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Original Article

Bismuth oxide nanoparticles induced oxidative stress-related inflammation in SH-SY5Y cell line

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ABSTRACT

Bismuth (III) oxide nanoparticles' (Bi_2O_3-NPs) unique physicochemical properties have attracted attention in biological, industrial, technological and medical fields. Concurrently, increasing numbers of studies revealing their potential toxic effects and possible toxicity mechanisms are ongoing. In this study, we assessed the toxic potentials of Bi_2O_3-NPs in human SH-SY5Y neuroblastoma cell line. After Bi_2O_3-NPs characterization using TEM, the cytotoxic potentials were evaluated by MTT and LDH assays. The induction of reactive oxygen species production was evaluated by H_2DCFDA. In order to evaluate the oxidative damages, the changes in antioxidant catalase and superoxide dismutase and glutathione levels were determined. The cellular death pathway and the role of immune response were studied by measuring the mRNA expression levels of related genes. Our results showed that Bi_2O_3-NPs decreased the cell viability through disruption on mitochondrial activity (IC50:77.57 µg/mL) and membrane integrity (LDH%50:16.97 µg/mL). At 50 µg/mL Bi_2O_3-NPs, the production of reactive oxygen species (ROS) was induced significantly as well as the catalase and superoxide dismutase levels. In immune response, the mRNA expression levels of interleukin (IL)-6 increased more than 1.5-fold in all doses; whereas, TNF-α, NF-κB and MAPK8 expressions remained unchanged. Consequently, Bi_2O_3-NPs induced oxidative stress-related inflammation via activation of pro-inflammatory cytokine, IL-6.

Keywords: Bismuth (III) oxide nanoparticles, neurotoxicity, oxidative stress

INTRODUCTION

Nanoparticles, chemical structures between 1-100 nm in size, are one of the most promising elements of a new era in technology and science. NPs-based products are growing rapidly in a variety of fields such as health and fitness, electronics and computers, cosmetics, home and garden, food and beverage as well as biomedical applications (Mnyusiwalla et al. 2003; EPA, 2007). The Nanotechnology Consumer Products Inventory report, released in October 2013, listed 1814 nano-containing consumer products, in which the largest group of these products (37%) contains metals and metal oxides NPs (Vance et al. 2015). NPs potentially cause harm to both humans and the environment due to their unique physicochemical properties, size, shape and solubility (Maynard et al. 2006). NPs can easily penetrate the cell membrane due to their very small size and alter the crucial functions of cells. Growing evidence shows that various molecular mechanisms such as DNA damage, oxidative stress, mitochondrial disruption, apoptosis resulting in morphological changes and eventually cell death are responsible for the toxic effects of NPs (Ray et al. 2009; Dhawan and Sharma, 2010; Iavicoli et al. 2013).

Bi_2O_3 is one of the significant metal oxides which has rapidly attracted attention, perhaps because of its use in technology, industry and biomedical sciences (Hyodo et al. 2000; Rabin et al. 2006; Taufik et al. 2011). Despite Bi_2O_3-NPs widespread usages and the increasing intentional or unintentional exposure, there is limited knowledge about their toxicity (Thomas et al. 2012; Hernandez-Delgadillo et

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al. 2013; Liman 2013). Our previous studies show that the toxicity of Bi_2O_3-NPs varies according to the cells type (Abudayyak et al. 2017). Previous data indicate that different metal based NPs highly induce neurotoxicity (Abudayyak et al. 2017b; Song et al. 2014).There is no *in vivo* or *in vitro* study which evaluates the toxic effects of Bi_2O_3-NPs in neuronal cells or systems, and there seems to be an urgent need to gather data. Therefore, this study aims to assess the neurotoxicity of Bi_2O_3-NPs in human neuroblastoma SH-SY5Y cells which are widely used in neurotoxicity research and preferred in the evaluation of metal-based NP toxicity (Abudayyak et al. 2017b; Choi et al. 2007; Chen et al. 2008, Kim et al. 2010). For this purpose, the cytotoxicity was determined by MTT and LDH assays, the ROS production was evaluated by H_2DCFDA, the levels of glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) were measured by ELISA kits, and the mRNA expression levels of inflammation related genes were determined.

