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*RESEARCH ARTICLE*

# Ultrafine Particles of Diesel Exhaust Induces Cytochrome P450 1A1 Mediated Oxidative Stress and DNA Damage in Cultured Blood and Lung Cells

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**ABSTRACT**

Attempts were made to investigate the role of cytochrome P450 1A1 (CYP1A1) on similarities in the generation of reactive oxygen species (ROS) and DNA damage in IM9, a human B lymphoblastic cell line with A549, the human lung adenocarcinoma epithelial cell line on exposure of diesel exhaust particles (DEP). A suspension of ultrafine particles (< 0.2 µM) of DEP (1mg/ml) in DMEM-F12 medium, at a concentration range of 1-100 µg/ml, was added to the cells for 6-48h. Expression studies revealed that DEP induced similar increase in the expression of CYP1A1, generation of ROS and DNA damage in both the cells. Pre-incubation with 3-methylcholanthrene (MC), a CYP1A1 inducer resulted in higher magnitude of induction of CYP1A1, ROS and DNA damage. This synergistic effect was lowered when α-naphthoflavone (α-NF), an inhibitor of CYP1A1 catalysed reactions, was added to these cells. Though the magnitude of alterations was lower in IM9 cells when compared to A549 cells, similarities in the alterations in blood and lungs cells has further suggested that blood lymphocytes can be used as a surrogate to monitor toxicity of vehicular emissions.

**Keywords:** Ultrafine particles, DEP, IM9, A549, CYP1A1, ROS, DNA damage

## 1. INTRODUCTION

Diesel exhaust particles (DEPs) consist of carbonaceous core to which combustion products including compounds such as polycyclic aromatic hydrocarbons (PAHs), quinones and transition metals are adsorbed[1]. DEPs with aerodynamic diameter of < 2.5 µm (PM 2.5) are of prime concern as because of greater surface area, they carry proportionally more chemicals and have the capacity to reach deep in the bronchial and alveolar regions of the lungs[2-5]. Studies have shown that organic compounds could be desorbed from DEP and bind to the cytosolic aryl hydrocarbon receptor (AhR). This binding to AhR is known to alter the expression of drug metabolising enzymes (DMEs) including cytochrome P450s (CYPs) and increase the generation of reactive oxygen species (ROS) leading to oxidative stress resulting in a variety of toxic manifestations[6-8]. DNA array studies have provided a mechanistic insight of the adverse effects of DEP in lungs. DEP was found to increase the expression of DMEs, inflammatory response genes, stress related genes thereby inducing pulmonary toxicity[9-10].

DEPs are primarily classified as group 1 human carcinogen (International Agency for Research on Cancer, IARC 2013). Exposure to DEP has been associated with increased risk of lung cancer[12-13]. *In vitro* studies have provided evidence for genotoxic potential of DEP and organic extracts of DEP[14-15]. DEP exposure has been associated with elevated levels of PAH- derived DNA adducts and 8-hydroxyguanosine (8-OHdG) in rat lungs contributing to DEP-mediated mutagenicity and carcinogenicity[16]. Significant association has also been reported for the increase in the formation of DNA adducts (8-OHdG) in lymphocyte of workers driving diesel forklift engines[17-18] showed that increased generation of ROS and lung injury after intratracheal instillation of DEP to mice was associated with a dose dependent increase in the expression of lungs CYP1A1 and a decrease in aryl hydrocarbon receptor suggesting that expression of lungs CYP1A1 could be used as biomarker of exposure to DEP.

Previous studies from our laboratory have shown that peripheral blood lymphocyte (PBL) CYPs can be used as a surrogate to monitor tissue expression of the drug metabolising enzymes[19-20]. Recent studies from our laboratory have further shown that trans-tracheal instillation of DEP induces a similar pattern of increase in the expression of PAH- responsive CYPs, anti-oxidant enzymes, their associated transcription factors, inflammatory signaling molecules as well as pro-apoptotic and anti-apoptotic genes in rat lungs and freshly prepared PBL[21-22]. Trans-tracheal instillation of DEP was also found to produce a similar increase in the expression of CYP1A1, 1A2 & 1B1, 2E1, glutathione S-transferases, GSTs (GSTPi, GSTM1 and GSTM2) and associated transcription factors in both, lungs and freshly prepared PBL isolated from the rats suggesting the suitability of using alterations in expression

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profiles of DMEs in PBL to predict exposure and toxicity of DEP[21-22].

Studies demonstrating that established human blood cell lines mimic the constitutive CYP expression status observed with primary blood lymphocytes have further suggested that PBL could be used for toxicity assessment[23-25]. However[24] reported that the inductive response of CYPs was not manifested in these blood cell lines that, in turn, could be attributed to various factors including the inducers used and the low levels of the expression of CYPs and other DMEs in PBL. In our efforts to demonstrate the suitability of using blood lymphocytes CYPs as a surrogate[19-21], attempts were now made to investigate the similarities in the effect of ultrafine particles of diesel exhaust (DE) on expression of CYP1A1, generation of reactive oxygen species (ROS) and DNA damage in IM9, the human B lymphoblastic cell line and A549, the human lung adenocarcinoma epithelial cell line. Using IM9 cells, studies were also carried out to explore the role of CYP1A1 in DEP mediated oxidative stress and DNA damage.

