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Original Article

"*IN VITRO* EVALUATION OF CYTOTOXICITY, OXIDATIVE STRESS, DNA DAMAGE AND INFLAMMATION INDUCED BY DIESEL EXHAUST PARTICLES IN HUMAN A549 LUNG CELLS AND MURINE RAW 264.7 MACROPHAGES"

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ABSTRACT

Objective: The aim of the present study was to examine the *in vitro* oxidative stress, cytotoxicity, inflammation and DNA damage induced by Diesel Exhaust Particles (DEPs).

Methods: Alveolar macrophages (murine RAW 264.7 cells) and cultured type II epithelium cells (human A549 lung cells) were exposed to various concentrations of Diesel Exhaust Particles for 24 h. The experiment was evaluated for cell viability, oxidative stress, cytotoxicity, inflammation and DNA damage parameters.

Results: The results showed that overall both cell lines had similar patterns in response to the oxidative stress, cytotoxicity, inflammation and DNA damage parameters induced by DEPs. Vehicle control showed no changes compared to the control. Both cells showed significant changes at the dose of 20 µg/mL and significant changes were found in cytotoxicity, oxidative stress, DNA damage and inflammation indexes.

Conclusion: Hence, exposure to DEPs resulted in dose-dependent toxicity in cultured A549 cells and RAW264.7cells and was closely correlated to increased inflammation and oxidative stress.

Keywords: Diesel Exhaust particles**,** Cytotoxicity, Oxidative stress, RAW 264.7 cells, A549 cells.

INTRODUCTION

Air pollution was found to be associated with increased respiratory and cardiovascular diseases [1]. Crucial components of air pollution include the Petrol Exhaust Particles (PEPs) and Diesel Exhaust Particles (DEPs) [2]. In automobile engines, both petrol and diesel fuels undergo combustion and produce Combustion Derived Nanoparticles (CDNPs) but diesel produces more particles per unit fuel than petrol and is by the far the most studied of the two regarding adverse health effects [3]. Majority of these particles have dimensions <1 µm, and most of these particles are known as ultrafine particles with dimensions < 0.1 µm [4]. These particles easily deposit at the bronchiolar and alveolar levels of the lung, due to their fine size and up to 33% of the inhaled fine particles are deposited in the respiratory tract [5]. The fine size and the related unique properties of these nanoparticles have noticeably increased their threat related to the environment and human health [6].

Studies revealed that on an equal horsepower basis diesel exhaust is 100 times more toxic than petrol exhaust [7], hence DEPs were chosen for this study. In various *in vitro* studies, it was proved that DEPs can enter into the epithelial cells [8,9]. Hence, the pulmonary epithelial cells are affected by atmospheric pollution and thus play a critical role in the physiology of pulmonary diseases [10].

Other *in vitro* studies established that different cell types took up DEPs and evoked toxic effects [10]. *In vitro* data on the cytotoxicity of DEPs have been controversial. The exact IC_{50} (50% Inhibitory Concentration) value has not been determined by previous studies. Effects associated with oxidative injury following DEP exposure included effects on protein expression, nuclear factor activation and gene transcription [6].

The present study aimed at exploring the *in vitro* cytotoxicity and the detailed mechanism of toxicity of DEPs on two cell lines, which were human lung alveolar type II epithelial cells (A549) and murine alveolar macrophages (RAW 264.7). We chose A549 cell lines since pulmonary toxicity was the most important health concern and macrophages were chosen, as they are the principal responders to diverse particles that initiate and propagate inflammatory reactions [11,12]. Epithelial cells are the main functional cells in the lung and A549 cell line is typically used for lung toxicity. The mechanism of cytotoxicity, inflammation and oxidative stress effects of DEPs on these two cell lines in comparison with each other have not been examined till date and has been revealed for the first time in the present study. This data highlights the importance to study the lethal effects of airborne PM and the associated oxidative stress and inflammatory effects.