MATERIALS AND METHODS

Chemicals

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) and the other chemicals were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). The cell culture mediums and all other supplements were purchased from Multicell Wisent (Quebec, Canada), and sterile plastic materials from Corning (Amsterdam, The Netherlands).

Cell culture and exposure conditions

SH-SY5Y cells (CRL-2266) were purchased from the American Type Culture Collection (ATCC). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) F-12 and Eagle's Minimum Essential Medium (EMEM) in a 1:1 ratio. The medium was supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U-100 µg/mL). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Bi_2O_3-NPs were freshly prepared at 1 mg/mL by suspending them in the culture medium, and sonicated at room temperature for 15 minutes immediately before exposure to avoid the aggregation/agglomeration of NPs. The treatment concentrations of Bi_2O_3-NPs were 50, 25, 12.5 and 6.25 µg/mL and the exposure duration was 24 h. All assays were done in triplicate on independent days. Also, qPCR was performed in triplicate for each cDNA sample as a technical check.

Nanoparticles characterization

Bi_2O_3-NPs (Cat. No. 631930) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Transmission electron microscopy (TEM) (JEM-2100 HR, JEOL, USA) was used to images the nanoparticles. For that, Bi_2O_3-NPs were suspended in a cell culture medium or distilled water and dropped on a copper grid prior to analysis (Abudayyak et al. 2017).

Evaluation of cytotoxic potential

The cytotoxicity of Bi_2O_3-NPs was determined by MTT and lactate dehydrogenase (LDH) assays. MTT assay is based on colour changes from yellow to purple-blue during the reduction of tetrazolium salt to insoluble formazan crystals by the mitochondrial succinate dehydrogenase enzyme (Van Meerloo et al. 2011). The extracellular release of LDH is an indicator of cell membrane damage. LDH activity can be measured via absorbance change as the decrease rate of reduced nicotinamide adenine dinucleotide (NADH) during pyruvate-lactate cycle (Fotakis and Timbrell, 2006; Han et al. 2011).

The cells were seeded at 10^5 cells/mL in 96-well plates. After the culture medium was discarded, serial dilutions starting from 250 µg/mL of Bi_2O_3-NPs were added to the wells. The culture medium and 1% sodium dodecyl sulphate (SDS) were used as negative and positive controls, respectively, for MTT assay. Triton X-100 was used as a positive control for 100% release of LDH in LDH assay. MTT assay was done according to Abudayyak et al. (2017). The LDH release was measured by Cytotoxicity Detection LDH Kit (Roche, Indiana, USA) according to the manufacturer's instructions. The optical densities (ODs) were read at 590 nm and 490 nm for MTT and LDH assays, respectively, using an Epoch microplate spectrophotometer system (BioTek, Vermont, USA). The half-maximal inhibition of enzyme activity (IC_{50}) was calculated as compared to the negative control whereas the half percentage of LDH release ($LDH\%_{50}$) was calculated as compared to the Triton-X 100.

Evaluation of pro-oxidant level

The potential of ROS induction in SH-SY5Y cells due to exposure to Bi_2O_3-NPs was evaluated using H_2DCFDA fluorescent probe by flow cytometer (Eruslanov and Kusmartsev, 2010). For this, the cells were treated with 50, 25, 12.5 and 6.25 µg/mL of Bi_2O_3-NPs. After a 24 h exposure period, the assay was done according to Oztas et al. (2019). The ROS dependent fluorescence intensity of $5x10^4$ cells was measured in FITC channel (excitation at 488 nm; emission at 530 nm) on an ACEA NovoCyte flow cytometer (San Diego, California, USA) and the results were expressed as the median of fluorescence intensity (MFI).

Evaluation of oxidative damage potential

The oxidative damage potential of Bi_2O_3-NPs was evaluated with determination of ROS production and measurement of GSH, CAT and SOD levels (Abudayyak et al. 2017). The cells were seeded at $5x10^5$ density in 3 mL culture medium into each well of 6-well plates and incubated overnight for cell attachment. They were then exposed to 50, 25, 12.5, 6.25 µg/mL of Bi_2O_3-NPs for 24h and culture medium as control. The suspended exposed cells were used for measurement of protein amount, GSH content and antioxidant enzyme levels. The amount of protein was measured by Bradford (1976) method. The GSH, CAT and SOD levels were determined by ELISA kit (SunRed Biological Technology, Shanghai, PRC), based on biotin double antibody sandwich technology, according to the manufacturer's instructions. The levels were calculated using a standard calibration curve, and the results were expressed as µmol, µg and µg per g protein for GSH, CAT and SOD, respectively.

