Characterization of coal fly ash nanoparticles and their induced in vitro cellular toxicity and oxidative DNA damage in different cell lines

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Coal combustion generates considerable amount of ultrafine particles and exposure to such particulate matter is a major health concern in the developing countries. In this study, we collected nano sized coal fly ash (CFA) and characterized them by scanning electron microscope-energy dispersive X-ray analysis (SEM-EDX), particle size analyzer (PSA) and transmission electron microscope (TEM), and investigated its toxicity in vitro using different cell lines. The imaging techniques showed that the coal fly ash nanoparticles (CFA-NPs) are predominately spherical shaped. The analyses have revealed that the CFA-NPs are 7-50 nm in diameter and contain several heavy metals associated with CFA particles. The studies showed significant amount of toxicity in all cell lines on treatment with CFA-NPs. The cytotoxicity and oxidative DNA damage caused by CFA-NPs were determined by inhibition of cellular metabolism (MTT), total intracellular glutathione (GSH), reactive oxygen species (ROS) and DNA fragmentation in cultured cell lines (Chang liver, HS294T and LL29). The cellular metabolism was inhibited in a dose-dependent manner in CFA-NPs treated cell lines. The CFA-NPs induced ROS and decreased the total intracellular glutathione with increased dose. Further, the CFA-NPs treated cells showed severe DNA laddering as a result of DNA fragmentation.

Keywords: Aerosol, CFA-NPs, Cytotoxicity, DNA fragmentation, Energy dispersive x-ray (EDX) analysis, Glutathione (GSH), MTT, Pollution, Reactive oxygen species (ROS), SEM, TEM, Ultrafine particles (UFP)

Coal is the main source of electric generation and industrial applications for its low cost and availability. Coal combustion generates sufficient amount of ultrafine particles (UFP) formed by mineral transformation during high-temperature combustion process. Exposure to coal fly ash particulate matter (CFA-PM) is a major health concern in countries, such as India and China. The CFA emitted from the thermal power plants are controlled by several methods viz., electrostatic precipitator, fabric filter, mechanical collectors and particle scrubbers¹². However, the collection efficiency of these technologies is not completely effective for the particles with an aerodynamic diameter in the range of 0.1-1μm¹³. The UFP are formed as a result of soot formation, vaporization of material matter and inorganic ash present in the coals. These vaporized particles nucleate to form large number of nano sized particles, which then grows through condensation and coagulation to form an accumulation mode of aerosol². Due to its extremely small size, UFP are inhaled and deposited deep inside the lung and evade many mechanism responsible for clearance of larger particles⁴.

Human health impacts of CFA (aerodynamic diameter <100 nm) released to the environment are gaining worldwide interest. These nano sized particles penetrate through the basic biological organs and result in inflammation of tissues and also alter the cellular redox balance towards oxidation, leading to cell death⁵. Many epidemiological studies associate exposure to ambient particulate matter (PM) to increased mortality and morbidity due to respiratory diseases, cardiovascular and/or cardiopulmonary diseases and liver and lung cancer⁶⁻¹². The CFA released into the environment remains airborne and gets deposited in the alveoli of lung leading to pneumoconiosis⁶,¹³,¹⁴. The elements present in the CFA are Al, C, Ca, Fe, K, Mg, Mn, Na, P, Si, Ti, and certain trace toxic heavy metals viz., As, Ba, Cd, Cr, Hg, Mo, Ni, Pb, Se, V, Zn which are primarily

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associated with the particle surfaces\textsuperscript{15-17}. The trace elements are found to be more toxic and effective components than that of the organic substances. The toxicity studies on airborne PM and fly ash have been performed on various organisms including human lymphocytes and pneumocytes\textsuperscript{18,19}. The \textit{in vitro} release of CFA with acellular generation of hydroxyl radicals and the amount of iron (Fe) release induces oxidative DNA damage and cytotoxicity in rat epithelial cells\textsuperscript{20}. The effect of size fractionated coal fly ash nano particles (CFA-NPs) showed greater ability to cause pulmonary inflammation and kill macrophages in cell culture compared to fine and coarse fractions\textsuperscript{21}. Okeson \textit{et al.} demonstrated that size and the material composition are the factors determining the toxicity of combustion derived PM in cultured rat epithelial cell lines\textsuperscript{22}.

