Study on the efficacy of dietary compounds to detoxify toxic metals *in vitro*

（試験管内で毒性金属を解毒するための食餌中化合物の有効性に関する研究）

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Course: Environmental Adaptation Science

Division: Environmental Science Development

Graduate School of Environmental Science,

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Dissertation in fulfillment of the requirement for the degree of
Doctor of Philosophy (Ph.D) in Environmental Science

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Contents

Chapter 1: General Introduction

Chapter 2: Cytotoxic effects of cadmium and zinc co-exposure in PC12 cells and the underlying mechanism

Chapter 3: Ameliorative effects of selenium on arsenic-induced cytotoxicity in PC12 cells via modulating autophagy/apoptosis

Chapter 4: Cytoprotective effects of lipoic acids against metal-(loids) induced toxicity in mammalian cells

Chapter 5: General conclusion and future research

List of abbreviations

ALA/LA	α-lipoic acid
As	Arsenic
ATSDR	Agency for Toxic Substances and Disease Registry
Cd	Cadmium
DHLA	Dihydrolipoic acid
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organizations
GSH	Glutathione
GSSG	Glutathione disulfide
GWQI	Groundwater Quality Index
Hg	Mercury
HI	Hazards Index
HQ	Hazards Quotients
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
MT	Mettalothionein
mTOR	Mammalian Target of Rapamycin
PARP	Poly (ADP-ribose) polymerase
Pb	Lead
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
WB	Western Blot
WHO	World Health Organizations

Abstract

In the era of globalization, environmental pollution has become a major human health threat. Due to heavy industrial activities, aggressive agricultural practices, unplanned mining, and natural disasters are contributing negatively to the environmental system. Almost every kind of pollutants in turn reaches to the food chain and creates burden for the human health with numerous diseases manifestations. In recent years, substantial human health risks were reported due to toxic metals through consumption of contaminated food stuffs, fishes, fruits, and water. We have also reported human health risk due to drinking from the arsenic (As) contaminated groundwater and from toxic metals (cadmium (Cd), chromium (Cr), and lead (Pb)) contained cow milk in Bangladesh. Moreover, the toxic metals induced health hazards are very concerning and so far there is no proper detoxification method available for biological systems. Therefore, in this research an environmental medicine approach to detoxify toxic metals was done using molecular and *in vitro* cell biological techniques. For instance, essential trace elements such as zinc (Zn) and selenium (Se) have been used to detoxify toxic metals such as Cd and As using PC12 cells which is well known as model cell line for fundamental molecular toxicology study. In addition, to that promising dietary supplements such as α-lipoic acid and dihydrolipoic acid (ALA and DHLA) have also been investigated against the metal-induced cytotoxicity in different cell lines including PC12 cells. The research hypothesis was 'dietary compounds may have potentials to detoxify toxic metals in cellular systems'.

In Chapter 2, Zn has been applied to detoxify Cd in PC12 cells following a simultaneous exposure pattern. Cd is one of the heavy toxic metals, which is also an inducer of cellular necrosis and apoptosis. Conversely, Zn is an essential trace element known to inhibit apoptosis induced by toxicants including Cd both *in vitro* and *in vivo*. However, the mechanism of Zn-mediated protection from Cd-induced cytotoxicity is not established yet. Thus, it was aimed to investigate the effects of Zn on Cd-induced cytotoxicity and apoptosis using PC12 cells. Cell viability and DNA fragmentation assays in PC12 cells exposed to Cd and/or Zn revealed that Cd (5 and 10 μM) alone induced significant cell death, and co-exposure to Zn (5, 10, and 100 μM) for 48 h had a protective effect. Assessment of intracellular glutathione (GSH) levels and lactate dehydrogenase (LDH) activity suggested that Cd (10 μM)-induced oxidative stress and disrupted cell

membrane integrity. Addition of Zn (10 and 100 μM) reduced Cd-mediated cytotoxicity. Moreover, changes in expression of the apoptotic factors, Bax, Bcl-2, Bcl-x, and cytochrome c were measured *via* western blotting analyses, and expression of caspase 9 was detected *via* reverse transcriptase polymerase chain reaction (RT-PCR). Western blots showed that Zn (10 and 100 μM) suppressed Cd-induced apoptosis (10 μM) by reducing cytochrome c release into the cytosol, and downregulating the pro-apoptotic protein, Bax. In addition, expression of caspase 9 was lower in Cd (5 μM)-treated PC12 cells when co-treated with Zn (2 and 5 μM). These findings suggest that the effective inhibition of Cd-induced apoptosis in PC12 cells by Zn might be due to suppression of mitochondrial apoptosis pathway and inhibition of Cd-induced production of ROS.

After Zn and Cd experiments, another combination of two metalloids (As and Se) was investigated. As is a well known toxicant responsible for human diseases, on the other hand, Se is an essential trace element with significant chemopreventive effects, anticancer potentials and antioxidant properties. Although previous studies have reported antagonism/synergism between As and Se in biological systems, the biomolecular mechanism is still inconclusive. Therefore, we hypothesized that co-exposure of Se with As may have suppressive effects on As-induced cytotoxicity in PC12 cells. Upon Se co-exposure with As increases cell viability, and suppresses As-induced oxidative stress. As-induced DNA fragmentation was also reduced by co-exposure of Se in PC12 cells. Furthermore, the western blotting analyses revealed that simultaneous exposure of both metals significantly inhibited autophagy which may further suppressed apoptosis through positively regulation of key proteins; p-mTOR, p-Akt, p-Foxo1A, p62, and expression of ubiquitin, Bax, Bcl2, NFκB, and caspase 3, although those are negatively regulated by As. In addition, RT-PCR analysis confirmed the involvement of caspase cascade in cell death process induced by As, and subsequent inhibition by co-exposure of Se with As. The cellular accumulation study of As in presence/absence of Se *via* inductively coupled plasma mass spectrometry confirmed that Se effectively retarded the uptake of As in PC12 cells. Finally, these findings imply that Se is capable to modulate As-induced intrinsic apoptosis pathway via enhancement of mTOR/Akt autophagy signaling pathway through employing antioxidant potentials and through inhibiting the cellular uptake of As in PC12 cells. So, it

can be concluded that homeostatic level of Se is dramatically essential to maintain the ionic balance in the body. In a situation where As exposure is prominent Se might be crucial to reduce the negative impacts of As in human health.

Likewise, we have investigated the protective role of ALA and DHLA against As, Cd and Pb stress in PC12 and Caco-2 cells. We found that both ALA and DHLA have significant cytoprotective effects against As, Cd and Pb toxicities in PC12 as well as Caco-2 cells. Cell viability of both cells was improved upon combined exposure of ALA-metals/DHLA-metals than the only metal-treated group. ALA showed cytoprotection at 250 μM concentration where as DHLA showed a similar effect at 50 μM. Boost up of the intracellular GSH level could be one of the mechanisms of the cytoprotection. We found a significant increase of GSH level in the co-treated group which was significantly lowered by the metal-treated group alone. Subsequently, DHLA (50 μM) showed potentials for the protection of DNA from possible damage caused by the metal exposure in PC12 cells. Significant damage of DNA was observed upon treatment with As, Cd and Pb. However, co-treatment with DHLA and metals lowered the damage intensity as visualized by agarose gel electrophoresis. Western blotting analysis reveals an upregulation of survival factor mTOR, Akt and downregulation of death marker Bax, cleaved PARP was induced by ALA in both PC12 and Caco-2 cells to protect the cells from As and Cd induced toxicity. ALA also induced Nrf2 upregulation thus to boost up the GSH defense to protect cells from oxidative damages. Similarly, DHLA also upregulated the expression of mTOR and Akt in PC12 cells in co-treated group with metals.

Chronic ingestion of trace levels of toxic metals such as As, Cd, and Pb may possess serious health hazards. And deficiency of essential trace elements could aggravate the deleterious effects. However, proper dietary intake of essential trace elements and dietary supplements may reduce the toxic effects of metals. This study suggests beneficial role of Zn, Se and ALA/DHLA against toxic metals *in vitro*, and recommended for further investigation using animal model.

Chapter 1: General Introduction

1.1 Background

Environment is one of the most important contributors to the human health issues such as disease manifestations. Human exposure to numerous environmental chemicals specifically pollutants and toxic chemicals have deleterious effects on health which contributes substantially to most diseases of major public health significance. Diseases depending on environments are those in general aroused or exacerbated by exposure to environmental stressors and include cancer, chronic lung disease, diabetes and neurodegeneration (Franco et al., 2009). Particularly, heavy metal pollution has been considering as a great environmental concern due to multiple effect in the food chain that posses severe toxic effects to biological systems. Still today there is no universal definition of heavy metals; however, a specific density of 5 g/cm^3 is widely recognized as heavy metal. Among the 92 naturally occurring metals about 30 metal-(loids) are being considered as potentially toxic to humans and animals (Morais et al., 2012). However, the ultimate tragedy of metal-(loids) toxicity is an unavoidable reality in environmental, ecological and nutritional point of view (Nagajyoti et al., 2010). Usually, heavy metals release into environment by both natural and anthropogenic routes

including; natural weathering of the earth's crust, mining, soil erosion, industrial discharge, urban runoff, sewage effluents, pests or diseases control agents applied to crops, air pollution fallout, and a number of others (Ming-Ho, 2005). Among the heavy metal-(loids), especially arsenic (As), lead (Pb), mercury (Hg) and cadmium (Cd) affect serious human health (Järup, 2003). Moreover, according to the US Agency for the Toxic Substances and Disease Registry (ATSDR, 2017), these four metals (As, Pb, Hg and Cd) rank among the top 10 priority hazardous substance (Priority List of Hazardous Substances (ATSDR, 2017) (Table 1.1).

Table 1.1 Rank of metal-(loids) in the ATSDR priority list in 2017.

Name of Substance	Rank in the list	Total points
Arsenic (As)	1st	1674
Lead (Pb)	2nd	1531
Mercury (Hg)	3rd	1458
Cadmium (Cd)	7th	1320

In addition to that World Health Organization (WHO) recommended limits for these metal-(loids) to be present in drinking water (Table 1.2). However, it may vary depending on the background value in the environmental system, availability of remediation technologies and socioeconomic status. The developed countries are more concern about the adverse effects of toxic metals than the developing/least developed countries.

Table 1.2 Sources, exposures and effects of As, Pb, Hg and Cd against human health.

Name	**Sources of exposures**	**Route of exposures**	**Tolerance limit**	**Associated diseases**	**References**
As	Mining, pesticide, pharmaceutical, glass and microelectronics, ground water.	Ingestion, inhLAtion and dermal contact	10 µg/L of drinking-water	Cardiovascular and peripheral vascular disease, developmental anomalies, neurologic and neurobehavioural disorders, diabetes, cancers.	WHO (2017), ATSDR, (2000), Tchounwou, (2003).
Pb	Mining, smelting, recycling, stripping of leaded paint, using leaded gasoline or leaded aviation fuel, leaded pipes, lead-glazed or lead-soldered containers; traditional cosmetics and medicines, toy,	Ingestion, inhLAtion and dermal contact.	Drinking water: 10 µg/L; PTWI: 25 µg/kg body weight; 5 µg/dL of blood (no lower limit)	Coma, convulsions, mental retardation and behavioural disorders, affect children's brain development, anaemia, hypertension, renal impairment; immunotoxicity and toxicity to the reproductive organs.	WHO (2017), EPA (2017)

	battery.				
Cd	Smoking, mining, smelting and refining of nonferrous metals, fossil fuel combustion, incineration of Cd-containing waste, phosphate fertilizers production, recycling, electric and electronic waste etc.	Ingestion, inhLAtion and dermal contact.	PTWI: 7 µg/kg body weight; Drinking water: 3 µg/L	Pulmonary edema, respiratory diseases, renal dysfunction, anemia, osteoporosis, cancer, neurological disorder, olfactory dysfunction; learning disability, hyperactivity and birth defects in children	IPCS (2005–2007), WHO (2010)
Hg	Mining, gold extraction, batteries, thermometers and barometers, electric switches, lamps (light bulbs) dental amalgam, cosmetics pharmaceuticals	InhLAtion, ingestion and dermal contact.	PTWI: 1.6 µg/kg body weight Drinking water: 6 µg/L	Neurological and behavioral disorders, tremors, insomnia, memory loss, neuromuscular effects, cognitive and motor dysfunction, kidney and immune effects, neurodevelopmental problems in the developing fetus.	WHO (2007), EPA (2017)

PTWI: Provisional tolerable weekly intake

A huge number of studies have been reported about the toxicity (acute and chronic) and toxicity mechanism of heavy metals using cellular and animal models. They have distinct way to affects biological systems; however, there are some common mechanisms of toxicity that include mimicry, oxidative damage, and adduct formation with DNA or proteins (Keil et al., 2011). For instance, As toxicity affects almost every organ system in the body including the brain (Tyler et al., 2014). A detail toxicokinetic features of As, Pb, Hg and Cd separately will be discussed in the later parts of this chapter. As stated before that the definition of heavy metal is vague and several criteria have been considered based on density, atomic weight or atomic number, or various chemical properties and toxicity (Duffus, 2002). Recently, 'toxic metal' instead of 'heavy metal' have already been proposed to avoid contradiction between toxic metals and essential trace metals.

Furthermore, these four (As, Pb, Hg and Cd) non-essential (toxic) metal/metalloid can mimic the essential trace elements such as zinc (Zn) by disrupting the cellular and enzymatic mechanisms (Keil et al., 2011). Thus, they might be at least one of the etiological factors for numerous environmental diseases such as impairment of cognitive development (e.g. developmental disorders), degenerative diseases of the nervous system, which would include Alzheimer's disease, Parkinson's disease, and multiple sclerosis (MS), problems with skeletal development and maintenance (e.g. osteoporosis), kidney

disorders, blood disorders and different forms of cancers. However, a line of evidences suggests that toxic metals induced alterations of biological homeostasis which leading to human health risk might be detoxified by environmental medicine. Environmental medicine is a multidisciplinary approaches that bridging among medicine, environmental science, chemistry and so on. It is to reduce environmental pollutions related human health issues, for example proper use of nutritional essential metal (Zn) and essential trace element (Se) may decrease toxic outcome of toxicants. However, this chapter aims to depict the potential mechanisms of toxic metals/metalloids in biological systems and to search for the potential dietary supplements for example Zn, Se, α-lipoic acid (LA) and dihydrolipoic acid (DHLA) in light of environmental medicine to detoxify toxic-metals induced deleterious effects on biological systems. Moreover, this chapter will also be discussing about the motivation of further investigations in the realm of environmental medicine against environmental illness.

1.2 Cd exposures and toxicokinetics

Cd is classified by International Agency for Research on Cancer (IARC) as Group I carcinogen to humans (IARC, 1993). According to WHO (2010) guidelines, a provisional tolerable monthly intake for Cd of 25 μg/kg body weight, whereas, drinking-water 3 μg/L and air 5 ng/m^3 (annual average). Biological limit values (BLVs) for Cd compounds are 1 μg/L in blood and 0.8 μg/L in urine (Hartwig et al., 2017). Sources of exposure to Cd are natural activities, such as volcanic activity, weathering and erosion, and river transport; as well as human activities such as tobacco smoking, mining, smelting and refining of nonferrous metals, fossil fuel combustion, incineration of municipal waste (Cd-containing batteries and plastics), manufacture of phosphate fertilizers, recycling of Cd-plated steel scrap, and electric and electronic waste (UNEP, 2008; WHO, 2010). Exposure route for Cd includes ingestion, inhalation and dermal absorption (ATSDR, 2012). Cd accumulation causes pulmonary edema, respiratory tract irritation, renal dysfunction, anemia, osteoporosis, cancer, birth defect and neurological disorder (Chen et al., 2008b; Lau et al., 2006). Olfactory dysfunction and neurobehavioral defects have been induced in Cd-exposed workers (Lau et al., 2006) and neurological disorders including learning disability and hyperactivity to children (Chen et al., 2008b). Parkinson's disease and Alzheimer's disease are believed to be associated with Cd (Chen et al., 2008a, 2008b).

Cd in intestine accumulates highly within the intestinal mucosa and transfers into the organism at a low rate by diffusive transfer (Elsenhans et al., 1997). The function of intracellular metallothioneins (MT) in Cd accumulation is controversial. Ohta and Cherian (1991) reported that though the intracellular MT does not affect the uptake of Cd from lumen, it may reduce both the release of Cd from the intestine and its deposition in liver. However, according to Liu et al. (2001), orally administrated Cd was not influenced in absorption and initial distribution by MT.

The main storage sites in the body for Cd are the liver and kidneys, especially the renal cortex (Jodan-Piedra et al., 2017). For never- and ever-smokers combined, the daily systemic uptake of Cd was estimated to be 0.0063 μg /kg body weight in men, with 35% increased uptake in women. And a daily uptake of for each pack-year per calendar year of smoking and the rate of urinary excretion from Cd accumulated in the kidney were estimated as 1.2 μg and 0.000042μg/day^{-} (Fransson et al., 2014).

Certain mineral deficiencies in the diet increases intestinal absorption and accumulation of Cd in organs. Intestinal absorption of Cd is influenced by body iron (Fe), Zn, dietary fiber, proteins and probiotics status. Park et al. (2002) indicated that the functional divalent metal transporter 1 (DMT1) protein which is the main intestinal transporters of Fe, was upregulated in the small intestine by body Fe depletion, and that upregulation probably increased Cd uptake from the gastrointestinal tract. Ryu et al. (2004) reported a correlation between expression of duodenal DMT1 or Ferroportin 1(FPN1, another intestinal transporter of Fe) and Cd body burden, which suggested a significant role of Fe transporters in Cd absorption. Vance and Chun (2015) reported that dietary and serum Zn in US adults are associated with Cd exposure and, influencing the absorption and accumulation of Cd. In adjusted regression models, a 10% increase in serum Zn was associated with a 2% decrease in blood Cd and a 4% increase in urinary Cd. House et al. (2003) showed that the bioavailability of Cd in rats was depressed when fiber-rich wholegrain wheat was part of the regular diet for rats. Increased intake of Zn in the wholegrain wheat lowered Cd absorption and retention. The role of proteins on gastrointestinal absorption of Cd seems to depend on the length of the treatment. Lower levels of Cd in the liver, kidney, and whole body were observed in mice fed high-protein diets for 24 hours before and after an oral dose of Cd; however, in long-term exposures the high protein diet was associated with high tissue levels of Cd (Revis and Osborne, 1984). Kojima et al. (1985) showed that the type of protein is also a

factor to be taken into account. They found that glycine and ovalbumin significantly depressed gastrointestinal absorption of Cd; however, gelatin did not have such effect (Kojima et al., 1985). Again, Lactobacillus plantarum CCFM8610 (a probiotic) reduced the intestinal absorption and accumulation of Cd in the liver and kidneys by increased fecal excretion after acute exposure (Zhai et al., 2013) and chronic exposure (Zhai et al., 2014).

1.3 As exposures and toxicological aspects

As is a natural component of the earth's crust, and is widely distributed throughout the environment in the air, water and land. It is highly toxic in its inorganic form. Exposure with As occurs occupationally in industries including mining, pesticide, pharmaceutical, glass and microelectronics, as well as environmentally from both industrial and natural sources. Drinking water and foods contaminated with As from natural rock formation, are the major source for As exposure (WHO, 2017). The recommended limit of As in drinking-water is 10 μg/L (WHO, 2017). Exposure with As occurs via ingestion, inhalation, dermal contact and the parenteral route to some extent (ATSDR, 2000). Keratosis or skin lesions are some of the most common and earliest nonmalignant effects related to chronic As exposure, and have been observed even at the exposure levels in the range of 0.005–0.01 mg/L of As in drinking water (Yoshida et al, 2004; Ng et al., 2003). As exposure associated with health effects include cardiovascular and peripheral vascular disease, developmental anomalies, neurologic and neurobehavioural disorders, diabetes, hearing loss, portal fibrosis, hematologic disorders (anemia, leukopenia and eosinophilia) and multiple cancers: increased mortality rates for cancers of the skin, lung, liver, urinary bladder, kidney, and colon in many areas of As pollution (Tchounwou et al., 2003; Abernathy et al., 2003).

Absorption and bioavailability of inorganic forms of As in animals have been studied. Juhasz et al. (2006) reported a high absolute bioavailability in swine as As(III) (~104%) and As(V) (92.5%), whereas Robert et al. (2002) found 74.5% absolute bioavailability for As(V) in monkeys. Absorbed inorganic As is transported to liver by the action of As(+3) methyltransferase (AS3MT) into a series of mono- and dimethylated metabolites with oxidation state +3 and +5, which may be bound to thiol groups (Thomas et al., 2007). The formation of methylated metabolites of inorganic As is not necessarily a detoxification process; however, intermediates and products formed

in this pathway may be more reactive and toxic than inorganic As. Dimethylated forms (DMA) are the final products of the process, and high presence of these forms indicates an adequate progress of the metabolic process. Contrarily, accumulation of monomethylated species (MMA), intermediate metabolites, or of inorganic As indicates a lower rate of metabolism. This metabolic process affects As distribution and excretion. Drobna et al. (2009) demonstrated that AS3MT knockout mice retained significantly higher percentages of inorganic As and showed slower whole body clearance compared to wild-type mice.

Studies in rodents have shown that the distribution of inorganic As depends on the oxidation state, dose, type of dosage and duration of the treatment. Kenyon et al. (2005; 2008) showed organ-specific differences in the distribution and methylation of inorganic As and its methylated metabolites after exposure to As(V). Normally, DMA is the main As product eliminated through urine. Kenyon et al. (2005) reported similar high amounts of DMA in cumulative 24h urinary excretion. Kenyon et al. (1997) showed also that Weanling female B6C3F mice on the Se excess diet excreted a significantly higher percentage of urinary As as inorganic As, with a significantly decreased ratio of organic to inorganic As compared to Se-sufficient mice, probably by decreased As methylation. Again, mice on the Se-deficient diet appeared to eliminate As(V), arsenite, and DMA in urine more slowly than Se-sufficient mice, indicating a relation between dietary Se and As metabolism. Gailer et al. (2000) revealed the presence of seleno-bis (S-glutathionyl) arsinium ion, $[(GS)2AsSe]^-$, formed from As(III), Se(IV), and glutathione (GSH) by structural studies of the bile of rabbits exposed to As(III) and Se(IV) using X-ray absorption spectroscopy. An essential vitamin, folic acid, also affects the toxicokinetics of inorganic As. Gamble et al., (2006) showed that after 12 weeks of folic acid supplementation to participants with low plasma folate, the proportion of total urinary As excreted as DMA increased, suggesting an improvement in As metabolism. Heck et al. (2007) reported that dietary intakes of cysteine, methionine, vitamin B12, calcium, and protein are inversely associated with the percentage of inorganic As in urine, indicating an increase in metabolic rate and greater excretion. Gonzalez et al. (1995) reported that phosphate poses a pronounced decrease in intestinal absorption of As(V) in rat, because of sharing the same transport mechanisms which is an active secondary carrier-mediated system depending on Na^+ and H^+ gradient. Villa-Bellosta and Sorribas (2010) and Calatayud et al. (2012) suggested that the sodium-dependent

phosphate transporters in the apical membrane of enterocytes- may intervene in absorption of As(V), both *in vivo* and *in vitro* studies. Clemente et al. (2016) showed that iron salts, sulfates of Fe(II) and Fe(III) reduced the solubility of inorganic As (86–99%) and DMA(V) (40–66%) in aqueous solution, as well as, reduced the bio-accessibility of As in rice and seaweed. Yu et al. (2016) demonstrated the effectiveness of Fe(III) in reducing bio-accessibility of As(III) (15–37%) using an *in vitro* dynamic gastrointestinal digestion model (SHIME). Bisanz et al. (2014) reported that consumption of probiotic yogurt containing Lactobacillus rhamnosus GR-1 had a significant protective effect against increase in As level in blood of an African population, indicating a correlation between probiotics and As level in plasma. However, As toxicity has multiface such as it depends on As speciation, specific doses, exposure patterns, duration of exposure, ionic status of cellular microenvironment and target organs.

1.4 Pb exposures and toxicological aspects

WHO has identified Pb as 1 of 10 chemicals of major public health concern. Pb is harmful even in extremely low amount, that there is no known safe blood lead concentration for human. Even blood Pb concentrations as low as 5 μg/dL, may be associated with decreased intelligence in children, behavioural difficulties and learning problems (WHO, 2017). Major sources of environmental contamination with Pb includes mining, smelting, manufacturing and recycling activities, and continued use of leaded paint, leaded gasoline and leaded aviation fuel (Cheng et al., 2010). More than three quarters of global Pb consumption are for the manufacture of lead-acid batteries for motor vehicles. Pb is also used in many other products, for example pigments, paints, solder, stained glass, lead crystal glassware, ammunition, ceramic glazes, jewellery, toys and in some cosmetics and traditional medicines. Drinking water supplied through Pb pipes or pipes joined with Pb solder may cause Pb contamination (Kumar and Puri, 2012; Etchevers et al., 2015). The pathway of exposure varies depending on their sources including ingestion (Naidoo et al., 2017), inhalation (Eqani et al., 2015), and dermal absorption (Filon et al., 2006). Children are more susceptible to Pb than adults, with exposure with Pb causing irreversibly effects on the cognitive performance, mental retardation and behavioural disorders (Hilary, 2001). Pb poisoning in adults can affect the peripheral and central nervous systems, kidneys, and blood pressure and can cause coma, convulsions and even death (Needleman, 2004). Pb

exposure also causes anaemia, hypertension, renal impairment, immunotoxicity and toxicity to the reproductive organs (WHO, 2017).

The effect of food intake on gastrointestinal absorption of Pb was measured in five healthy men, eating constant Pb diets, and found that absorption of Pb was increased when ingested without food (Rabinowitz et al., 1980). Pb absorption was 10.3% for food Pb; 8.2% for tracers (nitrate, cysteine complex, or sulfide) ingested with food; and 35% for tracers ingested without food. It was indicated that components of diet have effect on Pb absorption (Rabinowitz et al., 1980). About 94% of the total absorbed Pb in adults is accumulated in the bones and about 73% in children (infant?); the rest is absorbed in soft tissues (liver, kidneys and brain). The half-life for Pb in blood and other soft tissues is about 28–36 days, but comparatively longer in bones (WHO, 1995). The release of bone Pb can continue to increase Pb concentrations in blood for years after the exposure ended (Ambrose et al., 2000).

Some minerals (Fe and Ca), vitamin C, fiber, macronutriens and probiotics can influence the intestinal absorption and accumulation in tissues. Bradman et al. (2001) compared blood Pb levels of iron-deficient and iron-replete children living in low, medium, or highly contaminated Pb in environments and reported a correlation between Fe-deficient diets and a higher concentration of Pb in blood. Bannon et al., (2002) demonstrated that Pb can be transported by DMT1, which transports both Pb and Fe because of their similar affinity, and in Fe deficiency the expression of this transporter in the intestine increases, which indirectly leads to an increase in the transport of Pb. This phenomenon can explain the reduction in absorption of Pb in Fe supplementation. Rosado et al. (2006) reported opposite result. Within a 3.5 km radius of a metal-smelter daily Pb-exposed school children with normal Fe status showed no significant improvement

Hernandez-Avila et al. (2003) showed that in a randomized, double-blind, placebo-control trial, among lactating women with relatively high Pb burden, calcium (Ca) supplementation was associated with a modest reduction in blood Pb levels (15–20%) over the course of lactation. Christakos (2012) demonstrated that in diets deficient in Ca there is an increase in synthesis of 1,25 dihydroxyvitamin D3 (1,25(OH)2D3), the major regulator of intestinal Ca absorption, which stimulates Ca2C transporter proteins at the intestinal level. Pb can be transported by these proteins, especially during their translocation inside the epithelium and in their passage across

the basolateral domain (Diamond, 2000) and thereby deficiency of Ca can increase intestinal transport of Pb.

Simon and Hudes (1999) suggested that high serum levels of vitamin C (ascorbic acid) are associated with prevalence of elevated blood Pb levels. However, Varnai et al. (2003) showed that ascorbic acid supplementation has no beneficial effect on Pb retention and even they reported to detect an increase in Pb concentration. Diets with different fiber sources (cellulose, pectin, guar gum, or carboxymethyl cellulose) and 0.1% of Pb for four weeks in rats showed that dietary fibers have an effect on intestinal absorption of Pb by increasing -excretion (Kim and Lee, 1990). Barltrop and Khoo (1976) reported that high fat diets increased blood and liver Pb concentration in rats fed with diets containing 0.075% $PbCl_2$, and suggested the possibility that Pb, fatty acids, and perhaps bile salts could form a soluble diffusible complex in the intestine, to be more readily absorbed.

Tian et al. (2012) showed that oral administration of both living and dead L. plantarum CCFM8661 offered a significant protective effect against Pb toxicity in mice blood and tissues by recovering blood δ-aminolevulinic acid dehydratase activity, decreasing the Pb levels in blood and tissues, and preventing alterations in the levels of glutathione, glutathione peroxidase, malondialdehyde, superoxide dismutase, and reactive oxygen species caused by Pb exposure. It was more effective when co-exposed with Pb, not after the exposure.

1.5 Hg exposures and toxicology

Hg is considered by WHO as one of the top ten chemicals or groups of chemicals of major public health concern. It occurs naturally and exists in various forms: elemental (or metallic); inorganic (e.g. mercuric chloride); and organic (e.g., methyl- and ethyl-mercury). These forms all have different toxicities and implications for human health. Hg can be released into environment from combustion of fossil fuels (specially coal), electricity-generating power stations, gold and mercury mining, manufacture of cement, pesticides, chlorine, caustic soda, mirrors and medical equipment, industrial leaks, dentistry, waste and corpse incineration, batteries, thermometers and barometers, electric switches, lamps (light bulbs), dental amalgam, cosmetics and pharmaceuticals. According to WHO (2007), provisional tolerable weekly intake is 1.6 μg/kg bodyweight/week for methylmercury in order to protect the developing fetus

from neurotoxic effects. WHO recommended guidelines are for water: 1 μg/L for total Hg, and for air: 1 μg/m^3 (annual average). The routes of exposure are ingestion, inhalation and dermal contact (EPA, 2017). Exposure with Hg causes harmful effects to human health, including cardiovascular and central nervous systems diseases, behavioral disorders, tremors, insomnia, memory loss, neuromuscular effects, motor dysfunction, neurodevelopmental problems in the developing fetus ataxia, speech impairment, visual field constriction, sensory disturbance, deafness, blindness, tremors, involuntary movements, mental retardation, coma, and death (Azevedo et al., 2012; EPA, 2017). These changes induced by Hg are known as Minamata disease or Russell-Hunter syndrome (Azevedo et al., 2012).

Studies on animals and humans showed that absorption of Hg(II) is variable and depends on the solubility of Hg salts (JECFA, 2011). Most of the inorganic Hg accumulations occur in kidney, especially the pars recta of the proximal tubule, because of the high bonding affinity between Hg ions and the thiol group(s) in proteins, peptides and amino acids (Zalpus, 2000). In intestine inorganic Hg accumulation is low and is excreted through feces. The absorption rate of organic Hg (CH_3Hg) is higher (80%) than inorganic Hg (JECFA, 2011). Highest absorption of organic Hg occurs in kidneys and liver, and 80-90% of it excreted in the form of inorganic Hg through faces (NRC, 2000).
Se, edible fat, fiber and probiotics are believed to have effect on the toxicokinetics of Hg. Orct et al. (2009) demonstrated that Hg concentration in organs, plasma and urine decreased with higher oral doses of Se when simultaneously exposed in suckling rats. However, opposite effect in mice has been reported by Magos and Webb (1977). They showed that significantly higher amount of Hg was observed in blood when exposed simultaneously with Se.

The effect of edible fat in toxicokinetics of Hg is contradictory. Højbjerg et al. (1992) reported that the type and amount of fats (cod liver oil, coconut oil, and soy oil) in the diet affects the whole-body retention and relative organ distribution of Hg in mice. However, Jin et al. (2007) claimed that dietary fats (soy oil, seal oil, docosahexaenoic acid, fish oil, or lard) may not have effect in brain/blood Hg distribution. Rowland et al. (1986) reported that incorporation of 15 or 30% bran fibers in the diet of mice decreased the total Hg concentration in the brain, blood and small intestine via a modification of the metabolic activity of the gut microflora; however, the proportion of Hg found in the mercuric form was significantly greater in

liver, kidneys and gut of mice fed bran. Bisanz et al. (2014) showed a significant reduction in Hg in blood of pregnant women treated with Lactobacillus rhamnosus GR-1, although it was not effective in children.

1.6 Overview of Zn and Se metabolism

It is important to discuss about the metabolic fate of nutritional trace elements to assess their homeostasis in the biological system. In this section Zn and Se metabolism is briefly discussed in animal body. Zn metabolism in human body can be described under four main processes such as absorption, transportation, homeostasis and excretion (Roohani et al., 2013). Through the carrier-mediated pathway Zn first absorbed in the small intestine; however, the exact level of absorbed Zn is difficult to determine as it is also excreted into the gut under normal physiologic condition (Cousins, 1985). The absorption rate is concentration dependent and usually also depends on different factors such as type of diets, phytate level and so on. It become free ions during digestion then these free ions bind with endogenously secreted ligands before transportation into enterocytes (Tubek, 2007). Zn transporters (ZnTs) and zip transporters are then facilitate the Zn mobilization into the cellular membranes. Usually, ZnTs lower the intracellular Zn availability by promoting Zn efflux from cells or into intracellular vesicles, on the other hand, zip transporters elevate the intracellular Zn availability by promoting extracellular Zn uptake and perhaps, vesicular Zn release into the cytoplasm (Sekler et al., 2007). Low molecular weight metal binding protein, MT may also be involved in Zn transportation as it may increase with Zn supplementation and decrease in Zn deficit situation; however, the mechanism of them is still unclear. DCT1 may also be involved in the Zn transportation process (McMahon and Cousins, 1998). After that the homeostatic level maintaining is markedly crucial for the Zn level in the cells. In biological system the adjustment of gastrointestinal absorption of Zn and intestinal excretion- operates the Zn level in the body. Rate of absorptions and excretion is maintained through a set of internal metabolic activity that eventually maintains the cellular homeostasis of Zn in either of Zn deficit and affluent condition (King et al., 2000). Gastrointestinal tract is the major route of Zn excretion; however, this Zn can be reabsorbed. The other means of excretion are through urinary and surface losses such as skin, hair and so on. Typically, Zn intake ranges from 107 to 231 μmol/day (equivalent to 14-30 mg/kg) in healthy human (Roohani et al., 2013). It

can be as low as 22 µmol/day and maximum as 306 µmol/day which can also be balanced by the metabolic system (Johnson et al., 1993).

Like Zn, another indispensable trace mineral Se is also needed for proper cellular functions in biological systems. However, among the four oxidation states (+6, +4, 0, -2) of Se, two (selenate: 6 and selenite: 4) are important for its biogeochemical and biological cycling (Sun et al., 2014). This two follows different biochemical pathways during uptake and metabolism. Sodium-sulfate cotransporters are mainly responsible for the transportation of selenate; however, passive diffusion into cell is the key absorption way for selenite (Bergeron et al., 2013; Park and Whanger, 1995). Once enters into the cells inorganic Se is biochemically metabolized to selenide (-2) (Spallholz, 1994). Studies reported that Se (+6) first undergoes enzymatic reduction to Se (+4) then it can be further metabolized non-enzymatically via GSH into Se (-2) (Ogra and Anan, 2009). Furthermore, it was proposed that Se (+4) usually reacts with GSH to form a seleno-diglutathione (GS-Se-SG) compound (Weiller et al., 2004). This compound is further subsequently converted to seleo persulfide (GSSeH) which finally decays (into elemental Se or GSH) or converted enzymatically into hydrogen selenide (H_2Se) in anoxic environment (Sun et al., 2014). However, Se (-2) is being used to biosynthesis of selenoproteins or for bio-methylation (methylselenol, dimethylselenide, trimethylselenonium cation). They have potential to be excreted from the cell/ body (Gailer, 2000). Sun et al. (2014) depicted this pathway using a diagram (Fig. 1.1).

