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To cite this article: Abdullah A. Alkahtane (2015) Indium tin oxide nanoparticles-mediated DNA fragmentation and cell death by apoptosis in human lung epithelial cells, *Toxicological & Environmental Chemistry*, 97:8, 1086-1098, DOI: [10.1080/02772248.2015.1086111](https://doi.org/10.1080/02772248.2015.1086111)

To link to this article: <http://dx.doi.org/10.1080/02772248.2015.1086111>



Accepted author version posted online: 25 Aug 2015.  
Published online: 29 Sep 2015.



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## Indium tin oxide nanoparticles-mediated DNA fragmentation and cell death by apoptosis in human lung epithelial cells

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*(Received 9 July 2015; accepted 12 August 2015)*

Indium tin oxide (ITO) nanoparticles (NP) have extensive applications in industrial fields, and concerns regarding their potential toxicity in humans and environmental impact have increased. Since exposure to ITO NP is mainly via skin and inhalation, this study was conducted utilizing human lung epithelial (A549) cell line. Cells were exposed to different concentrations of the ITO NP for 24 and 48 hr. A severe cytotoxic response of ITO NP was observed as evident by the (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and neutral red uptake assays after 48 hr exposure. ITO NP significantly reduced glutathione levels with a concomitant increase in lipid hydroperoxide levels, superoxide activity, and reactive oxygen species (ROS) generation after exposure. A significant induction in caspase activity and formation of condensed chromosomal bodies was also observed after ITO NP (10 or 25  $\mu\text{g/ml}$ ) exposure. Furthermore, a significant induction in DNA damage was observed by the Comet assay in cells exposed to ITO NP. Our data demonstrate that ITO NP display cytotoxic and genotoxic potential. However, increase in ROS levels and oxidative stress leading to oxidative DNA damage and condensed chromosomal bodies formation, suggests involvement of apoptosis. Thus, ITO NP-mediated effects on cell viability indicate cytotoxicity, and therefore, exposures need to be carefully monitored in the industrial sector.

**Keywords:** NRU; indium tin oxide nanoparticles; A549 cells; oxidative stress; apoptosis; DNA damage

### Introduction

Nanotechnologies based on the chemical, mechanical, optical, magnetic, and biological properties of nanomaterials are being increasingly used in a wide range of industries, and commercial products (EPA 2007; Zhao and Castranova 2011). Indium tin oxide (ITO) nanoparticles (NP) are widely used in manufacturing of touchscreen phones, televisions, solar panels, and related high technology applications. However, workers are exposed to ITO NP during production, which has led to an emerging occupational syndrome termed indium lung disease (Badding et al. 2014). Due to growing number of applications, there is an increasing risk of environmental exposure to NP (Gomes et al. 2012; Almeida-Silva et al. 2014). ITO NP were found to produce interstitial pulmonary diseases in workers (Chonan, Taguchi, and Omae 2007). The rapid growth and development of nanotechnology have resulted in increased exposure to these nanostructures, leading to inhalation, ingestion, injection, and absorption of nanomaterials (Oberdörster, Oberdörster, and Oberdörster 2005; Oberdörster et al. 2015). Due to the elevation in investment and production of goods at the nanoscale, combined with the increased occupational exposure of

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workers and consumers, it is of great importance to assess the safety of these nanomaterials (Hu et al. 2009; Snyder-Talkington et al. 2012).

NP impacts are still a matter of exploration and the actual knowledge on the effects of nano-sized pollutants on biological systems remains incomplete (Singh et al. 2009; Magdolenova et al. 2012). These effects need to be evaluated in order to provide a scientific basis for a safe development of nanotechnology. Due to their small size, NP cross biological barriers to reach different tissues, and according to their surface and size accumulate in many different organs (Li and Chen 2011; Oberdorster et al. 2015).

The genotoxic potential of nanomaterial is of particular concern since the changes of the genetic material have potential for cell death, tissue malfunction, cancer development, and reproductive adverse effects (Magdolenova et al. 2012; Guo et al. 2012; Demir et al. 2013). Liu et al. (2012) reported indium compounds induced toxicity due to reactive oxygen species (ROS) generation. Some metals are capable of producing ROS through various reactions with hydrogen peroxide (Li et al. 2014), and cellular effects of ROS production include damage to proteins, lipids, and DNA. Wang et al. (2011) reported that NP-induced oxidative stress as evaluated by increasing levels of lipid peroxidation (LPO), ROS, and decreasing intracellular glutathione (GSH). Oxidative stress plays an important role in cellular signaling, inflammatory, genotoxicity, and proliferative responses (Borm, Schins, and Albrecht 2004). This study was designed to assess the oxidative stress biomarkers including GSH, ROS, and LPO in response to ITO NP exposure and determine whether apoptosis was potential mechanism underlying ITO NP effects in A549 cells.