## 2. MATERIAL AND METHODS

### 2.1 Particle Preparation and Characterization

Diesel Exhaust particle (DEP) Standard Reference Material, SRM 2975, generated from forklift engine was procured from the U.S. National Institute of Standards and Technology(NIST, Gaithersburg, MD). DEP was suspended in complete culture medium (RPMI or DMEM) at a concentration of 1mg/ml followed by sonication at 100 megahertz for 5 minutes[21]. Characterisation studies using dynamic light scattering and phase analysis light scattering (PALS) in a Zetasizer Nano-ZS, Model ZEN3600 (Malvern instruments Ltd., UK) revealed that hydrodynamic size distribution and zeta potential of DEP were found to be in the ultrafine range of 184± 20.0 nm and -30.2±4.0 mV, respectively.

### 2.2 Cell Culture and Treatment

A549 (ATCC no. CCL-185TM), the human lung adenocarcinoma epithelial cell line used in the study was procured from National Centre for Cell Sciences, Pune (India) and IM9 (ATCC no.CCL-159 TM), the human B lymphoblastic cell line, was obtained from American Type Culture Collection (Rockville, MD). A549 and IM9 cells were cultivated in DMEM F-12 and RPMI 1640 culture media respectively. The culture media were supplemented with 10 % fetal bovine serum (FBS), 0.2 % sodium bicarbonate ($NaHCO_3$), 1ml/100ml medium of 1 % antibiotic-antimycotic at $37^0$C in 5 % $CO_2$-95 % atmospheric air under high humid conditions. After attachment of the cells, suspension of DEP (1mg/ml) in DMEM-F12 medium, diluted to a concentration range of 1-100 µg/ml was added to the cells for 6-48 hrs.

For cytotoxicity studies, cells were seeded in 96-well plates (10,000 cells per well). The cells were then exposed for 96 hrs to different concentrations of DEP. For studying DNA damage, cells were seeded in six-well plates (Corning, International Medical, Brussels). After the exposure, the culture medium was removed and the cells were washed with PBS and trypsinised. The cells were centrifuged for 5 min at 350 X g and resuspended in 200 ml of PBS (final cell suspension).

### 2.3 MTT Assay

The MTT assay, which provides an indication of mitochondrial integrity and activity and interpreted as a measure of percent cell viability, was carried out following the method of Pandey[26], *et al.* In brief, cells ($1x10^4$) were allowed to adhere for 24 hrs under high humid environment in 5 % $CO_2$- 95 % atmospheric air at 37 °C in 96-well culture plates. The medium was aspirated and A549 or IM9 cells were subjected to selected concentrations of DEP or 3-methylcholanthrene (MC), an inducer of CYP1A1/1A2 catalysed reactions or α-naphthoflavone (α-NF), an inhibitor of CYP1A1/1A2 catalysed reactions for 6-96 h in fresh medium. A fixed concentration of 10 µl/well of tetrazolium bromide salt (5mg/ml of stock in PBS) was added in 100 µl of cell suspension and plates were incubated for 4 h. At the end of incubation, the reaction mixture was carefully taken out and 200 µl of DMSO was added to each well. The plates were kept on rocker shaker for 10 min at room temperature and then analysed at 550 nm using Multi-well micro-plate reader (Synergy HT, Bio-Tek, USA). Untreated sets were also run under identical conditions and served as basal control.

For identifying the role of CYP1A1 in DEP mediated genotoxicity, a flask containing A549 or IM9 cells were pre-incubated with MC (4 µm for A549 and 15 µm for IM9, based on MTT assay) for 12 h and then exposed to DEP for 6 h (A549) or 12 h (IM9) along with MC. The cells after incubation were processed accordingly for Comet assay, ROS generation and mRNA expression of CYP1A1. For studying the protein expression of CYP1A1, a flask of cells after pre-incubation were incubated with DEP for 48 h and then processed for immuno-cytochemistry. Another batch of cells was also exposed to α-NF (20 µm for A549 and 10 µm for IM9) together with DEP. The concentrations of inducer (MC) and inhibitor (α-NF) were based on the previous published reports[27-28].

### 2.4 DNA Damage

Comet assay was carried out in A549 and IM9 cells to study DNA damage using the method of Bajpayee[29], *et al.* with some modifications. Slides were prepared in duplicate and lysed overnight in lysing solution (pH 10.0). Electrophoresis was conducted for 15 min at 0.7 V/cm and 300 mA at 4°C in chilled electrophoresis solution (1 mM $Na_2EDTA$ and 300 mM NaOH, pH > 13). All the steps were performed under dimmed light to avoid additional DNA damage. Following electrophoresis, excess alkali was neutralised and this was repeated three times. Slides were then stained with ethidium bromide (20 µg/ml, 75 µl/ slide) and processed for analysis using an image analysis system Komet 5.0 (Kinetic Imaging, Liverpool, UK) attached to a fluorescent microscope (Leica, Germany).

### 2.5 Quantitative Real Time-PCR (qRT-PCR) Analysis

Total RNA was isolated from control or treated A549 and IM9 cells using TRIzol reagent according to manufacturer's protocol. For qRT-PCR, cDNA was synthesised by High-Capacity cDNA Reverse Transcription Kit (RT) (Applied Biosystems, USA) as described[30]. The PCR reaction mixture for CYP1A1 or and β-actin in 20 µl contained 1X Taq-Man Universal PCR Master Mix (Applied Biosystems, Foster city, California, USA), 10 pM of each gene primer, 4 pM of each gene probe, 2 µl cDNA and nuclease-free $H_2O$. TaqMan assays for each gene target were performed in triplicate on cDNA samples in 96- well optical plates on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C.