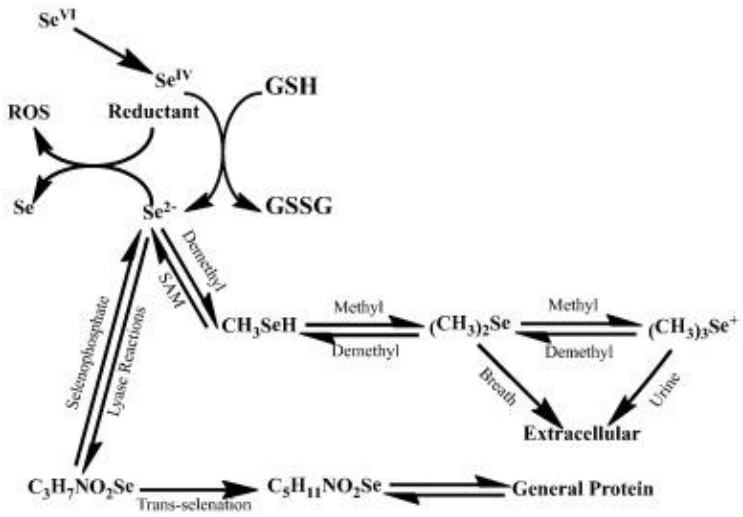

Fig. 1.1 Selenium metabolism pathways adopted from Sun et al. (2014). Se^{VI} = selenate; Se^{IV} = selenite; Se^{2-} = selenide; GSH = glutathione; GSSG = oxidized glutathione; SAM = *S*-adenosylmethionine; CH_3SeH = methylselenol; $(CH_3)_2Se$ = dimethyselenide;

$(CH_3)_3Se^+$ = trimethylselenonium cation; $C_3H_7NO_2Se$ = selenocysteine; and $C_5H_{11}NO_2Se$ = selenomethionine.

1.7 Zn in detoxification of Cd

Zn is a nutritionally essential trace element, and is known to involve in diversified structures and functions of many macromolecules such as in enzymes to regulate cellular processes and cellular signaling pathways (Jarosz et al., 2017). It is also well known for its antioxidant and anti-inflammatory activities and for enhancement of the formation of low molecular weight metal binding proteins such as MT. In this section, we discussed about the interaction of Zn and Cd in terms of antagonism/synergism and the underlying biomolecular mechanism/s in biological systems. There are a lot of studies have been reported in this issue both *in vitro* and *in vivo*; however, we intended to focus on the studies published since 2008 and afterwards.

Many studies have already reported that oxidative stress induction is one of the pivotal pathways for Cd-toxicity. Thus, the antioxidant property of Zn has been studying since last few decades to combat Cd-toxicity both in cellular level as well as level in organism. For instance, Amara et al. (2008) studied the antioxidant role of Zn on Cd-induced toxicity in Wistar rat model. They reported that Cd induced significant oxidative stress in the rat testis and gonads via increasing malondialdehyde (MDA) (+46%), mettalothionein (+200%), 8-oxodGuo (+71%) concentrations in testis, and decreasing GPx (–30%), CAT (–32%), mitochondrial Mn-SOD (–34%), cytosolic Cu, Zn-SOD (–32%) activities in the gonad (Amara et al., 2008). However, Zn treatment ($ZnCl_2$, 40 mg/L) significantly improved the antioxidant activities by maintaining GPx, Cu, Zn-SOD, Mn-SOD, lowering MDA in the testes similar as the control group (Amara et al., 2008). Similarly, Jihen et al. (2009) reported that Zn restored the Cd-induced depletion of total SOD, Cu, Zn-SOD, GPx and Cu, Zn-SOD/GPx ratio in the rat liver(Jihen et al., 2010). Later on, Jihen et al. (2011) reported also that high exposure of Cd (200 mg/L) and Zn (500 mg/L) together or alone induced significantly higher accumulation of Cd and Zn in livers of male rats. Moreover, Cd-induced a significant depletion of GSH, GSH/GSSG, Cu, Zn-SOD and GPx levels as well as rose up the levels of Cd/Zn and GSSG without affecting Cu, Zn-SOD/GPx, and Mn-SOD in the liver (Jihen et al., 2011). On the other hand, Cd-Zn co-treatment significantly increased the activity of GSH, GPx,

GSH/GSSG ratio, and reduced the level of GSSG in rat liver. Therefore, only an indirect ameliorative effect of Zn on Cd-induced toxicity was stated by the study (Jihen et al., 2011). Similarly, higher Cd retention in the kidney tissue due to Zn and Cd combined exposure has been suggested as one of the key mechanisms of Zn protection against Cd-induced toxicity in rat liver (Jihen et al., 2010).

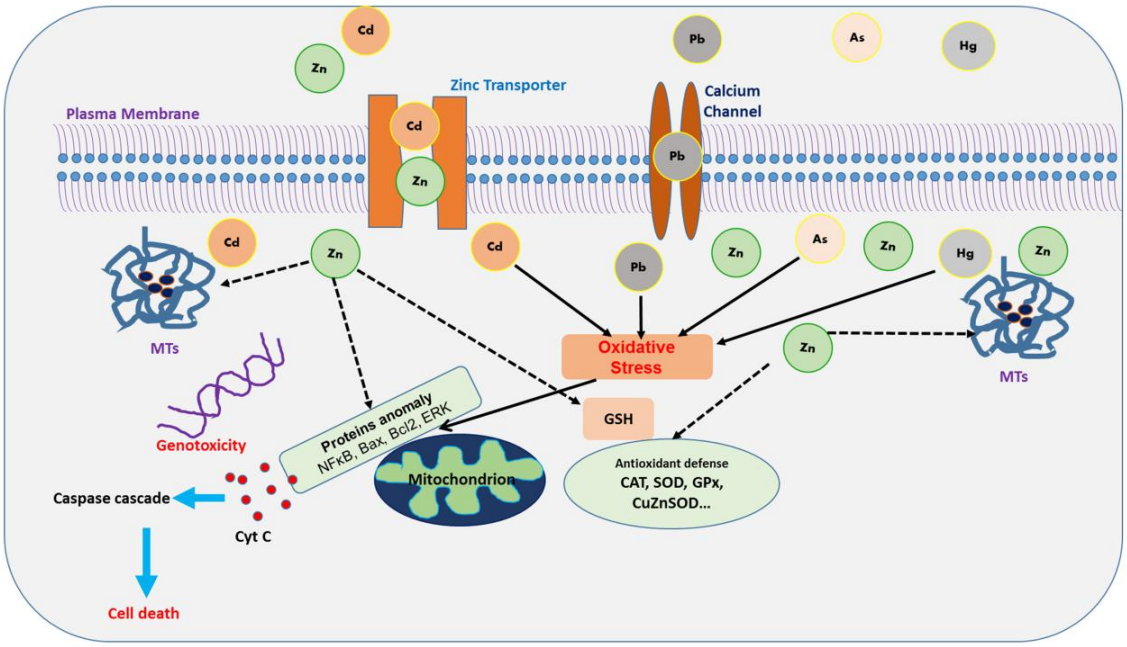

Fig. 1.2 Possible mechanism/s exerted by zinc against toxic metals in biological systems. Here, Cyt C; cytochrome c, MT; metallothionein, CAT; catalase, SOD; superoxide dismutase.

In another study, Galazyn-Sidorczuk et al. (2012) reported that Zn (30 mg/L) protected rat from Cd-toxicity *via* restoring GPx activity and increasing the level of Se content because Cd exposure was significantly lowered the Se content in serum, liver and in kidney. However, at low exposure of Cd (5mg/L) the protection was independent of GPx activity but at high exposure (50 mg/L), it was GPx dependent. Moreover, Zn pretreatment was found to protect the kidney of Cd-challenged rat by improving oxidative damage and by formation of MT due to excess Zn supplementation (Jemai et al., 2010). However, in combination treatment of Zn and Cd a significant decreased serum Zn level was observed (Jemai et al., 2010).

Furthermore, Zn (30 mg/L) was reported more effective than Zn (60 mg/L) against Cd (5 and 50 mg/L) induced liver toxicity in rat model, because the increasing

dose of Zn synergistically increased the necrosis in liver (Rogalska et al., 2011). However, it must be noted that both doses of Zn protected Cd-induced alternations in the structure and function of liver in rat model (Rogalska et al., 2011). Moreover, Rogalska et al. (2009) also studied the role of Zn exposure on Cd-induced changes in lipid metabolism of a rat model. It was reported that Cd (5 and 50mg Cd/L) exerted a significant changes in lipid profile by elevating the concentrations of FFA, TCh, LDL, LPO, F2-IsoP, oxLDL (spelled out and show abbreviations in parenthesis), and by lowering the level of spelled out (PL) and high-density lipoprotein (HDL) in exposed rat serum; however, Zn supplementation at 30 and 60 mg/L dose protected from all the Cd-induced changes except F2-IsoP (partial improvement) in the lipid status of serum of the studied rat. In addition, Zn supplementation (30 and 60 mg/L) has also been found to protect Cd-induced (5 and 50 mg/L) bone damages in rat (Brzóska and Rogalska, 2013). In this study, Zn was found to improve oxidative stress (GPx, SOD, CAT, glutathione reductase (GR) and GSH) induced by Cd through restoring protein, DNA, and lipid oxidation in the bone, and also Zn supplementation inhibited from the Cd-induced increase in soluble RANK legand (sRANKL) level and the sRANKL/osteoprotegerin (OPG) ratio, as well as lowered in OPG level both in the bone and serum of studied Wistar rat (Brzóska and Rogalska, 2013). In addition, Zn was also found to lowered the alkaline phosphatase, calcium, phosphorus, magnesium, on the other hand, elevated the albumin concentration which are negatively affected by Cd (0.5, 1 nad 2 mg/kg) exposure in Wistar rat (Najafi et al., 2016). These bone metabolism properties are exceedingly important, and Zn was found to protect/restore them from Cd intoxication in rat.

Recently, we have also reported a Zn protection against Cd-induced oxidative stress by improving GSH level in PC12 cells (Rahman et al., 2017). Moreover, Cd-induced (5 and 10 μM) mitochondrial apoptosis *via* cytochrome c release into cytosol, deregulating Bax/Bcl2 ratio and upregulating caspase 3, 9 expressions was reduced by the Zn (5-100 μM) exposure simultaneously in PC12 cells (Rahman et al., 2017). Zhang et al. (2014) reported protective effects of Zn against Cd-toxicity in Madin-Darby bovine kidney epithelial cells via reduction of apoptotic cell death, DNA damage, and production of ROS. Moreover, the co-exposure of Zn (10 μM) and Cd (10 μM) showed upregulation of MT-1 and -2 and their mRNA levels in kidney epithelial cells (Zhang et al., 2014). In addition, Cd-induced toxicity was reported in human BJAB cells, and found

a dose dependent reduction of cell viability and DNA damage (Nemmiche and Guiraud, 2016). It also found that the Cd-accumulation was attributed with the production of 8-hydroxy-2′?-deoxyguanosine adducts as well as upregulated MTF1 expression and accompanied with the Zn transporters, DMT1 and ZnT1, thus the involvement of Zn transporters might be claimed strongly in the process of Cd-toxicity induction in BJAB cells (Nemmiche and Guiraud, 2016). Recently, in a study on HepG2 cells it was found that Cd treatment enhanced the uptake of Zn and Fe regardless of the presence or absence of n-3 fatty acids; however, Cu was unchanged (Sampels et al., 2017). In addition, the expressions of MT-1g and MT-1m were upregulated due to excess exposure of Cd in HepG2 cells (Sampels et al., 2017).

The risk of reproductive toxicity due to Cd exposure has been reported in both animal and human populations through increasing of infertility as well as formation of cancers in the reproductive tissues (El-Demerdash et al., 2004; Goyer et al., 2004). Messaoudi et al. (2010b) reported that a significant alteration in the testicular tissues was induced by Cd (200 mg/L, drinking water) exposure such as lowering of plasma testosterone level, Zn concentrations (in plasma and testis), increasing of oxidative stress and expression of MT-1 and MT-2 genes (Messaoudi et al., 2010b). However, combined exposure of Cd (200 mg/L, drinking water) and Zn (500 mg/L, drinking water) restored the testosterone level, SOD activity, lowered Cd testicular uptake and reduced the expression level of MT-1 and MT-2 genes (Messaoudi et al., 2010b). Furthermore the co-treatment could partially modulate histological changes, lipid peroxidation, and Zn depletion compared to the only Cd-treated rat (Messaoudi et al., 2010b). A similar kind of protective effects of Zn co-treatment with Cd was found against testicular injury due to Cd in Wistar rat by reducing lipid peroxidation, improving sperm properties, and histopathology of testis (Babaknejad et al., 2017). The role of two isoforms of MT, CeMT-1 and CeMT-2 possessed distinct affinity to Cd and Zn in *C. elegans*; however, both of the proteins showed equal affinity for Zn (Zeitoun-Ghandour et al., 2010). In a recent study, it was stated that Cd inhibited estrogen signaling in zebrafish brain via inhibition of estradiol (E2) induced transcriptional activation of estradiol receptors (ERs), and increased Aro-B expression in protein and gene level (Chouchene et al., 2016); although Cd alone did not affect the ERs but Zn exposure altered the antagonistic effects of Cd on E2 induced ERs at protein level *in vivo* and *in vitro* (Chouchene et al., 2016). Ameliorative role of Zn on Cd-induced

reproductive toxicity was studied *via* testing of testis development following a prenatal and postnatal exposure of Cd (50 mg/L) with/or without Zn (60 mg/L) in rat model (Chemek et al., 2016). A significant Cd toxicity was reported on testis development. However, they also found a disruption in Zn metabolism due to Cd exposure, and dietary supplementation of Zn protected from Cd-toxicity at exceedingly early development of rat (Chemek et al., 2016). It was also explored that Zn protection against Cd-toxicity via reduction of Cd accumulation and elevation of Zn availability for the offspring rat (Chemek et al., 2016). Recently, Chemek et al. (2018) studied Cd- induced reproductive toxicity in male rat and potential reversal by Zn supplementation. A significant reduction of reproductive organ weight, anomalous testicular histology and sperm count were induced by in utero and lactational Cd exposure. Moreover, rat testicular disheveled-associated activator of morphogenesis 1 (DAAM1) expression was also inhibited by Cd-exposure both in protein and mRNA levels (Chemek et al., 2018). However, these changes in the reproductive organs were almost completely reversed by the supplementation of Zn in the rat (Chemek et al., 2018). Furthermore, Cd-induced (2.2 mg/kg per day) testicular toxicity was also inhibited by Zn (2.2 mg/kg per day) exposure *via* improving the antioxidant defense and anti-inflammatory activities of Zn (Bashandy et al., 2016). Using of blood rheological properties as one of the early disease markers, a study has been conducted on the influence of Zn on Cd-induced changes in the rheological properties of male rat (Moussa et al., 2016). They found a restoration of rheological property for blood serum viscosity by Zn co-exposed with Cd in rat which was significantly increased by Cd-alone, whereas Cd alone did not show any significant effect on the %torque and shear stress at the lower shear rates (200–600 s–1); however, at higher rate Cd did significant changes (Moussa et al., 2016). It can be attributed with the Cd displacement by Zn or at least a competitive inhibition at the binding site of the element. Therefore, Zn has also been studied extensively against Cd toxicity and along with the antioxidant and scavenging properties like Se. In addition, it was considered that Zn induced the formation of metal binding low molecular weight protein MTs which are a key player in the detoxification of Cd by Zn supplementation. Also a combination of Zn and Se could have been more effective than the separated one, and requires more investigation to distinct the individual functions both in cellular and organisms levels.

1.8 Se in the detoxification of As

Se and As are two metalloids with peculiar features such as both were reported as anti-cancer as well as cancer inducing agents. Their metabolic interactions have been investigated by numerous scientists since long ago. Two types of interactions between Se and As have been hypothesized by Sun et al. (2014). Firstly, a low concentration exposure of Se could be antagonistic against As toxicity by excretion of As-Se compound [(GS3)2AsSe], and the second one was to induce synergistic toxicity with As at high dose exposure through reacting with S-adenosylmethionine and glutathione, and modifying arsenite methyltransferase. Therefore, in this section, we focused on the aforementioned hypothesis to further explore the critical interactions in toxicological points of view between Se and As, and their interplay in organisms and cellular level.

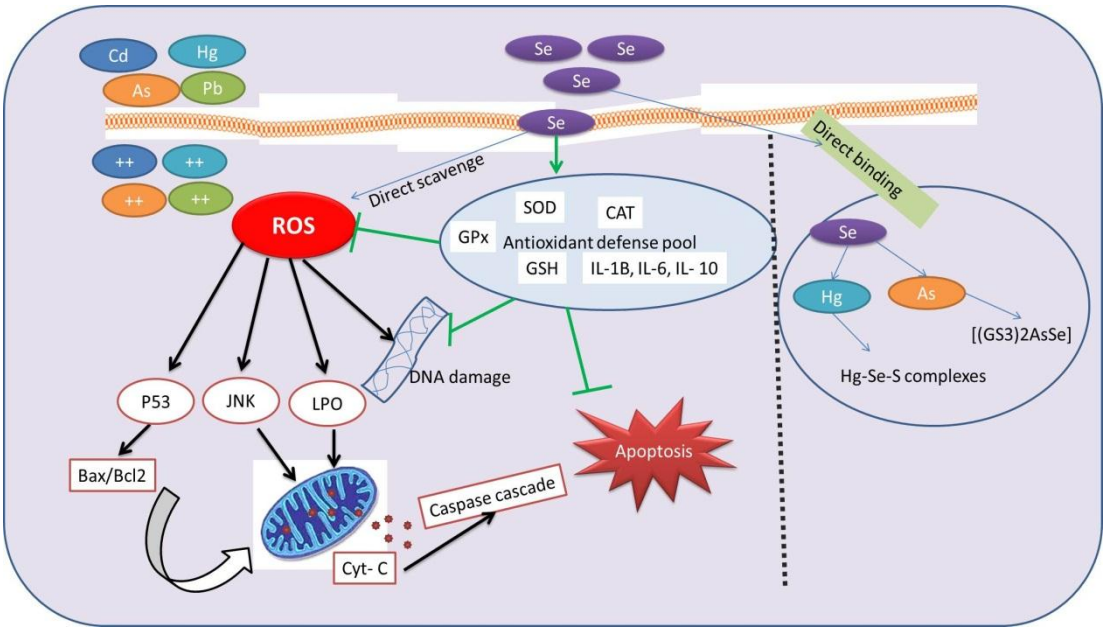

Fig. 1.3 A schematic diagram for molecular mechanism/s depicting antagonism between selenium and toxic metals. Here, ROS-Reactive oxygen species; GPx-Glutathione peroxidase; LOP-Lipid peroxidation; Cyt-C-Cytochrome C; IL-Interleukin

Since 1939, after the report of Moxon and DuBios (1939) on a rat model study which found a low exposure of As (III) (5 mg/kg oral administration) completely reversed the toxicity of Se (IV) (5 mg/kg oral administration) in rat liver, it has been drawing the attention of scientific community to investigate more. Later on, it was found that Se (IV) significantly decreased the As (III)-induced malformations in pregnant hamsters

(Holmberg and Ferm, 1969). Since then, the antagonism between As and Se had gain sufficient fuel to explore the molecular mechanism/s behind it. Recently, many research groups including us have reported alleviating effects of Se against As toxicity both *in vitro* and *in vivo*. For instance, Chitta et al. (2013) studied a combined effect of As (III) and selenomethionine (SeMet) (100 μM) in HEK293 (human kidney cells), and reported As (30 μM) induced ROS, protein and DNA damage which were detoxify by the As-Se Met combination to confer HEK293 survival. Similar antioxidant effects of Se against As toxicity was also previously reported by Selvaraj et al. (2012) in poeciliopsis lucida hepatocellular carcinoma line 1 (PLHC-1) cells. They found to reduce DNA damage and to inhibit apoptosis induced by As. We further investigated the cytoprotective role of Se (10 μM) against As (10 μM) using PC12 cells upon simultaneous exposure for 48 h (Rahman et al., 2018). It was reported that the combination of As and Se showed significant reduction of DNA damage, improved antioxidant protection, inhibition of cellular apoptosis/autophagy compared to As-treated PC12 cells. Previous researches already reported that a quite similar methylation pathway shares by Se and As (Sun et al., 2014). It was also found that they can inhibit the excretion of their methylation metabolite (Kenyon et al., 1997), and thus a hypothesis was proposed to form a As-Se compound which could possibly possess less toxicity compared to their sole toxicity (Fig. 2.4). Gailer et al. (2000) was the pioneer to announced for the first time such a new As–Se compound named as selenobis (S-glutathionyl) arsinium ion $[(GS)2AsSe]^-$, that can readily be excreted from hepatocytes to bile. Another study reported similar As-Se compound which is formed in erythrocytes and went out to enter into the blood (Manley et al., 2006).

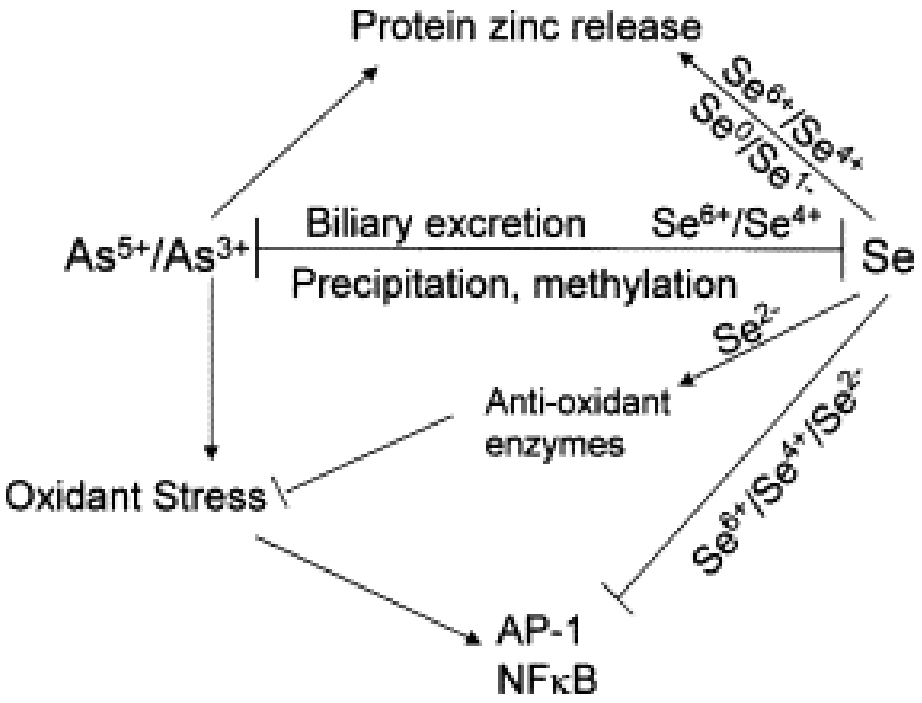

Fig. 1.4 Cellular metabolic pathway depicting the antagonism between Se and As by direct and indirect interactions (Zeng et al., 2005). Where, Arrows represent induction, and single capped lines represent inhibition of pathways; the double capped line represents the mutual inhibition of Se/As bioactivity through the increase of As/Se biliary excretion, the formation of Se–As precipitate, and the modification As/Se methylation pathways (Zeng et al., 2005).

In addition to that, another As–Se compound $[(CH3)2As(Se)2]^{-}$ was also observed (Gailer et al., 2002; 2003). Recently, after the phase III clinical trial ''Selenium in the Treatment of Arsenic Toxicity and Cancers'' As-Se antagonism was reported using animal model (hamsters) to localize at the tissue and organ levels (Ponomarenko et al., 2017). After 30 min of exposure via injection, As-Se co-exposure group showed a significant co-localization of both species in different organs such as liver, gall bladder, small intestine (Ponomarenko et al., 2017). Finally, they also found the formation of seleno bis-(S-glutathionyl) arsinium ion, and it was excreted via bile into intestinal tract. Furthermore, role of dietary Se in As detoxification was studied by Sah et al. (2013). They reported that Se enriched diet (high Se lentil; 0.3 mg/kg) induced higher fecal and urinary As excretion and lower renal As residues in As-treated rat while improving the glutathione level as well as health indicators of rat. Similarly, a high Se (0.3 mg/kg) lentil diet was also reported to rescue from As-induced atherosclerosis in mouse (Krohn

et al., 2016). They found plaque formation in the sinus and in the aortic arch of mice due to As was reduced/abolished upon the Se-fortified lentil diet; however, As-induced lesions were not changed in either Se deficit or adequate group.

Protection from oxidative damage induced by As have also been reported as a detoxification pathway by Se supplementation. For instance, Rossman and Uddin (2004) pointed out that Se able to protect As-induced oxidative damage of DNA through the selenoproteins upregulation sucg as GPx and thioredoxin reductases. We also reported Se-induced upregulation of GPx1 and increased GSH level in PC12 cells (Rahman et al., 2018; Hossain et al., 2018). Moreover, As-induced (10 μM) decreasings of GSH level and GPx1, and increasings of MDA and DNA damage were reversed in the co-exposure of As (10 μM) and Se (10 μM) in PC12 cells (Rahman et al., 2018). In addition, significant reduction of ROS generation was reported due to low level of As and Se co-exposure in NB4 cells was reported by Wang et al. (2015). Furthermore, it was also found that Se and As co-exposure significantly reduced the concentration of As accumulation in NB4 cells (Wang et al., 2015) and in PC12 cells (Rahman et al., 2018). In a study on hepatocytes, 100 μM of As induced loss of cell viability, increasing intracellular ROS production, decreasing GSH/GSSG ratio and decreasing of enzymatic activities of GPx and SOD (Jamwal et al., 2017). They also found antagonistic effects of Se against As toxicity *in vitro*. On reproductive toxicity in hamsters, it was reported that As accumulated in the placenta and fetus, increasing fetal mortality, non-viable fetuses and resorptions (Sampayo-Reyes et al., 2017). Co-exposure with Se (100 mg/L) significantly reduced teratogenic damage due to As. Moreover, Se significantly influenced As biotransformation via lowering the monomethyl arsonic acid (MMA)/In As index and elevating dimethyl arsonic acid (DMA)/MMA (Sampayo-Reyes et al., 2017).

Besides many investigations on antagonistic relationship between As and Se, there have been a significant studies reflected the synergistic relationship between them. Mutual inhibitions of their metabolites were found in different studies. Based on those reports, Sun et al. (2014) hypothesized that 'As and Se can mutually inhibit the formation of their methylated metabolites, resulting in more retention of inorganic and/or monomethyl As and/or Se in tissues'. In addition, a line of epidemiological evidences suggested synergistic relationship between As and Se, and demonstrated that the deficiency of dietary Se could enhance the toxicity and cancer risk due to As (Spallholz et al., 2004; Kolachi et al., 2011; Hsueh et al., 2017). However, these critical interactions

between Se and As, their exactrole and functions in biological systems, and their interactions warrant further investigations in human exposure level (Bode and Dong, 2002; Pilsner et al., 2011; Janasik et al., 2017). Complexity, nutritional and other biomolecular status, individual behaviors, and health conditions in human are all possibly effects the interactions between As and Se, thus, further investigation are needed in human levels to explore the complete interaction scenarios.

1.9 Overview of α- lipoic and dihydrolipoic acid

α-lipoic acid (LA) is an organo-sulfur compound also known as thioctic acid or 1,2-dithiolane-3-pentanoic acid. It is usually produced in the body and essential for the aerobic metabolism. It is furthermore a naturally occurring compound presents in almost all prokaryotic and eukaryotic cell types (Fei et al., 2016). It contains two sulfur atoms at C6 and C8 connected with a disulfide bond that makes it oxidized. It has one chiral center (C6) which makes it exist in two enantiomeric states R and S-enantiomeric forms (Shay et al., 2009). Of the two enantiomers, only R-enantiomer can covalently conjugate to conserved lysine residues through an amide linkage, thus being considered as the real essential cofactor for biological systems, as described in early reports (Dörsam et al., 2016). The reduced form of LA is called dihydrolipoic acid (DHLA) which contains a pair of thiol groups, and here again, only the R-enantiomer is biologically active (Fig. 1.2).

LA can be found in almost all foodstuffs, although the amount is quite low. A higher amount of LA is usually found in kidney, heart, liver, spinach, broccoli, yeast extract, dried egg powder, fresh egg yolk, fresh potatoes, and canned peas (Durrani et al., 2010). This compound naturally is always covalently bound and not easily available from dietary sources. Furthermore, it is present in an extreamely low amount in a nutrient supply. For example, the purification of LA to determine its structure used an estimated 10 tons of liver residue, which yielded 30 mg of LA (Reed, 2001). Thus, the supplementation of LA is almost completely derived from the chemically synthesized sources. However, almost all physiological requirements of LA is met up via *de novo* synthesis, and its role as a cofactor is well defined as compared to the oral intake although it can be taken up with the diet.

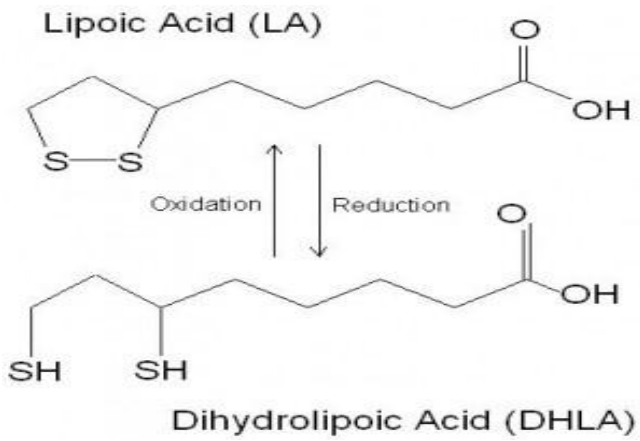

Fig. 1.4 Chemical structure of α-lipoic acid (LA) and dihydrolipoic acid (DHLA)

Although in a quite low quantity, the common dietary sources of LA are muscle meats, kidney, liver, fruits and vegetables as reported earlier (Durrani et al., 2010). However, the use of food supplements makes up a primary source of LA intake, ranging from 50 to 600 mg per day (Shay et al., 2009). LA uptake from the diet is a crucial factor to study in its metabolism. Previous studies reported multimodal transporters to be responsible for LA uptake. In CaCo2 cells, the LA could traverse rapidly into the cell monolayer, although depending on pH (Takaishi et al., 2007). Na^+-depended multivitamin transporter has been reported to be involved in the transport of LA *in vitro* (Balamurugan et al., 2005). Due to the various mechanisms involved in LA transport, the bioavailability of LA varies depending on the state of the compound as well as whether it is taken with a meal or as a sole supplement.

1.10 α-lipoic and dihydrolipoic acids as antioxidants/prooxidants

LA and DHLA react with different free radicals, which possess an unpaired electron. The resulting thiol radicals are very short-lived and therefore difficult to detect, although confirming observations have been done with electron spin resonance (Mottley and Moson, 2001). Thiol radicals can react with DHLA radicals, reshaping LA and DHLA. Reduced glutathione (GSH) is the primary antioxidant to react with free radicals, and having the main formation of a glutathionyl radical, it is found intracellularly at millimolar concentrations. LA radicals can be eliminated by reacting with GSH. Two glutathionyl radicals then form oxidized glutathione (GSSG), and thus the free radicals are removed. GSSG is then reduced to GSH by glutathione reductases (GR)

and NADPH (Fig. 1.3). Interestingly, LA/DHLA is the redox pair's ability to strengthen the antioxidant defense of the cell by restoring oxidized reaction products of, e.g., vitamin C, vitamin E, and GSH (Golbidi et al., 2011). For instance, in HepG2 cells, LA was reported to regenerate GSH *via* the Nrf2/ARE signaling pathway and thus alleviates Cd-induced cell toxicity (Zhang et al., 2017). Macias-Barragan et al. (2017) have reported a similar *de novo* synthesis and recycling pathway used by Cd and LA to balance GSH in HepG2 cells. Furthermore, LA regulates numerous signal transduction pathways that are highly susceptible to oxidative stress such as nuclear factor erythroid 2-related factor (Nrf2) (Chen et al., 2015). This applies to GSH/GSSG, whose default redox potential -0.24V is higher than that of LA/DHLA (-0.32V) so that DHLA can directly reduce GSSG.

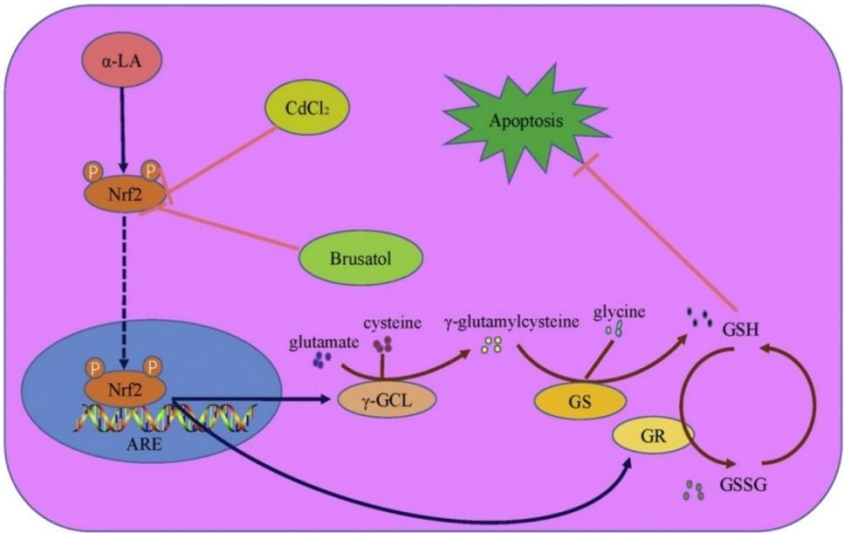

Fig. 1.5 Regeneration pathway of glutathione by LA under Cd stress in HepG2 cells (Zhang et al., 2017)

Despite the fact that LA and DHLA have proven to be excellent antioxidants; however, under special circumstances, pro-oxidant effects of LA and DHLA have been observed such as ROS production and peroxidation of lipids. For example, Bhatti et al. (2005) reported that LA has anti-oxidant effects on streptozotocin-induced diabetic rats as it attenuated albuminuria, glomerulosclerosis, tubulointerstitial fibrosis, superoxide (O_2^-) anion generation, and kidney expression of NADPH oxidase subunits p22phox and p47phox (Bhatt et al., 2005). However, in non-diabetic rats, LA was reported to show pro-oxidant effects *via* reversing the oxidative stress factors such as increased superoxide anion generation and NADPH oxidase subunits P22phox and p47phox

expression in kidney (Bhatti et al., 2005). Furthermore, Nur et al. (2017) reported a pro-oxidant effect of LA in combination with cisplatin to modulate apoptosis and oxidative stress in MCF-7 breast cancer cells. In their study, they found increased level of apoptosis, mitochondrial membrane depolarization, ROS production, lipid peroxidation, PARP1, caspase 3 and 9 expression levels in simultaneous LA (0.05 mM) and cisplatin (0.025 mM)-co-treatment group in MCF-7 cells although cell viability, reduced glutathione, and glutathione peroxidase (GPx) values were decreased by the treatments (Nur et al., 2017). They proposed that apoptosis and oxidant effects of cisplatin were increased by activation of TRPV1 channels, but its action on the values was further increased by LA (Nur et al., 2017). It can be said that type of oxidative stress and physiological status at least in part, determined the pro-oxidant role of LA and DHLA; however, the ability to function as either anti- or pro-oxidants. However, further research is warrant to explain the exact scenario upon which they reverse their functions against oxidative stress.