mRNA expression levels of cell death and inflammation genes

The mRNA expression levels of cell death and inflammation related genes (interleukin-6 [IL-6], mitogen-activated protein kinase 8 [MAPK8], nuclear factor kappa B [NF-κB] and tumor

necrosis factor-α [TNF-α]) were determined using LightCycler 480 Probes Master and Catalog Assays (Roche, Mannheim, Germany) on the Roche RealTime LightCycler 480 II platform according to the manufacturer's instructions and Oztas et al. (2019). Cells were seeded at $5x10^5$ density in 3 mL culture medium into each well of 6-well plate and incubated overnight, then, exposed to 50, 25, 12.5, 6.25 µg/mL of Bi_2O_3-NPs for 24h and culture medium as control. The RNA isolation was performed using High Pure RNA Isolation Kit (Roche, Mannheim, Germany) in a benchtop laminar flow hood. After RNA quantity and purity were checked, cDNA synthesis was carried out using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. β-actin [ACTB] was used as a housekeeping gene, and the results were expressed as the fold-change of the control.

Statistical analysis

Data was expressed as mean ±standard error (SE). The significance was calculated by one-way ANOVA Post Hoc Dunnett t-test using IBM SPSS v. 20.0 for Windows (IBM Corp.; Armonk, NY, USA). A two-tailed $p<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Nanoparticles characterization

The particle size and size distribution of Bi_2O_3-NPs in both distilled water and cell culture medium were analysed by TEM (Figure 1). The calculated average particle diameter in water was 149.7 nm. The agglomeration- aggregation could explain the increase in the average diameter (182.2 nm) of the particles after dissolution in cell culture medium (Abudayyak et al. 2017).

Evaluation of cytotoxic potential

Bi_2O_3-NPs decreased the cell viability through disruption on mitochondrial activity and membrane integrity which are determined by MTT and LDH assays, respectively. The IC_{50} values

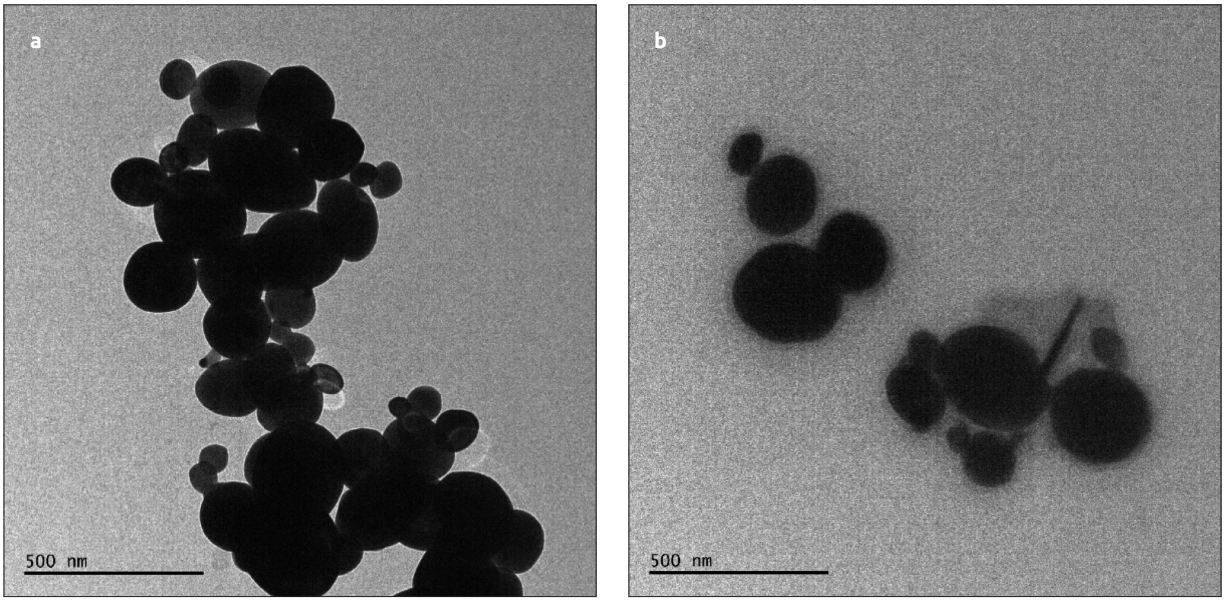


Figure 1. a, b. The Characterization of Bi_2O_3-NPs in (a) distilled water and (b) cell culture medium by TEM analysis.

