) and 48 h post-incubations (Fig. 5c) indicated strong positive correlation of loss of MMP as a function of increasing the concentration of $\text{Bi}_2\text{O}_3\text{NPs}$ at both incubation time points.

Apoptosis

The apoptotic effects of $\text{Bi}_2\text{O}_3\text{NPs}$ in MCF-7 cells were confirmed using different parameters such as chromosome condensation, caspase-3 activity, phosphatidylserine translocation, and apoptotic and necrotic cell sorting by using a flow cytometer. Untreated (UTC) cells showed normal nucleus, whereas the apoptotic cells showed fragmented chromatin with blue color fluorescence (Fig. 6a). Moreover, the current result revealed significant upregulation of caspase-3 enzyme activity in treated cells as compared with untreated cells (Fig. 6b). Induction of caspase-3 enzyme activity led to cleavage of specific substrates which in turn led to cell death. Time- and dose-dependent effects were remarkably observed as caspase-3 activity increased from 57 to 74% and from 87 to 93% at 24- and 48-h incubation time points, respectively. The leakage of phosphatidylserine from treated MCF-7 cells is an indicator of early apoptosis (Fig. 7). The leakage of phosphatidylserine was significantly increased in a dose- and time-dependent manner. The percentage of apoptotic vs. necrotic cells after exposing was measured using Annexin V-FITC and 7-AAD fluorescent stains. Flow cytometry analysis revealed that $\text{Bi}_2\text{O}_3\text{NPs}$ (20 $\mu\text{g/mL}$) induced apoptosis and necrosis as compared with control (Fig. 8a).

Discussion

Few studies have reported on the hazardous effect of bismuth compounds. The toxicity of nano-sized bismuth oxide ($\text{Bi}_2\text{O}_3\text{NPs}$) differs from bismuth compound due to size, large surface area, and physiochemical nature. The present study was designed to investigate the cytotoxicity and apoptosis of $\text{Bi}_2\text{O}_3\text{NPs}$ in MCF-7 cells. The size of $\text{Bi}_2\text{O}_3\text{NPs}$ was 100 nm according to the supplier; however, the size and shape of nanoparticles were determined by SEM, TEM, and DLS. The measured size of $\text{Bi}_2\text{O}_3\text{NPs}$ was more than the original (supplier) size. In comparison with measurements in the dry