1.11 Research motivation

"Environmental medicine could be the sustainable solution for environmental diseases" can be simply stated as my motivation of current research. In this very section some important facts behind the research motivation is depicted. Bangladesh has been facing a disaster of groundwater arsenic contamination since it was first detected in 1993 by Department of Public Health Engineering (DPHE). Extensive monitoring study was conducted to determine As in groundwater. About 70 (59 districts out of 64 districts) millions people are being affected by As pollution in Bangladesh. Recently, we also reported As contamination in groundwater of two major districts in Bangladesh namely Manikganj (Rahman et al., 2016) and Gopalganj (Rahman et al., 2017) with elevated risk of health hazards, especially about children. Although recent study showed that people are now more likely to avoid the As-contaminated groundwater than before but the hazards still persist (Chakraborti et al., 2015). It is now spreading risk by diversified ways such as irrigation with As-contaminated water dispersed the toxicants through foodstuffs. Moreover, besides drinking water (Islam et al., 2017) toxic metals such as Cd, Cr, and Pb has been also reported in the food stuffs such as in rice, vegetables (Islam et al., 2015) and cow milk (Muhib et al., 2017). We have also assessed the health risk due to the toxic metal in drinking water and milk through determination

of hazards index (HI) (Rahman et al., 2017; Muhib et al., 2017). Not only in Bangladesh but the toxic metal-(loids) exposures and its potential risks to public health is a global concern. In this regards, the lack of awareness, proper education, lack of nutrition and poor baseline information could aggravate the scenario to worsen. For example, epidemiological study found that the As toxicity is increased where the body Se status is deficit in Bangladesh. There are intervention options to combat toxic metal-(loids) exposure and to effect beneficial roles in human; however, depending on magnitude and severity its almost impossible to achieve the ultimate benefits from them. Once it enters into the body, it is difficult to find a cure of it. So far no proper therapeutics is available to completely detoxify the metal-(loids) toxicity in biological systems. However, we have to search for perfect therapeutics.

Our previous researches on toxic metal-(loids) in drinking water and other foodstuffs with significant risks to human health have greatly motivated me to investigate the fundamental mechanisms of toxic metal-(loids) in biological systems and to search for potential environmental cure to reduce the deleterious effects of toxic metal-(loids).

Therefore, it can be hypothesized as "dietary trace elements/other supplements may have protective roles on toxic metal-(loids) induced deleterious effects on biological systems".

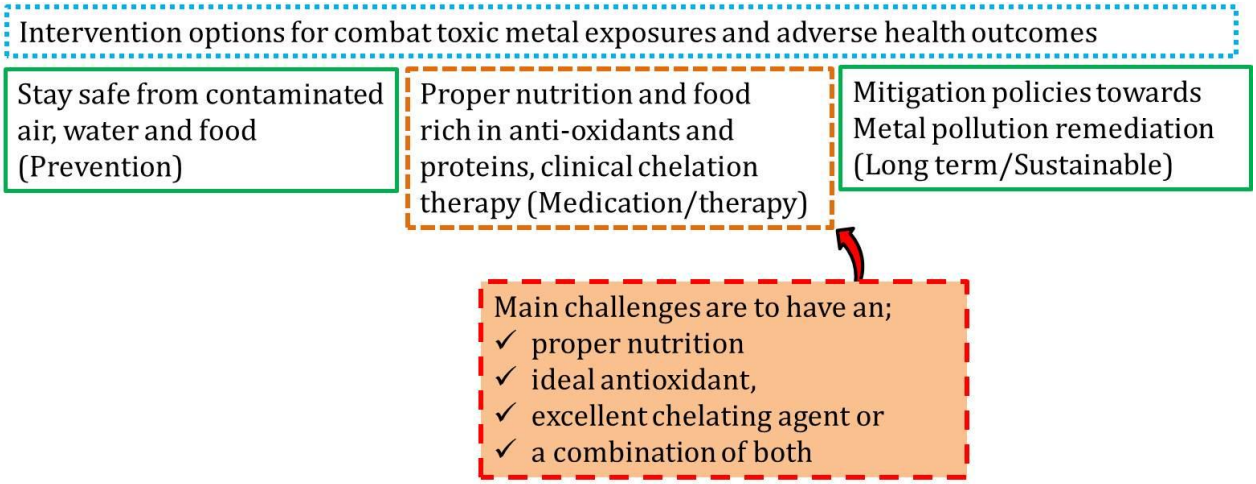

Fig. 1.6 Intervention options for toxic metals exposure to human

1.12 Aim and Objectives

The main aim of this research is to elucidate the fundamental mechanism/s of metal-(loids) induced cytotoxicity and their reversals with dietary supplements using cell biology and molecular biology techniques. To achieve the goal, a set of specific objectives is outlined as follows:

a. Make an overview of existing literatures to assess the recent progresses and to specify the necessity of fundamental investigation using cellular techniques.
b. To investigate the cytoprotective potentials of essential trace elements (Zn and Se) and dietary compounds (α-lipoic acid/dihydrolipoic acid) against As, Cd, Pb and Hg induced toxicity *in vitro*.
c. To check the cytotoxic effects of essential trace element (Zn and Se) on mammalian cells.
d. To elucidate the molecular mechanism/s behind the essential dietary compounds induced cytoprotection against metal-(loids) burden.

1.13 Outline of the thesis

This thesis comprises of six consecutive chapters including the first chapter on general introduction. In Chapter 1, a comprehensive background matter was discussed on effects of toxic metals on human and other biological systems, their sources, tolerable limits and toxicokinetics. Moreover, some mechanistic interactions were also discussed between Zn-Cd and Se-As in biological system. In addition, brief overview and aim of the study were also presented for the dietary elements along with dietary supplements and their roles and metabolisms. Then, Chapter 2 will be presenting the protective roles of Zn against Cd-induced toxicity using PC12 cells. Where, the cytoprotective potentials of Zn will be depicted by means of biomolecular mechanisms using *in vitro* cell culture methods. Moreover, one article has been published with the findings of this chapter in peer reviewed international journal, "Chemico-biological Interactions". Furthermore, ameliorative effects of Se against As induced cytotoxicity will be discussed in Chapter 3. Here involvement of autophagy along with apoptotic cell death was discussed for the first time using PC12 cells. This new finding has also been published in "Chemosphere". After that in Chapter 4 the effects of α-lipoic and dihydrolipoic acid will be presented against the toxic effects of As, Cd, Pb and to some extent of Hg using PC12 and Caco-2 cells. Then in Chapter 5 an overall discussion will be

taking place. This chapter is intended to summarize all the findings in a common platform with focusing distinct molecular insights. After the Chapter 5, there will be section/s on the ongoing and future researches based on the current theme of this thesis. Where, very interesting and outstanding research findings will be shown briefly but succinctly.

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Chapter 2: Cytotoxic effects of cadmium and zinc co-exposure in PC12 cells and the underlying mechanism

Abstract

Cadmium (Cd^{2+}) is a well studied inducer of cellular necrosis and apoptosis. Zinc (Zn^{2+}) is known to inhibit apoptosis induced by toxicants including Cd^{2+} both *in vitro* and *in vivo*. The mechanism of Zn^{2+}-mediated protection from Cd^{2+}-induced cytotoxicity is not established. In this study, we aimed to understand the effects of Zn^{2+} on Cd^{2+}-

induced cytotoxicity and apoptosis using PC12 cells. Cell viability and DNA fragmentation assays in PC12 cells exposed to Cd^{2+} and/or Zn^{2+} revealed that Cd^{2+} (5 and 10 μM) alone induced significant cell death, and co-exposure to Zn^{2+} (5, 10, and 100 μM) for 48 h had a protective effect. Assessment of intracellular free sulfhydryl levels and lactate dehydrogenase activity suggested that Cd^{2+} (10 μM) induced oxidative stress and disrupted cell membrane integrity. Addition of Zn^{2+} (10 and 100 μM) reduced Cd^{2+}-mediated cytotoxicity. Changes in expression of the apoptotic factors Bax, Bcl-2, Bcl-x, and cytochrome c were measured *via* western blot and expression of caspase 9 was detected *via* reverse transcriptase polymerase chain reaction. Western blots showed that Zn^{2+} (10 and 100 μM) suppressed Cd^{2+}-induced apoptosis (10 μM) by reducing cytochrome c release into the cytosol, and downregulating the proapoptotic protein, Bax. In addition, expression of caspase 9 was lower in Cd^{2+} (5 μM)-treated PC12 cells when co-treated with Zn^{2+} (2 and 5 μM). These findings suggest that the effective inhibition of Cd^{2+}-induced apoptosis in PC12 cells by Zn^{2+} might be due to suppression of mitochondrial apoptosis pathway and inhibition of Cd^{2+}-induced production of reactive oxygen species.

2.1 Introduction

Cadmium (Cd^{2+}) is one of the most toxic heavy metals due to its prolonged biological half life, low rate of excretion and high accumulation capacity in soft tissues. It is a widespread toxicant of occupational and environmental concern because environmental levels have risen steadily with increased (about 13,000 tons/year) production of Cd^{2+} for nickel-cadmium batteries, pigments, chemical stabilizers, metal coatings, and alloys (Rafiq et al., 2014; Rani et al., 2014). Major sources of human exposure to Cd^{2+} include i) occupational: primary metal industries, battery production industries, chemical stabilizer industries, among others and ii) non-occupational: cigarette smoking and consumption of contaminated foods and water (Satarug and Moore, 2004). The toxic effects of Cd^{2+} have been extensively studied in *in vivo* and *in vitro* systems. Cd^{2+} affects metabolic processes including energy metabolism, membrane transport, and protein synthesis. It may also act on DNA directly or indirectly by interfering with gene control and repair mechanisms (Beyersmann and Hechtenberg, 1997; Beyersmann and Hartwig, 2008). Numerous studies have shown that Cd^{2+}

damages mammalian organs including the lungs, kidneys, testes and the cardiovascular, hematopoietic, and nervous systems (Siu et al., 2009; Sabolić et al., 2010). Cd^{2+} exposure has been reported as a causative factor in the progressive neurodegenerative disorder amyotrophic lateral sclerosis (ALS). The link between Cd2+ exposure and ALS may include reduced neuronal expression of copper-zinc superoxide dismutase (Cu/Zn-SOD) (Bar-sela et al., 2001). Occupational Cd^{2+} exposure was found to negatively affect psychomotor function and memory of workers and cause olfactory dysfunction and neurobehavioral defects (Baxter et al., 2006; Kim et al., 2005). Increasing evidence indicates that Cd^{2+} disrupts integrity of the mitochondrial membrane (Lopez et al., 2006). Recently, it has been shown that Cd^{2+} can disrupt the blood-brain barrier and enter the brain (Shukla et al., 1996; Li et al., 2003). Neuronal exposure to Cd^{2+} eventually triggers release of mitochondrial proteins such as cytochrome c into the cytosol. These mitochondrial proteins activate catabolic hydrolases, which cleave important cellular targets resulting in cell death. Cd^{2+} toxicity may be associated with production of reactive oxygen species (ROS), making mitochondria key targets for Cd^{2+} toxicity. High levels of ROS have been suggested to increase blood-brain barrier permeability, induce tubulin alterations, and perturb synaptic transmission (Baxter et al., 2006). It has been proven that Cd^{2+} can induce apoptosis *via* the mitochondrial pathway in cell lines (Szuster-Ciesielska et al., 2000; Long et al., 2008; Jiang et al., 2014). At low and moderate concentrations in cell culture systems (e.g., 0.1-10 μmol/L), Cd^{2+} causes apoptosis. At higher concentrations (>50 μmol/L), necrosis becomes evident (Templeton and Liu, 2010).

Zinc (Zn^{2+}) is a well-known essential trace metal belonging to group IIB in the periodic table (as does Cd^{2+}), and is a required cofactor for various essential enzymes. By contrast, Cd^{2+} has no known physiological or biochemical functions in organisms (Templeton and Liu, 2010). Multiple biological macromolecules contain Zn^{2+} as a structural component, and Zn^{2+} is a major regulator of normal human development (Uriu-Adams and Keen, 2010). According to the Protein Data bank, more than 2700 enzymes, including hydrolases, transferases, oxidoreductases, ligases, isomerases and lyases contain Zn^{2+} (Andreini and Bertini, 2012). Additionally, Zn^{2+} exhibits antioxidant properties and can activate metal-binding proteins/chaperones. It is also capable of binding to and inhibiting oxidation of reduced glutathione (GSH) under oxidative stress

conditions (Oteiza, 2012). Several studies have reported a therapeutic role of Zn^{2+} in treating Cd^{2+} toxicity *in vivo* and *in vitro*. For example, Zn^{2+} inhibits Cd^{2+}-induced apoptosis and ROS production in HeLa cells and bovine aorta endothelial cells (BAECs) (Barbieri et al., 1992; Jiang et al., 2014); Zn^{2+} supplementation improves biochemical characteristics of distal femur and femoral diaphysis in male rats chronically exposed to Cd^{2+} (Brzóska et al., 2008). Enhanced Zn^{2+} consumption prevents alterations in lipid metabolism induced by Cd^{2+} in male rats (Rogalska et al., 2009); and Zn^{2+} protects rats against Cd2+-induced hepatotoxicity (Rogalska et al., 2011). Zn^{2+} affects some enzymes involved in DNA metabolism and inhibits apoptosis *via* its effect on transcriptional factors activated during apoptosis (Formigari et al., 2007). Studies have shown that Zn^{2+} plays a crucial role in maintenance of the cellular redox balance *via* several molecular mechanisms, including modulation of oxidant production and oxidative damage (Aimo et al., 2010), regulation of GSH metabolism, induction of metallothionein (MT), and scavenging of oxidants (Maret, 2011).

The effects of Zn^{2+} discussed above led us to test the hypothesis that Zn^{2+} might have critical regulatory effects on the pathways through which Cd^{2+} induces toxicity in PC12 cells. The presence of multiple metals in the environment and biological systems, and the possibility of simultaneous exposure to multiple metals have led to an increased interest in these studies. Currently, cytological and *in vivo* studies on the effects of simultaneous exposure to similar compounds, such as Cd^{2+} and Zn^{2+}, are limited. PC12 cell line is a rat pheochromocytoma clonal cell line, which has been used as a neuron model in molecular biology. Cd^{2+} is considered a potential etiological factor in neurodegenerative diseases and the PC12 cell line has been selected as a neuron model upon differentiation to study the underlying mechanisms. The objective of this study was to understand the effects of Zn^{2+} on Cd^{2+}-induced toxicity, and to investigate its regulatory role in mechanisms underlying Cd^{2+}-induced apoptosis in PC12 cells. Finally, we aimed to unveil the underlying molecular mechanism by which Zn^{2+} inhibits Cd^{2+}-induced apoptosis after co-exposure by measuring cell viability, DNA integrity, leakage of lactate dehydrogenase, intracellular levels of GSH, and changes in expression of apoptotic factors at the mRNA and protein level using PC12 cells.

2.2 Materials and methods

2.2.1 Materials

PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's medium (DMEM), ribonuclease A (RNase), ethidium bromide, and peroxidase-conjugated avidin were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was bought from HyClone (Rockville, MD, USA). SV total RNA isolation system and RT-PCR kit were purchased from Promega (Madison, WI, USA). High pure PCR product purification kit and proteinase K were purchased from Roche Diagnostics (Mannheim, Germany). Biotinylated goat anti-mouse IgG whole antibody and ECL western blotting detection reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Polyclonal antibodies against Bcl-2 (Cat#PC68, Oncogene), Bax (BIS, bs-0127R, BIOSS), beta-actin (GTX 109639, GeneTEX) were purchased. Anti-cytochrome c monoclonal antibody was purchased from BD Biosciences Pharmingen (San Jose, CA, USA). Trypan blue solution (0.4%) was purchased from Bio-Rad (Hercules, CA, USA). The DNA 7500 assay and RNA 6000 nano assay kits were purchased from Agilent Technologies (Waldbronn, Germany). All other chemicals were of analytical grade.

2.2.2 Cell culture

PC12 cells were cultured in DMEM supplemented with 10% FBS in a humidified incubator at 37 °C with 5% CO_2. The cells were pre-incubated in 25-cm^2 flasks for 24 h; then, the medium was replaced with serum/serum-free DMEM with or without various concentrations of $CdCl_2$ and $ZnCl_2$, or with a mixture of both chemicals, and the cells were incubated for 48 h. When the medium was replaced with serum-free medium, cells were washed twice with serum-free DMEM. The desired concentration for treatment was selected by exposing PC12 cells to Cd^{2+} (0, 2, 5, 10, 20 μM) and Zn^{2+} (0, 2, 5, 10, 20, 100, and 500 μM) separately, and then the final combination was decided. The selected concentration for Cd^{2+} was 10 μmol, whereas Zn^{2+} was used at 10, 100, and 500 μM.

2.2.3 Cell viability

Cell viability was determined using trypan blue exclusion assay. PC12 cells were seeded at a density of 1×10^5 cells/flask and pre-incubated for 24 h. Then, the cells were treated with Cd^{2+} (0, 2, 5, 10, and 20 μM and 0, 5, 10, 100, and 500 μM) and Zn^{2+}

(0, 2, 5, 10, and 20 μM and 10, 100, and 500 μM) separately; in addition, they were co-exposed to Cd^{2+} (10 μM) and Zn^{2+} (10, 100, and 500 μM). The cells were then incubated for 48 h. Total cells and trypan blue-stained cells were counted using a Bio-Rad automated cell counter (Hercules, CA, USA). Cell viability was expressed as percentage of the counted trypan blue-stained cells. Each experiment was carried out at least in triplicate to ensure biological reproducibility and statistical validity.

2.2.4 Cytotoxicity assay

2.2.4.1 Lactate dehydrogenase (LDH) activity assay

Cytotoxicity was assessed by measuring the activity of LDH in the treatment medium using a nonradioactive cytotoxicity assay kit (Promega) as described by Kihara et al. (2012). PC12 cells (1×10^5 cells/flask) were cultured in the medium with/without Cd^{2+} (0, 10 μM) or Cd^{2+} (10 μM) + Zn^{2+} (10, 100, and 500 μM) for 48 h. After 48-h incubation, 50 μL of the medium was transferred to a 1.5-mL tube, and then 50 μL of a substrate mixture containing tetrazolium salts was added to the tube. After 30-min incubation at room temperature (25 ºC), 50 μL of the stop solution was added, and the amounts of formazan dye formed were determined by measuring the absorbance at 490 nm using a DU-65 spectrophotometer (Beckman, CA, USA). LDH activity was expressed as LDH activity/1×10^6 cells. This experiment was carried out in triplicate for ensuring reproducibility.

2.2.4.2 Measurement of intracellular free sulfhydryl (SH) levels

Intracellular free SH levels were investigated as previously described by Kihara et al. (2012). Cells (1×10^5) were pre-incubated for 24 h. Then, they were exposed to Cd^{2+} (10 μM) or Cd^{2+} (10 μM) + Zn^{2+} (10–500 μM) for 48 h. The cells were harvested, washed with 1× phosphate-buffered saline (PBS), added to 150 μL of a lysis buffer, and then incubated at room temperature (25 ºC) for 10 min. Two freeze-thaw sonication cycles were performed in order to rupture the cell membranes, and the resultant solution was centrifuged at 1,500 rpm for 10 min to collect the supernatant. The total protein contents were measured spectrophotometrically by using protein assay dye reagent (Bio-Rad, Hercules, CA, USA). Intracellular free SH levels were determined using 2.5 μM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, pH 7). DTNB (final concentration; 200μM) was added to the cell lysate, and then the absorbance was measured at 412 nm

by using a DU-65 spectrophotometer (Beckman, CA, USA). The concentration of free SH in PC12 cells was determined using a molecular coefficient factor of 13,600 per cell number (1×10^5). The experiment was carried out in triplicate to ensure mechanical reproducibility.

2.2.5 Isolation of genomic DNA from PC12 cells

After treatment of PC12 cells with various concentrations of $CdCl_2$ and $ZnCl_2$, the genomic DNA was isolated using high pure PCR template preparation kit according to the manufacturer's instruction as described by Kawakami et al. (2008). After 48-h incubation, the cells were harvested using a scraper. Then, the obtained cells were centrifuged at 1,500 rpm for 5 min to remove the supernatant. After addition of 3 mL of PBS, the mixture was centrifuged again at 1,500 rpm for 5 min to wash the cells. The obtained solution containing DNA was mixed with 2 μL of 500 μg/mL RNase and incubated for 15 min at 37 °C. After incubation, 500 μL of ethanol and 20 μL of 3 M NaOAc buffer (pH 4.5) were added for ethanol precipitation, and the solution was allowed to stand overnight in a freezer to precipitate the DNA. On the next day, DNA was separated using microcentrifugation at 15,000 rpm for 8 min, and then washing with 70% ethanol at the same speed for 3 min was carried out. Then, the DNA sample was dried for approximately 10 min, and DNA concentration was measured after reaction with 1× Tris/Borate/EDTA (TBE) using a UV-visible spectrophotometer.

2.2.6 Agarose gel electrophoresis of genomic DNA

The ladder pattern of DNA was analyzed *via* agarose gel electrophoresis. The DNA was collected from PC12 cells after treatment with Cd^{2+} (0, 2, 5, 10, and 20 μM) and Zn^{2+} (0, 2, 5, 10, and 20 μM) for 48 h. Approximately 3–5 μg of DNA with the loading dye was subjected to electrophoresis on a 1.5% agarose gel. Electrophoresis was carried out for 40 min at 100 V in 1.5% of agarose gel by using a submarine-type electrophoresis system (Mupid-ex, Advance, Tokyo, Japan). To visualize the DNA strand breaks, the gel was soaked in ethidium bromide solution for 5–10 min. Images of the agarose gel were taken under UV illumination using a ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). To evaluate cell apoptosis, the fluorescence intensity of DNA in the gel was analyzed by a software named Quantity one. Apoptosis was expressed as the ratio of DNA to the DNA ladder. This experiment was conducted at least in triplicate.

2.2.7 Determination of gene expression *via* RT-PCR

Gene expression of caspase 9 was detected using the access RT-PCR kit and access RT-PCR introductory kit according to the manufacturers' instructions. Total RNA in PC12 cells treated with 5 μM Cd^{2+} and 0, 2, 5 μM Zn^{2+} for 48 h was extracted using SV total RNA isolation kit. The PCR primers for caspase 9 used were similar to those described by Kawakami et al. (2008). The detailed sequences of the primers, expected sizes of PCR products, annealing temperatures, and cycles are summarized in Table 4.1. The PCR conditions were as follows: 48 °C for 45 min and 94 °C for 2 min. The cycles were as follows: 94 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min. Final extension was carried out for 7 min at 68 °C. Forty cycles were performed, and an annealing temperature of 60 °C was used. *β*-Actin was selected as an internal control. PCR products were verified using a DNA 7500 assay with an Agilent 2100 Bio analyzer according to the manufacturer's instructions (Santa Clare, CA, USA). This experiment was carried out at least in triplicate.

2.2.8 Western blot analysis for determination of protein expression

Cytochrome c release in the cytosol was quantified using cytochrome c release apoptosis assay kit (Merk-Millipore, Darmstadt, Germany). PC12 cells were cultured in 5 mL of DMEM containing 10% FBS, 0, 10 μM Cd^{2+}, and 0, 10, 100, and 500 μM Zn^{2+}. After 48-h treatment, the cells were harvested and centrifuged at 1,500 rpm for 5 min to remove the supernatant. After addition of 10 mL of ice-cold PBS, the mixture was centrifuged again at 1,500 rpm for 5 min. After removal of the supernatants, the cells were resuspended in 150 μL of cytosol extraction buffer mix (1× cytosol extraction buffer containing 1 mL of protease inhibitor cocktail and 2 mmol/L DTT). The mixture was allowed to stand on ice for 10 min, and subsequently the cells were disrupted by sonication for 30 s using a Sonicator 250 (Branson). To remove the unbroken cells, the lysed cells were centrifuged at 3,000 rpm for 5 min. The supernatant was transferred into a 1.5-mL tube and centrifuged at 11,000 rpm for 30 min. The obtained final supernatant was collected as the cytosolic fraction for cytochrome c analysis. For determination of Bcl-2, Bax, and Bcl-x, β-actin lysis buffer (100 mM HEPES, 1 M NaCl, 300 mM EGTA, 0.1 M PMSF, 100 mM Na_3VO_4, 10 mM Na_2MgO_4, 100 mmol/L 2-glycerophosphoric acid, 1 mM $MgCl_2$, 100 mM DTT, 100 mM NaF, and triton X-100) was used to collect the protein fraction after two cycles of sonication followed by

centrifugation at 1500 rpm for 10 min. After that, the protein concentration was determined spectrophotometrically by using protein assay dye reagent (Bio-Rad, Hercules, CA, USA). Then, the equal amount (25 μg) of protein was separated via polyacrylamide gel electrophoresis (12.5–15%), and the electrophoresed proteins were transferred to nitrocellulose membranes with a semidry blotting system, type-AE6678 (ATTO, Tokyo, Japan). The membranes were incubated overnight at 4 °C in 5% skimmed milk as a blocking agent. The membranes were incubated for 60 min at 37 °C with the primary antibodies, washed three times, and then incubated with the secondary antibody for 60 min. After washing for five times (each 3 min), the protein bands were visualized using enhanced chemiluminescence. The images of the detected bands were analyzed using a ChemiDoc XRS (Bio-Rad, USA). Each experiment was conducted at least in triplicate to ensure reproducibility.

2.2.9 Statistical analysis

All data are expressed as the mean ± standard error of mean (SEM). Statistical analyses were performed using single-factor analysis of variance (ANOVA) followed by unpaired Student's *t*-test.

Table 4.1

Primers used in RT-PCR analyses of caspases 9 and *β*-actin.

Primer's name	Sequence (5'→3') (bp)	annealing site	Length (bp)	T_m (°C)	Cycle (number)
Bact F	ATGGATGACGATATCGCTG		19	61.1	40
Bact R	ATGAGGTAGTCTGTCAGGT		19	53.1	40
Casp 9F	CAAAGGAGCAGAAAGTAGTGAAG	411-433	23	60.95	40
Casp 9R	GAGGAAGGGCAGAAGTTCAC	687-668	20	62.73	40

2.3 Results

2.3.1 Cell viability

To examine whether zinc (Zn^{2+}) affected cadmium (Cd^{2+}) toxicity, the viability of PC12 cells exposed to 0, 10 μM Cd^{2+} and to 10, 100, and 500 μM Zn^{2+} for 48 h separately, as well as to a combination of both was measured *via* trypan blue staining (Fig. 4.1). The

cell viability of PC12 cells was not reduced after treatment with Zn^{2+} 10 μM and 100 μM for 48 h, but significantly decreased upon exposure to Zn^{2+} 500 μM (Fig. S1). Cell viability significantly reduced after exposure to Cd^{2+} (10 μM), whereas it significantly increased after co-exposure to Cd^{2+} (10 μM) and Zn^{2+} (10, 100 μM). However, it decreased upon exposure to Cd^{2+} (10 μM) and Zn^{2+} (500 μM) (Fig. 3.1). For comparison between the control, Cd^{2+}-, Zn^{2+}-, and Cd^{2+}+ Zn^{2+} (1:1)-treatment groups, cell viability was also studied after 48-h exposure (data not shown). It was found that Zn^{2+} had no effect on cell viability (<100 μM), whereas Cd^{2+} caused a significant decrease in cell viability (2, 5, 10, and 20 μM), and the Cd^{2+} + Zn^{2+}-treatment group showed significantly higher cell viability than the Cd^{2+}-treatment group. The cell viability study confirmed that the combined exposure to Cd^{2+} and Zn^{2+} resulted in significantly higher cell viability than Cd^{2+} treatment only. On the basis of these results (Fig. 2.1), the treatment groups were decided as follows: control group (no treatment), Cd^{2+}-treatment group (10 μM), Cd^{2+} (10 μM) + Zn^{2+} (10 μM)-treatment group, Cd^{2+} (10 μM) + Zn^{2+} (100 μM)-treatment group, Cd^{2+} (10 μM) + Zn^{2+} (500 μM)-treatment group.

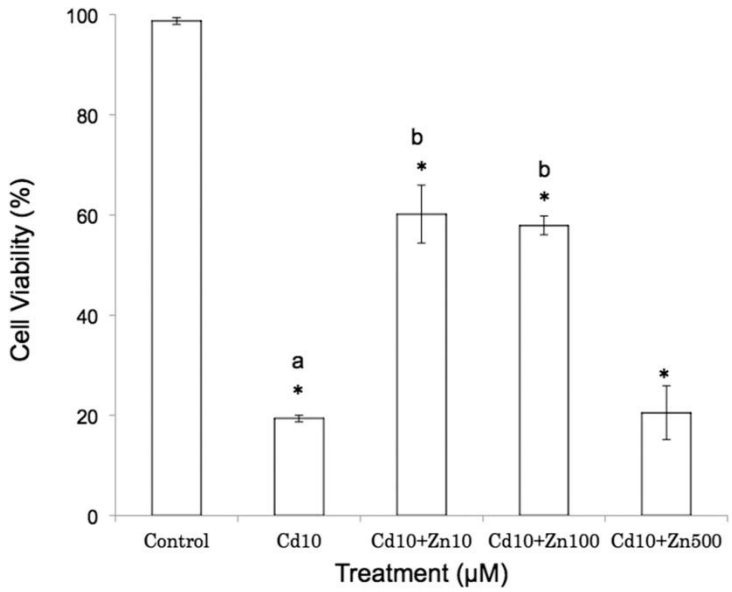

Fig. 2.1 Viability of PC12 cells with and without Cd^{2+}/Cd^{2+} + Zn^{2+} treatment at different concentrations after 48-h incubation measured *via* trypan blue staining method. Each experiment was conducted five times independently to ensure biological reproducibility. Cells co-exposed to 10 μM Cd^{2+} and 10–500 μM Zn^{2+}. Error bars indicate the mean ± SEM (n = 5), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference (p <0.05) between [a] and [b].

2.3.2 LDH activity after combined exposure to Cd^{2+} and Zn^{2+}

To investigate the combined effects of Cd^{2+} and Zn^{2+} on cytotoxicity, LDH activity assay was carried out in the culture medium of PC12 cells after treatment with/without Cd^{2+} (10 μM) and Zn^{2+} (10, 100, and 500 μM). The results showed that there was a significant difference between the treatment groups ($p < 0.05$). LDH activity significantly increased after treatment with 10 μM Cd^{2+} only (Fig. 2.2). However, after combined exposure to 10 μM Cd^{2+} along with 10 and 100 μM Zn^{2+}, a reduction in LDH activity was observed, and increase in Zn^{2+} concentration to 500 μM resulted in further increase in LDH activity. These results suggested that Zn^{2+} had protective effects against the cytotoxicity induced by Cd^{2+} (10 μM) in PC12 cells up to a certain concentration. Our findings are in agreement with the results of the cell viability study (Fig. 2.1).

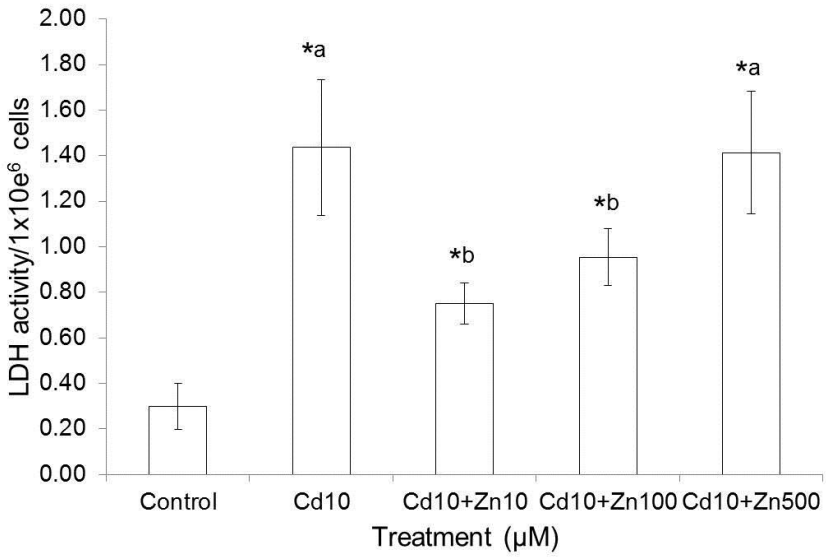

Fig. 2.2 LDH activity in the culture medium of PC12 cells with and without Cd^{2+}/Cd^{2+}+Zn^{2+} treatment at different concentrations after 48 h measured by non-radiative cytotoxicity assay kit. Each experiment was conducted three times for reproducibility. Error bars indicate mean ± SEM (n=3). Error bars indicate the mean ± SEM (n = 3), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between a and b.

In addition, to confirm whether effects of Zn^{2+} on Cd^{2+} toxicity are observed in other cell line, we have done same experiments using HepG2 cell, a well-differentiated

hepatocellular carcinoma cell. Zn^{2+} has recovered Cd^{2+} toxicity in HepG2 cells (data not shown) as well as PC12 cells.

2.3.3 Effects of simultaneous exposure of Cd^{2+} and Zn^{2+} on intracellular free SH levels

Glutathione (GSH), a major thiol component of the cellular antioxidant system, has been shown to play an important role in protecting the cells against ROS, such as free radicals and peroxides (Alfonso et al., 2003). GSH is usually oxidized to GSSG after stress stimuli, particularly oxidative stress.

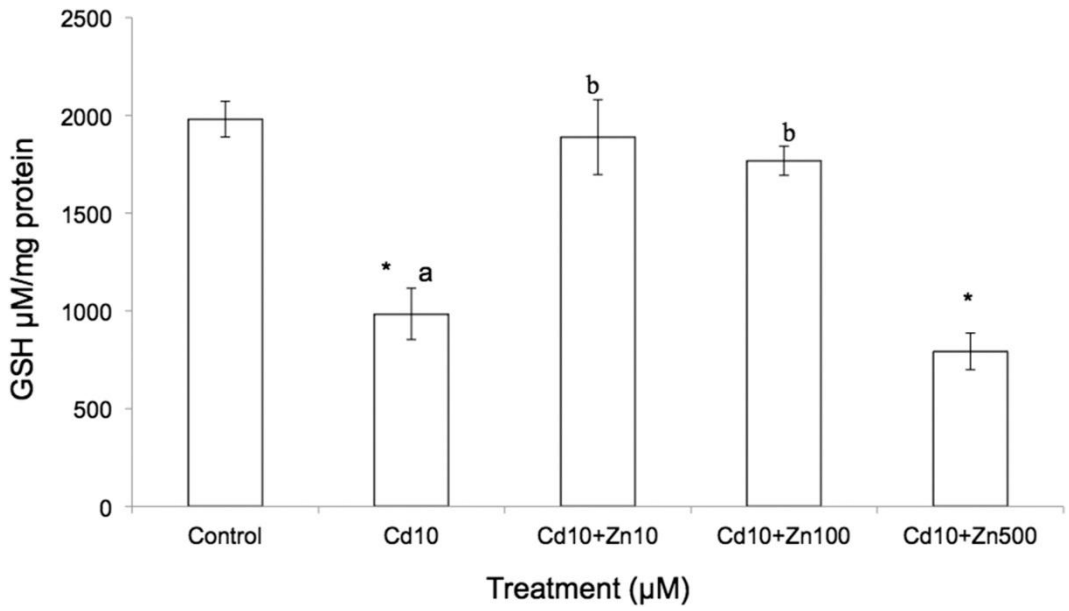

Fig. 2.3 Intracellular free-SH levels in PC12 cells with and without Cd^{2+}/Cd^{2+}+Zn^{2+} treatment at different concentrations after 48 h determined *via* DTNB assay method. Each experiment was carried out three times independently for ensuring reproducibility. Error bars indicate the mean ± SEM (n = 3), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between a and b.

To examine the stress status in PC12 cells after combined exposure of Cd^{2+} and Zn^{2+}, intracellular free SH levels were determined in cell lysates after treatment with/without Cd^{2+} and Zn^{2+} separately and in combinations. A significant reduction in free SH levels was observed after treatment with Cd^{2+} (10 μM) compared to the control group **(**Fig. 2.3**).** After combined exposure to Cd^{2+} (10 μM) and Zn^{2+} (10 or 100 μM), the levels of intracellular free SH increased significantly ($p < 0.05$) compared to the Cd^{2+} (10 μM)-treatment group alone (Fig. 2.3). However, free SH level in the Cd^{2+} (10 μM) + Zn^{2+} (500 μM)-treatment group decreased to the same level as in the Cd^{2+} (10 μM) treatment group. These results indicated that Zn^{2+} (up to 100 μM) could reduce oxidative stress induced by Cd^{2+} in PC12 cells. This finding is also in accordance with the cell viability results (Fig. 2.1).