MATERIALS AND METHODS

Collection and characterization of DEPs

Light duty multi-cylinder diesel and petrol engines (ALLMECH Pvt Ltd) operating on standard diesel and petrol fuel at a speed of 1500rpm was used to collect DEPs, as previously described by Sagai *et al*., 1993[13]. The average diameter of the collected particles was less than 2.5µm in size. The morphological analysis was done using High Resolution Transmission Electron Microscope (HR-TEM) (JEOL 3010). The elemental analysis for DEPs was performed using High Resolution Scanning Electron Microscopy with Energy Dispersive X-ray Analysis (HRSEM/EDX) (FEI Quanta FEG 200).

Cell culture and exposure to exhaust particles

A549 (Adenocarcinomic human alveolar basal epithelial cells) and RAW 264.7 cell lines (Murine macrophage alveolar cells) were used for the determination of cytotoxic end points. The cell lines were purchased from National Centre for Cell Science, Pune. Cells were grown in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) with 100 µg/mL streptomycin and 100 units/mL penicillin in a humidified atmosphere (5% CO2 and 95% air) at 37 °C (SLIM CELL incubator). Cells were regularly passaged and maintained. Cells were exposed to DEPs at concentrations of 25, 50, 100, 200 and 500 µg/ ml (Table 1). DEPs were suspended in sterile normal saline (NaCl 0.9 %) containing Tween 80 (0.01 %) for all further experiments. To minimize aggregation, particle suspensions were always sonicated (Clifton Ultrasonic Bath, Clifton, New Jersey, USA) for 15 min and vortexed before their incubation with cells [14].

Cell Viability

The percentage cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay[15]. RAW 264.7 cells were seeded onto 96-well plates , maintaining a density of 2.0×10^4 cells per well in 200 µL culture medium and incubated for 24 h before exposure to DEP solution for another 24 h. This was followed by the addition of 20 µL MTT (0.5 mg/mL) to each well followed by incubation for 4h at 37 °C. The culture medium was carefully aspirated and dissolved with 100 µL Dimethylsulfoxide (DMSO). This was allowed to stand for 10 mins. Optical density (OD) was read using an ELISA plate reader (BIO-RAD, USA) at absorption wavelength of 570 nm. The percentage viability of A549 cells was determined in a similar procedure with minor change in which the cells were seeded at a density of 3×10^5 cells per well in a 100 µL culture medium. Each experiment was performed in triplicates. The IC_{50} value was determined.

Table 1: Different concentrations of DEPs used to study Cell viability (MTT assay)

Exhaust Particles	Cell lines	Concentrations (µg/ml)
DEPs	A549 RAW 264.7	25, 50, 100, 200 and 500 µg/ ml

Measurement of NO (Nitric Oxide), TP (Total Protein) and LDH (Lactate Dehydrogenase) in cell culture supernatant fluids

For the measurement of NO, TP and LDH 1/25th, 1/10th and 1/5th IC_{50} values of A549 and RAW 264.7 cells were taken as different concentrations of DEPs (D1, D2, D3) as denoted in table 2. The maximum concentrations were taken based on the relevant concentration of $PM_{2.5}$ in urban air[16]. A549 and RAW 264.7 cells were seeded into 6-well plates at a density of 2.0×10^5per mL in 2.5 mL culture medium were allowed to proliferate attach and cover around 80% of the plate surface area before the treatment with DEPs (3 different concentrations each) for 24 h. 0.9% Nacl with 0.01% tween 80 was taken as vehicle control for all further experiments. After 24 hours of exposure, the culture supernatant was collected to determine the levels of NO (Biovision Inc), TP (Bio-rad, USA) and LDH (Sigma-Aldrich, India). The TP levels were measured using an assay Coomassie Brilliant Blue protein assay kit. The LDH activity was determined spectrophotometrically in the presence of lactate by observing the reduction of NAD^+ at 340 nm. The NO levels were determined using a nitric acid reductase kit according to the manufacturer's protocol.