### 2.6 Enzymatic Analysis

The activity of 7-ethoxyresorufin-O-deethylase (EROD), a marker of CYP1A1 and 1B1catalysed reactions was determined in A549 or IM9 cells by the method of Parmar[31], *et al.* with slight modification. Briefly, A549 or IM9 cells were grown to its almost full confluency at 37 °C and then exposed to DEP or MC or α-NF. After treatment, cells were washed with PBS (pH 7.4) and collected in a tube using a cell scraper and lysed using a sonicator at a frequency of 60 MHz / 10 s. The process was continued for seven times to completely lyse the cells. The lysate was then used for EROD assay. Levels of resorufin in the supernatant was measured using a Cary Eclipse Fluorescence spectrophotometer at excitation wavelength of 550 nm and emission wavelength of 585 nm with a slit width on 10 nm each and integration time of one second.

### 2.7 Immuno-cytochemical Analysis

Control or treated IM9 and A549 cells ($1 \times 10^6$ cells/ml) were seeded on cover slips in culture media. Cells were then allowed to adhere for 24 hr under high humid environment in 5 % $CO_2$ – 95 % atmospheric air at 37 °C. After 24 hr, media was removed and cells were washed with PBS and fixed with 4 % paraformaldehyde at 37°C for 20 min, followed by washing with PBS three or four times. Cells were further incubated with 0.5% $H_2O_2$ (w/v) in methanol for 1 h. After washing the cells with PBS, cells were incubated in blocking buffer containing 0.02 % Triton X100 and 0.1 % BSA in PBS for 15 min and then incubated with 1:100 dilution of polyclonal antibody of CYP1A1/1A2 (primary antibody). After incubation with primary antibody, the cells were washed with PBS and incubated with anti-rabbit FITC labelled secondary antibody (1:1000 dilution) for 1 h and then counterstained with DAPI (nuclear stain) containing antifade. The cells were observed under fluorescence microscope (Leica Qfluro Standard, Leica Microsystems Imaging Solutions Ltd. Germany). Experiments were performed at least three times, and on average 20 fields were evaluated for double blind scoring on each slide.

### 2.8 Determination of ROS

ROS production was determined by the method of[32] using dichloro dihydrofluoresce in diacetate (DCFDA), a non-fluorescent probe which is converted into highly fluorescent (2', 7' dichlorofluorescein) molecule in presence of ROS. Briefly, cells ($3X10^5$) were placed on six well cell culture plates and allowed to adhere for 24 h in $CO_2$ incubator at 37 °C. The medium was then replaced with the complete medium containing desired concentration of DEP or MC or α-NF for the given time period. After treatment, cells were detached from wells and spun for 5 min at 1,000 rpm and again resuspended in phosphate buffer saline (PBS) containing 10 µm DCFDA in the dark at 37 °C for 30 min. After 30 min, the cells were pelleted and resuspended in PBS and analysed by multiwell microplate reader (Snergy HT, Biotek USA) with an absorbance of 488 nm and emission of 525 nm. DCF-DA has been widely used as a marker for oxidative stress and has been suggested to be a good indicator of the overall oxidative status of the cell[33], though recently inadequacy of relying on probes fluorescence to detect radicals and oxidant has also been discussed[34-35].

For flow cytometry analysis, lungs and lymphocyte cells ($3X10^5$) in culture medium were placed on six well cell culture plates and allowed to adhere for 24 h in $CO_2$ incubator at 37 °C. The culture medium was then replaced with the complete medium containing desired concentration of DEP or MC or α-NF for the given time period. After treatment, cells were detached from wells and spun for 5 min at 1,000 rpm and again resuspended in phosphate buffer saline (PBS) containing 10 µm DCFDA in the dark at 37 °C for 30 min. After 30 minutes incubation the DCF fluorescence in cells was measured by flow cytometry. Flow cytometric analysis was carried out using a FACS Canto II flow cytometer (BD Biosciences, USA) and 10,000 events were acquired for analysis. Light scatter signals were plotted in a linear mode and fluorescence signals were plotted in logarithmic mode. Results were expressed as percentage of DCF fluorescence measured on FITC channel.

### 2.9 Statistical Analysis

Students `t' test was employed to calculate the statistical significance between control and treated groups. $p<0.05$ was considered to be significant when compared with the controls.

## 3. RESULTS

### 3.1 Particle Characterstistics

The hydrodyanamic size and zeta potential of DEP in tissue culture medium was approximately 184± 20.0 nm and -30.2±4.0 mV respectively.

### 3.2 Cytotoxicity

The cytotoxicity of DEP, as measured by MTT assay in A549 or IM9 cells show that viability of cells is decreased as the duration of exposure and concentration of DEP is increased in both the cells (Table 1). In case of A549, the cells start losing viability when DEP at the concentration of

**Table 1.** Cell viability, as assessed by MTT assay, of DEP in A549 and IM9 cells

| Concentration | 12 h | | 24 h | | 48 h | | 96 h | |
|---|---|---|---|---|---|---|---|---|
| | A549 | IM9 | A549 | IM9 | A549 | IM9 | A549 | IM9 |
| Control | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| DEP(0.001µg/ml) | 97.1 | 95.3 | 96.3 | 96.2 | 97.3 | 95.1 | 91.1 | 98.3 |
| DEP(0.1µg/ml) | 95.7 | 90.6 | 93.8 | 94.4 | 91.89 | 93.4 | 89.8 | 89.8 |
| DEP(10 µg/ml) | 92.8 | 89.1 | 90.1 | 92.5 | 78.37 | 88.5 | 70.1 | 88.1 |
| DEP(50 µg/ml) | 91.1 | 85.9 | 90.4 | 50.4* | 70.4 | 34.4* | 69.8 | 18.6* |
| DEP(100 µg/ml) | 91.4 | 61.1 | 88.9 | 29.2* | 50.4* | 15.2* | 35.44* | 10.4* |

Data are mean ±SEM of three independent experiments, with six replicates per sample.