2.3.4 DNA fragmentation analysis using agarose gel electrophoresis

To clarify whether apoptosis was induced in PC12 cells treated with Cd^{2+}, and to examine the effect of Zn^{2+} on Cd^{2+} toxicity, the genomic DNA extracted from PC12 cells treated with various concentrations of Cd^{2+} (0, 2, 5, 10, and 20 μM) and/or Zn^{2+} (0, 2, 5, 10, and 20 μM) for 48 h was electrophoresed using 1.5% agarose gel. The morphological characteristics of apoptosis are frequently accompanied by multiple cleavages of DNA resulting in fragments of 180–200 base pairs. The oligonucleosomal fragments can be visualized as a characteristic DNA ladder following agarose gel electrophoresis (Woodgate et al., 1999). This DNA ladder pattern was observed for the cells treated with more than 5 μM Cd^{2+} (Fig. 2.4A). These results indicated that apoptosis was induced by Cd^{2+}. In addition, DNA ladder pattern was significantly reduced upon addition of Zn^{2+} (1:1) in the medium containing Cd^{2+} (Fig. 2.4B), which confirmed the suppressive effects of Zn^{2+} on Cd^{2+}-induced cytotoxicity and/or apoptosis.

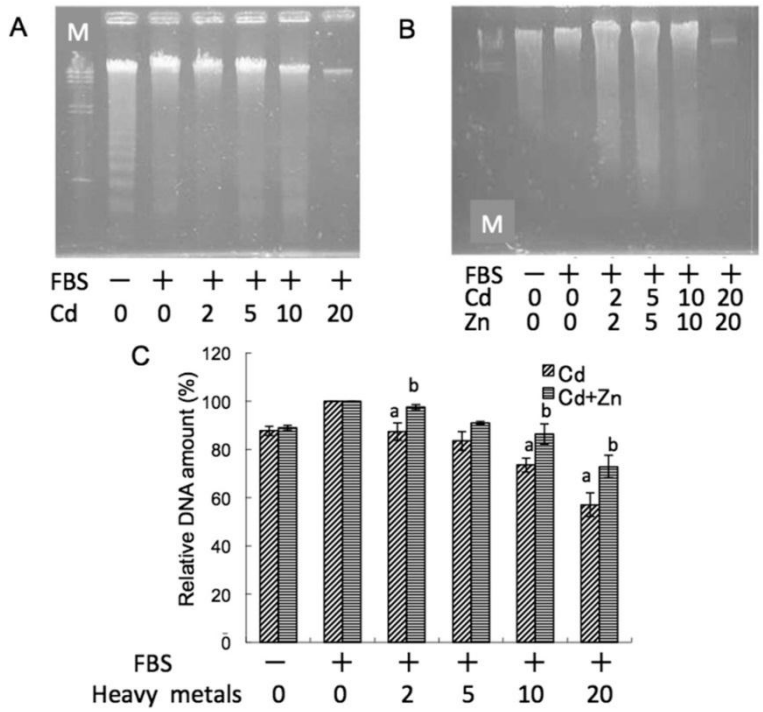

Fig. 2.4 Agarose gel electrophoresis of genomic DNA extracted from PC12 cells cultured in the medium with and without Cd^{2+}/Cd^{2+}+Zn^{2+} for 48 h. It was conducted four times to confirm biological reproducibility. A) DNA from the cells treated with 0-20 μM Cd^{2+}, B) DNA from the cells treated with 0-20 μM Cd^{2+} and 0-20 μM Zn^{2+}, C) the fluorescent intensity of genomic DNA extracted from PC12 cells cultured in the medium for 48 h after treatment with 0-20 μM Cd^{2+} and 0-20 μM Zn^{2+}. Lane M in the Figs. A and B means λ DNA digested with *Hind*III for the marker. The values are calculated following formula; (DNA/ DNA and DNA ladder) ×100. Error bars indicate mean ± S.E.M. (n=4). Significant difference between a and b is observed ($P<0.05$).

To evaluate the degree of apoptosis in the cell, the fluorescence intensity of DNA in the gels was analyzed. The relative intensity of DNA ladder after treatment with Cd^{2+} + Zn^{2+} was higher than that after treatment with Cd^{2+} (0, 2, 5, 10, and 20 μM) only (Fig. 2.4C). These results indicated that apoptosis induced by Cd^{2+} (5, 10, and 20 μM) was suppressed by Zn^{2+} (2, 5, 10, and 20 μM).

2.3.5 Western blot analysis for determination of protein expression

To examine whether Zn^{2+} could affect the proapoptotic environment induced by Cd^{2+} treatment, western blot analysis of the lysate of PC12 cells treated with Cd^{2+} (10 μM) or with Cd^{2+} (10 μM) + Zn^{2+} (10, 100, and 500 μM) was performed to assess the protein expression of Bax, Bcl-2, Bcl-x, β-actin, and cytochrome c. The western blot results are shown in Fig. 2.5(A-D). As shown in Fig. 2.5A, the proapoptotic Bax significantly increased after treatment with 10 μM Cd^{2+} compared to the control group.

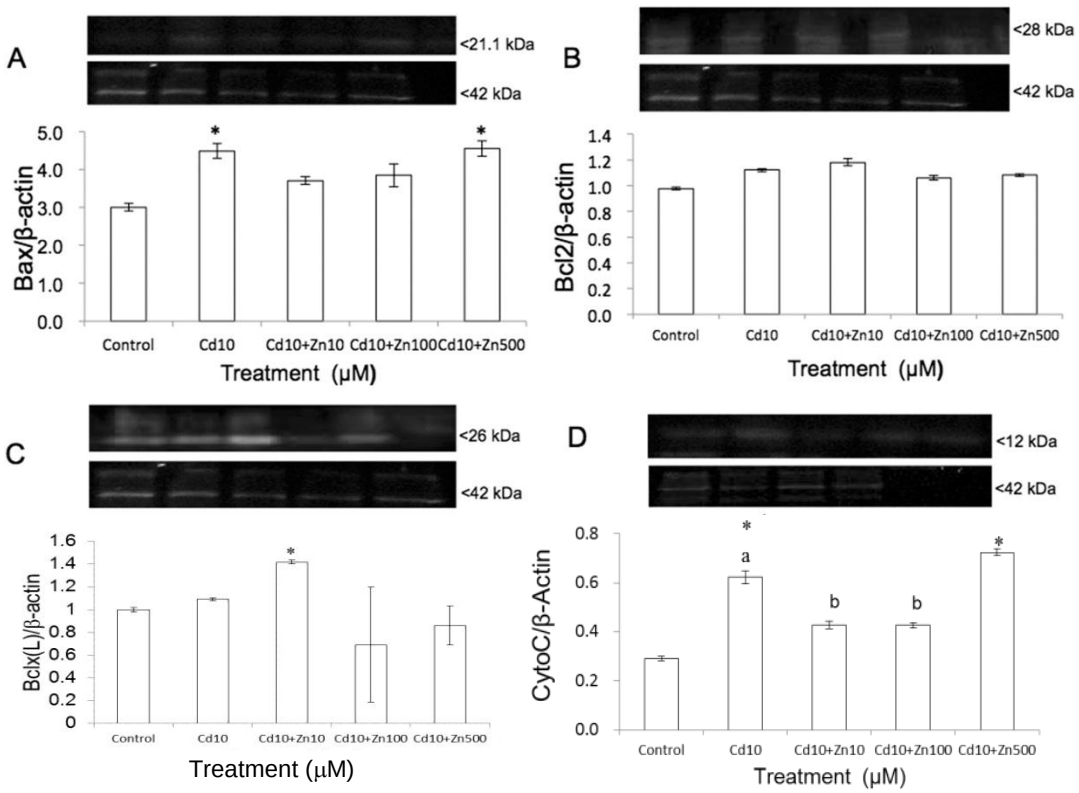

Fig. 2.5 Western blot analyses for the relative contents of A; Bax, B; Bcl2, C; Bclx(L) and D; cytochrome c 48 h after treatment with and without Cd^{2+}/Cd^{2+}+Zn^{2+} treatment. Each experiment was conducted three times separately to ensure reproducibility. Immunostaining bands are present above the bar diagram for respective protein. M means Error bars indicate the mean ± SEM (n = 3), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference (p <0.05) between a and b.

However, after combined exposure to Cd^{2+} (10 μM) + Zn^{2+} (10, 100 μM), Bax protein expression was reduced, whereas it increased again after co-treatment with Cd^{2+} (10 μM) + Zn^{2+} (500 μM). In addition, the levels of the antiapoptotic protein, Bcl-2

were relatively unchanged after both treatments (Fig. 2.5C); however, the levels of Bcl-x were found to be lower in the Cd^{2+} (10 μM) + Zn^{2+} (10, 100 μM)-treatment group compared to the Cd^{2+} (10 μM)-treatment group and Cd^{2+} (10 μM) + Zn^{2+} (500 μM)-treatment group. From these results, it was supposed that there was a net imbalance in Bax/Bcl-2 ratio, which indicated the occurrence of apoptosis in PC12 cells after treatment with Cd^{2+}, whereas co-exposure to Cd^{2+} and Zn^{2+} resulted in reduction in proapoptotic Bax protein level. Consequently, the release of cytochrome c into the cytosol was also investigated (Fig. 2.5D). Results showed that the cells treated with Cd^{2+} (10 μM) only exhibited high content of cytochrome c, which was reduced after co-treatment with Cd^{2+} (10 μM) + Zn^{2+} (10, 100 μM); however, co-exposure to Cd^{2+} (10 μM) + Zn^{2+} (500 μM) further increased cytochrome c levels. From these results, it was suggested that Cd^{2+} mainly induced the mitochondrial pathway of apoptosis, and Zn^{2+} suppressed Cd^{2+}-induced cytochrome c release upon simultaneous exposure. To investigate the effects of the low exposure level, we examined the effects of co-exposure to Cd^{2+} (5 μM) + Zn^{2+} (2, 5, 10, and 20 μM). We observed that Zn^{2+} (2, 5, 10, and 20 μM) suppressed cytochrome c release induced by Cd^{2+} (5 μM) (data not shown). Thus, it was confirmed that Cd^{2+} induced apoptosis in PC12 cells *via* activation of the mitochondrial pathway, whereas Zn^{2+} (10, 100 μM) had a suppressive effect upon co-exposure with Cd^{2+} (5, 10 μM).

2.3.6 Expression of caspase 9

To investigate the mechanism of apoptosis induced by Cd^{2+} and the effects of Zn^{2+} on the apoptotic pathways, changes in apoptotic factors of PC12 cells after treatment with Cd^{2+} and Zn^{2+} were determined using RT-PCR. The mRNA expression levels of caspase 9 in PC12 cells treated with Cd^{2+} (0, 5 μM) and Zn^{2+} (2, 5 μM) for 48 h are shown in Fig. 2.6. Caspase 9 mRNA levels were significantly ($p<0.05$) higher in PC12 cells treated with Cd^{2+} (5 μM) compared to those in the control. Co-treatment with Zn^{2+} (2, 5 μM) and Cd^{2+} (5 μM) resulted in the significant ($p<0.05$) reduction in caspase 9 mRNA levels to the same level as that in the control cells.

2.4 Discussion

This study was conducted to test the hypothesis that co-exposure to the essential trace element, Zn^{2+}, will antagonize the cytotoxicity and apoptosis induced by the toxic

heavy metal Cd^{2+} in PC12 cells, a widely studied neuron model. The two metals were selected because of their similar chemical characteristics and the high possibility of co-exposure.

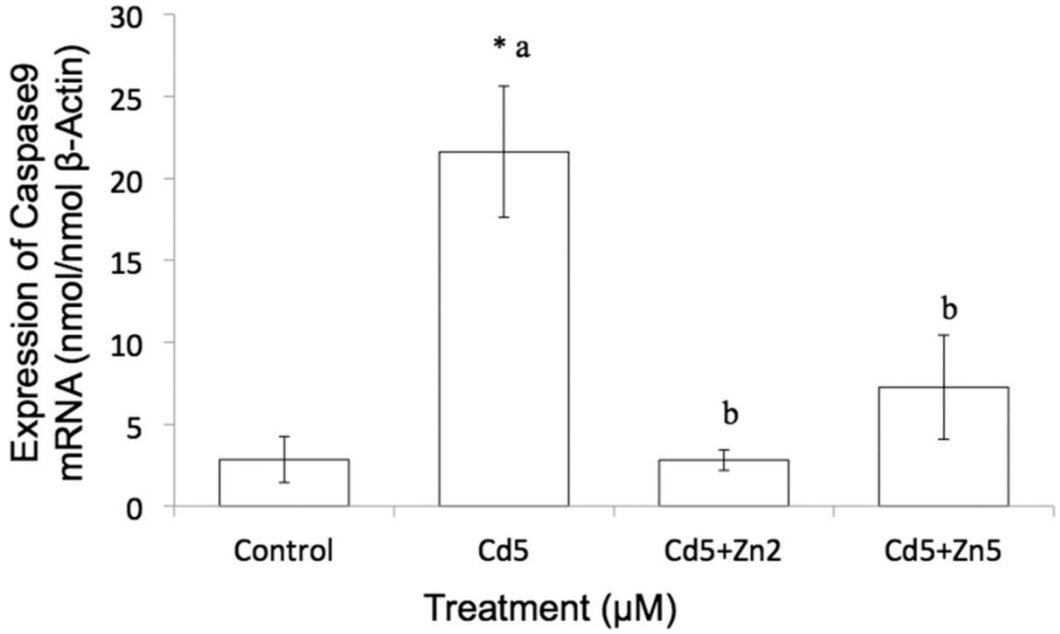

Fig. 2.6 Expression levels of caspase 9 mRNA in PC12 cells cultured in the medium containing 5 μM Cd^{2+} and 0-5 μM Zn^{2+} for 48 h using of RT-PCR method. Expression level of caspase 9 mRNA is expressed as nmol / nmol of *β*-actin which used as an internal control. Error bars indicate the mean ± SEM (n = 4), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference (p <0.05) between [a] and [b].

The present study showed that Cd^{2+} (5 μM) induced apoptosis in PC12 cells. Similar results have been observed in previous studies using HeLa cells and BAECs (Szuster-Ciesielska et al., 2000). Cd^{2+}-induced DNA fragmentation and formation of apoptotic bodies have been observed by several investigators under different experimental conditions, including Cd^{2+}-induced apoptosis in the testis of freshwater crab (Wang et al., 2011) and in pancreatic β-cells (Chang et al., 2013). Our results also showed DNA fragmentation in PC12 cells exposed to Cd^{2+} (Fig. 2.4). Various mechanisms could be involved in Cd^{2+}-induced DNA damage, including activation of Ca^{2+}-dependent endonucleases resulting in DNA fragmentation and interference with the DNA repair process (Yuan et al., 2013; Lohman and Beyersmann, 1993). In addition, Cd^{2+} toxicity could be partly due to oxidative stress induced by ROS produced in the cells in response to metal ions (Hassoun and Stohs, 1996; Fotakis and Timbrell, 2006). Cd^{2+} induces the

expression of hemeoxygenase enzyme, which generates ROS, in various cell types (Ossola and Tomoro, 1997; Srisook et al., 2005). Some evidence suggests that Cd^{2+}-MT complexes can induce ROS, which in turn cause DNA damage in cells (Muller et al., 1994). Intracellular levels of free SH groups determined in this study confirmed that Cd^{2+} (10 μM) exposure induces oxidative stress, and that co-exposure to Zn^{2+} (10 or 100 μM) prevented this effect (Fig. 2.3). In our study, co-exposure to Zn^{2+} (10 or 100 μM) significantly increased the level of GSH relative to that found in cells treated with Cd^{2+} only. A further increase in Zn^{2+} concentration (to 500 μM) reduced the level of GSH. These results indicate that Cd^{2+}-induced oxidative stress decreases in the presence of Zn^{2+} in PC12 cells, which confirms the antioxidant effects of Zn^{2+} in cells exposed to Cd^{2+}. The reduction of Cd^{2+}-induced oxidative stress by Zn^{2+} may also be due to the combined influence of both metal ions as described in a previous study (Brzóska et al., 2008). Supplementation with Zn^{2+} may also diminish the inhibitory potency of Cd^{2+} on the antioxidant enzymes SOD, peroxidase, and catalase. Furthermore, LDH activity in cell culture medium significantly increased after treatment with Cd^{2+} (10 μM). Simultaneous exposure to Zn^{2+} (10 or 100 μM) and Cd^{2+} (10 μM) markedly inhibited leakage of LDH into the culture medium of PC12 cells (Fig. 2.2). Again, increasing the concentration of Zn^{2+} to 500 μM increased the leakage of LDH into the culture medium. These findings suggest that Zn^{2+} might act as a scavenger of ROS produced by Cd^{2+}; alternatively, Zn^{2+} could compete with Cd^{2+} for accumulation sites in PC12 cells. The viability of PC12 cells exposed to Cd^{2+} decreases in a concentration-dependent manner (data not shown). However, when cells are co-exposed to Zn^{2+} (10 or 100 μM), their viability significantly increases (Fig. 2.1). A further increase in Zn^{2+} concentration (to 500 μM) synergistically promoted Cd^{2+} (10 μM)-induced apoptosis. Similarly, DNA fragmentation caused by Cd^{2+} was suppressed by addition of Zn^{2+} (Fig. 2.4). These results suggest that apoptosis induced by Cd^{2+} in PC12 cells is inhibited by the addition of Zn^{2+} up to 100 μM. This finding agrees with the results of previous studies, which showed that addition of Zn^{2+} significantly inhibits Cd^{2+}-induced apoptosis in HeLa cells and reduces ROS production in BAECs (Szuster-Ciesielska et al., 2000). Previous studies have also shown that Zn^{2+} ions can inhibit DNA fragmentation and apoptosis induced by other stimuli in multiple *in vivo* and *in vitro* biological systems.

To clarify the mechanism by which Zn^{2+} suppresses Cd^{2+}-induced apoptosis, western blot (Fig. 2.5A-D) and RT-PCR (Fig. 2.6) analyses were carried out. The results indicated that Cd^{2+} (10 μM)-mediated increased expression of caspase 9 and mitochondrial release of cytochrome c were suppressed by addition of Zn^{2+} (2, 5, 10 or 100 μM). Apoptosis can be triggered by the activation of death receptors (extrinsic pathway) or induction of mitochondria-mediated signaling pathways (intrinsic pathway). The intrinsic apoptotic pathway is regulated by members of the Bcl-2 protein family, which also play a central role in regulating mitochondrial membrane permeability (Adams and Cory, 2001; Chipuk and Green, 2008). Cytochrome c is a key regulator of the intrinsic pathway, activating caspase-dependent apoptosis. Release of cytochrome c from the mitochondria to the cytosol depends on certain factors, including mitochondrial membrane potential (MMP) and membrane integrity. The proapoptotic Bcl-2 family member Bax can interact with the mitochondrial permeability transition (MPT) pore components, particularly the voltage-dependent anion channel (VDAC), to release cytochrome c into the cytosol. In addition, it can alter the MMP and initiate swelling of mitochondrial organelles. Under conditions of cellular homeostasis, cytochrome c binds to the inner mitochondrial membrane in association with the anionic phospholipid, cardiolipin. Studies have suggested that dissociation of cardiolipin from cytochrome c could be the critical step in its release into the cytosol (Gogvadze and Orrenius, 2006; Tuominen et al., 2002). Several studies have shown that changes in levels or chemical structure of cardiolipin can create a soluble pool of cytochrome c within the mitochondrial inter-membrane space, which can promote release of cytochrome c into the cytosol upon permeabilization of the outer mitochondrial membrane (Petrosillo et al., 2001; Ott et al., 2002). In our study, the increased level of cytochrome c in the cytosol upon Cd^{2+} (10 μM) exposure suggests that Cd^{2+} influences MMP in PC12 cells. The release of cytochrome c from mitochondria into the cytosol accelerates activation of caspase 9, initiating a downstream caspase cascade, ultimately leading to cell death (Jiang et al., 2014). In this study cytosolic levels of the proapoptotic proteins Bax and cytochrome c increased upon Cd^{2+} (10 μM) exposure, and co-treatment with Zn^{2+} (10 or 100 μM) significantly suppressed these increases. A further increase in Zn^{2+} concentration to 500 μM has an opposite effect in Cd^{2+} (10 μM)-treated cells. One explanation is that co-treatment with Zn^{2+} might ameliorate Cd^{2+}-induced

production of excess ROS in PC12 cells. Previous studies reported similar effects in Zn^{2+} on Cd^{2+} exposure in different cell lines.

In the present study, we showed the suppressive effects of Zn^{2+} on Cd^{2+}-induced apoptosis in PC12 cells for the first time. Zn^{2+} is a redox-inactive metal, which is considered to be an important part of the cellular antioxidant system. The ability of Zn^{2+} to maintain cellular redox balance can be attributed to several possible molecular mechanisms, including regulation of oxidant production, induction of oxidative damage, induction of the Zn^{2+}-binding protein, MT, involvement in regulation of GSH metabolism (Mackenzie et al., 2011), and direct or indirect regulation of redox signaling. The normal cellular amount of Zn^{2+} is very small, and most of it strongly binds to proteins and other molecules, including GSH. One study showed that 5–10 pmol of Zn^{2+} is present in the cytosol and nucleus of resting PC12 cells (Jiang et al., 2014). Zn^{2+} can modulate cellular oxidant production, and bind to SH groups to protect them from oxidation. In our present study, Cd^{2+} (10 μM) induced oxidative stress, leading to the cell death and this may be due to displacement of Zn^{2+} bound to thiol groups (Figs. 2.1 and 2.2) . Co-treatment with Zn^{2+} (10 or 100 μM) suppressed cell death and LDH activity relative to that in cells treated with Cd^{2+} only, possibly due to the increased supply of Zn^{2+}. Zn^{2+} may also modulate oxidant levels by binding to SH groups of thiols. In addition, it may enhance/initiate synthesis of metal binding proteins such as MT. In neural cells, Cd^{2+} exposure is reported to significantly decrease Zn^{2+} content in proteins, hinder the enzymatic activity of Cu/Zn-SOD, and induce significant conformational changes in proteins that ultimately lead to neural cell apoptosis (Huang et al., 2006). In this study, we observed a significant increase in levels of GSH in co-treated cells (Cd^{2+} 10 μM + Zn^{2+} 10 or 100 μM) compared to that in Cd^{2+}(10 μM)-treated cells. Therefore, Zn^{2+} (10 or 100 μmol) supplementation effectively protects PC12 cells against Cd^{2+} (10 μM)-induced oxidative stress, possibly through modulation of intracellular GSH levels. Moreover, Cd^{2+} can induce MT synthesis, resulting in the formation of intracellular Cd^{2+}-MT complexes, which can cause apoptosis. Similar to Cd^{2+}, Zn^{2+} exposure can also initiate synthesis of MT. Although we did not investigate the involvement of metal-binding proteins/chaperones, this possibility cannot be neglected. It can be speculated from our study that co-treatment of PC12 cells with Cd^{2+} and Zn^{2+} can affect the formation and chemistry of MT. Additional studies are needed to evaluate this hypothesis. Suzuki et al.

(1990) showed that co-treatment with Cd^{2+} and Zn^{2+} salts might increase synthesis of MT in different organs, including the liver, kidneys, and pancreas, relative to that reported for treatment with Cd^{2+} or Zn^{2+} alone. When cells are exposed to Cd^{2+} and Zn^{2+} simultaneously, both metals compete for MT binding sites. We measured caspase mRNA levels after exposure to Cd^{2+}, or Cd^{2+} and Zn^{2+}. Caspase 9 mRNA levels significantly ($p<0.05$) increased after treatment with Cd^{2+} (5 μM); however, the levels decreased significantly ($p<0.05$) upon co-treatment of Cd^{2+}-treated cells with Zn^{2+} (2 or 5 μM). Thus, Zn^{2+} effectively inhibited Cd^{2+}-induced expression of caspase 9, possibly explaining its mechanism of inhibiting Cd^{2+}-induced apoptosis in PC12 cells. Previous studies have shown similar results, suggesting that Zn^{2+} can inhibit maturation of caspase 3, an apoptotic protease, which acts upstream of the endonuclease in apoptotic cell death (Aiuchi et al., 1998). In addition, Kumari et al. (2011) showed that prepubertal dietary Zn^{2+} deficiency induced apoptotic changes in the testes of Wistar rats. Our results indicate that Cd^{2+} induces apoptosis *via* the mitochondrial pathway, and Zn^{2+} supplementation inhibits this induced cell death process. It has also been reported that Zn^{2+} can inhibit activation of caspase 9 in different cells (Formigari et al., 2013). Additionally, it is known that Zn^{2+} reduces Cd^{2+} accumulation in biological systems both *in vivo* and *in vitro*. Low concentrations of Zn^{2+} may inhibit activation of caspase 9, while high concentrations inhibit accumulation of Cd^{2+} in cells. Therefore, our findings confirm the antioxidative effects of Zn^{2+} against Cd^{2+}-induced oxidative stress and its inhibitory effects against Cd^{2+}-induced apoptosis in PC12 cells. A schematic pathway is proposed to explain the molecular mechanism behind the Zn protection against Cd-toxicity in PC12 cells (Fig. 2.7).

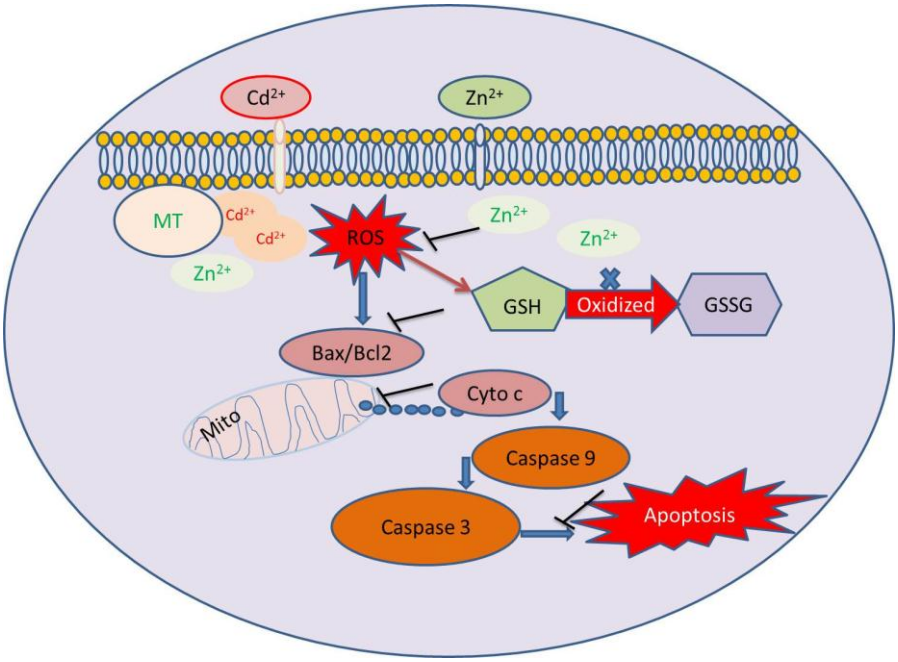

Fig. 2.7 Scheme of proposed molecular mechanism for Zn protection against Cd-toxicity in PC12 cells

2.5 Conclusion

Our present findings indicate that Zn co-treatment protected PC12 cells against Cd induced oxidative stress, loss of cell membrane integrity, DNA fragmentation, and ultimately, apoptotic cell death via the mitochondrial pathway. In addition, Zn^{2+} and Cd^{2+} may cooperatively induce synthesis of metal binding proteins/chaperones to neutralize the possible adverse effects of Cd^{2+} in neuron-like PC12 cells.

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Chapter 3: Ameliorative effects of selenium on arsenic-induced cytotoxicity in PC12 cells *via* modulating autophagy/apoptosis

Abstract

Arsenic is well known toxicant responsible for human diseases including cancers and neurological disorders. On the other hand, selenium is an essential trace element with significant chemopreventive effects, anticancer potentials and antioxidant properties. Although previous studies have reported antagonism/synergism between arsenic and selenium in biological systems, the biomolecular mechanism/s is still inconclusive. Therefore, to elucidate the molecular phenomena in cellular level, we hypothesized that co-exposure of selenium with arsenic may have suppressive effects on arsenic-induced cytotoxicity in PC12 cells. We found that selenium in co-exposure with arsenic increases cell viability, and suppresses oxidative stress induced by arsenic. Consequently, DNA fragmentation due to arsenic exposure was also reduced by arsenic and selenium co-exposure in PC12 cells. Furthermore, the western blot analyses revealed that simultaneous exposure of both metals significantly inhibited autophagy which may further suppressed apoptosis through positively regulation of key proteins; p-mTOR, p-Akt, p-Foxo1A, p62, and expression of ubiquitin, Bax, Bcl2, NFκB, and caspase 3, although those are negatively regulated by arsenic. In addition, reverse transcriptase polymeric chain reaction analysis confirmed the involvement of caspase cascade in cell death process induced by arsenic and subsequent inhibition by co-exposure of selenium with arsenic. The cellular accumulation study of arsenic in presence/absence of selenium *via* inductively coupled plasma mass spectrometry confirmed that selenium effectively retarded the uptake of arsenic in PC12 cells. Finally, these findings imply that selenium is capable to modulate arsenic-induced intrinsic apoptosis pathway via enhancement of mTOR/Akt autophagy signaling pathway through employing antioxidant potentials and through inhibiting the cellular uptake of arsenic in PC12 cells.

3.1 Introduction

Arsenic is ubiquitous in the environment, and it is released *via* both geochemical and anthropogenic processes. It is considered to pose the most significant potential threat to human health based on frequency of occurrence, toxicity and human exposure through entering into the food chain mostly *via* contaminated water and food in many parts of the world (Smedley and Kinniburgh, 2002; Rosen and Liu, 2009). Arsenic exposure is associated with numerous disease regime including cardiovascular and peripheral vascular diseases, neurological disorders, alzheimer disease and associated disorders, diabetes mellitus, and various forms of cancer(Gong and O'Bryant, 2010;Naujokas et al., 2013; Sun et al., 2014). In the environment, arsenic exists mainly in the inorganic forms, arsenate (+5), arsenite (+3) and other possible forms which are elemental arsenic (0) and arsine (-3). Arsenite (+3) is believed mostly responsible for the induction of epidemiological toxicity by producing reactive oxygen species (ROS) (Shi et al., 2004; Wang et al., 2001) and it also exerts genotoxicity (Gentry et al., 2010). In the body, arsenic is transformed through i) reduction; by reductases in the presence of glutathione (GSH)/ other thiols, and ii) methylation by methyltransferase in the presence of S-adenosylmethionine(Gailer et al., 2000; Aposhian et al., 2004). It causes cytotoxicity in different cells *via* several pathways mainly by producing ROS upon exposure. It also produces excess ROS by inducing NADPH oxidase (Chou et al., 2004). It is well established that ROS induces cytotoxicity through mitogen activated protein kinases (MAPKs) and tumor necrosis factor (TNF). These pathways modulate diverse cellular functions including cell proliferation, differentiation, and apoptosis (Shen and Liu, 2006; Ventura et al., 2004).Furthermore, arsenic also induces cytotoxicity *via* affecting the status of tumor-suppressor protein 53 (p53) as well as nuclear factor-κB (NFκB), which finally modulates the transformation and regulation of cell growth, apoptosis and others (Ryan et al., 2001; Wang et al., 2015).

Selenium is an essential nutrient element with potential chemopreventive and anticancer effects (Patrick, 2004). It is a member of the group VIA (metalloid) with four oxidation states in the environment; selenate (+6), selenite (+4), elemental selenium (0), and selenide (-2) (Tinggi, 2003). It is a component of many selenoproteins such as glutathione peroxidases and thioredoxinreductases with important biological functions

(Letavayová et al., 2008). These selenoproteins are well known for their antioxidant and detoxification functions (Zeng et al., 2005). There have been several proposed mechanisms for chemoprotective effects of selenium such as oxidant protection, altered carcinogen metabolism, enhanced immune surveillance, regulation of apoptosis, and inhibition of angiogenesis (Zeng et al., 2005). However, these chemoprotective effects of selenium can be altered due to presence of other elements, including As, in the environment as well as in foods (Ganther, 1999). Previous studies suggested that a proper dose of selenium can prevent cancers in animals and humans (Ganther, 1999; Clark et al., 1996).Conversely, it can also exert toxicity at slightly above the homeostatic dose (Zhang et al., 2014). For instance, high dose of selenium (5-20 μM) could induce the accumulation ROS and the apoptosis in NB4 cells (Li et al., 2003; Zuo et al., 2004; Guan et al., 2009). Furthermore, Se antagonize to alleviate arsenic toxicity by modifying cytotoxicty, genotoxicity and oxidative stress both *in vitro* and *in vivo* (Chitta et al., 2013; Selvaraj et al., 2012; Sah et al., 2013; Ponomarenko et al., 2017; Jamwal and Niyogi, 2017); however, the molecular mechanisms behind that is remain inconclusive. In addition, epidemiological evidences of synergistic effects of arsenic and selenium explained that the deficiency of dietary selenium could enhance the toxicity and cancer risk due to arsenic (Spallholz et al., 2004; Kolachi et al., 2011; Hsueh et al., 2017).

Furthermore, a line of evidence has demonstrated critical interactions between arsenic and selenium to modulate their functions in biological systems by each other and their interactions warrant further investigations (Bode and Dong, 2002; Pilsner et al., 2011; Janasik et al., 2017). However, there is still existing contradictions as both synergistic and antagonistic toxicity between arsenic and selenium specially on their molecular mechanism/s. Their role varies depending on the specific objects and duration of exposures and extents of doses. For example, in human leukemia (HL-60) cells, Se can induce both apoptosis (3 μM) and necrosis (>3 μM) while arsenic has antagonistic effects in combination treatment with selenium (Zeng, 2001); in APL cells, selenium has dose dependent effects on arsenic-induced apoptosis and cell differentiation (Wang et al., 2015) only up to very small concentration (4 μM) ;in acute promyelocytic leukemia (NB4) cells, selenium can induce apoptosis at high dose (5-20 μM) (Li et al., 2003), selenite reduces arsenite induced toxicity in hepatocytes of

rainbow trout by restoring thiol balance and boosting up antioxidant defense enzyme (SOD) (Jamwal and Niyogi, 2017) but the cell death and subsequent biomolecular mechanism/s needs further investigations.

Thus, although there have been previous studies about the combined effects of As and Se, there are still discrepancies regarding the cytoprotective mechanisms of Se on As-induced cytotoxicity. Particularly, there is still no study on the possible involvement of autophagy (type II cell death) in apoptosis induced by As and its subsequent inhibition by Se. Therefore, in this study we hypothesized that upon co-exposure, Se might induce some ameliorative effects on As-induced cytotoxicity in PC12 cells possibly by regulating autophagy/apoptosis. The interrelationship between As and Se is critically complex, and in combination treatment they may act as both antagonistically and synergistically. Therefore, our present study has been carried out to explore the status of cell growth, oxidative stress, and possible cell death mechanisms in light of apoptosis and autophagy signaling pathway for the first time using rat pheochromocytoma cells (PC12 cells).

3.2 Materials and methods

3.2.1 Materials

PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's medium (DMEM),ribonuclease A (RNase), ethidium bromide, peroxidase-conjugated avidin,$NaAsO_2$ (As^{3+}), and Na_2SeO_3 (Se^{4+}) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was bought from Biosera (Kansas City, MO, USA). SV total RNA isolation system and RT-PCR kit were purchased from Promega (Madison, WI, USA). High pure PCR product purification kit and proteinase K were purchased from Roche Diagnostics (Mannheim, Germany). Biotinylated goat anti-mouse IgG whole antibody and ECL western blotting detection reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Polyclonal antibodies against Bcl-2 (Cat#PC68, Oncogene), Bax (BIS, bs-0127R, BIOSS), beta-actin (GTX 109639, GeneTEX), anti-Foxo1A (phospho s256, ab131339, Abcam), Akt, p-Akt (Ser473), antibody, caspase3, mTOR, ubiquitin, ERK1 (Cell signaling technology), GPx1 (ab22604, Abcam), p62, NF-κB p65 (D14E12 CST)

werepurchased. Trypan blue solution (0.4%) was purchased from Bio-Rad (Hercules, CA, USA). The DNA 7500 assay and RNA 6000 nano assay kits were purchased from Agilent Technologies (Waldbronn, Germany). All other chemicals were of analytical grade.

