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NANO TITANIUM INDUCES CYTOTOXICITY AND OXIDATIVE STRESS IN HUMAN LUNG CELLS

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ABSTRACT

The titanium dioxide nanoparticles (TNP) are widely using in skin care, biosensing and many other commercial applications. TNP, 82% anatase / 18% rutile, with primary average diameter of 22 ± 6 nm (TNP 20), 87 ± 16 nm (TNP100). So TNP 20 and TNP 100 cell-particle interactions with human cells and human exposure risk are gaining much importance due to their extensive pharmaceutical and nanobased applications. The potential high-risk exposure for TNP generally inhalation route was the most considered. So, the present study investigates the in vitro cytotoxicity and oxidative stress upon exposure to human lung epithelial cells (A549) using 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). The oxidative stress bioindicators like lactate dehydrogenase (LDH) leakage, glutathione (GSH), lipid peroxidation (TBARS) levels were estimated. Both the TNP 20 and TNP 100 were decreased the cell viability and caused cell membrane damage via increased LDH in A549 cells for 48 h post exposure. The TNP exposure to lung cells resulted in decreased GSH levels and increased TBRAS levels were indication of oxidative stress development. The TNP 20 have shown significant toxicity against all tested oxidative stress parameters when compare to TNP 100 and quartz treated cells. Finally, the results indicating that the tested both the TNP were induced cytotoxicity and oxidative stress in A549 post exposed cells. So, the in vitro data could help in risk/hazard assessment of TNP in relevance to human toxicology.

KEY WORDS

TNP 20, TNP 100, MTT Assay, Cytotoxicity, Oxidative Stress

INTRODUCTION

Titanium dioxide nanoparticles (TNP) are used extensively in numerous commercial applications such as sunscreens, pigments, paints, cosmetics, plastics, paper, an anticaking or whitening agent (Wang *et al*., 2014). As their production volume and multiple applications in various industrial sectors have been raising safety concerns in the present nanoworld (Bhatt and Tripathi, 2011). As of now very few authors have screened the TNP toxicity using human cell line. They include TNP 26 nm induce DNA damage and cell cycle arrest in human alveolar cells, A549 (Kansara *et al*., 2015).

Rollerova *et al*., 2015 studied some aspects of toxicity and future developments related to TNP. Harikiran *et al*., 2015 reported that the gold NP 10, 25 nm sized were produced cytotoxicity and oxidative stress in liver, Hep G2 cells. Short-term exposure to low doses of nanosized TNP and potential modulatory effects on intestinal cells was studied by Ammendolia *et al*., 2017. Simone *et al*., 2016 revealed that the bulk and nano titanium induces subtle changes after their exposure to human astrocytes, D384 and skin, HaCaT cells. Role of TNP size, shape and surface area that would affect the toxicity was studied.

The emerging trends of nanotoxicology area in public health and disease from agri-food to nanotherapeutic applications (Banerjee *et al*., 2016). Indeed, it is important to predict the possible toxicity effects of TNP in human lung cells due to their high-volume

production, wide spread use of applications and high risk of occupational exposure. So, the occupational exposure to TNP was producing unknown health complications in the humans and animals. Due to their nano size, their entry into the lungs via inhalation was much focused in several studies since the last decade (Piperigkou *et al*., 2016). The TNP interaction with lung tissue (primary target) via inhalation route was studied for possible toxicity effects. The objective of the present study was cell-particles interaction, cytotoxicity and oxidative stress induction using MTT assay and determination of oxidative stress parameters upon post exposure to A549 cells for 48 h with various dose ranges by *in vitro.* To evaluate the potential mechanisms of cytotoxicity, different types of oxidative stress parameters including cell membrane damage (LDH leakage assay), reduced glutathione (GSH assay), lipid peroxidation product (TBARS assay) levels quantitatively determined and were compared to control and NQTZ (positive control) treated cells.

MATERIALS AND METHODS

Test Particles and Chemicals

The test poly ethylene glycol (PEG) coated TNP 20 and TNP 100 were purchased from Sigma Aldrich, USA. Quartz (QTZ) particles (>100 nm; 99.94% purity) were obtained from Berkely Springs, West Virginia, USA. The fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), 1% L-glutamine, 1% penicillin-streptomycin antibiotic solution, phosphate buffer saline (PBS) and trypsin-EDTA 0.25% were obtained from Himedia, Mumbai, India. The MTT was purchased from Sigma-Aldrich, USA. All the biochemical assay kits were purchased from BioVision, USA.

