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Abstract:

Cadmium (Cd) is one of the well-known highly toxic environmental and industrial pollutants. Cd first accumulates in the nucleus, and later interacts with zinc finger proteins of anti-apoptotic genes; inhibit the binding of transcriptional factors and transcription. However, the role of Cd in oxidative stress and apoptosis is less understood. Hence the present study was undertaken to unveil the mechanism of action. A549 cells were treated with or without Cd and cell viability was measured by MTT assay. Treatment of cells with Cd show reduced viability in a dose dependent manner with IC_{50} of 45µM concentration. Cd significantly induces the reactive oxygen species (ROS), Lipid peroxidation followed by membrane damage with the leakage of LDH. Cells with continuous exposure of Cd deplete the antioxidant super oxide dismutase (SOD) and glutathione peroxidase (GSH-Px) enzymes. Further, analysis of the expression of genes involved in apoptosis show that both the extrinsic and intrinsic apoptotic pathway were involved. Death receptor marker TNF-α, executor Caspase-8 and pro-apoptotic gene (Bax) were induced, while anti-apoptotic gene (Bcl-2) was decreased in Cd treated cells. FACS analysis further confirms the induction of apoptosis in Cd treated A549 cells.

Key words: Cadmium; ROS; SOD; Apoptosis; FACS; RT-PCR.

Introduction

Cadmium (Cd) is a soft bluish-white heavy metal that exhibit extremely toxic effect in industrial workplaces. It is absorbed or inhaled but poorly excreted and hence accumulated in the human system with a long half-life. Although, the International Agency for Research on Cancer (IARC) classified Cd as a carcinogen with category 1 , it is not directly genotoxic, non-mutagenic in bacteria, but show weak mutagenic property towards mammalian cells (Dally and Hartwig, 1997). However, Cd is known to induce inflammation and death of organelles and finally cell death. Metals mainly accumulate in liver and kidney and present as a complex bound to metal-binding protein metallothionein (MT), Metal-MT complex is a temporary detoxifying mechanism of the metal shown by humans and mammals (Waalkes, 2000). However, acute exposure of humans to Cd fumes may cause flu like symptoms including chills, fever and muscle ache. Occupational exposure to humans with Cd takes place in industrial factories such as zinc (Zn) smelters, battery manufacturing units, metal recovering factories, Cd refining companies, paint and pigment production units. Exposure may also occur via other anthropogenic factors like waste incineration and fossil fuel combustion.

Cd is also found to be a major component of tobacco due to the hyper accumulating characteristics of *Nicotiana tabacum* plants. Hence, Cd accumulation occur in tobacco leaves and concentration range between 1 and 2 μg/g dry weight and that may result in the presence of Cd up to 1 μg per cigarette. Furthermore, the Cd oxide generated during smoking along with the metal either deposited in lung tissues or absorbed into the systemic blood circulation (Satarug and Moore, 2004). Thus the smokers will have 4–5 times higher Cd and its oxide levels in lungs as well as in blood and 2–3 times greater amount of metal in the kidneys than non-smokers. High Cd concentrations lead to accumulation in all tissues impairing normal functions of tissues and organs.

Cd enhances oxidative burst in cells and affects cellular DNA (Pathak and Khandelwal, 2006b). Thus Cd induces the production of cellular reactive oxygen species (ROS), particularly singlet oxygen, hydroxyl radicals, superoxide radical ($^{\cdot}O_2$) and hydrogen peroxide (Lopez *et al.*, 2006). Tissue increased levels of malondialdehyde (MDA) and the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are the proven indicators of oxidative stress (Pathak and Khandelwal, 2006b, Pathak and Khandelwal, 2006a). However, the induced production of ROS is indirect, as Cd is not a redox-active metal and cannot itself direct the Fenton type of reactions. Further, it does not bind to DNA and does not stably interact with DNA. Hence Cd is not directly genotoxic: (Dally and Hartwig, 1997). However, the induction of ROS by Cd lead to oxidative stress within cells by reacting with macromolecules and cause damages, such as mutations in DNA, destruction of protein structure and function. Increased $^{\cdot}O_2$, and peroxidation of lipids as well as alteration in gene expression may lead to apoptosis (Valko *et al.*, 2006). To neutralize the $^{\cdot}O_2$ and free radicals the cell may induce the change in the antioxidant status by enhanced production of non-enzymatic antioxidants (Stohs and Bagchi, 1995). Thus, the continuous exposure of cells/tissues to heavy metals lead to constant production of free radicals and that may deplete the enzymatic and non-enzymatic anti-oxidants and results in diseased status of the tissues or organs.

Apoptosis is a programmed cell death associated with cell shrinkage, plasma membrane blebbing, chromatin condensation, DNA fragmentation and formation of apoptotic bodies that can be taken up and degraded by neighboring cells without triggering any inflammatory reaction (Robertson and Orrenius, 2000). Apoptosis occur either by extrinsic or intrinsic (mitochondrial-dependent) pathways (Putcha *et al.*, 2002). In extrinsic apoptosis, external signals or ligands interact with a receptor present in plasma membrane, initiating a cascade of events that lead to apoptosis. The death receptor family includes tumor necrosis factor

receptor (TNFR), Fas, death receptors, and decoy receptors. These receptors contain an extracellular cysteine-rich ligand binding domain and an intracellular death domain. Once a ligand binds to the receptor, an interaction occurs with death domain adaptor proteins, such as FADD, RIP, DAX, and TRADD. These proteins activate caspases and other signaling pathways, such as MAP kinase and NF-κB. Caspases, which are aspartate-specific cysteine proteases, are cytoplasmic proenzymes that play an important role in initiation and effector phases of apoptosis (Nicholson and Thornberry, 1997). Based on their substrate specificity and function, caspases can be organized into (i) initiator caspases such as caspases-8, -9, and -10, which initiate and amplify a death signal and (ii) effector caspases such as caspases-2, -3, -6, and -7, which degrade a variety of vital cellular components (Cryns and Yuan, 1998). Following accumulation of Cd in the nucleus, the metal interacts with zinc finger proteins or domain of anti-apoptotic genes and inhibits the binding of transcriptional factors and blocks the transcription.

