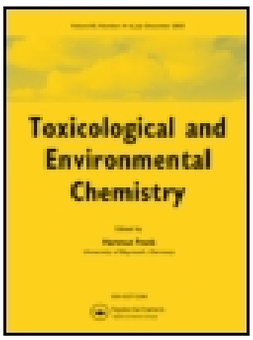

Toxicological & Environmental Chemistry

ISSN: 0277-2248 (Print) 1029-0486 (Online) Journal homepage: http://www.tandfonline.com/loi/gtec20

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To cite this article: Saud Alarifi, Daoud Ali & Saad Alkahtani (2016): Involvement of mitochondrial dysfunction in nanosized lead oxide induced cellular damage in human lung alveolar epithelial cells, Toxicological & Environmental Chemistry, DOI: 10.1080/02772248.2016.1234177

To link to this article: http://dx.doi.org/10.1080/02772248.2016.1234177

TOXICOLOGICAL & ENVIRONMENTAL CHEMISTRY, 2016
VOL. 0, NO. 0, 1–11
http://dx.doi.org/10.1080/02772248.2016.1234177

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Involvement of mitochondrial dysfunction in nanosized lead oxide induced cellular damage in human lung alveolar epithelial cells

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ABSTRACT

High levels of industrial lead (Pb) exposure have decreased in the last 10 years as an outcome of removal of the metal from gasoline and paints. However, environmental Pb exposures remain extensive and may be correlated with adverse human health outcomes. The present study was designed to examine molecular mechanisms underlying cytotoxicity of lead oxide nanoparticles (PbONPs) on human lung alveolar epithelial (A549) cells. When A549 cells were incubated with PbONPs, the production of reactive oxygen species was enhanced as observed by 2',7'-dichlorodihydrofluorescein diacetate. PbONPs significantly reduced proliferation of A549 cells and increased caspase3 activity. In addition, exposure of PbONPs decreased levels of glutathione, and increased lipid peroxide levels and activities of superoxide dismutase and catalase. Exposure of PbONPs enhanced DNA damage as evidenced by tail DNA (%) and olive tail moment. Taken together, these finding indicated that PbONPs diminished cell proliferation and increased apoptotic cell death of A549 cells.

KEYWORDS

Lead oxide nanosphere; A549 cells; DNA damage; apoptosis

Introduction

With the development of nanotechnology, there is a tremendous growth of the application of nanomaterials which increases the risk of human exposure to these nanomaterials (Kisin et al. 2007; Zhao and Castranova 2011). The production and use of engineered nanomaterials (NM) are constantly expanding due to exploitation of unusual properties exhibited by materials at the nanoscale, making them less than 100 nm in at least one dimension (Salata 2004; Kermanizadeh et al. 2016). NPs may include transition metals and metal oxides, silica, carbon compounds (single-and multi-walled carbon nanotubes and fullerenes), nanocrystals and quantum dots, among others (Dreher 2003; Alaraby et al. 2016). The enhanced presence of NMs in the environment requires an assessment of potential toxicities and biological interactions (Zhao and Castranova 2011; Kermanizadeh et al. 2016).

Lead (Pb) is an abundant environmental toxicant and is distributed in the environment as metallic Pb (Bellinger 1994). Exposure to Pb may occur through multiple routes,

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including consumption of contaminated foods, water and house dust (Carneiro, Evangelista, and Barbosa 2013; Lopes et al. 2015). Martins Jr. et al. (2015) reported genetic polymorphisms in ALAD and GPx activities in Brazilian battery workers due to effects of Pb exposure. Spivey (2007) noted that exposure to Pb occurred due to food-borne pollutants and tap water. Metallic Pb is known to adversely affect kidney, reproductive organs, nervous and hemopoetic system (Baranowska-Bosiackaa et al. 2009; Liu et al. 2014; Counter, Buchanan, and Ortega 2015; Pollack et al. 2015).

Some investigators suggested that oxidative stress is the main mechanism involved in Pb-mediated toxicity. This is characterized by the production of enhanced levels of reactive oxygen species (ROS). Katsuyama, Matsuno, and Yabe-Nishimura (2012) reported that ROS are generated by NADPH oxidase or mitochondrial electron transport chain in cells and are implicated in differentiation and proliferation of cells and stability of genome. Ostrovsky et al. (2009) demonstrated oxidative stress and ROS stimulate cellular damage comprising apoptosis and DNA fragmentation.