Cell culture and treatment

Human lung (A549) cells were revived from National Center for Cell Sciences (NCCS), India with a job number 1615. The flasks were checked for their fungal and bacterial contamination. The passage number 18 cells were used. Lung cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin antibiotic solution. Cells were seeded at 2, 50,000 cells/flask in a total volume of 10 mL. After achieving confluence, cells were trypsinized and seeded into 96 well plates at the rate of 3.0 x 10^4 cells/0.1 mL. The cell cultures were maintained in a 5% CO_2 incubator (WTC Binder, Germany) at 37^{0}C. The test TNP were prepared as suspensions in PBS as solvent with < 1% PEG as stabilizer. The different concentrations 1-300ug/mL were used for screening of possible toxicity effects in human lung cells.

CYTOTOXICITY ASSAY

The effect of test TNP on the cellular proliferation and viability was determined by using MTT assay method (Tomankova *et al*., 2015). The yellow tetrazolium salt was reduced by dehydrogenase enzymes present in mitochondria of metabolically active cells, to generate reducing equivalents, NADH and NADPH. The lung cells were seeded into 96 well plates and add test TNP suspensions (100 µL, in quadraplate) in media. The microtiter plate was incubated at 37^{0}C for 48 h in 5% CO_2 incubator and 20 µL of MTT (5 mg/mL) was added to each well. The plate was again incubated for 2 h. The formazan product was dissolved in DMSO (80 µL) and followed by aluminum foil wrapping to prevent the oxidation of the dye. The plate was placed on a rotary shaker for 2 hours for proper mixing. The absorbance was recorded using ELISA reader at 470 nm. The test absorbance was compared with that of solvent control to get the cell viability (Bhikku *et al*., 2014).

OXIDATIVE STRESS PARAMETERS ESTIMATION

The lung cell extract was prepared after cell loading into a 96 well plate at a density of 3.0 x 10^4 cells/well in 100 µL of culture medium. To each well, 100 µL of increasing concentrations of test TNP suspensions were added in quadraplate. The microtiter plate was incubated in 5% CO_2 incubator at 37^{0}C for 48 h. The supernatants were transferred into fresh 96 well plates and assayed according to the manufacturer's protocol for estimation of LDH, GSH and TBARS levels respectively.

LACTATE DEHYDROGENASE (LDH) LEAKAGE ASSAY

LDH is a soluble zinc containing enzyme present in the cytosol. It is released into surrounding culture medium upon cell injury or lysis during apoptosis and necrosis. LDH leakage into the culture medium can be used as a sign of cell membrane integrity, and therefore used as a measurement of cytotoxicity.

This assay measures cell death in response to chemicals or environmental pollutants using a coupled two step reaction. In the first step, LDH enzyme catalyzes the reduction of NAD^+ to generate NADH and H^+ by oxidation of lactate to pyruvate. In the second step of reaction, diaphorase uses the newly generated NADH and H^+ to catalyze the reduction of a tetrazolium salt to colored formazan which absorbs strongly at 490-520

nm. The quantity of formazan generated is proportional to the amount of LDH leakage into the culture medium for measurement of oxidative stress (Harikiran *et al*., 2016).

GLUTATHIONE (GSH) ASSAY

Glutathione is chemically a tripeptide (γ-glutamylcysteinyl glycine), distributed in plants, animals and human beings. This assay employs an optimized enzymatic recycling process, using glutathione reductase, for the quantification of GSH. The sulphydryl group of GSH reacts with 5,5'-dithio-*bis*-2-nitrobenzoic acid (DTNB) and generates a yellow colored TNB (5-thio-2-nitrobenzoic acid). The mixed disulfide generated is reduced by glutathione reductase to recycle the GSH and produce more TNB. The quantity of TNB generation is directly proportional to the concentration of GSH in the cell sample. The absorbance of TNB was measured at 405- 414 nm and GSH levels were estimated in the cell sample (Shukla *et al*., 2015).

LIPID PEROXIDATION ASSAY

Malondialdehyde (MDA) was formed on lipid peroxidation naturally. The MDA content was measured indirectly in terms of thiobarbituric acid reactive substances (TBARS). The determination of TBARS is a well-established method for extent of lipid peroxidation. This assay was a simple, reproducible and standardized tool for estimation of lipid peroxides in cell lysates. The TBARS were measured colorimetrically at 530-540 nm under high temperatures and acidic conditions (Chellappa *et al*., 2015).