## **Materials and methods**

### ***Chemicals and reagents***

ITO NP (Product No. 544876 and APS <50 nm), GSH, 5, 5-dithio-bis-(2-nitrobenzoic acid) (DTNB), MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], neutral red dye, 2, 7-dichlorofluorescein diacetate (DCFH-DA), JC-1 probe (5, 5', 6'6-tetrachloro, 1',3,3'-tetraethylbenzimidazolcarbocyanine iodide), and 4', 6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich. Fetal bovine serum (FBS), penicillin-streptomycin, Dulbecco's modified eagle medium/F-12 (DMEM/F-12) medium were purchased from Invitrogen Co. (Carlsbad, CA, USA). All other chemicals were purchased from commercial sources.

### ***Characterization of ITO NP***

Shape and size of ITO NP in the stock dispersion were determined by field emission transmission electron microscopy (FETEM). After sonication and stabilization, the TEM samples were prepared by drop coating of the stock suspension on carbon-coated copper grids. The films on the grids were dried using a tissue paper prior to measurement. Evaluation was performed by FETEM (JEOL model 2100 F) at an accelerating voltage of 200 kV. In addition, distribution of ITO NP and zeta-potential of the dispersion in DMEM was evaluated by dynamic light scattering (Malvern Instruments Ltd., Herrenberg, Germany).

### ***Cell culture***

A549 cells were procured from American-type culture collection Rockville, MD, USA, preserved and subcultured in the lab. A549 cells were cultured in DMEM/F-12 medium

supplemented with 10% FBS and 100 unit/ml penicillin-streptomycin at 5% CO<sub>2</sub> and 37 °C. At 85% confluence, cells were harvested by using 0.25% trypsin and subcultured into 75 cm<sup>2</sup> flasks, 6-well plates, and 96-well plates. Cells were allowed to adhere to the surface for 24 hr prior to treatment.

### ***Cell morphology***

After treatment of ITO NP for 24 and 48 hr, size and shape of A549 cells was observed by using an inverted microscope (Leica DMIL).

### ***MTT assay***

MTT assay was used to investigate mitochondrial function as described by Mosmann (1983).

### ***Neutral red uptake (NRU) assay***

The neutral red uptake (NRU) assay was conducted according to the method of Borenfreund and Puerner (1984).

### ***Intracellular ROS generation***

ROS generation was evaluated using DCFH-DA, which is oxidized to DCFH in the presence of ROS (Sauer et al. 2003).

### ***Oxidative stress biomarkers***

A549 cells at a final density of  $6 \times 10^5$  in a 75 cm<sup>2</sup> culture flask were treated to ITO NP at 0, 1, 10, 25, or 50 µg/ml for 24 or 48 hr. After treatment, cells were scraped and washed twice with chilled PBS. The harvested cell pellets were lysed in cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1% Triton, and 2.5 mM sodium pyrophosphate]. Cells were centrifuged at 15,000 *g* for 10 min at 4 °C and supernatant (cell extract) maintained on ice until assayed for oxidative stress biomarkers. Protein was measured by Bradford (1976) method, using bovine serum albumin as the standard.

### ***LPO levels assay***

LPO levels were determined by measuring formation of malondialdehyde (MDA) using the method of Ohkawa, Ohishi, and Yagi (1979). Absorbance was measured at 532 nm and expressed in nmol MDA/hr/mg protein using a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

### ***GSH estimation***

GSH levels were quantified using Ellman's reagent (1959). The assay mixture contained phosphate buffer, DTNB, and cell extract. The reaction was monitored at 412 nm and the amount of GSH was expressed in terms of nmol GSH/mg protein.

### ***Measurement of superoxide dismutase (SOD) activity***

SOD activity was estimated employing a method described by Kakkar, Das, and Viswanathan (1984). One unit of SOD enzyme activity is defined as the amount of enzyme required for inhibiting the chromogen production (560 nm) by 50% in 1 min under assay conditions and expressed as specific activity in units/min/mg protein.

### ***DAPI staining for chromosome condensation***

Chromosome condensation in A549 cells due to ITO NP exposure was observed by DAPI staining. DAPI solution was used to stain the exposed cells in eight chamber slides and the slides were incubated for 10 min in the dark at 37 °C. Images of the nucleus were captured using a fluorescence microscope (Nikon).

### ***Caspase-3 assay***

The activity of caspase-3 was determined from the cleavage of the caspase-3 substrate I (N-acetyl-DEVD-p-nitroaniline). The p-nitroaniline was used as the standard. Cleavage of the substrate was monitored at 405 nm and specific activity expressed in picomoles product (nitroaniline) per min/mg of protein.