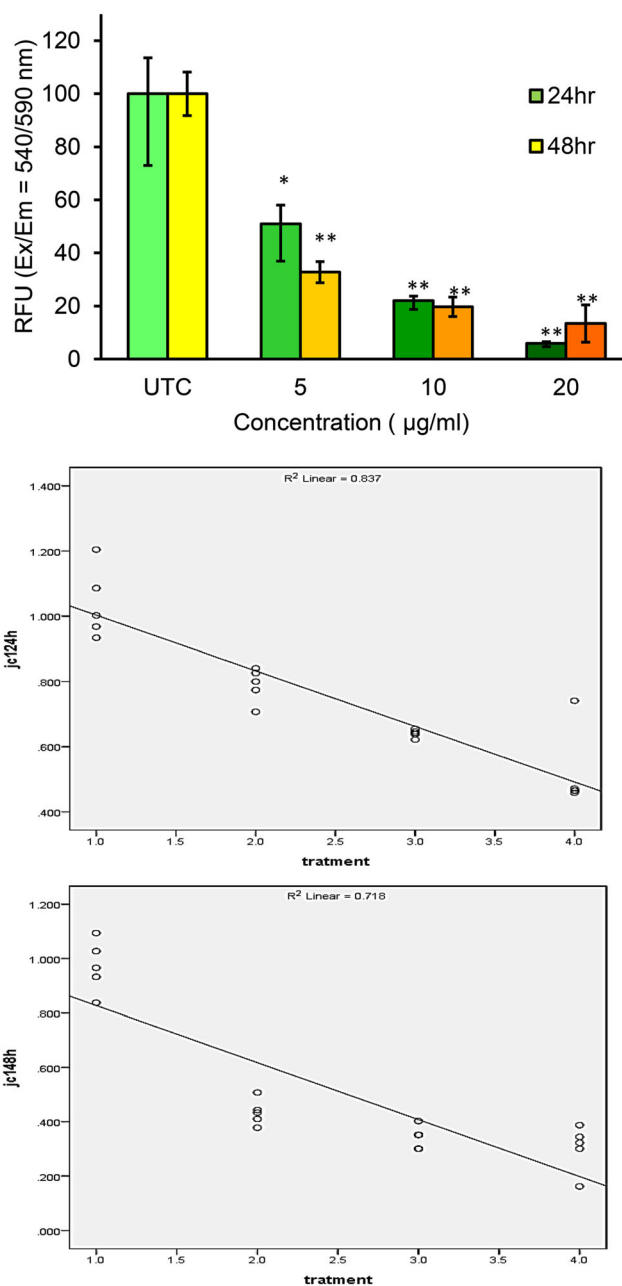


Fig. 5 a Loss of mitochondrial membrane potential determined due to $\text{Bi}_2\text{O}_3\text{NP}$ exposure on MCF-7 cells for 24 h and 48 h, as determined by JC-1 fluorescence dye. b Pearson correlation of JC1 24 h. c Pearson correlation of JC1 48 h. * $p < 0.05$ and ** $p < 0.01$ vs. control

phase, the average particle sizes and size distributions of particles, measured by DLS, were enhanced when measured in aqueous media. Due to high specific surface area and energy level, nanoparticles have the propensity to form micro-size particles that are more stable in the environment (He et al. 2010). To protect the formation of agglomeration, the suspension of nanoparticles was prepared freshly and sonicated prior to the experiment. Strayer et al. (2015) reported that nanoparticles induced more toxic effects due their size, shape, and surface chemistry.