Measurement of intracellular SOD (Superoxide Dismutase), GSH (Glutathione) and MDA (Malondialdehyde)

A549 and RAW 264.7 cells were seeded into 6-well plates at a density of 2.0×10^5per mL in 2.5 mL culture medium and allowed. to proliferate, attach and cover around 80% of the plate surface area before the treatment with DEPs (3 different concentrations each) for 24 h (as in Table 2). After 24h treatment, the cells were washed with ice-cold PBS followed by trypsinization and immediate disruption by continuous frozen-thaw process (three times). The cell lysates was collected, centrifuged and stored at -20 °C for the determination of intracellular SOD, GSH and MDA (Sigma Aldrich, India) using commercial kits following the manufacturer's instructions.

Measurement of intracellular Hydrogen Peroxide (H_2O_2) formation

A549 and RAW 264.7 cells were seeded into 6-well plates at a density of 2.0×10^5per mL in 2.5 mL culture medium and allowed to proliferate attach and cover around 80% of the plate surface area before the treatment with DEPs (3 different concentrations each) for 1 h (as in Table 2). The intracellular H_2O_2 was determined by the chemiluminescence (CL) method using horseradish peroxidase (HRP). After 1h treatment, the cells were collected, rinsed with ice-cold PBS and followed by trypsinization. For the radical measurement, the cell lysates were centrifuged, washed and suspended in two different epindorf's containing 400 µL Phosphate Buffered Saline (PBS). To this 8 µL of horseradish peroxidase and4 µL luminol were added. Chemiluminescence was measured at 25 °C for each second from the 0th to 9th second with a Luminometer apparatus (Zylux, U. S.).

Table 2: Different concentrations of DEPs used to study NO, TP, LDH, SOD, GSH, MDA and H_2O_2

Exhaust Particles	Cell lines	Concentrations (µg/ml)	
		Denotation	Concentrations used for the study(µg/ml)
DEPs	A549	D1	4
		D2	10
		D3	20
	RAW 264.7	D1	4
		D2	10
		D3	20

Measurement of cytokine secretion in A549 and RAW 264.7 cell lines

A549 and RAW 264.7 cells (10^6 cells/mL) were seeded and incubated with 20 µg/ml of DEPs. DEPs were suspended in sterile normal saline (NaCl 0.9 %) containing Tween 80 (0.01 %). The setup was incubated with DEPs for 12 and 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. After 12 and 24 h, the quantitative measurement of pro-inflammatory cytokines (IL-8, TNF-α and IL-6) was performed in the supernatants. Supernatants were collected by centrifugation of the culture medium at 2500 rpm for 20 min at 18 °C and assayed for and TNF-α, IL-6 and IL-8 using commercial ELISA assay kits (ebiosciences, USA), according to the manufacturer's recommendations. The samples and standards were all run in triplicates and the data were analyzed.

Statistical Analysis

All experiments were performed in triplicates. The results were expressed as mean± SD. Data were analyzed by standard statistical analysis one-way ANOVA with Duncan's test for multiple comparisons to determine significance between different groups. The results were considered statistically significant if 'p' value was.05 or less.

RESULTS

Characterization of DEPs

The HR-TEM and EDAX analysis revealed the presence of nanoparticles with Carbon as the major element.