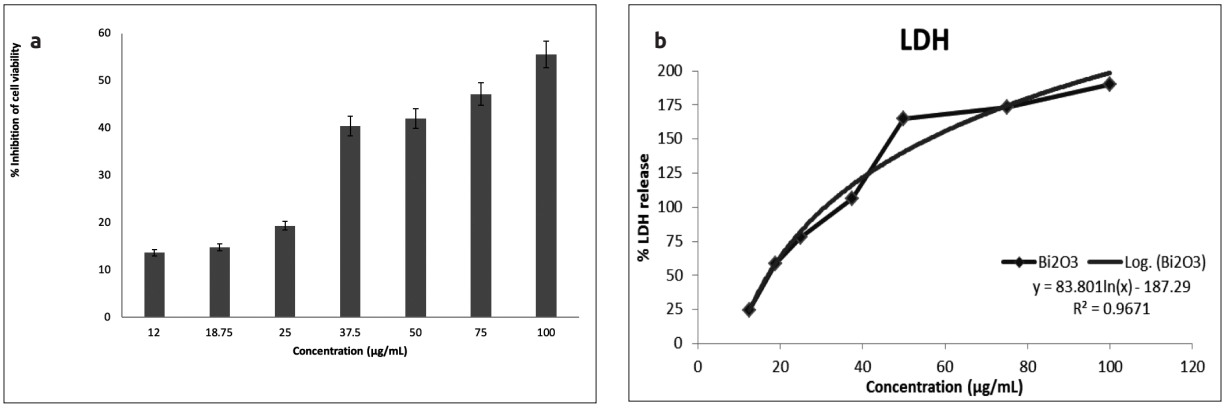


Figure 2. a, b. Inhibition of cell viability and extracellular release of LDH by MTT and LDH assays, respectively.

Inhibition of cell viability (a) and LDH% release (b, grey line) were measured in the cells exposed to Bi_2O_3-NPs (12.5-100 µg/mL). $LDH\%_{50}$ (b) was calculated according to the formula of the logarithmic (red line) curves. The IC_{50} values of Bi_2O_3-NPs were 77.57 µg/mL by MTT assay and $LDH\%_{50}$ was 16.97 µg/mL by LDH assay. Error bars represent ±standard deviation.