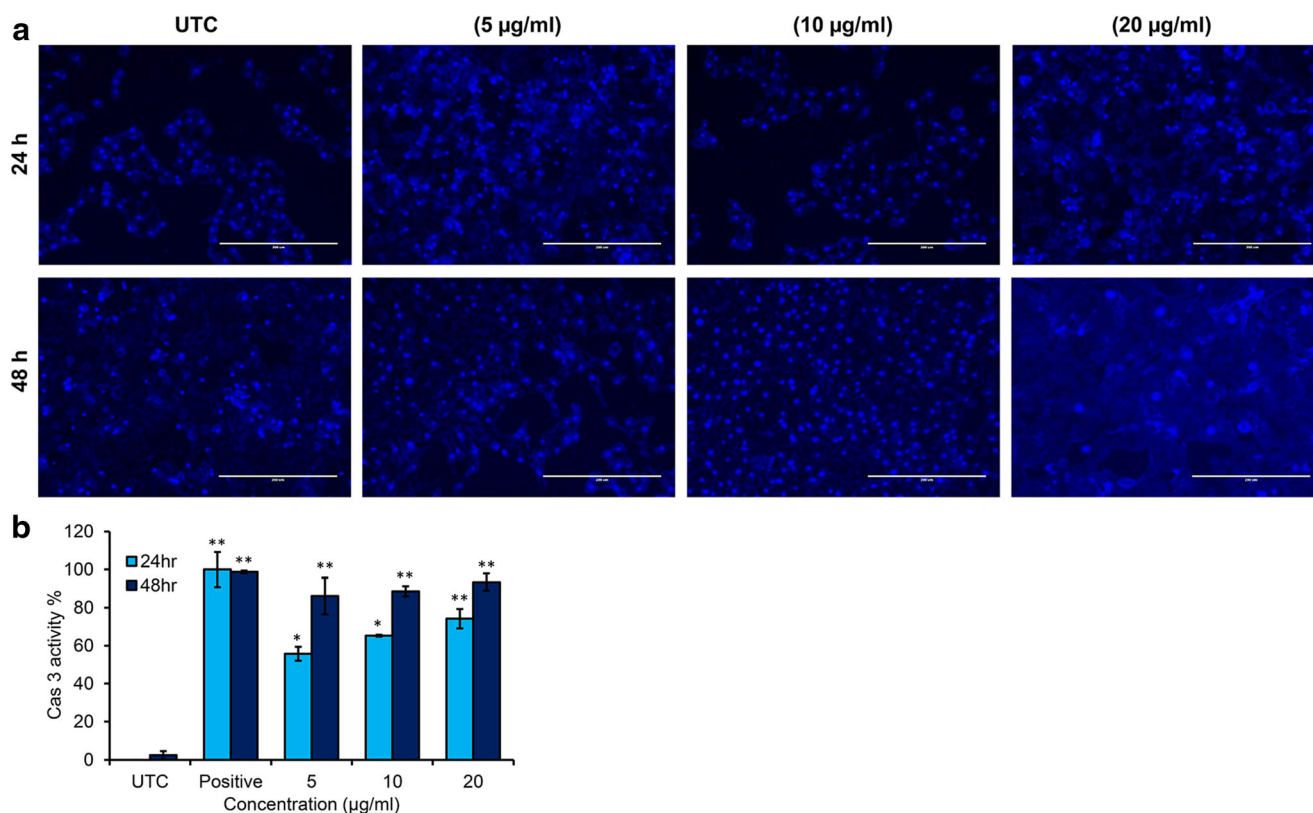


Fig. 6 **a** Chromosomal condensation in MCF-7 cells after exposure to nanoparticles for 24 to 48 h. **b** Induction of caspase-3 activity in MCF-7 cells after exposure to nanoparticle for 24 h and 48 h. Each value represents the mean \pm SE of three experiments. * p < 0.05 and ** p < 0.01 vs. control

In the current experiment, we aimed to identify the cytotoxic mechanism of $\text{Bi}_2\text{O}_3\text{NPs}$ either necrosis or apoptosis in the dose- and time-dependent manner. Therefore, we

performed MTT assay that targets mitochondrial function and the NRU assay that tested lysosomal activity in MCF-7 cells. $\text{Bi}_2\text{O}_3\text{NPs}$ increased the caspase-3 activity and the

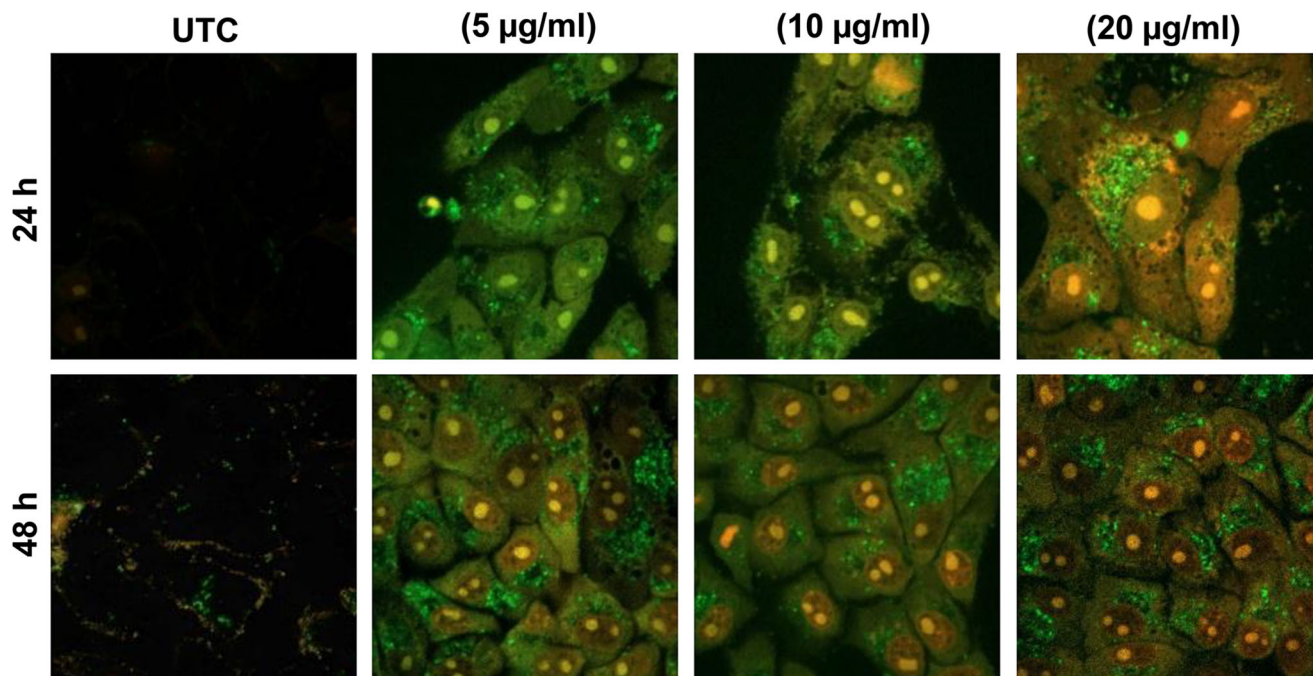


Fig. 7 Phosphatidylserine (PS) translocation in MCF-7 cells after exposure to various concentrations of nanoparticles for 24 to 48 h. Each value represents the mean \pm SE of three experiments