3.2.2 Cell culture

PC12 cells were cultured in DMEM supplemented with 10% FBS in a humidified incubator at 37 °C with 5% CO_2. The cells were pre-incubated in 25-cm^2 flasks for 24 h; then, the medium was replaced with serum DMEM with or without various concentrations of $NaAsO_2$ (As^{3+}), and Na_2SeO_3 (Se^{4+}), or with a mixture of both chemicals, and the cells were incubated for 48 h. The desired concentration for treatment was selected by exposing PC12 cells to As^{3+} (0, 5, 10, 20, and 40 μM) and Se^{4+} (0, 5, 10, 20, and 40 μM) separately, and then the final combination was decided. The selected concentration for co-exposure of $As^{3+}$and Se^{4+} was 10 μM.

3.2.3 Cell viability

Cell viability was determined using trypan blue exclusion assay. PC12 cells were cultured at a density of about 1 × 10^5 cells/flask and pre-incubated for 24 h in DMEM culture medium. Then, the cells were treated with As^{3+} (0, 5, 10, 20 and 40μM) and Se^{4+} (0, 5, 10, 20 and 40 μM) separately; in addition, they were co-exposed to As^{3+} (10 μM) and Se^{4+} (10 μM). The cells were then incubated for another 48 h. Total cell and trypan blue-stained cells were counted using a Bio-Rad automated cell counter (Hercules, CA, USA). Cell viability was expressed as percentage (%) of the counted trypan blue-stained cells. Each experiment was carried out at least six times to ensure biological reproducibility and statistical validity.

3.2.4 Lactate dehydrogenase (LDH) activity assay and visualization of cell wall integrity

Cytotoxicity was assessed by measuring the activity of LDH in the treatment medium using a nonradioactive cytotoxicity assay kit (Promega) as described by Rahman et al. (2017) and Kihara et al. (2012). In briefly,PC12 cells (1 × 10^5 cells/flask) were seeded in the medium with/without As^{3+}/Se^{4+} (0 and 10 μM) orAs^{3+}(10 μM) + Se^{4+} (10 μM) for 48 h. After 48-h incubation, 50 μL of the medium was transferred to a 1.5-mL tube, and then 50 μL of a substrate mixture containing tetrazolium salts was added to the tube. After 30-min incubation at room temperature (25°C), 50 μL of the stop solution was

added, and the amounts of formazan dye formed were determined by measuring the absorbance at 490 nm using a iMark™ immunoplate Reader (BioRad; Hercules, CA, USA). LDH activity was expressed as LDH activity/1 × 10^6 cells. This experiment was carried out in triplicate for ensuring reproducibility.

3.2.5 Measurement of oxidative stress markers

3.2.5.1 GSH level

Intracellular free-SH levels were investigated as previously described by Rahman et al. (2017) and Kiharaet al. (2012). PC12 cells (1 × 10^5) were pre-incubated for 24 h. Then, cellswere exposed to Se^{4+} (10 μM), As^{3+} (10 μM) orAs^{3+} (10 μM) + Se^{4+} (10 μM) for 48 h. The cells were harvested, washed with 1× phosphate-buffered saline (PBS), added to 150 μL of a lysis buffer, and then incubated at room temperature (25°C) for 10 min. Two freeze-thaw sonication cycles were performed in order to rupture the cell membranes, and the resultant solution was centrifuged at 1,500 rpm for 10 min to collect the supernatant. The total protein contents were measured spectrophotometrically by using protein assay dye reagent (Bio-Rad, Hercules, CA, USA). GSH levels were determined using 2.5 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, pH 7) assay kit. DTNB (Final concentration: 200 μM) was added to the cell lysate, and then the absorbance was measured at 412 nm by using a DU-65 spectrophotometer (Beckman, CA, USA). The concentration of free SH in PC12 cells was determined using a molecular coefficient factor of 13,600 per cell number (1 × 10^5). The experiments were carried out in triplicate to ensure mechanical reproducibility.

3.2.5.2 Malondialdehyde (MDA) Assay

Formations of MDA were determined to check the index of lipid peroxidation. PC12 cells (1 × 10^5) were pre-incubated for 24 h. Then, they were exposed to As^{3+} (10 μM), Se^{4+} (10 μM) or As^{3+}(10 μM) + Se^{4+} (10 μM) for 48 has above mentioned. The cells were harvested, washed with 1×PBS, added to 150 μL of a lysis buffer, and then incubated at room temperature (25°C) for 10 min. Two freeze-thaw sonication cycles were performed in order to rupture the cell membranes, and the resultant solution was centrifuged at 1,500 rpm for 10 min to collect the supernatant. The total protein content was then measured spectrophotometrically via protein assay dye reagent (Bio-Rad, Hercules, CA USA). The contents of lipid peroxidation were measured as thiobarbituric

acid reactive substances (TBARS) or specifically, as malondialdehyde by using Northwest Life Science Specialties (NWLSS) Malondialdehyde Assay kit (NWLSS; Vancouver, Canada) following the protocol attached. The butylated hydroxyltoluene (10 μL) was mixed to cell lysate (100 μL), and then, 250 μL of 1M phosphoric acid and 250 μL of TBA were added to the mixture. The mixture was incubated at 60 °C for 1 h, and then centrifuged at 10,000 g for 3 min. After that, the supernatant was used to measure the absorbance at 532 nm using a DU-65 spectrophotometer (Beckman, CA, USA). The experiment was carried out at least triplicate to ensure the biological reproducibility.

3.2.6 Arsenic uptake by PC12 cells

Arsenic contents in PC12 cells treated with coexposure of both metals were measured with an ICPE-9000 inductively coupled plasma mass spectrometry (ICP-MS) (Shimazu; Kyoto, Japan). Before the measurement, the PC12 cells treated with each or both metals were digested with 1 M nitric acid along incubation with 70°C for 1 h. The digested samples were filtered through a 0.25 μm pore membrane and diluted with deionized water for the measurement. For ensuring reproducibility the experiment was carried out triplicate.

3.2.7 Isolation of genomic DNA from PC12 cells

After treatment of PC12 cells with various concentrations of arsenic and selenium, the genomic DNA was isolated using high pure PCR template preparation kit according to the manufacturer's instruction. After 48-h incubation, the cells were harvested with scraper. Then, the obtained cells were centrifuged at 1,500 rpm for 5 min to remove the supernatant. After addition of 3 mL of 1× phosphate-buffered saline (PBS), the mixture was centrifuged again at 1,500 rpm for 5 min to wash the cells. The obtained solution containing DNA was incubated with 5 μg/mL RNase for 15 min at 37 °C. After incubation, ethanol and 3 M NaOAc buffer (pH 4.5) were added for ethanol precipitation, and the solution was allowed to stand overnight in a freezer to precipitate DNA. On the next day, DNA was yielded using micro-centrifuge at 15,000 rpm for 8 min, and then washed the precipitate with 70% ethanol at the same speed centrifugation for 3 min. Then, the DNA sample was dried for approximately 10 min, and DNA concentration was measured after adding of 1× Tris/Borate/EDTA (TBE) using a GeneQuant (GE Health Care; South East England, UK).

3.2.8 Agarose gel electrophoresis of genomic DNA

The fragmentation of DNA was analyzed *via* agarose gel electrophoresis. A 4.0 µg of the obtained DNA with the loading dye was subjected to electrophoresis on a 1.5% agarose gel. Electrophoresis was carried out for 40 min at 100 V by using a submarine-type electrophoresis system (Mupid-ex, Advance, Tokyo, Japan). To visualize the DNA strand breaks, the gel were soaked in ethidium bromide solution for 5–10 min. Images of the agarose gel were taken under UV illumination using a ChemiDoc XRS (Bio-Rad; Hercules, CA, USA). To evaluate amount of fragmentation, the fluorescence intensity of DNA in the gel was analyzed by a software named Quantity one. Amount of intact DNA was expressed as the ratio of DNA density to the fragmented DNA density. This experiment was conducted at least in triplicate for biological reproducibility.

3.2.9 Western blot analysis for determination of protein expression

PC12 cells were cultured in 5 mL of DMEM containing 10% FBS, 0–10 µMAs^{3+}, and 0–10 µM Se^{4+} or both for 48-h. The procedure of western blot analysis was done according to Rahman et al., 2017 briefly as, the total protein in PC12 cells was extracted by ice-cold lysis buffer (2 mM HEPES, 100 mMNaCl, 10 mM EGTA, 0.1 µM PMSF, 1 mM Na_3VO_4, 0.1 mM Na_2MgO_4, 5 mM 2-glycerophosphoric acid, 10 µM $MgCl_2$, 2 mM DTT, 50 µMNaF and 1% triton X-100). The extracted protein concentration was measured spectrophotometrically using protein assay dye reagent (BioRad). The total protein (30 µg) from each sample was separated by 12.5/15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis and then transferred onto nitrocellulose membrane via semi-dry transfer method. After that, the membrane was blocked by 5% skim milk at 4°C for 24 h. Then, the membrane was sequentially incubated with desired primary and secondary antibodies. Finally, the protein on the nitrocellulose membrane was visualized using enhanced chemiluminescence and the image of the detected band was analyzed using a ChemiDoc XRS (Bio-Rad, USA). The intensities of the bands were as the ratio to that of β-Actin. All of the experiments were conducted at least in triplicate to ensure reproducibility.

3.2.10 Reverse transcription-polymerase chain reaction (RT-PCR) for gene expression

Gene expression of caspase 9 was examined after PC12 cells being exposed to As^{3+} (10 μM), Se^{4+} (10 μM) /both for 48 h following the method described by Rahman et al. (2017). Total RNA in PC12 cells was collected by using the SV total RNA isolation kit. The PCR primers used and the PCR conditions were the same as previously described by Kawakami et al. (2008) and Rahman et al. (2017). The PCR conditions were as follows: 48 °C for 45 min and 94 °C for 2 min. The cycles were as follows: 94 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min. Final extension was carried out for 7 min at 68 °C. Total of 40 cycles were performed, and an annealing temperature of 60 °C was used. β-Actin was selected as an internal control. PCR products were verified using a DNA 7500 assay with an Agilent 2100 Bio analyzer according to the manufacturer's instructions (Santa Clare, CA, USA). This experiment was carried out in triplicate for reproducibility.

3.2.11 Statistical analysis

All data are expressed as the mean ± standard error of mean (SEM). Statistical analyses were performed using single-factor analysis of variance (ANOVA) followed by unpaired Student's *t*-test *via* MS excel 2007 program.

3.3 Results

3.3.1 Effects of As and Se on the viability of PC12 cells

The effect of 48 h exposure of As^{3+} (0-40 μM) or Se^{4+} (0-40 μM) on the viability of PC12 cells was measured using trypan blue staining method (Fig. 3.1A). The viability of PC12 cells was significantly ($p<0.05$) reduced after treatment with 10 μM to 40 μM of As^{3+}dose-dependently (Fig. 3.1A). On the other hand, a significant ($p<0.05$) decrease in cell viability was observed in the Se^{4+}-treated group at 20 and40 μM concentration levels (Fig. 3.1A). Furthermore, to check the effect of co-exposure of arsenite and selenite, PC12 cells were exposed to As^{3+} (10 μM) and Se^{4+} (10 μM) separately and simultaneously for 48 h (Fig. 3.1B). The co-exposure of As^{3+} (10 μM) and Se^{4+} (10 μM) significantly ($p<0.05$) increased the viability compared to the As^{3+}-treated group alone. This finding suggested the cytoprotective effect of Se^{4+} (10 μM) on As^{3+} (10 μM)-induced cell death upon co-exposure. Starting from this point, the study was carried out focusing on co-exposure of As^{3+} (10 μM) and Se^{4+} (10 μM) for 48 h.

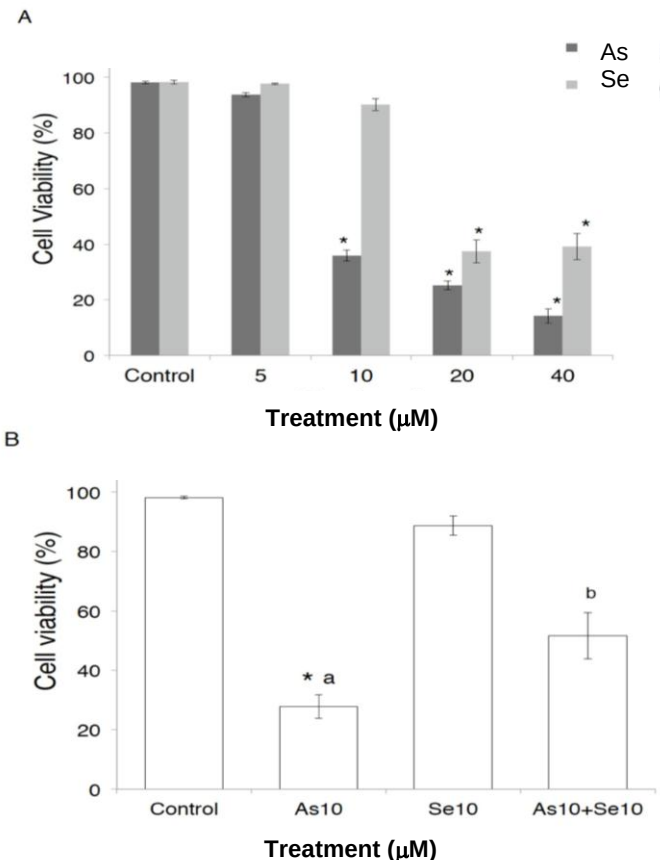

Fig. 3.1 Viability of PC12 cells using trypan blue exclusion method. (A) PC12 cells treated with different concentrations of $As^{3+}$and $Se^{4+}$for 48 h. (B) PC12 cells treated withAs^{3+} (10 μM) and Se^{4+} (10 μM) separately or combined for 48 h. Each experiment was conducted 6 times independently to ensure reproducibility. Error bars indicate mean ± SEM (n=6), asterisk * indicates significance at p<0.05 compared to the control group, and [a], [b] denotes significance at p<0.05between groups.

3.3.2. Effect of As and Se on membrane integrity in PC12 cells

The leakage of LDH into the cell culture medium is one of the indications for the disruption of the cell membrane integrity and subsequently cell death. In this study, we measured the LDH activity in the cell culture medium for PC12 cells after being treated with As^{3+} (10 μM), Se^{4+} (10 μM), and co-exposure of the both for 48 h (Fig. 3.2A).The LDH activity was markedly increased in the medium due to treatment with As^{3+} (10 μM) than the control group, whereas, no significant change was observed in Se^{4+} (10 μM)-treated group compared to the control group. However, the simultaneous exposure of As^{3+} (10 μM) and Se^{4+} (10 μM) showed significant decrease in LDH activity compared to the treatment upon As^{3+} (10 μM) alone at p<0.05. In addition, the image of trypan blue

stained cells represents the damaged cell wall which also in agreement with the LDH activity results (Fig. 3.2B).

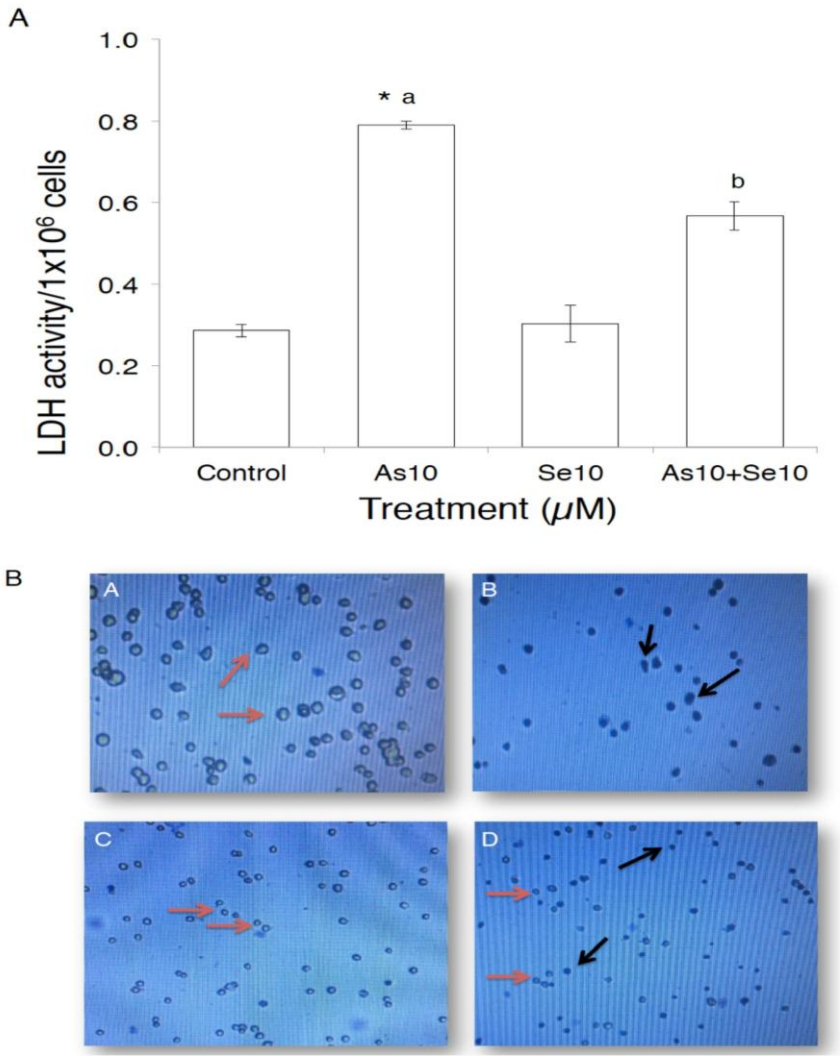

Fig. 3.2 Status of cell wall integrity of PC12 cells upon exposure to arsenic and selenium. (A) Lactate dehydrogenese (LDH) leakage into the culture medium of PC12 cells with/without As^{3+}(10 μM) and Se^{4+}(10 μM) treatment after 48 h measured by non radiative cytotoxicity assay kit. (B) Visualization of trypan blue staining after different treatments; a. control, b. arsenic, c. selenium, d. both arsenic and selenium. In the photograph, red and black arrows mean intact and damage cell walls, respectively. Each experiment was conducted at least three times for reproducibility. Error bars indicate mean ± SEM (n=3), * denotes significance at p<0.05 compared to control group and [a], [b] indicates significance at p<0.05 among groups.

3.3.3 Effects of As and Se co-exposure on oxidative stress markers

3.3.3.1 Effects on intracellular levels of GSH and glutathione peroxidase (GPx1)

The intracellular level of GSH is very important to maintain the functions of cellular antioxidant defense system. The reduction of GSH level indicates anomaly in cellular oxidative homeostasis. In this study, GSH level was measured in PC12 cells upon exposure to As^{3+} (10 μM), Se^{4+} (10 μM) and/ in combination of both for 48 h (Fig. 3.3A).GSH contents in the cells treated with As^{3+} (10 μM) significantly decreased compared to those in the control group and Se^{4+} (10 μM)-treated group. In contrast, Se^{4+} (10 μM) markedly increased the GSH level in PC12 cells compared to the control group. However, the co-exposure of As^{3+} (10 μM) and Se^{4+} (10 μM) to PC12 cells showed significantly increase GSH levels compared to As^{3+} (10 μM)-treated group alone. It was indicated that the co-exposure reduced oxidative stress induced by As^{3+} (10 μM) in PC12 cells. Furthermore, GPx1, one of the major anti-oxidant selenium-containing enzymes was also checked which uses GSH as a reductant *via* western blotting analysis (Fig. 3.3B). The results corroborate with the result of GSH. Thus, selenium effectively reduced the arsenic induced oxidative stress in PC12 cells.

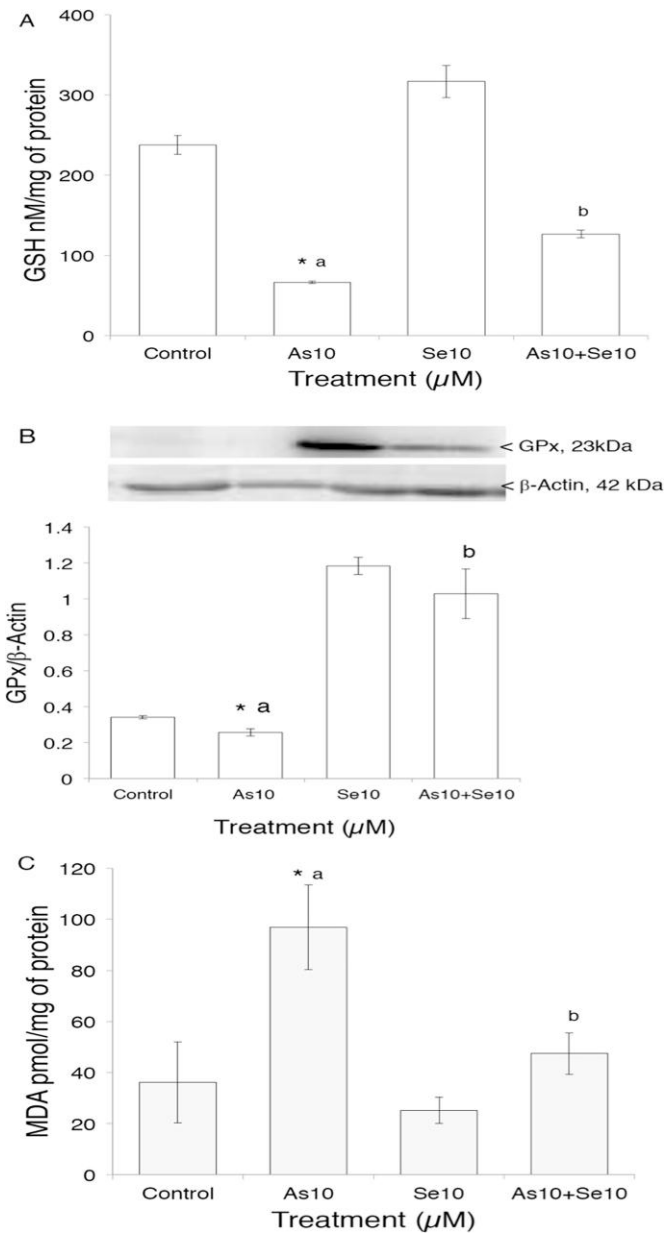

Fig. 3.3 Oxidative stress marker in PC12 cells after treatment with arsenic and selenium. (A) Intracellular glutathione (GSH) level in PC12 cells after exposure of As^{3+} and/Se^{4+} for 48 h *via* DTNB assay. (B) Glutathione peroxidase (GPx1) assay after treatment with As^{3+} and/Se^{4+} for 48 h through western blot analysis. (C) Lipid peroxidation level in PC12 cells after treatment with/without As^{3+} and/Se^{4+} for 48 h *via* malondialdehyde (MDA) assay. Each experiment was repeated 4 times to ensure biological reproducibility. Error bars indicate mean ± SEM (n=4),* indicates significance at p<0.05 compared to control group and[a, b] indicate significance at p<0.05 between treated groups.

3.3.3.2 Effect on lipid peroxidation

MDA is one of the low molecular weight end-products of lipid hydroperoxide decomposition. It is often measured as an index of lipid peroxidation. To clarify changes of MDA, PC12 cells were treated with As^{3+} (10 μM) and Se^{4+} (10 μM), and both of them in coexposure for 48 h (Fig. 3.3C). A significant (p<0.05) increase level of MDA was found in As^{3+} (10 μM)-treated PC12 cells to compared with that in the control group. There is no significant difference of MDA level was observed between the cells treated with Se^{4+} (10 μM) and without metals. However, upon coexposure of As^{3+} (10 μM) and Se^{4+} (10 μM) markedly (p<0.05) reduced the MDA level compared to the As^{3+} (10 μM)-treated group alone. These results supported a tendency from those of GSH and GPx1 (Figs. 3.3A and B).

3.3.4 Effect of As and Se on DNA

To investigate the status of genomic DNA, agarose gel electrophoresis was carried out after the PC12 cells being exposed to different concentrations of As^{3+} and/or Se^{4+} for 48 h (Fig. 3.4A).

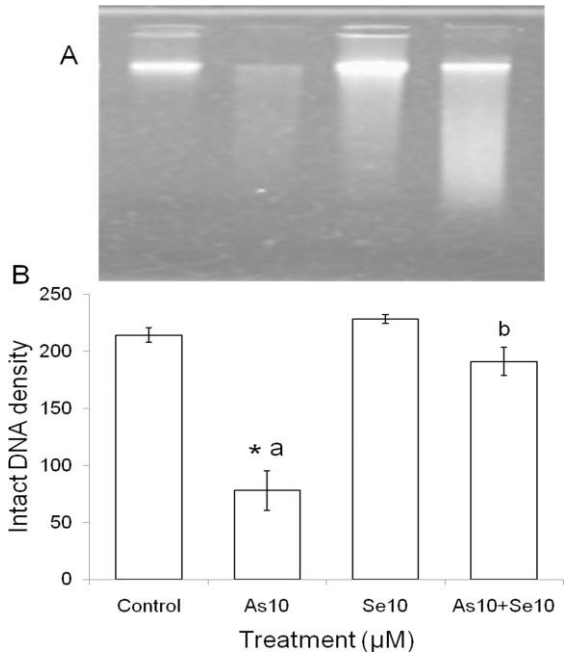

Fig. 3.4 Agarose gel electrophoresis of genomic DNA extracted from PC12 cells treated with and/without As^{3+} and/ Se^{4+} for 48 h. This was done three times independently for reproducibility. (A) DNA electrophoresis after treated with As^{3+} and/ Se^{4+}. (B) DNA band intensity for fragmentation detection. Error bars indicate mean ± SEM (n=3),* indicates significance at p<0.05 compared to control group and [a, b] indicates significance at p<0.05 between treated groups.

The gel analysis showed a dramatic decrease of intact DNA in As^{3+} (10 μM)-treated cells, where as a little smearing observed in Se^{4+} (10 μM)-treated cells. However, upon the coexposure of As^{3+} (10 μM) and Se^{4+} (10 μM), fragmentation was clearly observed in the form of dense smearing. In addition, the DNA band density measurement showed the similar trend like as gel scanner image. A significant decrease in DNA band density was found in As^{3+} (10 μM)-treated cells compared to the control cells (Fig. 3.4B). The coexposure of As^{3+} (10 μM) and Se^{4+} (10 μM) revealed a significantly higher intensity than alone As^{3+} (10 μM)-treated cells. The coexposure of arsenic and selenium is shown significantly ($p<0.05$) reduce the DNA damage induced by As^{3+} (10 μM) in PC12 cells.

3.3.5 Effects of As and Se on the regulation of apoptosis/autophagy related factors in PC12 cells

In this study, we analyzed the expression of key factors for possible elucidation of autophagy and/or apoptosis mechanism by western blot method. Results showed that As^{3+} (10 μM) downregulated significantly the contents of autophagy related proteins; mTOR, p-mTOR, Akt, p-Akt and p-Foxo 1A, and upregulated the expressions of p62 and ubiquitin (Fig. 3.5 and Fig. 3.6). On the other hand, expressions of anti-apoptotic proteins Bcl2, NFκB and ERK1 were significantly decreased by As^{3+} (10 μM) as well as increased the expressions of pro-apoptotic proteins, Bax and caspase-3 (Fig. 3.7). However, Se^{4+} (10 μM) did not regulate significantly the expression of these factors. However, upon coexposure of Se^{4+} (10 μM) showed significant antagonistic effects with As^{3+} (10 μM) on the regulation of all the proteins related to autophagy and apoptosis. These results suggested that Se^{4+} (10 μM) hindered the As^{3+} (10 μM)-induced apoptosis/autophagy upon coexposure in PC12 cells.

3.3.6 Expression of caspase 9 in PC12 cells by RT-PCR

To elucidate the mechanism of apoptosis induced by arsenic and the effects of selenium on the apoptotic pathways caused by arsenic, changes of caspase 9 mRNA expression in PC12 cells after treatment with As^{3+}(10 μM) and Se^{4+} (10 μM) for 48 h were determined using RT-PCR. Results showed that caspase 9 mRNA levels in PC12 cells treated with As^{3+} (10 μM) were significantly ($p<0.05$) higher than those in the control cells. Co-treatment with Se^{4+} (10 μM) resulted in the significant reduction

(p<0.05) of caspase 9 mRNA levels as compared with to those in the control cells (Fig. 3.8).

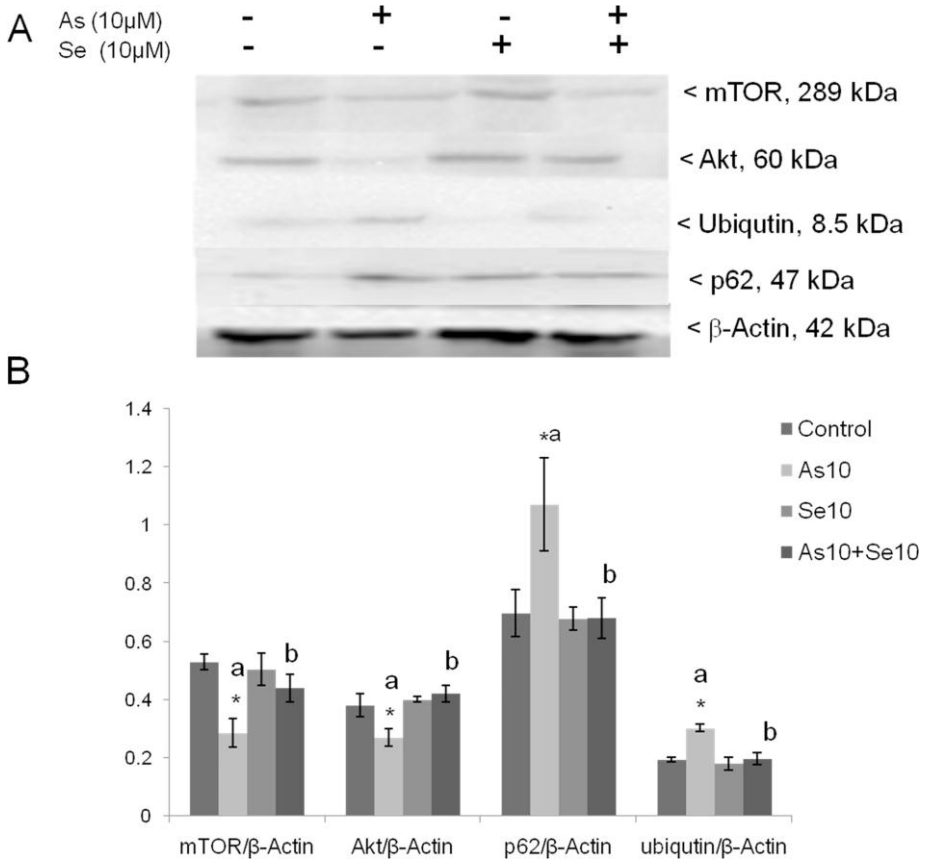

Fig. 3.5 Western blot analysis for key proteins related to autophagy in PC12 cells after being exposed to As^{3+} and/ Se^{4+} for 48 h. (A)Immunoblotting for treatment with and/ without As^{3+}/Se^{4+}. This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. (B) Relative density of each protein band to β-Actin, error bars indicate mean ± SEM (n=3), * denotes significance at p<0.05 compared to control group; [a, b]indicates significance at p<0.05 among treatment groups.

3.3.7 Effects of Se^{4+} on As uptake in PC12 cells

The uptake of arsenic in PC12 cells was measured using an ICP-MS (Watanabe et al., 2011). Results showed that upon coexposure of Se^{4+} (5 and 10 μM) significantly inhibited the uptake of As^{3+} (5 and 10 μM) into the PC12 cells (Fig. 3.9). This result can explain reason why As^{3+}-induced apoptosis/autophagy in PC12 cells was reduced by coexposure of Se^{4+}.

3.4 Discussion

There are similarities between arsenic and selenium in terms of their metabolic fates and chemical properties (Zeng et al., 2005). In addition, both of them have anticancer activity as well as cytotoxicity to a certain level of exposure however, selenium has lower cytotoxicity, genotoxicity and oxidative toxicity than arsenic. Recently, combination therapy is getting scientific attractions to treat diseases such as cancers, neurodegenerative disorders and so on (Wang et al., 2015; Hsueh et al., 2017) for ensuring mutual benefits on the target objects. Unlike low concentrations, high dose of selenium could produce reactive oxygen species (ROS) that synergizes with arsenic toxicity. In this view point, we have carried out this research to find out the upper limit of possible protective effects of selenium on arsenic exposure and also to elucidate the underlying molecular mechanisms for the first time using in PC12 cells.

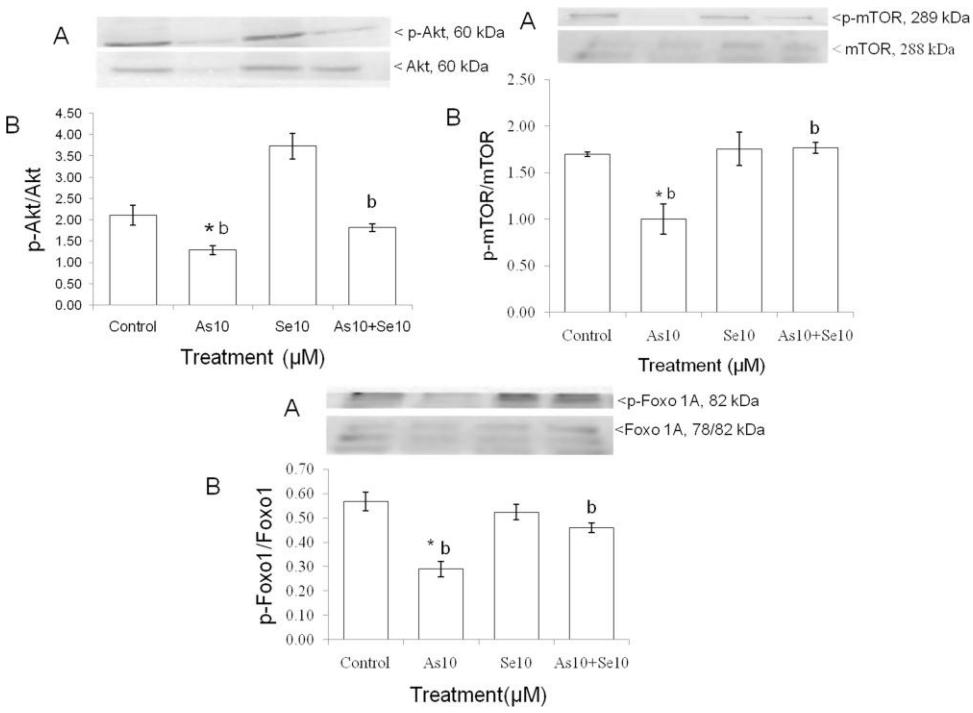

Fig. 3.6 Western blot analysis for activated form of autophagy related proteins in PC12 cells after As^{3+} and Se^{4+} co-/exposure for 48 h. (a) Immunoblotting for treatment with and/ without As^{3+} /Se^{4+}. This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. (b) Relative density of each protein band to active counter part, error bars indicate mean ± SEM (n=3), * denotes significance at p<0.05 compared to control group; [a, b] indicates significance at p<0.05 among treatment groups.

To maintain cellular homeostasis, cells consistently produce free radicals and ROS, and these are subsequently neutralized by cellular antioxidant defense systems such as GPx,GSH, superoxide dismutase, vitamins and flavonoids (Urso and Clarkson, 2003). Cellular damages occur upon the disruption of cellular oxidative homeostasis which leads to the increase level of stress markers such as LDH and even cell death. In our study, we found that the viability of PC12 cells (Fig. 3.1) and the leakage of LDH into the culture medium (Fig. 3.2) were significantly affected by As^{3+} (10 μM) exposure. However, co-exposure of As^{3+} (10 μM) and Se^{4+} (10 μM) improved both of the parameters, while Se^{4+} (10 μM) alone did not show significant changes upon 48 h exposure in PC12 cells. Furthermore, we examined possible oxidative stress by determining the GSH, GPx1 and MDA (Fig. 3.3). Our results demonstrated that As^{3+} (10 μM) exposure induced oxidative stress in PC12 cells through reduction of GSH, GPx1 and increase of MDA as in other cell line such as NB4 (Wang et al., 2015) and primary hepatocytes of rainbow trout (Jamwal and Niyogi, 2017). However, all of the markers are significantly improveduponSe^{4+} (10 μM) co-exposures with As^{3+} (10 μM) in PC12 cells (Fig. 3.3).GPx1, a selenoprotein is one of the vital members of antioxidant enzyme group, and potentially expresses to protect the cells in oxidative stress condition (Roy et al., 2014). GPx1 also activates and highly expresses in selenium supplementation condition thus, selenium is considered as a pivotal determinant of this protein activity and expression to strengthen the cellular defenses both *in vivo and in vitro* (Schnabel et al., 2008).