10 µg/ml was added to the cells for 96 hr (Table 1). Exposure of DEP at the concentration of 100 µg/ml for 48 and 96 hr in A549 cells was found to be cytotoxic. In case of IM9 cells, DEP was cytotoxic when exposed at the concentration of 50 µg/ml for 24 hr and above (Table 1).

### 3.3 DEP Induced DNA Damage in A549 and IM9 Cells

Exposure of different concentrations of DEP caused significant concentration dependent DNA damage in A549 or IM9 cells as assessed by Olive tail moment (OTM), a parameter of Comet assay (Table 2). Maximum DNA damage (1175 %) was observed when DEP at the concentration of 100 µg/ml was added to the cells. However, at this concentration, mortality of the cells was also observed. Based on the ability of DEP to induce DNA damage as well as its cytotoxicity, 12.5 µg/ml concentration of DEP was used in A549 cells and 25 µg/ml in IM9 cells to investigate the similarities in the alterations of DEP mediated induction of CYP1A1 in the lungs and lymphocyte cells as well as to study DEP induced ROS generation and DNA damage and to identify the role of CYP1A1 in DEP induced DNA damage.

**Table 2.** Effect of non-cytotoxic dose of DEP on DNA damage, as assessed by Comet assay, in A549 and IM9 cells

| | A549 | | IM9 | |
|---|---|---|---|---|
| OTM/µg/ml | | % increase | OTM | % increase |
| Control | 0.87 ±0.11 | | 0.81±0.10 | |
| 3.12 | 0.92 ±0.5 | 5.7 | 0.85±0.11 | 4.93 |
| 6.25 | 1.4±0.19 | 60.9 | 0.92±0.14 | 13.5 |
| 12.5 | 1.9±0.2* | 118.4 | 1.01±0.13 | 24.69 |
| 25 | 3.34±0.5* | 283 | 1.52±0.16* | 87.6 |
| 50 | 8.1± 2.0* | 831 | 1.78±0.19* | 119.7 |

Data are mean ±SEM of three independent experiments. For each experiment the Olive tail moment of 50 cells was calculated. *p<0.05 compared to control

### 3.4 Effect of Pretreatment of CYP1A- inducer on DEP Mediated Induction of CYP1A1

Exposure of DEP for 6 hrs in A549 cells resulted in a significant increase (20 fold) in the mRNA expression of CYP1A1. Several fold higher increase in the mRNA expression of CYP1A1 (129- folds) was observed when A549 cells were exposed to MC for 18 hrs (Table 3). When the MC pretreated cells were exposed to DEP, a still higher increase in the mRNA expression of CYP1A1 (170-folds) was observed. This synergistic effect of MC and DEP in the expression of CYP1A1 was lowered (75-folds) when α-NF, an inhibitor of CYP1A1 catalysed reactions, was added to these cells (Table 3).

Likewise, exposure of IM9 cells with DEP for 6 hrs increased the mRNA expression of CYP1A1 (1.64 folds), though the increase was several fold lower when compared to A549 cells. Exposure of MC (15µM) to the IM9 cells for 12 hrs resulted in a relatively higher magnitude of induction (2.84 folds) of CYP1A1. When DEP was added to the cells, pre-exposed to MC, a still higher increase in the mRNA expression of CYP1A1 (2.91 fold) was observed when compared to the cells exposed to MC or DEP alone (Table 3). This synergistic effect of MC and DEP in the expression of CYP1A1 was lowered (1.77 folds) when α-NF was added to these cells (Table 3).

Immunocytochemical studies have further demonstrated that the DEP mediated increase in CYP1A1 mRNA in A549 or IM9 cells is associated with an increase in CYP1A1 protein. A549 or IM9 cells, when incubated with monoclonal antibody raised against human CYP1A1 (primary antibody) and the secondary antibody labelled with FITC showed positive staining (green fluorescence) for CYP1A1 as observed by fluorescence microscopy (Figs. 1 and 2). The intensity of the staining was greater in A549 cells indicating higher basal expression of CYP1A1 in these cells when compared to IM9 cells. Superimposition of fluorescence exhibited by FITC with DAPI, the nuclear

**Table 3.** Effect of 3-MC, DEP and α-NF on the relative mRNA expression of CYP1A1 in A549 and IM9 cells

| | A549 | IM9 |
|---|---|---|
| Control | 1.00±0.25 | 1.00±0.13 |
| α-NF | 1.20±0.15 | 1.09± 0.17 |
| DEP | 20.0± 4.5* | 1.64±0.14* |
| MC | 129.0± 20.0* | 2.84± 0.22* |
| MC+DEP | 170.0± 32.0* | 2.91± 0.21* |
| MC+ DEP+ α-NF | 75.0± 10.2* | 1.77± 0.18* |

Each reaction was performed in triplicate on cDNA samples in 96 well optical plates The threshold cycle value ($C_t$ value) of each sample was normalised with $C_t$ value of endogenous control (β- actin) (Δ $C_t$). Fold change is calculated from $\Delta\Delta C_t$ value of each sample$\Delta\Delta C_t$=$\Delta C_t$ of treated-$\Delta C_t$ of control. p<0.05 when compared with the controls. All the values are mean± S.E. of 6 animals. *p<0.05 when compared to the controls.
Data are mean ± SEM of three independent experiments.
Concentration of α-NF was 20 µm for A549 cells and 10 µm for IM9 cells.
Concentration of DEP was 12.5 µg/ml for A549 cells and 25µg/ml for IM9 cells.
Concentration of MC was 4 µm for A549 cells and 15 µm for IM9 cells
*p<0.05 compared to control.