Statistical Analysis

All the data were analyzed and expressed as the percentage of control. Statistical analysis was performed using one-way ANOVA followed by Dunnett test. The statistical significance was assigned at *p<0.05, $p<0.01, #p<0.001 versus control cells. NQTZ used as a positive control.

RESULTS

The cell viability reduction was measured by using MTT assay. The lung cells were exposed to TNP 20 and TNP 100 for 48 h, results showed that TNP 20 induced significant cytotoxicity at the doses 100 and 300 µg/mL shown in Figure 1.

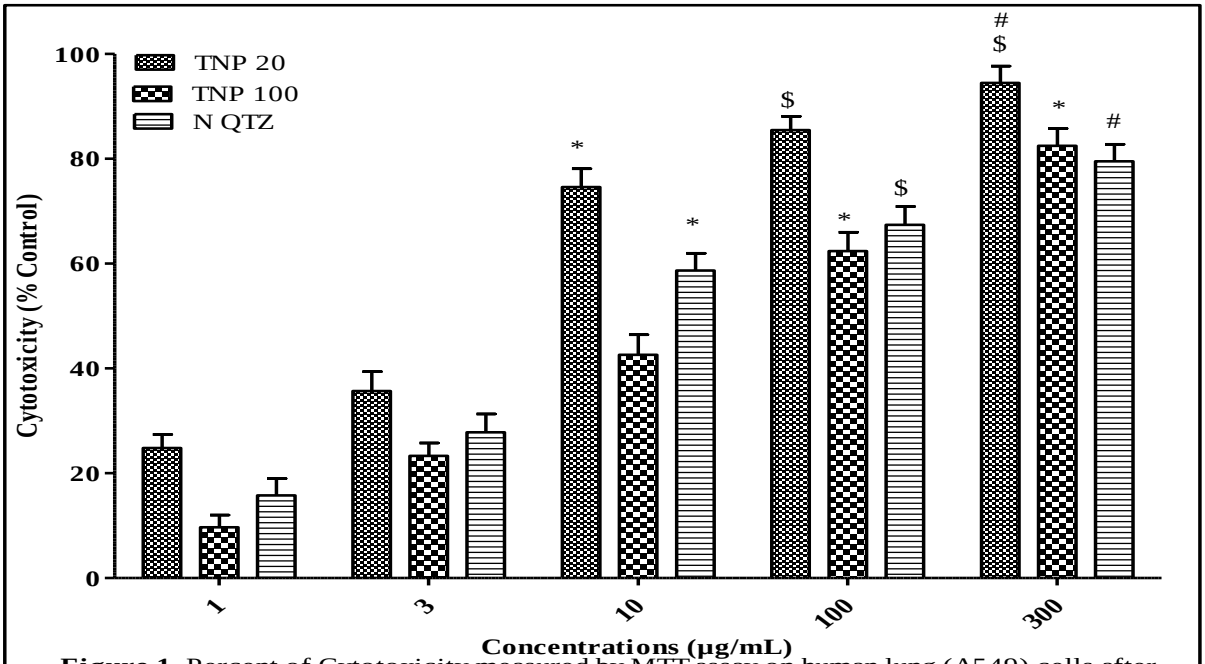

Figure 1. Percent of Cytotoxicity measured by MTT assay on human lung (A549) cells after post exposure to TNP 20, TNP 100 with concentrations (1 - 300 µg/mL) for 48 h. Data as mean ± standard deviation (S.D.) n = 4. Stastical analysis was performed using one-way ANOVA followed by Dunnett test. *p<0.05, #p<0.001, $p <0.01 versus control.