The potential for adverse effects of lead oxide nanoparticles (PbONPs) is a specific concern because damage to genetic material may induce carcinogenesis, mutagenesis and, ultimately, cell death. The aim of this investigation was to determine levels of generation of ROS and oxidative stress as potential genotoxic mechanism attributed to PbONPs in A549 cells.

Materials and methods

2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), glutathione, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red (NR) uptake dye, culture media and fetal bovine serum were purchased from Sigma (St. Louis, MO, USA).

Characterization of nanoparticles

Suspension of PbONPs was prepared in Dulbecco's modified eagle medium (DMEM) (1 mg/mL) and sonicated in sonicator for 30 min at 40 W capacity at room temperature. The diameter of PbONPs was determined by using transmission electron microscope (TEM). The hydrodynamic size of PbONPs was measured by dynamic light scattering (DLS) (Nano-Zeta Sizer-HT, Malvern Instrument, UK).

Cell culture and treatment of nanoparticles

A549 cells purchased from NCCS, Pune, India, were cultured in DMEM/F-12 medium at 37 °C in CO_2 (5%) incubator. Cells (85% confluence) were harvested and cultured into 96-well plates and 25-cm^2 flask at different concentrations (0, 5, 10 or 50 μg/mL) of PbONPs.

Morphological analysis of cells

The shape and size of A549 cells were observed using an inverted microscope (Leica DMIL) after exposure of PbONPs for 24 hours.

Cell viability test

Cell viability test was measured by two methods: (1) MTT assay and (2) NR uptake, according to Alarifi et al. (2013).

Mitochondrial membrane potential (MMP)

Evaluation of MMP due to exposure of PbONPs in A549 cell over 24 hr was carried out according to JC-1 mitochondrial membrane potential kit (Cayman Chemical Ann Arbor, MI).

ROS evaluation

ROS generation A549 cells were evaluated using DCFH. A549 cells were cultured into 96-well plates with black bottomed (Nun, Thermo Scientific, Germany) and 24 hr later exposed for 12 and 24 hr to PbONPs. ROS was quantified by recording at 529 nm of dye, DCFH by Glomax® multidetection system (Promega, Madison, WI). Three independent experiments were performed, and in each experiment, 10 wells were used as replicates. Results are represented as the fold increase in ROS levels of PbONPs-exposed A549 cells as compared to untreated cells.

Antioxidant enzymes evaluation

A549 cells were exposed to PbONPs in culture flask (75 cm^2) for over 24 hr. After 12 and 24 hr, the cells were removed and placed in lysis buffer. Protein content was measured by Lowry et al. (1951). Catalase (CAT) enzyme was quantified from alteration of H_2O_2 to H_2O (Aebi 1984) and superoxide dismutase (SOD) (Marklund and Marklund 1974).

Levels of thiobarbituric acid reactive substances (TBARS) and glutathione (GSH)

Cell lysates were used for the determination of lipid peroxidation. Malondialdehyde is a product of lipid peroxide and was evaluated as TABRS (Buege and Aust 1978). GSH was measured according to Carlsberg and Mannervik (1985).

Chromosome condensation

For determination of chromosome condensation, 4,6-diamidnio-2-phenylindole (DAPI) marker was used. Solution of DAPI was added to A549 cells in chambered slide and A549 cells were present for 15 min at 37 °C in the dark. Microphotographs of nucleus were captured by fluorescence microscope (Dhar-Mascareno, Carcamo, and Golde 2005).

Caspase-3 activity

Twenty-four hours of A549 cells exposure with or without PbONPs cells were cleaned thrice and reseeded in culture media. Caspase-3 activity determined by caspase-3

(Red-DEVD-FMK, Calbiochem, San Diego, CA) detection kits and Glomax® multi detection system (Promega). The method was used as described by the manufactures.

DNA damage determined by SCGE assay

The single cell gel electrophoresis (SCGE) assay was performed by using the method of Ali, Ray, and Hans (2010).

Statistical analysis

Experiments were done independently in triplicate for each experiment. Data were analyzed through one-way analysis of variance followed by Student's-*t* test to determine statistical significance. A $p < 0.05$ was considered significant.

