Study of the Effects of Cadmium Toxicity on Regulation of Small GTPases, HSP70 and Apoptosis in Human Alveolar Cells

THESIS

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Under the Supervision of
Dr. Angshuman Sarkar

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

CERTIFICATE

This is to certify that the thesis entitled **"Study of the Effects of Cadmium Toxicity on Regulation of Small GTPases, HSP70 and Apoptosis in Human Alveolar Cells"** and submitted by **Geethanjali Ravindran,** ID No **2011PHXPF007G** for the award of Ph.D. of the Institute embodies original work done by her under my supervision.

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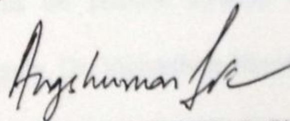

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I dedicate this thesis to my family

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ABBREVIATIONS AND SYMBOLS

BLAST	**Basic Local Alignment Search Tool**
BSA	**Bovine serum albumin**
CAS	**Chemical Abstracts Service**
Ca	**Calcium**
$CaCl_2$	**Calcium Chloride**
CBB	**Coomassie Brilliant Blue**
CDC42	**Cell division control protein 42 homolog**
$CdCl_2$	**Cadmium chloride**
Cu	**Copper**
DMEM	**Dulbecco's Modified Eagle Medium**
DMSO	**Dimethyl sulfoxide**
DMT1	**Divalent Metal ion Transporter 1**
DNA	**Deoxyribonucleic acid**
dNTPs	**Deoxy Nucleotide Tri Phosphate**
EDTA	**Ethylenediaminetetraacetic acid**
EFSA	**European Food Safety Authority**
ER	**Endoplasmic reticulum**
EtBr	**Ethidium bromide**
FBS	**Fetal bovine serum**
Fe	**Iron**
g	**Gram**
GTP	**Guanosine-5'-triphosphate**
GI	**gastro intestinal**
HCL	**Hydrochloric acid**
IARC	**International Agency for Research on Cancer**
JECFA	**Joint FAO/WHO Expert Committee on Food Additives**
kg	**kilogram**
L	**Litre**
MDM2	**Mouse double minute 2 homolog**
MT	**Metallothionein**
Mn	**Manganese**
mg	**Milligram**
MTF1	**Metal regulatory transcription factor 1**
NADP	**Nicotinamide adenine dinucleotide phosphate**
NCBI	**National Center for Biotechnology Information**
NFκB	**Nuclear factor κB**
OSHA	**Occupational Safety and Health Administration**
Pb	**Lead**

PBS	**Phosphate-buffered saline**
PMSF	**phenylmethylsulfonyl fluoride**
ppm	**parts per million**
PTMI	**Provisional tolerable monthly intake**
PTWI	**Provisional tolerable weekly intake**
RPM	**Rotations Per Minute**
RT-PCR	**Reverse Transcriptase PCR**
SDS-PAGE	**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis**
TAE	**Tris Acetate EDTA**
TBS	**Tris Buffered Saline**
TMB	**3,3',5,5'-Tetramethylbenzidine**
TNFα	**Tumor necrosis factor Alpha**
USEPA	**The United States Environmental Protection Agency**
WHO	**world health organisation**
ZIP8	**zinc transporter 8**
Zn	**Zinc**
µg	**Microgram**
µM	**Micro Molar**

ABSTRACT

Cadmium is an age old chemical element whose elemental as well as compound forms find many applications in day to day human life. They are used in pigments, paints, semiconductors, batteries, corrosion resistant coatings, alloys, and even in nuclear reactors, to name a few. But along with these advantages comes a disadvantage, in the form of its toxicity. Cadmium and its compounds are known to be quite detrimental to living beings. Almost every organ in our body is affected by cadmium. Even though the occurrence of cadmium in the earth's crust is only about 0.15 ppm, we are exposed to this heavy metal through air, water and food. Cadmium enters our respiratory system through polluted air, our digestive system through polluted water and edible consumables (plants can absorb cadmium from the soil via their roots) and finds its way to various tissues and organs through the circulatory system. Its half life in living systems is estimated to be about 20-30 years. This aids the bio magnification of this heavy metal in the human body.

There is a significant amount of cadmium in cigarettes. Average amount of cadmium per cigarette is in between 0.5 to 1.5µg. Pulmonary absorption of cadmium is higher (about 16% of inhaled cadmium) compared to its gastrointestinal absorption (about 5% of ingested cadmium).

Even though the biological effects of cadmium are well studied, the cellular and molecular mechanisms underlying cadmium toxicity are still under development. This study aims to look into various molecular mechanisms involved during cadmium toxicity in human lung adenocarcinoma cell line- A549, since lung is the first organ to be affected by cadmium through cigarette smoke, polluted air as well as dust particles.

The first chapter deals with the effect of cadmium (given in the form of cadmium chloride, $CdCl_2$) on the viability and morphology of A549 cells and its subsequent effect on the expression of genes involved in the regulation of cell morphology. It is known that cadmium interferes with the dynamics of actin filaments – the main component of cytoskeletal structures called microfilaments. The viability of A549 cells exposed to various concentrations of cadmium chloride was measured by total viable cell count after vital staining and also by resazurin cell viability assay. Expression of the genes involved in the induction of various actin- filled cellular processes like filopodia, lamellipodia and stress fibres, that is *cdc42, rac1* and *rho*A was studied in cadmium exposed A549 cells. Following this, the expression of these cellular processes was examined by fluorescent studies involving Phalloidin conjugated FITC and are compared with expression of *cdc42*, *rhoA* and *rac1*. The stress induced expression of HSP70 in cadmium treated A549 cells was also studied- both, at mRNA as well as protein level.

Second chapter undertakes the study of the interaction between vitamin B_{12} analog- methylcobalamin (MeB_{12}) and $CdCl_2$, and its subsequent effect on the A549 cells. Methylcobalamin is a reduced form of vitamin $B_{12.}$ It is also a known antioxidant, used in the treatment of various neurological disorders and cellular toxicities. The aim of this study was to find out whether MeB_{12} helps in the reduction of cadmium induced stress in A549 cells. A549 cells were grown in media containing two different concentrations of MeB_{12} (50 & 100 µg/ml) for 48 hours before exposing them to different concentrations of cadmium chloride (0-10µg/ml) and were compared with the A549 cells that were similarly exposed to cadmium, but grown in regular media. Microscopic analysis, viability assays and nuclear damage studies revealed that MeB_{12} had a synergistic effect on cell death along with cadmium which has not been reported earlier.

Studies on the mRNA expression of genes involved in the signalling pathways of inflammatory response and programmed cell death showed a huge variation between the cellular signalling in A549 cells grown in MeB_{12} containing media and those grown in normal media following the exposure to cadmium.

Third chapter involves a comparative study of the effect of cadmium on various cell lines including A549, HEK293 and HCT116. In this chapter, the difference in the degree of cytotoxicity induced by the heavy metal cadmium on three transformed cell lines with wild type P53; A549 (human lung adenocarcinoma), HEK293 (human embryonic kidney cells) and HCT116 (human colon cancer cells) was examined and compared it with the response of a *P53* knock (-/-) out line of HCT116 (HCT116*P53*-/-), to see whether these cellular responses followed some pattern in the molecular level.

In all the cell lines viability decreased with increase in cadmium concentration, but with varying degree. *p*53 mRNA was gradually down regulated in all the three cell lines with increasing cadmium dosage, but with different extents. The *p*53 knock out cell line (HCT116p53-/-) was the most sensitive towards cadmium. HEK 293 cell line showed similar level of cadmium sensitivity as (HCT116p53-/-) and had the highest degree of *p53* mRNA down regulation in response to cadmium. On the other hand A549 and HCT116 cell lines showed better tolerance towards cadmium. They also had the lowest degree of *p53* mRNA down regulation. This suggested a correlation between P53 expression and cadmium sensitivity. Further study into P53 protein expression and level of P53 phosphorylation showed that A549 and HCT116 resisted P53 degradation by phosphorylation while degree of P53 phosphorylation was very low in HEK293 cells, in the presence of cadmium.

Chapter 1:

INTRODUCTION

AND REVIEW OF LITERATURE

It always seems impossible until it's done

-Nelson Mandela

1.1 HEAVY METAL TOXICITY

"Heavy metal" is a collective term for the larger metallic elements that are toxic to living organisms and the environment (Tchounwou et al, 2012). Included in this category are naturally occurring elements with relatively higher atomic weight and density. They find various applications in our daily life, which result in their extensive dispersal in the environment. Their accumulation in the environment as well as in biological system and their negative effect on both, raise concerns. Metals are inevitable part of day to day human life. From the time cave men discovered copper to the most modern present, metals have found applications in every walk of our life. In spite of their importance in human life our biological system did not agree with all of them. When some of them are highly essential for our biological existence others are harmful to our body. For example iron in the form of Fe^{2+} is essential for our body while lead (Pb) even at lower concentrations is detrimental.

Heavy metals that are classified as extreme toxicants include cadmium, chromium, lead, arsenic and mercury. Various factors affect the degree of toxicity of a heavy metal, including the dosage or concentration, duration, exposure route, the chemical form of the metal, the age, diet and gender of the affected people and even their genetic makeup (Tchounwou et al, 2012). These metallic elements have no constructive purpose in the human body and once exposed they affect the whole body causing multiple organ damage. The United States Environmental Protection Agency, (USEPA) and the international agency for research on cancer, IARC have classified these metals as potential carcinogens (Tchounwou et al, 2012).

Cadmium is an age old systemic toxicant which can adversely affect almost all organs in our body, when exposed. Unlike the heavy metals, lead, arsenic, mercury and chromium, cadmium is more frequent in our life. We do not encounter the other heavy

metals as much as we do cadmium, which is present in our food, drinking water and the air we breathe. Acute cadmium exposure can be detected and treated quickly, detection of chronic exposure due to consumption of cadmium contaminated products for a long duration of time is possible only when visible symptoms break out and by then it will be too late. Studying the short term as well as long term effects of this toxicant in human body, down to cellular and molecular level has been interesting and work on this will give an insight into how this metal manifests its toxicity and how our cells respond to it. This in turn may help in developing our knowledge on how it can be detected and treated safely.

1.2 HISTORY OF CADMIUM

Cadmium, Cd, (CAS registry number 7440-43-9) is the 48th element in the periodic table. It is a transition metal which belongs to group 12. It is a natural element which exists in a divalent state. Two German chemists, Fredrick Stromeyer and Karl Samuel Leberecht Hermann independently discovered cadmium in 1817, as an impurity in the ore of zinc (Cobb, 2012). It is an element of poor abundance. The name cadmium is derived from "cadmia" latin name for calamine (zinc carbonate). Soon after its discovery, owing to the numerous desirable properties of the metal and its compounds, they found various applications in our day to day life. Cadmium compounds are used as pigments and paints. Cadmium is applied as a corrosion resistant coating for other metals since it is resistant to decay. Cadmium hydroxide is used as an electrode material in Nickel-Cadmium batteries. Cadmium alloys have a lot of electrical applications. Cadmium rods are used in nuclear reactors to control the rate of fission reaction. In the late 1900s the usage of cadmium highly decreased due to the environmental and health regulations owing to this hazardous nature. China is the largest producer of cadmium in the world (Friberg, Nordberg and Vouk, 1979).

1.3. CADMIUM EXPOSURE AND ABSORPTION

The abundance of cadmium in the earth's crust is about 0.15 parts per million (ppm) (Wedepohl, 1995). It is present throughout the atmosphere, water and land. Main access routes of cadmium in the biological system are through food, air and water. Cadmium intrudes its way into our body through contaminated food, water and polluted air. Cadmium enters the food chain through plants, as plants absorb cadmium from soil through their roots (Sarkar et al, 2013). Even though metallic cadmium as a whole is insoluble in water, there are many soluble cadmium compounds contaminating water bodies, mainly due to mining activities and industrial effluents (Sarkar et al, 2013). Usage of fossil fuels, smelting, and incineration of solid waste as well as natural means like volcanic eruption, discharges cadmium into the atmosphere (Sarkar et al, 2013).

The half life of cadmium in the biological system is about 10-30 years (Sarkar et al, 2013). Oral absorption of cadmium through the gastro intestinal (GI) tract is only about 5% of the ingested cadmium while pulmonary absorption is quite high -10 to 50% (Patrick, 2003). Even though the absorption of cadmium through digestive tract is comparatively lower, deficiency of dietary proteins, calcium, zinc, iron, copper etc can cause increased gastrointestinal abortion of cadmium. For example, the infamous *Itai-itai* disease of the 1960s hit coastal areas of Japan after the water bodies got contaminated by cadmium due to decades of mining activities which continued up to World War II. Malnourished village people contracted the disease on consuming cadmium-contaminated rice, characterised by severe pain in the spine and joints (Sarkar et al, 2013; Nogawa et al, 2004)

Occupational exposure and cigarette smoking are the two main reasons for the entry of cadmium through respiratory tract. Cigarette smoke is one of the major sources of

pulmonary cadmium exposure. Burning one cigarette releases about 1-2µg of cadmium of which about 0.1 to 0.2 µg enters the blood stream via alveolar lining (Sarkar et al, 2013; Patrick, 2003). About 1to 2µg of cadmium is absorbed by an active smoker per pack of cigarettes. Smokers have comparatively higher blood cadmium level than non-smokers (Sarkar et al, 2013).

1.3.1. Permissible limits of cadmium

The concentration of cadmium in blood depends on the amount of cadmium taken in our body through food and water. Everyday dietary intake of cadmium by a person differs from country to country and ranges from 10-35 µg per person (WHO, 2011).

According to World Health Organisation's 2004 guidelines on drinking water quality, the critical limit of cadmium in drinking water is 3µg/L (WHO 2004). Occupational Safety and Health Administration (OSHA), in the United States has set an average of 0.0005 and 1.0 mg per m^3 of workplace air for 8 hours a day, 40 hours a week, depending on the cadmium compound. This is attributed to the extremely long half-life of cadmium in the living system, which is calculated to be between 10 to 30 human years, an average of about 20 years (Martelli et al, 2006).

The provisional tolerable monthly intake (PTMI) or provisional tolerable weekly intake (PTWI) is actually calculated for a toxicant that has no constructive purpose in our body and is defined as the acceptable level of toxic metal that can be ingested monthly or weekly over a lifetime without appreciable health risk (Nevárez, Leal, and Moreno, 2015). The PTWI value set for cadmium was as high as 400–500 µg/person/week in the beginning (Satarug, Garrett, Sens, and Sens, 2009) which was calculated based on a critical renal concentration of 100–200 µg cadmium/g wet kidney cortex weight, reached after taking 140–260 µg of cadmium/day for > 50 years or 2,000 mg over a

lifetime (WHO 1989). PTWI values are calculated by assuming an oral absorption of 5% (retention about 0.5 to 1.0 µg of cadmium per day from food) and a daily excretion rate of 0.005% of total cadmium taken up by the human body. Based on this model, toxico kinetic studies predict that over a period of 50 years the renal cortical cadmium level of 50 µg per g of wet kidney cortex weight could be attained with the cadmium intake of 1 µg/kg body weight/day (Satarug et al, 2003; Satarug et al, 2000).

Critical concentration of a toxic agent is defined as the dose at which the said toxicant gives rise to adverse effect in our body. It is measured mostly as the concentration of the toxicant in kidney, since it is an organ of high importance. World health organisation has established 200µg cadmium/g of renal cortex as critical renal cadmium concentration. Studies have found a correlation between cadmium eliminated in the urine and that present in the kidney and from that, it is concluded that 200µg cadmium/g of renal cortex corresponds to about 10 µg cadmium/g creatinine in urine (Friberg, 1984). Based on these calculations 7 µg cadmium/week/kg body weight or 25 µg cadmium/kg body weight per month was set by the Joint Food and Agricultural Organisation, FAO/WHO Expert Committee on Food Additives, JECFA, as the PTMI for cadmium. But in order to assure better customer safety, Panel on Contaminants in the Food Chain, EFSA (European Food Safety Authority) has kept a PTWI of 2.5 µg/kg body weight for cadmium.

Studies show that, the average cadmium intake by people around the world is lower when weighed up against the WHO's standard for tolerable cadmium intake (Joint Expert Committee on Food Additives (JECFA) of WHO set tolerable cadmium intake of 25 µg per kg body weight per month). In fact, cadmium intake levels are declining over the past 20 years. It was averaged to about 15 µg/kg bodyweight per month in the

1960s but decreased to about 5 µg/kg body weight per month, far below WHO JECFA standard established in 2010.

1.3.2. Cadmium toxicity

Cadmium toxicity can be acute or chronic (long term exposure). Acute poisoning results from exposure to a very high concentration of cadmium or its compounds and its symptoms appear faster, usually within 24 hours and include shortness of breath, general weakness, fever etc gradually resulting in pulmonary edema, pneumonia, respiratory failure and death. The term "cadmium blues" refers to the flu- like symptoms that include fever, head ache, muscle pain, leading to tracheo-bronchitis, pneumonitis and pulmonary edema is associated with acute exposure to cadmium fumes if the exposure is severe (Zaidi, Wani and Khan, 2012). Oral intake of large amount of cadmium results in sudden poisoning and damages the liver and the kidneys (Sikary et al, 2015).

Breathing air polluted with cadmium, over time, manifests in the form of respiratory and renal disorders. Cadmium is classified as a class one carcinogen by IARC. Its compounds are also carcinogenic (Liu et al, 2009)

Exposure to low doses of cadmium over a long period of time (Chronic toxicity) results in its increased accumulation in the kidney cortex (Sarkar et al, 2013; Klaassen et al, 1999; Gonick, 2008).

Cadmium which is absorbed by the epithelial lining of GI tract as well as lung alveoli enters the blood vascular system and it binds to plasma proteins like albumin or to erythrocytes itself. It gets deposited in various tissues and organs in our body including

bone, pancreas, kidney, liver etc, majority being in the liver and the kidney (Godt et al, 2006)

In the liver it binds to metallothionein (MT), a low molecular weight, cysteine-rich metal binding protein (Klaassen et al, 1999). Intracellular cadmium also induces the synthesis of this protein by activating the transcription factor, MTF1 (Metal regulatory transcription factor 1) (Klaassen et al, 1999).

The cadmium/ metallothionein complex or the CdMT complex is released gradually from the liver makes its way to other organs like kidneys and bones. There it accumulates, eventually leading to renal disorders and conditions like osteoporosis and osteomalacia (Jarup et al, 1998). Alpha-l-microglobin or beta-2-microglobin level in urine is often elevated and is an indicator of early cadmium-induced renal damage (Wittman and Hu, 2002).

1.3.3. Transport and storage of cadmium

After entering our circulatory system, cadmium, as any other toxicant, accumulates mainly in the liver where it forms complex with Metallothionenins, - CdMT complexes. Over the time the CdMT complexes are released slowly and gradually from the liver into the blood stream where they end up in various tissues and organs. Distribution of cadmium in the body depends on the chemical form of this element. Increased accumulation of Cd^{2+} ions in the liver, kidney and bones occurs after the exposure to cadmium in the form of inorganic salts (eg $CdCl_2$) than from the cadmium present in conjunction with metallothionein (CdMT). Cadmium salts like $CdCl_2$ accumulates mainly in the liver; whereas, CdMT in the kidney. Over time cadmium starts accumulating in various tissues and organs in our body -the liver, pancreas, kidneys, spleen, thymus, heart, bones, lungs, salivary glands and even in reproductive organs

like testis and prostate. But owing to the high metallothionein concentration about 50% of the cadmium found in the body is stored in the liver and the kidneys (WHO, 2011; Waalkes and Klaassen, 1985; Siddiqui, 2010). On inhalation, cadmium particles, enter the olfactory system and travel to the olfactory bulb through primary olfactory neurons and accumulates there. (Sunderman, 2001)

In smokers cadmium accumulates after inhalation, in the lungs. Even though the pulmonary epithelium can efficiently block toxic materials, cadmium is found to pass through alveolar cells and enter the circulation (Bressler et al, 2004).

Major source of cadmium exposure for a healthy non smoking adult is the intake of cadmium-containing food and water which is about 30 μg per day. The low pH in the digestive tract helps in the transport of cadmium by the proton-metal co transporter DMT1. (Sarkar et al, 2013) This metal transporter is partly regulated by iron. So diet deficient in iron will lead to increased absorption of cadmium from food. (Bressler et al, 2004)

1.4. MECHANISM OF CADMIUM TOXICITY

There is not a thorough understanding of molecular mechanism behind toxic effects of cadmium. Mainly, non protein bound free cadmium is toxic while the cadmium in cells bound to metallothionein is non toxic (Patrick, 2003). So far it is known to cause DNA damage in the cells, causes oxidative damage and protein misfolding. It interferes with antioxidant enzymes like catalase, copper/zinc-superoxide dismutase, manganese-superoxide dismutase etc (Casalino et al, 2002). It can also cause alterations in thiol proteins, inhibition of energy metabolism including cellular respiration and oxidative phosphorylation by tampering mitochondrial function, alter membrane structure and function, induce the expression of a number of stress responsive genes and affect

activities of several enzymes (Korach, 1998). It is known to substitute for zinc in metalloenzymes (Houston 2007). Cadmium generated free radicals in the cellular system cause oxidation of lipids or peroxidation of the phospholipids in the cell membrane, resulting in cell damage consequently leading to tissue damage and organ failure. Studies on rats have shown that cadmium induced lipid peroxidation occurs in liver, kidney, heart, brain, lung, as well as testes (Ercal et al, 2001).

Free radicals generated by cadmium can also lead to the expression of inflammatory chemokines and cytokines (Dong et al, 1998), causing DNA damage, alteration of DNA repair mechanisms, leading to mutagenic changes eventually resulting in cancers (Fowler, 1978).

Although metallothionein is known to help the cellular system with detoxification of cadmium, thereby reducing cellular damages caused by the heavy metal, it also can cause cadmium induced kidney damage. This happens when the cadmium-metallothionein complex leaks into blood and dissolves in plasma, releasing free cadmium ions. These ions get deposited in the liver, and taken up by the kidney which can also be reabsorbed in the proximal tubules. Even though these free cadmium ions complex with newly synthesized metallothionein, if there is an insufficient supply of metallothionein or other antioxidant defence and detoxification systems(like glutathione) in the kidney, they will damage renal cell membranes (Klaassen et al, 1999). Studies in metallothionein knockout mice shows that compared to their wild type counterparts, these mice are much more susceptible to renal injury and hepatotoxicity resulting from chronic cadmium toxicity (Klaassen et al, 1999). Normally cadmium is eliminated in bile in complex with glutathione. Glutathione is the cofactor of the enzyme glutathione peroxidase which is a selenium-dependent enzyme. Cadmium can

displace selenium there by lowering the activity of glutathione peroxidase which has been seen in cadmium-exposed workers (Wasowicz et al, 2001).

Transmembrane transporters, DMT1 or Divalent Metal ion Transporter 1, Voltage gated Ca^{2+} Channels, Zip8 which are meant for divalent metal cations like zinc, iron, calcium etc is used by cadmium for cellular entry. Intracellular cadmium can displace divalent metal cations like Fe, Mn, Cu, Zn etc from metal containing proteins rendering them non-functional and leading to misfolding of the protein or eventual production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) leading to oxidative damage followed by cell death (Sarkar et al, 2013). Cadmium binds to metallothionein displacing Zn^{2+} which in turn binds to MTF1 transcription factor, translocating it to the nucleus to initiate transcription of its target genes by binding to the metal response elements- MRE in their promoter region. Intracellular cadmium binds to specific receptors on ER membrane leading to the release of calcium. This will either activate caspases leading to apoptosis or activate certain kinases and phospahtases resulting in transcription of cell cycle genes leading to cell proliferation or mitogenisis. The fate of the cell on the entry of cadmium that is whether it should undergo programmed cell death or mitogenesis depends on the concentration of cadmium. Normally cadmium at very low cellular concentration is mitogenic while at higher concentration, is cytotoxic (Sarkar et al, 2013).

The intracellular effects of cadmium and the cellular damages caused by it are summarised in figure 1.1 & 1.2.

