INTEGRATED IN VITRO - OMICS BASED APPROACH TO ASSESS THE HEAVY METALS AND THEIR BINARY MIXTURES TOXICITY IN HIPPOCAMPAL HT-22 CELL LINE

Venkatanaidu Karri

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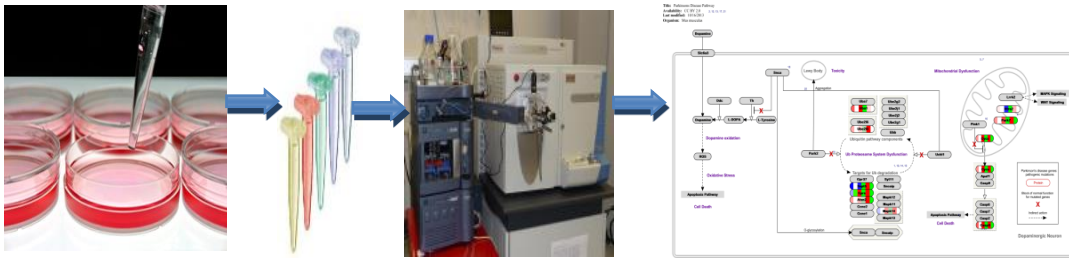

DOCTORAL THESIS
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DOCTORAL THESIS

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UNIVERSITAT ROVIRA I VIRGILI

Tarragona, 2018

FAIG CONSTAR que aquest treball, titulat "**Integrated In vitro - Omics based Approach to assess the Heavy metals and their Binary mixtures toxicity in Hippocampal HT-22 cell line**", que presenta **Venkatanaidu Karri** per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament d'Enginyeria Química d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "**Integrated In vitro - Omics based Approach to assess the Heavy metals and their Binary mixtures toxicity in Hippocampal HT-22 cell line**" que presenta **Venkatanaidu Karri** para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Ingeniería Química de esta universidad.

I STATE that the present study, entitled **"Integrated In vitro - Omics based Approach to assess the Heavy metals and their Binary mixtures toxicity in Hippocampal HT-22 cell line"**, presented by **Venkatanaidu Karri** for the award of the degree of Doctor, has been carried out under my supervision at the Department of Chemical Engineering of this university.

Tarragona, 2nd of Feburary, 2018

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I DEDICATE THIS THESIS TO MY MOTHER LATE THAUDAMMA

Abbreviations

AD= Alzheimer's disease

APP= Amyloid precursor protein

As= arsenic

ATSDR= Agency for Toxic Substances and Disease Registry

BBB= blood brain barrier

Cd= cadmium

CDC= centre for disease control

CNS= central nervous system

ETC= Electron transport chain

LTP= long term potentiation

MeHg= methylmercury

NMDA= N-methyl D-aspartate

Pb= lead

PD= Parkinson's disease

UPS= Ubiquitin proteasome System

WHO= World Health Organization

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Summary

Many environmental pollutants have been associated with human diseases. In recent years, exposures to hazardous metals, such as Pb, Cd, As, and MeHg are shown significant toxicological effects in brain. Our current knowledge of heavy metals (Pb, Cd, As, and MeHg) induced adverse health effects is mainly limited to the individual level. The scientific community increasingly recognizes the adverse effects associated with metal mixtures are a serious concern. One of the major challenges for metals toxicology is to find how mixtures exert their effects on living organisms, due to mixtures toxicity studies on hippocampus is very limited. The regulatory frameworks such as REACH in the EU are becoming more critical regarding the use of animal testing. Therefore, rational approaches are needed to assess the potential toxicity of mixtures by using the new tools, which should incorporate alternative animal experimental tools. Recent advancement in *in vitro*, and omics techniques may allow an accurate understanding of metal mixtures toxicity mechanism.

The advancement and development of *in vitro* and omics methods make them powerful tools for evaluation of single metals, and their binary mixtures exposure related early cellular changes and also the mode of toxicity on hippocampal cell lines. The main goal of this thesis was taking advantage of in vitro, label free proteomic technologies and system biology tools for assessing comparative toxicity signature profiling of heavy metals (Pb, Cd, As, and MeHg) and their binary mixtures on the mice HT-22 hippocampal cell line. Thus, hippocampus relevant in vitro toxicity studies are could be more rational for understanding the potency of metal.