Various toxicity studies have demonstrated relationship between the ambient PM and human health; however, still the knowledge is limited for CFA-NPs. This area requires further extensive examination, since earlier studies have not sufficiently considered the characterization of CFA-NPs, surface associated transition metals and mechanistic aspect of CFA induced toxicity in terms of ROS production, DNA and oxidative damage and cell death in human cell lines. Due to extensive production and wider application, there is a heavy discharge of CFA-NPs into the atmosphere, and it is a potential risk to humans. Therefore, here we investigated the toxicity response at \textit{in vitro} cellular models to understanding the mechanism of action and its functional response to CFA-NPs.

**Materials and Methods**

\textit{Collection of coal fly ash (CFA)}—The CFA was collected using respirable dust sampler (APM 460). The dust sampler was placed near to coal fired boilers exhaust chimneys. The ambient air laden with CFA suspended particulates enters the system through the inlet pipe in the dust sampler. As the air passes through the system, non-respirable dust is separated from the air stream by centrifugal force acting on the solid particles. These solid particulates were collected separately in the sampling bottle. Only the fine dust particles forming the respirable fraction of the total suspended particulate (TSP) passes through the cyclone and is carried by the air stream to the filter paper placed between the top cover and filter adaptor assembly. The respirable dust is retained by the filter paper and the carrier air exhausted from the system through the blower. The particles collected on silicon filter paper allowing a measurement of respirable fractions of TSP of CFA. The CFA particles were collected for several days to carry out the experiments. The particles collected using these respirable dust samplers are the suspended PM which escapes through the chimneys and distributed into the atmosphere. In this study, we specifically collected the CFA-PM, which escapes into the atmosphere. The samples were collected from the power plant located about 25 Km, north of Chennai, Tamil Nadu, India. This power plant consumes Bituminous coals for power generation.

\textit{Scanning electron microscopy-energy dispersive X-ray (SEM-EDX) analysis}—The surface morphology and chemical composition of the collected CFA were characterized using high resolution scanning electron microscope (FEI Quanta FEG 200), which was additionally equipped with energy dispersive X-ray analysis (EDX) for analyzing chemical composition. The fine CFA-NPs were mounted on an aluminium stub using double sided carbon tape. The sample was then coated with gold sputter coating unit at 10 Pa vacuum for 10 S (SC7620, Japan). The acceleration potential used was 30 KV and the image was captured at desired magnification.

\textit{Particle size analyzer (PSA) and transmission electron microscope (TEM) analysis}—The CFA-NPs were determined using Malvern zeta sizer (Malvern Instrument Inc, London, UK). The CFA particles were diluted and uniformly dispersed in water. The sample was analysed for average size distribution of particles. TEM (Philips CM120 series at an accelerating voltage of 120 kV) analysis was performed to measure the size and the shape of the CFA-NPs. The samples were prepared by dispersing the CFA in water and drop-coated onto a copper grid covered with the carbon film. The CFA coated grids were allowed to stand for few minutes. The excess solution was removed and dried.