In intrinsic pathway the signal involve the binding of ligand TNF-α or Fas to their receptors lead to the activation of the protease caspase-8 which either directly cleaves and activates the effector caspases, or indirectly activate the down-stream caspases through the cleavage of BH3- the only protein Bid (Luo *et al.*, 1998). However, during the induction of intrinsic pathway there will be decreased expression of anti-apoptotic signals such as Bcl-2 and translocation of pro-apoptotic signals such as Bax and Bak to mitochondria. These events lead to the release of cytochrome c and other apoptosis-inducing factors from mitochondria into cytosol that trigger the subsequent activation of procaspase-9 and downstream apoptotic effectors (Crompton, 2000). Earlier studies report that the toxic effect of Cd involves the non-genotoxic or indirectly genotoxic events and is a poor mutagen (Waalkes, 1996). Cadmium *in vivo* shown to induce apoptosis in testis, Various organs in rat (Xu *et al.*, 1999) and also in *in vitro* several mammalian cells (Fujimaki *et al.*, 2000) including chicken lymphocytes (Liu *et*

al., 2014b). In alveolar cells of rat lungs Cd show moderate increase in ROS with increased expression of p53 and Bax but not protected by anti-oxidants treatment (Lag *et al.*, 2002) while in the promonocytic U937 cells antioxidants prevented cadmium induced apoptosis (Galan *et al.*, 2001). Glutathione / catalase inhibited Cd induced apoptosis in rat c6 glioma cells (Watjen and Beyersmann, 2004). Selenium decreased Cd induced brain damage in the chickens (Liu *et al.*, 2014a). Further in human myeliodal cells catalase decreased the apoptosis induced by Cd (Sancho *et al.*, 2003). Depletion of catalase suppressed Cd induced apoptosis in lung tumor cell line (Chuang *et al.*, 2003, Waisberg *et al.*, 2003). These controversial findings promoted us to study the role of Cd metal in induction of oxidative stress and apoptosis using lung A549 cells as an *in vitro* model system. However, the potential mechanism of Cd in oxidative stress and apoptosis in lung is poorly understood. To study the toxic effect of Cd metal by ROS, the levels antioxidant enzymes membrane damage by LPO and its effect on the expression of apoptotic genes was also analyzed. Further, FACS analysis was carried out to confirm the induction of apoptosis by the Cd metal.

Materials and Methods:

Materials:

Human lung epithelial cells (A549 cells) were purchased from NCCS (Pune, India); TRIzol, lithium lactate, iodonitrotetrazolium chloride (INT), phenazine methosulphate (PMS), malondialdehyde (MDA), glutathione reductase were purchased from Sigma-Aldrich (St.Louis, USA). Fetal bovine serum (FBS), penicillin, streptomycin, glutamine, RPMI 1640 medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and trypan blue, fluorescein diacetate , sodium dodecyl sulphate, acetic acid, sodium carbonate, epinephrine, NADH, sodium Azide, NADPH, sodium acetate,

Phosphate buffer saline (PBS) were purchased from Himedia (Mumbai, India). Cadmium was used as Cadmium chloride and was procured from Sisco research laboratory (Mumbai, India), Annexin V-FITC apoptosis detection kit I (Thermo Fisher, Oregon, USA), Oligo dTs and superscript reverse transcriptase were obtained from Invitrogen Bio Services India Pvt. Ltd (Bengaluru, India); Taq DNA polymerase (1 U/µl) was purchased from Merck-Millipore (Mumbai, India). Oligos forward and reverse primers (Table 1) were designed and purchased from Sigma-Aldrich (St. Louis, USA).

Culturing of A549 Cells:

A549 cells were cultured with or without fetal bovine serum (10 %) in basal RPMI 1640 medium. Growth and maintenance of cells under *in vitro* conditions with respect to temperature, oxygen and carbon dioxide concentration, pH, osmolarity and nutrients were standardized and maintained in the medium (RPMI-1640) supplemented with 10 % fetal calf serum, penicillin (100 u/ml) and streptomycin (100 u/ml) under the atmosphere of 5 % CO_2, 95 % air in a humidified carbon dioxide incubator at 37 $^{\circ}$C (Patil *et al.*, 2015).

Cell viability (MTT) assay:

Cell viability, MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out as per the protocol described earlier with slight modifications (Babu *et al.*, 2013). The MTT assay involves the reduction of the soluble yellow dye (MTT) to an insoluble purple formazan salt. Cells were cultured in a sterile 96 wells plate (5 x 10^3 cells/well) in 100 µl media and incubated overnight for attachment. The cells were treated with or without different concentrations of Cd (10 µM - 100µM) for 48 h, and at the end of reaction 50 µl of MTT dye (5 mg/ml) was added to each well and incubated further for 4 h at 37 °C in a CO2 incubator. The formazan products formed in cells were dissolved in DMSO

(100 µl) and absorbance was measured at 540 nm using multimode plate reader (Perkin Elmer).

Reactive oxygen species assay:

ROS produced in cells were measured by a real time assay as per the protocol described earlier (Periyakaruppan *et al.*, 2007). A549 cells ($3X10^3$ cells/ well) were cultured in a black colored 96 wells plate, and incubated overnight for attachment. Cells were washed with phosphate buffered saline (PBS) and treated with 10 µM DCF for 3 h in 1N NaOH and incubated further for 4 h at 37 °C in a CO_2 incubator, Further the cells were washed with PBS, incubated with or without different concentration of Cd (20 µM -100 µM) in media for different time interval. Fluorescence was read using a multimode plate reader at excitation wavelength of 485 nm and emission of 527 nm.