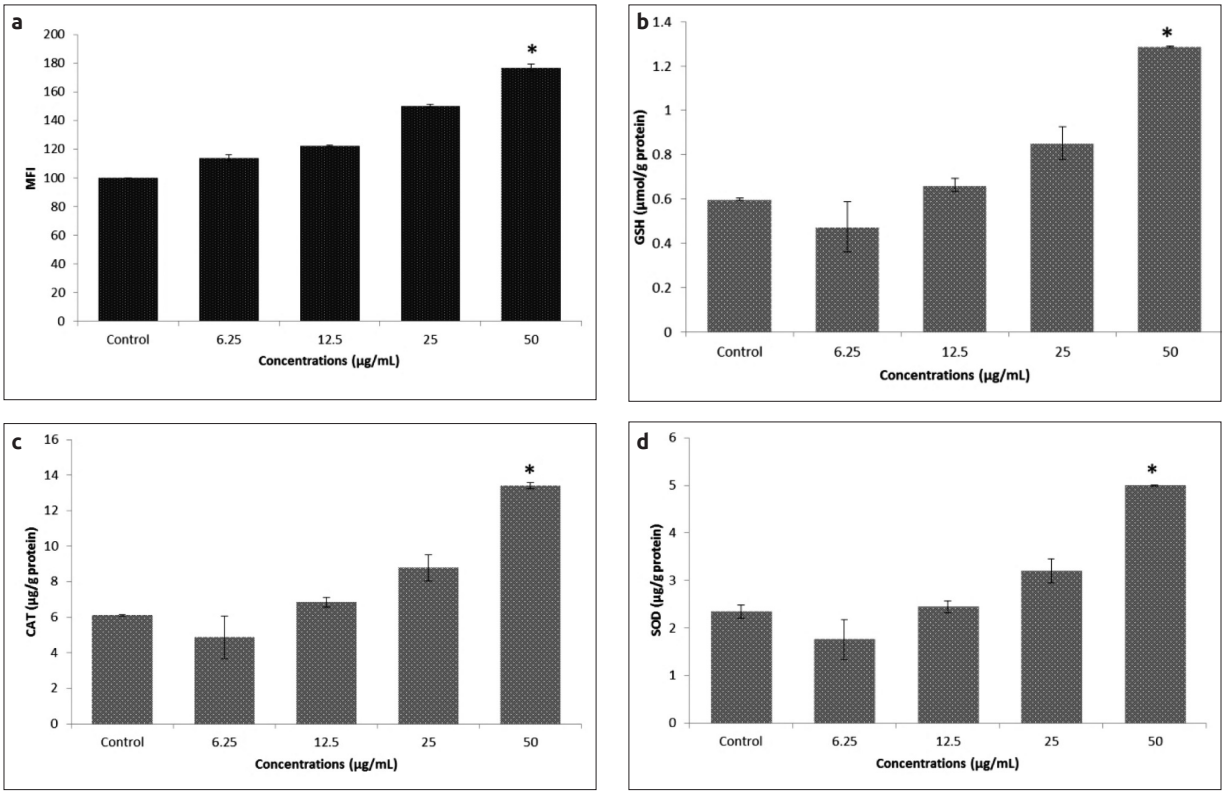


Figure 3. a-d. The pro-oxidant levels induction and oxidative damage potential of Bi_2O_3-NPs.

Pro- oxidant ROS production (a) and the levels of GSH (b), CAT (c) and SOD (d) were evaluated in cells exopsed to 6.25-50 µg/mL Bi_2O_3-NPs for 24 h. The ROS production was expressed as MFI and GSH, CAT and SOD levels were expressed as µmol, µg and µg per g protein, respectively. Cells exposed to culture medium used as control. The error bar represents ±standard error, one-way ANOVA Post Hoc Dunnett t-test was performed and *p<0.05.