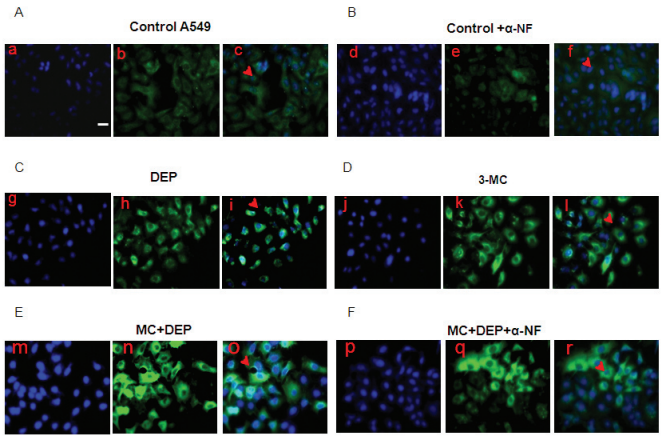


**Figure 1.** Immunocytochemical detection of CYP1A1 in A549 cell line isolated from (A) control, (B) α-NF, (C) DEP,(D) MC (E) MC+DEP(F) MC+DEP+α-NF treated cells. a, b and c represents control A549. d, e ,f, g, h, I, j, k, l, m, n, o, p, q, and r represents treated A549. a, d, g, j, m, and p shows dapi staining(blue,nuclear stain).b, e, h, k, n and q show immunoreactivity in the control and treated A549 with anti CYP1A1(green FITC).c, f , I, l, o, and r represents overlay of the two images control and treated cells, respectively. Scale 40 µm .

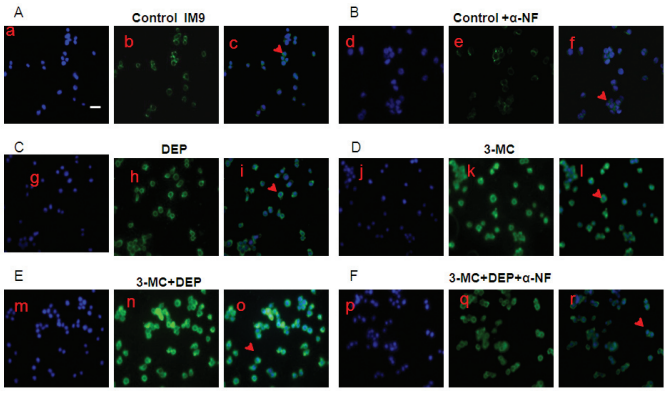


**Figure 2.** Immunocytochemical detection of CYP1A1 in IM9 cell line isolated from (A) control, (B) α-NF, (C) DEP,(D) MC (E) MC+DEP(F) MC+DEP+α-NF treated cells. a, b and c represents control A549. d, e ,f, g, h, I, j, k, l, m, n, o, p, q and r represents treated IM9. a, d, g, j, m, and p shows dapi staining(blue,nuclear stain).b, e, h, k, n & q show immunoreactivity in the control and treated IM9 with anti CYP1A1(green FITC).c, f , I, l, o, and r represents overlay of the two images control and treated cells, respectively. Scale 40 mm .

stain revealed that the CYP1A1 mediated fluorescence was localised in the cytoplasm of the cell (Figs.1 and 2). Likewise, cells (A549 or IM9) isolated after exposure with MC revealed intensity of much higher magnitude in A549 cells when compared to IM9 cells. As observed with the control cells, superimposition of fluorescence exhibited by FITC with DAPI revealed that the CYP1A1 mediated fluorescence was localised in the cytoplasm of the cell (Figs. 1 and 2). Exposure of DEP to A549 or IM9 cells also increased the intensity of positive staining for CYP1A1, though the magnitude of increase was less in these cells (A549 or IM9) when compared to the cells exposed to MC. Further, the cells isolated after exposure of MC and DEP showed marked increase in the expression of CYP1A1 as characterised by increase in the intensity of fluorescence in these cells. The increase in the intensity of positive staining for CYP1A1 was higher in the cells exposed to the combination of MC and DEP when compared to MC or DEP alone (Figs. 2, 3). The exposure of cells with α-NF along with MC or MC and DEP resulted in reduced positive staining in both, A549 or IM9 cells when compared to the cells exposed to MC or DEP alone.