Firstly, we assumed that the mode of actions of individual metal toxicity can be used as a baseline for understanding potential mechanisms associated with mixture (Pb, Cd, As, and MeHg) in hippocampus region. We showed that single metals cytotoxic effects and potency in different exposure conditions, in a Pb<Cd<As<MeHg as well as in genotoxicity and apoptosis in mice HT-22 hippocampal cells. We have also shown that metal mixtures interaction in by utilizing the dose addition, response addition models in Chapter 3. These findings give significant evidence of the metal mixtures neurotoxic activity and their potential interactions depend on the composition of elements, cell line sensitivity.

However these studies have drawback to understand the underlying mechanism. Recent proteomic research has provided evidence that toxic chemical induce the expression of characteristic stress-related proteins in cells/organ. This opens the possibility of using these protein expression profiles (signatures) for the detection of heavy metals and their mixtures toxicity profile in HT-22 cells. The purpose of the chapter 4, 5 is to investigate the underlying mechanisms of the heavy metals toxicity by using a proteomic and system biology tools.

Summary

This enables to gain a global understanding of the way in which metal single (Pb, As, MeHg) and their mixtures response in the HT-22 cells related to neurodegenerative disease. The outcomes indicates that the single metals and their mixtures impact in critical molecular pathways such as ubiquitin proteasome system (UPS), mRNA splicing, oxidative stress and mitochondrial dysfunction and that these alterations also have a relation on the neurodegenerative diseases (Alzhmer's disease, Parkinson's disease). The multi-faceted comparison of the cellular responses to Pb+MeHg, Pb+As, and MeHg+As allowed us to get better insights into the responses of the interaction profile in protein level. In our results, Pb+MeHg induce detectable perturbation of the cellular functions than other two mixtures Pb+As, MeHg+As. We confirm that by analyzing complete cellular proteome and neurodegeneration related pathways, all of the evidence suggests that disruption occur by metal mixtures exposure is more hazardous than single metal exposure in HT-22 cells.

The present study therefore provides an efficient and practical proteomics approach for generating scientific data to characterize and predict metal mixture toxicity and to support risk assessment of environmental mixtures. In future, refinement is needed in this research to validate quantitative risk assessment and to support the assumptions; these may include testing for some specific proteins dose–response curves, determining whether interaction assumptions are applicable or not for describing mixture risk.

Introduction

Over the last three decades there has been increasing global concern over the impacts on public health attributed to environmental pollution, in particular, the global burden of disease (Falco et al., 2006). The World Health Organization (WHO) estimates that about one quarter of the diseases occur due to prolonged exposure by environmental chemicals (Nadal et al., 2004; Esteban-Vasallo et al., 2012; Alves et al., 2014; Mari et al., 2014;; Rovira et al., 2015; Vilavert et al., 2015). Approximately five million well known chemicals are present in the environment, only 80,000 are used in today (CDC, 2015). Most of these chemical exposure related diseases are not easily detected and may be acquired during childhood and manifested later in adulthood (Martorell et al., 2011). In recent years, there has been an increasing ecological and global public health concern associated with environmental contamination by heavy metals (Morais and Garcia, 2010). Also, human exposure have been risen dramatically as a result of an exponential increase of their use in several industrial, agricultural, domestic, and technological applications (Cui et al., 2005). Heavy metals such as lead (Pb), cadmium (Cd), arsenic (As), and methylmercury (MeHg) are among the top 10 hazardous agents in the substance priority list of 2013, prepared by the United States Environmental Protection Agency (EPA) and the Agency for Toxic Substances and Disease Registry (ATSDR) (ATSDR 2012). In natural environments, heavy metals occur at low concentrations (Rovira et al. 2015; Vilavert et al. 2015). Heavy metals are released into the environment from metal smelting and refining industries, scrap metal, plastic and rubber industries, various consumer products, and from burning of waste containing these elements (Eqani et al., 2016; Ha et al., 2017; Li et al., 2017; Oken et al., 2016; Tang et al., 2017; Xu et al., 2016). In environment, these metals are not degraded and persist for many years, poisoning humans through inhalation, ingestion, and skin absorption (Cobbina et al., 2015). The issues concerned with heavy metals pollution is continuously reviewed by federal agencies such as the EPA, ATSDR and the World Health Organization (WHO) (WHO, 2010). Although, considerable progress has been made with regard to their monitoring and regulation, leading to the decrease in the release of these toxic metals, their concentrations still exceeds the allowable limits in many regions of the world (Yu et al., 2011). Such an increase in environmental concentration poses an alarming concern towards their negative impact on human health (Caserta et al., 2013). In general, heavy metals (Pb, Cd, As, and MeHg) affect most of the organs in human body (Goyer and Clarkson, 2001), but the nervous system was found to be the most sensitive target (Angelica and Fong, 2014). These metals are prevalent in the environment and have evidence of neurotoxicity (Gundacker and Hengstschläger, 2012). The common neurodevelopmental toxic effects caused by these heavy metals include altered neurobehavioral development, neurocognitive deficits, mental retardation, muscular weakness, poor coordination of motor and sensory responses (Wright and Baccarelli, 2007). It is well accepted that humans are routinely exposed to multiple metals simultaneously or sequentially (Hart et al. 1989; Luo et al. 2009; Viaene et al. 2000). There is evidence that the toxicity of individual metals may