Lipid peroxidation assay:

Quantification of lipid peroxidation in Cd treated cells was determined by the detection of thiobarbituric acid-reactive malondialdehyde (MDA), as per the protocol described earlier with slight modifications (Wise *et al.*, 2005). Overnight cultured A549 ($4x10^5$ cells/well) cells were seeded in a 6-wells plate were treated with or without different concentrations of Cd (20 µM-100 µM) for 24 h and the cell lysate was prepared using lysis buffer. Lysate containing equal amount of protein (100 µl) was added to 800 µl of lipid peroxidation assay mix, (Assay mix: 0.4 % (w/v) thiobarbituric acid, 0.5 % (w/v) sodium dodecyl sulfate and 9.4 % (v/v) acetic acid, pH 3.5), and incubated for 1 h at 95 °C, Samples were cooled to room temperature, centrifuged at 14,000 x g for 10 min, and the absorbance was read at 532 nm using spectrophotometer.

Lactate dehydrogenase assay (LDH):

LDH in the media was quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD+ reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product which was measured at 490 nm. The formazan product formed is directly proportional to the amount of LDH released into the medium, which reflects the cytotoxic effect. Overnight cultured A549 ($4x10^5$ cells/well) cells were seeded in a 6-wells plate were treated with or without different concentration of Cd (20 µM -100 µM), and incubated further for 24 h in an incubator at 37 °C with the supply of 5 % CO_2. Cell-free supernatant (50 µl) from each well was transferred to a 96-wells plate in triplicates and 150 µl of LDH-assay reaction mixture was added. Once the color was developed the optical density was measured at a wavelength of 490 nm using a microplate reader (Ramirez *et al.*, 2014).

Superoxide dismutase assay (SOD):

Total SOD activity was determined according to the method described earlier (Misra and Fridovich, 1972). Overnight cultured A549 ($4x10^5$ cells/well) cells were seeded in a 6-wells plate were treated with or without different concentrations of Cd (20 µM-100 µM) for 24 h. Cell lysate was prepared using lysis buffer, (0.5 mM Tris Hcl, 10 mM EDTA, 0.1 % SDS and 0.5 mg/ml Proteinase K). To the cell lysate (100 µl) 880 µl carbonate buffer (0.05 M Na_2CO_3, pH 10.2, 150 mM NaCl) and 30 mM epinephrine in 0.05 % acetic acid was added and the absorbance was measured for 4 min at 480 nm in a spectrophotometer. The amount of enzyme that results in 50 % inhibition of epinephrine auto oxidation is defined as one unit of enzyme activity. Results were plotted as µM of SOD oxidized/µg of protein by Cd treated samples against control.

Glutathione peroxidase assay:

GSH-Px assay was carried out as per the protocol described earlier (Kankofer, 2002). Overnight cultured A549 (4 x 10^5 cells/well) cells were seeded in a 6-wells plate and were treated with or without different concentrations of Cd (20 µM-100 µM) and incubated for 24 h in an incubator at 37 °C with the supply of 5 % CO_2. Cell lysate was prepared using lysis buffer (0.5 mM Tris Hcl, 150 mM NaCl, 0.1% SDS and 0.5 mg/ml Proteinase K) and to 100 µl of cell lysate 2.8 ml of glutathione reaction mixture was added . GSH-Px reaction mixture contained 100 µl of NADPH solution (8.4 mM), 10 µl glutathione reductase (GSSG-R, 100 U/mg protein/ml), 10 µl sodium azide (1.125 M), 100 µl glutathione (0.02 M, GSH) made up with phosphate buffer (0.05 mol/l, pH 7.0) The enzyme reaction was initiated by the addition of 100 µl of 0.022 M H_2O_2 to the GSH-Px reaction mixture. Rate of GSSG formation was measured by decrease in the absorbance at 340 nm, as NADPH was oxidized to NADP+, using spectrophotometer.

RNA isolation and semi-quantitative RT-PCR analysis:

A549 cells (3×10^5 cells/well) in a 6-wells plate were treated with or without Cd (35 and 45 µM were used based on IC_{50} values taken from cell viability assay) and incubated for 48 h in an incubator with the supply of CO_2.Total RNA was isolated from A549 cells using TRI-Reagent and as per the protocol described earlier (Sharma and Richards, 2000) and manufacturer's protocol. In brief, the cells were washed with ice cold PBS and lysed with 1 ml of TRI Reagent. Cells were scraped and then passed repeatedly through a pipette tip to completely lyse the cells. Lysate was then transferred to an Eppendorf tube and allowed to stand at room temperature for 10 min. To this 100 µl of chloroform was added and mixed vigorously and further allowed to stand at room temperature for 15 min, centrifuged at 12,000 rpm for 10 minutes at 4 °C. Aqueous upper layer was transferred to a new Eppendorf

tube and the RNA was precipitated by adding equal volume of isopropyl alcohol and centrifuged at 12,000 rpm for 12 min. RNA pellet was washed with 70 % ethanol and RNA was re-precipitated with 0.3 M sodium acetate (pH 5.2) and 2.5 volume of chilled ethanol, centrifuged at 10,000 rpm for 12 min. The pellet was dissolved in 100 µl RNase free DEPC treated water. RNA concentration was determined by measuring the absorbance at 260 nm and purity was assessed by measuring the absorbance at 260 nm/280 nm. The quality of the RNA was assessed on 1 % MOPS-formaldehyde gel.