### ***Alkaline single-cell microgel electrophoresis assay***

The alkaline single-cell microgel electrophoresis assay was applied to detect DNA strand breaks and alkali labile as well as incomplete excision repair sites in single cells (Ali et al. 2011).

### ***Statistical analysis***

At least three independent experiments were carried out in duplicate for each evaluation. Data were expressed as mean ( $\pm$ SEM) and analyzed by one-way analysis of variance. The  $p < 0.05$  was considered statistically significant.

## **Results**

### ***ITO NP***

The image of ITO NP as shown in Figure 1(a) demonstrates the most of the particles were in spherical and some of them rectangular shape.

### ***Morphological changes of A549 cells***

Figure 2 illustrates the morphology of untreated and ITO NP-treated A549 cells. A549 cells exposed to 50  $\mu$ g/ml ITO NP displayed altered into spherical shape and detached from surface (Figure 2(b)).

### ***ITO NP-induced cytotoxicity in A549 cells***

Mitochondrial function (MTT reduction) and lysosome activity (NRU) were utilized as cytotoxicity end points. MTT results confirmed a concentration- and time-dependent

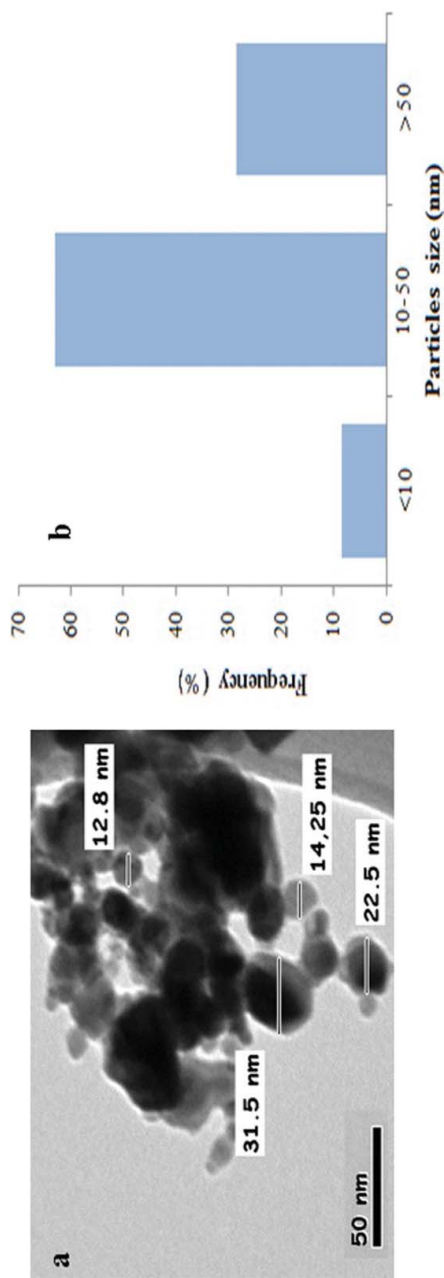


Figure 1. Characterization of ITO NP. (a) TEM image. (b) Percentage of size distribution of ITO NP generated by TEM image.

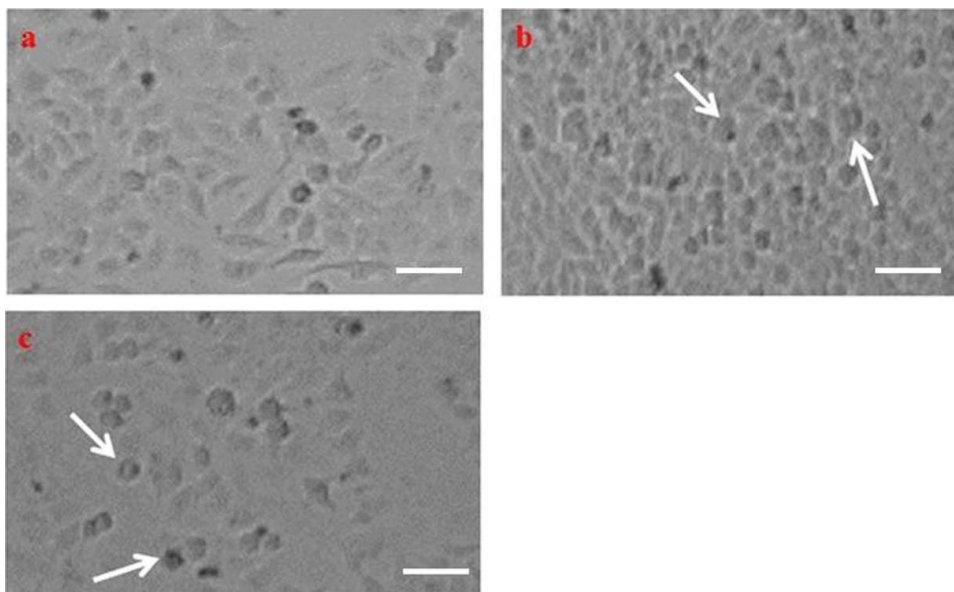


Figure 2. Morphology of A549 cells. (a) Control. (b) At 50  $\mu\text{g/ml}$  of ITO NP for 24 hr. (c) At 50  $\mu\text{g/ml}$  of ITO NP for 48 hr. Scale bars (—) 100  $\mu\text{m}$ .