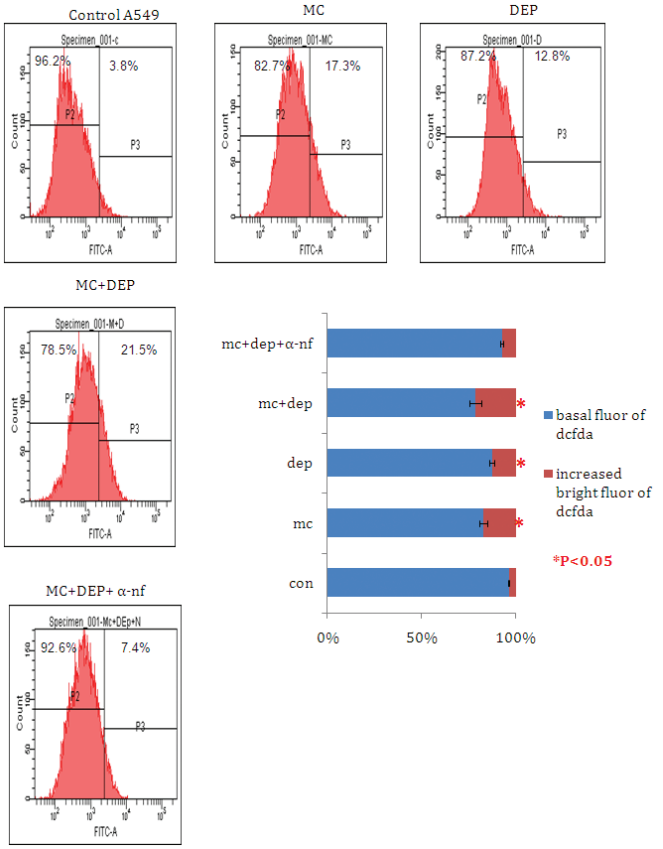


**Figure 3.** Reactive oxygen species production (ROS) by 3-MC(b),DEP(c), MC+DEP (d)and MC+DEP+ α-NF(e) in cultured A549 cell line by flow cytometry. (P2: Basal fluorscence,P3:increased fluorescence).f) % change in ROS formation as measured by flow cytometry. All values are the mean of three individual experiments. Significant changes are calculated by Student's t test. *p < 0.05.

The activity of EROD, a marker for CYP1A1 isoenzyme, was significantly increased (380 %) in A549 cells exposed with DEP. Exposure of DEP to the cells also resulted in an increase (100 %) in the activity of EROD. Exposure of DEP to these cells along with MC led to a still higher increase (500 %) in the enzyme activity when compared to the cells exposed with DEP or MC alone. Exposure of α-NF to the cells treated with MC and DEP reduced the extent of induction (150 %) in the EROD activity (Table 4). Likewise, when IM9 cells were exposed to MC, activity of EROD

**Table 4.** Effect of 3-MC, DEP and α-NF on EROD activity in A549 and IM9 cells

| | A549 | | IM9 | |
|---|---|---|---|---|
| **Category** | **Specific activity#** | **% increase** | **Specific activity#** | **% increase** |
| Control | 0.10± 0.01 | | 0.08±0.01 | |
| α-NF | 0.11± 0.01 | 10 | 0.082±0.07 | 2.4 |
| DEP | 0.20± 0.02* | 100 | 0.14±0.01* | 75 |
| MC | 0.48±0.05* | 380 | 0.21±0.02* | 162.5 |
| MC+DEP | 0.60±0.07* | 500 | 0.28±0.04* | 250 |
| MC+ DEP+ α-NF | 0.25± 0.01* | 150 | 0.17±0.02 | 112.5 |

Data are mean ± SEM of three independent experiments.
Concentration of α-NF was 20 µm for A549 cells and 10 µm for IM9 cells.
Concentration of DEP was 12.5 µg/ml for A549 cells and 25µg/ml for IM9 cells.
Concentration of MC was 4 µm for A549 cells and 15 µm for IM9 cells
# pmoles resorufin/min/mg protein
*p<0.05 compared to control.

was significantly increased (162 %) in these cells while exposure of DEP resulted in relatively smaller increase (75 %) in the activity of EROD (Table 4). Exposure of DEP to the cells pre-incubated with MC led to a still higher increase (250 %) in the enzyme activity when compared to the cells treated with MC alone. Exposure of α-NF to the cells treated with MC and DEP showed a much smaller increase (112.5 %) in the EROD activity when compared to the cells treated with MC+DEP (250 %) or MC (162.5 %) alone (Table 4).

### 3.5 Involvement of Reactive Oxygen Species (ROS) in DEP Induced Toxicity

Spectrophotometric studies showed that treatment of A549 cells with DCFDA showed a considerable increase (136 %) in ROS formation in the cells exposed with DEP when compared to control cells. Significant increase (210 %) was observed in the generation of ROS in the cells exposed to MC while a still higher increase (384 %) in the formation of ROS was seen when the cells, pre-incubated with MC, were exposed to DEP (Table 5). Interestingly, when α-NF was pre-incubated along with MC and the cells were then exposed to DEP, the formation of ROS was significantly reduced (142 %) when compared to the cells exposed to MC and DEP (384 %) or MC (210 %) alone (Table 5). Similarly, as seen with lung A549 cells, IM9 cells also showed statistically significant increase in the formation of ROS when exposed to DEP (67 %) or MC (116 %). Incubation of cells, pre-exposed to MC, with DEP showed a higher increase (159 %) when compared to the cells exposed to MC or DEP alone. Likewise, pre-incubation of α-NF along with MC and DEP resulted in significantly reduced (87 %) formation of ROS when compared to the cells exposed to MC and DEP (159 %) or MC (116 %) alone (Table 5).

Flow cytometric data revealed that similar to that seen with conventional multiplate reader, a considerable increase in ROS formation was observed in DEP exposed A549 cells (12.8 %) when compared to the control cells (3.8 %) (Fig. 3). Significant increase was also observed in the generation of ROS in the cells exposed to MC (17.3 %) while a still higher increase in the formation of ROS was seen when DEP was added to the cells, pre-incubated with MC (21.5 %) (Fig. 3). Interestingly, when α-NF was pre-incubated along with MC and the cells were then exposed to DEP, the formation of ROS was significantly reduced (7.4 %) when compared to the cells exposed to MC and DEP or MC alone (Fig. 3). Similarly, as seen with lung A549 cells, IM9 cells also showed statistically significant increase in the formation of ROS when exposed to DEP (13.1 %) or MC (15.4 %). Addition of DEP to the cells pre-incubated with MC, showed a higher increase (17 %) when compared to the cells exposed to MC or DEP alone. Likewise, pre-incubation of α-NF along with MC and DEP resulted in significantly reduced formation of ROS (9.7 %) when compared to the cells exposed to MC and DEP or MC alone (Fig. 4).