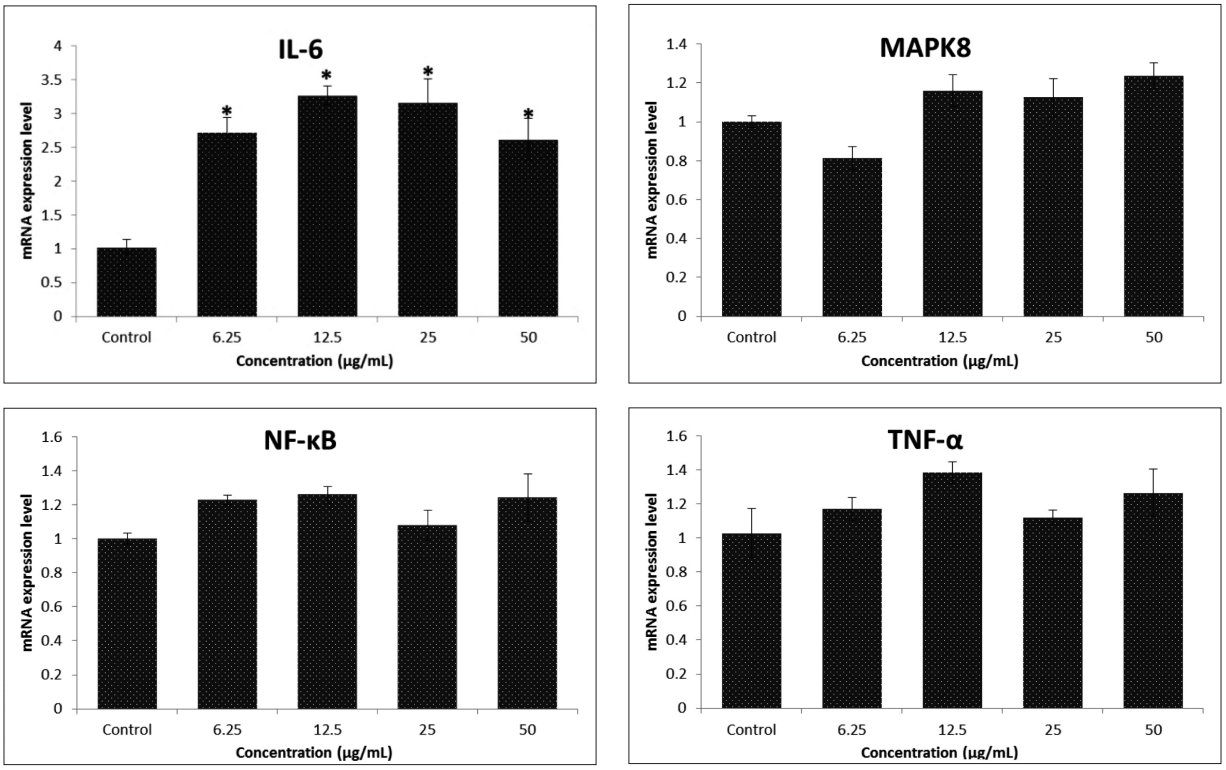


Figure 4. mRNA expression levels of cell death and inflammation related genes.

Cells were treated with various concentrations of Bi_2O_3-NPs (6.25-50 µg/mL). The results were expressed as the relative fold-change. Cells exposed to culture medium used as control, the error bar represents ±standard error one-way ANOVA Post Hoc Dunnett t-test was performed and *p<0.05.

of Bi_2O_3-NPs were 77.57 µg/mL by MTT assay (Figure 2a) and LDH%$_{50}$ was 16.97 µg/mL by LDH assay (Figure 2b).

Assay of pro-oxidant levels

The potential of Bi_2O_3-NPs to induce the production of ROS was evaluated by flow cytometer using H_2DCFDA fluorescent probe. The results showed an increase in ROS levels in a dose dependent manner (Figure 3a). The ROS production was significantly induced over 1.5-fold in the highest concentration (50 µg/mL) ($p<0.05$).

Oxidative damage potential

The oxidative damage potential of Bi_2O_3-NPs was evaluated by measuring the levels of GSH, CAT and SOD. Bi_2O_3-NPs induced oxidative damage in a dose-dependent manner. Additionally, GSH content, CAT and SOD activity were significantly higher with approximately 2-fold change at 50 µg/mL Bi_2O_3-NPs than control ($p<0.05$) (Figures 3b-d).

Cell death and inflammation related gene expressions

The regulations in cell death and inflammation related genes were determined in Bi_2O_3-NPs treated SH-SY5Y cells using specific hydrolysis probes on a qPCR platform (Figure 4). Bi_2O_3-NPs increased the mRNA expression levels of IL-6 more than 1.5-fold in all doses ($p<0.05$); whereas the changes in expression levels of MAPK8, NF-κB and TNF-α were insignificant.

DISCUSSION

Toxicity of metal-based NPs is still an emerging topic because they may have a quite different toxicity profile depending on their shape, size, coating, surface charge etc. than their macro-sized compounds or salts. Although, a few reports indicated that Bi_2O_3-NPs showed toxic effects in several cell lines, there is no data about the neurotoxicity potential and related molecular mechanism of Bi_2O_3-NPs. Differentiated or undifferentiated SH-SY5Y cells are widely used *in vitro* models in the neuroprotection or neurotoxicity studies due to their morphological, neurochemical and electrophysiological similarities to neurons (Xie et al., 2010). Therefore, the toxicity profile of Bi_2O_3-NPs in SH-SY5Y cells was evaluated and focused on oxidative stress related mechanisms.