cytotoxicity after exposure to ITO NP in A549 cells (Figure 3(a)). Results of NRU assay are shown in Figure 3(b). A concentration- and time-dependent decline in viability of cells exposed to ITO NP for 24 or 48 hr was noted. However,  $\text{IC}_{50}$  values by MTT assay was 47  $\mu\text{g/ml}$  and in NRU assay was greater than the highest concentration of ITO NP for 48 hr.

#### *ITO NP-induced ROS generation and oxidative stress*

Results in Figure 4 demonstrate ITO NP-induced rise in intracellular ROS generation in A549 cells. ITO NP-produced oxidative stress was further evidenced by depletion of GSH (Figure 5(b)) and elevation of LPO levels and SOD activity (Figure 5(a) and 5(c)).

#### *Chromosomal condensation and caspase-3 activity*

Chromatin condensation was determined by DAPI staining. A549 cells treated with ITO NP for 48 hr produced chromatin condensation (Figure 6). Caspase-3, which plays a key role in the apoptotic pathway of cells, was induced by treatment with ITO NP (Figure 6 (d)). In A549 cells treated with 1, 10, or 25  $\mu\text{g/ml}$  concentrations of ITO NP over 24 or 48 hr, the activity of caspase-3 was increased in concentration- and time-dependent manner.

#### *DNA fragmentation/damage*

Cells treated with ITO NP at 1, 10, 25, or 50  $\mu\text{g/ml}$  exhibited significantly higher DNA damage than controls. The highest DNA damage was recorded at 50  $\mu\text{g/ml}$  ITO NP (Figure 7).

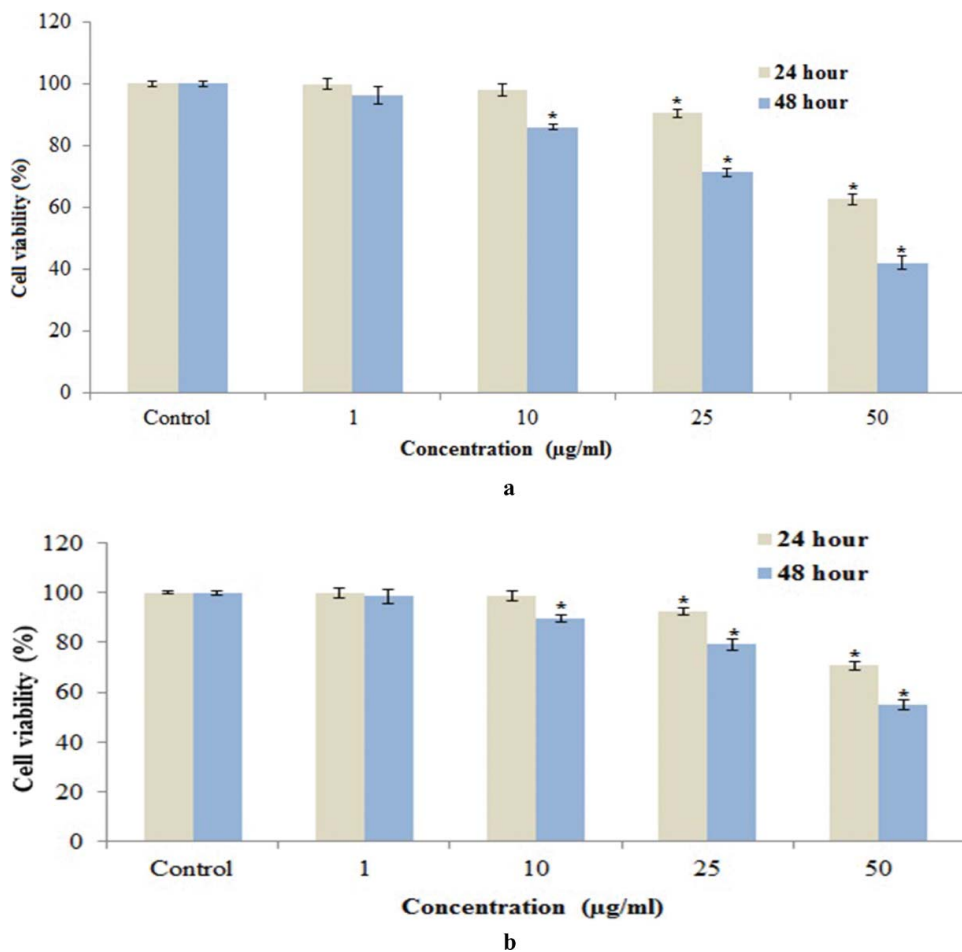


Figure 3. Cytotoxicity of ITO NP in A549 cells for 24 or 48 hr, as assessed by (a) MTT and (b) NRU tests. Each value represents the mean  $\pm$  SEM of three experiments. \* $p < 0.05$  vs. control.