Cytotoxicity

Cytotoxicity of DEPs was evaluated by cell viability and changes in biochemical factors such as NO, TP and LDH present in cell culture supernatants. In this research, both A549 cells and RAW 264.7 macrophages showed identical patterns in response to the cytotoxicity of DEPs. The control and vehicle control showed no changes in cell viability or an increase in NO generation, TP content and LDH activity. Cytotoxicity end point. (IC_{50} value), at which the concentration of DEPs resulted in 50 % inhibition of cell lines after 24h exposure to DEPs was found to be 100 µg/ml (Figure 1a) and the relative cell viability percentage was found to be 50.60 and 51.02 respectively. The effects of DEP incubation on NO secretion in supernatant fluids were investigated. As shown in Figure 1b, compared to control in DEP incubated A549 cells and RAW 264.7 cells significant increase was found in 10 (D2) and 20 (D3) µg/ml treatment, which was 1.36 and 1.84 times higher than the control in A549 cells and 1.37 and 1.86 times higher than the control in RAW 264.7 cells. DEPs incubation increased the total protein content in both cell lines in a dose-dependent manner. Significant differences were observed in 4 (D1), 10 (D2) and 20 (D3) µg/ml treatments of A549 and RAW 264.7 cells incubated with DEPs. In A549 cells, DEP incubation was 1.30, 1.57 and 1.59 times higher than the control. In RAW 264.7 macrophages, DEP incubation was 1.33, 1.54 and 1.58 times higher than the control (Figure 1c).

Increased LDH activity is a sign of membrane leakage. DEP incubation with RAW 264.7 macrophages and A549 cell lines resulted in the significant increase in 10 (D2) and 20 µg/ml (D3) treatments compared with control. In RAW 264.7 macrophages 10 (D2) and 20 µg/ml treatments (D3) were 1.16 and 1.32 times higher than the control Similar results were obtained for A549 cell lines; D2 was 1.18 times and D3 was 1.33 times higher than the control values as shown in Figure1d.

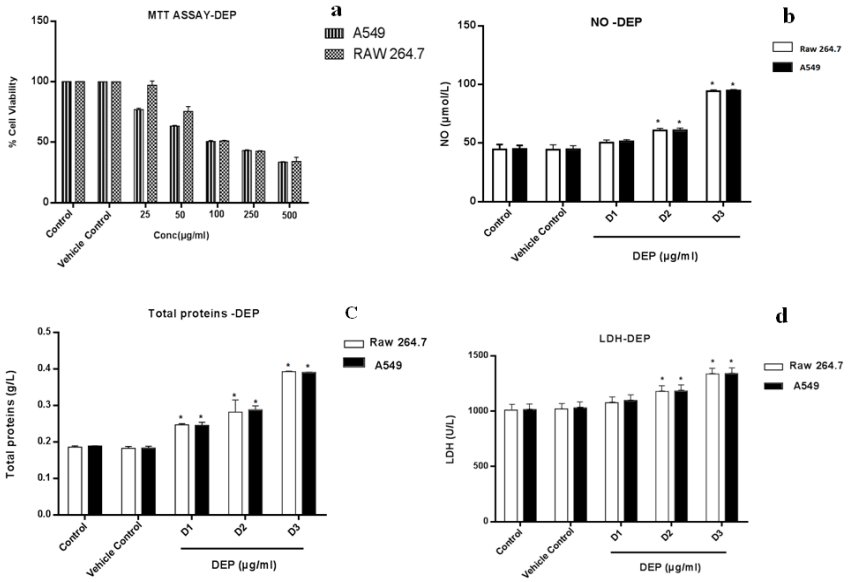

Fig. 1: (a) Cell viability of DEPs exposed to human A549 lung cells and murine RAW 264.7 macrophages after 24 h exposure was determined by MTT assay. Data are expressed as percentage of the control, mean ±SD of three repeated DEP experiments at the concentration of 25, 50, 100, 250 and 500 µg/ml respectively, (b) Effect of Cytotoxicity of DEPs to human A549 lung cells and murine RAW264.7 macrophages after 24 h exposure by measurement of levels of Nitric oxide (NO), (c) Total Protein (TP) and (d) Lactacte dehydrogenase acitivity (LDH) in culture supernatant fluids. Data are expressed as mean±SD of three repeated DEP experiments at different concentrations of DEPs.* Denotes a significant difference from the control (* p<0.05).