Reverse transcription of RNA and PCR analysis of apoptotic genes were carried out as per the protocol described earlier (Sharma *et al.*, 1999). In brief total RNA (2 µg) was reverse transcribed using Superscript III First strand synthesis Invitrogen kit. The cDNA was subjected to 30 cycles of PCR using specific forward and reverse primers of genes applying appropriate annealing temperatures as indicated in (Table 1) in a gradient Eppendorf thermo cycler. Amplified PCR products were analyzed on 1 % agarose gel using 1X TAE buffer. Expression of β-actin mRNA was used as a positive control and for normalization (Patil *et al.*, 2016).

Apoptosis Detection by Annexin V-FITC and Propidium Iodide Staining:

Annexin V-FITC apoptosis detection kit I (Thermo Fisher, Oregon, USA.) was used to sort live cells and apoptotic cells by flow cytometry as per the protocol described earlier (Allen *et al.*, 1997). Briefly, A549 cells (1×10^6 cells/well) in a 6-wells plate were treated with or without different concentrations of Cd (35 µM and 45 µM) for 24 h. After incubation, both floating and attached cells were harvested by trypsinization. Later, cells were washed with PBS and were suspended in Annexin-V reaction mixture for 15-20 mins in dark and further analyzed using a Becton Dickinson FACS Aria III flow Cytometry (Scientific Instruments Centre, Indian Institute of Science, Bengaluru).

Statistical analysis:

Experimental data shown as mean ± standard deviation from three independent experiments. Statistical analysis was done by Student's t test and one-way ANOVA followed by post hoc tukey test. Difference between control and Cd treated cell samples were considered significant if the level was *P < 0.05, **P < 0.005.

Results:

Cd inhibited the viability of A549 cells:

A549 cells were treated with or without different concentrations of Cd (10 μM -100 μM) for 48 h and the cell viability was determined by MTT assay. Results show that there was a dose dependent decrease in the cell viability by 48 h and as low as 10 μM Cd reduces the cell viability by more than 20 % and maximum of more than 70 % decreased in cell viability was observed at 100 μM with IC_{50} value of 45 μM of Cd metal (Fig. 1).

Cd induces ROS in A549 cells:

A549 cells were treated with or without different concentrations of Cd (20 μM - 100 μM) and incubated for different time intervals (0 - 150 mins). Total ROS produced in cells were measured by real time assay. Results show that the ROS was induced by Cd in a dose dependent manner (Fig. 2a). The Cd metal also induced ROS as a function of time (Fig. 2b). Cd as low as 20 μM concentration significantly induced ROS by 15 % and maximum of 60 % increase was observed at 100 μM concentration of the metal. The increase in ROS was found to be significantly increased by as early as 30 min and increased with time at different concentrations until 120 min. Maximum of ROS was produced at 100 μM concentration of Cd and by 120 min incubation. However, at later time interval at 150 mins the ROS produced

declined compared to 120 min and at 80 μM or 100 μM of Cd (Fig. 2). The decline in ROS at 150 min may be due to the depletion of antioxidants present in cells.

Cd induces Lipid peroxidation in A549 cells:

A549 cells treated with or without Cd (20 μM -100 μM) for 24 h show increased levels that reflects the measure of LPO. LPO was found to be significantly increased in a dose dependent manner with different concentrations of Cd compared to control. Maximum lipid peroxidation of more than three folds was observed with the cells treated with 100 μM concentration of Cd (Fig. 3). Results suggested that the elevated levels of enzyme lipid peroxidase may cause lipid peroxidation and membrane damage leading to cell death by Cd treatment. A549 cell death by treatment of Cd was also reflected with the decreased cell viability as measured by MTT assay.

Cd induces LDH activity in the medium from lung cells:

A549 cells were treated with or without different concentrations of Cd (20 μM-100 μM) for 24 h and the concentration of LDH in the medium were measured. Increased levels of the LDH in the medium reflect the membrane damage. The results show that the treatment of cells with Cd for 24 h caused membrane damage with the increased levels of LDH in the medium in a dose dependent manner. Maximum of more than 2 fold increase in the LDH was observed at 100 μM concentration of Cd (Fig. 4), suggested that the metal induces lipid peroxidation, membrane damage and LDH leakage pronouncing the toxic effect of the metal.

Cd treatment depletes the Glutathione peroxidase (GSH-Px) enzyme activity in A549 cells:

A549 cells were treated with or without different concentrations of Cd (20μM- 100μM) for 24 h and cell lysates were analyzed for GSH-Px activity. GSH-Px enzyme activity was

significantly decreased in the cells treated with Cd (Fig. 5) in a dose dependent manner. Cells treatment with as low as 20 μM concentration of Cd decreases the GHS-Px enzyme activity by 11 % and maximum of 35 % decrease in the enzyme activity was observed at 100 μM concentration of the metal ion (Fig. 5) The decrease in GSH-Px activity may be one of the major factor responsible for the oxidative stress due to Cd treatment.

Cd depletes SOD in A549 cells:

A549 cells were treated with or without different concentrations of Cd (20 μM-100 μM) for 24 h. Cell lysates were prepared and subjected to total SOD analysis. Results show that the cells treated with Cd decrease the SOD activity in a dose dependent manner. As low as 20 μM concentration of Cd treated show more than 31% decrease in the SOD enzyme activity and almost 50 % decrease in enzyme activity was observed at 100 μM concentration of the metal (Fig. 6).