Figure. 1.1: Intracellular effects of cadmium

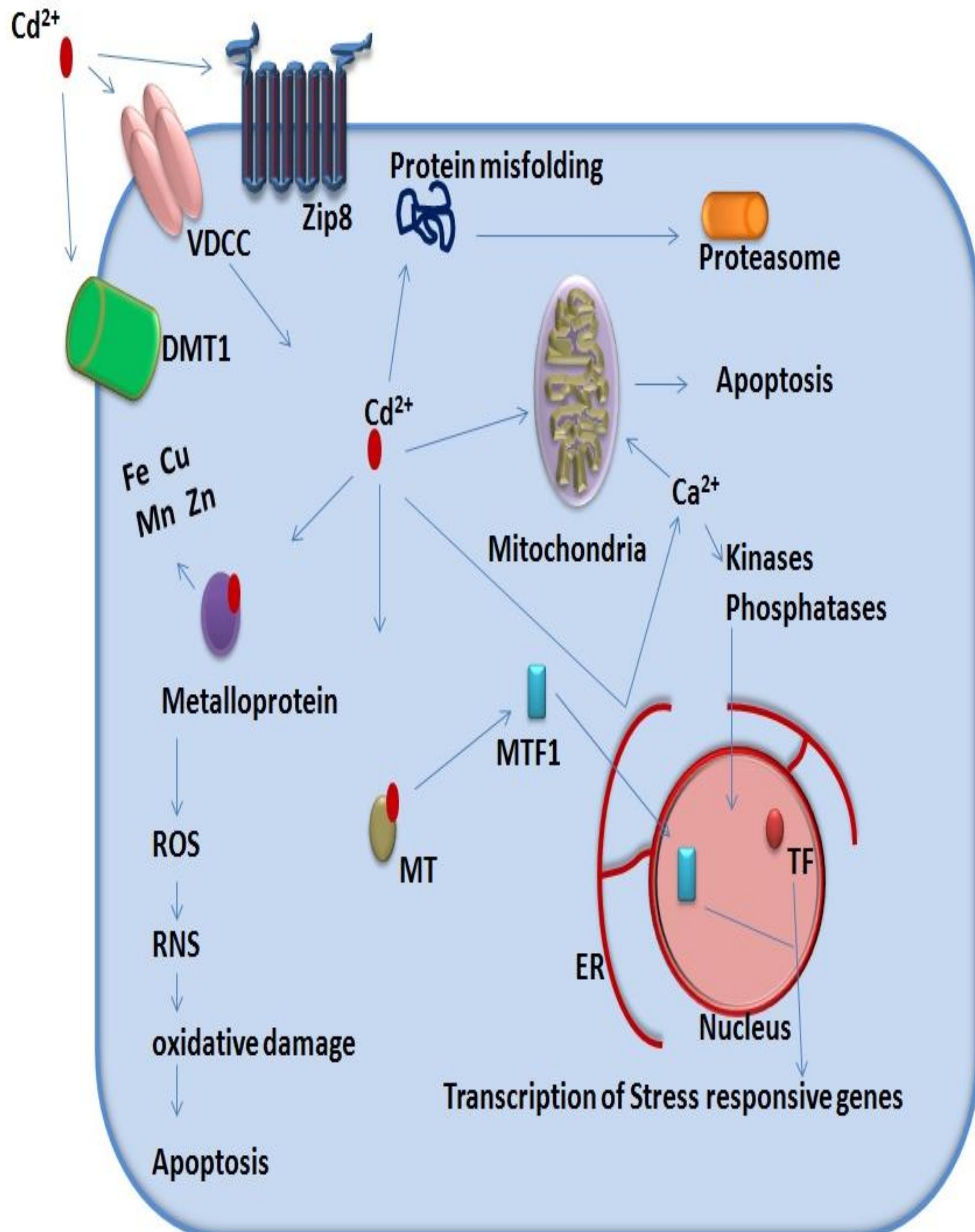


Figure .1.2.

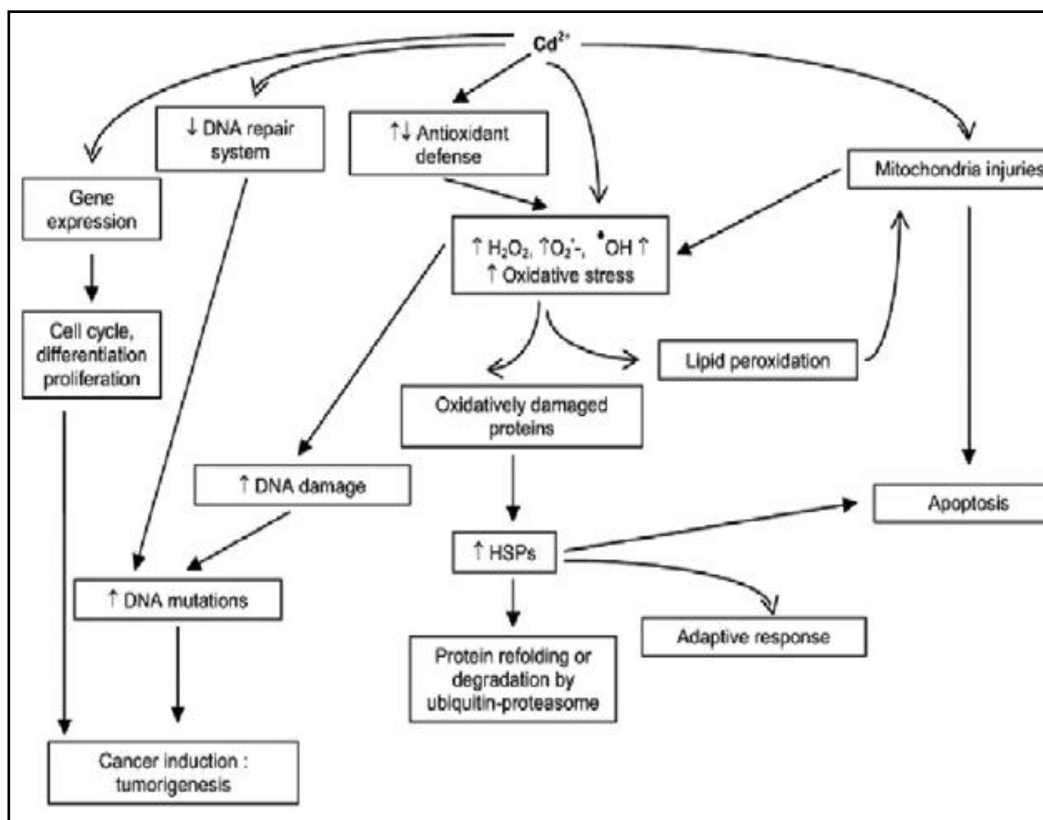


Figure.1.2. Cellular damages caused by cadmium: Divalent cadmium ion enters the cell through voltage gated calcium channels in the plasma membrane. Once inside the cell, it binds to cytoplasmic proteins and other cellular biomolecules. At cytotoxic dosage, cadmium can inhibit the syntheses of various biomolecules like DNA, RNA, and protein. Cadmium can deplete the antioxidant, glutathione in the cell, can react with sulfhydryl groups in proteins and also can replace zinc in certain metalloproteins, leading to protein misfolding and eventual proteasomal degradation all of which will result in the production of reactive oxygen species such as superoxide ion, H_2O_2, and OH- radical. These free radicals cause oxidative damage and induce lipid peroxidation leading to membrane damage. It can also cause DNA damage and chromosomal aberrations. This will alter the cellular homeostasis, eventually leading to cellular demise in a programmed fashion. Cadmium at sub lethal doses causes genotoxicity by interfering with the DNA repair mechanism there by directly acts as a mutagen. It up regulates many cell signalling pathways which lead to increased mitogenesis.

1.5. CELLULAR DEFENCE AGAINST CADMIUM TOXICITY

‘Antioxidants’ are the first line of defence against cadmium stress in an animal cell. Glutathione is the main antioxidant involved in the intracellular detoxification against cadmium. On exposure to cadmium, cellular defence initiates at the genomic level by up regulation of the biosynthesis of sulfhydryl compounds like metallothionein and glutathione, both of which can act as protective metal chelators helping in the elimination of the heavy metal from the cell (Ochi et al, 1988). Proteomic studies in U937 cell line revealed calbindin, a 28 KD, calcium binding protein as a secondary cadmium –responsive protein capable of offering resistance to cadmium induced apoptosis (Jeon et al, 2004). But studies have shown that at higher dosages cadmium can reduce the level of glutathione and react with protein sulfhydryl groups, resulting in the production of reactive oxygen species (Nigam et al, 1999; Xu et al, 2003). This leads to lipid peroxidation, oxidative stress, DNA damage and changes in cellular calcium and sulfhydryl homeostasis. Studies have also shown that metabolic precursors like different forms of vitamin B_{12} as well as folic acid can significantly improve the synthesis of glutathione in autistic patients (James et al, 2008). Therefore checking the effects of these kinds of precursors in enhancing the antioxidant level in the cells during cadmium induced stress can help in augmenting the cellular defence against the metal toxicity.

Metallothionein is a zinc-concentrating low molecular weight protein rich in cysteine, located in the golgi membrane. It is synthesised primarily in the liver and stored there. Inside the hepatic cytosol it complexes with cadmium, there by sequestering it from other cellular proteins and organelles, thus reducing the cadmium induced cellular damage. This also prevents cadmium from depleting glutathione in the cell. Studies show that over expressing metallothionein in neonatal mice, which have higher

metallothionein levels, naturally exhibit more resistance to cadmium induced liver damage (Liu et al, 1996). Metallothionein, like glutathione can scavenge free radicals like, hydroxyl and superoxide species and can function like microbial superoxide dismutase (Thornalley and Vasak, 1985).

1.6. CELLULAR STRESS AND APOPTOSIS

During cellular stress normal physiological functions are impaired, cellular structures undergo damage, and eventually lead to cell death. The cell death happens through an ordered and highly conserved pathway of self-destruction, called apoptosis (Kannan and Jain, 2000). Apoptosis is a type of programmed cell death in multicellular organisms. This process is involved in the essential removal of cells during embryogenesis and maintains proper cell number in multicellular organisms. It is also initiated in response to various stress stimuli like hyperthermia, anoxia, UV irradiation, chemotherapeutic drugs, etc. (Mosser et al, 1997)

Apoptosis induced by cellular stress follows an array of biochemical processes involving mitochondrial cytochrome C, apoptotic protease activating factor 1-Apaf-1 and proteases known as caspases (Beere et al, 2000). Apoptotic cell death is managed by the activation of caspases, a family of aspartate specific cysteine proteases. Due to ROS production and release of Ca^{2+} from endoplasmic reticulum, cytochrome C is released from mitochondria. Cytochrome C binds to Apaf-1 in the cytoplasm. Cytochrome C triggers oligomerization of Apaf-1 forming apoptosome complex which recruits and activates procaspase-9. Caspase-9 then activates caspase-3 and caspase-7, which in turn initiate programmed cell death through the selective proteolysis of key protein substrates. Various factors like exposure to ultraviolet irradiation, toxic heavy metals, aerosols, xenobiotics, herbicides, and other exogenous factors can induce the

prodcution of ROS in the cell leading to oxidative damage (Sinha et al, 2013). This oxidative damage may trigger apoptosis based on the interplay between pro and anti apoptotic regulatory proteins. They are a group of related proteins called Bcl2 family which governs the mitochondrial membrane permeability (Basu and Haldar, 1998). Anti apoptotic member of the family -Bcl2 confers resistance to programmed cell death. Pro apoptotic member Bax, a protein homologous to Bcl2, promotes apoptosis by competing with Bcl2. Bax proteins form homodimers and induce cell death, while Bcl2 forms heterodimer with bax leading to cell survival. Bcl2 and Bax are transcriptional targets of nuclear transcription factor p53, whose activation induces cell cycle arrest or apoptosis in response to DNA damage. Cell survival as well as death is controlled by regulation of the interaction between these molecules (Basu and Haldar, 1998).

1.7. CELL MORPHOLOGY AND RHO GTPASE SIGNALING

. Cytoskeleton is the structural protein- filamental network in the cell, responsible for regulating the cell morphology, intracellular transport, cell migration etc. It gives mechanical support to the cells to carry out essential cellular functions like division and movement, apart from giving shape to the cell. In a eukaryotic cell the cytoskeleton is composed of microfilaments (made of the protein actin), intermediate filaments (composed of various numbers of structural proteins like lamins, keratins etc) and microtubules (made of the protein tubulin) (Wickstead and Gull, 2011). Rho GTPases are a family of small GTP-binding proteins involved in the regulation of signal transduction involving actin dynamics during cytoskeleton organization, migration, transcription, and proliferation (Hall and Nobes, 2000). The three main members of Rho GTPase family are Rho, Rac and Cdc 42. They control the reorganization of actin cytoskeleton in response to various stimuli (Tapon and Hall, 1997). Rho induces the

formation of stress fibers which is an assembly of contractile actin-myosin filaments. It is also responsible for the formation of focal adhesion complexes, a macromolecular assembly which acts as a connection between cell and the extracellular matrix**.** Rac activation causes the formation of lamellipodia or membrane ruffles - an assembly of a meshwork of actin filaments at the cell periphery. Cdc42 is responsible for the induction of filopodia which are nothing but actin-rich surface protrusions (Tapon and Hall, 1997). Nuclear factor-κB is the hallmark molecule of inflammation and cellular stress. It is shown that RhoA, Rac1 and CDC 42 regulate NF-κB dependent transcription in response to various stress stimuli (Perona et al, 1997). Donne et al in 1997 demonstrated that cadmium at high concentration denature actin filaments and precipitate actin, but at lower concentrations regulate actin polymerization, more effectively than $MgCl_2$ and $CaCl_2$ by replacing Ca^{2+} in the filamental structure (DalleDonne et al, 1997).

1.8. HSP70 AND CELLULAR STRESS

All cells, prokaryotic as well as eukaryotic, produce heat shock proteins or HSPs in response to stress. They confer resistance and tolerance towards stress and speed up the recovery process (Beere et al, 2000). They are a family of highly conserved proteins and functions as molecular chaperons helping in protein folding. Their expression is regulated by transcription factors called Heat Shock Factors or HSFs (Sorger, 1991).The cyto protective effect of HSPs include preventing protein aggregation and disaggregation of proteins by refolding damaged proteins.

HSP70 family is one of the best characterized among the HSP gene family. HSP70 also regulate the progression of apoptosis induced by various kinds of stimuli, through numerous mechanisms (Cotter et al, 2000; Stankiewicz et al, 2005). It is found to inhibit cytochrome C release from mitochondria (Stankiewicz et al, 2005). It also

indirectly prevents activation of pro apoptotic gene *bax* (Stankiewicz et al, 2005). HSP70 also protects the cells from ER stress- induced apoptosis by interacting with Endoplasmic reticulum stress sensor protein IRE1alpha (inositol-requiring enzyme 1alpha). HSP70 may also inhibit apoptosis by inhibiting the functional assembly of apoptosome complex by associating with Apaf-1(Beere et al, 2000).

1.9. METHYLCOBALAMIN (MeB_{12})

A vitamin is a nutrient which the organism requires in limited amount but is important for vital bodily functions. Vitamins can be fat soluble (vitamins A, D, E and K) as well as water soluble (vitamins B and C). Among these, B vitamins are essential for cellular metabolism and erythropoiesis. They include Vitamin B_1 or thiamine, Vitamin B_2 or riboflavin, Vitamin B_3 or niacin, Vitamin B_5 or Pantothenic acid, Vitamin B_6 or pyridoxine, Vitamin B_7 which is biotin, Vitamin B_9 which is folic acid and Vitamin B_{12} which are cobalamins. They are all chemically distinct.

Cobalamin is the chemical name of vitamin B_{12}, one of the eight B vitamins. It is essential for the functioning of the nervous system and also for the formation of red blood cells. Other than bacteria and archea none of the living systems are capable of producing cobalamins (Bertrand et al, 2015). Only these microbes have the enzymes required for the synthesis of vitamin B_{12}, but several food items are natural sources of B_{12} because of bacterial symbiosis.

Figure.1. 3.

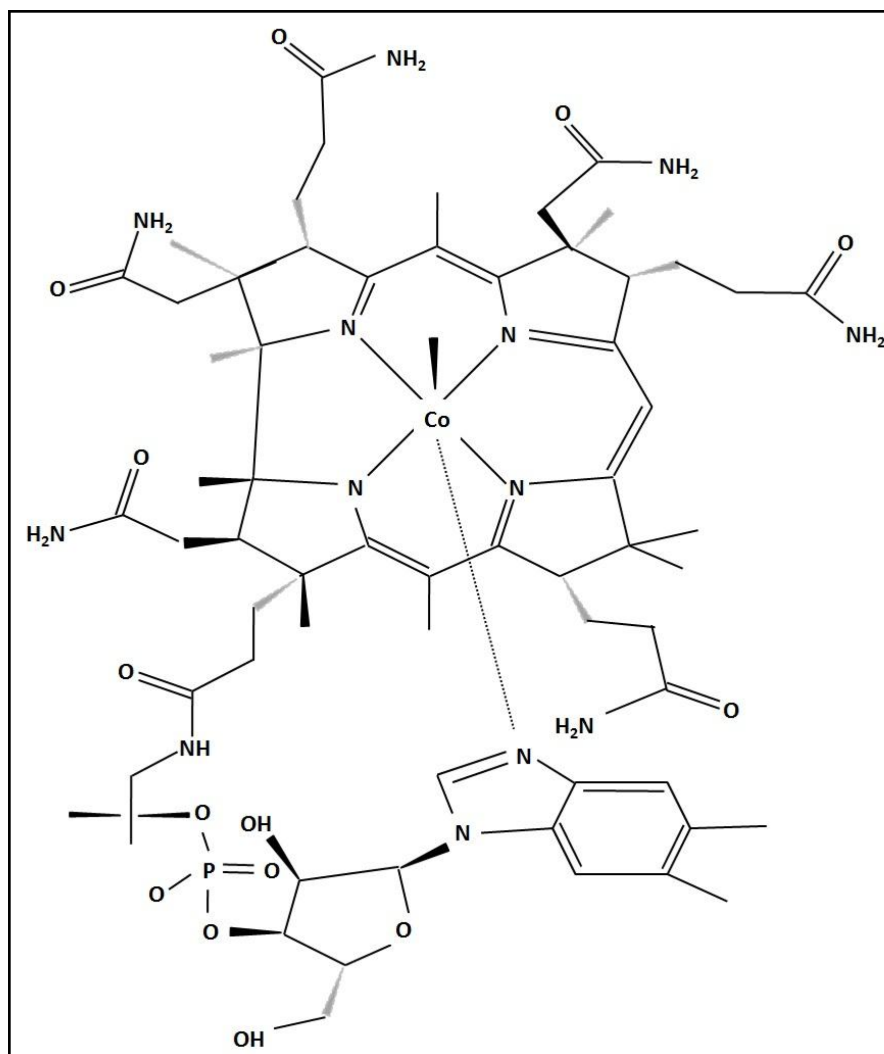


Figure.1. 3.CHEMICAL STRUCTURE OF METHYLCOBALAMIN (Mehmood et al, 2015)

Cobalamins are of various chemical forms and contain a cobalt ion in the centre of a planar tetra-pyrrole ring called a corrin ring. These include Cyanocobalamin, Hydroxocobalamin (B_{12a}), Methylcobalamin (MeB_{12}), Adenosylcobalamin, all of which differs from one another in the upper axial ligand attached to the cobalt ion. In the case of methylcobalamin the ligand is a methyl group. It is the coenzyme form of vitamin B_{12} that participates in homocysteine metabolism where it acts as the cofactor of the enzyme methionine synthase. Methionine synthase catalyzes the production of

methionine from homocysteine. MeB_{12} is employed as an intermediate carrier of methyl group from methyltetrahydrofolate to homocysteine, releasing 4 folate groups along with the formation of a methionine molecule (Drennan et al, 1994). In our body during vitamin B_{12} deficiency, low level of methylcobalamin leads to the deficiency of folate, popularly known as the 'folate trap' (Murray, Granner, Mayes and Rodwell,1996). Deficiency of Vitamin B_{12} and folic acid leads to an increased level of toxic homocysteine, since both of them are the coenzymes in the enzymatic conversion of the latter to methionine. Accumulation of homocysteine has been linked with increased risk of several disorders of the nervous system like Alzheimer's disease (McCaddon et al, 1996; Clarke et al, 1998), multiple sclerosis (Baig and Ali, 1995) cardiovascular disease (Araki et al, 1993), as well as chronic fatigue syndrome/fibromyalgia (Regland et al, 1997).

Methylcobalamin is more important for homocysteine disposal than folic acid. Conversion of homocysteine to methionine using methylcobalamin also generates ample amount of SAM (S-adenosyl methionine), the body's most important methyl donor- a positive effect linked to its effective role in protection from neurotoxicity (Akaike et al, 1993; Kikuchi et al, 1997).

Most of the vitamin B_{12} in the body is stored as adenosylcobalmin in the liver and is converted to the methylcobalamin, as and when required. The active forms of Vitamin B_{12} in our body adenosylcobalamin, and methylcobalamin, are produced by two distinct metabolic pathways. Adenosylcobalmin ends up in the mitochondria where as methylcobalamin is found in the cytoplasm, and is the vitamin B_{12} form predominating in the blood and in other body fluids. Adenosylcobalamin is the coenzyme of methylmalonyl coenzyme A mutase (MCM) that catalyzes the isomerization of

methylmalonyl-CoenzymeA to succinyl-CoenzymeA. Cobalamin deficiency mainly results in the accumulation of both homocysteine and methylmalonyl-CoA (MMA) while accumulation of only homocysteine happens during folate deficiency (McMullin et al, 2001). This is because cobalamin is essential for the folate dependent conversion of homocysteine to methionine and also for the folate independent conversion of methylmalonyl-CoenzymeA to succinyl-CoenzymeA.

The transport and intracellular processing of cobalamin in our body is summarised in figure1. 4. Cyanocobalamin is the most common form of vitamin B_{12}. It has a cyanide group (-CN) attached to the central cobalt ion, whereas in methylcobalamin it is a methyl (-CH3) group. Under normal conditions natural B_{12} of our body does not contain much cyanocobalamin form; neither does it occur naturally in plants and animals. Only during cyanide poisoning or in the case of chronic smoking, cyanocobalamin levels are raised in our body.

Vitamin B_{12} supplements contain cyanocobalamin rather than methylcobalamin. This is because charcoal was used to filter extracts for the isolation of vitamin $B_{12.}$ The traces of cyanide in the charcoal convert all natural forms of B_{12}, into a stable cyanocobalamin form. Because of which the vitamin B_{12} coenzymes and their metabolic roles were unknown for years (Herbert, 1988).

Only 1% of orally ingested Vitamin B_{12} usually gets absorbed by gastro intestinal epithelium and enters the bloodstream. Sublingual administration is more recommended since absorption through oral mucosa is a better way to increase blood Vitamin B_{12} levels. Methylcobalamin is the most common naturally circulating form of vitamin B_{12} in blood plasma. If we take cyanocobalamin our body has to remove the cyano group and reduce the cobalt ion so that it can bind to the methyl group. There are many

advantages of taking methyl B_{12} as a supplement over ordinary B_{12} supplements. The body does not have to use its resources and energy to reduce it to methylcobalamin since it's already there. Methylcobalamin is the ultimate reduced form of vitamin B_{12} possible, making it a very good reducing agent (antioxidant).

Figure.1.4

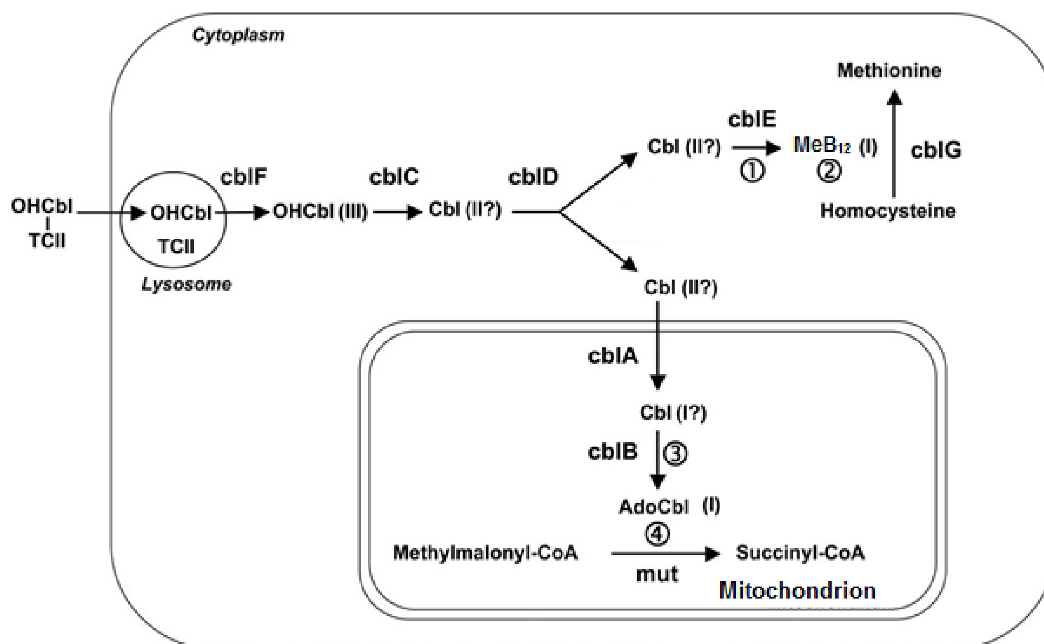


Figure.1.4. cobalamin processing in the cell: cobalamin, Cbl; transcobalamin, TCII; hydroxocobalamin, OHCbl; adenosylcobalamin, AdoCbl [Adapted from Suormala et.al. 2004].