In the present study, Bi_2O_3-NPs showed cytotoxicity in SH-SY5Y cells through disruption on mitochondrial activity and membrane integrity in a dose-dependent manner. Based on the IC_{50} value (77.57 µg/mL) and LDH%$_{50}$ value (16.97 µg/mL), it can be suggested that cell membrane is more vulnerable to Bi_2O_3-NPs in SH-SY5Y cells; since, the concentration caused the extracellular leakage of LDH to be lower. Similarly, Abudayyak et al. (2017) reported that Bi_2O_3-NPs showed cytotoxicity in several cell lines in which IC_{50} values ranged from 35.11-96.55 µg/mL and cell sensitivity to cytotoxic damage ranked at NRK-52E < Caco-2 ~ A549 < HepG2. Cornelio et al. (2011) reported that Bi_2O_3 caused slightly cytotoxic effect in murine periodontal ligament and rat osteosarcoma cells at 100 mg/mL. In a study by Song et al. (2014), they reported a cytotoxic potential of bismuth ferrite NPs in PC-12 rat neuronal cells. In a recent research by Akbarzadeh et al. (2018), it was noticed that Bi_2O_3-NPs and Folate-Conjugated Bi_2O_3-NPs induce cell death in KB nasopharyngeal carcinoma and A549 cell lines. Similar results were reported by Ahamed et al. (2019) and Bogusz et al. (2018) in MCF-7 human breast cancer and 9L malignant gliosarcoma cell lines. By contrast, Hernandez-Delgadillo et al. (2013) reported that aqueous colloidal Bi_2O_3-NPs did not induce cytotoxicity in Vero cells at ≤ 1 mg/mL. It should be considered that the controversial results could be associated with the selected cell types, with the cytotoxicity assays as well as with Bi_2O_3 size and formulation.

Toxicity of metallic NPs could be related with two main ways release of ions and oxidative stress (Seabra and Duran, 2015). NPs may interact with cellular proteins and enzymes resulting in increased ROS production which may cause DNA damage, lipid peroxidation, apoptosis and necrosis (Arora et al. 2012; Elsaesser and Howard, 2012). It is well known that oxidative stress plays an important role in many complications such as diabetes, atherosclerosis, cancer as well as aging. Since, NPs may induce excessive ROS production in the nervous system with their ability to cross blood brain barrier, neurodegenerative diseases such as Parkinson and Alzheimer could be a consequence of NP toxicity (Sorg 2004; Schrand et al. 2010; Afanas'ev 2011). Ahamed et al. (2019) reported oxidative stress mediates the cytotoxic effects of Bi_2O_3-NPs in MCF-7 cells. They also indicated the role of ROS in the oxidative stress. In the present study, Bi_2O_3-NPs induced oxidative damage in a dose-dependent manner through increasing in ROS production, GSH content, CAT and SOD activity. Similar to our results, both macro- and nano-structured Bi_2O_3 induced oxidative stress in cultured human blood cells and in HepG2, NRK-52E, Caco-2 and A549 cells (Abudayyak et al. 2017; Geyikoglu and Turkez, 2005), respectively.

It seems that NPs may induce ROS production by either innate immune response or autocatalysis (Love et al. 2012). Considering the bivious relationship between oxidative stress and inflammation, profound changes in mRNA expression levels of pro-inflammatory cytokines as key elements of immune response can be attributed as a consequence of oxidative stress (Marklová, 2007; Park and Park, 2009). Oxidative stress can trigger signalling pathways of MAPK8, and transcription factors such as NF-kB and TNF-α; and finally release of pro-inflammatory cytokines and some critical chemokines (Roach et al. 2002; Shishodia and Aggarwal, 2002). Ahamed et al. (2019) indicated the role of Bax/Bcl-2 pathway in cell death. Several reports indicated that NPs such as titanium dioxide (Monteiller et al. 2007), carbon black (Niwa et al. 2008) and crystalline silica (Rao et al. 2004) induced strong pro-inflammatory responses; however, in the present study Bi_2O_3-NPs induced pro-inflammatory response is evaluated for the first time. In the present study, Bi_2O_3-NPs increased the mRNA expression levels of IL-6, an important factor for the coordination of the innate and acquired immune response. MAPK8, NF-κB and TNF-α remained unchanged and it should be considered that these signalling pathways also play a crucial role in oxidative stress-mediated apoptosis and/or necrosis (Chandra et al., 2002). Considering the previous study (Abudayyak et al., 2017) reported that Bi_2O_3-NPs induce apoptosis in HepG2 and NRK-52E cells and necrosis in Caco-2 and A549 cells. This study is limited in that apoptotic/

necrotic cell status was not checked with any other methods such as Annexin V/Propidium iodide staining.

CONCLUSION

In the fields of drug delivery and toxicology, the application of NPs has been studied intensively in the past few decades. Potential toxic effects of metal oxide-based NPs have received growing attention and not been completely addressed. Preliminary data from *in vitro* experiments can potentially provide precautionary hazard identification about toxic effects on the people who come into contact with NPs. Obtained data will lead the way to safe usage of NPs in drug delivery and industrial applications.

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Conflict of Interest: The authors have no conflict of interest to declare.

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