## Discussion

This study was designed to assess the cytotoxic and genotoxic effects of ITO NP in A549 cells with concerns of possible adverse effects and genotoxic potential. Our results demonstrated that ITO NP-induced cytotoxic and apoptotic effects in A549 cells. Data indicated that the mode of cell death involved apoptosis via ROS-triggered mitochondrial pathway as evidenced by cleavage of caspase-3 and chromosome condensation.

The capacity of some NP to produce cytotoxicity assays is well known (Snyder-Talkington et al. 2012). Monteiro-Riviere, Inman, and Zhang (2009) suggested that cytotoxicity of NP should be assessed with two or more independent test systems for validating the findings. Xia et al. (2007) reported pulmonary epithelial cells exhibited greater susceptibility to particle-induced cell death than macrophages. Our results also indicate that ITO NP-produced cell death in human lung epithelial cells. An apoptotic response was observed but reduced A549 cell viability appeared to involve caspase-induced apoptosis (Figure 6). Tanaka et al. (2010) showed that intratracheal instillation of ITO particles in hamsters resulted in significant necrotic debris within alveolar macrophages, which may be a result of necrotic epithelial cell death.



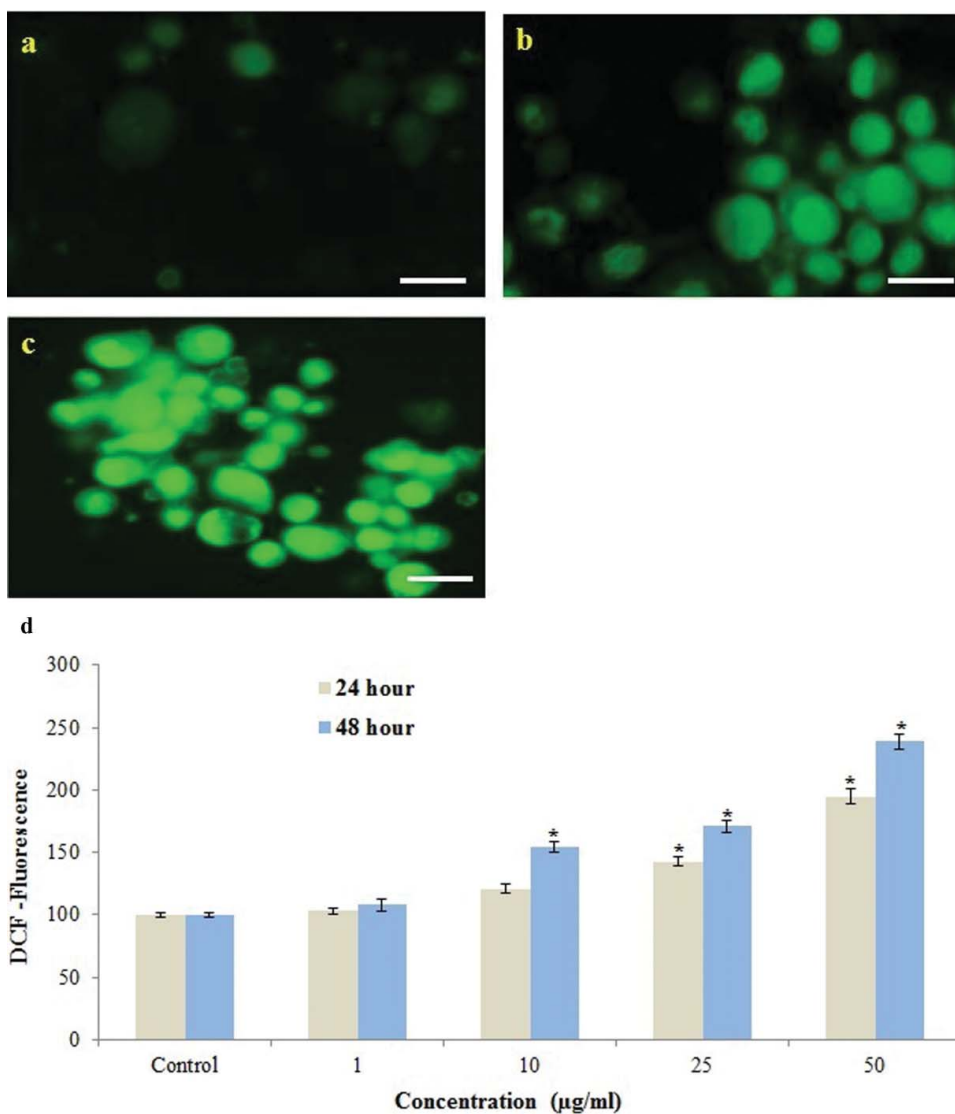


Figure 4. ITO NP-induced ROS in A549 cells. (a) Control. (b) At 50 µg/ml of ITO NP for 24 hr. (c) At 50 µg/ml of ITO NP for 48 hr. (d) % ROS production at various concentrations of ITO NP in A549 cells. Images were snapped in phase contrast cum fluorescence microscope (Nikon, model 80i). Each value represents the mean  $\pm$  SEM of three experiments. \* $p < 0.05$  vs. control. Scale bars (—) 50 µm.

Cytotoxicity of ITO NP was measured by two methods including MTT and NRU to enhance reliability of our observations. The reduction of cell viability is in agreement with findings of Badding et al. (2014) in RAW 264.7 and BEAS-2B cells for ITO compounds. Nel et al. (2006) reported that LPO and oxidative stress is one of the most important mechanisms underlying toxicity related to NP. This was attributed to its small size and large surface area, which is generally thought to produce the ROS and oxidative stress (Xia et al. 2006). The ITO NP in this study also produced elevated intracellular ROS