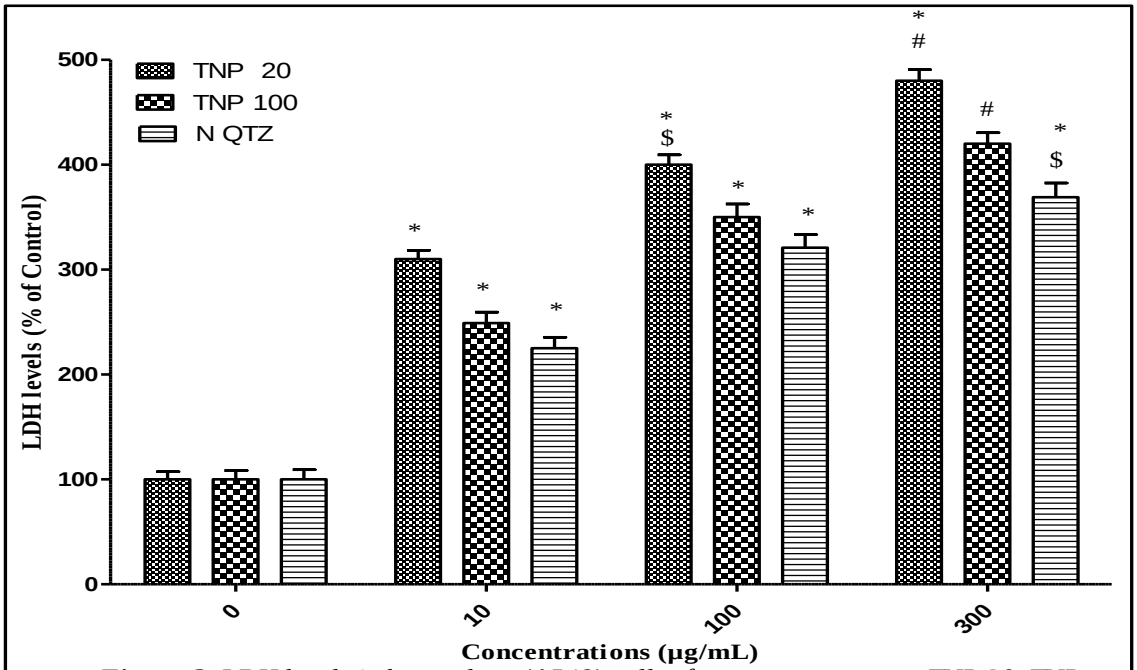

Figure 2. LDH levels in human lung (A549) cells after post exposure to TNP 20, TNP 100 with concentrations (10 - 300 µg/mL) for 48 h. Data as mean ± standard deviation (S.D.) n = 4. Stastical analysis was performed using one-way ANOVA followed by Dunnett test. *p<0.05, #p<0.001, $p <0.01 versus control.