Results

Characterization of PbONPs

The average hydrodynamic diameter and zeta potential of PbONP suspension in milli-Q water assessed by DLS were 153 nm and-11 mV. The mean size observed by TEM was 40 nm (Figure 1).

Shape of A549 cells

Figure 2 illustrates the morphological changes in untreated and PbONP-exposed A549 cells for 12 and 24 hr. PbONPs (50 μg/mL)-exposed A549 cells altered the spherical shape and the cells became detached from the outer layer of the culture flask (Figure 2(B,C).

Cell viability and mitochondrial membrane potential (MMP)

PbONP-induced toxicity in A549 cells was observed as MMP and NR uptake method. MMT results showed a concentration and time-dependent cytotoxic effect due to PbONPs in A549 cells (Figure 3(A)). NR uptake data were similar to the MTT

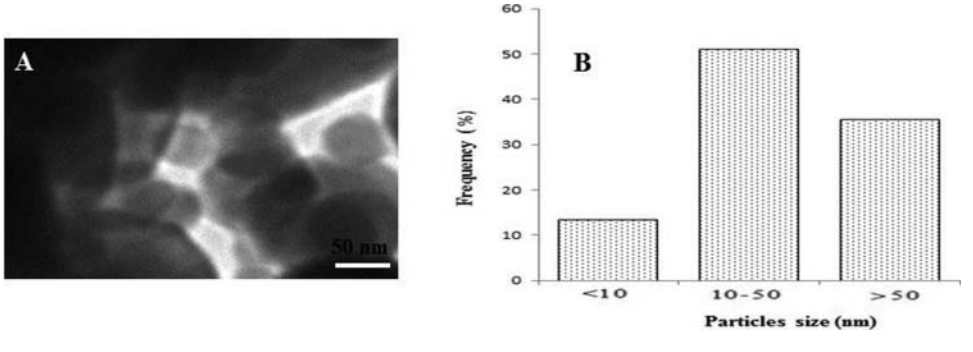

Figure 1. Characterization of PbO nanoparticles A: TEM image; B: percentage of size distribution of PbO nanoparticles.

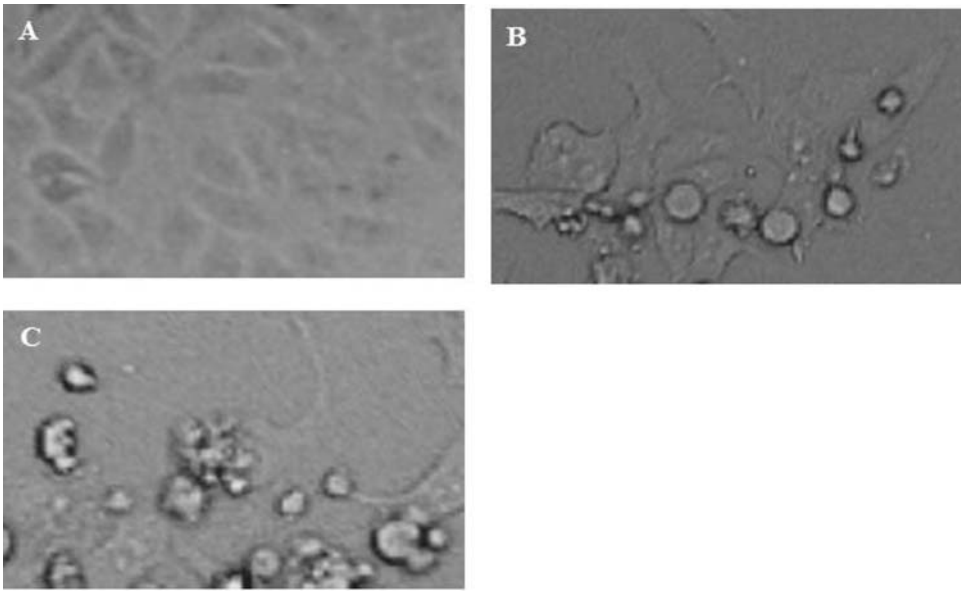

Figure 2. Morphology of A549 cells: A: control; B: at 50 μg/mL of PbO nanoparticles for 12 hr; C: at 50 μg/mL of PbO nanoparticles for 24 hr.

observations (Figure 3(B)). MMP was decreased after 24-hr treatment with PbONPs in A549 cells (Figure 4). Degeneration in MMP was demonstrated in a concentration-dependent manner with significant decline at 50 μg/mL of PbONPs.