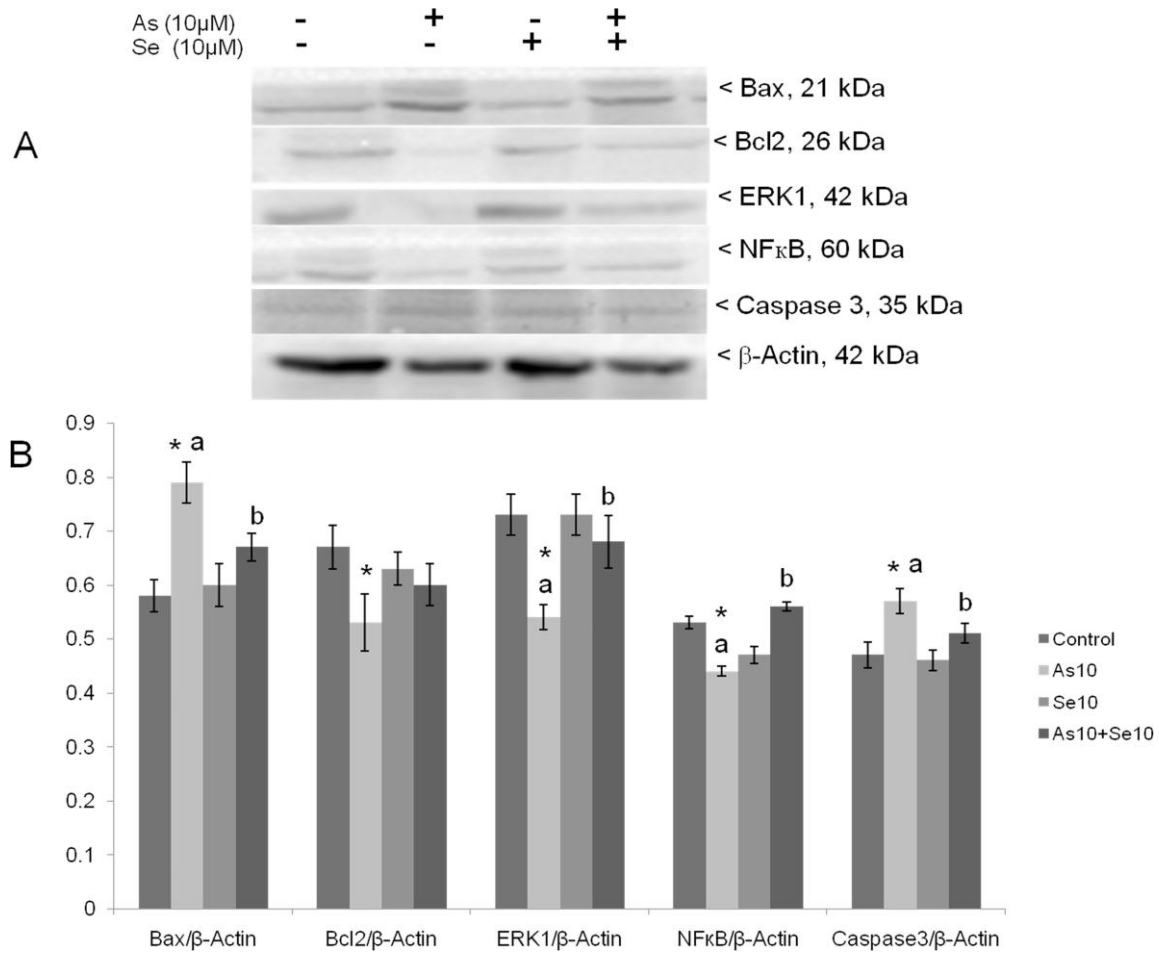

Fig. 3.7 Western blot analysis for some apoptosis related proteins in PC12 cells after being exposed to As^{3+} and/ Se^{4+} for 48 h. (A)Immunoblotting for treatment with and/ without As^{3+}/Se^{4+}. This experiment was conducted at least three times for reproducibility and only representative images (cropped) are provided. (B) Relative density of each protein band to β-Actin, error bars indicate mean ± SEM (n=3), * denotes significance at p<0.05 compared to control group; [a,b]indicates significance at p<0.05 between treatment groups.

We found that after selenium exposure the expression of GPx1 significantly increased, and co-exposure of arsenic and selenium also increased expression of GPx1 compared to only arsenic treatment and control group. This means that increased antioxidant enzymes due to presence of selenium in PC12 cells can protect the cells from arsenic-induced oxidative stress. Thus, our study demonstrated that the exposure of selenium acts as an antioxidant to reduce arsenic induced oxidative stress in PC12 cells. Different cellular systems have distinct capacity to deal with external stimuli. However, the protective role of selenium against arsenic induced oxidative stress in

PC12 cells could be a matter of interest to clarify neurological disorders due to acute or chronic arsenic exposure under selenium deficit condition (Kolachi et al., 2011), and to design the possible therapeutic agents for the disease.

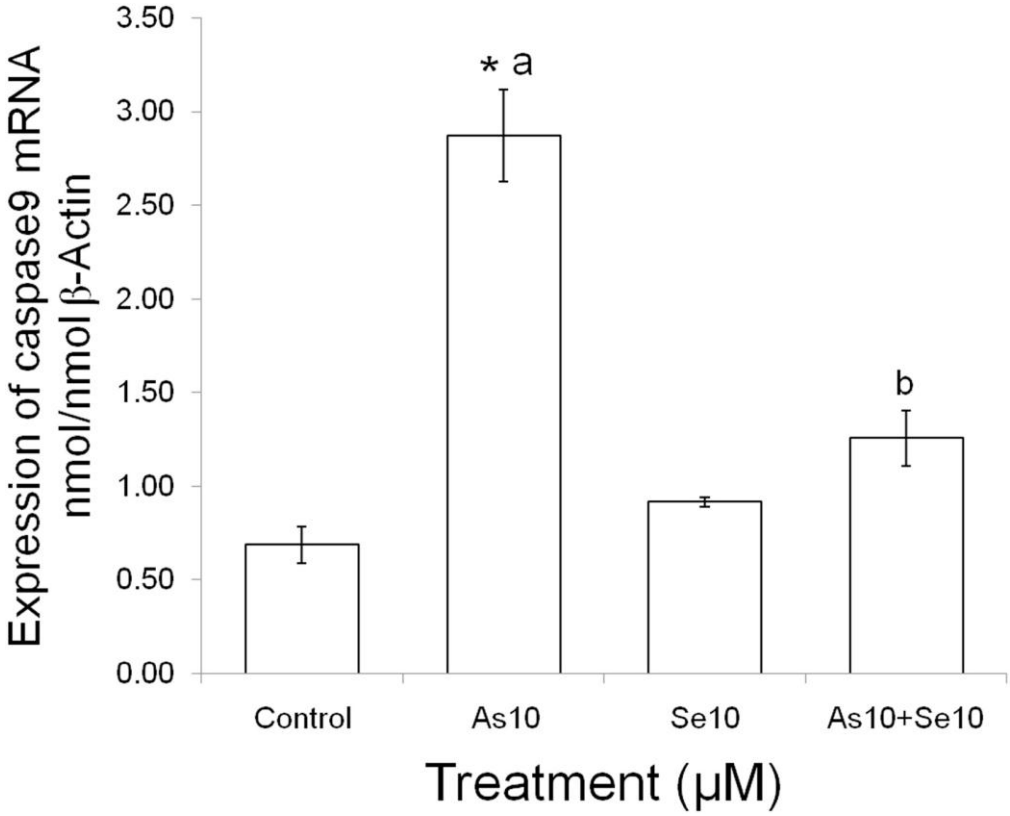

Fig. 3.8 Expression levels of caspase 9 mRNA in PC12 cells cultured in the medium containing As^{3+} and Se^{4+} for 48 h using of RT-PCR method. Expression level of caspase 9mRNA is expressed as nmol / nmol of *β*-actin which used as an internal control. Error bars indicate the mean ± SEM (n = 3), * denotes significance at p< 0.05 compared to control. There is also a significant difference (p<0.05) between [a]and[b].

Arsenic can induce apoptosis and necrosis depending on exposure concentrations in different cell lines (Zeng, 2001; Wang et al., 2015). It was assumed by Gailer et al. (2000) as follows; "lethal dose of arsenic can be overcome by otherwise lethal/sub-lethal dose of selenium". However, in this study for the first time we are intended to figure out the possible effects of arsenic in higher concentration than IC_{50} (As^{3+} 10 μM) with/without the presence of selenium in PC12 cells focusing on both apoptosis (Type I) and autophagy (Type II) cell death process. Apoptosis and autophagy can be

independent of each other as well as can be cumulative. In arsenic-induced apoptosis, Bax, Bcl-2, NF-κB, caspase 3, caspase 9 and ERK1 have been reported to play crucial roles (Cai et al., 2000; Lemarie et al., 2006).

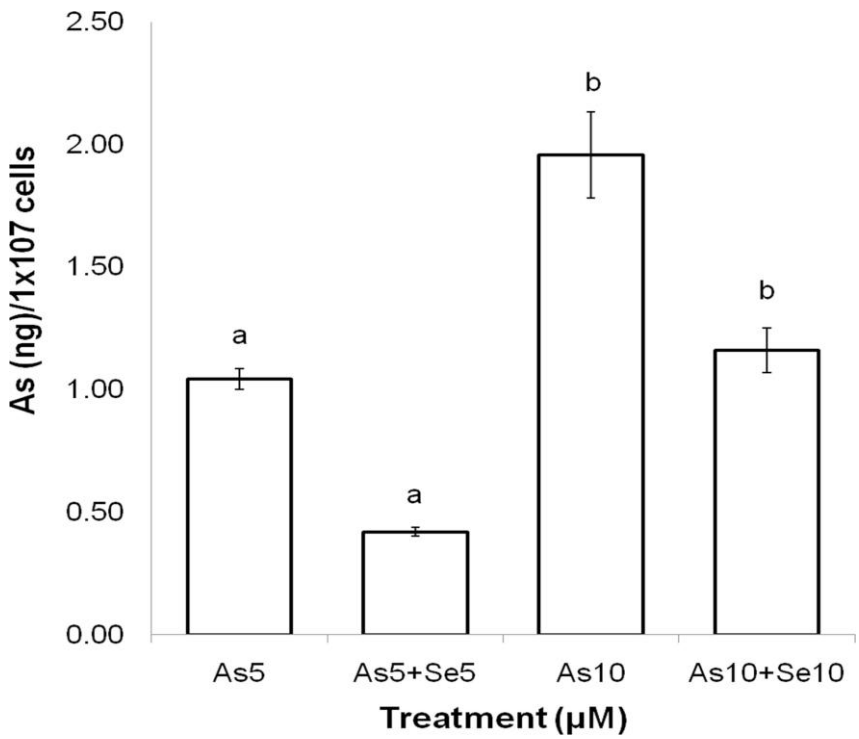

Fig. 3.9 Effects of selenium on PC12 cell uptake of arsenic *via* ICP-MS analysis. PC12 cells exposed to different concentrations of As^{3+} and Se^{4+} for 48 h. This experiment was carried out three times for reproducibility. Error bars indicate the mean ± SEM (n = 3), [a, b] denotes significance at p< 0.05 between groups.

These factors were chosen to explore the potential mechanism of apoptosis induced by As^{3+} (10 µM), Se^{4+} and/or both (As^{3+}+Se^{4+}) in PC12 cells. Upregulated expression of pro-apoptotic proteins Bax, caspases 3, 9 and downregulated expression of anti-apoptotic proteins Bcl-2, NF-κB, and ERK1 have observed in As^{3+} (10 µM) treated group of PC12 cells (Fig. 3.7). The upregulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2 suggested that As^{3+} induced apoptosis in PC12 cells by intrinsic mitochondrial mediated pathway (Lemarie et al., 2006; Rahman et al., 2017). The down-regulation/inhibition of NF-κB is also considered as a key to the induction of apoptosis in other cell lines (Mathas et al., 2003). Moreover, the increased levels of caspase 9 mRNA expression and caspase3protein expression indicated the involvement of caspase

dependent pathway of apoptosis induced by arsenic in the PC12 cells (Figs. 3.7 and 3.8). However, in co-exposure selenium effectively inhibits/reduces arsenic-induced apoptosis through down regulation of pro-apoptotic proteins Bax, caspases3, 9, and by upregulation of anti-apoptotic proteins Bcl2, and NF-κB following the intrinsic apoptosis pathway (Wang et al., 2015; Zhu et al., 2011).

In addition, apoptosis can also be induced *via* autophagy; in this process, ROS inhibits the autophagy related pro-survival factors such as mTOR andAkt in the downstream of the PI3K/mTOR/Akt signaling pathway (Roy et al., 2014). This inhibition stimulates upregulation of pro-apoptotic Bax and/or downregulation of anti-apoptotic Bcl-2 and Bclx (Singh et al., 2012). Finally, the dyshomeostasis between Bax and Bcl2 activates the caspase cascade through upregulation of caspases 3 and 9 to induce apoptosis (Li et al., 2015). Furthermore, autophagy can also contributes to the accumulation of excess ROS by selectively increasing the degradation of major enzymatic ROS scavengers (Yu et al., 2006; Roy et al., 2014). This consideration supports our findings. The roles of ROS in autophagy induction have been reported by several authors (Roy et al., 2014; Singh et al., 2012). Autophagy involves the formation of autophagosomes within cytoplasm which can sequester cytosolic constituents.

Antioxidants are well known to diminish the formation of autophagosomes and consequently the degradation of autophagy related proteins such as mTOR, Akt and others (Scherz-Shouval et al., 2007). It was reported that PI3K/mTOR/AKT pathway played a crucial role in the early stage of autophagosome formation which may further lead to autophagic cell death (Tassa et al., 2003; Petiot et al., 2000; Roy et al., 2014).To check the possible involvement of autophagic cell death we examined autophagy related proteins within the same treatment regime of arsenic and selenium in PC12 cells and our findings are presented in Figs. 3.5 and 3. 6. A significant reduction in p-mTOR, p-Akt and p-FoXo 1A expression effectively contributes to the downstream of the PI3K/mTOR/AKT autophagy signaling pathway.

In our study, arsenic significantly downregulated the expression of p-mTOR/mTOR, p-Akt/Akt and p-FoXo 1A/FoXo 1A; however, co-exposure with selenium reduces these downregulation induced by arsenic in the PC12 cells.

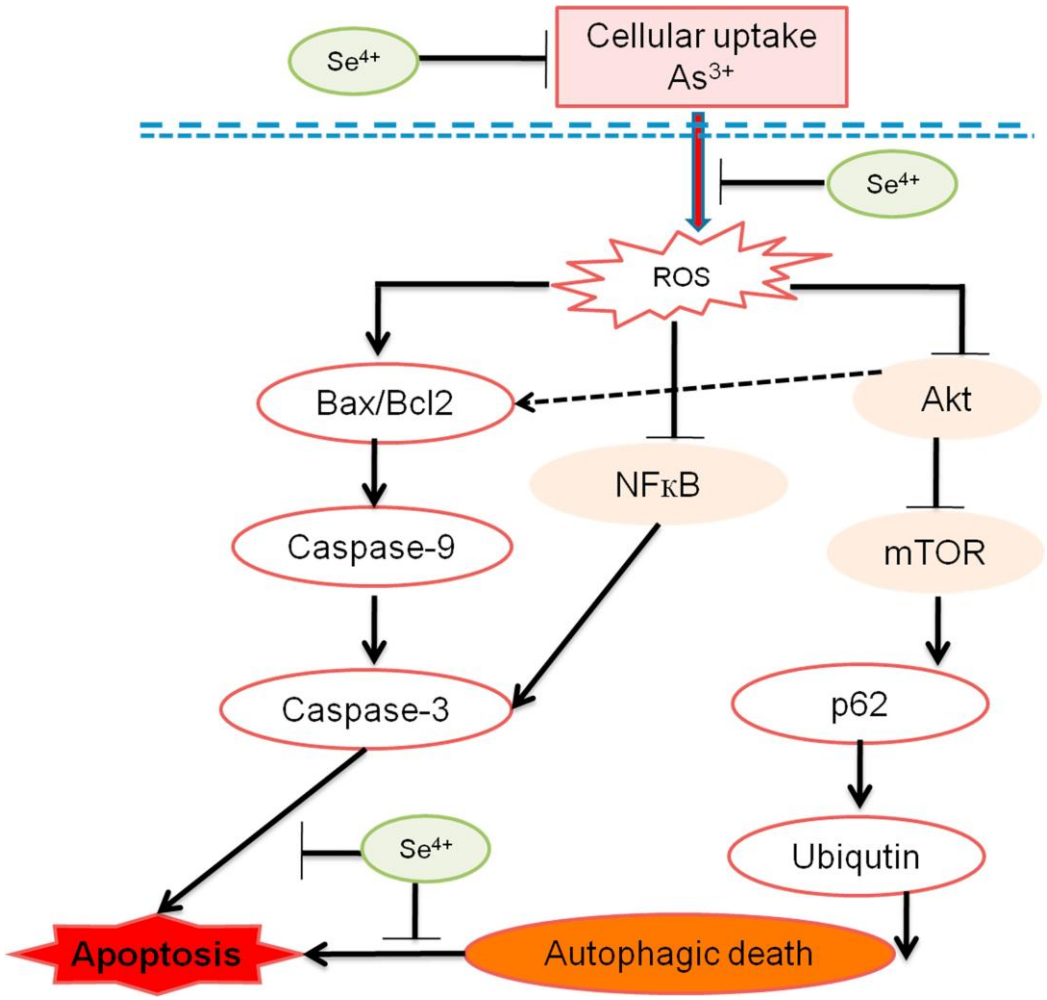

Fig. 3.10 A schematic diagram for potential molecular mechanism/s in PC12 cells upon arsenic and selenium co-exposure.

Moreover, arsenic induced-accumulation of p62 and ubiquitin upregulation is reduced by selenium and arsenic co-exposure in PC12 cells. Thus, arsenic is found to downregulate the autophagy related factors which may in turn contribute to increase more intracellular ROS production that may triggers the downregulation of NF-κB and finally ended up with induction of apoptosis. In addition, the inhibition of mTOR/Akt might enhance the increased expression of Bax and decreases the expression of Bcl2 protein. At the end, these reactions activate the caspase cascade through upregulation of caspases 3 and 9 to induce intrinsic apoptosis in PC12 cells. Selenium was found effective to reduce arsenic-induced autophagy/apoptosis in PC12 cells. Furthermore, it was also found that coexposure of selenium with arsenic can significantly reduce the

cellular accumulation of arsenic in PC12 cells (Fig. 3.9).This phenomenon has also been described by Zeng et al. (2005). In previous study, it was reported that upon acute exposure of selenium and arsenic in rabbit a new compound containing arsenic and selenium was found through spectrophotometrically as the seleno-bis(s-glutathinyl) arsinium ion[(GS)2AsSe]− (Gailer et al., 2000),which could readily excrete from the organ through the intestinal tract (Ponomarenko et al., 2017).The formation of the complex and subsequent excretion from the cells may also contribute to detect lower arsenic content in presence of selenium in PC12 cells upon co-exposure. Therefore, it can be concluded that arsenic exposure may lead to initiate the PI3K activation along with ROS production in PC12 cells, which modulates the downstream of PI3K/mTOR/Akt signaling pathway and also mutually contribute to the induction of apoptotic cell death in PC12 cells (Fig. 3.10). However, selenium inhibits arsenic-induced PC12 cell deaths in co-exposure may be i) by reactivating the mTOR/Akt signaling pathway; ii) by suppressing the apoptosis through downregulation of Bax and upregulation of anti-apoptotic factors; and finally, iii) by retarding the cellular accumulation of arsenic.

3.5 Conclusion

Selenium showed antioxidant properties by protecting PC12 cells from arsenic-induced oxidative stress upon simultaneous exposure. In addition, selenium also confirmed the suppression of arsenic-induced autophagy and apoptosis in PC12 cells by positively regulating the expression of key proteins related to the designated cell death processes. These findings may contribute to establish the role of selenium in ameliorating arsenic-induced toxicity in humans and animals and possibly design metal-based anti-cancer/anti-tumor therapeutics using multiple components.

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Chapter 4: Cytoprotective effects of lipoic acids against metal-(loids) induced toxicity in mammalian cells

Abstract

α-lipoic acid (ALA) is an endogenous dithiol compound with significant antioxidant properties. It is reduced enzymatically to form dihydrolipoic acid (DHLA) and together plays important role in mitochondrial energy metabolism. In this study, ALA and DHLA were applied to reduce toxic metal (Cd, As, Pb and Hg)-induced toxicity in PC12 and Caco-2 cells as simultaneous exposure. ALA and DHLA significantly decreased the Cd (5 μM), As (5 μM), Pb (5/50 μM)-induced cell death in PC12 cells. However, upon combined exposure ALA did not reduce the Hg-induced cell death in PC12 cells. Moreover, ALA (250 μM) protected Cd, As and Pb-induced cell death in Caco-2 cells. Subsequently, ALA reduced the lactate dehydrogenase activity in the culture

medium for PC12 and Caco-2 cells as well as DHLA (50 μM) also showed the similar effects in PC12 cells against Cd, As and Pb. The study of intracellular GSH level revealed that ALA and DHLA recovered the antioxidant defense ability which was lowered by exposure of metals in PC12 cells and Caco-2 cells. In Addition, DHLA (50 μM) protected PC12 cells from As (5 μM), Cd (5 μM) and Pb (5 μM) induced DNA damages upon co-exposure scenario. Furthermore, western blot analysis showed that ALA upregulated the survival related proteins expression mTOR, Akt, Nrf2 in PC12 cells that were downregulated by As or Cd exposure. Subsequently, ALA downregulated the expression of p53 protein induced by each As and Cd in PC12 cells. On the other hand, Akt and Nrf2 were upregulated upon co-exposure of As/Cd with ALA in Caco-2 cells, although Akt and Nrf2 were downregulated by the As or Cd exposures. Moreover, As and Cd induced upregulation of Bax and cleaved-PARP-1 downregulated by ALA in Caco-2 cells. These findings suggest that ALA and DHLA are capable of reducing the toxic effects of As, Cd and Pb in both PC12 and Caco-2 cells. The protection may be due to enhancement of the antioxidant defense by reducing cellular oxidative stress and the inhibition of apoptosis related cell death in both cell lines.

4.1 Introduction

α-lipoic acid (ALA/LA), one of the dithiol compounds often reduced enzymatically to dihydrolipoic acid (DHLA), and gained much interest due to their potential role in free radical scavenging, able in chelating metals and restoring intracellular glutathione (GSH) levels when toxicants accretion, environmental insults and senescence were occurred by environmental pollutants such as heavy metals.(Shay et al., 2009; Holmquist et al., 2017; Tibullo et al., 2017). Recently, ALA has been used in multivitamin formulations, food supplements, anti-aging formulas, and even in human and pet food recipes, as an asserted antioxidant compound (Shay et al., 2009; El-Senousey et al., 2017).

As ALA also showed functional pleiotropism with different signal transduction pathways, in the dysfunction of which leading to relevant pathologies, its use as a potential therapeutic agent would appear quite promising (Sudeesh et al., 2011; Sanders et al., 2017; Tibullo et al., 2017). One study showed that ALA is capable of efficiently scavenging free radicals *in vivo* (Castañeda-Arriaga and Alvarez-Idaboy, 2014). In its reduced form, as DHLA, it can scavenge reactive free radicals *via* hydrogen

transfer mechanism (Castañeda-Arriaga and Alvarez-Idaboy, 2014). Furthermore, DHLA keeps GSH and protein thiols in their reduced forms. Dietary intake of ALA has been considered to be clinically safe, becauseALA enters biological membranes easily due to its affinity with membrane lipids, as well as the blood-brain barrier (BBB), which confers to ALA a potential neurodegenerative preventive activity (Rochette et al., 2013; Venigalla et al., 2016; Kan et al., 2017). Unlike many other antioxidants, after ALA formed upon reaction with various pro-oxidants and free radicals, it can easily reconstitute DHLA. Furthermore, ALA and DHLA are amphipathic molecules and can, therefore, work both in aqueous and in hydrophobic environments, explaining that they can counteract oxidation of lipids, proteins, and DNA (Thannickal and Fanburg, 2000; Koloverou et al., 2017). However, some controversial results indicate that DHLA can also act as a cancer promoter under certain conditions (Wang et al., 2017), presumably when combined with tamoxifen (Belmont-Diaz et al., 2015). Therefore, the context where DHLA operates is particularly important (Muzio et al., 2012).

In recent years, ALA has been studied extensively as a biological antioxidant, a detoxification agent, and an anti-diabetes medicine, and moreover it has also been claimed to ameliorate age-associated cardiovascular, cognitive and neuromuscular deficits besides to be implicated as a modulator of various inflammatory signaling pathways (Smith et al., 2004; Suh et al., 2004; Shay et al., 2009; Demr et al., 2014; Kocaoğlu et al., 2017; Sanders et al., 2017).

In addition, in recent years environmental chemicals have increased attention, and have become a big concern in the field of oxidative stress and disease-promoting actions due to their frequent exposures through food chains, general air, and water pollution. Especially the toxic metals cadmium (Cd), lead (Pb), arsenic (As) and mercury (Hg) represent hazardous environmental contaminants, and they may interact with and affect the potential benefit of ALA and DHLA. To examine further possible role of ALA and DHLA against these toxic metals and metalloids we conducted *in vitro* study using mammalian cells (PC12 and Caco 2). Furthermore, a comparative cytoprotective potential was also accounted focusing the mechanisms behind it. In this chapter, crucial roles of ALA and DHLA are sufficient discussed.

4.2 Materials and method

4.2.1 Materials

PC12 cells were purchased from the American Type Culture Collection (USA and Canada) and Caco-2 cells (RCB0988) were kindly donated by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Dulbecco's modified Eagle's medium (DMEM), ribonuclease A (RNase), ethidium bromide, and peroxidase-conjugated avidin were purchased from Sigma (St. Louis, MO, USA). Eagle's minimum essential medium (EMEM) was purchased from Wako (Japan). Fetal bovine serum (FBS) was obtained from Biosera (Kansas City, MO, USA). SV total RNA isolation system and RT-PCR kit were purchased from Promega (Madison, WI, USA). High pure PCR product purification kit and proteinase K were purchased from Roche Diagnostics (Mannheim, Germany). Biotinylated goat anti-mouse IgG whole antibody and ECL western blotting detection reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Polyclonal antibodies against specific proteins such as Nrf2, mTOR, Akt, cleaved PARP-1, Bax (BIS, bs-0127R, BIOSS), beta-actin (GTX 109639, GeneTEX) were purchased. Trypan blue solution (0.4%) was purchased from Bio-Rad (Hercules, CA, USA). The DNA 7500 assay and RNA 6000 nano assay kits were purchased from Agilent Technologies (Waldbronn, Germany). All other chemicals were of analytical grade.

4.2.2 Cell culture

PC12 cells were cultured in DMEM supplemented with 10% FBS in a humidified incubator at 37°C with 5% CO_2, where as Caco-2 cells were cultured in EMEM medium supplemented with 10% FBS and 1% non-essential amino acids (NEAA) under same incubation condition. The cells were pre-incubated in 25-cm^2 flasks for 24 h; then, the medium was replaced with fresh medium with or without various concentrations of Cd, As, Pb, Hg, ALA and DHLA or with a mixture of them, and the cells were re-incubated for another 48 h. The desired concentration for treatment was selected by exposing PC12 and Caco-2 cells to several concentrations of each agents for example Cd^{2+} (0, 2, 5, 10, 20 μmol) separately, and then the final combination was decided. The selected concentrations for Cd^{2+}, As^{3+}, Pb^{2+}, ALA and DHLA were 5, 5, 10 or 50, 250 and 50 μM respectively. DHLA effects on toxic agents were studied only using PC12 cells but effects of ALA were examined using both PC12 and Caco-2 cells.

4.2.3 Cell viability

Cell viability was determined using trypan blue exclusion assay. Cells (PC12 and Caco-2) were seeded at a density of 1 × 10^5 cells/flask and pre-incubated for 24 h. Then, the cells were treated with Cd^{2+}, As^{3+}, Pb^{2+}, ALA and DHLA separately as well as co-exposed to Cd^{2+} , As^{3+}(5 μM), Pb^{2+}(10/50 μM) and ALA (250 μM)/DHLA (50 μM). The cells were then incubated for 48 h. Total cells and trypan blue-stained cells were counted using a Bio-Rad automated cell counter (Hercules, CA, USA). Cell viability was expressed as percentage of the counted trypan blue-stained cells. Each experiment was carried out at least in triplicate to ensure biological reproducibility and statistical validity.

4.2.4 Lactate dehydrogenase (LDH) activity assay

Cytotoxicity was assessed by measuring the activity of LDH in the treatment medium using a nonradioactive cytotoxicity assay kit (Promega) as described by Kihara *et al.* [28]. PC12 and Caco-2 cells (1 × 10^5 cells/flask) were cultured in the medium with/without Cd^{2+} , As^{3+}(5 μM), Pb^{2+}(10/50 μM) and ALA (250 μM)/DHLA (50 μM) for 48 h. After 48 h incubation, 50 μL of the medium was transferred to a 96-well plate and then 50 μL of a substrate mixture containing tetrazolium salts was added to each of the sample well in the 96-well plate. After 30-min incubation at room temperature (25°C), 50 μL of the stop solution was added, and the amounts of formazan dye formed were determined by measuring the absorbance at 490 nm using a microplate reader (Bio-Rad, CA, USA). LDH activity was expressed as LDH activity/1 × 10^6 cells. This experiment was carried out in triplicate for ensuring reproducibility.

4.2.5 Measurement of GSH levels

Intracellular glutathione (GSH) levels were investigated as previously described by Kihara et al. (2012). Cells (1 × 10^5) were pre-incubated for 24 h. Then, they were exposed to Cd^{2+}, As^{3+} (5 μM), Pb^{2+} (10/50 μM) and ALA (250 μM)/DHLA (50 μM) for 48 h. The cells were harvested, washed with 1× phosphate-buffered saline (PBS), added to 150 μL of a lysis buffer, and then incubated at room temperature (25°C) for 10 min. Two freeze-thaw sonication cycles were performed in order to rupture the cell membranes, and the resultant solution was centrifuged at 1,500 rpm for 10 min to collect the supernatant. Intracellular free SH levels were determined using 2.5 μmol/L 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB, pH 7). DTNB (final concentration; 20 μM) was added to the cell lysate, and then the absorbance was measured at 412 nm by using a microplate reader (Bio-Rad, CA, USA). The concentration of intracellular GSH in cells

was determined using a molecular coefficient factor of 13,600 per cell number (1×10^5). The experiment was carried out in triplicate to ensure mechanical reproducibility.

4.2.6. Isolation of genomic DNA and Agarose gel electrophoresis

After treatment of PC12 and Caco-2 cells with above mentioned chemicals, the cells were harvested. Then, the obtained cells were centrifuged at 1,500 rpm for 5 min to remove the supernatant. After addition of 3 mL of PBS, the mixture was centrifuged again at 1,500 rpm for 5 min to wash the cells. The genomic DNA was isolated using high pure PCR template preparation kit according to the manufacturer's instruction as described by Kawakami et al. (2008). The obtained solution containing DNA was mixed with 2 µL of 500 µg/mL RNase and incubated for 15 min at 37°C. After incubation, 500 µL of ethanol and 20 µL of 3 M NaOAc buffer (pH 4.5) were added for ethanol precipitation, and the solution was allowed to stand overnight in a freezer to precipitate the DNA. On the next day, DNA was separated using microcentrifugation at 15,000 rpm for 8 min, and then washing with 70% ethanol at the same speed for 3 min was carried out. Then, the DNA sample was dried for approximately 10 min, and DNA concentration was measured after reaction with 1× Tris/Borate/EDTA (TBE) using a UV-visible spectrophotometer. The ladder pattern/intact DNA was analyzed *via* agarose gel electrophoresis. Approximately 3–5 µg of DNA with the loading dye was subjected to electrophoresis on a 1.5% agarose gel. Electrophoresis was carried out for 40 min at 100 V in 1.5% of agarose gel by using a submarine-type electrophoresis system (Mupid-ex, Advance, Tokyo, Japan). To visualize the DNA strand breaks/degradation, the gel was soaked in ethidium bromide solution for 5–10 min. Images of the agarose gel were taken under UV illumination using a ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). To evaluate cell apoptosis/DNA damage, the fluorescence intensity of DNA in the gel was analyzed by a software named Quantity one. This experiment was conducted at least in triplicate.

4.2.7 Western blot analysis for determination of protein expression

Cells were cultured in 5 mL of DMEM/EMEM containing 10% FBS, with or without above mentioned treatment for 48-h. The procedure of western blot analysis was done according to Rahman et al., 2017 briefly as, the total protein in cells was extracted by ice-cold lysis buffer (2mM HEPES, 100 mMNaCl, 10 mM EGTA, 0.1 µM PMSF, 1 mM Na_3VO_4, 0.1mM Na_2MgO_4, 5mM 2-glycerophosphoric acid, 10µM $MgCl_2$,

2mM DTT, 50 μMNaF and 1% triton X-100). The extracted protein concentration was measured spectrophotometrically using protein assay dye reagent (BioRad). The total protein (30 μg) from each sample was separated by 12.5/15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis and then transferred onto nitrocellulose membrane via semi-dry transfer method. After that, the membrane was blocked by 5% skimmed milk at 4°C for 24 h. Then, the membrane was sequentially incubated with desired primary and secondary antibodies. Finally, the protein on the nitrocellulose membrane was visualized using enhanced chemiluminescence and the image of the detected band was analyzed using a ChemiDoc XRS (Bio-Rad, USA). The intensities of the bands were as the ratio to that of β-Actin (internal control). All of the experiments were conducted at least in triplicate to ensure reproducibility.

4.3 Results

4.3.1 Combined effects of toxic metal-(loids) and ALA/DHLA on cell viability

The cell viability of PC12, Caco-2 and MCF-7 cells after exposure to ALA (10-1000 μM) for 48 h were determined using trypan blue exclusion method and results are presented in Fig. 4.1. PC12 cells showed no significant changes against the applied concentration range. However, in Caco-2 and MCF-7 cells at 500 μM or more concentration of ALA significantly reduced the viability from the control group. Furthermore, MCF-7 cells showed more susceptibility against ALA treatment than the Caco-2 cells (Fig. 4.1). On the other hand, DHLA up to 150 μM for 48 h showed no significant changes in viability of both cell lines (data not shown). Thus, 250 and 50 μM dose was decided for ALA and DHLA, respectively, for further combined study with toxic metal (Cd, As, or Pb) using PC12 and Caco-2 cell lines. To examine the combined effect of Cd, As or Pb with ALA, PC12 and Caco-2 cells were exposed to both of chemicals (binary mixture) for 48 h. Results showed that in both PC12 and Caco-2 cells Cd (5μM), As (5μM) and Pb (5/50 μM) induced significant cell death compared to the control and solvent control (Fig. 4.2A).