Cd inhibits Bcl-2 and induces Bax, TNF-α and caspase-8 mRNA levels in A549 cells:

A549 cells were treated with Cd (35 μM and 45μM) and the expression of pro-apoptotic gene Bax, anti-apoptotic gene Bcl-2, TNF-α and caspase-8 were analyzed by semi-quantitative RT-PCR and β-actin was used as a positive control. Results show that the Cd at 35 μM significantly increases the pro-apoptotic gene Bax by 2.7 fold and with 45 μM induced by 3.3 fold. Cd also induces the expression of TNF-α receptor by 2.4 fold and 4.7 fold with 35 μM and 45 μM respectively. Upon activation of TNF-α, the downstream caspase-8 expression was also activated and found to be increased by 2 and 3 folds following the treatment of cells with 35 μM and 45 μM concentration of metal ion respectively. However, Cd at 35 μM and 45μM concentration treated decreased the expression of anti-apoptotic Bcl-2 by 9% and 14% respectively. Results suggested that the Cd metal induces apoptosis of A549 cells (Fig. 7).

Cd induces apoptosis in A549 cells:

A549 cells were cultured overnight and then incubated with and without different concentrations of Cd (35μM and 45μM) for 24 h. Cells were subjected to Annexin V-FITC assay and FACS analysis to assess apoptosis. Results show that the treatment with Cd (35 μM and 45μM) for 24 h induces apoptosis in A549 cells. Control cells show little or no apoptosis (Fig. 8a), while treatment with Cd (35 μM) concentration induces apoptosis by more than 40% and dead cells accounts for 4 % (Fig. 8b), cells treated with Cd (45 μM) induces apoptosis and dead cells increase with concentration reached more than 57 % (Fig. 8c) and Fig. 8d shows the bar graph representation of FACS analysis of control and treated A549 cells.

Discussion:

The generation of ROS occurs naturally in all most all the cell types, when NADH+ is partially oxidized in electron transport chain (ETC). Heavy metals (Pb, Hg, Cr etc) particularly iron are capable of inducing oxidative stress both under *in vivo* and *in vitro* conditions Dick et al. (2003). Free redox-active metals directly enhance the production of OH^- (hydroxyl) radicals and H_2O_2 through the Fenton reaction (Angshuman, 2013). Studies also show that, Cd induces oxidative stress in cells and tissues of both animals and plants (Thevenod, 2009). However, Cd is unable to catalyze the redox reactions directly in biological systems, but probably increase the concentration of free redox-active metals such as Fe (II) and Cu (II) by replacement with various metalloproteins or by changes in mitochondrial membrane potential or by inhibiting the flow of electrons from reduced ubiquinone to cytochrome c in ETC (Seifried *et al.*, 2007).

The susceptibility of the lung to Cd toxicity occurs through inhalation route which is often encountered during employee's occupational exposure. However, very few studies have been reported on the mechanism of toxicity of Cd in lung. This may be due to the non availability of animal model system, although limited toxic studies were carried out using rodent models. Further the detailed toxic mechanism is not worked out because of limited studies in humans. Hence, the present study was undertaken to use human lung epithelial A549 cells as a model system to understand the toxicity and mechanism of action of Cd. Results of our study show that the Cd decreases the cell viability of A549 cells in a dose dependent manner suggested the toxic effect of the metal ion. Similar induction of oxidative stress with decreased viability of lung cells and toxicity was also observed with uranium metal (Periyakaruppan *et al.*, 2007). Cd appears to be more effective which requires only 0.1mM as against 1mM concentration of uranium for maximum decrease of (80 %) viability in cells. Results of our study also show that Cd induces oxidative stress in a dose dependent manner which also correlates well with the increase in time. The addition of Cd in the early induction of ROS in organs/tissues like liver, kidney, lung etc., with increase in ROS leading to cell death was reviewed extensively (Nair *et al.*, 2013) and therefore in the present study Cd followed a similar outcome leading to reduced viability of A549 cells. Increased ROS also induce LPO in A549 cells that may lead to cell membrane damage followed by cell death with reduced viability. Results of our study also show that the leakage of LDH confirms membrane damage by Cd metal. Further, Cd toxicity found to be associated with the activation of apoptotic pathways due to oxidative stress (Yao *et al.*, 2006). Continuous exposure of cells to Cd results in the induction of oxidative stress and depletion of antioxidant defense system. GSH-Px is one of the major antioxidant enzymes present in the cells to counteract oxidative assaults and cellular redox potential is largely determined by glutathione (GSH), which accounts for more than 90% of cellular non-protein thiols (Meister, 1994). Therefore,

decrease in the activity of the enzyme GSH-Px might be a major factor responsible to increase in the oxidative stress by Cd in lung cells. Our study show that there was a decrease in the level of GSH-Px along with the SOD enzymes to overcome the oxidative stress in Cd treated cells, which clearly indicates a correlation between loss of antioxidant enzymes and increase in the oxidative stress. Two SOD enzymes one is localized in the mitochondria and another in cytosol of cells reduce and neutralizes of ROS (oxygen free radicals). Depletion of SOD enzyme may be due to transcriptional inactivation or due to proteolysis. Similar loss of SOD enzyme activity by proteolysis was observed in the treatment of primary cultures of astrocytes with strausosporinein (Ahlemeyer *et al.*, 2001).

Our studies show that Cd induces the expression of TNF-α the death receptor in a dose dependent manner. TNF-α was chosen as a marker of cellular apoptotic activity. It was shown that both Co2+ and Cr3+ induce TNF-α secretion in a dose and time dependent manner (Catelas *et al.*, 2003). Studies reported that human neuroblastoma cells treated with CdTe QD nanoparticles induced apoptosis associated with the increased Fas expression (Choi *et al.*, 2007). Cd also found to induce the expression of caspase-8 in a dose dependent manner. Earlier studies show that Cd induces caspase-8 and Caspase-3 in many different cell lines, including myocardial, smooth muscle, neuroglial, renal proximal tubule, and lymphoma cells, and inhibition of caspase-3 prevents cadmium-induced apoptosis (Pulido and Parrish, 2003). Studies also show that the uranium induces the caspase-8 and caspase-3 activity in lung epithelial cells (Periyakaruppan *et al.*, 2009).