Oxidative stress

Induction of oxidative stress by PbONPs was evaluated by the estimation of the ROS (Figure 5), lipid peroxidation, GSH, SOD and CAT (Figure 6) in A549 cells. Data demonstrated that PbONPs produced enhanced intracellular ROS production in a concentration- and time-dependent fashion (Figure 5). PbONP-induced oxidative stress was further evidenced by the reduction of GSH (Figure 6(B)) and increased lipid peroxides, SOD and CAT concentration- and time-dependent manner (Figure 6 (A,C,D).

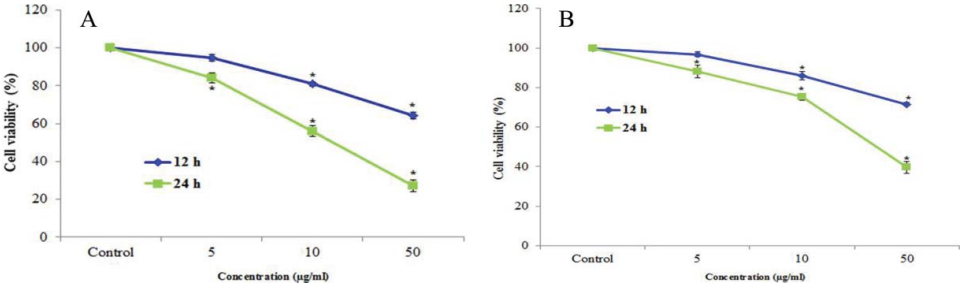

Figure 3. Cytotoxicity of PbONPs in A549 cells for 12 and 24 hr, as measured by (A) MTT and (B) NRU tests. Each value represents the mean ± SE of three experiments.$^{*}p < 0.05$ vs. control.

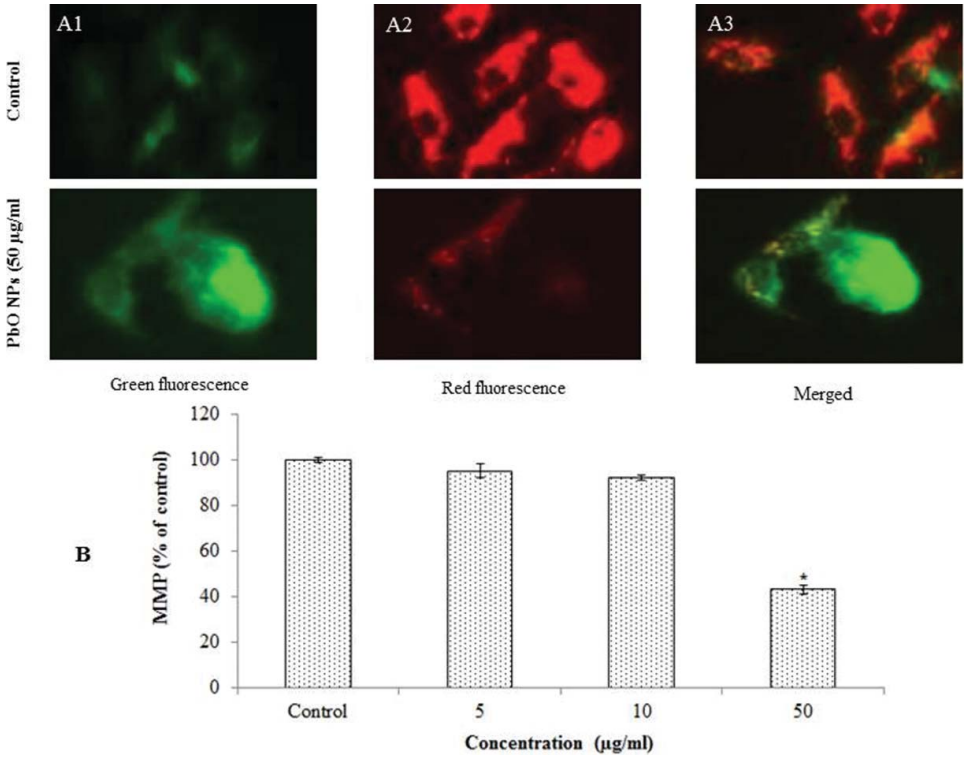

Figure 4. Effect of PbONPs on the MMP of A549 cells. Representative images in fluorescence microscope. A1 appears green fluorescence (JC-1 monomer); A2 appears red fluorescence (JC-1 aggregate); A3 appears merged fluorescence (green and red); B: ratio of MMP (%). $^{*}p < 0.05$ vs. control.