\textit{Cell culture}—Chang liver (Human liver), HS294T (human skin cells) and LL29 (AnHa) (Human Caucasian diploid lung) cell lines were tested in all the experiments. Cell lines were purchased from National Centre for Cell Science (NCCS), Pune. Cells were cultured in DMEM (Dulbecco’s modified eagle medium) supplemented with 10 % FBS (fetal bovine serum), D-Glucose and antibiotics at 37°C under the humidified atmosphere containing 5% CO\textsubscript{2}.
**Determination of cell viability**—The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to determine the cell viability in CFA-NPs treated cell lines following the protocol as described previously. The cell lines (Chang liver, HS294T and LL29) were plated separately in 96-well plates at a concentration of 1 × 10⁵ cells/well. After incubation for 24 h at 37°C at 5% humidity of CO₂ incubator, cells were washed and treated with different concentrations of CFA-NPs (12.5-1000 μg/ml). The serum free MTT labeling reagent (0.5 mg/ml) was added and incubated for 4 h at 37°C in a CO₂ incubator. The assay plates were shaken intermittently at 2 h interval in a portable rocker in the CO₂ incubator to reduce the NPs agglomeration. The 50 % inhibitory concentration value (IC₅₀) of the CFA-NPs treated cells were obtained for untreated (control) cell lines. The formula Percentage cell viability = OD of sample/OD of Control x 100 was used to calculate the cell viability.

**Estimation of total glutathione (GSH)**—The glutathione content was estimated as described by Mishra et al. The respective cell lines (Chang liver, HS294T and LL29) were plated in six-well dishes (106 cells/well). After 24 h, the medium was replaced and cells were incubated in fresh serum-free medium containing different concentrations (12.5-1000 μg/ml) of CFA-NPs for 24 h at 37°C at 5% humidity of CO₂ incubator. The assay plates were shaken intermittently at 2 h interval in a portable rocker in the CO₂ incubator to reduce the NPs agglomeration. The untreated cells served as the control. To extract cellular GSH, the treated cells were then dispersed using a sonicator by two 20 s bursts. An aliquot of sonicate was taken for protein determination. The glutathione determination is based on the enzymatic recycling assay of the 5,5-dithio-bis (2-nitrobenzoic acid)-glutathione disulfide reductase (DTNB-GSSG reductase) with some minor modifications. The absorbance was measured at 412 nm, and the activity was calculated based on standard calibration curve plotted using GSH standard.

**Determination of reactive oxygen species (ROS)**—Intracellular ROS production was quantified by using fluorescent probe DCFH-DA according to previously described method. The various concentrations of CFA-NPs (control, 12.5, 250 and 1000 μg/ml) treated cell lines (Chang liver, HS294T, LL29) were incubated for 24 h at 37°C in CO₂ incubator. The assay plates were shaken intermittently at 2 h interval in a portable rocker in the CO₂ incubator to reduce the NPs agglomeration. DMEM was replaced by PBS with glucose (5.5 mM) and the cells were treated with 1 μM of CM-H₂DCFH-DA (2,7-dichlorofluorescin-diacetate) for 30 min at 37°C in dark. The fluorescence was recorded at 495 nm excitation and 530 nm emissions by a luminescence fluorescent microscope (Labomed, India). The treated cells were observed in 10x resolution and recorded for fluorescence quantification using ImageJ image pixel analysis software.

**DNA fragmentation assay**—DNA fragmentation was used to determine the induction of apoptosis by observing the DNA fragmentation. Briefly, the cell lines (Chang liver, HS294T, and LL29) were seeded in 12-well plates with a cell load of 2 × 10⁵/well and treated with various concentrations of CFA-NPs (control, 100 and 250 μg/ml). The CFA-NPs treated cell lines were incubated for 24 h at 37°C in CO₂ incubator. The assay plates were shaken intermittently at 2 h interval in a portable rocker in the CO₂ incubator to reduce the NPs agglomeration. The concentrations were selected based on significant toxicity by CFA-NPs as assessed by MTT assay. The total DNA was extracted from treated cells using phenol: chloroform method. The extracted DNA was electrophoresed on 1.2 % agarose/EtBr gel at 5V. After electrophoresis DNA fragments were analyzed with UV illuminator at 312 nm.