depend on the presence of other metals; so that, effect of mixtures on human health is a currently an active area of research (Farina et al., 2013; Montgomery, 1995). These combined metals exposure can increases the diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) in population (Basha et al., 2005; Cholanians et al., 2016; Kasner et al., 2013; Prakash et al., 2015; Tolins et al., 2014; Vahidnia et al., 2008; Weiss et al., 2002). Lot of research has been done in the past to study their various toxic effects, mechanisms and factors involved. Some of the basic information is discussed in the following sections.

Human health and environment

Environment plays a major role in human health and well-being (He et al., 2005). Human exposure to contaminated environment causes illness, and that condition is known as environmental diseases (Järup, 2003). Environmental exposures to chemical, physical and biological agents may contribute to diseases in susceptible individuals along with personal factors like diet, smoking, etc. (Ferré-Huguet et al., 2008). The environmental factors contribute to 25-33% of the global burden of diseases and 80% of all cancers (Knol et al., 2009). Among the various environmental factors, exposure to heavy metals in occupational, indoor and outdoor environment is increasingly recognised as an important contributor to human diseases (Risher and Dewoskin, 1999; Caserta et al., 2013; Yu et al., 2011).

1. Heavy metals (Pb, Cd, As, and MeHg) exposure

An improper disposal of chemicals into the environment can contaminate water, land, or air, and impact the environment and human health (Wang and Du, 2013). Many hazardous waste sites and industrial facilities have been contaminated for decades and continue to affect the environment (Bilos et al., 2001). Exposure is defined as a contact over time and space between a person and one or more biological, chemical or physical agents. Among various pollutants, toxic heavy metals contribute to a great proportion of soil and water pollution and cause major problems to human beings (Anawar et al., 2002; Ng et al., 2003; Wasserman et al., 2004). Their multiple industrial, domestic, agricultural, medical, and technological applications have led to their wide distribution in the environment, raising concerns over their potential effects on human health and the environment (Mauriello et al., 2017; Persoons et al., 2014). The most common routes of metal exposure are dermal, inhalation, and ingestion routes from both occupational and environmental exposures (Chowdhury et al., 2016; Tóth et al., 2016). Their toxicity depends on several factors including the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of exposed individuals (Bolan et al., 2017; Deshommes et al., 2016; Khan et al., 2013; Le Bot et al., 2016; Nadal et al., 2016). Heavy metals are considered systemic toxicants that are known to induce multiple organ damages, even at lower levels of exposure (Clarkson and Magos 2006; Fujimura et al., 2009).