Activity of caspase-3 and chromosome condensation

Figure 7(B) shows condensed chromosome due to PbONP exposure compared with control (Figure 7(A)). Caspase-3 enzyme activity involved in the apoptotic pathway of cells was induced after the treatment of PbONPs (Figure 7(C)). After exposure with PbONPs for over 24 hr, caspase-3 activity rose according concentration and time dependent manner.

DNA strand breakage

DNA damage was assessed as percent tail DNA (Figure 8(A)) and olive tail moment (Figure 8(B)). A549 cells exposed to PbONPs showed higher DNA damage than control. The highest DNA damage was observed at PbONPs (50 µg/mL) in A549 cells (Figure 8).

Discussion

Results of the present study demonstrated adverse effects of PbONPs to A549 cells. Previously, Lopes et al. (2015) reported that exposure to Pb posed a risk to human health (Mason, Harp, and Han 2014). Several investigators also demonstrated a concentration-dependent cytotoxic effect of Pb in different cell systems. In the current study, 50 µg/mL

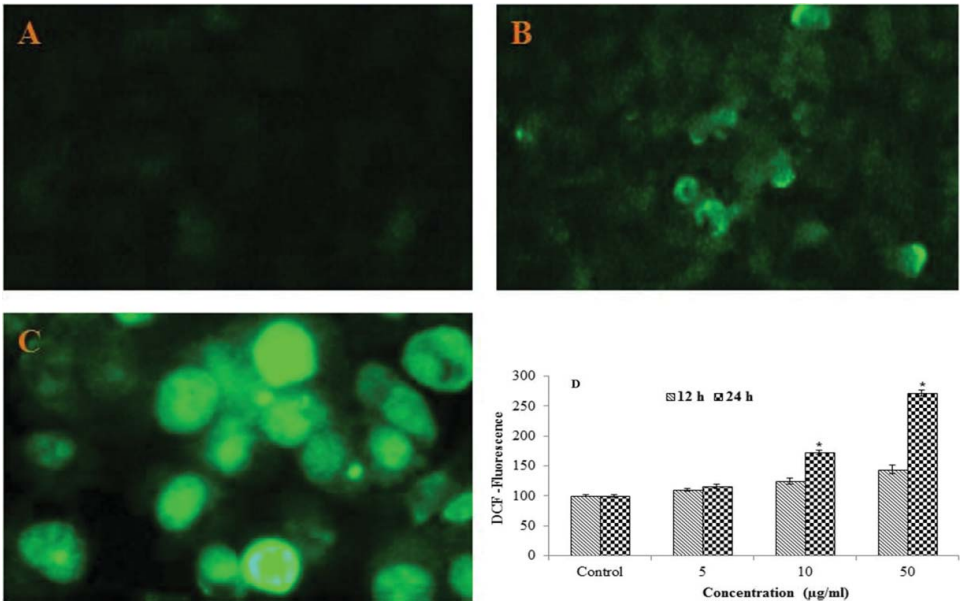

Figure 5 A–C: Effect of PbO nanoparticles on ROS generation in A549 cells. A: control; B: at 50 µg/mL of PbO NPs for 12 hr; C: at 50 µg/mL of PbO NPs for 24 hr; D: % ROS production due to PbO NPs in A549 cells. Each value represents the mean ± SE of three experiments.$^{*}p < 0.05$ vs. control.