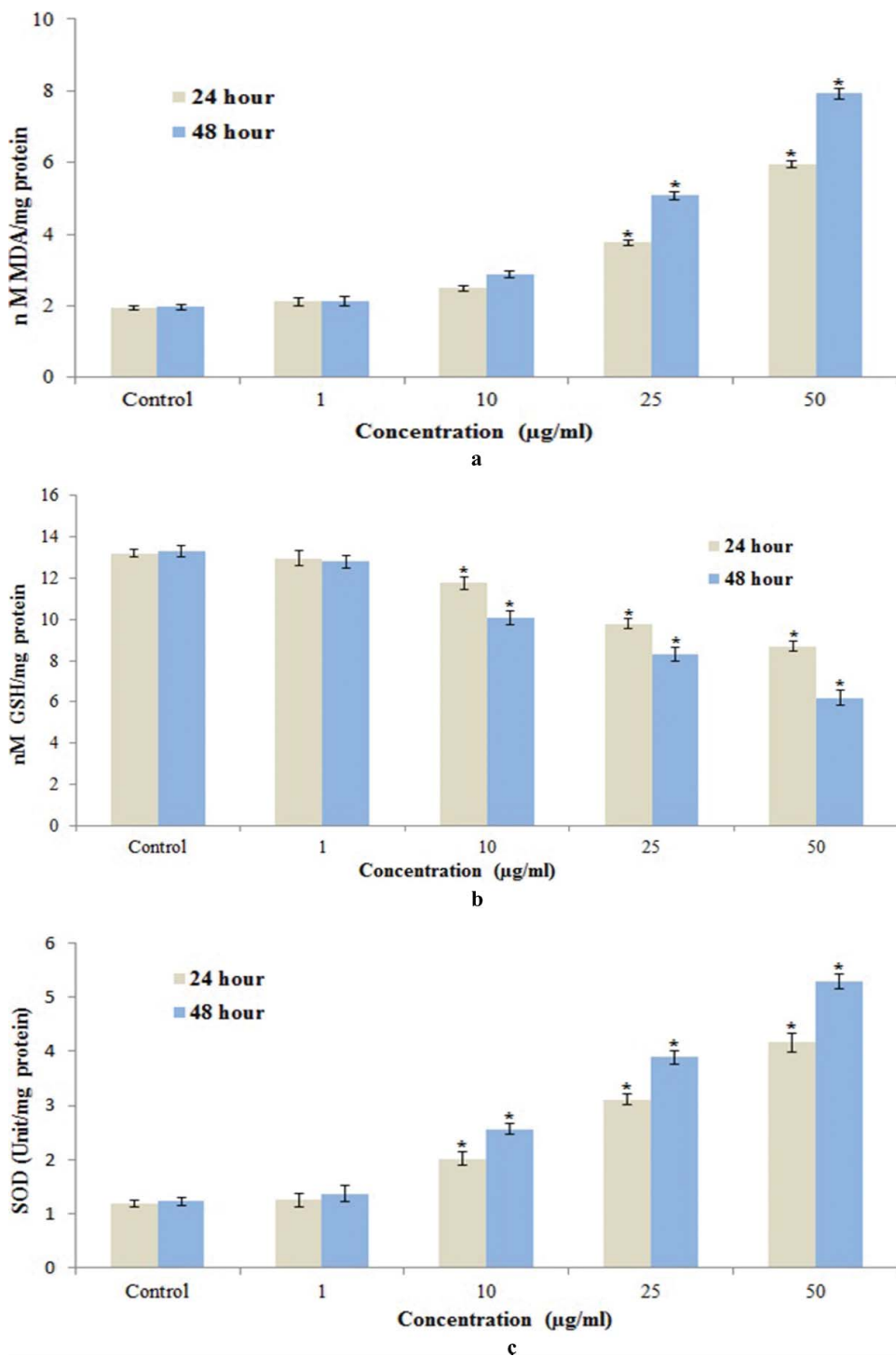


Figure 5. (a) Levels of LPO, (b) GSH, and (c) SOD in A549 cells after treatment to ITO NP for 24 or 48 hr. Each value represents the mean  $\pm$  SEM of three experiments. \* $p < 0.05$  vs. control.

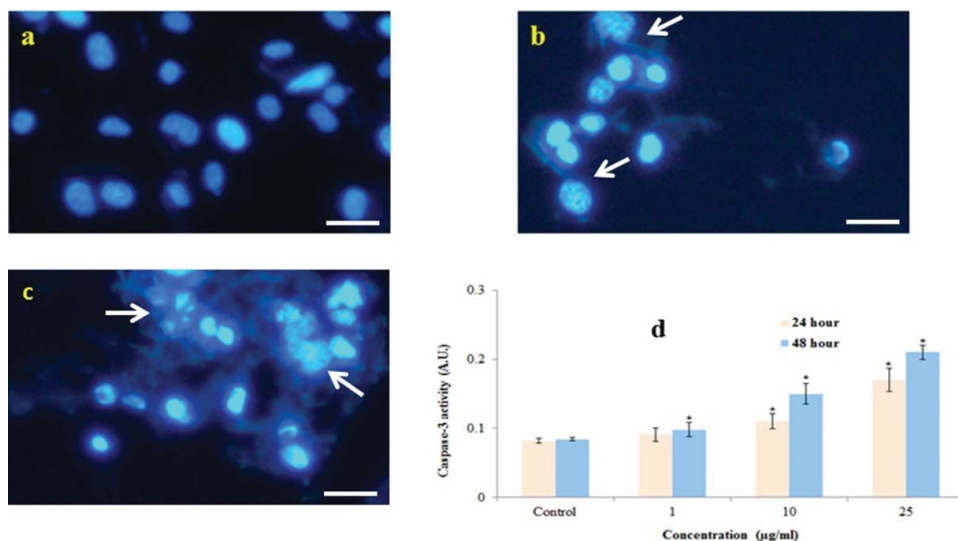


Figure 6. Chromosomal condensation and induction of caspase-3 activity in A549 cells after treatment to ITO NP. (a) Control. (b) At 25 µg/ml of ITO nanoparticles for 24 hr. (c) At 25 µg/ml of ITO NP for 48 hr of ITO nanoparticles. (d) Caspase-3 activity. Each value represents the mean  $\pm$  SEM of three experiments. \* $p < 0.05$  vs. control. Scale bars (—) 50 µm.

levels. ROS typically include the superoxide radical, hydrogen peroxide, and the hydroxyl radical which produce damage to cellular components including DNA damage and ultimately apoptotic cell death (Ott et al. 2007; Rana 2008). Our results are in agreement with other studies which examined the effects of ITO compounds on RAW 264.7 and BEAS-2B cells (Badding et al. 2014).

An increase in LPO and SOD levels associated with decrease in GSH levels in A549 cells after treatment with ITO NP, denotes indication of oxidative stress. LPO may further give rise to more free radicals and damage biomolecules like DNA, protein, and lipids in conjunction with ROS. The depletion of GSH in ITO NP-exposed cells combined with elevated levels of LPO and SOD indicate that oxidative stress may be the primary mechanism for toxicity attributed to ITO NP in A549 cells. ITO NP also lead to the generation of free radicals after interaction with cells components such as mitochondrial damage. NP-induced LPO and oxidative stress resulted in DNA damage and apoptosis (Kang et al. 2008). Our findings are consistent with other results indicating metal oxide NP have the potential to induce DNA damage (Eom and Choi 2009).

ITO NP-induced cell death observed in this study may occur via two distinct modes: apoptosis and necrosis, which are distinguishable by morphological and biochemical features. DAPI staining of ITO NP-treated A549 cells resulted in chromosomal condensation and fragmentation which is another morphological hallmark of apoptosis. Chen and Mikecz (2005) reported that NP due to their small size are capable of reaching the nucleus and interacting with DNA. They may also exhibit an indirect effect on DNA through their ability to generate ROS. This DNA damage may either induce carcinogenesis or cell death, thus disrupting normal cell functions.