Oxidative stress

Dose-dependent effects were found in all the intracellular oxidative stress parameters. Compared to the control, significant SOD reductions of 58% and 40% in RAW 264.7 macrophages as well as 57% and 39% in A549 cells were observed after 24 h incubation with 10 (D2) and 20 µg/ml (D3) DEPs (Figure 2a). Similar results were also found in GSH after incubation of cells with DEPs. DEP treated A549 cells resulted in 91% and 68% reductions in GSH levels compared to the control, when treated with 10 (D2) and 20 µg/ml (D3) DEPs. Similarly, in RAW 264.7 macrophages incubation with 10 (D2) and 20 µg/ml (D3) DEPs resulted in significant reduction of 90% and 68% in GSH levels when compared to the control (Figure 2b).

The intracellular malondialdehyde content (MDA), a product of lipid peroxidation was measured. Significant elevations in MDA levels were found in both cell lines after 24 h incubation with 4 (D1), 10 (D2) and 20 (D3) µg/ml DEPs when compared to control. In RAW 264.7 macrophages, significant elevation was found to be 1.40, 1.54 and 1.59 times higher than the control levels. Elevation was also found in A549 cells after DEP treatments. In A549 cells, the elevation was found to be 1.43, 1.53and 1.63 times higher than of the control (Figure 2c). The effect of DEPs on radial formation was examined by measuring intracellular H_2O_2. Compared to the control, H_2O_2 content accomplished a less than 2-fold significant elevation in D2 and D3 treatments in both RAW 264.7 macrophages and A549 cells (Figure 2d).

DNA damage by apoptosis

The Fluorescent stain of DAPI (4', 6-diamidino-2-phenylindole) binds strongly to the AT rich region of the Genetic material. A549 cells and. RAW 264.7 macrophages were incubated for 12h and 24h with DEPs. Cell death was examined by a fluorescent microscope. In RAW 264.7 macrophages incubation with DEPs for 12h and 24h resulted in significant increase in DNA damage compared to the control (42% and 43 %) in contrast to this, A549 cells showed a significant increase in DNA damage of 15% and 16% when compared to the control (Figure 3).

Inflammatory response

DEPs induced pro-inflammatory cytokines (TNF-α, IL6 and IL8) in lung A549 cells and RAW 264.7 macrophages at 12 h and 24h incubation with DEPs. Treatment of RAW 264.7 macrophages with DEPs at 12 and 24 h showed the significant increase of TNF-α by 21% and 23% compared to the control. A549 cells treated with DEPs showed significant increase of 23% and 22% when compared with control (Figure 4a).

DEP treated RAW cell lines at 12h and 24h showed a significant increase of IL-6 by 19%, 14% compared to the control whereas. DEP treated A549 cells showed a significant increase of IL-6 by 21% and 15% respectively (Figure 4b). The DEP treatment showed the significant increase in IL-8 by 17%, 15% in RAW cells and 20%, 14% in A549 cells respectively (Figure 4c).

DISCUSSION

The physiochemical and environmental factors influence nanoparticle toxicity [17, 18]. Our previous HR-TEM and EDAX studies on DEPs demonstrated the presence of nanoparticles in the range of 15.0 to 35.0 nm with "C" (Carbon) as the major element [6]. Totlandstal *et al.*, 2010 [19] stated that the occurrence of organic fractions such as carbon in DEPs is the major factor leading to the production of ROS, which in turn induces DNA damage and inflammation. Cytotoxicity results indicated that at low concentrations DEPs changed the cell metabolism resulting in toxicity. Size and composition are the major factors that influence changes in cellular metabolism. Previous studies revealed that different nanoparticles were capable of crossing culture barriers and hence leading to cytotoxicity. Results of our study indicated an increase in levels of LDH, TP and NO in culture supernatants. They are indicators of cell membrane damage and cytotoxicity. Studies showed that NO was elevated 1000 times under inflammatory

conditions. NO, can be oxidized, reduced and complexed with various biomolecules hence leading to local tissue or cell damage [20]. Our studies demonstrated considerable cytotoxicity and cell membrane damage contributed by DEPs.