### 3.6 Role of CYP1A1 in DEP Mediated DNA Damage

Comet assay showed that exposure of DEP (12.5 µg/ml for 6 hrs) or MC (4 µM for 18 hrs) alone to the A549 cells induced DNA damage as evident by increase in tail moment when compared to the damage seen in control cells (Table 6). However, exposure of DEP to the cells, pre-incubated with MC, further increased the DNA damage as assessed by the olive tail moment (394 %) when compared to the cells exposed to DEP (174 %) or MC (284 %) alone. Further, exposure of α-NF to the cells incubated with DEP and MC significantly reduced the DNA damage (191 %) when compared to the cells exposed to DEP+MC (394 %). Addition of α-NF to the cells alone did not produced any significant DNA damage as evident by the alterations in the olive tail moment assessed by Comet assay (Table 6).

Similar to that seen in A549 cells, Comet assay data showed that exposure of DEP (25 µg/ml for 12 hr) or MC (15 µM for 12 hr) to the IM9 cells induced DNA damage as evident by an increase in tail moment when compared to that seen in control cells (Table 6). A further increase in DNA damage (137 %) was observed in the cells pre-incubated with MC and then

**Table 5.** Effect of 3-MC, DEP and α-NF on ROS production in A549 and IM9 cells

| | A549 | | IM9 | |
|---|---|---|---|---|
| **Category** | **Fluorescence** | **% Increase** | **Fluorescence** | **% Increase** |
| Control | 116± 9.8 | | 41.4± 5.7 | |
| α -NF | 123± 10.2 | 6 | 43.2± 5.4 | 4 |
| DEP | 274± 33.6* | 136 | 69.1± 6.2* | 67 |
| MC | 359± 37.0* | 210 | 89.4± 8.1* | 116 |
| MC+DEP | 562± 45.1* | 384 | 107.4± 10.4* | 159 |
| MC+DEP+α-NF | 281± 14.8* | 142 | 77.4± 5.5* | 87 |

Data are mean ± SEM of three independent experiments.
Concentration of α-NF was 20 µm for A549 cells and 10 µm for IM9 cells.
Concentration of DEP was 12.5 µg/ml for A549 cells and 25µg/ml for IM9 cells.
Concentration of MC was 4 µm for A549 cells and 15 µm for IM9 cells
*p<0.05 compared to control.

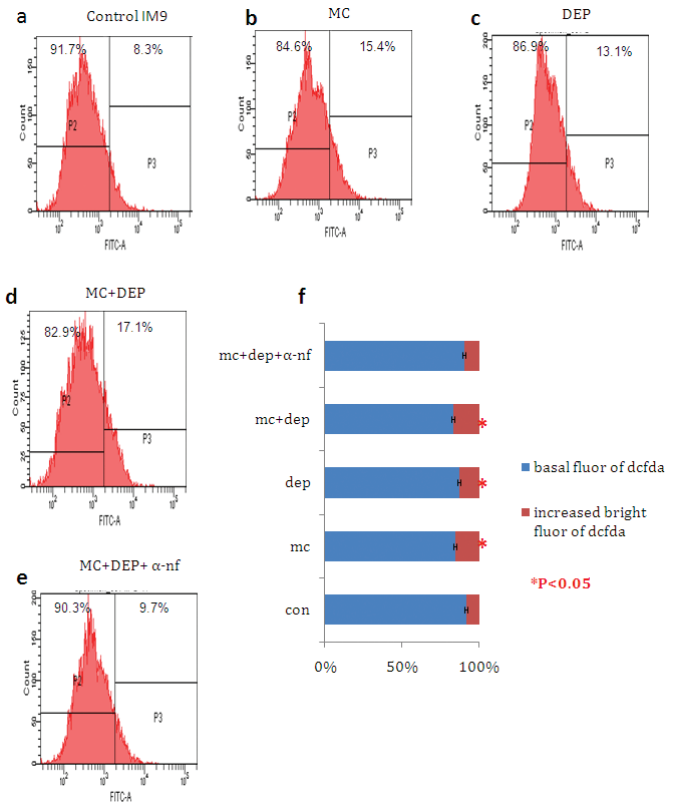


**Figure 4.** Reactive oxygen species production (ROS) by 3-MC(b),DEP(c), MC+DEP (d)and MC+DEP+ α-NF(e) in cultured IM9 cell line by flow cytometry. (P2: Basal fluorscence,P3:increased fluorescence).f) % change in ROS formation as measured by flow cytometry. All values are the mean of three individual experiments. Significant changes are calculated by Student's t test. *p < 0.05.

**Table 6.** Genotoxic effects of DEP in MC induced A549 and IM9 cells with and without CYP1A1 inhibitor α-naphthoflavone

| | A549 | | IM9 | |
|---|---|---|---|---|
| **Category** | **OTM** | **% increase** | **OTM** | **% increase** |
| Control | 0.91± 0.2 | | 0.87± 0.1 | |
| α-NF | 1.10± 0.1 | 21 | 0.89± 0.2 | 2 |
| DEP | 2.52± 0.3* | 174 | 1.49± 0.14* | 71 |
| MC | 3.53±0.5* | 284 | 1.71± 0.2* | 97 |
| MC+DEP | 4.52± 0.9* | 394 | 2.07± 0.22* | 138 |
| MC+ DEP+ α-NF | 2.71± 0.2* | 191 | 1.52± 0.11* | 75 |

Data are mean ± SEM of three independent experiments.
Concentration of α-NF was 20 µm for A549 cells and 10 µm for IM9 cells.
Concentration of DEP was 12.5 µg/ml for A549 cells and 25µg/ml for IM9 cells.
Concentration of MC was 4 µm for A549 cells and 15 µm for IM9 cells
For each experiment the olive tail moment of 50 cells was calculated.
*p<0.05 compared to control.

exposed to DEP. As evident from Table 6, exposure of α-NF to the cells pre-incubated with DEP and MC significantly reduced the DNA damage (75 %) when compared to the cells exposed to MC alone (97 %) or cells exposed to DEP+MC (137 %). Addition of α-NF to the IM9 cells alone did not produce any significant DNA damage as evident by the alterations in the olive tail moment (Table 6).