Thus results of our study suggested that the extrinsic pathway of apoptosis was induced by Cd metal treatment. However mitochondrial route is activated in response to various internal or external stimuli, which cause a change in mitochondrial permeability. Disruption of the mitochondrial membrane potential results in the release of pro-apoptotic factors, such as cytochrome c, from the mitochondria into the cytosol, which activate caspase 9 and then

caspase 3 (Asara *et al.*, 2013). Our study also shows that the treatment of Cd metal induces the expression of pro-apoptotic gene Bax with the decrease in the expression of anti-apoptotic gene Bcl-2 suggested that the intrinsic pathway of the apoptosis is also activated. Earlier studies CdTe-QD treatment also appears to increase Bax and decrease Bcl-2 levels, induces programmed cell death in human neuroblastoma cells (Choi *et al.*, 2007) which are in agreement with our studies suggested the induction of intrinsic apoptosis pathway (Dejean *et al.*, 2006). Further Bcl-2 found to be a key inhibitor of apoptosis and it's over expression blocks the translocation of cytochrome c from mitochondria to cytosol, thus preventing cells from undergoing apoptosis (Yang *et al.*, 1997). Our study show that cells treated with Cd decreases the expression of anti-apoptotic Bcl-2 gene, suggested the entry of Cd treated A549 cells into apoptosis pathway. Conversely, over-expression of the Bax gene and its translocation to mitochondria shown to promote the release of cytochrome c into cytosol, leading to activation of effector caspases and subsequently lead to apoptosis (Finucane *et al.*, 1999). Earlier studies show that the uranium induces the leakage of cytochrome c from mitochondria to cytosol, leading to apoptosis in lung epithelial cells. Similar mechanism of release of cytochrome c from mitochondria to cytosol may occur with Cd treated A549 cells, and that need to be analyzed.

In addition, our studies also show that Cd treated A549 cells exhibited elevated extra cellular annexin V binding, as well as an increase in uptake of PI, providing further evidence for the induction of apoptosis. Apoptosis occurs via death receptor-dependent (extrinsic) or mitochondrial (intrinsic) pathways. The extrinsic pathway is triggered by the activation of death receptors, such as Fas and TRAIL (DR4, DR5), which activate the initiator caspase-8 followed by the cleavage of the executioner caspase-3.

To conclude, A549 cells treated with Cd induces ROS and causes membrane damage with the leakage of LDH. Oxidative stress and apoptosis was induced by Cd due to depletion of

antioxidant enzymes. Thus our study provides an *in vitro* model system to analyze small antioxidant molecules such as GSH, N-acetyl cysteine, vitamin C etc. and some plant extracts, to understand the counter activity that may be useful in the treatment of toxic effect of Cd and other heavy metals.

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Declaration of Interest:

The authors report no declarations of interest.

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Table:

Table 1: Sequence of primers used for the PCR amplification

Gene	Primer sequence (5′ - 3′)	Annealing temp. (°C)	Product size (bp)	References
Bcl-2	F: AGATGTCCAGCCAGCTGCACCTGAC	62	365	(Babu *et al.*,
	R:AGATAGGCACCCAGGGTGATGCAAGCT			2013)
Bax	F: AAGCTGAGCGAGTGTCTCAAGCGC	61	366	(Babu *et al.*,
	R: TCCCGCCACAAAGATGGTCACG			2013)
Caspase-	F: GATATTGGGGAACAACTGGAC	58	366	Present Study
8	R: CATGTCATCATCCATGGAGAACACT			
TNF-α	F: CAAGCCTGTAGCCCATGTTGTAGC	58	430	(Patil *et al.*, 2016)
	R: ATCCCAAAGTAGACCTGCCCAGAC			
β-actin	F: TACCACTGGCATCGTGATGGACT	62	516	(Babu *et al.*,
	R: TCCTTCTGCATCCTGTCGGCAAT			2013)

Columns 3 and 4 *show annealing temperatures and the size of the amplified products.*
F *forward,* ***R*** *reverse*