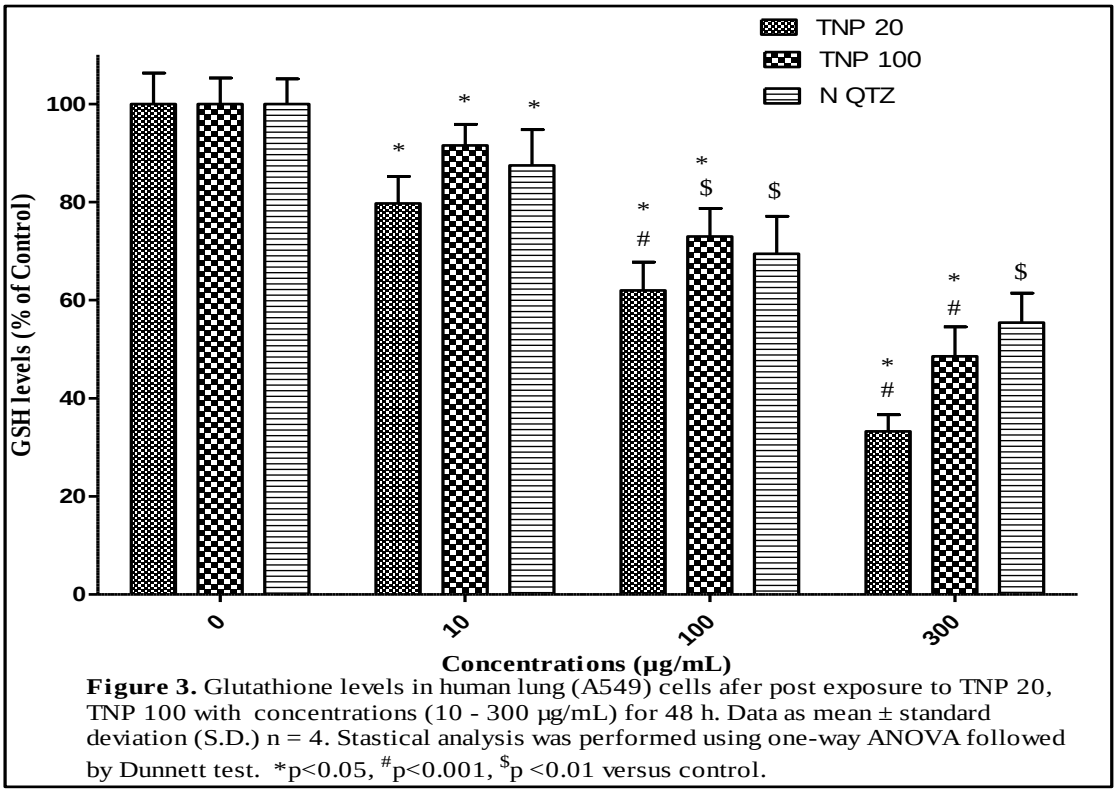

Figure 3. Glutathione levels in human lung (A549) cells afer post exposure to TNP 20, TNP 100 with concentrations (10 - 300 µg/mL) for 48 h. Data as mean ± standard deviation (S.D.) n = 4. Stastical analysis was performed using one-way ANOVA followed by Dunnett test. *p<0.05, #p<0.001, $p <0.01 versus control.

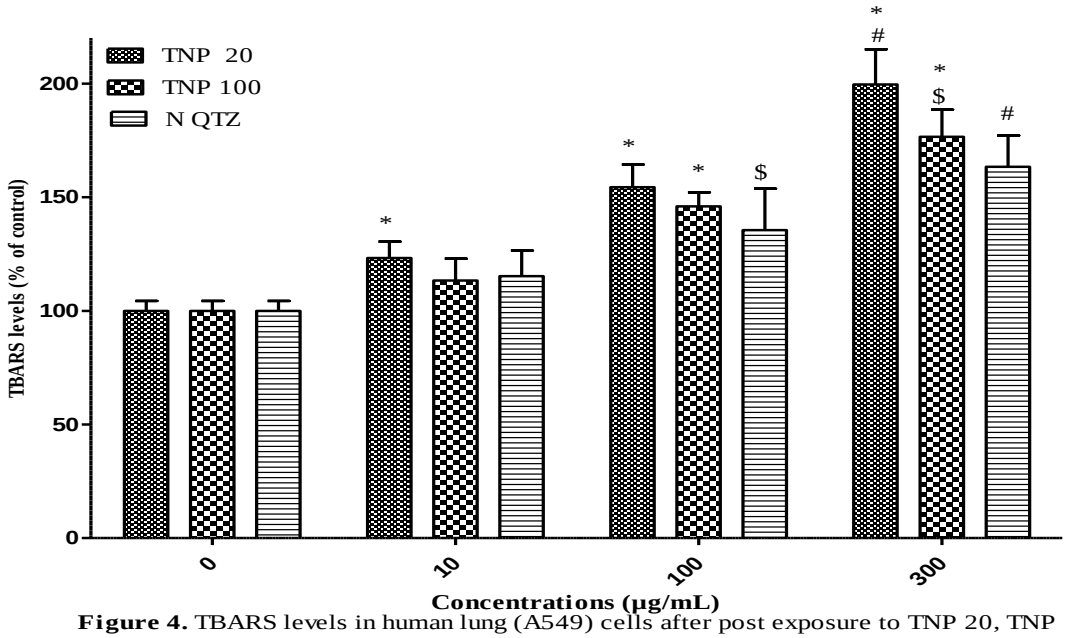

Figure 4. TBARS levels in human lung (A549) cells after post exposure to TNP 20, TNP 100 with concentrations (10 - 300 µg/mL) for 48 h. Data as mean ± standard deviation (S.D.) n = 4. Stastical analysis was performed using one-way ANOVA followed by Dunnett test. *p<0.05, #p<0.001, $p <0.01 versus control.

The cell viability was decreased in a dose dependent manner. Both TNP were significantly (p<0.001) decreased the cell viability when compared with control cells. TNP 20 showed highest cell viability reduction 94.41 % at 300 (p<0.001) µg/mL than TNP 100 and NQTZ. The cell viability at lower concentrations 1 and 3 µg/mL were reduced non- significantly.