## 4. DISCUSSION

Consistent with our previous reports indicating similarities in the expression of CYPs in PBL with the tissue enzymes[19-20], the present study has provided evidence for basal expression of PAH-responsive CYP1A1 in IM9 cells as observed in lung derived cells. Krovat[21], *et al.* have also shown that the constitutive pattern of expression of xenobiotic metabolising CYPs were maintained in the blood cell lines, though the responsiveness of some of these CYPs was less when compared to freshly prepared blood lymphocytes or tissue derived cells. Expression and enzymatic studies have demonstrated that exposure of MC was found to increase the mRNA expression of CYP1A1 in IM9 cells which was associated with an increase in the protein expression of CYP1A1 and EROD activity in cultured blood cells. Similar response following exposure of MC, though of a higher magnitude, was observed in A549 cells. Further, as observed in the tissues[36-39], exposure of MC was found to increase the formation of ROS in IM9 or A549 cells indicating similarities in the responsiveness of blood derived cells with the tissues.

Similar to that seen in freshly prepared PBL[21,22,40-42], the present study has shown that exposure of DEP to IM9 cells significantly increased the mRNA and protein expression of CYP1A1. A similar increase, though of a much higher magnitude, was observed in A549 cells and has also been reported after exposure of DEP or organic extracts of DEP[6,38,40,43-45]. A greater magnitude of increase in the expression of CYP1A1 in IM9 or A549 cells exposed to MC prior to DEP have shown that like in the tissues, PAH metabolising CYPs, enriched following exposure of MC, catalyze the metabolic activation of PAHs present in DEPs in the blood cells. Likewise, greater magnitude of inhibition in the expression of CYP1A1 in IM9 or A549 cells exposed to α-NF and MC prior to DEP as compared to the cells exposed to MC or DE alone, have further provided evidence that inhibition of the levels of CYP1A1 leads to the decrease in the metabolic activation of PAHs that may account for the lesser increase in the expression of these CYPs in these cells.

Further evidence for the role of CYP1A1 in activation of PAHs adsorbed to DEPs was provided by the present data indicating increased generation of ROS in IM9 or A549 cells exposed to MC prior to DE. DEP has been shown to induce ROS generation via metabolic activation of organic compounds such as PAHs adsorbed on DEP through CYP1A1, microsomal P450 reductase and quinine reductases[41,46]. The enrichment of PAH metabolising CYP1A1 by MC may further increase the formation of ROS in these blood cells when co-exposed to DEPs[6,47]. Likewise, the decrease in the

formation of ROS in both IM9 and A549 cells co-exposed to both, MC and α-NF, prior to the exposure of DEP have demonstrated that the depletion of the levels of CYP1A-isoenzymes by α-NF may account for the decrease in the generation of ROS from ultrafine particles of DE.

That the DE induced increase in the expression of CYP1A, which, in turn, increases the generation of ROS may lead to toxic manifestations was demonstrated in the present study indicating oxidative DNA damage, as reflected by Comet assay, in the blood cells exposed to ultrafine particles of DE. Studies have convincingly shown the association between particle size, ROS production and oxidatively damaged DNA[48-49]. Studies have shown that ROS such as hydrogen peroxide ($H_2O_2$), hydroxyl radicals (.OH), superoxide ($O_2$.-) and singlet oxygen($^1O_2$) as well as reactive nitrogen species (RNS) including nitric oxide and peroxynitrite generated by DEPs have an impact on DNA damage and are considered as a major determinant of genotoxic properties of DEP[7,50]. Comet assay demonstrating increased DNA damage in IM9 or A549 cells exposed to MC prior to DEP has shown that CYP1A1 is involved in the increased generation of ROS resulting in increased DNA damage in these cells. *In vivo* and *in vitro* studies have also shown that bioactivation of PAHs present in DEPs by CYP1A isoenzymes is partially responsible in mediating genotoxic response as seen through DNA strand breaks or through formation of DNA adduct (8-OHdG) formation in exposed cells[8,48,51-53]. The reduction in the extent of DNA damage following preincubation of A549 or IM9 cells with α-NF, has further demonstrated the role of PAH metabolising CYP1A1 in the metabolic activation and toxicity of DEP.

## 5. CONCLUSION

Present study has shown that as reported in PBL, CYP1A1 is expressed in blood cells and its responsiveness to PAHs is retained under *in vitro* conditions. Ultrafine particles of DE were found to induce the expression of PAH-responsive CYP1A1 in IM9, the human B lymphoblastic cell line and A549, the lung derived cells. Our data also provided evidence that CYP1A1 catalysed metabolic activation of the PAHs, adsorbed to DEPs, leads to ROS generation resulting in DNA damage in IM9 and A549 cells. Since the concentrations used are often encountered in ambient environment[54], the present data has provided support to our previous reports that blood lymphocytes can be used as a surrogate to monitor toxicity of airborne toxicants particularly fine and ultrafine DEP.

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**Conflict of Interest :** None

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