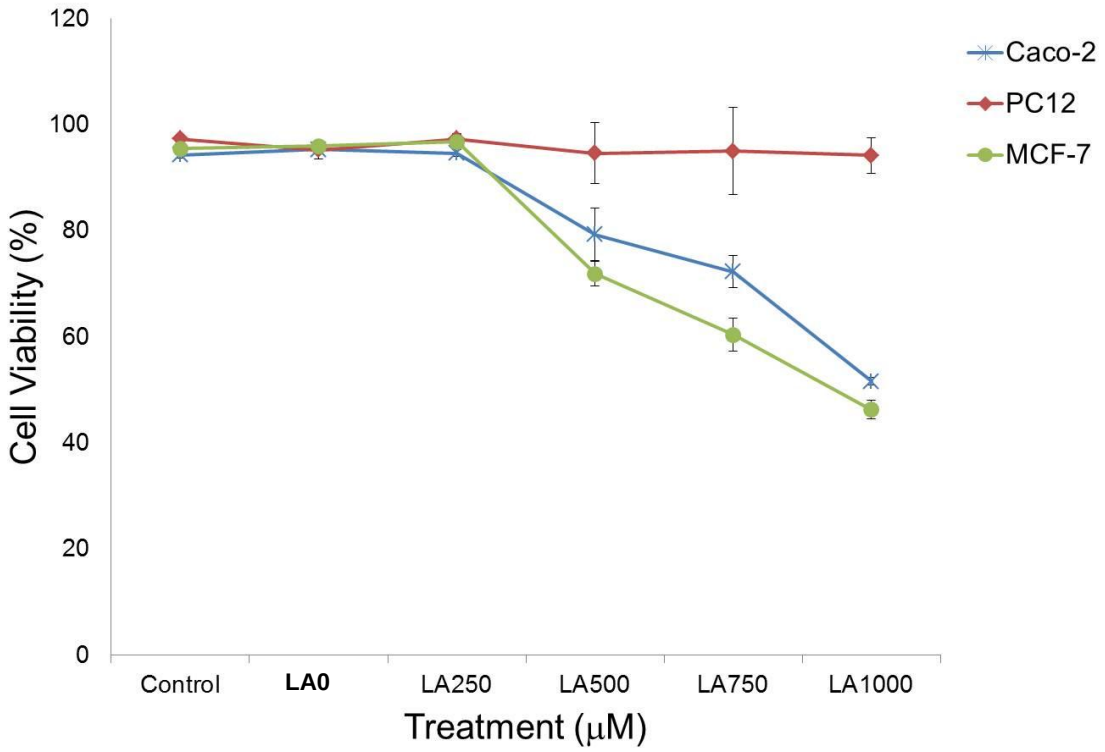


Fig. 4.1 Cell viability of mammalian cells after LA exposures for 48 h measured using trypan blue exclusion method. Error bars indicate mean ± SEM (n=5).

For PC12 cells, a reduction of cell viability to 45%, 42% and 6.6% were observed due to 48 h exposure of As (5 μM), Cd (5 μM) or Pb (50 μM), respectively. Where as, in Caco-2 cells exposed with As (5 μM), Cd (5 μM) or Pb (50 μM) the cell viabilities were declined to 44%, 50% and 53%, respectively, in response to the similar exposure of the toxic metals as in PC12 cells (Fig. 4.2B). However, in co-exposure with ALA (250 μM) and toxic metals the cell viability increased significantly compared to the only metal treated group for both PC12 and Caco-2 cells (Figs. 6.2A and B). In addition, DHLA (50 μM) showed cytoprotection by significantly increasing cell viability in combination with As (5 μM), Cd (5 μM) and Pb (5 μM) in PC12 cells (Fig. 4.3). However, ALA did not protect PC12 cells from Hg (2.5/5 μM) exposure in co-exposure (data not shown). In the study, we further did not carry out the Hg exposure experiments.

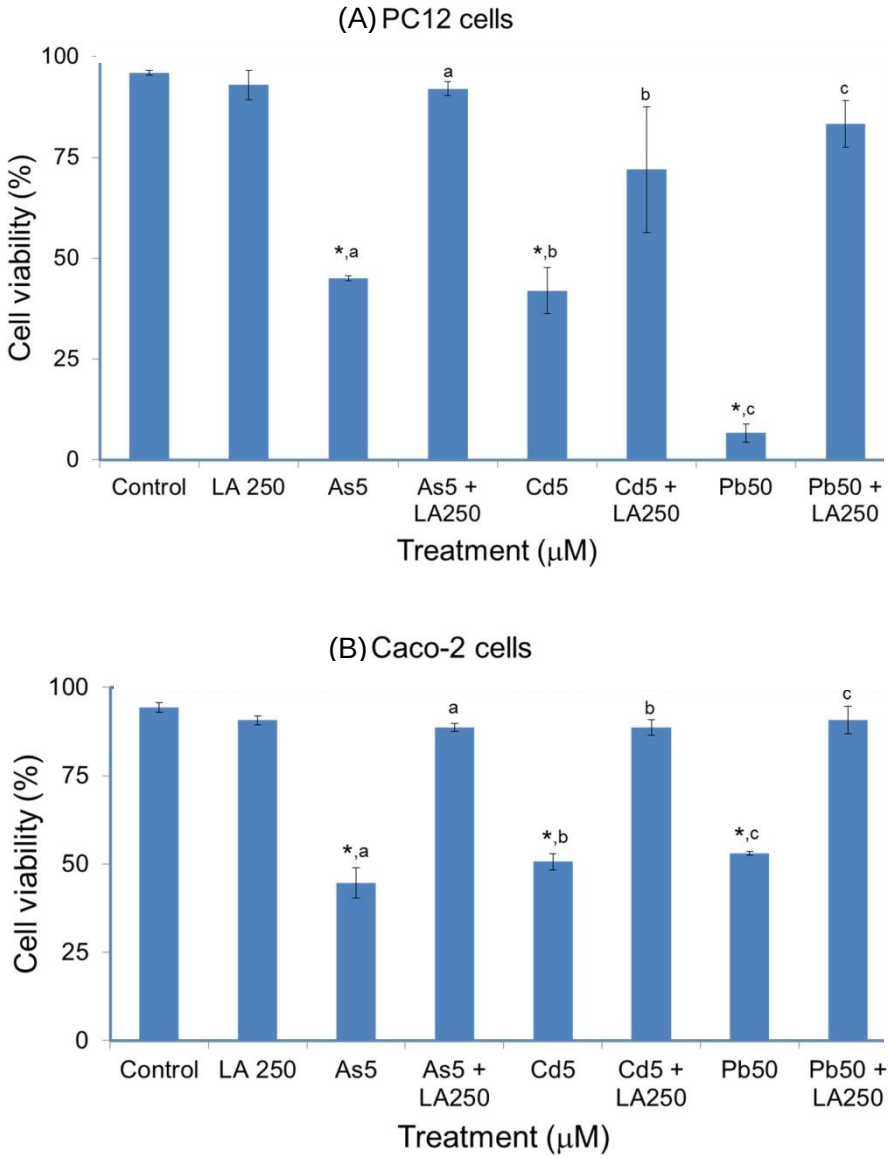


Fig. 4.2 Cell viability of PC12 cells (A) and Caco-2 cells (B) after exposure to toxic metals and/or ALA for 48 h of incubation measured by trypan blue exclusion method. Error bars indicate mean ± SEM (n=5). Asterisks * denotes significant difference with $p<0.05$ and there is also significant difference between similar letter at $p<0.05$.

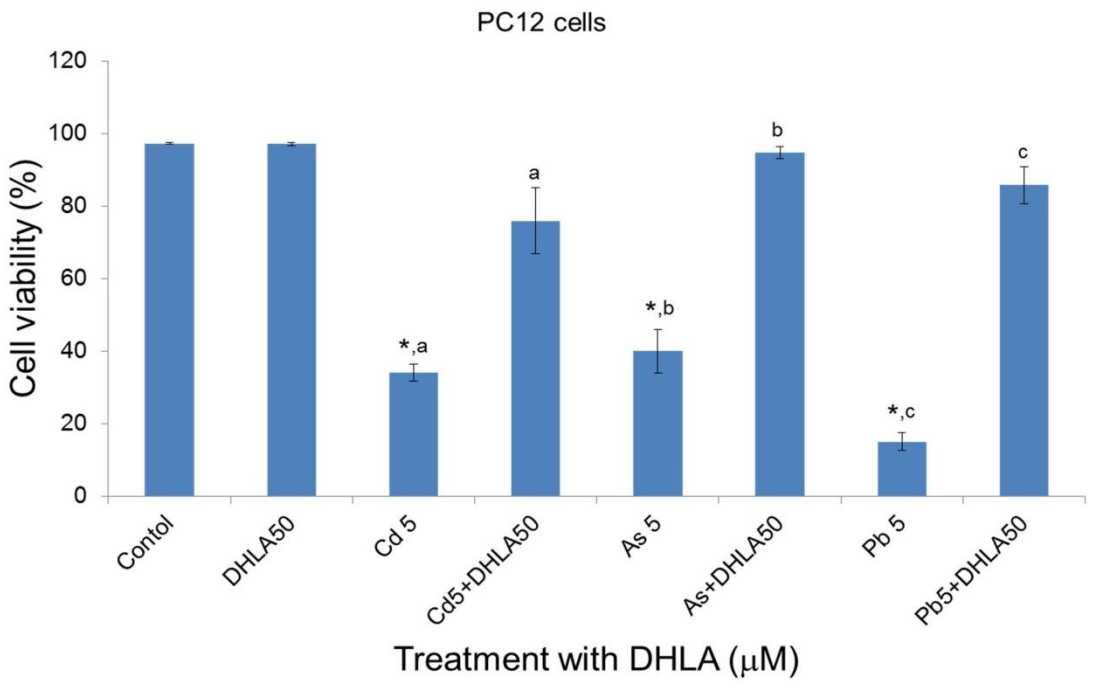


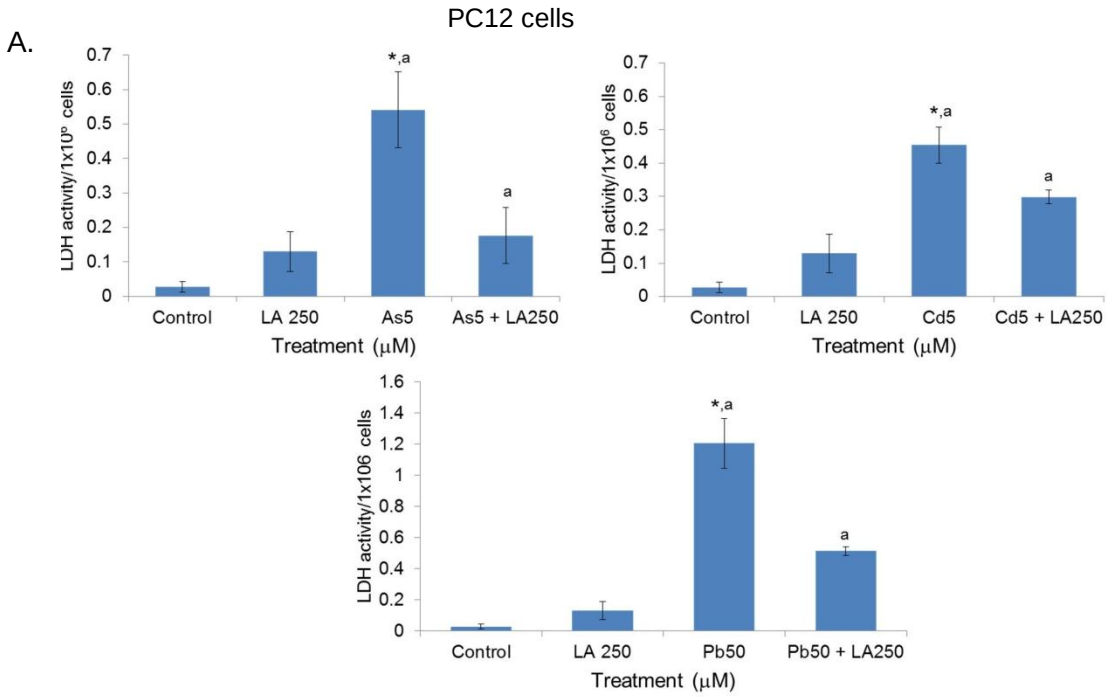
Fig. 4.3 Cell viability of PC12 cells after treatment with toxic metals and/or DHLA for 48 h determined by trypan blue exclusion method. Error bars indicate mean±SEM (n=6) and asterisks * denote significant at $p<0.05$ compared to control and similar letters are also significantly differs at $p<0.05$.

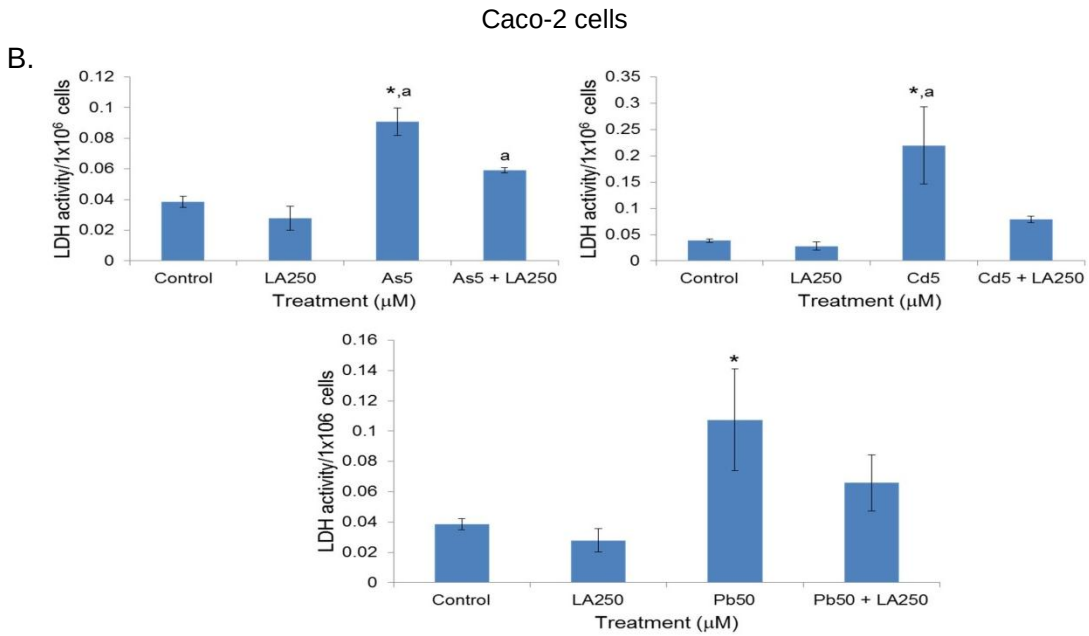
Where, a remarkable increase in cell viability was observed after combined treatment of DHLA with toxic metals; Cd (μM) (34%) to Cd (μM) and DHLA (50μM) (76%); As (5 μM) (40%) to As (5μM) and DHLA (50 μM) (94.8%); Pb (5 μM) (15%) to Pb (5 μM) and DHLA (50 μM) (85.83%) as shown in Fig. 4.3.

4.3.2 Combined effects of metal-(loids) and ALA/DHLA on LDH activity

LDH activity was measured in the cell culture medium after being exposed/co-exposed to Cd, As, Pb, ALA and DHLA for 48 h. Both PC12 and Caco-2 cells showed significant increased of LDH activity in their respective culture medium in response to metal burdens (Figs. 4.4A-C). However, co-exposure to toxic metals and ALA/DHLA showed a significant decreased of LDH activity than the metal treated group alone in PC12 cells as well as in the Caco-2 cells (Figs. 4.4A-C). These results corroborate the finding of cell viability under similar experimental condition for both of the studied cell lines. The

damaged cell membrane was significantly lowered in the co-treated group (toxic metals + ALA/DHLA) than the metal treated group alone.





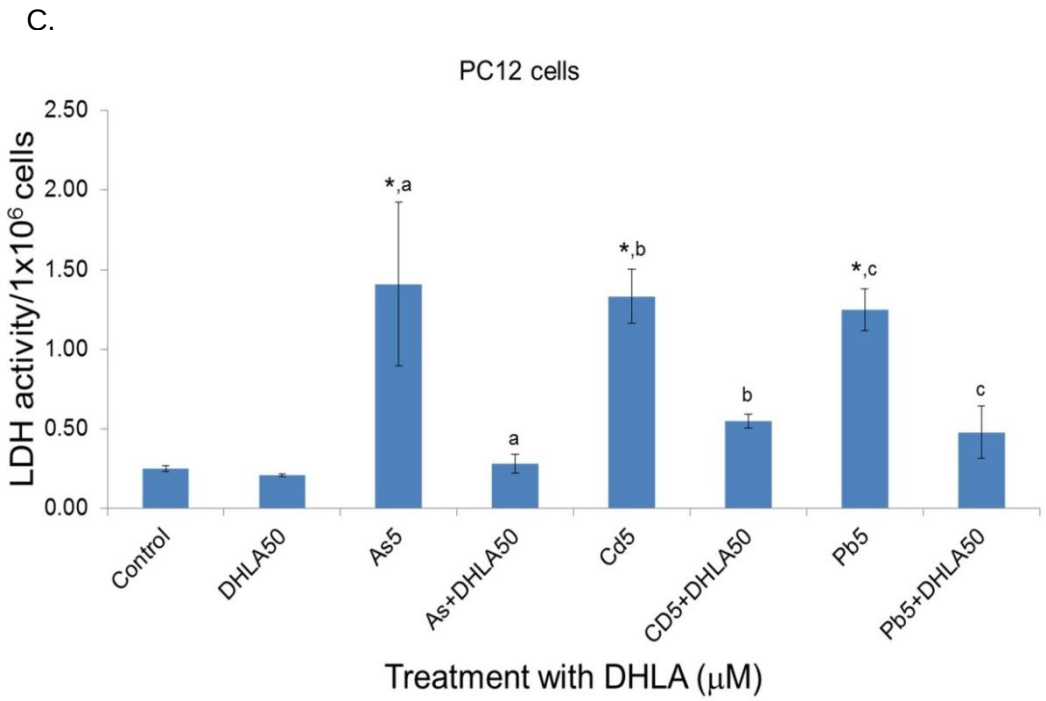


Fig. 4.4 Lactate dehydrogenase (LDH) activity in cell culture medium after exposure to toxic metals and/or ALA (A, B) and DHLA (C) for 48 h of exposure. Error bars indicate mean±SEM (n=4) and asterisks * denote differs significantly compared to control and similar letters indicate significant difference at $p<0.05$.

4.3.3 Combined effects of metal-(loids) and ALA/DHLA on GSH level

Intracellular GSH level was measured following the above mentioned metal exposure with/or without ALA/DHLA in PC12 and Caco-2 cells. As, Cd and Pb exposures showed significant decreases in reduced GSH level in both of the cell lines (Figs. 4.5A-C). ALA and DHLA showed significant inhibition of GSH oxidation due to As, Cd or Pb exposure upon co-exposure scenario. On the other hand, ALA also showed similar protection against metal induced GSH depletion in Caco-2 cells (Fig. 4.5B). A severe depletion of GSH level was observed upon exposure of toxic metals in both PC12 and Caco-2 cells. Therefore, means possible increase of oxidative stress due to burden of toxic metals in cells. However, the exposure of ALA and DHLA was found to boost up the GSH defense for both PC12 and Caco-2 cells against toxic metals stress. Although, only ALA and DHLA treated PC12 cells showed an increased level of GSH but it was not significant in case of ALA treated group alone.

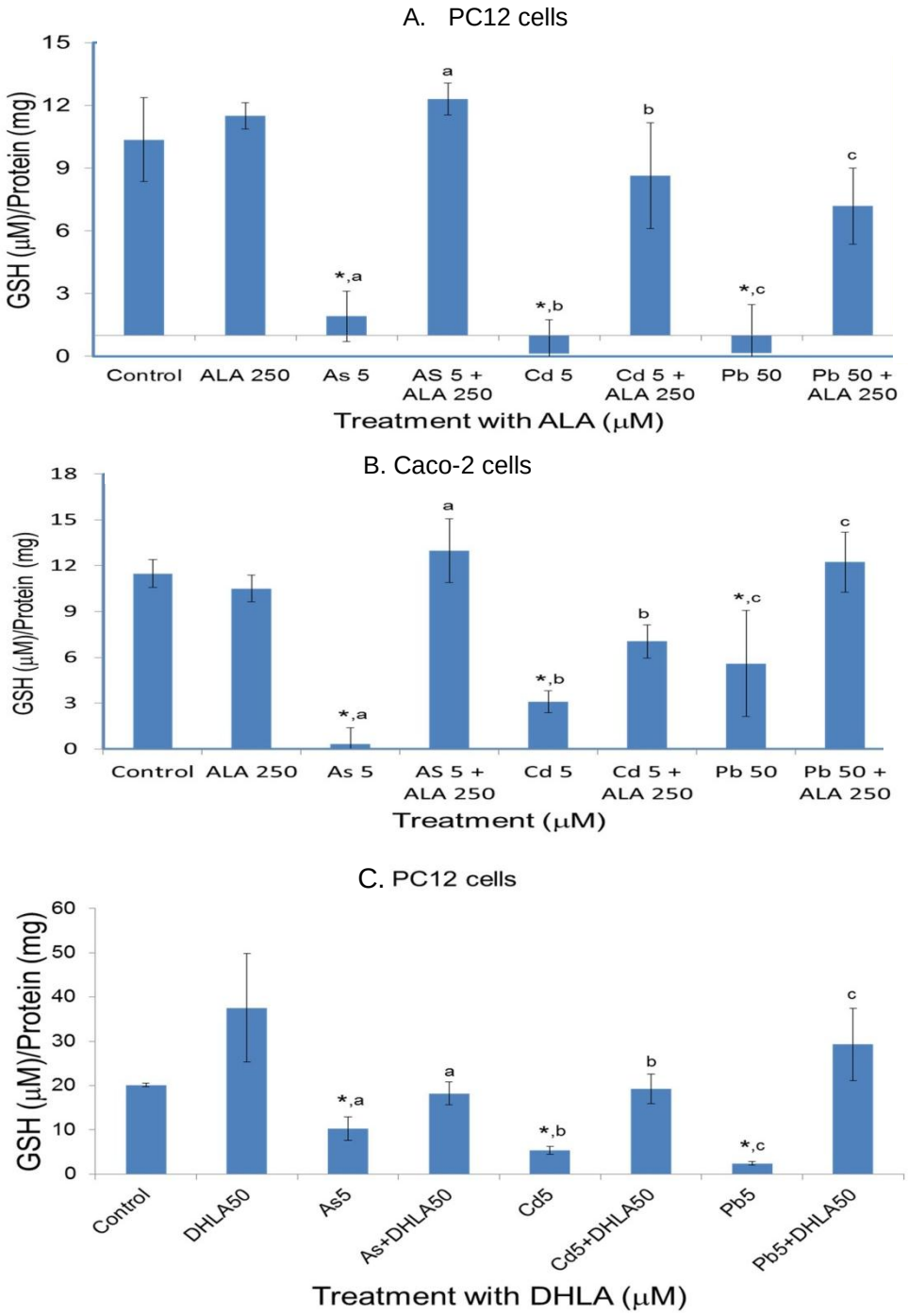


Fig. 4.5 Intracellular levels of GSH upon exposure to toxic metals and/or ALA measured by DTNB assay in Caco-2 and PC12 cells (A-B) and metals/DHLA in PC12 cells (C). Error bars indicate mean±SEM (n=3) and asterisks * denote significant at $p<0.05$ compared to control, similar letters indicate also significant at $p<0.05$.

4.3.4 Combined effects of metal-(loids) and DHLA on genotoxicity

The results from agarose gel electrophoresis revealed that the cellular DNA was damaged due to As, Cd or Pb exposure in PC12 (Fig. 4.6). Co-exposure of metals with DHLA showed significant increase of intact DNA density compared to metal treated group alone. DNA damage was also characterized by ladder/smearing pattern in PC12 cells due to As and Cd. This result indicated that metal induced genotoxicity was significantly inhibited by DHLA as well as ALA produced also the similar results (data not shown).

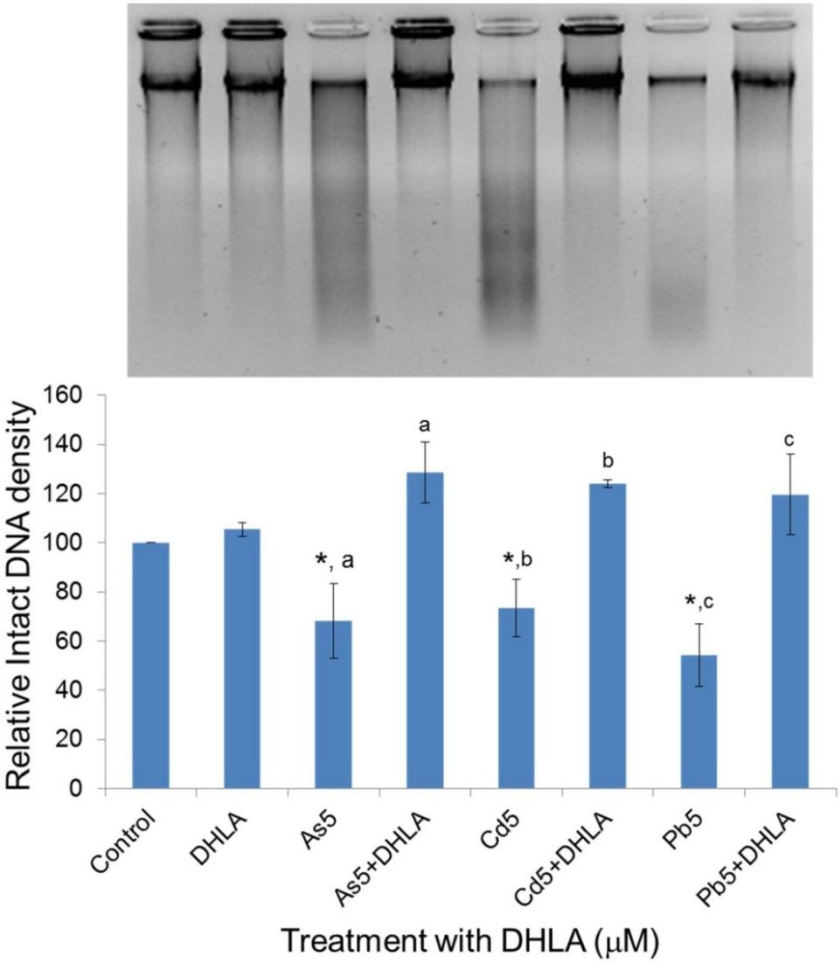


Fig. 4.6 DNA electrophoresis taken by agarose gel method (upper part) and DNA density measurement (lower part) calculated by image J software after co-treatment with metals and DHLA in PC12 cells. Error bars indicate mean±SEM (n=4). Asterisk * denotes difference is significant at $p<0.05$ compared to control and similar letter mentions significant difference as well at $p<0.05$.

4.3.5 Protein expression

Protein expression was determined by western blot analysis for PC12 and Caco-2 cells after co-treatment with "As and ALA", and "Cd and ALA". Immunoblot results are presented for PC12 cells in Fig. 4.7. In PC12 cells, ALA (250 μM) upregulated the expression of protein Nrf2 compared to the control. Furthermore, ALA upregulated the expression of mTOR, Akt and Nrf2 which were downregulated by the exposure of As (5 μM) or Cd (5 μM) in PC12 cells (Fig. 4.7). However, although As and Cd induced p53 upregulation, this increases of p53 was downregulated by the treatment of ALA in PC12 cells (Fig. 4.7).

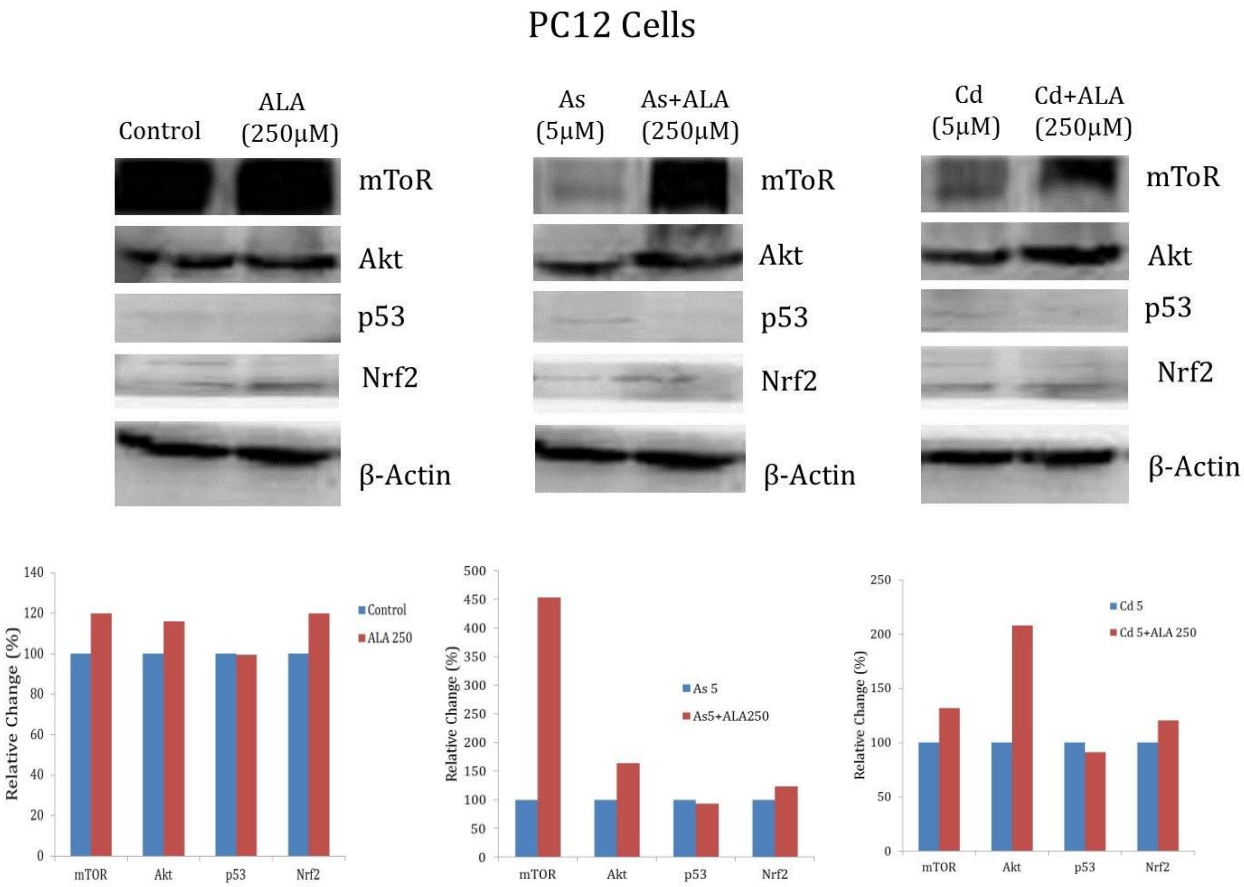


Fig. 4.7 Western blot analysis of protein extracted from cells after treatment with metals/metals with ALA in PC12 cells for 48 h. Representative images (cropped) are presented from two independent experiments. Relative change of band density is presented in percentage (%) of control, As 5 and Cd 5 alone after average of two experiments.

On the other hand, protein expressions after treatment with metals/metals and ALA in Caco-2 cells were shown in Fig. 6.8. From the Immunoblot image it was observed that metal induced upregulation of pro-apoptotic Bax and poly (ADP-ribose) polymerase-1 (cleaved-PARP-1) protein was downregulated by ALA co-exposure (Fig. 4.8). Moreover, metal-induced downregulation of protein Nrf2 and Akt was also upregulated upon coexposure of ALA in Caco-2 cells (Fig. 4.8). However, for both of the cell lines, ALA induced upregulation of Nrf2 protein (Figs. 4.7 and 8).

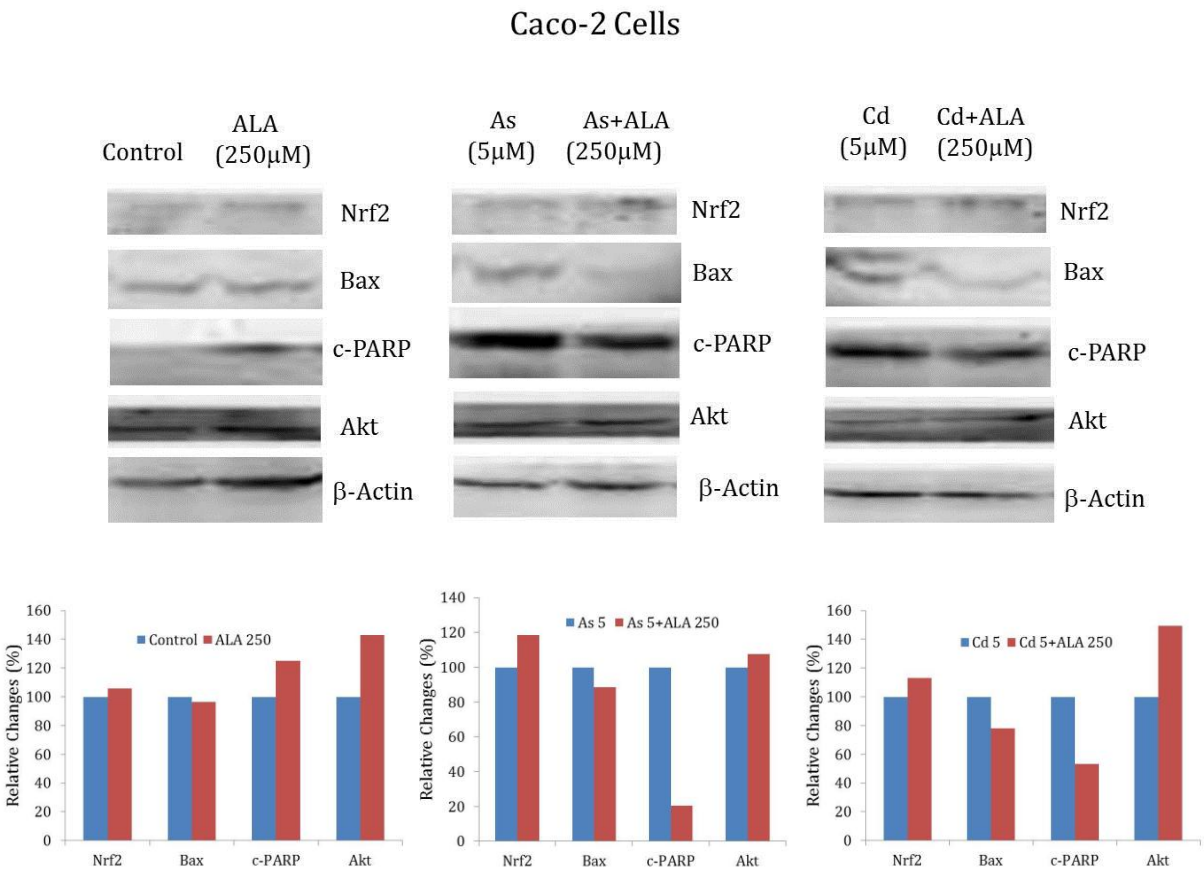


Fig. 4.8 Western blot analysis of protein extracted from Caco-2 cells after being treated with metals/ metals and ALA for 48 h. Representative images (cropped) are presented from two independent experiments. Relative change of band density is presented in percentage (%) of control, As 5 and Cd 5 alone after average of two experiments.

4.4 Discussion

Despite the prooxidant potential at high dose level of α-lipoic acid (ALA/LA) at low dose level it has antioxidant role as well in oxidative stress condition. In Sprague-

Dawley rats intraperitoneally administrated ALA (100 mg/kg) for 14 days induced oxidative stress and cytotoxicity (Cakatay and Kayali, 2005). In this study, we found that ALA at 250 (μM) did not have any toxic effects in PC12, Caco-2 and MCF-7 cells in terms of cell viability (Fig. 4.1). Moreover, compared to the control experiments ALA did not induce oxidative stress in PC12 and Caco-2 cells. These results are in consistent with the finding of a study on that colorectal cancer cells did not affected by ALA (200 μM) by Dorsam et al. (2015). However, they reported an IC_{50} value of ALA for Caco-2 cells is 517 (μM) for 48 h of exposure (Dorsam et al., 2015). In this study, a significant reduction in cell viability was observed upon exposure to As (5μM), Cd (5μM) and Pb (5/50μM) in PC12 and Caco-2 cells (Figs. 4.2 A-B). Similarly, As and Cd induced reduction of cell viability in PC12 cells was also reported in our previous study (Rahman et al., 2017; Rahman et al., 2018). Moreover, As and Cd can produce oxidative stress by generating intracellular ROS, and oxidation of cellular reduced GSH that may be challenged to the homeostasis of biological macro-molecules of the cells such as proteins, nucleic acids and lipids as reported by many researchers including our research group (Hossain et al., 2018, Rahman et al., 2018).