Three soluble Cobalamin-binding proteins are known to be involved in the uptake and transport of Cobalamins in humans: (Mathews et al, 2007; Wuerges et al 2006)

1) Haptocorrin (HC)

2) Intrinsic factor (IF)

3) Transcobalamin (TC)

The main dietary source of cobalamin is animal products (Hsing et al, 1993 46). Milk products mainly contain hydroxy and methyl cobalamin while meat contains adenosyl as well as hydroxycobalamin. The requirement of cobalamin for an adult is about 1mg

per day. The first transport protein Haptocorin or HC is a glycoprotein secreted by salivary glands. It is also known as transcobalamin 1 (TCN1) or R protein. Its function is to safely transport acid sensitive vitamin B_{12} through the low pH environment of stomach to duodenum. Haptocorin gets digested by pancreatic enzymes, releasing vitamin B_{12} in the small intestine where the conditions are alkaline, which then binds to intrinsic factor (Fedosov, 2011). Parietal cells of the stomach secretes Intrinsic Factor (IF) which is a glycoprotein that helps in the absorption of cobalamin in the small intestine (Howard et al, 1996).The epithelial cells (enterocytes) of the ileum will absorb the intrinsic factor cobalamin complex, inside of which cobalamin dissociates from the intrinsic factor and binds to transcobalamin II (TCN2). The cobalamin-transcobalamin II complex moves out of epithelial cells enters circulatory system to reach the liver eventually (Fedosov, 2011). The receptors for transcobalamin (TCblR) are expressed by all mammalian cells. This receptor captures cobalamin-transcobalamin II complex from circulation and by absorptive endocytosis brings them into the cell. Still inside acidic endosomes the receptor dissociates from the complex and is recycled back to the cell surface. The complex is separated inside the lysosomal vesicles by degradation of transcobalmin by proteolytic enzymes. The cobalmain is exported to the cytosolic space through the lysosomal transporter cblF, where it then binds to the cblC (MMACHC) chaperone. The cblC (MMACHC) chaperone cuts the β-axial ligand of the cobalamin. From the cytosol cblC -bound Cobalamin is directed to the mitochondria through cbl D gene product (even though this role of this transporter is still under study) for Adenosylcobalamin synthesis (through cblA and cblB), or to cytosolic methionine synthase for Methylcobalamin synthesis (through cblE and cblG).

Methylcobalmin tablets are used for the treatment of neurological disorders like diabetic Neuropathy (Yaqub et al, 1992) and peripheral neuropathy (Jian-bo et al 2013).

Various studies have reported its role as a neurotrophic or growth-promoting factor for nerve cells (Mori et al, 1991; Mikhailov et al,1983), a desirable effect in the case of regeneration of damaged central and peripheral nervous tissues in disorders such as amyotrophic lateral sclerosis (Okada et al, 2010) and diabetic peripheral neuropathy (Kikuchi et al, 1997). MeB_{12} showed protective effects against neurotoxicity in rat retinal cells on administration of chronic (for a period of 48-96 hours) dosage (Mori et al, 1991). Physiological level of MeB_{12} in human body is equal to that of vitamin B_{12}. Methylcobalamin is shown to be effective in treating diseases resulting from deficiency of Vitamin B_{12} like pernicious anaemia (Izumi et al, 2013). It is shown to be effective in the treatment of various other diseases like HIV (Weinberg et al 1995) cancer (Tsao and Myashita 1993) etc. It is shown to methylate arsenic, along with selenium and methionine thereby helping our system to eliminate the heavy metal (Patrick, 2003; Zakharyan and Aposhian, 1999).

1.10. GAPS IN EXISTING RESEARCH

Even though cadmium toxicity is an age old phenomenon, the exact mechanism of this metal induced toxicity is not clearly understood. There is only vague information on cellular defence against this element. Response mechanism of a cell against a particular toxicant differs from cell type to cell type. Cadmium is said to reduce cellular immunity by attacking the antioxidant defence of the cell, but there are still debates on this matter. So far no particular study has been under taken to see what is the clear demarcation in the cellular response with respect to difference in cadmium concentration, since cadmium at low concentration can be mitogenic but at higher dosages is cytotoxic. How cadmium is properly eliminated from the cell and how the antioxidants indigenous to the cell can help the cell from the metal induced toxicity is still not very clear.

Cytoskeleton is responsible for cellular dynamics like the intracellular transport of vesicles and cell organelles, cell movement and overall cellular integrity. The modifications induced by cadmium, on cell morphology as well as on the cell signalling involving the Rho family of GTPases, are not well understood. A detailed study on this signalling pathway will reveal the changes occurring in the cell during cadmium induced stress which in turn will give an idea on the status of cellular integrity during adverse conditions.

Members of HSP70 protein family are one of the first stress response proteins synthesized by cells in response to cadmium toxicity. Actual mechanism by which they protect the cells from the adverse condition is still vague. The mechanism also varies from cell type to cell type. A detailed study on HSP70 induction and related signalling during cadmium induced stress will provide a greater insight into the possible effects of cadmium on signal transduction and stress-related pathways in the cell.

Vitamins are micronutrients, essential for vital bodily functions. They are thought to be very protective of the cellular system. Compared to its counter parts, Vitamin B_{12} analog, methylcobalamin is a recent player in the human health department. This began with unveiling the positive effect of vitamin B_{12} on nervous system and search for an effective and non toxic analog of vitamin B_{12}. Unlike adenosyl cobalamin, methylcobalamin is readily bio available, and methyl group in methylcobalamin is non toxic compared to cyano group in cyanocobalamin. Study on the effect of this chemical on human system is still in its infancy. Most of the studies are focussed on its effect on nervous system. Not many studies are undertaken to explore the interactions of this vitamin with various tissues and cell types in our body.

1.11. OBJECTIVES

The proposed research aims at:

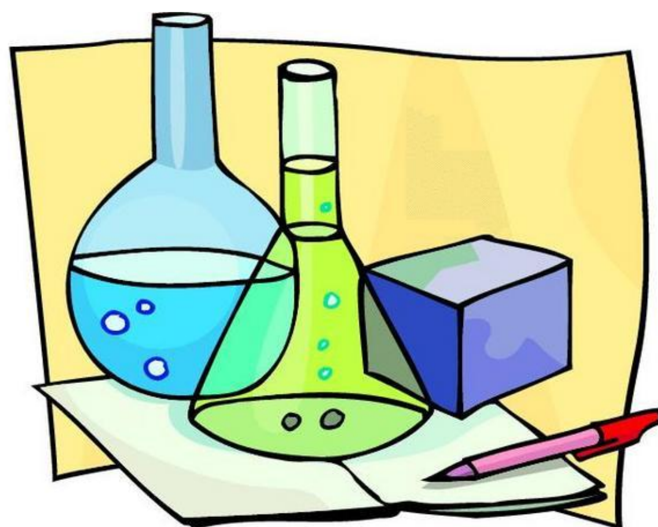
1. To study the effect of cadmium on the morphology of human alveolar cells and its effect on expression and regulation of small GTPases.

2. To study the effect of cadmium induced expression of 'Heat shock protein 70 (HSP70) and its signaling cascade.

3. To study the involvement of cadmium stress in apoptotic pathway.

Chapter 2:

MATERIALS AND METHODS



Science is nothing but trained and organized common sense

-Thomas Huxley

2.1 MATERIALS

All the cell culture materials including culture media and reagents used in the study namely, DMEM, FBS, Antibiotic-antimycotic solution (100 X) and Trypsin 1:250 powder were either purchased from HiMedia Laboratories or Sigma-Aldrich, Co., USA or Life Technologies (Gibco BRL, USA). Plastic wares for animal cell culture- cell culture treated T flasks (25cm2 , 75cm2), Cell culture treated 6 well plates, and 24 well plates, Conical centrifuge tubes (15ml, 50ml), cryovials were purchased from Corning USA. Reagents for testing cell viability -trypan Blue dye was purchased from HiMedia Laboratories and Resazurin sodium salt from Sigma-Aldrich, Co.,USA.

Anti HSP70 and anti beta-actin antibodies were purchased from Sigma-Aldrich, Co.,USA. Secondary antibodies (Anti-mouse HRP secondary antibody) were purchased from Banglore Genei Pvt Ltd., India. PVDF membrane was purchased from life technologies. Protein molecular weight markers were purchased from Banglore Genei Pvt Ltd., India.

TRIzol® LS Reagent for RNA extraction was purchased from Invitrogen, USA. First strand cDNA synthesis kit for RT-PCR (Tetro cDNA synthesis kit) was purchased from Bioline, USA. PCR reagents namely Taq DNA Polymerase enzyme, dNTPS, PCR Buffers as well as DNA markers (100bp and 50 bp) were purchased either from Bioline, USA, Invitrogen, USA, and Chromus biotech, India. PCR primers for reverse transcriptase PCR experiments were synthesized by Integrated DNA Technologies, USA.

Fluorescent dyes –Hoechst33342 and Phalloidin conjugated Fluorescein Isothiocyanate, FITC were purchased from Sigma-Aldrich, Co.,USA,

Other routinely used chemicals were purchased from HiMedia Laboratories, Sigma-Aldrich, Co.,USA, Thermo Fisher Scientific, Merck KGaA, Germany.

All the cell lines A549, HCT116, HCT116p53-/-, HEK293 were obtained from National Centre for Cell Science, NCCS, Pune, India.

2.1.1 Buffers and Solutions

2.1.1a Cell culture:

Complete medium: DMEM: 90%, FBS: 10%, Antibiotic-antimycotic solution (100 X): Final concentrations of 0.1 X.

Trypsin solution: 0.25 % Trypsin in DMEM (without FBS)

Freezing medium: 90% FBS, 10%DMSO

2.1.1b Cell count and viability:

Trypan blue solution: 0.4% trypan blue powder dissolved in 1X PBS

Resazurin solution: 1mM in 1X PBS

2.1.1c Protein extraction:

Protein Extraction Buffer (PEB):

TrisCl (pH 7.4) - 20mM
EDTA (pH 8) - 1mM
PMSF -1mM
Triton X-100 -0.1%
Protease cocktail: 1 tablet per 100 ml

2.1.1d Bradford assay:

Bradford reagent (5X): 50 mg of CBB G-250 was dissolved in 25 ml absolute ethanol in a dark bottle while stirring. 50 ml of orthophosphoric acid was added with continuous stirring. The volume was made up to 100 ml with distilled water added drop wise using Pasteur pipette. The final solution (wine red coloured) was stored at 4°C.

2.1.1e SDS-PAGE:

Gel solution, 30%: 29.2 g Acrylamide and 0.8 g N'N'-bis-methylene-acrylamide was dissolved in distilled water and the volume was made up to 100 ml Stored in the dark, at 4° C.

Tris-HCl pH 8.8 (1.5 M): 18.17g Tris base was dissolved in distilled water. pH was adjusted to 8.8 using 1N HCL. The volume was made up to 100 ml with distilled water. Stored at 4^0C.

Tris-HCl pH 6.8(0.5 M): 6.05g Tris base was dissolved in distilled water. pH was adjusted to 6.8 using1N HCL. The volume was made up to 100 ml with distilled. Stored at 4^0C.

Ammonium Per Sulfate, APS (10%): 0.1 g of ammonium persulfate in 1ml of distilled water. Stored at 4^0C.

Sodium Dodecyl Sulphate, SDS (10%): 10 g of SDS was added in 70ml of distilled water and dissolved by heating to 55^0 C in a water bath. Volume was made up to 100 ml with distilled water. Stored at room temperature.

Gel Running Buffer 1X: (Tris-glycine electrophoresis buffer)

Tris base: 25 mM
Glycine: 190 mM
SDS: 0.1%

2X /Sample buffer (10ml): (Laemli, 1970)

0.5M Tris-Cl (pH 6.8)	-2.5ml
10 %(W/V) SDS	-4ml
β-mercaptoethanol	-1ml
Glycerol	-2ml
0.05% bromophenol blue	-0.5ml

The dye solution was made without β-mercaptoethanol and Aliquoted in vials (900μl in each vial). The aliquots were stored at -20^{0}C. 100μl β-mercaptoethanol was added to the 900 μl aliquot just before use.

Gel staining solution:

0.125% CBB R- 250 in destainer I

Destainer I:

Methanol: 50%

Glacial acetic acid: 10%

Distilled water: 40%

Destainer II:

Methanol: 10%

Glacial acetic acid: 10%

Distilled water: 80%

Gel storage solution:

7% glacial acetic acid in distilled water

2.1.1f Western Blot:

Transfer Buffer 1X:

Tris base: 25 mM

Glycine: 190 mM

Methanol: 20%

Ponceau stain (50 ml 0.1%):

0.05 g - ponceau stain powder

500 μl – Glacial Acetic Acid

49.5 ml – distilled water

PBS 1X (pH 7.4) 1L:

NaCl : 8g

KCl: 0.2g

Na_2HPO4: 1.44 g

KH_2PO4: 0.24g

TBS (pH 7.5) 1L:

Tris base: 6.05g
NaCl: 8.76g

Blocking solution: 3% BSA in 1X PBS

Color developer solution: 1X TMB/H_2O_2

Primary antibodies:

Primary mouse HSP70 - Monoclonal anti-HSP70 antibody (SIGMA)

Primary mouse β Actin - Monoclonal anti-β Actin antibody (SIGMA)

Secondary antibody: Anti-mouse HRP secondary antibody (GENEI)

2.1.1g Reverse Transcriptase -PCR:

TE buffer (pH 8.0):

Tris-Cl (pH 8.0): 100 mM

EDTA (pH 8.0): 10 mM

TAE stock 50X: (for 1L)

242 g of Tris base

57.1 ml of glacial acetic acid

100 ml of 0.5M EDTA (pH 8.0)

Made up to 1 L with distilled water

Agarose gels:

2% agarose in 1X TAE

1.2% agarose in 1X TBE

DNA gel loading dye (6X):

Bromophenol blue: 0.25%

Xylene cyanol FF: 0.25%

Glycerol in water: 30%

EtBr staining solution:

Stock: 1mg/ml in distilled water

Working solution: 0.5µg/ml

Table.2.1. PCR Primer list:

Gene Name (annealing temperature)	**Primer Sequence**
CDC 42(50^{0}C)	Forward primer 5’ AGA GGA AAT ACG AGG GGT GGT 3’ Reverse primer 5’ CCT GAC TGG TCC CCA TGT TG 3’
RAC (60^{0}C)	Forward primer 5’GCC AAT GTT ATG GTA GAT GGA 3’ Reverse primer 5’TTA AGA ACA CAT CTG TTT GCG 3’
RHOA (55^{0}C)	Forward primer 5’CCT TGT CTT GTG AAA CCT TG3’ Reverse primer5’ ACT GGT AGC AAG ATG ACT TC3’
HSP70 (50^{0}C)	Forward primer 5’ ACA CGA ATC CCT GCG GTA AA3’ Reverse primer 5’ AGC AGG CGA TAA GAT GGC AC3’
NF-κB (50^{0}C)	Forward primer 5’ATC CAT ATT TGG GAA GGC CTG AA3’ Reverse primer5’GTA TGG GCC ATC TGT TGG CAG 3’
MT1 (55^{0}C)	Forward primer 5’CCT GCA CCT GCA CTG GCT CCT G 3’ Reverse primer 5’ TGG GCA CAC TTG GCA CAG CTC AT 3’
MT2 (50^{0}C)	Forward primer 5’ CAA AGG GGC GTC GGA CAA G 3’ Reverse primer 5’ ATA GCA AAC GGT CAC GGT CAG 3’
P53 (50^{0}C)	Forward primer 5’TGC TCA AGA CTG GCG CTA AA3’ Reverse primer 5’CAA TCC AGG GAA GCG TGT CA3’

BCL2 (60^0C)	Forward primer 5'GAA CTG GGG GAG GAT TGT GG 3'
	Reverse primer 5'GGC AGG CAT GTT GAC TTCAC3'
BAX (70^0C)	Forward primer 5'GGC CCT TTT GCT TCA GGG TTT C3'
	Reverse primer 5'CAG TCG CTT CAG TGA CTC GG 3'
18SrRNA (50^0C)	Forward primer 5'GTA ACC CGT TGA ACC CCA TT3'
	Reverse primer 5' CCA TCC AAT CGG TAG TAG CG 3'

2.1.1h Fluorescent Staining

Hoechst dye stock: 50 mg/ ml in distilled water

Phalloidin FITC conjugates stock: 0.1mg/ml in Dimethyl sulfoxide (DMSO)

2.1.2 Cell lines:

2.1.2a A549: A549 cell line was developed from adenocarcinoma of human alveolar basal epithelial cells by D. J. Giard, et al. in 1972 through the removal and culturing of cancerous lung tissue in the explanted tumour of a 58-year-old Caucasian male. The pulmonary epithelium is made of Type I and Type II alveolar epithelial cells. A549 cell line is useful for studying Type II alveolar cells. A549 cells are suitable model for exploring the functions of alveolar Type II cells in the metabolic and macromolecule processing as well as mechanisms of drug delivery through the pulmonary epithelium. TP53 gene is wild type in this cell line (Giard et al, 1973; Foster et al, 1998; Stellavato et al, 2011).

2.1.2b HEK293: Human Embryonic Kidney 293 is a transformed cell lineage originally established from human embryonic kidney cells with sheared adenovirus which resulted in an insert consisting of 4.5 kb from the left arm of the viral genome,

incorporated into human chromosome 19. It was developed in Alex van der Eb's laboratory in Leiden, The Netherlands, in 1973. This cell line has wild type TP53 gene.

2.1.2c HCT116: human colorectal carcinoma HCT116 cell line is one of the three strains of malignant cells derived from a male with colorectal carcinomas. They are adherent cells with epithelial morphology and have mutation in codon 13 of the *ras* proto-oncogene. TP53 gene is wild type in this cell line.

2.2 METHODS

2.2.1.Cell culture: Storage and maintenance: Cell lines were maintained in Dulbecco's Modified Eagle's Medium (GIBCO, HIMEDIA) containing 10% Fetal Bovine Serum, FBS (SIGMA,HIMEDIA) supplemented with 0.1% antibiotic antimycotic solution in humidified 5% $C0_2$ condition at 37^0C. Monolayers were passaged (split ratio1:4) once they reached about 90% confluency. Cell lines were cryopreserved for future studies, at -80^0C in freezing medium (90% FBS and 10% Dimethyl sulfoxide, DMSO).

2.2.2. Preparation and exposure to Cadmium Chloride: Cadmium chloride ($CdCl_2.H_2O$; HIMEDIA) stock was prepared in sterile double distilled water and was sterilized by filtration. After plating equal number of cells they were exposed to various concentrations of cadmium chloride. Cells were harvested for downstream applications 24 and 48 hours after cadmium treatment.

2.2.3. MeB_{12} and Cadmium Treatment: A549 Cells at a seeding density of $2X10^4$ cells/ml where grown in media containing 50 and 100 µg/ml MeB_{12} for 48 hours while maintaining one set of cells in regular medium. After 48 hours, these 3 experimental sets were exposed to different concentrations of $CdCl_2$ in the following order –control (no $CdCl_2$), 2.5, 5 and 10µg/ml $CdCl_2$. After 24 hours of treatment still surviving attached cells were harvested and subjected to various studies.

2.2.4. Total viable Cell count: Total viable cell count for every experiment was carried out after the required period of cadmium treatment. The monolayer was washed with 1X PBS and cells were trypsinized and pelleted down. Three different dilutions of cell suspensions were taken from each test and the total viable cell count was determined using haemocytometer (Tiefe Depth Profondeur, Marienfeld, Germany) by trypan blue dye exclusion method.

2.2.5. Cell morphological analysis: Following cadmium exposure (24 and 48 hours) the cells were observed under inverted optical microscope (Nikon eclipse TS 100) and the pictures were captured using compact digital camera (Nikon COOLPIX22).

2.2.6. Cytotoxicity assay: For assessing the cytotoxic effect of cadmium on A549 cells, cells were plated in 24 well plates and were exposed to different doses of cadmium. Resazurin dye solution was added to each well in an amount equal to 10% of the culture medium volume. This was followed by an incubation of 3 hours until blue coloured resazurin is reduced to red resorufin observed by the change in media colour. The dye conversion was measured spectrophotometrically by monitoring the decrease in absorbance at a wavelength of 600 nm. The absorbance was measured at a reference wavelength of 690 nm and was subtracted from the 600 nm measurement (Vega-Avila and Pugsley, 2011).

2.2.7. RNA Extraction: Total RNA was extracted from the control and experimental cells as per the manufacturer’s (Invitrogen, USA) protocol using TRIzol® LS Reagent. 1ml of the TRIzol® LS reagent was added directly to the monolayer after removing the media and the cells were homogenized by repeated pipeting. The homogenate was then transferred to a fresh sterile vial and was incubated with chloroform for 10 minutes followed by centrifugation at 12000 rpm for 10 minutes. The separated aqueous layer was carefully transferred to a new vial and the RNA was precipitated using isopropanol. The precipitated RNA was pelleted down by centrifugation, washed with 70% ethanol and then dissolved in sterile double distilled water which was followed by incubation at 60^0C for 3 minutes. After the incubation total cellular RNA was quantified spectrophotometrically at 260 nm wavelength and then was stored at -80^0C for future applications.

2.2.8. cDNA Synthesis: First strand cDNA was synthesized from RNA samples (2 µg) using the cDNA synthesis kit for RT-PCR (Tetro cDNA synthesis kit) from Bioline, USA, as per specifications provided in the kit.

2.2.9. Primer designing and Synthesis: The cDNA sequence for each gene was obtained from The Reference Sequence (RefSeq) database, NCBI. The primers specific for each template was designed using NCBI primer blast soft ware. The list of primers used along with their respective gene name is given in section 2.1.1g.

2.2.10. Reverse Transcriptase PCR: PCR amplification of a given cDNA was carried out using specific primers (1 mM each) designed for it using NCBI Primer BLAST. The PCR was carried out for 30 cycles (97^0 C for 2 min, 94^0 C for 1 min, 50^0 C for 2 min, 70^0 C for 1 min) in a thermocycler (BIORAD).

2.2.11. Protein Extraction and Estimation of protein by Bradford's method

Total cellular protein was extracted from non exposed controls and cadmium exposed test cells, using lysis buffer (Sarkar et al, 2002).The monolayer was washed with 1X PBS and the cells were pelleted down after trypzinisation. The pellet was then homogenized in lysis buffer using a tissue homogenizer followed by spinning at 2500 rpm at 4^0C. The supernatant containing the soluble protein was transferred to a new sterile vial and protein concentration of the cell extracts were determined using Bradford's micro estimation method (Bradford, 1976).

2.2.12. SDS PAGE Analysis

The extracted proteins were analyzed on SDS PAGE under reducing conditions followed by coomassie brilliant blue Staining**.** Samples containing equal quantity of proteins were denatured in Laemmli buffer (Laemmli, 1970) for 3-5 minutes at 100^0C and were resolved in a 10% SDS Polyacrylamide gel. The 5% stacking gel was overlaid on the separating gel of 10% polyacrylamide with an acrylamide: Bis ratio of 29.2:0.8.

The running buffer consisted of 0.025 M Tris, 0.2 M glycine, and 0.1% (w/v) SDS. The gels were run in a MINIKIN gel apparatus at a constant voltage of 80V. Protein bands were visualized using coomassie blue staining method.

2.2.13. Western Blot Analysis

Following SDS PAGE, proteins were electrophoretically transferred to a charged PVDF membrane using 1X transfer buffer. The transferred protein bands were visualized in the membrane using Ponceau S stain. After washing off the stain the membrane was incubated in 1XPBS for 5 minutes before being transferred to blocking buffer followed by overnight incubation at 4^0C. After blocking the membrane was incubated in primary antibody solution for 4-6 hours at room temperature. After incubation the blot was washed for 5 minutes in 1XPBS-T, followed by two washes in 1XPBS. The blot was then incubated in HRP conjugated secondary antibody for 1 hour. After the incubation the protein of interest was visualized by colour development using 1X TMB/H_2O_2 followed by termination of the colour development using 0.1N H_2SO_4

2.2.14. Fluorescent staining with Phalloidin FITC

Stock solutions of phalloidin conjugate was made in DMSO at a concentration of 0.1 mg/ml. Final staining solution in buffer is in the concentration range of 0.1 μM. Cells were washed with 1X (PBS) and were fixed for 5 minutes in 3% formaldehyde solution in 1XPBS. The fixed monolayer was then washed extensively in 1X PBS and then dehydrated with acetone, permeabilized with 0.1% TRITON X-100 in PBS, followed by PBS wash. Cells are stained with fluorescent phalloidin conjugate solution in PBS (containing 1% DMSO from the original stock solution) for 40 minutes at room temperature followed by Washing several times with PBS to remove unbound phalloidin conjugate. Stained cells were visualized under Nikon eclipse TS 100

fluorescence microscope at 20 X magnification with an excitation wavelength of 495 nm and an emission wavelength of 519 nm.

2.2.15. Fluorescent staining with Hoechst 33342

Nuclear fragmentation was detected by Hoechst 33342 staining. The monolayer was washed twice with 1XPBS. Cells were fixed in 10% formaldehyde and dehydrated using 90% methanol. After fixing and dehydrating the monolayer was washed once and maintained in 1XPBS. Hoechst 33342 (final concentration-1µg/ml) was directly added to it by gently shaking at 4°C for 5 min. Stained nuclei were visualized under Nikon eclipse TS 100 fluorescence microscope at an excitation wavelength of 366 nm and an emission wavelength of 480 nm.