PbONPs inhibit more than 50% A549 cell proliferation using both NR an MTT assays. Although MTT and neutral red uptake (NRU) tests are two methods to measure the cytotoxicity of A549 cells, Lanone et al. (2009) reported that MTT test was more sensitive than NRU test to evaluate NP toxicity. In this study, it was found that the EC_{50} value of PbONPs through MTT test was less sensitive than that of NRU test. Our results are in accordance with the finding of Davoren et al. (2007) for toxicity due to single-walled

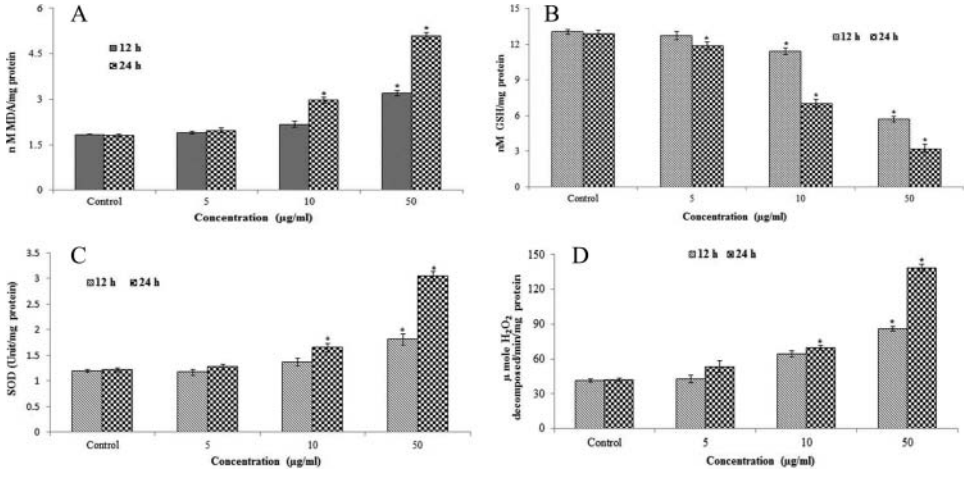

Figure 6. Effect of PbO nanoparticles on (A): lipid peroxide; (B): glutathione; (C): superoxide dismutase; and (D): catalase in A549 cells. Each value represents the mean ± SE of three experiments.$^{*}p < 0.05$ vs. control.

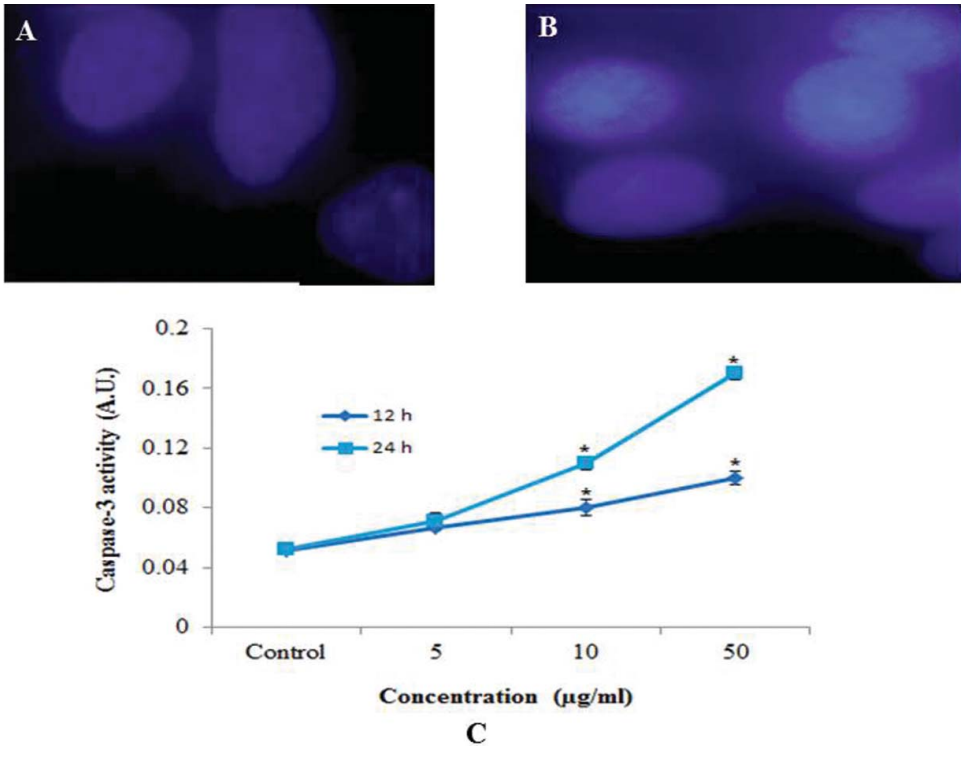

Figure 7. Chromosomal condensation and induction of caspase-3 activity in A549 cells due to PbO nanoparticles. A: control; B: at 50 μg/mL for 24 hr; C: caspase-3 activity. Each value represents the mean ± SEM of three experiments. $^{*}p < 0.05$ vs. control.