The genotoxic potential of ITO NP in A549 cells was examined by the comet assay which is capable of detecting single as well as double DNA strand breaks and alkali labile sites even at low levels of DNA damage (Collins 2004). Martinez et al. (2003) noted that

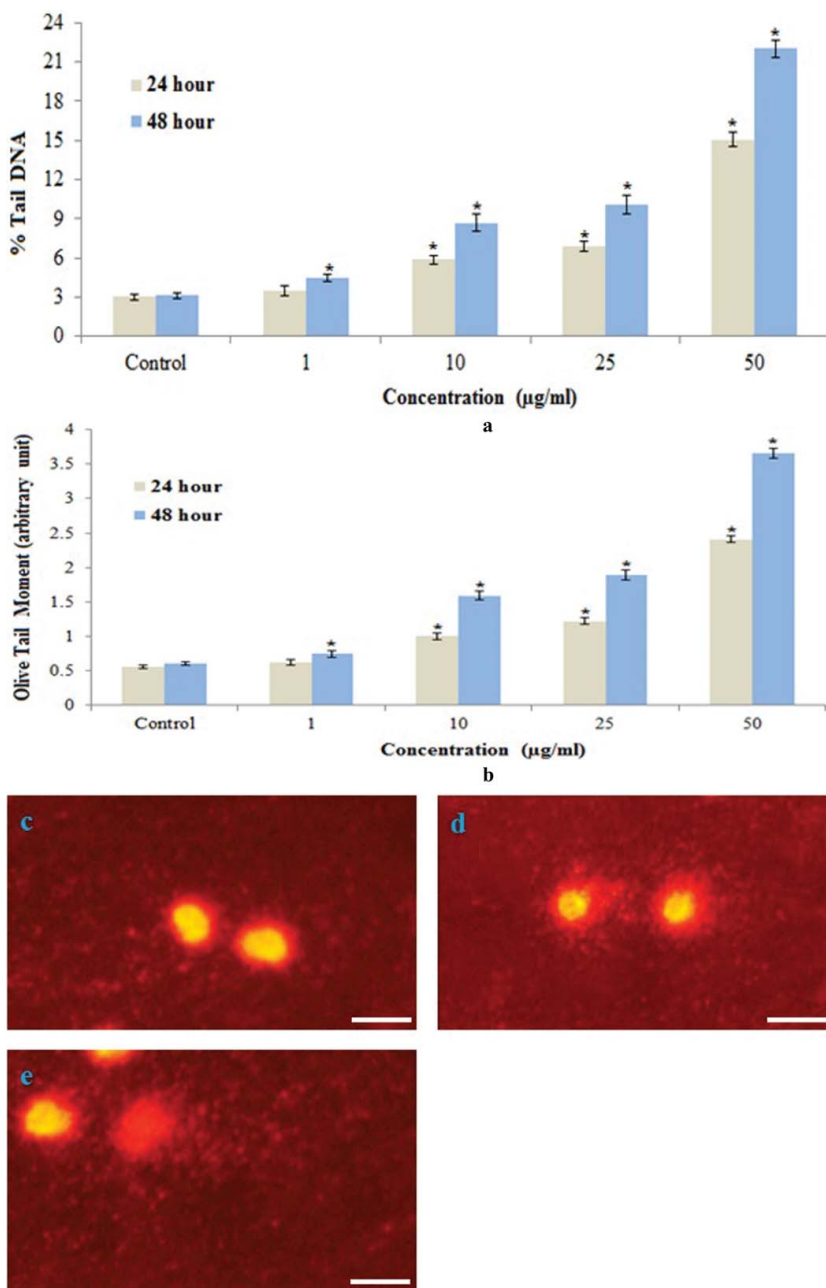


Figure 7. DNA impairment in A549 cells due to ITO NP. (a) Tail DNA (%). (b) Olive tail moment. (c) Control cell. (d) At 50 µg/ml of ITO NP for 24 hr. (e) At 50 µg/ml of ITO NP for 48 hr. Each value represents the mean  $\pm$  SEM of three experiments. \* $p < 0.05$  vs. control. Scale bars (—) 50 µm.

ROS are involved in DNA damage inducing damage to both purine and pyrimidine bases as well as the DNA backbone. Our results demonstrated that ITO NP induce apoptosis and DNA impairment in A549 cells, which may be mediated through the ROS and oxidative stress.

## Acknowledgments

The author would like to extend his appreciation to Deanship of Scientific Research, College of Science Research Center, King Saud University, Saudi Arabia.

## Disclosure statement

No potential conflict of interest was reported by the author.

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