**Results**

**CFA-NPs characterization**—The morphological analysis of CFA-NPs is shown in Fig. 1A and it represents the presence of homogeneous spherical shaped particles. The EDX spectrum (Fig. 1B) of CFA-NPs reveals the presence of several major elemental constituents such as Al, C, Ca, Fe, Mg, Mo, Na, S Si and other trace elements such as Ar, Ba, Cd, Cr, K, Mn, Nd, Pb, Rh, Se, Sr, Ti, V, Zn. The Fig. 2A that depicts the particle size analysis of CFA-NPs shows the uniform distribution of CFA-NPs with an average size about 50 nm. The TEM image of CFA-NPs (Fig. 2B) shows the presence of spherical shaped particles with size range 7-30 nm.

**CFA-NPs cytotoxicity**—CFA-NPs showed significant toxicity in all the tested cell lines as shown in Fig. 3. In initial concentrations of 12.5, 25 and
50 µg/ml the CFA-NPs treated liver cells were inhibited up to 22% of cell viability. The loss of cell viability was observed at increased concentration and cell death up to 80% in liver cells. Similarly, in skin and lung cells also, the initial concentration of CFA-NPs showed similar significant inhibition compared with the control. The percentage of cell viability is shown in Fig. 3. The IC$_{50}$ value of CFA-NPs treated liver, skin and lung cells were found at 94, 66 and 177.1, respectively.

**CFA-NPs effects on intracellular GSH**—The cellular toxicity was measured by level of reduced glutathione (GSH) as shown in Fig. 4A. In liver cells,
@ 12.5 μg/ml, the CFA-NPs treated cells inhibited the cellular GSH concentration up to 6 %, which was significantly decreased to that of the control cells (Fig. 4A). In higher concentrations, such as 100, 250, 500 and 1000 μg/ml, the inhibition was significant with the level of GSH at 35, 37, 38 and 43 %, respectively. Similarly, in skin cells @ 12.5 and 25 μg/ml, the CFA-NPs treated cells inhibited the level of GSH concentration up to 6 %, and at higher concentrations they showed significant inhibition up to 37 % (Fig. 4B). However, the CFA-NPs treated lung cells inhibited the level of GSH up to 37 % at higher dose treatment (Fig. 4C).

CFA-NPs effects on ROS production—Intracellular ROS generation induced by CFA-NPs at different concentrations (12.5, 250 and 1000 μg/ml) in Chang liver, HS294T and LL29 cells as examined using a DCFH-DA fluorescence probe is shown in Fig. 5. The ROS generation was significant in all the tested concentrations and showed a concentration dependent increase in levels of fluorescence (Fig. 5 a-d of A, B and C). An increase of 1.5 fold was observed in ROS generation in CFA-NPs treated liver cells compared to control cells (Fig. 5A). In skin cells, the level of ROS was not significantly altered, though raised up to 1.2 and 1.3 folds (Fig. 5B). Whereas, the lung cells showed more sensitive response to administration of CFA-NPs; significant increase up to 2 fold at 1000 μg/ml concentration (Fig. 5C). Among the tested cell lines, the liver cells showed more damage at low concentration of CFA-NPs.

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**Fig. 4**—Effect of CFA-NPs on the intracellular glutathione. [Cell lines (A) Chang liver; (B) HS294T; and (C) LL29 were exposed to various concentrations of CFA-NPs (0, 12.5, 25, 50,100,250,500 and1000 μg/ml) for 24 h and analysed for intracellular GSH concentration. The CFA-NPs treated cell lines showed decrease in the level of GSH compared to untreated cell lines [(A) Chang liver; (B) HS294T; and (C) LL29 in a dose dependent manner. All three CFA-NPs treated cell lines showed similar level of cell toxicity].