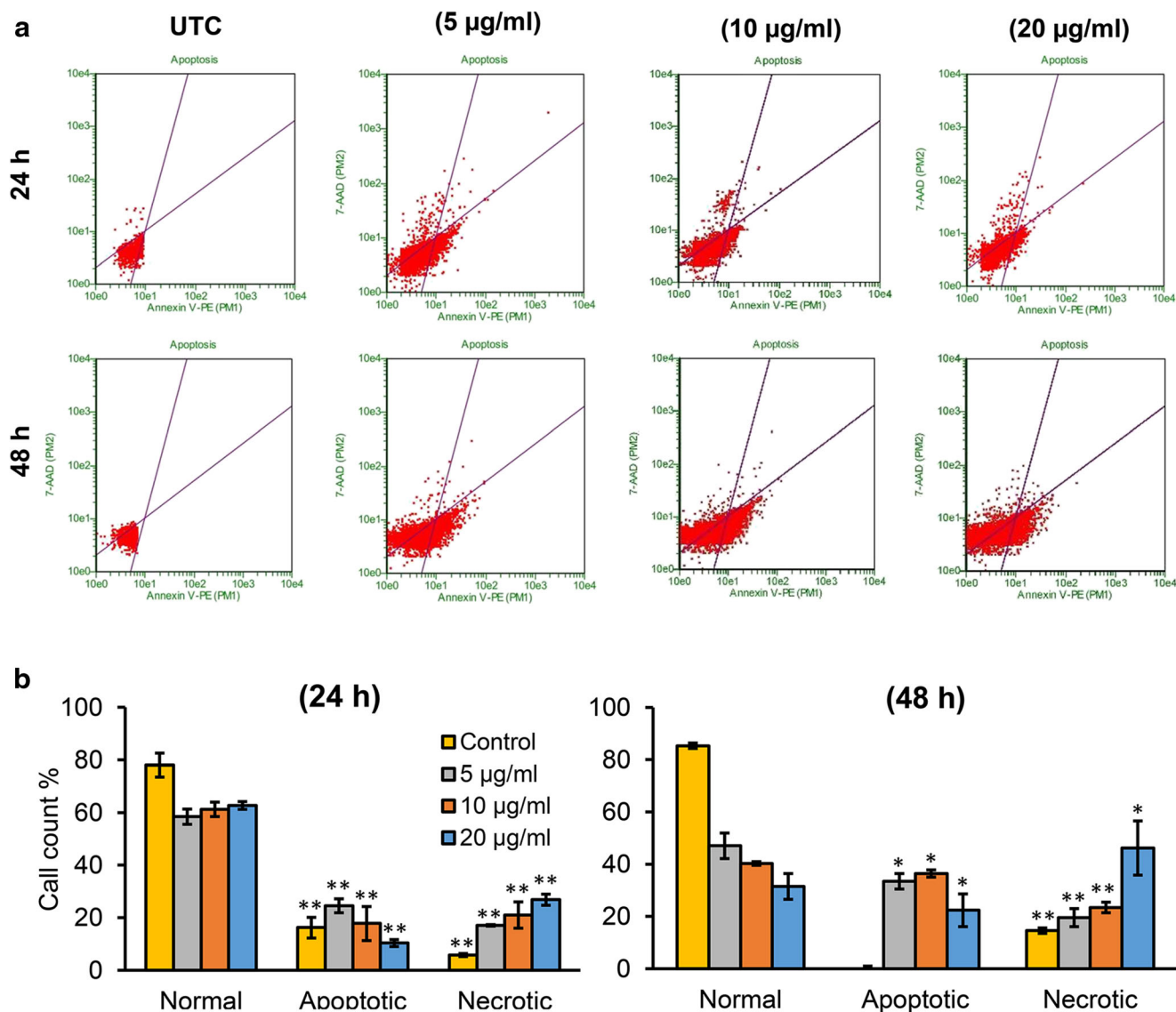


Fig. 8 Various concentrations of $\text{Bi}_2\text{O}_3\text{NPs}$ induced apoptosis and necrosis in MCF-7 cells. **a** Annexin V-FITC and 7-AAD fluorescence were measured using flow cytometer with PI and Annexin V-FITC filters,