Fig. 1

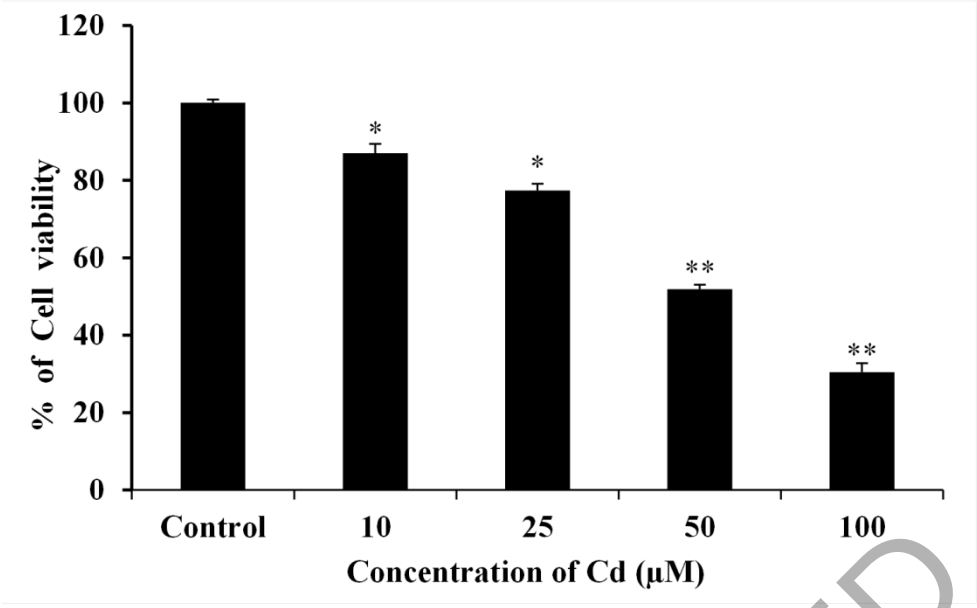


Fig. 2

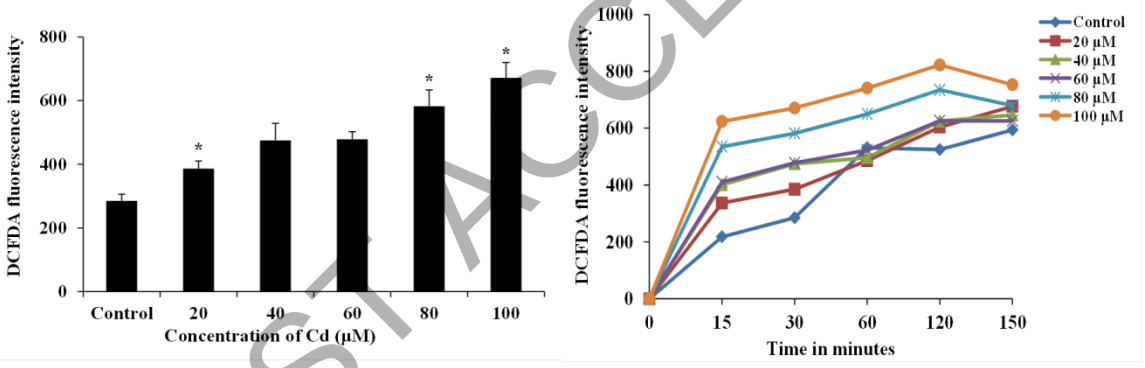
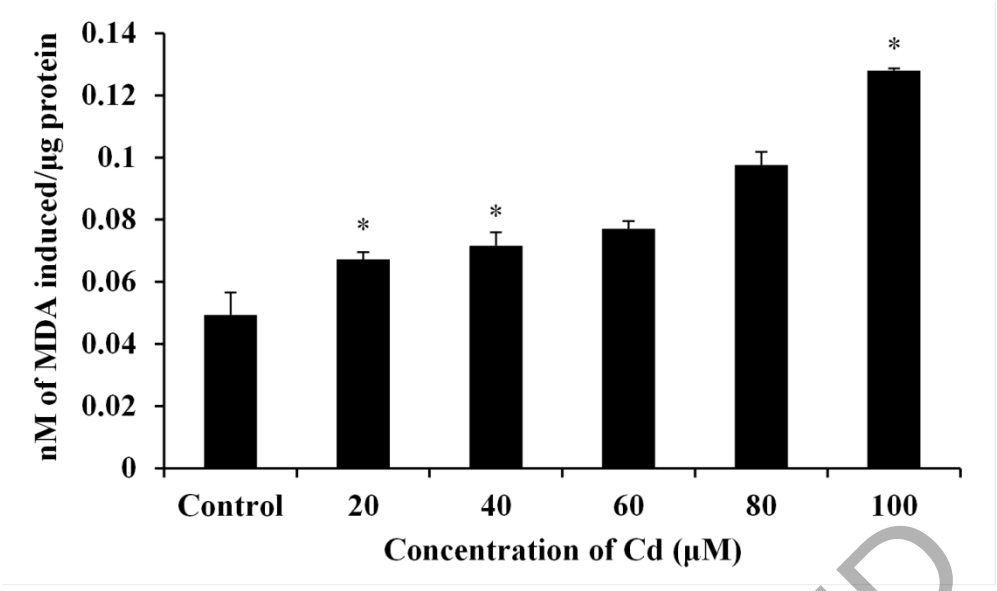


Fig. 3



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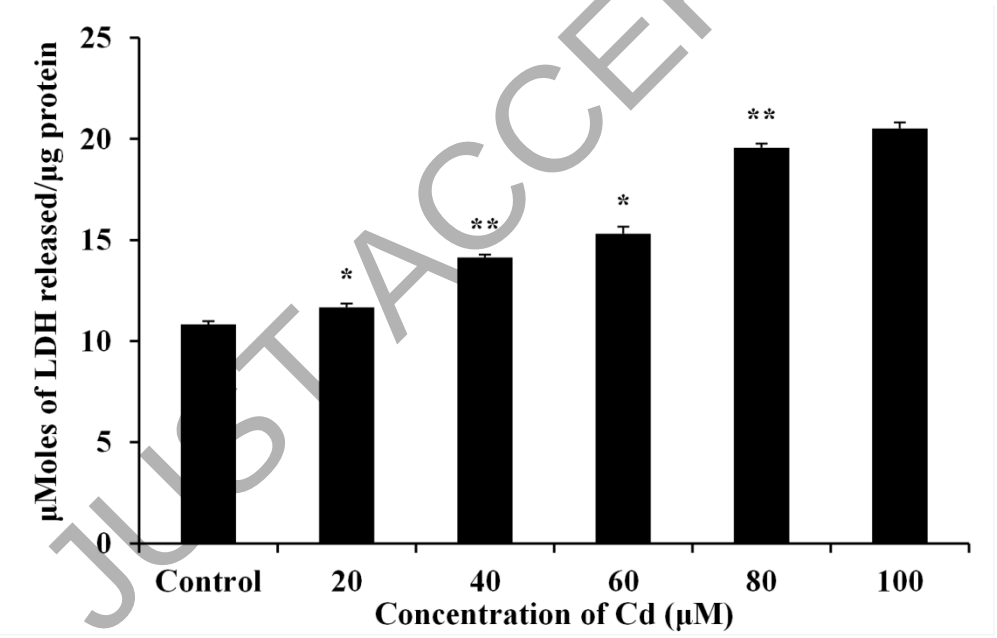