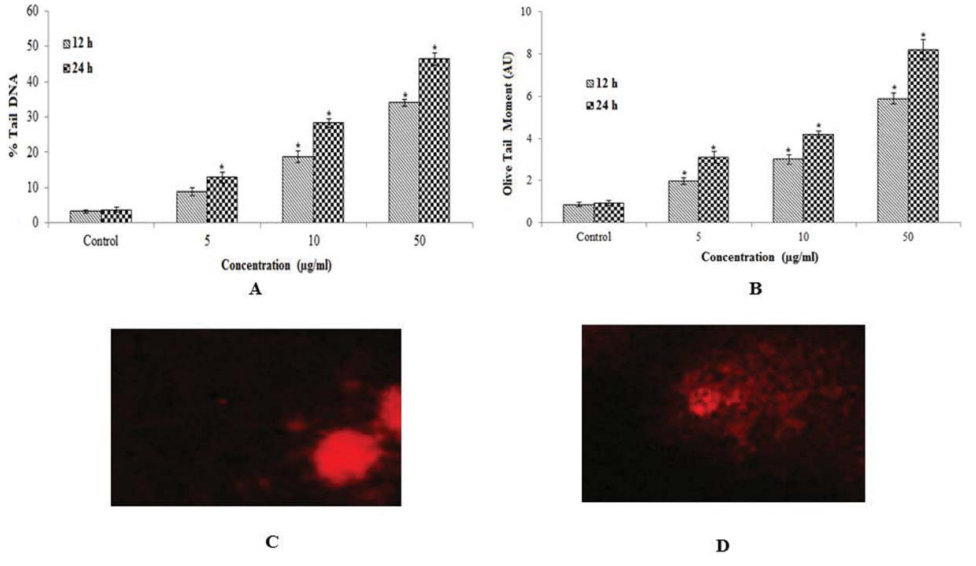

Figure 8. Effect of PbO nanoparticles on DNA damage in A549 cells. A: tail DNA (%); B: olive tail moment C: control cell; D: at 50 μg/mL for 24 hr. Each value represents the mean ± SE of three experiments. $^{*}p < 0.05$ vs. control.

carbon nanotubes for A549 lung cells. Both tests were conducted carefully and confirmed that no NP was detected in wells of culture plate when recording the optical density for MTT and NR tests. The NR assay is based upon uptake of NR dye in lysosome while MTT is based upon the metabolic activity of mitochondria, which might account for the differences. In both tests, PbONPs produced increased cytotoxicity frequency in A549 cells.

The main feature of apoptosis involves cleavage of cell protein through caspase-3. The activity of caspase-3 was increased at concentration of PbONPs after 12- and 24-hr treatments of A549 cells. Damage of chromosome associated with caspase-3 activity occurs in the late stage of apoptosis and measured with DAPI dye (Bai et al. 2010). DAPI staining produced brighter fluorescence at higher concentration of PbONPs indicating late apoptosis (Figure. 7). The late apoptotic cells are consumed by phagocytes *in vivo*. Phagocytosis does not occur *in vitro* and thus late-stage apoptotic cells are transformed into necrotic ones (Gill and Dive 2000). In this study, it was found that PbONPs adhere to the cell membranes of A549 cells. PbONPs enter A549 cells and cell death and apoptosis were detected in PbONP-exposed cells. PbONPs produced significant increased generation of ROS. In agreement with Lewinski, Colvin, and Drezek (2008), oxidative stress is well defined in NP toxicity; PbONPs also produced oxidative stress.

Taken together it may be concluded that PbONPs induced morphological changes and cytotoxicity in concentration- and time-dependent manner. PbONPs induced ROS generation which decreases antioxidant level and apoptotic cell death in A549 cells. Further ROS generation resulted in DNA damage which contributed to cell death.

Acknowledgments

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the research Group Project No. 185 RGP-180.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Deanship of Scientific Research at King Saud University [project number RGP-180].

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