2.2.16. Construction of Isobologram

To see the type of interaction displayed by the $CdCl_2$ and MeB_{12}, a classic isobolograhic technique was used. When two chemicals are mixed together, the resulting interaction between them can be additive, synergistic and antagonistic. When their combined effect is equal to that of the sum of their individual potencies, then the interaction is termed additive, when it is greater the interaction is synergistic and when it is lesser, antagonistic[Lee,2010; Tallarida, 2012].

Isobologram is a graph in Cartesian coordinates where each axes represent the concentrations of a particular chemical (here MeB_{12} in X axis and $CdCl_2$ in Y axis). Isobole is a line that joins dose pairs that produce a fixed response. Here we have selected half the maximum response, so the intercepts are individual IC_{50} values of MeB_{12} and $CdCl_2$. In brief, A549 cells in 24-well plates were exposed in triplicate to increasing concentration of each chemical or both the chemicals using a constant ratio($CdCl_2$: MeB_{12}=1:2) combination design for 48 hours. Cell viability after 48 hours was measured by resazurin assay. From the experimental values a dose matrix was

constructed in excel and entered in chalice analyzer software to construct the isobologram.

The line of additivity follows the isobole while the Cartesian coordinates denoting synergy will be below the line of additivity and that representing antagonism will be above it [Zhao et al, 2014].

The evaluation of synergy, construction of dose matrix and isobologram was done using online software tool named Chalice Analyzer.

2.2.17. Methylation specific PCR (MSP)

The MSP primers for MT1A and MT2A genes were designed using the online software tool, MethPrimer. Genomic DNA was extracted from control and treated A549 cells using a Genomic DNA isolation Kit (Bioline, USA). Equal amount of genomic DNA from each sample was subjected to bisulfite modification using Nucleo-pore® DNA Methylation Kit as per instructor's manual. Methylation-specific PCR for the MT1A and MT2A genes was carried out with primers specific for the methylated and unmethylated promoter DNA sequences of the two genes (sequences are shown in Table. 1.2). The reaction mixture was incubated at 95°C for 5minutes, followed by 30 cycles of 94°C for 30 seconds, annealing temerature for 30 seconds, 72°C for 1 minute seconds, and a final extension at 72°C for 5 minutes. PCR products were visualized on 2% TAE agarose gels, by ethidium bromide staining.

Table.2. 2. List of MSP primers

Gene Name		Primer Sequence
MT1A	Forward M primer	ATA GGG ATA GGT AAG GCG ATA GC
	Reverse M primer	CGA TCG ACG TAA TAC AAA ACG TA
	Forward U primer	TGG ATA GGG ATA GGT AAG GTGA TAG T
	Reverse U primer	CCC A ATC AAC ATA ATA CAA AAC ATA
MT2A	Forward M primer	GAG TCG TAA GTG ATT TAG CGC
	Reverse M primer	TTC GCT AAA ACT TAA AAA AAA CGT A
	Forward U primer	TGG AGT TGT AAG TGA TTT AGT GTG G
	Reverse U primer	TTC ACT AAA ACT TAA AAA AAA CAT A

2.2.18. Statistical analysis

Data are expressed as the mean ± standard deviation. The significance of observed differences was evaluated using the two-tailed t test. Probabilities of $p< 0.05$ were regarded as statistically significant.

Chapter 3:

EFFECT OF CADMIUM ON THE MORHOLOGY AND VIABILITY OF HUMAN ALVEOLAR CELLS

The important thing is to never stop questioning

-Albert Einstein

3.1 INTRODCUTION

One of the main sources of exposure to cadmium by the pulmonary epithelium is the cigarette smoke. Studies show that one cigarette contains about 1-3µg of cadmium (Ashraf 2011; Ashraf 2012; Bernard 2008). About 7-10% of this cadmium enters the biological system; rest is trapped in different parts of cigarette, posing health as well as environmental hazards which put both active as well as passive smokers at risk (Ashraf 2012).

In this chapter the effect of cadmium on *in vitro* cultured human A549 cells is studied. Reports have shown that cadmium can interfere with the dynamics of actin microfilaments in various cell types (DalleDonne et al, 1997; Go et al, 2013). This chapter deals with the connection between these changes in actin dynamics induced by cadmium and the expression of cell morphology regulating genes *cdc42, rac1* and *rho*A.

The cells were exposed to increasing concentration of cadmium chloride ($CdCl_2$) -0, 2.5, 5, 7.5,10 and 15µg/ml, for 24 hours. The extent of changes in cell viability, morphology and expression of cell morphology regulating genes were compared with that of non- treated control.

The cells showed decrease in cell viability as a function of increasing cadmium concentration. Microscopic analysis revealed vast variance in cell shape in cadmium treated cells compared to that of the control. mRNA expression studies on cell morphology regulating small GTPase genes *cdc42*, *rac1* and *rhoA* revealed that the expression is in comparison with the corresponding morphological changes exhibited by the cells.

3.2. RESULTS

3.2.1. Effect of cadmium on A549 cell viability

A549 cells showed decrease in viability with increasing cadmium concentration (Figure 3.2.1 A and B). Both assays- total viable cell count with trypan blue and resazurin cell viability assay gave comparable results. The viability of cells in non template control as well as saline control was almost equal. 24 well plates showing resazurin reduction in $CdCl_2$ treated A549 cells as well as in non -treated controls is shown in Figure.3.2.1.C.

3.2.2. Effect of cadmium on A549 cell morphology

A549 cells exposed to increasing concentration of $CdCl_2$ exhibited vast difference in their morphology compared to the non treated control cells (Figure.3.2.2.A and B). As the concentration of $CdCl_2$ increased cells appeared stressed with thinner longer cell-connections spread in all direction. Most of them had stress granules (Figure.3.2.2.B). At higher $CdCl_2$ dosage like 10 and 15 μg/ml cells appeared smaller; many of them were round and came out of the attachment and were floating in the media. The morphology of the cells in control remained the same (Figure.3.2.2.B).

Figure. 3.2.1

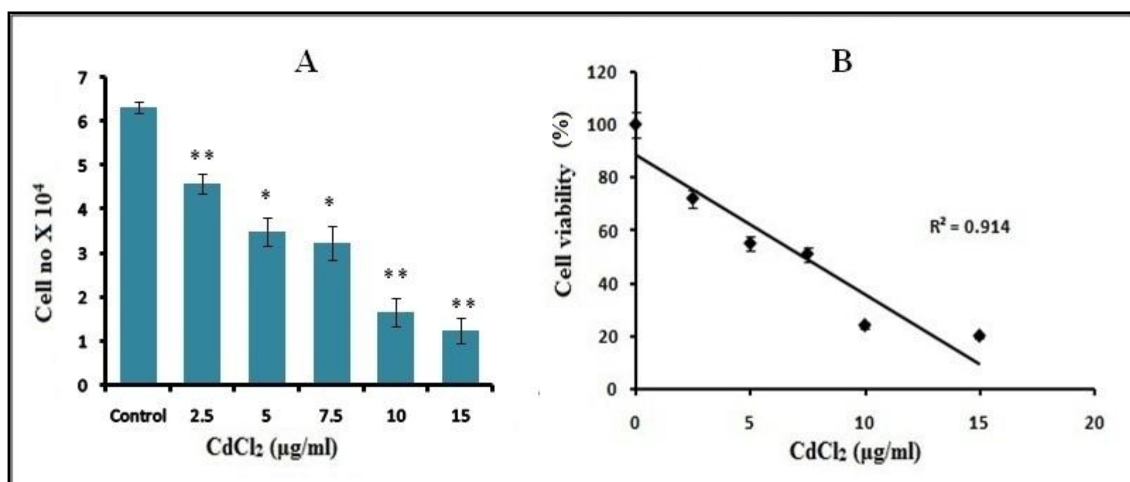


Figure. 3.2.1. A. Total viable Cell count: Decrease in cell number with increasing concentration of cadmium chloride for 24hours. Calculated IC_{50} value for cadmium in A549 cells was 9.1±.0486. Data expressed as mean ±SD. Values represent results from a two-tailed Student's t test. *p<0.05 and ** p<0.01. **B. Resazurin Reduction Assay:** Increase in cytotoxicity with increase in cadmium concentration (for 24 hours).

Figure.3.2.1. C

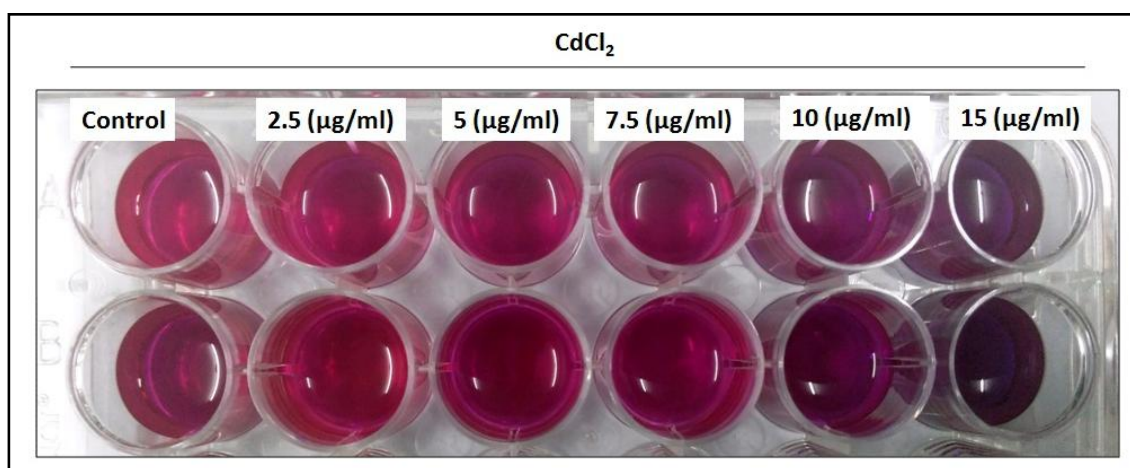


Figure.3.2.1. C. Resazurin Assay (In duplicates). 24 well plate showing resazurin reduction in $CdCl_2$ treated A549 cells compared to the non- treated controls.

Figure.3.2.2.A

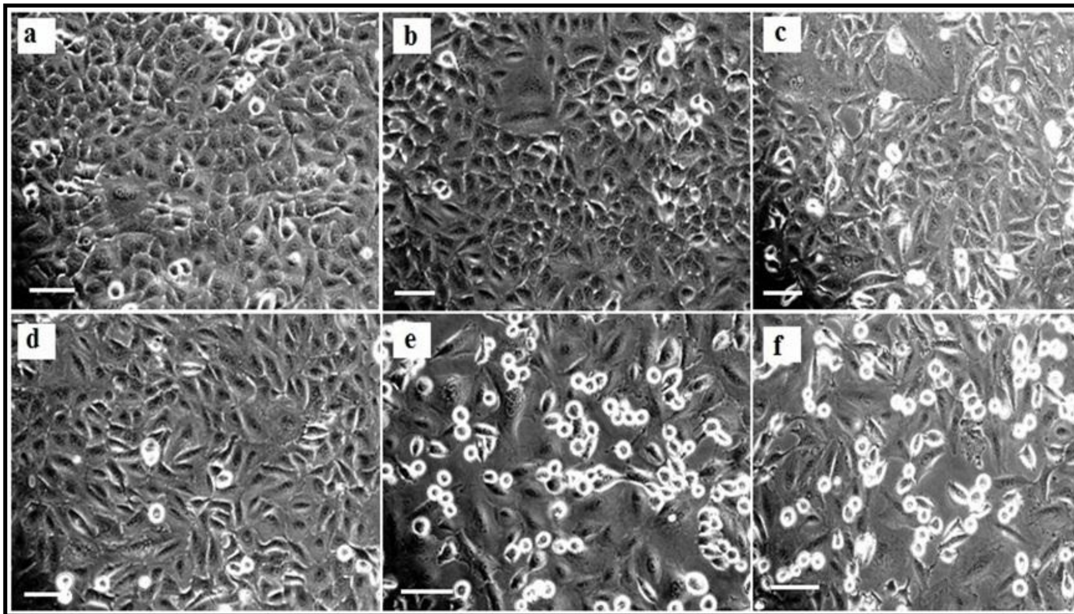


Figure.3.2.2.B

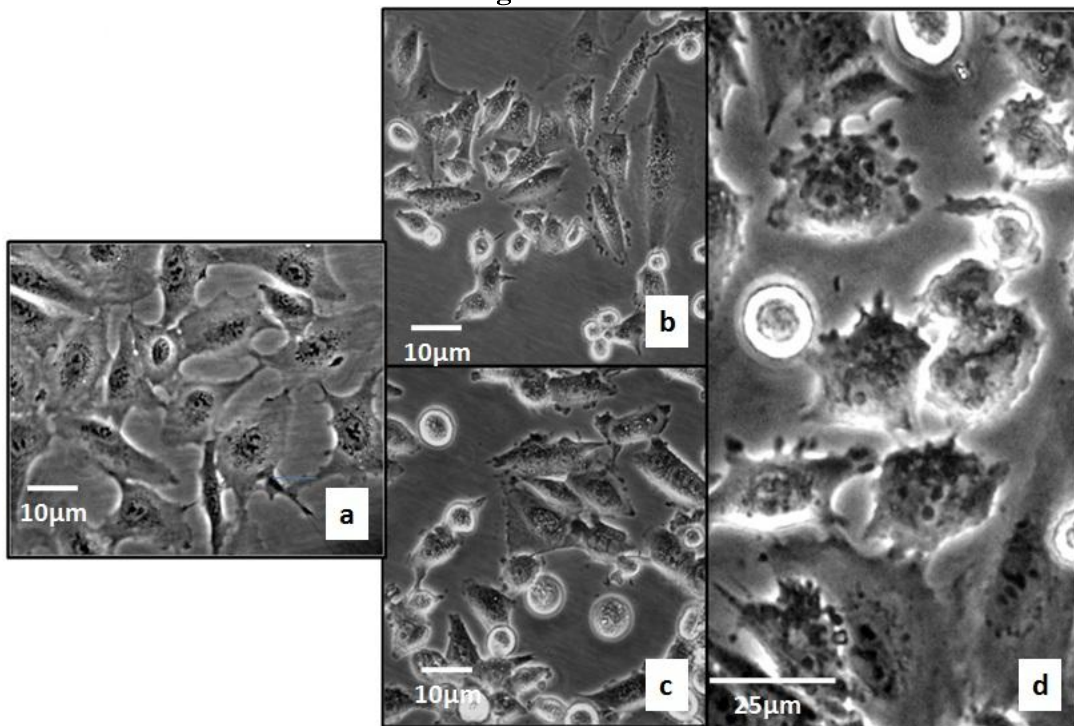


Figure.3.2.2. Effect of cadmium on A549 cell morphology. A: Micrograph of A549 cells exposed to various concentration of $CdCl_2$ (Scale 20µm). In the picture (a), (b), (c),(d),(e), (f) corresponds to non treated control (NTC) 2.5, 5.0, 7.5, 10 and 15 µg/ml of $CdCl_2$.. **B:** Enlarged morphology of control as well as cadmium treated A549 cells. a: control cells; b, c & d: cells exposed to15 µg/ml of $CdCl_2$.

3.2.3. Effect of cadmium on small GTPase expression and actin dynamics in A549 cells

Cadmium treated cells showed different degree of *rac1*, *rhoA* and *cdc42* mRNA expression in response to increasing concentration of cadmium compared to that of the non treated control (Figure. 3.2.3. A). *cdc42* mRNA level increased with increase in cadmium concentration initially up to a cadmium exposure of 7.5 µg/ml, then came with further increase in the concentration of exposed cadmium. *rac1* maintained same level of expression in cells up to an exposed cadmium concentration of 5 µg/ml and then the expression level came down below basal level in cells with an exposure of above 5 µg/ml $CdCl_2$. *rhoA* had similar expression pattern as *rac1*with the exception that its level started going below the basal level in cells when exposed to a cadmium concentration of 5 µg/ml of cadmium.

The actin dynamics of A549 cells was studied by fluorescent staining using phalloidin conjugated to FITC. More cellular connections as well as projections were visible in cadmium treated cells compared to that in control cells. Cells exposed to 10 µg/ml of *cdc42* appeared to be smaller with long projections thrown out in all directions compared to the control cells.

Figure.3.2.3.A

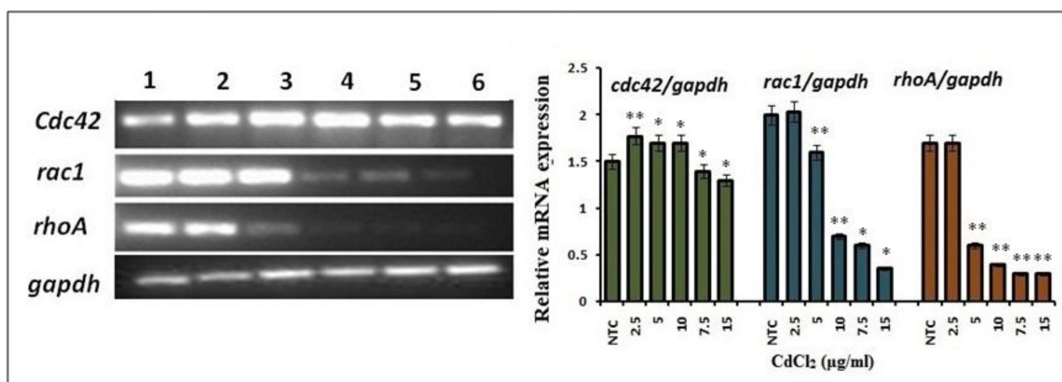


Figure.3.2.3.A. RT-PCR Profile of small GTPases: C-control,1-2.5µg/ml $CdCl_2$, 2-5.0 µg/ml $CdCl_2$, 3-7.5 µg/ml $CdCl_2$, 4-10 µg/ml $CdCl_2$, 5-15 µg/ml $CdCl_2$. Data expressed as mean ±SD. Values represent results from a two-tailed Student's t test. *p<0.05 and ** p<0.01 and ***p<.0001.

Figure.3.2.3.B

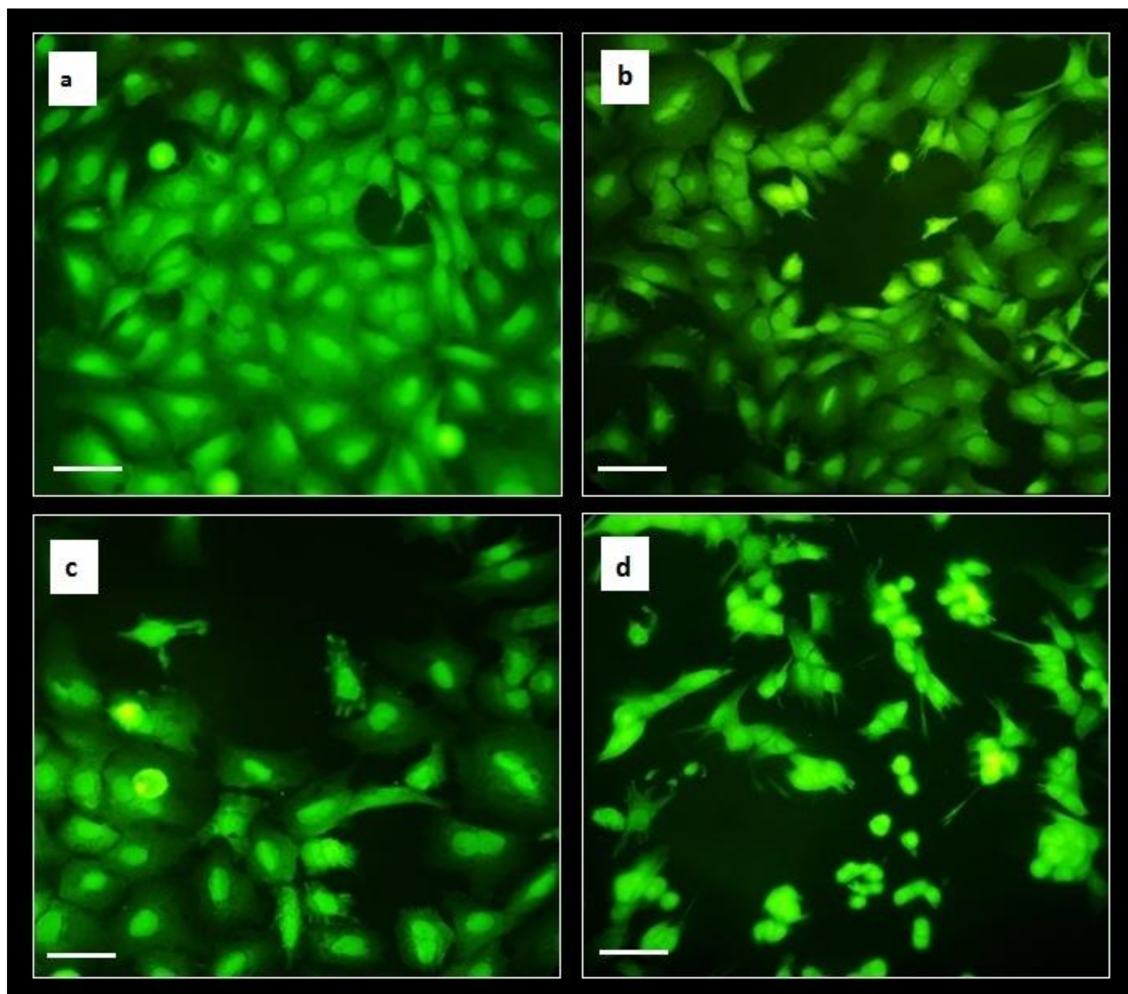


Figure.3.2.3.B. Study of Actin dynamics using phalloidin FITC. (a)-control, (b)-2.5µg/ml $CdCl_2$, (c)-5.0 µg/ml $CdCl_2$, (d)-10 µg/ml $CdCl_2$. Scale 10µm.

3.2.4. Effect of cadmium on the expression of Heat Shock Protein – HSP70 on A549 cells.

RT-PCR analysis of Heat Shock Protein, HSP70 showed an increase in mRNA expression with $CdCl_2$ exposure, compared to that in control cells till a concentration of 7.5 µg/ml. In cells exposed to 10µg/ml the expression of HSP70 mRNA came down below the basal level. Interestingly protein expression showed a steady, linear increase in the level of stress induced HSP70 in A549 cells with increase in $CdCl_2$ dosage.

(Figure.3.3.4). Though the *HSP70* mRNA level increased with cadmium dosage it came below the basal level on exposure to10µg/ml of $CdCl_2$.But the protein level increased with increase in $CdCl_2$ concentration and remained steady even at an exposure of 10µg/ml of $CdCl_2$ even after the decline in the level of its mRNA.

Figure.3.2.4

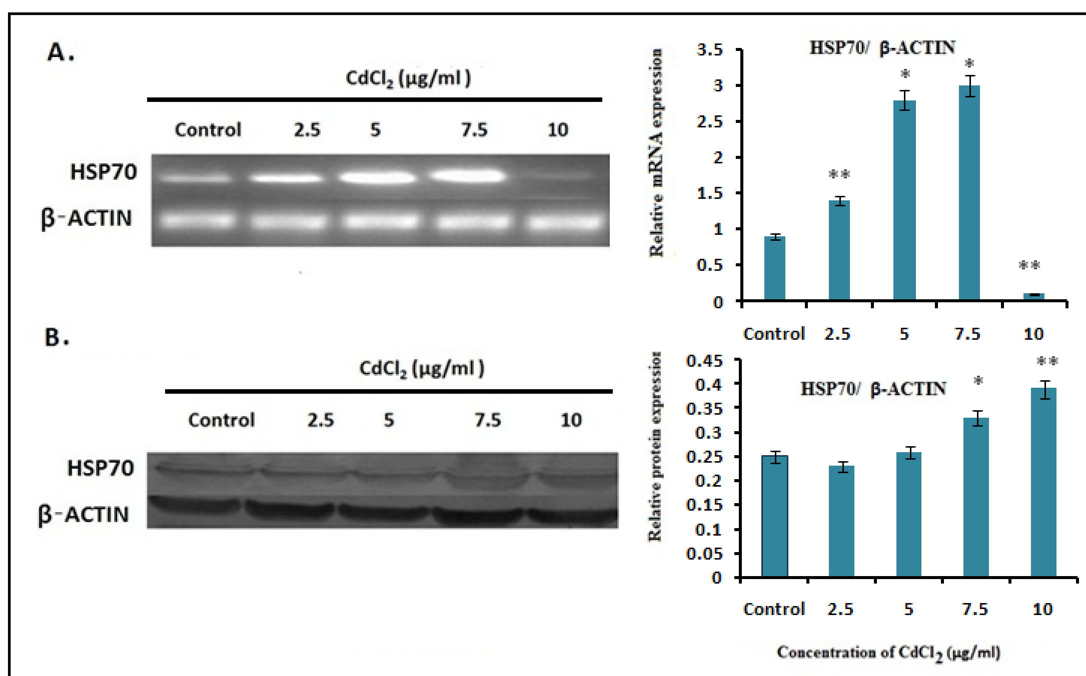


Figure.3.2.4. Expression of HSP70 in $CdCl_2$ treated A549 cells. A. RT PCR result of HSP70 in $CdCl_2$ treated A549 cells compared to the non treated control.
B Western Blot analysis of HSP70 expression in $CdCl_2$ treated A549 cells compared to the non treated control. Data expressed as mean ±SD. Values represent results from a two-tailed Student's t test. *$p<0.05$ and ** $p<0.01$.