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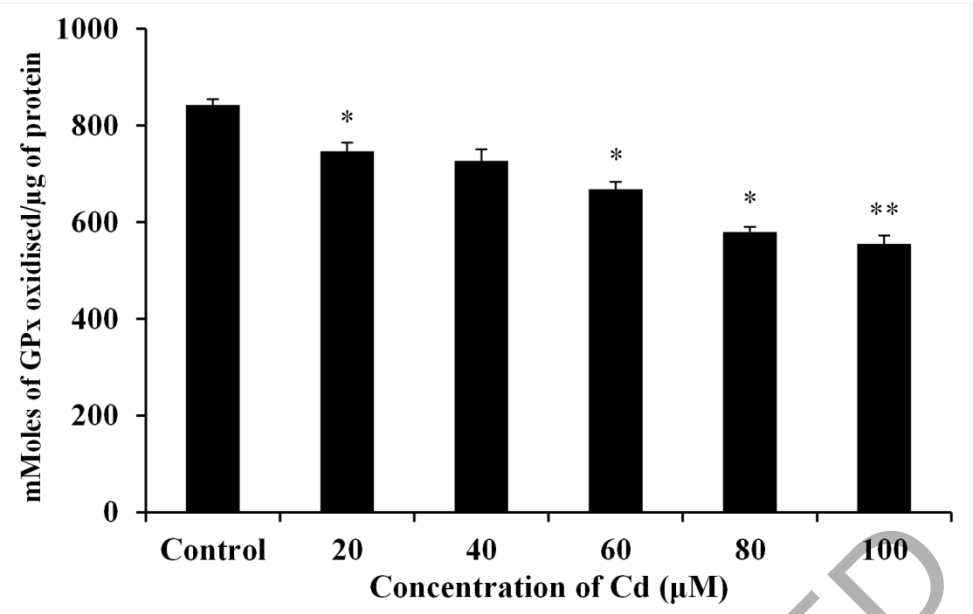

Fig. 6

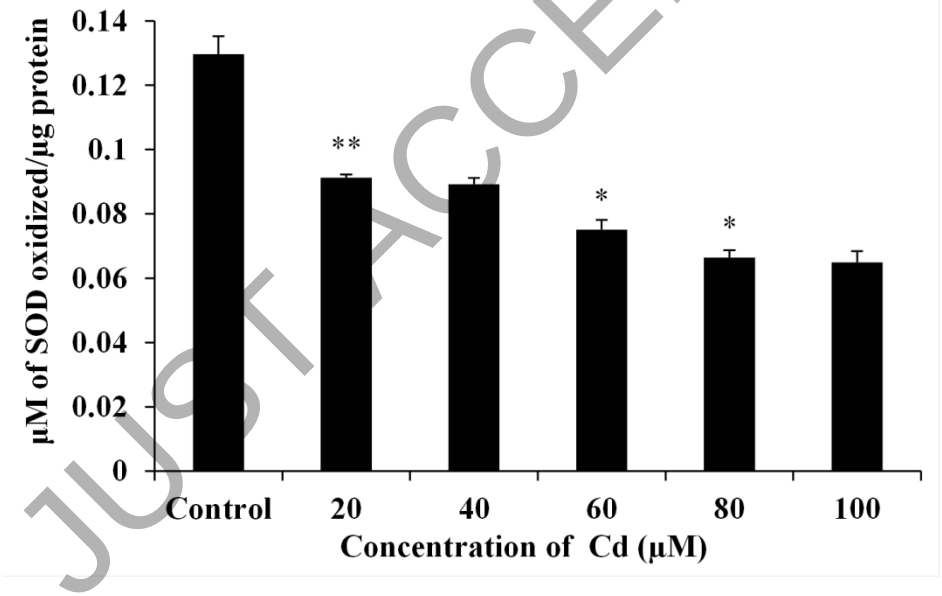

Fig. 7

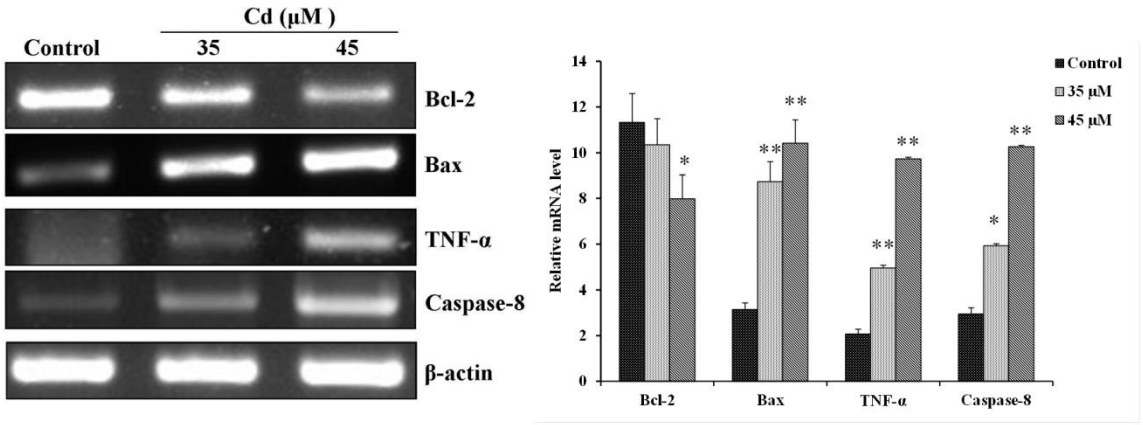

Fig. 8