ALA can scavenge numerous free radicals such as reactive oxygen and nitrogen species (Guven et al., 2008). Level of intracellular GSH was decreased upon exposure to As, Cd or Pb in PC12 and Caco-2 cells. However, co-exposure of these metals with ALA significantly increased the level of GSH in both of the studied cell lines (Fig. 4.5). DHLA also showed similar effects in PC12 cells (Fig. 4.5). Therefore, it can be said that metals induced oxidative stress can be reduced by ALA/DHLA in co-exposure for PC12 as well as in Caco-2 cells. Regeneration of reduced GSH can be a possible mechanism of the cytoprotection against metal induced oxidative stress. For instance, Cd may directly form conjugate with thiol group in GSH to induce GSH-Cd conjugate which could readily be excreted out from the cells thus depleting the reduced GSH (Rai et al., 2003). ALA and DHLA increased level of GSH in co-exposure of metals may be the key mechanisms to resist against metal induced oxidation of GSH and thus protecting the PC12 and Caco-cells (Fig. 4.5). Lawal and Ellis (2010) reported that the excessive ROS production along with oxidation of reduced GSH might be very crucial player in cadmium-induced cytotoxicity in three human cell lines; HepG2 cells, 1321N1 cells and HEK293 cells. Similarly, As also induced the depletion of GSH in PC12 cells as like Cd. Therefore, Cd and As-induced cytotoxicity could be at least partially related to the lowering of

intracellular GSH that may disrupt the reduced GSH/GSSG. Our present finding also corroborate with the study finding of Bharat et al. (2002) showing ALA protected against oxidative stress and cytotoxicity induced by preventing depletion of GSH contents in dopaminergic PC12 cells. Moreover, leakage of LDH induced by metal was also reduced by the simultaneous exposure of ALA or DHLA in both PC12 and Caco-2 cells (Fig. 4.4). These results clearly indicate the reduction of cell membrane damage in presence of ALA and DHLA with metals.

Nrf2-ARE signaling pathway has been elucidated to be involved in reduced GSH synthesis processes. Lines of evidence have reported that Nrf2 is localized in the cytoplasm where it remains with Keap1 under homeostasis. However, it is activated upon response to oxidative and electrophilic stimuli including ROS, some antioxidants, heavy metals and certain disease processes (Jeyapaul and Jaiswal, 2000), which induce phosphorylation of Nrf2. Therefore, Nrf2 dissociates from Keap1, and then translocates into the nucleus, binding to the antioxidant response element (ARE) and inducing the transcription of genes encoding GR (Kaspar et al., 2009; Wang et al., 2007). In our study, it was found that upon response to the ALA the expression of Nrf2 is increased in protein level in both PC12 and Caco-2 cells. Subsequently the As and Cd treated group showed downregulation of the Nrf2 protein. However, co-exposure with metals and ALA showed again upregulation of the Nrf2 protein attributing the ALA induced antioxidant/ROS scavenging events in both PC12 and Caco-2 cells (Figs. 4.7 and 8). Our findings are corroborated with the study conducted in HepG2 cells by Shi et al. (2016). Where, they found the downregulation of pNrf2/Nrf2 upon Cd exposure, and this downregulation was reversed by coexposure with Cd and ALA in HepG2 cells (Shi et al., 2016; Macias-Barragan et al., 2017). Moreover, the upregulated expression of p53, and downregulated expression of mTOR and Akt upon As and Cd exposure might be characterized by the cellular apoptosis in PC12 cells (Dorsam et al., 2015, Rahman et al., 2018). However, the co-exposure of ALA reversed the situation in PC12 cells. Furthermore, in Caco-2 cells As and Cd induced cleavage of PARP-1 and upregulation pro-apoptotic Bax expression. It confirms the intrinsic apoptosis and ALA co-exposure subsequently reduced the expression of cleaved PARP-1 and Bax in Caco-2 cells. A similar cleaved PARP-1 induction was reported in Caco-2 cells to explain apoptosis (Dorsam et al., 2015). The normal function of PARP-1 is the routine repair of DNA damage by adding poly (ADP ribose) polymers in response to a variety of cellular

stresses (Chaitanya et al., 2010). PARP-1 is one of several known cellular substrates of caspases, and cleavage of the PARP-1 by caspases is considered to be a hallmark of apoptosis (Kaufmann et al., 1993). Finally, in PC12 cells a severe DNA damage was done by As, Cd or Pb which was reduced upon co-exposure of DHLA with metals (Fig. 4.6). DNA fragmentation induced by Cd or As is in agreement with our previous findings in PC12 cells (Hossain et al., 2018; Rahman et al., 2018). Therefore, metals were found to increase cellular oxidative stress and subsequent alterations in the biomolecules such as proteins, DNA and GSH in both PC12 and Caco-cells, and significant ameliorative effects were observed by the treatment of ALA/DHLA. Thus, ALA and DHLA can detoxify metals toxicity in PC12 and Caco-2 cells by reducing oxidative stress, activating Nrf2, inhibiting DNA fragmentations and protecting from apoptosis induced cell death process (Fig. 4.9).

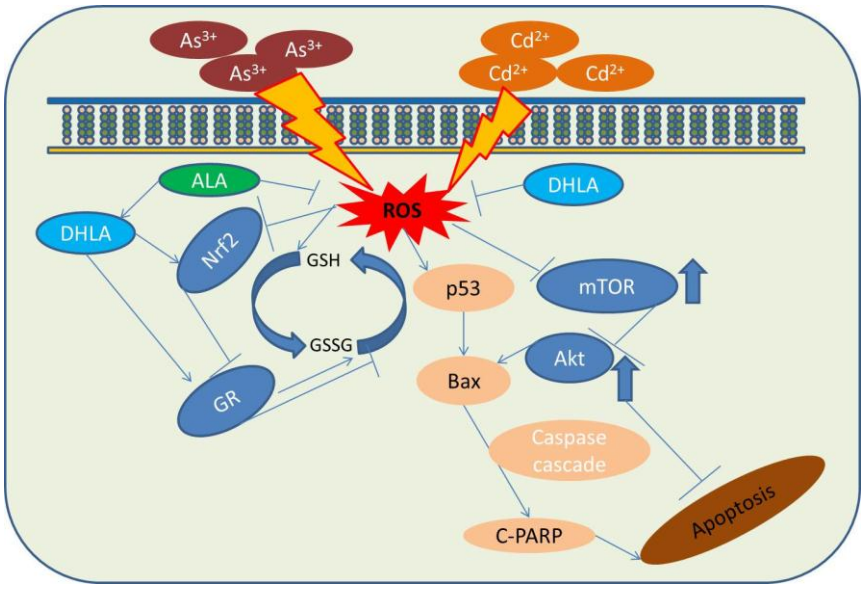


Fig. 4.9 Schematic diagram depicting possible molecular pathway of ALA/DHLA against metal toxicity

4.5 Conclusion

In this study, significant cytoprotective effects of ALA and DHLA was reported against Cd, As, and Pb induced toxicity in PC12 and Caco-2 cells. Strengthening of the cellular antioxidant defense by ALA and DHLA could be crucial way to get rid of the metal induced cellular burden in PC12 and Caco-2 cells. However, the regeneration of reduced GSH trough the Nrf2-ARE signaling pathway might be involved in the antioxidant defense regime. Subsequently, the ALA induced reduction of cell death may

be due to the reduction of apoptosis through mitochondrial pathway. DHLA also proved to protect PC12 cells from metal induced DNA fragmentation. Finally it can be concluded that ALA/DHLA has protective roles against toxic metal induced cellular damage in PC12 and in Caco-2 cells.

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Chapter 5: General conclusion and future research

5.1 General Conclusion

This research has been started with some specific objectives which are majorly related to environmental toxicants (toxic metals) and their deleterious roles in biological systems. Environmental pollution caused by metals has become a global concern due to unavoidable industrial pollution sources, increasing population, excessive pressure on natural resources, and numerous human health complications. Every compartments of the environment (Air, Water, Soil, and Anthrosphere) are affected by the negative anthropogenic activities as well as some forms of natural disaster which in turn put challenges towards dynamic environmental balance. Thus, pollution resulted as an obvious outcome. It is observed that compared to the developed countries developing and least developed countries are vulnerable to environmental pollution. Specifically countries like Bangladesh, Vietnam, China, India, Argentina, and Mexico have been facing severe environmental challenges. Fast growing economic activities with less concern about environmental burden aggravated the issues of public health concern due to environmental pollution.

Drinking water, food stuffs, soil and air all are now being polluted specially with different industrial chemicals such as toxic metals, pesticides, particulate matter (PM_{10}, $PM_{2.5}$), hazardous organics, and toxic gases. It is exceedingly important to assess the pollution scenario in respect of human health in vulnerable areas. For instance, we reported that due to As contamination in groundwater a substantial health risk is presumed to the people in contaminated areas in central part of Bangladesh. We also

reported the presence of toxic metals (Cd, Cr, and Pb) in cow milks in Bangladesh. In addition, researchers also reported toxic metals (Cd, Cr, Pb, and Hg) contamination in various food stuffs such as rice, vegetables, fruits, and fishes with considerable human health risks all over the world (Jafarian-Dehkordi and Alehashem, 2013; Shaheen et al., 2016). All these evidence indicate contamination of food chain with different levels of toxic metals. Therefore, toxic metals can easily enter into the human body and imposing serious health concerns. Recent studies reported toxic metals as at least one of the partial etiological factors of some diseases including cancers, diabetes, and neurological disorders (Bjørklund et al., 2018). However, there is no specific medication to reduce the deleterious effects of toxic metals after entering into the body. In addition, the status of metal body burden in mass people is always neglected and in conventional medical education also showed controversial system to focus on metals in the body. As a result, the dark site of the excess and/or deficient level of metals in the body has been coming out with several negative outcomes. For instance, a recent study stated that Se deficiency has a crucial role in the ionic homeostasis in the muscle of the chicken. Where, due to Se deficiency some essential micronutrients were found to be less than the normal chicken on the other hand toxic metals such as Cd were found to be higher in the muscle compared to the control (Yao et al., 2017).

Since the last couple of decades scientists are looking for suitable remediation process of toxic metals in the biological systems under chronic and acute exposure conditions. Application of metal chelator, use of antioxidants has been studying as some of the ways to fight back metals in the body. There is another approach which has been drawing the attention of toxicologists to reduce the toxic effects of metals by applying some nutrients including trace metals. For example, Zn and Se have been extensively studying to detoxify Cd, Hg, and As in animals and in plants. These studies report promising results but the mechanism/s behind the protection is still to be well understood.

Therefore, it is very important to understand the fundamental mechanism/s of metal toxicity in biological system to find therapeutics and to reduce the toxic effects. As stated earlier, a line of evidence reported the potentials of trace elements such as Zn, Se and other antioxidants against deleterious effects of toxic metals in organisms and in cellular level. Thus, to elucidate the role of Zn and Se against Cd and As, this research mainly focused on the *in vitro* cellular techniques to establish possible protection

mechanism/s. Moreover, a very popular dietary supplements α-lipoic acid (ALA) and it's reduced form dihydrolipoic acid (DHLA) was also examined against metal toxicity using cell models.

Significant modulatory effects of Zn and Se were pointed out against the toxic effects of Cd, Pb, As and Hg in both animal and cellular levels. These protections are manifested due to the antioxidant as well as direct ROS scavenging properties of Se and Zn. In some ways, protection due to Zn and Se against toxic metals is indistinct; however, there is some dissimilarity as well. Se exerts protection in more diversified ways than Zn because of the multiple Se species. On the other hand, Zn provokes the formation of metallothionein (MT) along with the antioxidant properties thus reduces the metal induced oxidative stress and lowers the metal availability through binding with MT by the target organs/cells. Moreover, the role of Zn in the Zn transporters and other metal transporter such as DCT1 may also play important role in the uptake and bioavailability of the metal. On the other hand, Se especially in the form of selenite, selenomethionine (SeMet) and to some extent seleocystine2 (SeCys2) is the mostly studied species of Se against metal toxicity especially against As and Hg. Se can form metabolic complexes to detoxify As and Hg as well as can boost up the antioxidant defense system either enzymatic or non-enzymatic. However, the specific effective doses of both of the essential trace metals (Zn and Se) are still to be decided to some extent especially for human health implications. Although both metals show promising potentials against toxicity; however, still many questions to be answered before considering them as clinically ideal detoxifiers against metals. For instance, the Zn-Hg relationship warrants extensive investigations in both animal and cellular levels. More focus should be given on the selection of doses to maintain the metabolic and cellular homeostasis of the essential trace metals such as Zn.

Moreover, in the environment it is difficult to find a contaminant discretely; rather a mixture of several compounds is used to stay heterogeneously. And sometimes, one compound is produced as a byproduct of the production process of other one such as Cd is produced in Zn smelting process. It is also reported that the Zn status is very crucial for organisms while exposing in a Cd polluted environment. Therefore, in the chapter 2, cytotoxic effects of Zn and Cd was reported as combined exposure to PC12 cells. Where, it is found that Zn (500 μM) at high concentration showed synergistic toxicity with Cd. However, lower concentration of Zn protected PC12 and HepG2 cells

from the Cd-induced cell death and membrane damage. However, in PC12 cells the protection due to Zn administration mainly depended on lowering the Cd-induced oxidative stress, DNA damage and finally the apoptotic cell death. Where, Cd-induced intrinsic apoptosis was reported in PC12 cells through increasing of Bax/Bcl2 ratio, the upregulation of cytochrome c into the cytosol and finally upregulation of caspases 3 and 9. Zn-induced significant positive alterations regulated the Cd-induced negatively regulated cytotoxic parameters. From this study, it can be suggested that the effective inhibition of Cd^{2+}-induced apoptosis in PC12 cells by Zn^{2+} might be due to suppression of mitochondrial apoptosis pathway and inhibition of Cd^{2+}-induced production of ROS. These findings particularly could be useful for the alleviation of Cd-toxicity and development of Zn-based therapeutics where Cd-exposure is in vulnerable condition.

Then, in the chapter 3 an antagonistic relationship of Se and As was reported using PC12 cells. Both Se and As are metalloids with peculiar properties, and roles in the biological system. For instance, As is well known toxicant responsible for human diseases where as Se is an essential trace element with significant chemopreventive effects, anticancer potentials and antioxidant properties. In this study, a combined exposure of both As and Se was performed, and found that Se in co-exposure with As improves cell viability, and reduces oxidative stress induced by As in the form of lipid peroxidation and GSH oxidation. Consequently, DNA fragmentation due to As exposure was also reduced by co-exposure (As and Se) in PC12 cells. Furthermore, protein analysis study using the western blot method revealed that co-exposure (As and Se) significantly inhibited autophagy which may further suppressed apoptosis through positively regulation of key proteins; p-mTOR, p-Akt, p-Foxo1A, p62, and expression of ubiquitin, Bax, Bcl2, NFκB, and caspase 3, although those are negatively regulated by As (Fig. 5.1).

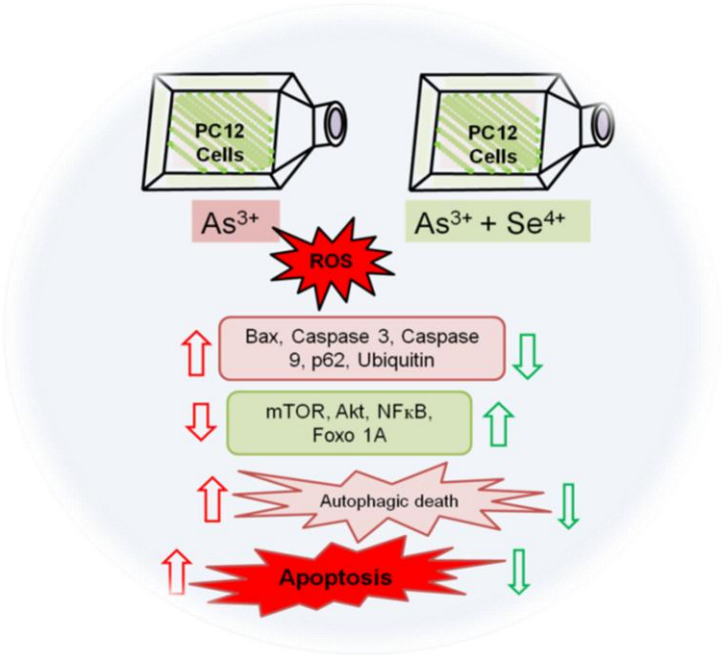

Fig. 5.1 Biomolecular mechanism of cytoprotection by Se against As-induced toxicity in PC12 cells

Moreover, reverse transcriptase polymeric chain reaction (RT-PCR) analysis confirmed the involvement of caspase cascade in cell death process induced by As and subsequent inhibition by co-exposure. The cellular accumulation study of As in presence/absence of Se using inductively coupled plasma mass spectrometry (ICP-MS) confirmed that Se effectively retarded the uptake/accumulation of As in PC12 cells. Thus, it can be concluded that Se is capable to modulate As-induced intrinsic apoptosis pathway *via* enhancement of mTOR/Akt autophagy signaling pathway through employing antioxidant potentials and through inhibiting the cellular uptake/accumulation of As in PC12 cells.

Furthermore, in chapter 4, the findings of the study in which two dietary supplements were applied simultaneously with As, Cd, Pb and Hg using PC12 and Caco-2 cells were reported. ALA or its reduced form DHLA was exposed with the aforementioned toxic metals. It was found the both supplements, ALA (250 μM) and DHLA (50 μM) effective reduce the cell death induced by each of the toxic metals in PC12 cells. And subsequent cell membrane damage was also significantly reduced by both of the supplements. However, in simultaneous exposure the ALA did not show any protective role against Hg in terms of cell viability. Both of the supplements boost up the

non-enzymatic antioxidant defense by increasing GSH levels as well as Nrf2 protein which was lowed due to toxic metal exposures in Caco-2 and PC12 cells. Furthermore, toxic metals induced DNA damage was reduced by DHLA in PC12 cells. However, in PC12 and Caco-2 cells, ALA modulates the expression of mTOR and Akt which are deregulated by As and Cd exposure, and in Caco-2 cells As and Cd-induced cleaved PARP was reduced by ALA exposure. Thus, ALA and DHLA have potential to counteract toxic metal toxicities. However, DHLA is more active against toxic metals than ALA. However, further investigation is warranted to depict the molecular mechanism behind the cytoprotection of ALA and DHLA against As, Cd and Pb.

Therefore, it can be concluded that nutrients (Zn and Se) particularly capable to ameliorate metal toxicity (Cd and As) in PC12 cells as well as in other mammalian cells. Furthermore, dietary supplements ALA and DHLA could also have cytoprotective effects against toxic metals. Thus, these dietary trace metals and supplements could be the potential target therapeutics for the deleterious effects of toxic metals in human body. However, animal level study is recommended further to investigate the similar potentiality of Zn, Se, and/or ALA/DHLA to propose as therapeutic model against acute and chronic exposure of toxic metals in human.

5.2 Future research: Investigation for a novel combination of dietary compounds for anticancer therapeutics

5.2.1Background

Scientists all over the world have been desperately searching for ideal anticancer therapy. Although there are several anticancer drugs such as cisplatin, 5-flurouracil (5-FU); however, their effectiveness and success rate is not out of the questions. For instance, in the treatment of colorectal cancer a multimodal therapy is required including surgical resection followed by chemotherapy using 5-FU in combination with oxaliplatin (a platinum based agent) or radiation therapy (Cunningham et al., 2010). However, the survival rate after 5-years of the treatments is accounts for only 10% (Kopetz et al., 2009). Therefore, it is highly required to find new ideas, combinations of therapies, concept to fight back against drug resistance of different cancers. The possible ways could be to stop the cancer cell metabolisms, and/or interference with the redox state of cancer cells (Hanahan and Weinberg, 2011).

ALA and it's reduced form DHLA have potentials to reduced oxidative stress by directly scavenging the ROS or by reducing their formation due to chelating with metal ions such as Fe^{2+}, and it can also enhance the GSH levels in the different cells (Rochette et al., 2013). Beside that ALA can also reduced the growth of several tumor cells such as H460 (human lung cancer) cells by producing ROS, HepG2 (human hepatoma) cells by inducing apoptotic cell death, and CRC (colorectal cancer) cells (Moungjaroen et al., 2006; Simbula et al., 2007; Dorsam et al., 2015). However, they all required quite a high level of doses to attain IC_{50} value which might be toxic for the normal cells. On the other hand, Se also showed some anticancer properties in different cell lines. For example, we reported Se-induced significant cell death in PC12 cells at a concentration of 10 μM. Any potential drug should be specific for its target action as well as should be less toxic for the applying environment to avoid side effects which is a major challenge. Therefore, we hypothesized that 'non-lethal dose of two compounds (Se and ALA/DHLA) might produce some lethality in the cancer cells but not in the normal cells'.

5.2.2 Objectives

- To examine the cytotoxic effects of ALA/DHLA and Se separately using PC12 cells, Caco-2 cells, and MCF-7 cells, and measure the IC_{50} value for each compound and cell line.
- Make a binary combination of Se and ALA or Se and DHLA to investigate the combined cytotoxic effects in above mentioned cancer cell lines.
- To elucidate the possible anticancer mechanism/s for the combination using cancer cell lines.

5.2.3 Methodology

After culturing the cells for 24 h, ALA, DHLA and Se will be added to the medium then let it incubated for another 24-48 h then harvest the cells and measure the subsequent cellular parameters using suitable methods (Fig. 5.2). The following figure is presenting the outline of the methods.

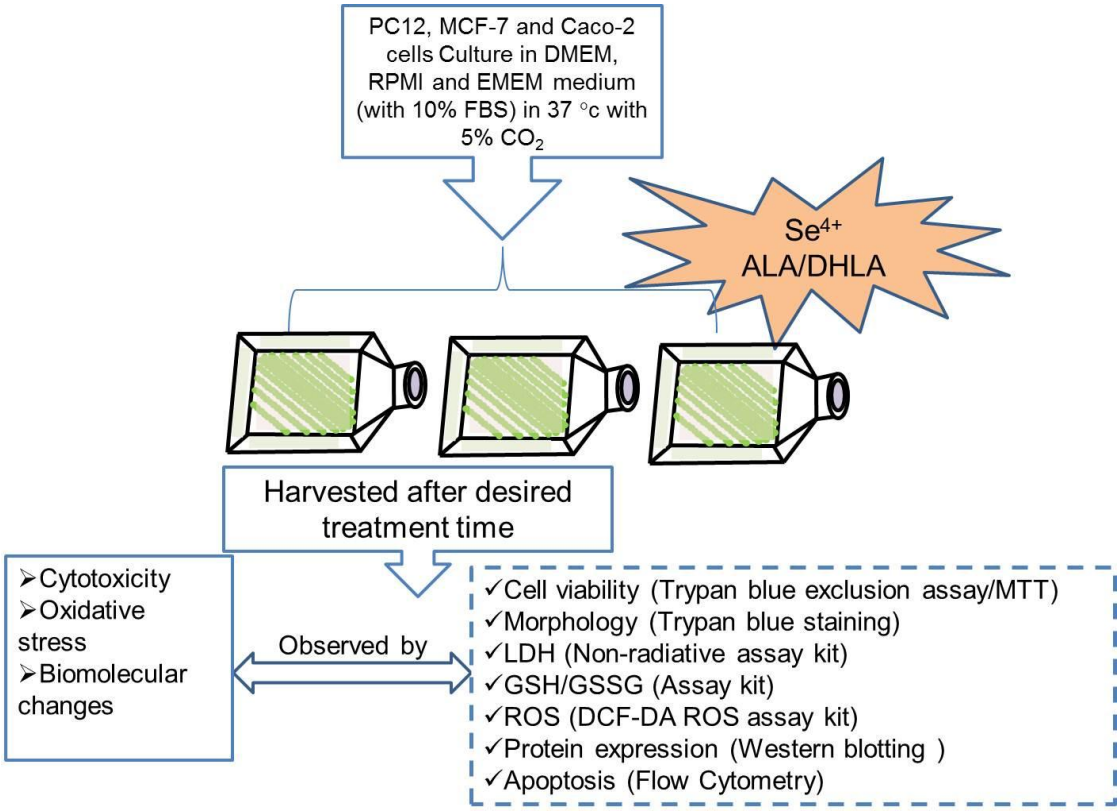

Fig. 5.2 Research outlines depicting methodologies to be followed.

5.2.4 Preliminary findings

5.2.4.1 Cell viability

To investigate the cytotoxic effects of ALA in PC12, Caco-2 and MCF-7 cells 250 to 1000 μM of ALA was added to the cell culture medium as final concentration, and incubated for 48 h then checked the cell viability using trypan blue exclusion method. The results are presented in Fig. 5.3 (Taken from chapter 4). From the results it can be stated that at ALA concentration 250(μM) either of the studied cells did not show any significant cell death. However, at 500 μM or more of ALA, Caco-2 and MCF-7 cell showed significant death compared to the control group. Moreover, the extent of cell death also significantly varied between Caco-2 and MCF-7 at 750 μM of ALA. It means that different cells showed distinct sensitivity toward ALA. In previous chapter, it was reported that lower than 10 μM of Se did not show significant cell death in PC12 cells. However, in Fig. 5.4 it can be observed that Caco-2 cells showed significant cell death at just more than 5 μM of Se and the IC_{50} value of Se for Caco-2 cells was calculated as 6.85 (μM) for 48 h of exposure.

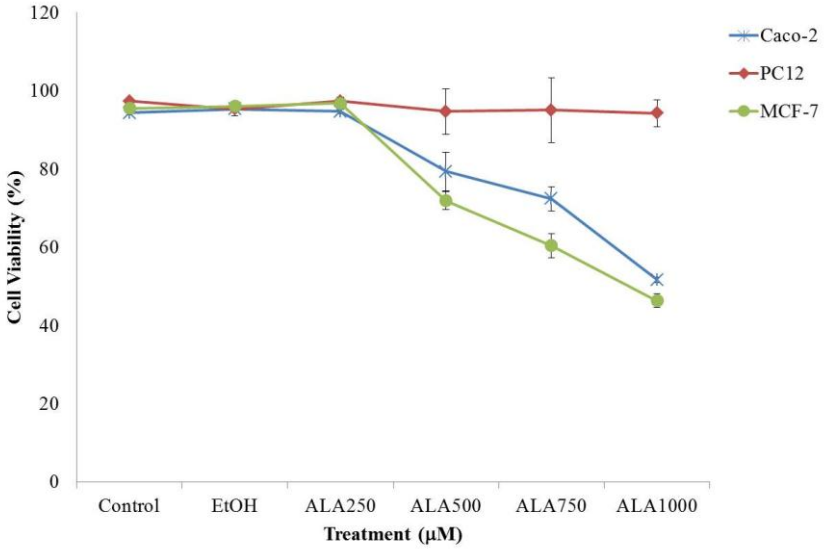

Fig. 5.3 Cell viability of different cell lines after treatment with ALA for 48 h measured using trypan blue exclusion method. Error bars indicate the mean ± SEM (n=4).

Considering the cell viability results of Fig. 5.3 and Fig. 5.4 a combined treatment was designed following the non-toxic dose of ALA and Se for PC12, Caco-2 and MCF-7 cells. Results are presented in subsequent Figs. 5.5-5.7, respectively.

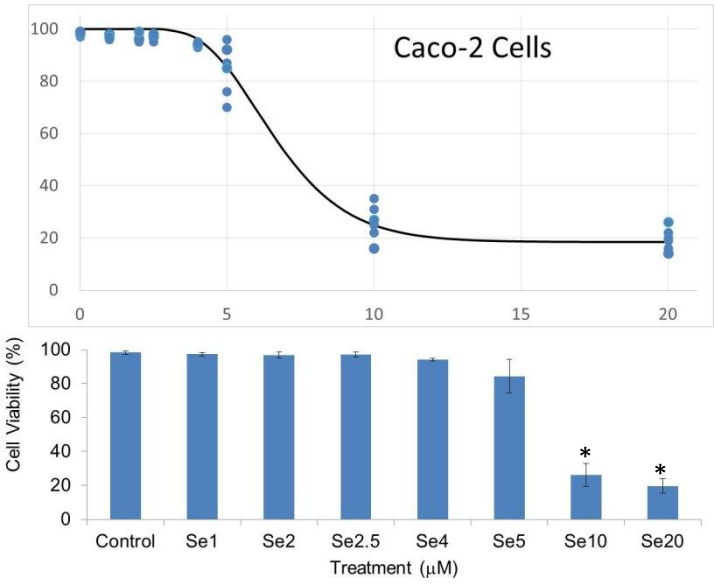

Fig. 5.4 Cell viability of Caco-2 cells against Se exposures for 48 h measured by trypan blue exclusion method. Error bars indicate mean ± SEM (n=6). Asterisk * indicates significant at $p<0.05$ compared to the control. Upper graph prepared by probit analysis to calculate IC50 value.

From Fig. 5.5 it is depicted that non-toxic dose of Se (2.5 and 5 μM) along with non-toxic dose of ALA (125 and 250 μM) showed remarkable cell death in PC12 cells. These combinations could have markedly promising meaning in terms of cytotoxicity induction in PC12 cells. The other cytotoxic parameters were also showed similar trends such as LDH leakage (data not shown). Further experiments will be conducted to explain the phenomenon behind this result.

Similarly, in case of Caco-2 cells the non-toxic dose of ALA and Se produced significant cell death after 48 h of incubation (Fig. 5.6). From preliminary results it can be assumed that a particular set of events could be responsible for these phenomena with the presence of both chemicals because they act as prooxidant rather than antioxidant. This event will be checked soon using suitable methods such a measuring the generation of ROS. Quite similar results also obtained by cell viability measurement in MCF-7 cells (Fig. 5.7). Where, 2.5 μM of Se with 125 and 250 μM of ALA induced significant cell deaths compared to their individual exposures.

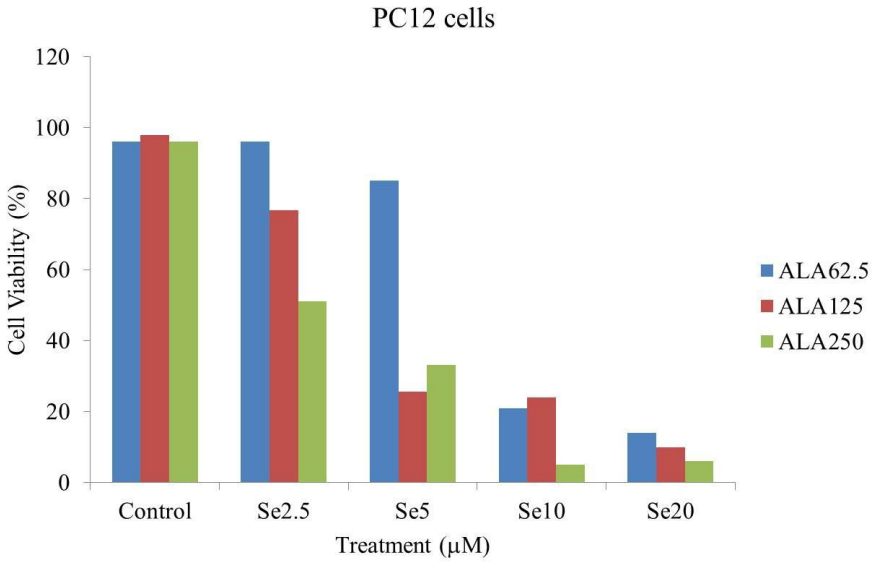

Fig. 5.5 Cell death analysis after treatment with ALA and Se in PC12 cells for 48 h using trypan blue exclusion method. This is the average of 2 independent experiments.

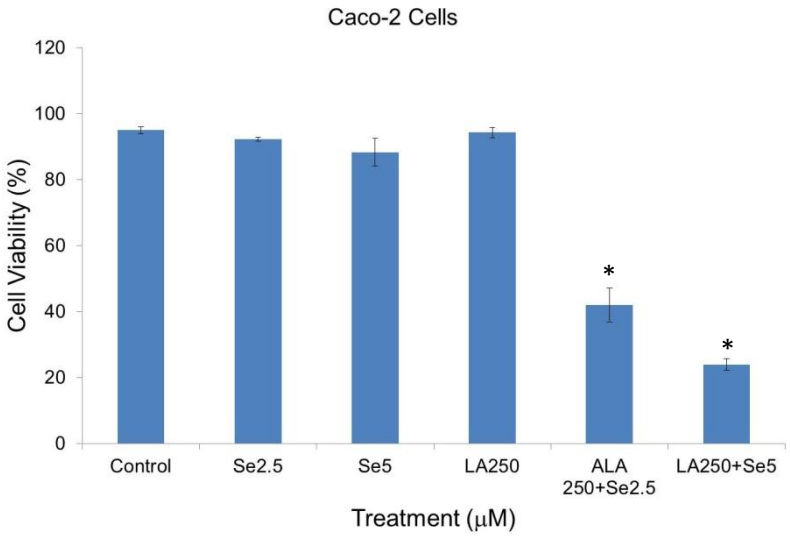

Fig. 5.6 Cell viability of Caco-2 cells after combined treatment with LA or ALA and Se for 48 h measured by trypan blue staining method. Error bars indicate mean ± SEM (n=4). Asterisk * indicates significant at $p<0.05$ compared to Se and ALA alone.

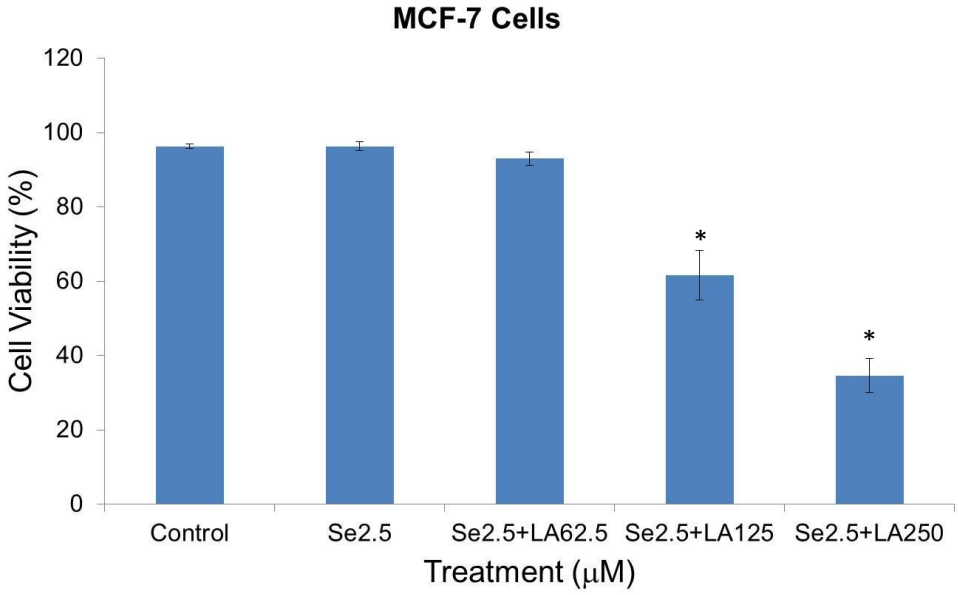

Fig. 5.7 MCF-7 cell viability after exposure to combination of LA or ALA and Se for 48 h using trypan blue exclusion method. Error bars indicate mean ± SEM (n=5). Asterisk * indicates significant at $p<0.05$ compared to the control/Se treated group alone.

5.2.5 Next experiments

Based on the cell viability results some further experiments have now been going on such as measuring the LDH activity to check membrane, and oxidative stress marker are now ready to check such as GSH, GSSG and ROS. Flow Cytometry will be carried out to identify the cell death type. Finally protein and gene expression of some target proteins will de conducted using western blot and RT-PCR methods.

To do so it can be assumed that about 4 to 6 months will be required to get a presentable outcome. This study is expecting outcomes to be published in high quality international journal. With the successful completion of the work it can be a novel combination to treat multiple cancers with minimizing the side effects and overcoming the resistance barrier by cancer cells.

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