3.3. DISCUSSION AND CONCLUSION

Studies on various cell lines have shown that cadmium causes cell death in a concentration dependent manner (Aimola et al, 2012; Kim et al, 2003) Cadmium has a significant effect on the cellular morphology of A549 cell line which can be related to the expression of cell morphology regulating genes like the members of small GTPases family, *rac1*, *rhoA* and *cdc42*.

Cadmium had a dose dependent effect on A549 cell viability. In response to increase in cadmium dosage cells appeared to be stressed and their viability decreased.

In resazurin assay the blue dye resazurin is reduced to pink resorufin by mitochondrial NADP/NADH dehydrogenase enzymes making this assay useful to quantify mitochondrial metabolic activity (Zhang et al, 2004, Abu-Amero and Bosley, 2005). Resazurin cytotoxicity indicated a decrease in overall mitochondrial respiration rate in cadmium exposed cells as compared to that in control. This shows cadmium can interfere with cellular respiration leading to impaired cellular metabolism and eventual cell death.

Apparent morphological changes were visible in the cadmium exposed cells compared to the control cells. As the concentration of cadmium increased more number of cells appeared rounded off and came out of attachment. The attached monolayer consisted of cells that looked stressed and their extensions thrown out in all directions. They were filled with stress granules and were disfigured compared to control cells.

Cells produce heat shock proteins in response to physiological as well as environmental stress; especially the HSP70 family. They help the cells to withstand stress, speed up the recovery process and confer resistance. The expression of HSP70 protein increased with cadmium dosage indicating the increased stress in cells induced by cadmium and corresponding cellular response to withstand that adverse condition. Even though the mRNA level decreased at higher $CdCl_2$ concentrations the protein level was steady. This might be because of the stabilization of protein while shutting down further mRNA synthesis to slow down cellular metabolism during adverse condition or the mRNA would have simply undergone degradation in the hostile toxic environment.

Expression studies on genes involved in the regulation of cell morphology i.e., *rac1*, *rhoA* and *cdc42* showed cells expressing mRNAs of these genes in varying degree in

response to cadmium concentration compared to that of non treated control cells. The interplay of *rac1* and *rhoA* genes regulates cell-cell adhesion as well as the attachment of cells to the substratum. Decrease in mRNA levels of *rac1* and *rhoA* gene with increase in cadmium concentration can be related to the rounding off of the cells. The up regulation of *cdc42* gene in cells exposed to a concentration of 5 and 7.5 µg/ml may be a transition stage in which cell requires lot of actin reorganisation for rounding up of cells. There was a significant increase in cellular projections like microspikes and filopodia in cells at these particular concentrations which might be because of increasing cell to cell communication for help in response to an adverse condition or can be a chemotactic response to migrate to a healthy environment; away from the toxic heavy metal. Small GTPases like *cdc42* and *rhoG* are found to help in cellular migration during chemotaxis (Monypenny et al, 2009). *rhoA* regulates the formation of actin stress fiber and focal adhesion assembly; *rac1* promotes lamellipodium or membrane ruffling formation; *cdc42* induces actin microspikes and filopodium formation (Wennerberg et al 2005). Fluorescent studies with phalloidin FITC clearly showed the increase in filopodia and microsikes projections in the cells expressing elevated levels of *cdc42* mRNA (Figure.3.2.3.B).

The effect cadmium has on the expression of cell morphology regulating small GTPase genes and the corresponding morphological variation was in concordance with their expression pattern. In conclusion cadmium induces considerable changes in cellular morphology by altering the expression of cell shape regulatory genes -small GTPases.

Chapter 4:

EFFECT OF VITAMIN B_{12} ANALOG, METHYLCOBALAMIN ON CADMIUM INDUCED STRESS IN A549 CELL LINE

Nothing in life is to be feared, it is only to be understood

-Marie Curie

4.1. INTRODUCTION

Methylcobalamin, (MeB_{12}) is a biologically active form of vitamin B_{12} (Miranda-Massari et al, 2011). It is chemically a cobalamin where the upper axial ligand linked to the cobalt ion is a methyl group. It is the main circulating form of vitaminB_{12} and is involved in the conversion of homocytsteine to methionine there by reducing plasma homocysteine levels (Koyama et al, 2002). It is known to improve neural health and functioning (Miranda-Massari et al, 2011; Watanabe et al, 1994; Nishimoto et al, 2015), hence can be used for the treatment of various neurological disorders (Okada et al, 2010; Kuwabara et al, 1999). It is also known to act as methyl donor in DNA methylation reactions and can regulate gene expression at higher concentrations (Kuwabara et al, 1999; Lcszkowicz et al, 1991).

In this chapter the effect of MeB_{12} on cadmium exposed A549 cells is studied. A549 cells were grown in media containing MeB_{12} to check whether it can help the cells resist cadmium induced stress. A549 cells were grown in media supplemented with two different concentrations of MeB_{12} (50 & 100 µg/ml) for 48 hours before exposing them to different concentrations of cadmium chloride (0-10µg/ml) for 24 hours. Microscopic analysis, cytotoxic assays and DNA damage studies revealed that MeB_{12} enhanced cadmium induced cell death. Studies on the expression pattern of mRNA levels of various genes involved in the signalling pathways of inflammatory response and programmed cell death showed a huge variation between the cellular signalling in the cells grown in containing media MeB_{12} compared to those grown in normal media following an exposure to cadmium.

4.2. RESULTS

4.2.1. Effect of MeB_{12} on the morphology and viability of A549 cells exposed to $CdCl_2$

A549 Cells at the exponential stage of growth were seeded in media supplemented with 50 and 100µg/ml MeB_{12} for 48 hours while maintaining one set of cells in regular medium. After 48 hours, these three experimental sets were exposed to various doses of $CdCl_2$ –control (non- treated cells), 2.5, 5 and 10µg/ml $CdCl_2$. After 24 hours of treatment cells were harvested and subjected to various studies like microscopic analysis, total viable cell count and resazurin assay [Refer. 2.2.3-2.2.6].

Cells in MeB_{12} treated sets (50 and 100 µg/ml) in comparison with cells in non treated sets showed more sensitivity towards cadmium as is evident from the IC_{50} values (Table.4.2.1). Results of resazurin assay indicate a dose dependent decrease in overall mitochondrial respiration rate in A549 cells, in response to cadmium. The decrease in mitochondrial respiration in response to cadmium was more in MeB_{12} treated cells, compared to the non treated cells. Also this sensitivity towards cadmium, increased with MeB_{12} concentration in the media (Figure.4.2.1.C & D). Microscopic analysis of the cells indicate that the cell death and stress induced morphology by cadmium was more in A549 cells in MeB_{12} sets. Also at a given cadmium concentration, cell viability decreased more with increase in MeB_{12} concentration in the media as is evident from the IC_{50} values (Figure.4.2.1.A & B) (Table.4.2.1). But the cells grown only in 50 and 100 µg/ml MeB_{12} showed similar degree of viability and similar morphology as the control A549cells (Figure 4.2.1. A- ii & iii and Figure 4.2.1.C & D) which shows MeB_{12} alone had no adverse effect on the cells.

MeB_{12} had no protective effect on cadmium induced stress in A549 cells as presumed. More over whatever adverse effect cadmium had on the cells, got aggravated in the presence of MeB_{12}. MeB_{12} alone appeared to have almost no effect on the cells. The reported antioxidant effect of MeB_{12} was not seen in the case of cadmium toxicity in A549 cells.

Figure.4.2.1.A

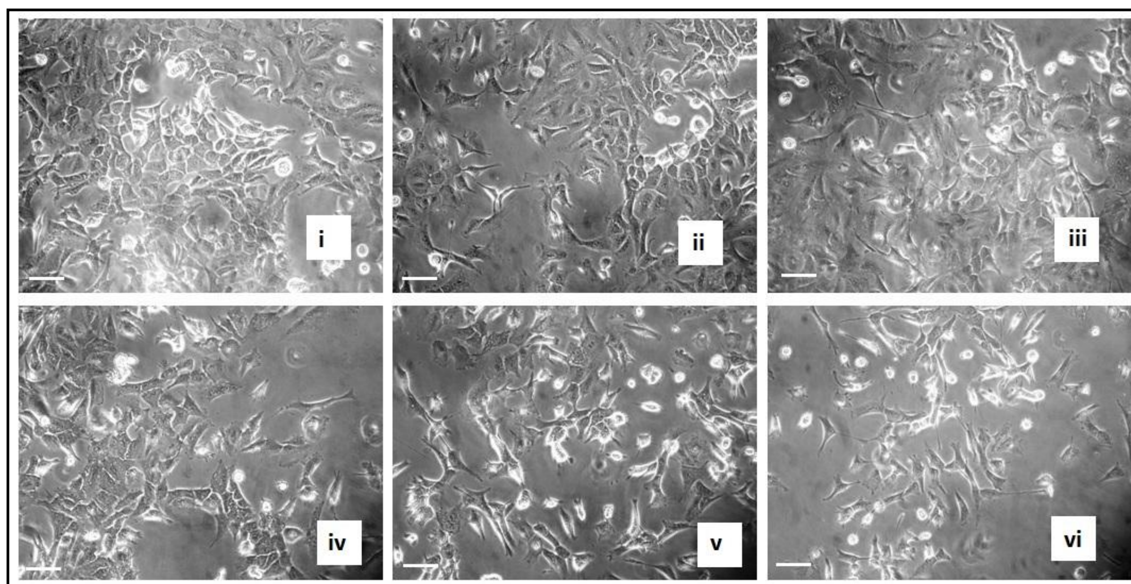


Figure.4.2.1.A. Effect of cadmium on A549 cell morphology.

In the picture A,
(a) and (b) correspond control cells and A549 cells in 10µg/ml of $CdCl_2$ respectively.
(c) and (d) correspond to control A549 cells grown in medium containing 50µg/ml MeB_{12} and A549 cells grown in medium containing 50µg/ml MeB_{12}, exposed to 10µg/ml of $CdCl_2$ respectively,
(e) and (f) correspond to control A549 cells grown in medium containing 100µg/ml MeB_{12} and A549 cells grown in medium containing 100µg/ml MeB_{12}, which is exposed to 10µg/ml of $CdCl_2$ respectively. Scale -10 µm.

Figure.4.2.1.B

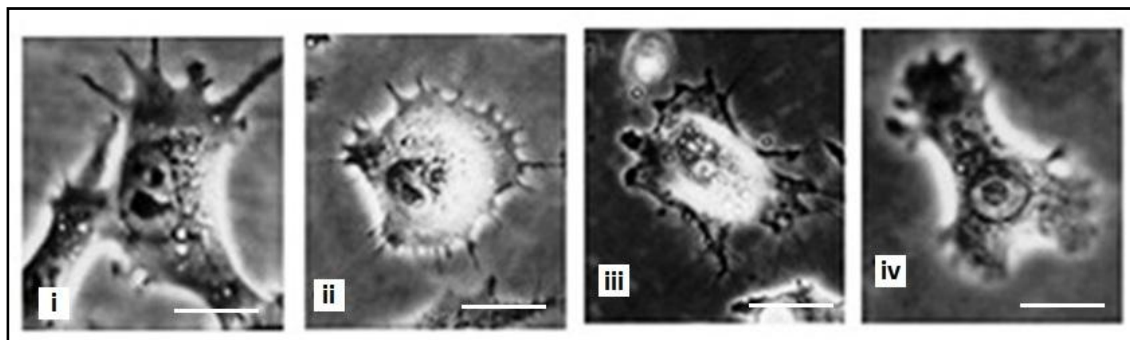


Figure.4.2.1.B Enlarged morphology of A549 cells exposed to 10µg/ml $CdCl_2$.

(i) - A549 cell grown in normal medium, (ii) - A549 cell grown in normal medium and exposed to 10µg/ml of $CdCl_2$, (iii)-A549 cell grown in 50 µg/ml MeB_{12} and exposed to 10µg/ml of $CdCl_2$, (iv) - A549 cell grown in 100 µg/ml MeB_{12} and exposed to 10µg/ml of $CdCl_2$. Scale bar -25 µm.

Figure.4.2.1.C

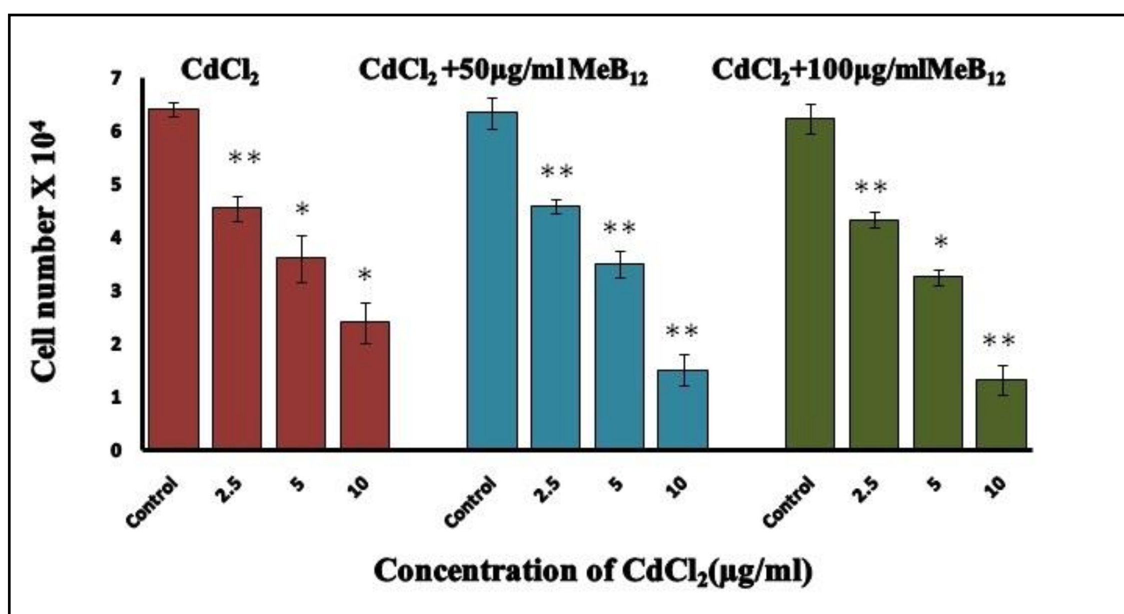


Figure.4.2.1.C. Total viable cell count: The data of three individual experiments are summarised in this figure. Values represent results from a two-tailed Student's t test. *$p<0.05$ and ** $p<0.01$.

Figure.4.2.1.D

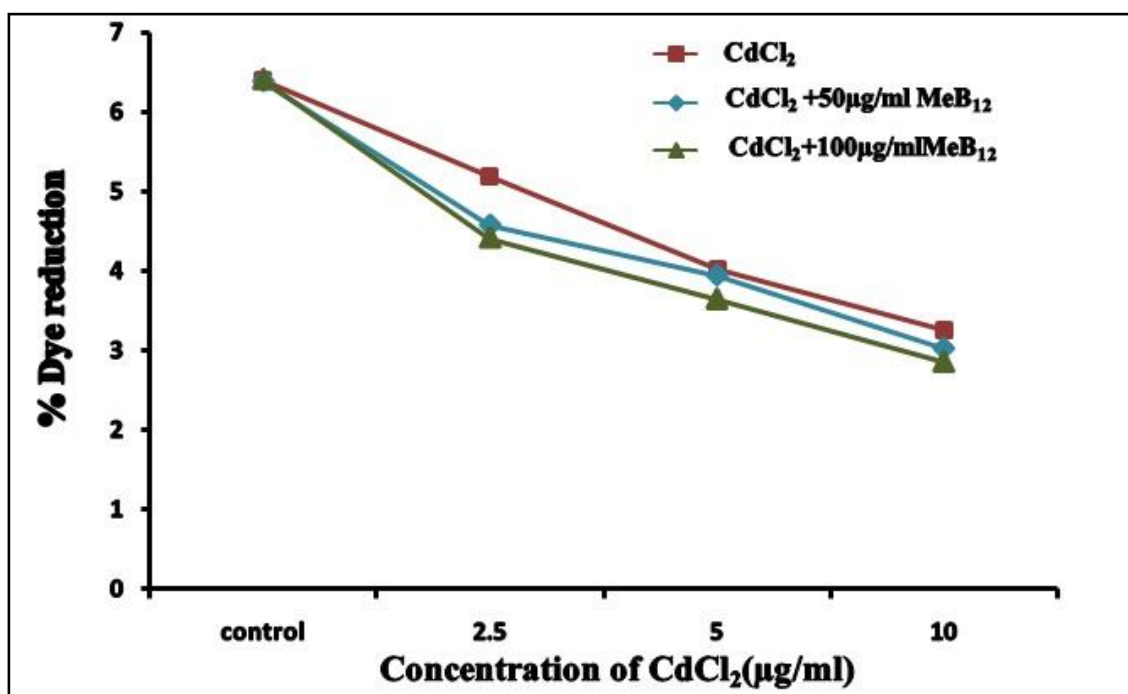


Figure.4.2.1.D. Cell viability assay: After 24 hours incubation at 37° C, the viability was measured using Resazurin reagent and related to untreated controls (set to 100%). The data of three individual experiments are summarised in this figure.

Table.4.2.1

Experiment Set	$CdCl_2$	$CdCl_2$+50µg/ml MeB_{12}	$CdCl_2$ +100 µg/ml MeB_{12}
IC_{50} values (µg/ml of $CdCl_2$)	9.2 ±0.841	8.5±0.543	7.8 ±0.657

4.2.2. Assessment of synergy between $CdCl_2$ and MeB_{12}

Since there was an increased degree of cell death when MeB_{12} is combined with $CdCl_2$ an isobolographic study was conducted to see whether these two chemicals exhibit synergy. Isobolographic analysis showed that $CdCl_2$ and MeB_{12} interact synergistically towards cell death in A549 cells. The IC_{50} value of $CdCl_2$ alone was 9.2 µg/ml, while

that of MeB_{12} was 920 μg/ml. But the same effect (IC_{50}) was achieved by the combinations of these two chemicals in various dose pairs below their individual IC_{50} values (Figure 4.2.2) there by confirming that the interaction between them is synergistic.

Figure.4.2.2

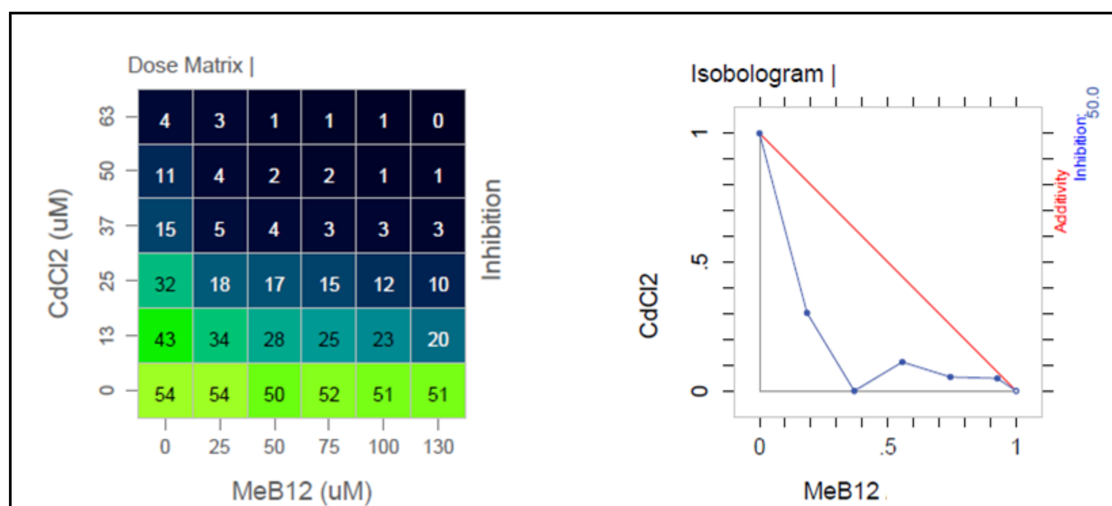


Figure.4.2.2. Dose matrix and Isobologram: Synergistic interaction between $CdCl_2$ and MeB_{12}, as measured by increase in cell death. A. Dose matrix showing Percentage increase in cell death, with MeB_{12} dose on horizontal axis and $CdCl_2$ dose on vertical axis. B. Isobologram showing dose pairs resulting in half the maximum cell death, IC_{50}.

4.2.3. Effect of MeB_{12} on cadmium induced nuclear damage

Nuclear damage induced by cadmium in A549 cells were measured by fluorescent staining with Hoechst 33342 dye. Cells were washed in PBS and fixed in 10% formaldehyde. After dehydration using 90% methanol, the monolayer was washed and stained with Hoechst 33342 (1 μg/ml). Stained nuclei were visualized under Nikon eclipse TS 100 fluorescence microscope [Refer. 2.2.15].

Cells in all the three experimental sets showed nuclear damage and chromatin condensation in response to cadmium. The extent of nuclear damage was more in

MeB_{12} treated cells and this effect increased with increase in MeB_{12} concentration i.e., more cells underwent nuclear damage in the experimental set where cells were treated with 100 µg/ml of MeB_{12}, when exposed to cadmium in comparison with the other two sets (Figure.4.2.3.A).

Figure.4.2.3

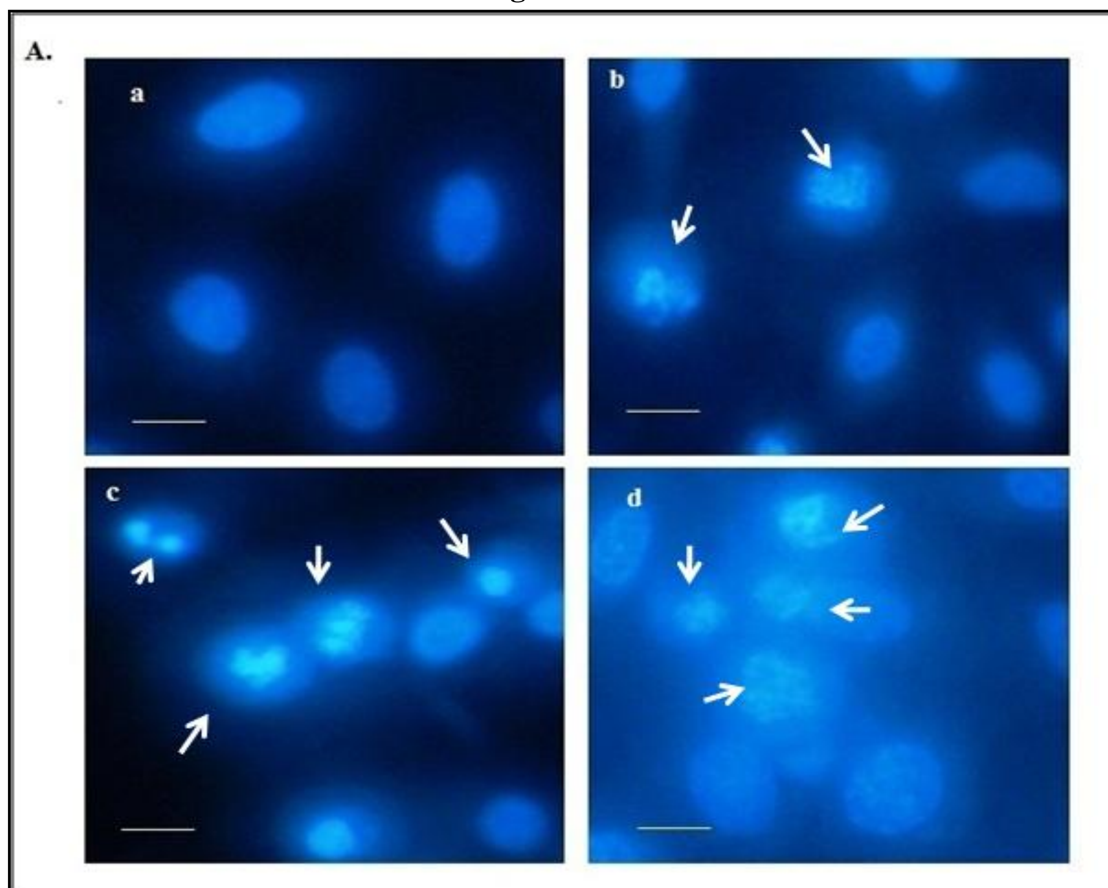


Figure.4.2.3. Nuclear Damage analysis using Hoechst staining:

In the picture A, (a) and (b) correspond control cells and A549 cells in 10µg/ml of $CdCl_2$ respectively.(c) and (d) correspond to control A549 cells grown in medium containing 50µg/ml MeB_{12} and A549 cells grown in medium containing 50µg/ml MeB_{12}, exposed to 10µg/ml of $CdCl_2$ respectively, (e) and (f) correspond to control A549 cells grown in medium containing 100µg/ml MeB_{12} and A549 cells grown in medium containing 100µg/ml MeB_{12}, which is exposed to 10µg/ml of $CdCl_2$ respectively. Scale -20 µm.