**Fig. 5**—The level of Intracellular reactive oxygen species (ROS) in CFA-NPs treated cell lines (A) Chang liver; (B) HS294T; and (C) LL29. [The ROS generated fluorescence images for various concentrations of CFA-NPs treated cell lines (a, Control; b, 12.5; c, 250; and d, 1000 μg/ml). The data represents the mean ± SD of three independent experiments.]
CFA-NPs effects on DNA damage—DNA laddering was determined in CFA-NPs treated cell lines and the DNA damage was observed and shown in Fig. 6. The CFA-NPs concentrations were selected based on IC_{50} value of MTT assay. The cells treated with various concentrations of CFA-NPs showed DNA damage in all the tested cell lines. Liver and lung cells showed severe DNA laddering (Fig. 6A & C), whereas in skin cell lines, the DNA damage was observed in streaked manner (Fig. 6B) which may be due to cell necrosis. The liver and lung cells (Fig 6A & C) demonstrated the apoptotic mediated cell death at higher tested concentrations. Both the concentrations of CFA-NPS (100 and 250 μg/ml) treated cells showed severe nuclear damage compared to untreated control cells.

Discussion

The epidemiological and experimental evidences on CFA induced inflammation and cancer available already are not adequate to explain the mechanism behind such actions. In this study, we used an efficient method to characterize the size and chemical composition of CFA-NPs specifically collected using respirable dust sampler from coal fired boilers. The CFA-NPs toxicity was investigated for in vitro responses using three cell lines. The nature and properties of coal differs with the quality of the coal, process of combustion and precipitation technique as well as place of origin. The SEM analysis of CFA (Fig. 1A) shows the presence of spherical shaped coal fly ash particles. The spherical shaped particles are distinctive for CFA which are produced due to coal combustion at high temperature through the process of decomposition, nucleation, coagulation and condensation of vaporized materials. The EDX analysis of CFA (Fig. 1B) revealed relative high contents of toxic heavy metals such as Cd, Co, Cr, Fe, Mn, Mo, Rh, Ti, V, which catalyzes the one-electron reductions of molecular oxygen necessary to produce ROS such as hydroxyl radical HO. This hydroxyl radical may damage the cell directly or may also activate the biochemical reaction that causes cell damage indirectly. The bioavailability of metals is a major primary factor determining the health effects due to particles. The water-soluble components are assumed as salts, which are efficiently removed from the respiratory tract while the insoluble components remain longer and induce severe tissue damage and toxicity to the lung.

The particle size analyser has shown that the average size of the CFA-NPs is 50 nm (Fig. 2A). TEM analysis confirmed the size range of the CFA-NPs to be 7-30 nm (Fig. 2B) in alignment with the earlier findings. The average size of the collected CFA particles ranged below PM 0.1 which was confirmed by PSA and TEM. Size is the major key factor determining the toxicity of CFA-NPs. The smaller is the size of the particle, the greater its surface area to its volume ratio, and higher its chemical and biological reactivity. There is an increase in toxicity with decreased PM diameter. Diabate et al. described that the ultrafine particles induces more significant generation of intracellular ROS compared to larger sized particles. Ober dorster et al. reported that the ultrafine particles of size 12-20 nm have ability to penetrate the alveolar epithelial lining and enter the lung interstitium to a greater extent than an equal mass of larger respirable particles about 200 nm was observed after 24 h following intratracheal instillation in rats. The level of persistence of the inhaled PM is a significant characteristic which determines the degree of inflammation and tissue damage.

In this study, we observed a dose dependent response of cell proliferation on treatment with various concentrations of CFA-NPs. After 24 h incubation with CFA-NPs, the viability of cells decreased in a dose dependent manner. At higher concentrations, the CFA-NPs treated cell lines (Chang liver, HS294T & LL29) showed decreased...
cell viability, and significantly increased cell toxicity. The chemical composition of CFA-NPs also has some impact on the inhibition of cell growth. Metals like iron, vanadium and zinc has toxicological effect which may inhibit the growth of cells\textsuperscript{17}. Okeson et al. reported that cellular metabolism is inhibited consistently in a dose-dependent manner by treatment with various concentrations of PM by MTT assay in lung alveolar type II epithelial cells (RLE-6TN cells)\textsuperscript{17}.