respectively. **b** % apoptotic and necrotic cells for 24 h and 48 h. Data represent average \pm SE of triplicate experiments. * p 0.05 compared with control. * p < 0.05 and ** p < 0.01 vs. control

numbers of condensed chromatin in treated MCF-7 cells. Furthermore, the leakage of phosphatidylserine was increased in treated cells as compared with untreated cells. The production of intracellular ROS and changed redox status are normal physiochemical phases in MCF-7 cells. ROS may be interacting with fatty acids of plasma membrane, causing lipid peroxidation (Gaetke and Chow 2003).

Excessive generation of intracellular ROS in treated cells occurred as a consequence of nanoparticles that promote oxidative damage and apoptosis in treated cells. This was followed by increased GSH concentration, as well as SOD and CAT activities. Abudayyak et al. (2017) has pointed that $\text{Bi}_2\text{O}_3\text{NPs}$ induced toxicity in various types of human cell lines. The oxidative stress assay consists of

SOD, catalase, and GSH; these are the defense tools to eliminate the oxidative stress in the cells. Xiong et al. (2014) suggested that mitochondria play an important role in apoptosis pathways, and the loss of mitochondrial integrity may be prevented by different apoptotic markers. $\text{Bi}_2\text{O}_3\text{NPs}$ weaken the mitochondrial membrane potential in MCF-7 cells. In this study, we found that cells, treated with $\text{Bi}_2\text{O}_3\text{NPs}$, had impaired mitochondrial membrane function, which results in high rate of apoptosis. Hüttemann et al. (2011) has reported that oxidative stress activates caspase enzymes via releasing cytochrome-c from the inter-membrane space into the cytoplasm. Our results showed caspase-3 activity and phosphatidylserine externalization, and apoptotic and necrotic cells were increased

after Bi₂O₃NP exposure in a concentration- and duration-dependent manner. These results were confirmed by fluorescence-activated cell sorting (FACS) using Annexin V-FITC and 7-AAD stains. In addition, this result showed that Bi₂O₃NPs damage the DNA, as this leads to the death of the cells. Mitochondria play a significant role in metal toxicity through inducing intracellular ROS generation (Keunen et al. 2011). Stewart et al. (2014) reported that nanoparticles increase ROS generation beyond the oxidative stress threshold of the cell, and finally resulting in apoptosis.

On the basis of the above finding, we conclude that Bi₂O₃NPs induce intracellular ROS generation and oxidative stress and provoked damage to the nuclear materials and organelles of MCF-7 cells. This study suggests that the use of Bi₂O₃NPs should be minimized because of its toxicity.

Authors' contributions Ali Alamer, Daoud Ali, Saud Alarifi, and Abdullah Alkahtane acquired and analyzed the data regarding the cytotoxicity of Bi₂O₃NPs. Mohammed AL-Zharani and Mohamed M. Abdel-Daim acquired and analyzed the data regarding physiochemical characterization of Bi₂O₃NPs and oxidative stress biomarkers. Gadah Albasher and Rafa Almeer acquired and analyzed the data regarding apoptotic markers. Abdulaziz Almalik and Ali H Alhasan contributed in writing the manuscript. Nouf K. Al-Sultan and Ali Alamer contributed in statistical analysis. Christos Stournaras, Saqib Hasnain, and Saad alkahtani were involved in the conception and design of the study and data interpretation, and critically revised the manuscript. All authors read and approved the final manuscript.

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Data availability The data generated or analyzed in this article are online and publicly available without request.

Compliance with ethical standards

Competing interests The authors declare that they have no conflict of interest.

Ethics approval and consent to participate Not applicable.

Consent to publish Not applicable.

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