4.2.4. Effect of MeB_{12} on cadmium induced expression of Rho GTPases- *cdc42, rac1* and *rhoA, hsp70, nfκb* genes and actin dynamics

Cadmium exposed A549 cells showed difference in morphology in all the three sets compared to their respective control cells. So the expression pattern of cell morphology regulating genes *cdc42, rac1* and *rhoA*, from the gene family of Rho GTPases was studied. RT PCR results indicate similar expression pattern of all these three genes in A549 cells grown in regular medium, in response to cadmium. All of them were up regulated in cells exposed to 2.5 and 5 µg/ml of $CdCl_2$ compared to the control, but came down to basal level in cells treated with 10 µg/ml of $CdCl_2$. The expression of these three genes varied in cells grown in MeB_{12} containing media in response to increasing dosage of $CdCl_2$. A549 cells grown in medium containing 50 µg/ml of MeB_{12}, in response to cadmium showed up regulation of *cdc42* and *rac1* mRNAs while *rhoA* expression remained fairly equal in all the conditions. Cell grown in 100 µg/ml of MeB_{12} showed over expression of *rac1* in response to cadmium while *cdc42* as well as *rhoA* expressions remained almost equal.

Actin dynamics of A549 cells were studied by fluorescent staining using Phalloidin conjugated FITC. A549 cells were washed in PBS and were fixed in formaldehyde solution. The monolayer was washed in PBS, dehydrated with acetone and permeabilized with 0.1% TRITONX-100 in PBS, followed by PBS wash. Cells were stained with fluorescent phalloidin conjugate solution in PBS for 40 minutes at room temperature followed by PBS wash to remove unbound phalloidin conjugate. Stained cells were visualized under Nikon eclipse TS 100 fluorescence microscope [Refer. 2.2.14].

Study of actin dynamics using fluorescent phalloidin FITC staining indicated the expression of various cellular processes like filopodia, lamellipodia, stress fibres etc (Figure.4.2.4.A).

hsp70 mRNA level remained fairly equal in both control and test cells in all the three experimental sets, even after cadmium treated cells appeared to be in stressed condition. The expression level of *nfkb* on the other hand, increased in cells grown in regular media in response to increasing cadmium concentration. The expression of these two mRNAs came down gradually in cadmium exposed cells grown in MeB_{12} containing media. The mRNA level went further down in cells grown in 100 µg/ml of MeB_{12} compared to A549 cells in the other two sets (Figure.4.2.4.B).

Figure.4.2.4. A

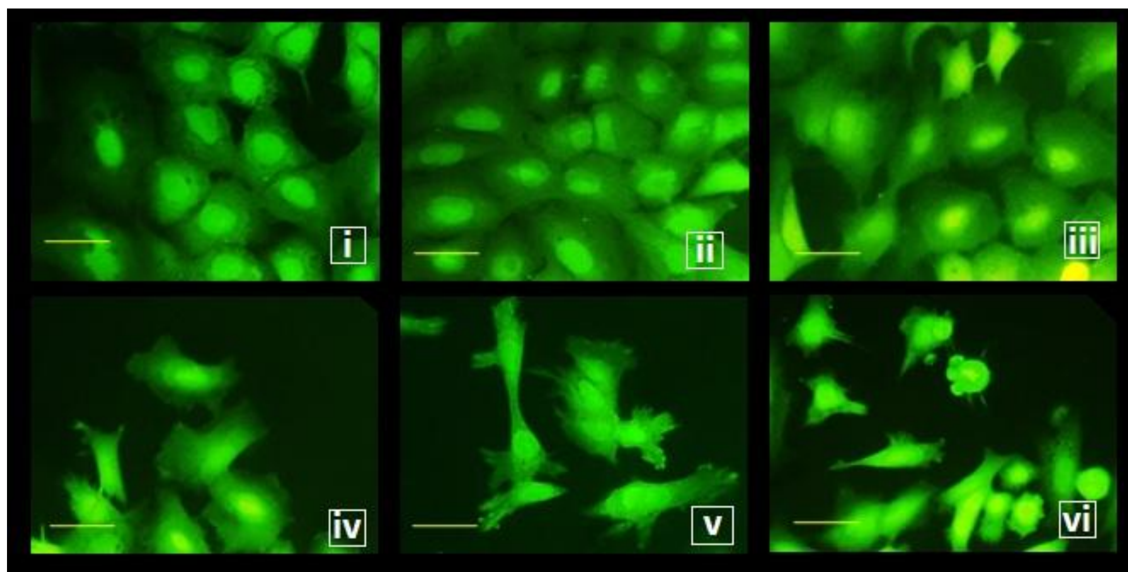


Figure.4.2.4.A. Effect of cadmium on A549 cell morphology.

In the picture A, (i) A549 cells grown in regular media, (ii) A549 cells grown in media supplemented with 50µg/ml of MeB_{12} (iii) A549 cells grown in media supplemented with 100µg/ml of MeB_{12} (iv) A549 cells (grown in regular media) exposed to 10µg/ml of $CdCl_2$, (v) A549 cells (grown in media supplemented with 50µg/ml of MeB_{12}) exposed to 10µg/ml of $CdCl_2$, (vi) A549 cells (grown in media supplemented with 100µg/ml of MeB_{12}) exposed to 10µg/ml of $CdCl_2$ .Scale bar- 20µm.

Figure.4.2.4. B

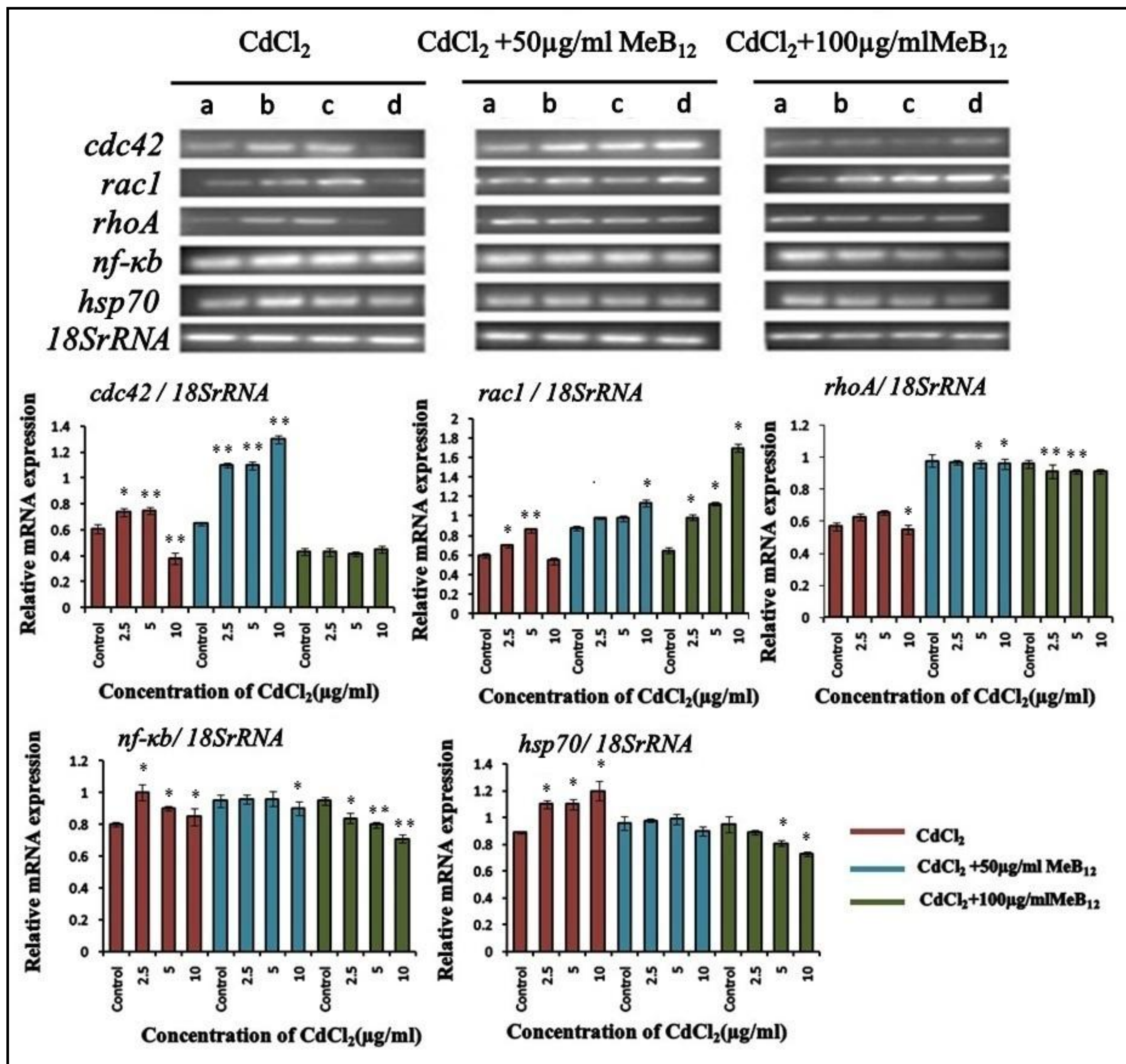


Figure.4.2.4. B. Expression Profile of *cdc42, rac1, rhoA, nfkb* and *hsp70*: Cells were grown in Media supplemented with 2 different concentrations of MeB_{12} (50 & 100 µg/ml) for 48 hours and both treated and non treated cells were exposed to increasing concentration of cadmium chloride (control, 2.5, 5.0, and10 µg/ml). After 24 hours total RNA was extracted, converted to cDNA and subjected to reverse transcriptase PCR against primers for *cdc42, rac1* and *rhoA, nfkb* and *hsp70*. Genes .(a)-control,(b)-2.5µg/ml $CdCl_2$, (c)-5.0 µg/ml $CdCl_2$, (d)-10 µg/ml $CdCl_2$. Data expressed as mean ±SD. Values represent results from a two-tailed Student's t test. *$p<0.05$ and ** $p<0.01$.

4.2.5. Effect of MeB_{12} on cadmium induced expression of apoptotic genes

Since nuclear damage was apparent in cells exposed to cadmium in all the three sets, mRNA expression of *p53* gene in them was also studied. A549 cells in all the three experimental sets showed down regulation of *p53* mRNA expression with increase in cadmium concentration. The extent of down regulation was more in cells exposed to cadmium, which are grown in MeB_{12} containing medium and this effect increased with increase in MeB_{12} concentration in the medium.

Even though clear nuclear damage was observed in cadmium exposed cells there was no corresponding increase in *p53* expression in response to it. More over there was a significant decrease in the level of *p53* mRNA expression, and it was more in cadmium exposed A549 cells grown in media supplemented with MeB_{12}.

Anti apoptotic gene *bcl2* showed significant increase in mRNA level in response to cadmium in the cells grown in normal medium, while pro apoptotic gene- *bax* mRNA level decreased with increasing cadmium.

In cells grown in MeB_{12} containing media the mRNA levels of both the genes were down regulated with increase in cadmium concentration. The degree of down regulation of *bax* was less in cells grown in 100 µg/ml of MeB_{12} while *bcl2* level was lesser even in the basal level (Figure 4.2.5).

Figure 4.2.5

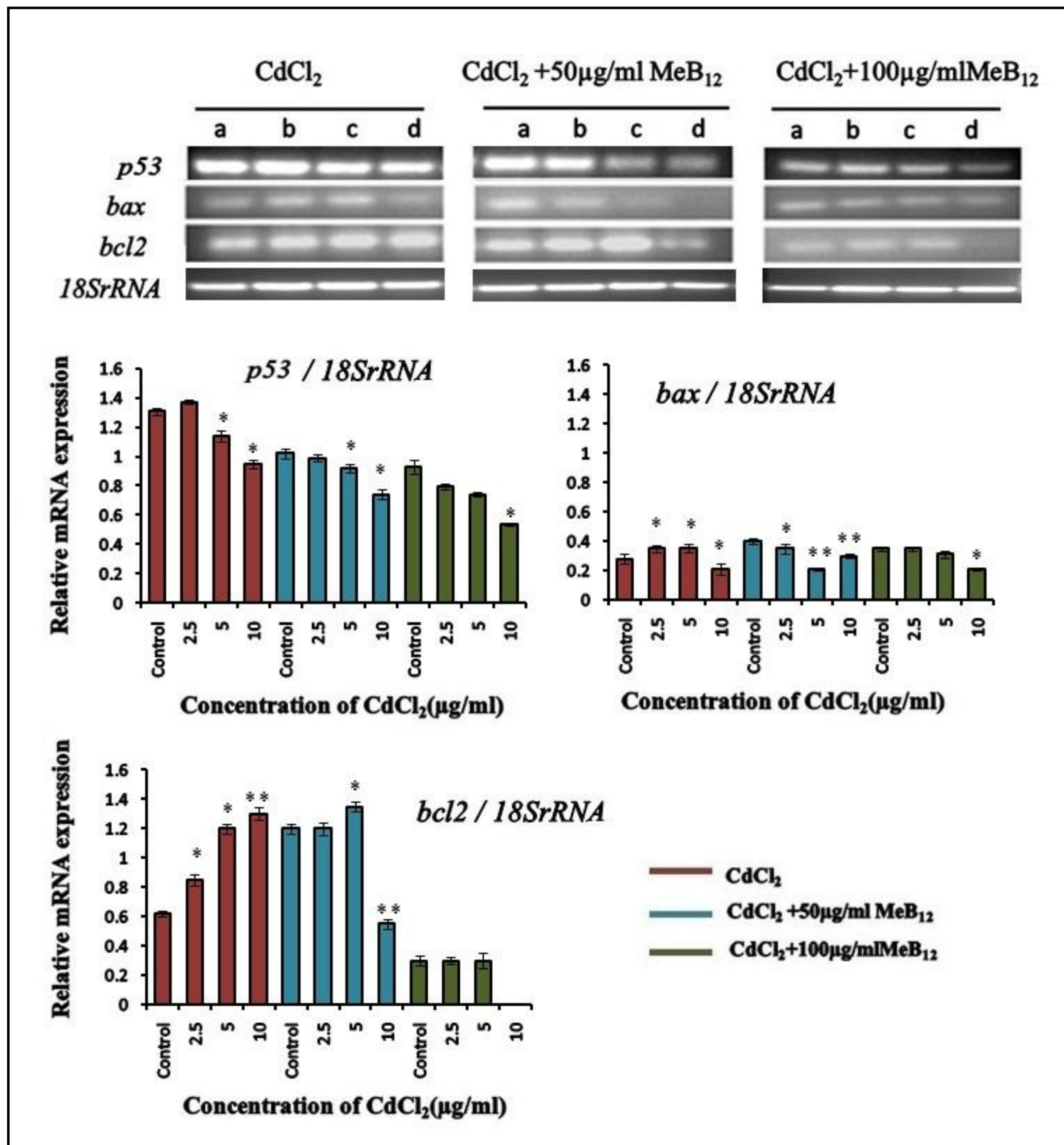


Figure 4.2.5. Expression profile of apoptotic genes: (a): control, (b): 2.5µg/ml $CdCl_2$, (c): 5.0 µg/ml $CdCl_2$, (d): 10 µg/ml $CdCl_2$. Data expressed as mean ±SD. Values represent results from two-tailed Student's t test. *p<0.05 and ** p<0.01 and ***p<.0001.

4.2.6. Effect of MeB_{12} on cadmium induced expression of metallothionien genes

Methallothionein gene subtypes - *mt1A* and *mt2A* showed slight but significant increase in mRNA levels in cells grown in regular medium with increasing concentration of cadmium. Interestingly, in cells grown in MeB_{12} containing media, with increase concentration of $MeB_{12,}$ the expression of both these genes decreased in response to cadmium dosage (Figure.4.2.6). *mt2A* gene appeared to be up regulated in cells grown in regular medium as we as in cells grown in 50 µg/ml of MeB_{12} on exposure to increasing cadmium concentration. But like *mt1A*, *mt2A* mRNA levels were too low, even in basal level in cells grown in 100 µg/ml of MeB_{12} (Figure.4.2.6).

Figure. 4.2.6

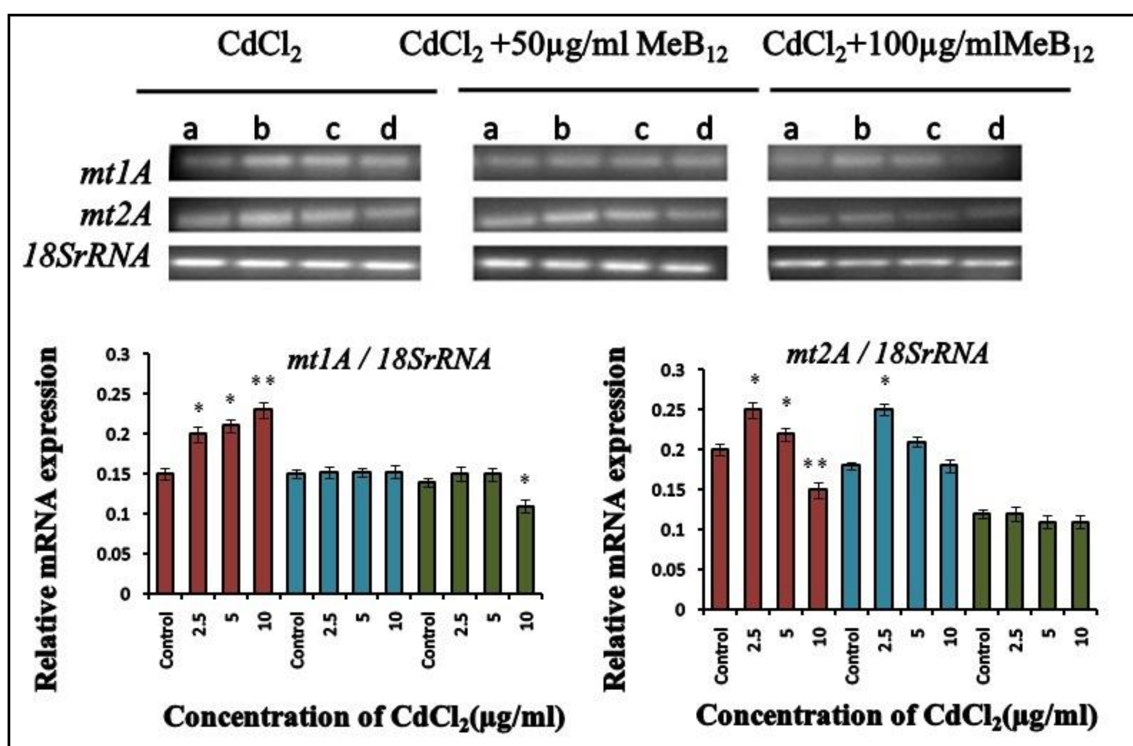


Figure. 4.2.6. Expression profile of metalothionein genes: a: control, b: 2.5µg/ml $CdCl_2$, c: 5.0 µg/ml $CdCl_2$, d: 10 µg/ml $CdCl_2$. Data are summarised in this figure and expressed as mean ±SD. Values represent results from two-tailed Student's t test. *p<0.05 and ** p<0.01

4.2.7. Promoter methylation studies of *MT1A* and *MT2A* genes

Promoter methylation of *MT1A* and *MT2A* genes was checked by methylation specific PCR after bisulfite modification of the genomic DNA. [Refer section 2.2.17]

Analysis of promoter methylation of metallothionein genes *MT1A* and *MT2A* revealed that there is already a significant amount of methylation in *MT1A* promoter sequence in A549 cells (Fig. 4.2.7). However, *MT2A* gene promoter appeared to be unmethylated. There was a slight degree of methylation in MeB_{12} treated A549 cells. Interestingly, this effect was further enhanced in the presence of cadmium. An increased level of methylation was observed in the promoter region of *MT2A* gene in the presence of $CdCl_2$ and MeB_{12} (Fig. 4.2.7).

Figure. 4.2.7

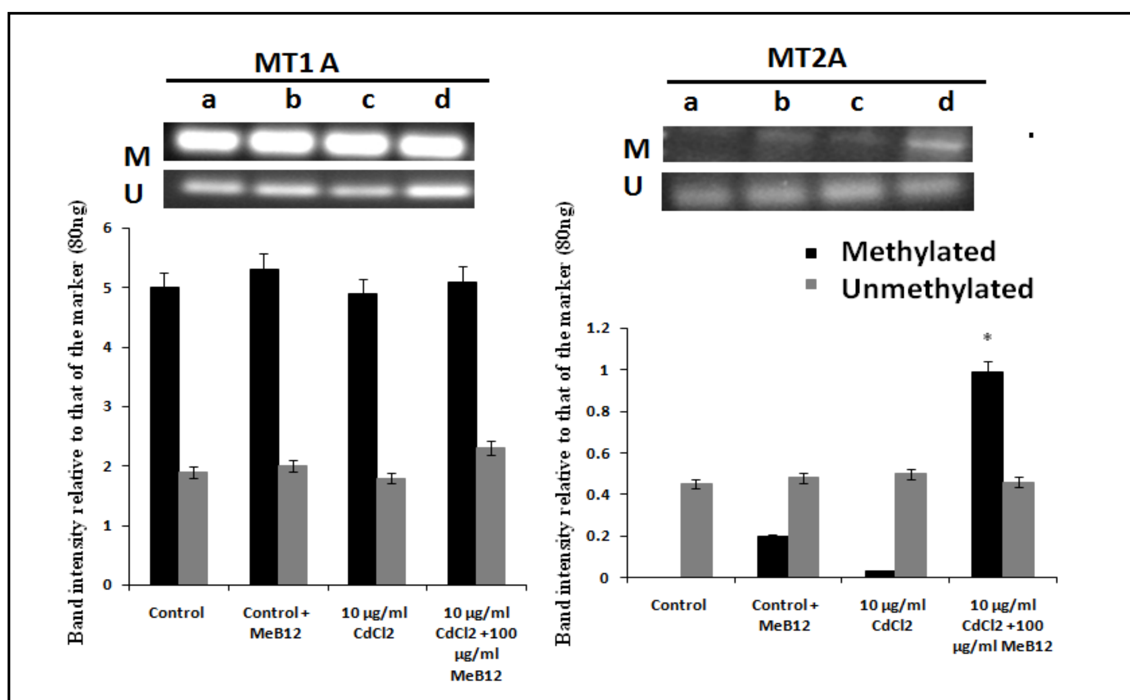


Figure. 4.2.7. Methylation specific PCR of MT1A and MT2A promoter sequences. a: control, b: 100µg/ml MeB_{12}, c: 10 µg/ml $CdCl_2$, d: 100µg/ml MeB_{12} +10 µg/ml $CdCl_2$. Data from three individual experiments are summarised in this figure and expressed as mean ±SD. Values represent results from two-tailed Student’s t test. *$p<0.05$

4.3. DISCUSSION AND CONCLUSION

This study was initiated to see whether MeB_{12} has any protective effect on cadmium induced cytotoxicity in A549 cell line since various reports had shown this effect of MeB_{12} on different types of cells suffering from different kinds of toxicity (Okada et al, 2010; Mikhailov et al, 1983; Mori et al, 1991).

The result from various studies explained above show that MeB_{12} has a lethal effect along with the heavy metal cadmium, on A549 cells. The overall expression pattern of the mRNAs of the genes involved in the cell signaling like *cdc42, rac1, rhoA*, *p53, bax, bcl2, nfκb* etc during cadmium toxicity was altered in the presence of MeB_{12}.

Cell death was linear with respect to increase in cadmium concentration in all the three experimental sets. Lower IC_{50} values in cells grown in MeB_{12} containing media shows that cadmium is lethal for them at a relatively lower concentration compared to the cells grown in regular medium.

Fluorescent staining with phalloidin FITC showed cells expressing characteristic actin filamental structures in response to the expression of their specific regulatory Rho GTPase genes -*cdc42, rac1*and *rhoA*. Rho GTPase genes control the behavior and organization of actin cytoskeleton and thus regulate the morphology of the cell (Hall and Nobes, 2000). *rac1* promotes the formation of lammellipodia or membrane ruffling- projections on the leading edge of the cell, made of actin (Ballestrem et al, 2000) *cdc42* induces the formation filopodia or microspikes - actin filled membrane projections that extend beyond the leading edge of lamellipodia (Krugmann et al, 2001)

and *rhoA* regulates the stress fiber formation which are actually contractile actin bundles (Boyer et al, 2003).

A549 cells grown in 50µg/ml MeB_{12} on exposure to 10µg/ml $CdCl_2$ showed increased level of *cdc42*. The cells appeared to have more filopodial projections along their membrane compared to the control cells. Cells grown in media containing 100µg/ml MeB_{12} had up regulated *rac1* and *rhoA* mRNAs on exposure to 10 µg/ml $CdCl_2$ and appeared to have comparatively more stress fibers and lamellipodia than control and other cadmium treated cells.

Up regulation of Rho GTPases, *rac1* and *rhoA* may be for activation of NFκB- a hall mark molecule of inflammatory response. RHOA -NFκB interaction is found to be important in cytokine (like TNFα) activated NFκB signalling processes (Xu et al, 2006)for the activation the NFκB response RAC1 is required (Boyer et al, 2003).