The cell protectant enzyme GSH is synthesized in the cytoplasm and migrates through circulatory system into different organs and sub cellular compartments\textsuperscript{38}. The GSH metabolism is a major mechanism for protection of cells against the agents which induce oxidative stress. It plays an important role in both scavenging ROS and detoxification of drugs and chemicals\textsuperscript{8,39}. Other antioxidant enzymes like Catalyse, SOD and GST also takes place in scavenging and detoxification process. In CFA-NPs treated cell lines; the level of glutathione was significantly reduced. The treated cells expressed decreased amount of GSH compared to untreated cells. The decrease in the GSH concentration is the progression of cell death in response to different apoptotic stimuli cell lines. The level of antioxidants were diminished due to excited stress and cellular injury developed by CFA-NPs. The damaged cells have lost its efficiency to defend itself against stress resulting in DNA Damage. The BEAS-2B cells on treatment with MAF98 fly ash showed both increase and decrease in the level of GSH concentration, which was an indicative of oxidative stress, leads to membrane dysfunction and DNA damage\textsuperscript{8}. The recent study showed that the oxidative stress by CFA particles leads to mobilisation of arachidonic acid and increase in level of GSH in RAW264.7 machophages\textsuperscript{40}.

The cellular oxidative stress was determined by the generation of ROS in intracellular cytoskeleton. The generation of ROS represents an early and sensitive cellular response to PM\textsuperscript{41}. The ROS generation occurs through the intrinsic properties of particles, such as transition metals which induce Fenton reactions or from surface charge\textsuperscript{2}. In this study, we found that, there was a dose-dependent increase in the level of ROS in CFA-NPs treated cell lines. The level of ROS was raised about 1.2 folds in all tested cell lines. The liver cell lines generated more level of ROS compared to other cell lines. The generation of ROS and induction of oxidative stress may be due to the particle surface and the metals associated to the surface\textsuperscript{8}. The increase in ROS level was due to decrease in intracellular GSH. ROS generation, such as hydroxyl radicals in cultured epithelial cells showed the release of pro-inflammatory cytokines such as IL-8 and IL-6\textsuperscript{42,43}. MAF98 fly ash treatment on rat lung epithelial cells showed increase in generation of ROS in dose-dependent manner; caused by water insoluble compounds present in the fly ash\textsuperscript{8}. The intracellular generation of ROS in PBMN cells treated with Fe (III) associated CFA-NPs showed a dose dependent increase in level of ROS\textsuperscript{32}.

Evaluation of apoptosis was carried out by determining the DNA laddering by DNA fragmentation assay, which is an indicative for last stage of apoptosis. The activation of Caspase-activated DNase cause the DNA damage and leads to apoptosis. In this study, CFA-NPs damaged the nucleus in the Lung and liver cell lines whereas necrotic based DNA damage was observed in the skin cells. The liver cells are found to be more sensitive and showed long DNA laddering compared to other cell lines. Studies showed that the PM induced the dose-dependent DNA damage and apoptosis in Alveolar epithelial cells\textsuperscript{44}. The PM [benzo[a]pyrene adsorbed on carbon black (CB+BaP)] treatment on cultured macrophages cells showed a time dependent expression and release of TNF-α and also induced cell death by DNA damage\textsuperscript{45}.

In conclusion, the CFA-NPs were characterized using appropriate techniques. The average size of the CFA-NPs lies below the range of PM 0.1. The elemental analysis showed there is considerable amount of toxic heavy metals present in CFA-NPs. The magnitude of cell response depends on particle size, composition and the time taken for observation. The CFA-NPs with surface adsorbed toxic heavy metals can act as cellular and DNA toxicant, capable of inducing inflammation, oxidative stress, DNA damage and cell death, which acts as an indicating event in triggering process for mutagenesis and carcinogenesis.

References