The cytotoxicity and oxidative stress was measured by cell membrane damage, determined through LDH leakage assay. Because, LDH is a cytosolic enzyme present in normal cells and cell membrane damage results it's leakage into the extracellular fluid. Lung cells exposed to TNP 20 and TNP 100 significantly increased the LDH levels shown in Figure 2. Both the TNP 20 and TNP 100 were increased the LDH leakage at doses 100 and 300 µg/mL concentrations significantly (p<0.001). The effect of TNP 20 on LDH levels observed that 2, 3, 4 folds increment were found at doses 10 (p<0.05), 100 (p<0.01), 300 (p<0.001) µg/mL significantly in A549 cells compared to control.

GSH is responsible for maintaining cellular oxidation-reduction homeostasis in cells; any changes in GSH levels leads to characteristic functional damage of cells. The reduced GHS levels of tested TNP 20 and TNP 100 were shown in Figure 3. A significant (p<0.001) depletion of GSH levels were found at 100 and 300 µg/mL in A549 cells for 48 h study. 4 folds decrement of GSH was observed for TNP 20 and 3½ folds for TNP 100 when compared to control.

The effect of TNP 20 and TNP 100 on lipid peroxidation, results were shown in figure 4 after post exposed to A549 cells for 48 h. Cells were exposed to 10, 100 and 300 µg/mL of test TNP for 48 h and the dose dependent increase of TBARS were observed. The results were indicating that there was a significant (p<0.001) enhancement in TBARS levels at 100 (p<0.01) and 300 (p<0.001) µg/mL concentrations of both the TNP compared to control cells.

DISCUSSION

In recent decades, TNP have been increased for their worldwide applications in cosmetics, food-industries, materials for air pollution control, pharmaceuticals and personal care products. The extensive production and use of TNP has increased risk for human exposure results in unknown health complications (Rehman *et al*., 2016). TNP enters into the human body in the form of skin care, nano-food or nanodrug delivery applications.

Due to their nano particle size, industrially released TNP can also be inhaled as air borne particles. So, safety concerns have been increasing about the possible health complications when exposed to TNP by humans and animals. Cells were exposed to different

concentrations (1-300µg/mL) among them lower doses (1and 3 µg/mL) represent environmental exposure, while higher ones considered the possibility of a pulmonary local accumulation of TNP. This study evaluated the potential toxicity of different doses of TNP in human respiratory cells. The alveolar epithelial cells can have direct contact with the inhaled TNP (Das *et al*., 2016).

So we have chosen A549 cell line as in vitro model to evaluate cytotoxicity and oxidative stress for 48 h exposure. The tested TNP 20 and TNP 100 induced cytotoxicity and oxidative stress in A549 cells. The MTT assay results clearly indicating that the tested TNP were reduced the cell viability significantly at higher doses 100 and 300 µg/mL after exposed to A549 cells for 48 h. The highest cell viability reduction 94.41 % was induced at the dose 300 ($p<0.001$) µg/mL for TNP 20. The TNP 20 and TNP 100 were reduced cell viability at lower concentrations 1 and 3 µg/mL in tested A549 cells for acute exposure (Rihane *et al*., 2016).

The cell membrane damage induced by TNP was done by LDH leakage assay. Because LDH is a cytosolic enzyme present in normal cells can release into surrounding extracellular fluid after cell membrane damage. The results showed that increased release of LDH into culture media after TNP exposure was in a dose-dependent manner. TNP 20 and TNP 100 have showed highest release of LDH compared to control and NQTZ treated cells at 48 h exposure period.

Kongseng *et al*., 2016 findings were similar to the results showing cytotoxicity towards A549 cells using MTT and LDH leakage assays in the present study. LDH leakage from cells into culture media is another evidence for penetration of nanoparticles into the cells and damage of cell membrane. The GSH levels were decreased drastically at the doses 100 ($p<0.01$) and 300 ($p<0.001$) µg/mL significantly. The cellular oxidative stress was determined by decreased GSH levels and increased TBRAS (Ahamed *et al*., 2016). The decreased cell viability and increased TBARS levels suggested that cell death was the primary cause of membrane damage by lipid peroxidation. The depleted GSH and increased TBARS significantly induce the oxidative stress in tested alveolar epithelial cells (A549).

CONCLUSION

So finally, we conclude that the tested TNP 20 and TNP 100 were induced cytotoxicity and oxidative stress due to the increased LDH, TBARS and followed by decreased GSH in A549 cells for acute exposure.

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Declaration of interest

The authors report that no conflict of interest

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