Heat shock proteins are members of protein family expressed by cells in response to adverse conditions (Ritossa, 1996). *HSP70* mRNA expression was fairly equal in control as well as treated cells in all the three sets. *Nfκb* was slightly up regulated in cells grown in normal medium, but showed a gradual decrease in expression in cells grown in MeB_{12} supplemented media, in response to cadmium. This shows that the inflammatory response was initiated in cells in normal media in the presence of cadmium while it was suppressed in cells grown in MeB_{12} supplemented media. In conclusion MeB_{12} appears to have suppressed the inflammatory response making the cells more vulnerable to cadmium induced stress and toxicity. More over it acted in synergy along with cadmium thereby by increasing cell death in A549 cells.

In response to DNA damage the level of P53 is known to rise drastically in a cell (Kastan et al, 1991). Here the *p53* gene expression in cells in all the three experimental

sets showed a gradual down regulation with increasing cadmium concentration. The down regulation of *p53* gene in these cells, in response to increasing cadmium concentration indicates that the programmed cell death (which can be P53 dependent or independent (Liebermann et al, 1995) involved in these cases may be through P53 independent pathway. Another plausible explanation for the *p53* mRNA down regulation is the possible degradation of mRNA in toxic cadmium environment. In this case P53 protein may be protected by post translation modification like phosphorylation and is available for mounting a stress response, making it possible the cell death to be P53 dependent.

A549 cells grown in regular medium expressed increased level of anti apoptotic gene *bcl2* in response cadmium. Elevated level of *bcl2* is one of the factors determining cell survival (Chang et al, 2007) and this would have helped them to resist programmed cell death to an extent. The expression of *bcl2* was gradually lowered in cadmium treated cells with increase in concentration. Cells grown in media containing 100µg/ml of MeB_{12} showed not only decreased expression of *bcl2* but higher level of *bax* expression.

Metallothioneins are small metal binging cysteine rich proteins induced in the cell in the presence of a metal. MT1 and MT2 are two major isoforms of this protein (Yagle and Palmiter, 1985), Expression of these isoforms is regulated by the transcription factor MTF1 (Metal-responsive transcription factor1). MTF1 binds to the metal response elements (MRE) located in the promoter region of Metallothionein genes (Yagle and Palmiter, 1985; Wimmer et al, 2005). Cadmium can induce the expression of MT1 and MT2 and they are found to play protective roles against cadmium induced cytotoxicity (Wimmer et al, 2005; Masters et al, 1994).

mRNA expression levels of these two isoforms increased in response to cadmium A549 cells grown in regular medium. In both the MeB_{12} sets the expression of these two isoforms were seen to be decreasing with increasing concentration of cadmium. Disruption of these two genes in mice has shown that it makes them susceptible to cadmium (Masters et al, 1994). These results show that MeB_{12} may be making the cells susceptible to cadmium by down regulating the mRNA expression of two metallothionein isoforms *mt1A* and *mt2A*.

Further studies on promoter methylation revealed that *MT2A* gene promoter is indeed getting methylated in the presence of MeB_{12}, and the level of methylation appeared to be enhanced in the presence of cadmium, providing more evidence on the synergy between these two compounds (Fig.4.2.7).

Cadmium enters the cell through protein channels, causes misfolding of proteins, DNA damage, oxidative stress etc; leading to programmed cell death. Elimination of cadmium from the cells involves the activation of the transcription factor MTF1 and its subsequent translocation to nucleus. In nucleus MTF1 binds to metal response elements (MRE) in the promoter region of its target genes like *mt1A* and *mt2A* and initiate their transcription. MT1 and MT2 proteins bind to cadmium there by sequestering it and preventing it from binding to cellular proteins and organelles, alleviating the cellular damages caused by cadmium. One of the mechanisms of down regulation of metallothionein genes transcriptionally is the methylation of the MREs or Metal Response Elements in their promoter region (Ghoshal et al, 2000). So being a methylating agent the possible mechanism of down regulation of metallothionein genes by MeB_{12} may be through methylation of promoter region of these genes (summarized in Figure.4.3).

Figure.4.3

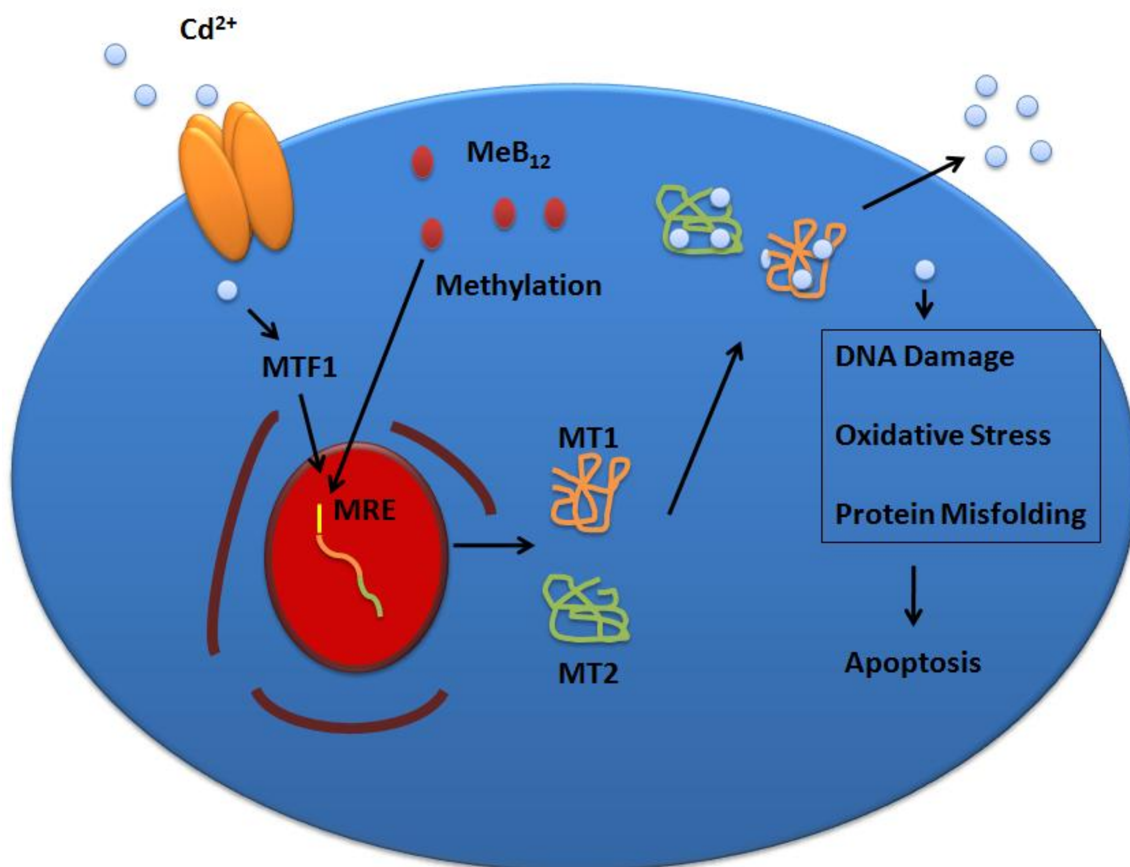


Figure.4.3. Schematic representation of possible role of MeB_{12} on increased cadmium sensitivity in A549 cells.

All these observations show that as the concentration of MeB_{12} increases, cells became more susceptible to programmed cell death on exposure to $CdCl_2$. To summarize MeB_{12} had no protective role against cadmium induced cytotoxicity in A549 cells. More over it acted as a sensitizer for cadmium, enhancing cell death by altering gene expression and cell signaling involved during this particular metal toxicity. It also seemed to suppress stress induced pathways making the cells more susceptible to metal induced cytotoxicity.

Chapter 5:

CELL SPECIFIC REGULATION OF P53 EXPRESSION, INDUCED BY CADMIUM

Chance favours the prepared mind

-Louis Pasteur

5.1. INTRODUCTION

The P53 pathway is a signaling network involving various intercommunicating genes and proteins in response to various intrinsic and extrinsic stress stimuli that can affect the cellular homeostasis which maintains the replication of DNA, the segregation of chromosomes and the cell division (Harris and Levine 2005). The P53 protein, which functions as a nuclear transcription factor undergoes post translational modifications and is activated, in response to the signal. In the nucleus P53 initiates the transcription of it target genes there by starting a signalling cascade. Eventually the signalling will either lead to cell cycle arrest, in the case of repairable DNA damage or programmed cell death, when the damage cannot be repaired. This will prevent the wrong information, caused by the infidelity of, DNA replication, genome stability and chromosome segregation from being passed on to the succeeding generations (Harris and Levine 2005).

When the cells are exposed to cadmium at concentrations lower than that can affect the viability of the cells, it can inhibit P53 from binding to the DNA. This leads to faulty DNA replication, due to the mistakes in DNA repair mechanism, resulting in cadmium induced carcinogenesis (Gaginis et al, 2006).

At cytotoxic concentrations, cadmium is found to induce P53 mediated apoptosis. Interfering with the activation of P53 was shown to inhibit apoptosis in cells exposed to cadmium (Luevano and Damodaran 2014).

In this study, we have tried to evaluate the difference in the degree of cytotoxicity induced by the heavy metal cadmium on three transformed cell lines with wild type P53 namely, A549 (lung adenocarcinoma), HEK293 (human embryonic kidney cells) and HCT116 (human colon cancer cells) and compared it with the

response of a *P53* knockout (-/-) line of HCT116 (HCT116*p53*-/-), to see whether these cellular responses followed some pattern in the molecular level.

5. 2. RESULTS

5.2.1. Effect of cadmium on the cell viability and integrity:

To study the effect of cadmium on the viability and integrity of the selected cell lines, cells from each cell line, in their exponential stage were seeded in 6 well plates (seeding density $3X10^4$ cells/ml).After 24 hours, the attached cells were exposed to increasing concentration of cadmium, in the form of cadmium chloride, $CdCl_2$ -0, 2.5, 5, 7.5,10 µg/ml. 24 hours after the exposure the cells were subjected to microscopic analysis [refer 2.2.5], total viable cell count [refer 2.2.4]and resazurin cell viability assay[refer 2.2.6].

Microscopic analysis showed that all the cell lines underwent stress in the presence of $CdCl_2$ which eventually led to cell death. In all the cell lines viability decreased with increase in $CdCl_2$ concentration. But each cell line showed different degree of tolerance towards this stress. The order of cell lines in the degree of tolerance was, HCT116 *p53*-/- < HEK293 < HCT116 < A549 (Figure 5.3.1.C).

Total viable cell count showed that cell death increased with increase in cadmium concentration in all the cell lines. Again the degree of tolerance exhibited by the cells varied among the cell lines. The order of cell lines in terms cell death was A549 < HCT116< HEK293 < HCT116 *p53*-/- (Figure 5.3.1.A).

The overall cellular energy metabolism decreased with increase in $CdCl_2$ concentration in all the cell lines. Again the level of reduction was different in each cell line. The order of cell lines in terms of mitochondrial activity was A549 > HCT116> HEK293 > HCT116 *p53*-/- (Figure 5.3.1.B).

From the above results it can be concluded that HCT116 *P53*-/- was the most sensitive cell line towards cadmium in this study. (IC_{50} 1.78 μg/ml of $CdCl_2$) (Table.5.3. 1). HEK293 (IC_{50-} 1.9 μg/ml of $CdCl_2$) showed almost equal level of response, while HCT116*p53*wt (IC_{50} – 7.2 μg/ml of $CdCl_2$) and A549 (9.6 μg/ml of $CdCl_2$ respectively) (Table. 5.2.1) showed higher tolerance level towards cadmium.

Table. 5.2.1

Cell lines	**A549**	**HCT116*p53*wt**	**HEK293**	**HCT116*P53*-/-**
IC_{50} values (μg/ml of $CdCl_2$)	9.6 ±.974	7.2±.212	2.3±.070	1.78±.084

Figure.5.2.1.A

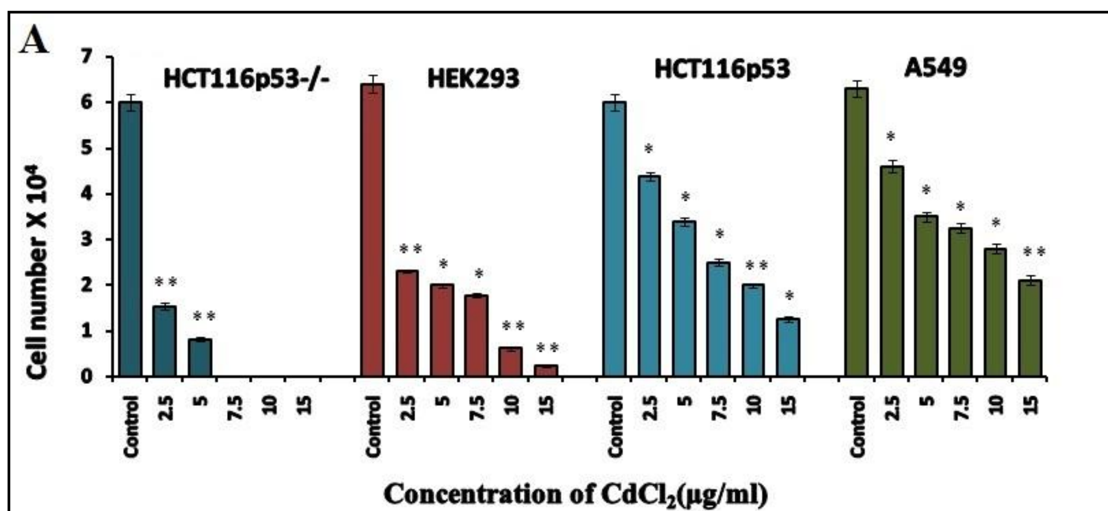


Figure.5.2.1. A. Total Viable Cell Count. Total viable cell count plot of HCT116*p53*-/- , HEK293, HCT116*P53*wt and A549 cell lines. Results are represented as mean± SD of three independent experiments**.** Values represent results from a two-tailed Student's t test. *p<0.05 and ** p<0.01 and ***p<.0001.

Figure.5.2.1.B

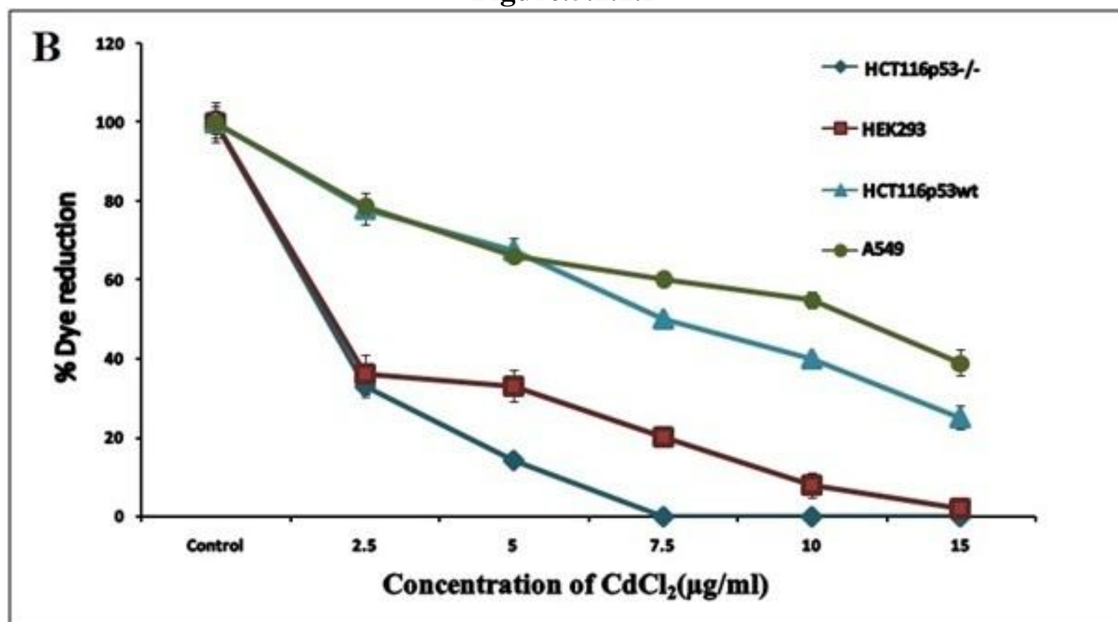


Figure.5.2.1.B. Cell Viability Assay. Concentration of $CdCl_2$ is plotted along horizontal axis and percentage of dye reduction in vertical axis. Results are expressed as mean± SD of three independent experiments.

Figure 5.2.1.C

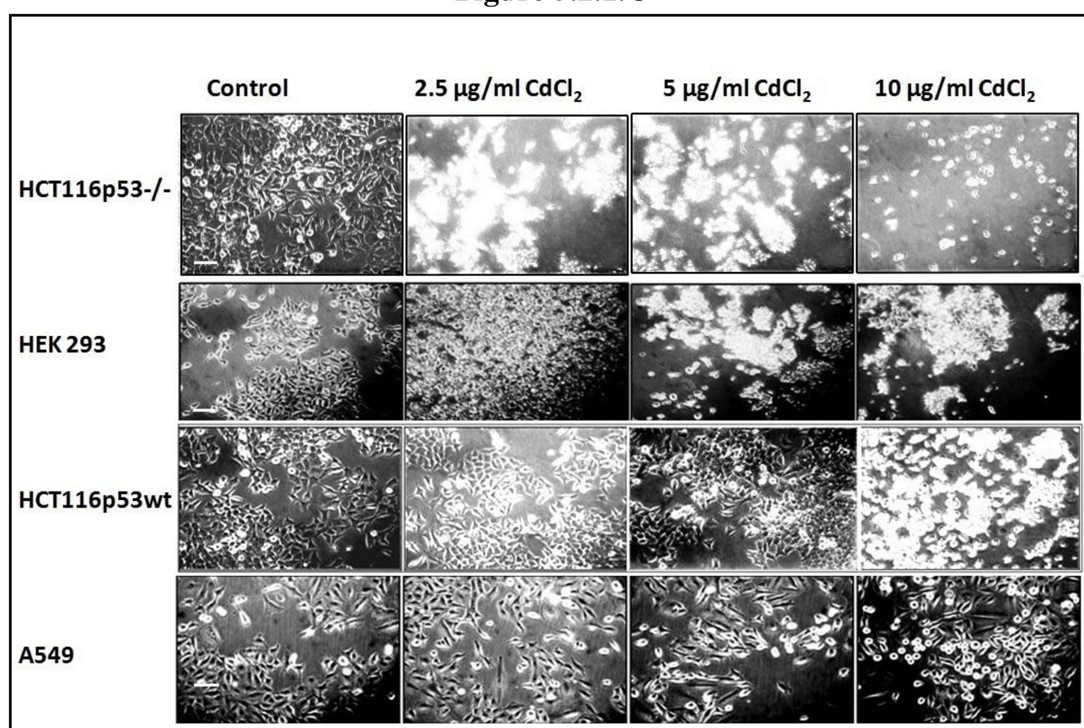


Figure5.2.1.C. Effect of cadmium on cell morphology and viability. (Representative images of cells observed microscope (Scale bar: 10µm).

5.2.2. Assessment of nuclear damage by Hoechst33342 staining:

Staining with Hoechst33342 revealed that exposure to $CdCl_2$ damaged the cellular nuclei. Chromatin condensation and nuclear fragmentation was visible in all the cell lines with exposure to increasing $CdCl_2$ concentration. (Figure.5.2.2). Nuclear damage was more in HEK293 cells compared to HCT116*p53*wt and A549 cells (Figure. 5.2.2. a, b, c & d). In the case of HEK293 cell line, cells in the maximum selected concentration of cadmium (10μg/ml), had all their nuclei distorted and were seen floating in the medium (Figure5.2.2.e & f).

Figure.5.2.2

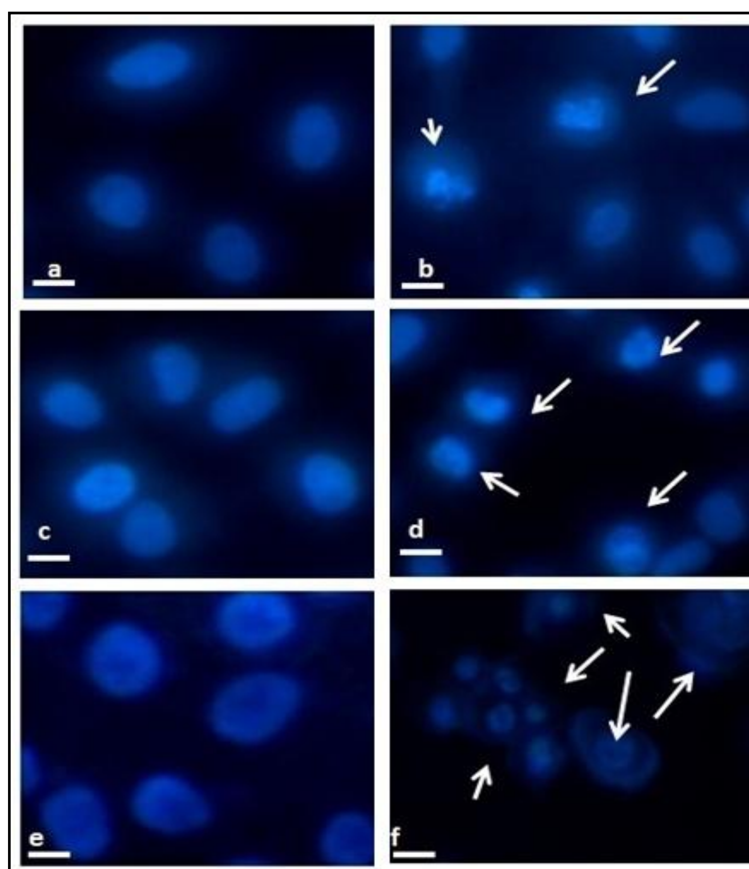


Figure.5.2.2. Nuclear staining using Hoechst33342 staining. (scale bar: 20 μm). In the figure, (a) and (b) correspond to cells in control and 10μg/ml of $CdCl_2$ respectively of A549 cells, (c) and (d) correspond to cells in control and 10μg/ml of $CdCl_2$ respectively of cells HCT116*p53*wt and (e) and (f) correspond to cells in control and

10μg/ml of $CdCl_2$ respectively of HEK293. The arrows point to the damaged cell nuclei in each figure, up on exposure to cadmium.

5.2.3. Expression profile of apoptotic genes in cadmium treated cells:

Since nuclear damage was observed on exposure to cadmium in all the cell lines, the expression profile of *p53* mRNA was measured in all of them. There was a dose dependent decrease in *p53* mRNA expression in all the three cell lines compared to their respective controls, though the extent of down regulation varied among them (Figure.5.2.3). In HEK293 cells, exposed to 2.5 μg/ml of $CdCl_2$, *p53* mRNA level was below that of the control and the level decreased further with increase in $CdCl_2$ concentration. HEK293 cells exposed to 10 μg/ml of $CdCl_2$ had *p53* mRNA level down to almost zero. Both HCT116*p53*wt and A549 cells showed better tolerance against cadmium induced *p53* mRNA down regulation. *p53* mRNA level came down to about 25% of that of the control in HCT116*p53*wt cells when exposed to 10 μg/ml of $CdCl_2$, while in A549 cells exposed to same concentration of $CdCl_2$ it was down to about 20% (figure 5.2.3).

Since all the cells showed some degree of cell death (dose dependent) when exposed to $CdCl_2$ the mRNA level of two important genes involved in programmed cell death- anti apoptotic gene *bcl2* and pro-apoptotic gene *bax* ,was measured.

In HEK293 cells there was a dose dependent decrease in *bcl2* mRNA level and a dose dependent increase in *bax* mRNA level compared to their control. *bcl2* level in these cells came down to about 20% that of their control on exposure to 2.5 μg/ml of $CdCl_2$ while there was about 30% increase in *bax* mRNA level when exposed to 5 μg/ml of $CdCl_2$ compared to that of the control. There was a dose dependent down regulation in both the mRNAs in HCT116*p53*wt cells, while in A549 cells *bcl2* mRNA level

increased with $CdCl_2$ exposure when *bax* showed a gradual down regulation in those same conditions (Figure.5.2.3).