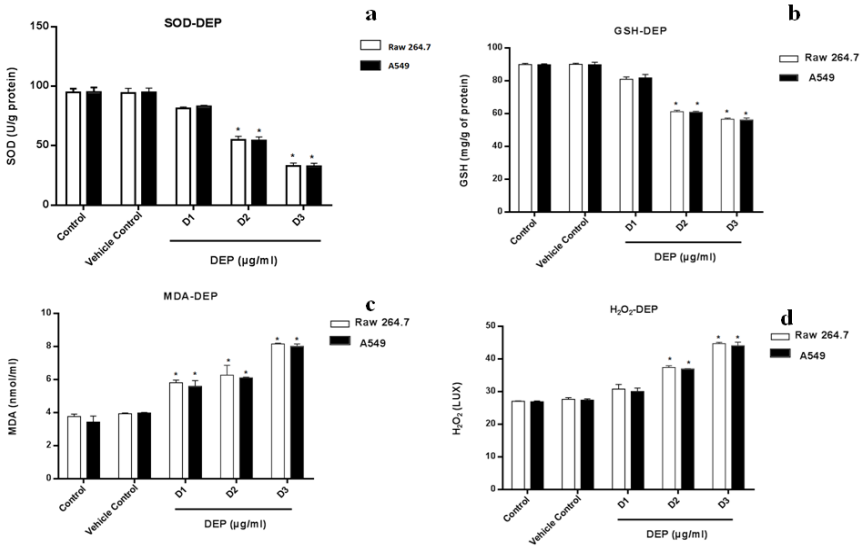

Fig. 2: (a) Oxidative stress induced by DEPs in human A549 lung cells and RAW264.7 macrophages after 24 h exposure by measurement of levels of Superoxide dismutase (SOD), (b) Glutathione (GSH), (c) malondialdehyde (MDA) and (d) hydrogen peroxide (H_2O_2) (after 1h exposure to DEPs), in culture supernatant fluids. Data are expressed as mean±SD of three repeated DEP experiments at different concentrations of DEPs.* Denotes a significant difference from the control (*p<0.05).

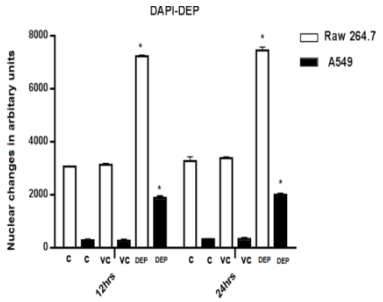

Fig. 3: DNA damage induced by DEPs in human A549 lung cells and RAW264.7 macrophages after 12 and 24 h exposure by measurement of apoptotic levels. Data are expressed as mean±SD of three repeated DEP experiments.* Denotes a significant difference from the control (* p<0.05).

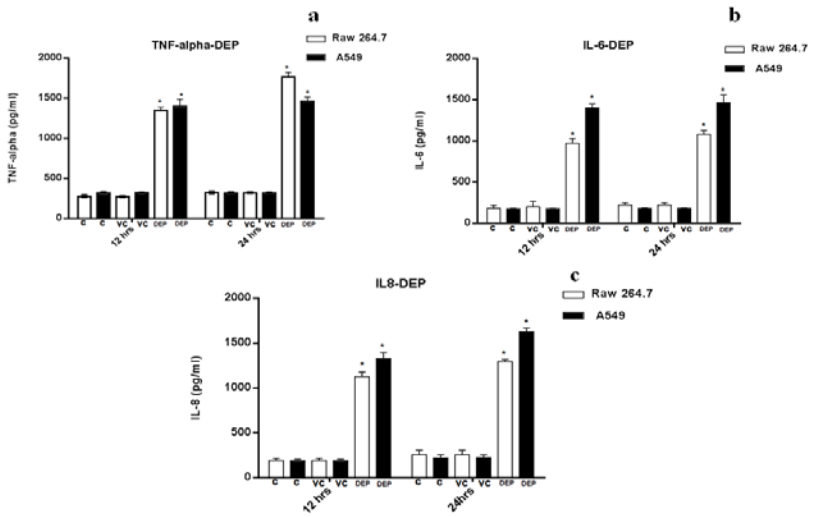

Fig. 4: (a) TNF-α, (b) IL-6 and (c) IL-8 induced by DEPs in human A549 lung cells and RAW264.7 macrophages after 12 and 24 h exposure to DEPs. Data are expressed as mean±SD of three repeated DEPs experiments.* Denotes a significant difference from the control (*p<0.05).

The reduced activity of antioxidants or exhaustion of free radical scavenging compounds on introduction of Particulate Matter (PM) leads to oxidative stress. Both SOD and GSH play a vital role in the regulation cell signaling, cell metabolism, cell proliferation, gene expression, immune response, cytokine production, nutrient metabolism, protein synthesis and anti-oxidant defense. Shvedovaa *et al*., 2008 [21] revealed that the depletion of SOD and GSH leads to oxidative stress. Studies also showed that depletion of GSH and hence oxidative stress, affects lung permeability [22]. Different studies demonstrated oxidation induced toxicity to biomolecules such as DNA and lipids in various cell lines exposed to air pollution particles [23].

Elevated levels of MDA in the current study, revealed cell membrane damage by the method of lipid peroxidation. Oxidative stress provoked by free radicals such as H_2O_2 is stated to be a vital mechanism of many acute and chronic infections and is currently evaluated to be one of the main reasons leading to the adverse health effects induced by airborne PM. Cachon *et al*., 2014 [24] revealed ROS production and changes in cell cycle metabolism contributing to oxidative stress in $PM_{2.5}$ exposed human bronchial cells.

Apoptosis or programmed cell death takes place in cells due to chromatin condensation, blebbing, cell shrinkage, chromosomal DNA and nuclear fragmentation [25]**.** Moller *et al*., 2010 [23] revealed that air pollutants have an increased DNA damaging capacity. In the present study, formation of ROS due to free radicals such as H_2O_2 was the key factor leading to DNA damage by DEPs.

In the current study, the levels of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α were found to be elevated on exposure to DEPs. Similarly, studies involving air pollution related particles such as $PM_{2.5}$ were seen to provoke inflammatory markers (cytokines) and hence led to oxidative stress and inflammation [26].

In the present study, we demonstrated that DEPs induced the reduction of anti-oxidant enzymes (GSH and SOD) in a dose dependent manner. The accumulation of H_2O_2 depleted intracellular GSH and SOD. Consequently, free radicals (H_2O_2) acted on biomolecules including proteins (TP), enzymes (LDH) and membrane lipids (MDA). Inflammation was also provoked by DEPs. Hence, production of H_2O_2 along with functional abnormalities of anti-oxidant mechanisms, the production of proteins and enzymes, inflammation, loss of cell viability and membrane disturbances demonstrated that oxidative stress was perhaps a major mechanism leading to the cytotoxicity of DEPs.

CONCLUSION

According to the results obtained in the present study, *in vitro* exposure to DEPs induced significant oxidative stress, together with membrane leakage, lipid peroxidation, cell inflammation and protein release, all of which may be the reason for cellular toxicity. These data revealed that the generation of free radicals play a key role in the mechanism of DEP toxicity. Future studies are therefore necessary to understand the mechanisms and the results of the oxidative stress *in vivo*, and the relationship between stress and the characteristics of PENPs.

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CONFLICT OF INTEREST

The authors report that there is no conflict of interest.

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