Figure.5.2.3

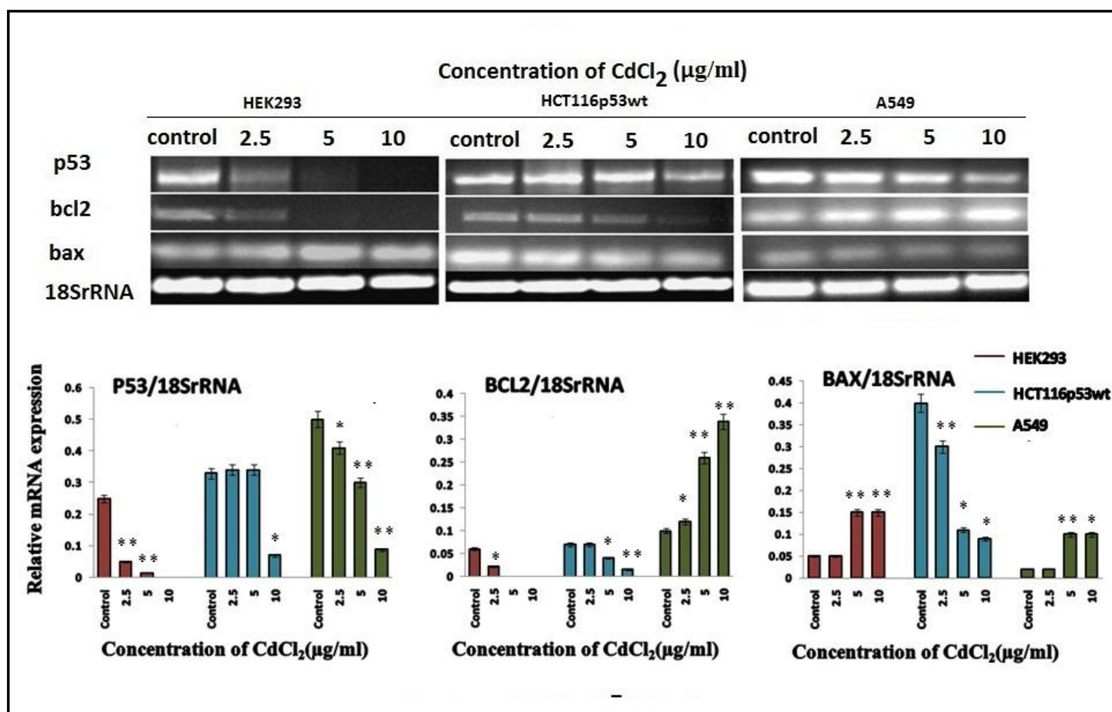


Figure.5.2.3. Expression of *p53*, *bcl2* and *bax*: Reverse Transcriptase PCR expression profile of *p53, bax* and *bcl2* mRNA is shown. Figure (a), (b) and (c) represent expression profile of *p53, bax* and *bcl2* mRNA in HEK293, HCT116*p53*wt and A549 cell lines respectively. Corresponding densitometric quantification profile represents the measurement of mRNA expression compared against the standard 18SrRNA (mean ± SD of three independent experiments). Values represent results from a two-tailed Student's t test. *$p<0.05$ and ** $p<0.01$ and ***$p<.0001$.

5.2.4. Expression of HSP70 in cadmium treated cells

Expression of HSP70 in the three cell lines after $CdCl_2$ treatment showed that the cell lines with comparatively better tolerance to cadmium like HCT116*p53*wt and A549 had slightly higher level of HSP70 in them compared to their control.

Figure.5.2.4

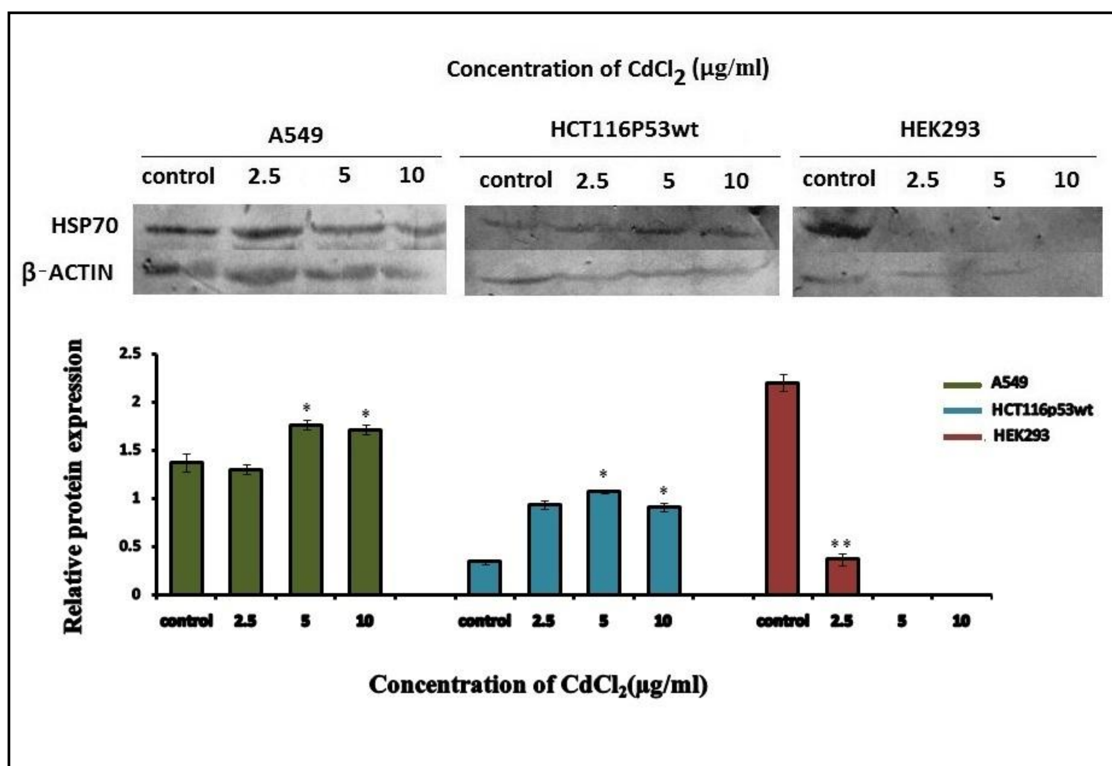


Figure.5.2.4. Expression of HSP70 in HEK293, HCT116*p53*wt and A549: Western blot results of HSP70 protein of control and cadmium exposed cells of HEK293, HCT116p53wt and A549. Corresponding densitometric quantitation profile represents the measurement of protein expression compared against the standard Beta actin protein. (mean ± SD of three independent experiments). Values represent results from a two-tailed Student's t test. *p<0.05 and ** p<0.01 and ***p<.0001.

5.2.5. Expression of P53 in cadmium treated cells

For the western blot analysis of P53 and phosphorylated P53 in cadmium treated cells total cellular protein was extracted and quantified, from each cell line after cadmium exposure for 24 hours as per the protocol described in section 2.2.11. Equal amounts of protein were resolved in 10% resolving gel, by SDS PAGE method and transferred to a PVDF membrane [refer 2.2.12-2.2.13]. After blocking in 3% BSA in 1X PBS for 1 hour the blots were incubated for 1 hour in primary antibody (dilution 1:2000 for anti P53, 1:4000 anti-P53 (phospho S15) antibody and 1:5000 for anti Beta actin). Following the washing the blots were incubated in secondary antibody (anti mouse IgG

dilution 1:4000) for 2 hours. The blots were developed by colour detection method using TMB/ H_2O_2 [refer 2.2.13].

A549 cells showed P53 protein profile similar to that of the corresponding *p53* mRNA level that is, expression decreasing slightly with increasing cadmium concentration. In these cells the phosphorylated p53 level on the other hand showed an up regulation with increase in cadmium exposure. HCT116*p53*wt cells had both P53 and phosphorylated P53 level up regulated with increase in cadmium exposure. HEK293 cells had approximately same level of P53 expression in control and cadmium exposed cells, but had decreased phosphorylated p53 levels with increase in cadmium dosage compared to that in their corresponding non treated control cells (Figure.5.2.5).

Figure.5.2.5

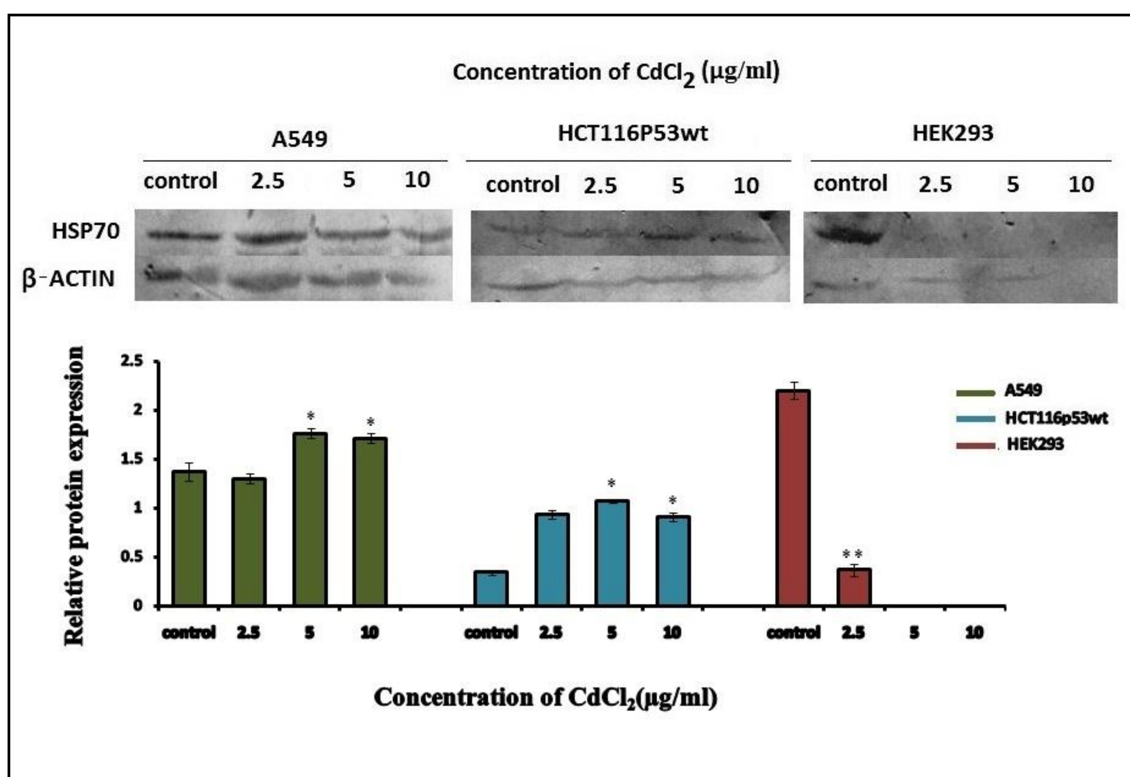


Figure.5.2.5. Expression of P53 and phospho53 in HEK293, HCT116p53wt and A549. (a): control, (b): 2.5µg/ml $CdCl_2$, (c): 5.0 µg/ml $CdCl_2$, (d): 10 µg/ml $CdCl_2$. Corresponding densitometric quantification profile represents the measurement of protein expression compared against that of the standard- beta- actin (mean ± SD of

three independent experiments). Values represent results from a two-tailed Student's t test. *p<0.05 and ** p<0.01 and ***p<.0001.

5.3. DISCUSSION AND CONCLUSION

Long term exposure to subtoxic concentration of cadmium can transform a normal cell to malignant cell. (Von Zglinicki et al, 1992 ;Habeebu et al, 1998; Shimoda et al, 2001). At cytotoxic concentrations it can induce DNA damage, cause oxidative damage, and impair cellular biosynthesis etc, eventually leading to the demise of the cell through programmed cell death (Yang et al, 2012; Hinkle et al, 1987; Feder and Hofmann 1999). Studies show that cadmium causes DNA damage (Fernandez et al, 2003; Badisa et al, 2007) and interferes with the DNA repairing mechanism. It is also known that it can inhibit the binding capacity of P53 transcription factor to the DNA (Meplan et al, 1999). During cadmium induced apoptosis activation of P53 is required and it was shown that inhibition of this activation was able to save the cells from apoptosis on exposure to cadmium (Aimola et al, 2012).

Because of this reason it is expected that a cell with no functional P53 will not be able to mount a response to cadmium and hence may be resistant to cadmium induced apoptosis. In fact it is reported that P53 mutation is the contributing factor for apoptotic resistance during cadmium induced carcinogenesis (Aimola et al, 2012).

But the results here show that transformed and malignant cells with no functional P53 that is HCT116*p53* -/- cells showed maximum sensitivity towards cadmium (IC_{50} 1.78 μg/ml) while its wild type counterpart had relatively higher tolerance (IC_{50} 7.2 μg/ml). Also the second most sensitive cell line in the study HEK293 had the lowest expression of P53 than A549 or HCT116*p53*wt cells at a given $CdCl_2$ concentration.

From this it can be assumed that P53 might be playing protective role in these transformed cells during cadmium induced cell stress. This assumption can be further

backed up by the fact, A549 (IC_{50} 9.8 µg/ml) cells which showed the maximum tolerance towards cadmium also had the minimum transcriptional repression of *p53* mRNA, followed by HCT116*p53*wt cells. HEK293 (IC_{50} value 1.9 µg/ml) cells with maximum sensitivity towards cadmium which had comparable responses to that of HCT116*P53* -/- cells, also had the highest level of *p53* transcriptional down regulation. The expression of transcriptional targets of P53 -Pro apoptotic gene *bax* and anti apoptotic gene *bcl2* (Moll et al, 2005; Miyashita et al, 1994) was measured to see the downstream effects of *p53* transcriptional down regulation. In HEK293 cells, even with the reduced *p53* expression, the level of pro apoptotic gene *bax* was higher and the expression of anti apoptotic gene *bcl2* was lower, compared to the control. *bcl2* was highly down regulated in the presence of cadmium and the pro apoptotic gene *bax* expression was up regulated with increasing concentration of cadmium, which is not seen in the absence of P53 expression, since P53 inactivation was found to inhibit apoptosis rather than promoting it. *bcl2* expression was down regulated in HCT116 *p53*wt cells too, but the extent of down regulation was not as much compared to that of HEK293, since *bcl2* down regulation started being apparent in HEK293 cells with an exposure to 2.5µg/ml of $CdCl_2$ while it started only at 10 µg /ml of $CdCl_2$ in HCT116*p53*wt cells. A549 cells showed a dose dependent up regulation of *bcl2* expression while *bax* was down regulated with increasing concentration of cadmium chloride, both in A549 as well as HCT116*p53*wt cells, which could have helped these cells to resist apoptosis.

To see what helped some cells to with stand and survive the toxic metal environment, the expression level of HSP70 in them was measured and compared to the corresponding non exposed control. During unfavourable conditions cells protect their internal environment from many kinds of damages, by stress induced expression of heat

shock proteins or HSPs (Tavaria et al, 1996). HSPs are ubiquitous proteins and they help the cells prevent induced denaturation of cellular proteins (Feder and Hofmann 1999). Many studies over the past have shown that HSP70 also regulate the progression of apoptosis induced by various kinds of stimuli [Cotter et al, 2000 72 & 73 Stankiewicz et al, 2005]. Many cell lines are found to use HSP70 as an anti apoptotic regulator interfering with the advance of programmed cell death, by stress induced expression of HSP70 proteins. But most of these studies show that HSP70 possibly inhibit apoptosis by interfering with the functional assembly of apoptosome complex by associating with Apaf-1 (Beere et al, 2000).

Studies so far have shown that inactivation of P5*3* signaling inhibits apoptosis. In this study it was observed that, the cells which resisted *p53* transcriptional down regulation survived cadmium induced cell death. This might indicate either an unknown protective role of functional P53 in transformed cells or the possibility of one of the mechanisms of cadmium induced cytotoxicity, being the transcriptional down regulation of P53.

In order to find out whether the above mentioned hypothesis is right, the activity of P53 was measured. P53 activation involves various post translational modifications, especially phosphorylation. Also P53 is known to be phosphorylated at serine15 and 37 residues, following DNA damage (Shieh et al, 1997; Siliciano et al, 1997). The level of Serine15 phosphorylation in P53 was measured using anti-p53 (phospho S15) antibody, in control and cadmium exposed A549, HCT116*p53*wt and HEK293 cells. In A549 as well as HCT116*p53*wt cells phospho P53 level was higher in cadmium treated cells compared to control, while HEK 293 cells had lower levels of phospho p53. Serine 15 residue phosphorylation is found to stabilize P53 and increase its half life. This is also found to prevent its interaction with MDM2 (Fiscella et al, 1998).

In conclusion phosphorylation at serine 15 residue in cadmium exposed A549 and HCT116*p53*wt cells would have stabilized P53 in protein level while its mRNA faced degradation in that condition. On the other hand, in HEK 293 cells, p53 level was maintained almost equal in control and cadmium exposed cells, in spite of its mRNA undergoing degradation. But these cells were unable to increase the level of phosphorylated P53. This might be the reason for HEK293 cells' increased sensitivity towards cadmium. In HEK cells P53 activation might be happening by some other post translational mechanism but, here it might be directly signaling for cell death. While in A549 and HCT116p53wt cells, increase in P53 stabilization indicates just growth arrest and possible repair of DNA damage.

SUMMARY AND CONCLUSION

Study of actin dynamics during cadmium exposure and gene expression of small GTPases *cdc42, rhoA* and *rac1* involved in actin dynamics revealed that what happened in the gene level had influenced the protein level. This in turn made it possible for the cell to react to the metal exposure in a way that the resulting response helped in minimising the chemical insult as much as possible. Those responses include morphological changes in cells (by altering the expressions of genes regulating actin dynamics like *cdc42, rhoA* and *rac1*) to minimize the surface area in contact with the chemical as well as increased expression of HSP70 protein that helps the cellular proteins from misfolding.

Examining the effect of vitamin B_{12} analog methylcobalmin- MeB_{12} in cadmium treated cells showed an interesting synergy between cadmium and MeB_{12}. The vitamin analog was found to facilitate the cadmium entry into the cells there by aggravating the cytotoxicity. The effect of this, in the molecular level, was the activation of programmed cell death pathway and suppression of inflammatory response so that the cells are unable to mount a response against the heavy metal.

Studies so far have shown that inactivation of P53 signaling inhibits apoptosis. In our study we saw that, the cells which resisted *p53* transcriptional down regulation survived cadmium induced cell death. This might indicate either an unknown protective role of functional P53 in transformed cells or the possibility of one of the mechanisms of cadmium induced cytotoxicity being the transcriptional down regulation of P53. Further studies on the transcriptional factors and other proteins that interact with P53 promoter will reveal the actual mechanism of its transcriptional down regulation.

Human cells employ a vast array of interconnected communication networks involving genes, proteins and other bio chemical molecules to respond to different physical and environmental conditions. To unravel these communication networks one by one in order to understand how they react to the heavy metal cadmium is the main objective of this work. So far this work has revealed many aspects of molecular mechanism of cadmium toxicity in different human cell lines as well as opened the door to an intriguing side of vitamin- metal interactions.

FUTURE SCOPE OF THE WORK

The present study on cadmium and its effects on various cell lines, in the molecular level, have paved the way for further studies in to the field of cell signaling past heavy metal exposure. The multi-cell line study here has shown that the expression of various genes involved in the signaling during cellular response to cadmium toxicity follows a pattern. This helps in the extrapolation of these results into various studies involving heavy metals similar to cadmium. We have seen an inverse correlation between degree of transcriptional repression of *P53* gene and cadmium toxicity which indicates a possible protective role of P53. An elaborated study on cell signaling involved in this response may reveal an interesting, previously unknown task of this transcription factor.

Studies on the effect of MeB_{12} on cadmium induced stress in A549 revealed an interesting property of this vitamin B_{12} analog- sensitizing the cells to cadmium. Verifying that this property is cancer cell specific and if this sensitization is possible for any cancer drug, might open a new idea for tumor specific cancer drug delivery using MeB_{12}.

P53 is the tumor suppressor protein that is mutated in majority of cancers. Present study (Chapter 3) shows that even wild type P53 functions differently in different types of cells under same environmental conditions. Studies on the expression pattern and function of wild type p53 in various cells will give an insight into the distinctive mechanisms of cellular responses towards damage and stress.

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LIST OF PUBLICATIONS

In Journal:

1. Sarkar, A. **Ravindran, G.** and Krishnamurthy. A Brief Review on the Effect of Cadmium Toxicity: from Cellular to Organ Level. International Journal of Bio-Technology and Research. ISSN 2013; 2249-6858. 3 (1): 17-36.

2. Ravindran, G, Chakrabarty, D and Sarkar, A. Cadmium toxicity causes alteration in mRNA expression pattern of Rho-like genes in human lung A549 cell line. Journal of Cell and Molecular Biology 11(1&2):13-20, 2013.

3. Ravindran, G, Chakrabarty, D and Sarkar, A. Cell specific regulation OF p53 mRNA expression induced by cadmium. Animal Cells and Systems. 21(1):23-30,2016.

Conference proceedings:

1. G. Ravindran, D. Chakrabarty and A. Sarkar. Cadmium toxicity induces morphological alteration in *in vitro* cultured human lung (A549) cell line, Seminar on advances in Zoology, Goa, 2012.

2. G. Ravindran and A. Sarkar, Cadmium Toxicity causes morphological Changes, production of stress granules and upregulation of HSP70 in human A549 cells cultured in vitro. 36th All India Cell Biology Conference, Mumbai, Pg: 163, 2012.

3. Geethanjali Ravindran, Snehal Nirgude, Dibakar Chakrabarty and Angshuman Sarkar. Synergistic effect of methylcobalamin on cadmium induced stress in human lung cell carcinoma (A549) cell line. TSICON 2013, Goa.

4. G Ravindran, D Chakrabarty and A Sarkar, Cadmium induced cytotoxicity: a cell specific stress response? 1st International Conference on Trends in Cell and Molecular Biology (TCMB) 2015, Goa.

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Master's dissertation: Clinical proteomics of *A.baumannii*- Biocomputational approach for drug target identification. January to May 2010, Institute of Bioinformatics and Biotechnology, IBB, Pune, India.

Bachelor's in Biotechnology from

Mercy College, Palakkad, affiliated to University of Calicut, Kerala, India.

Score: 79.5%

FELLOWSHIPS & AWARDS

Fellowship of Council of Scientific and Industrial Research (CSIR), Govt of India, from 2011-2016

OTHER RESPONSIBILITIES

Teaching assistant for Bachelor's and Master's laboratory courses from 2011- present.

Reviewer in the journal *Human & Experimental Toxicology.*

RESEARCH INTERESTS

Molecular mechanisms and cellular signalling involved in cancer, cancer therapeutics and drug development.

Study of Epigenetic modifications involved in various cellular events and diseases.

RESEARCH EXPERIENCE

Doctoral research: Studied the degree of toxicity of cadmium chloride in various cell continuous human cell lines like A549, HCT116, HEK 293. Various techniques used in this study include; cell viability studies using trypan blue dye exclusion assay, measurement of mitochondrial activity using resazurin assay, study of various gene expression patterns using RT PCR, study of promoter methylation using bisulfite modification and methylation specific PCR, fluorescent studies using Hoechst 3332 to measure nuclear damage and that with phalloidin conjugated FITC for visualizing actin dynamics and western blotting for measuring protein expression.

Master's dissertation: Clinical proteomics of *A.baumannii*- Biocomputational approach for drug target identification. January to May 2010, Institute of Bioinformatics and Biotechnology, IBB, Pune, India.

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Dr. Angsuman Sarkar obtained his Ph.D. degree from Department of Biotechnology, University of Pune, India in 2004. He went for Post Doctoral training in Cold Spring Harbor Laboratory, New York, USA for 3 years. After finishing there he joined Department of Molecular Biology and Microbiology in Case Western Reserve University in Cleveland, USA as a senior research fellow. Later on he was offered Associate ship in the Cancer Biology Department in Cleveland Clinic Foundation, Cleveland, USA. He joined Department of Biological Sciences, BITS Pilani K K Birla Goa Campus, Goa in September, 2009. Besides teaching the core courses at the UG and PG level Dr. Sarkar has been involved in teaching advance courses like Animal Cell Technology and Cancer Biology.

Member of Scientific Body:

1) Life member for ‘Indian society for Cell Biology’.
2) Life member for “Toxinological Society of India’.
3) Yearly member for ‘American Association of Cancer Biology’, USA.
4) Yearly member for Genetics Society, USA.

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►Awarded “Appreciation Certificate” by Memorial Sloan Sloan-Kettering Cancer Center, USA, in 2008 for outstanding leadership and commitment in the Cancer Research in 2009.

►Awarded Research Associateship by Department of Cell and Molecular Biology [CWRU, Cleveland, USA in 2007.

►Offered a Fellowship by Cold Spring Harbor Laboratory (CSHL), New York, USA for Post Doctoral Training in 2004.

►Published a paper in one of the high ranking Cell Press Journal about the role of some proteins in maintaining germ line stem cells niche in 2007, this paper was subsequently selected as top 20 cited papers in USA for the year 2007.

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Post Doctoral Research Experience (From 2004 till 2009 in USA):

1) Cold Spring Harbor Laboratory, New York, USA, Molecular Developmental Biology.

2) Case Western Reserve University; School Micro Biology and Molecular Biology, Cleveland, OH, USA.

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►2004 - 2004: Rsearch Associate (DBT, Govt. of India) in the Department of Biotechnology, Govt. of India, University of Pune. Pune, India.

►2002 – 2004: Senior Research Fellow, awarded by Council of Scientific and Industrial Research (CSIR) New Delhi, Govt. of India.

►1999 – 2002: JRF (CSIR) as well as DBT fellow.

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PUBLICATIONS

Publication 1

Publication 2

Publication 3

Abstract 1

Abstract 2

Abstract 3