1.2. Lead (Pb)

Pb is an environmentally abundant heavy metal, and its toxic effects create health impacts among adults and children (Cecil et al., 2008). Ingestion of Pb contaminated food, water, and paints and inhalation are the major routes of exposure (Li et al., 2013; Chaumont et al., 2012; Flora et al., 2012; Järup, 2003; Khoury and Diamond, 2003). The other sources are household paints, occupational exposure from workplaces, Pb used in toys, batteries, and cosmetics (Guilarte and Miceli, 1992). Inhalation exposure to Pb is a much more efficient route of absorption than ingestion (Baranowska-Bosiacka et al., 2012). Pb is known to accumulate in bones, marrow, kidneys, and liver and brain tissues (Shukla and Chandra, 1987). Pb exposure causes toxicity to various organs, including kidney, liver, nervous system, hematopoietic, endocrine, and reproductive systems (Aschner et al., 2007; Maynard et al., 2005; Sanders et al., 2009). Pb has long been recognized as a developmental neurotoxicant that can interfere with the developing brain, resulting in functional impairment. The nervous system is a major concern; Pb causes encephalopathy particularly in children (Aschner et al., 2007; Maynard et al., 2005; Sanders et al., 2009). The exposure to Pb creates adverse effects on intellectual and neuromotor performance, especially in children and adolescents(Jusko et al., 2008). Pb exposures in the workplace are responsible for a wide range of health impacts (Wright and Baccarelli, 2007).

1.2. Cadmium (Cd)

The main routes of exposure to Cd are via inhalation of cigarette smoke, and ingestion of food (Järup et al., 1998; Perceval et al., 2002). The other sources are occupational exposure to metal industries, workplaces and contaminated food (Nordberg, 2009). Among these sources, smoking is the major contributor and in the non-smoking population ingestion of food is the major sources (Bilos et al., 2001). Cd exposure reduces the pulmonary function and causes emphysema, osteoporosis, and kidney damage (Shukla and Chandra, 1987). Chronic occupational exposure of Cd leads to slowing psychomotor functions of the brain (Viaene et al., 2000). In vivo experimental studies, reported that Cd disrupts the hippocampus region of the brain that has an effect on the memory region (Luo et al., 2009).

1.3. Arsenic (As)

As compounds are widespread mainly due to various man-made activities and contamination in water and soil (Ng et al., 2003; Wasserman et al., 2004). As exposure can occur through ingestion of contaminated drinking water (Abernathy et al., 2003Tseng et al., 2005; Wang et al., 2007), and via inhalation, which is particularly important for certain occupational exposure (Jadhav et al., 2015; Mandal, 2017; Singh et al., 2015). As is rapidly absorbed, distributed and stored in the body organs such as liver, kidney, heart, and lungs (Kruger et al. 2009). This metal easily crosses the blood brain barrier (BBB) and is accumulated in the brain (Rodríguez et al., 2003). Chronic inorganic As exposure has been associated with increased incidence of vascular and

cardiovascular disease, cancers of the skin, lung, and neurological effects (Luo et al. 2009). Various epidemiological reports have shown that As metal exposure can alter cognitive function, particularly learning and memory during childhood (Luo et al., 2009; Rodriguez et al., 2002; Tyler and Allan, 2014).

1.4. Methylmercury (MeHg)

Mercury is perhaps the most frequently encountered heavy metal in the human because of its multiple routes of human exposure (Davidson et al., 2008; Murata et al., 1999). The common forms are inorganic mercury, ethylmercury and methylmercury. The most concerning form is MeHg (Aschner and Aschner, 1990). MeHg is produced by aquatic bacterial methylation of elemental or inorganic mercury (Tchounwou et al., 2003). The MeHg subsequently accumulates in fish and shellfish throughout the food web (Clarkson and Magos 2006; Fujimura et al., 2009). Humans can be exposed to MeHg through consumption of fish and other seafood (Crespo-López et al., 2009). MeHg accumulates in kidneys, neurological tissue, and liver; exhibits toxic effects, including neurotoxicity, nephrotoxicity (Angelica and Fong, 2014). It is well known that ingested MeHg can interact with proteins and enzymes due to its strong affinity for sulphur, causing organ dysfunction and a devastating effect on the whole central nervous system, particularly the developing brain (Ceccatelli et al., 2010; Johansson et al., 2007; Yee and Choi, 1996). The MeHg affects many different areas of the brain and their associated function (Debes et al., 2006). Exposure of MeHg to fetal brain is more susceptible to mercury-induced damage than the adult brain (Clarkson et al., 2003). In vitro studies in animals have shown that MeHg disrupt the biochemical processes believed to be involved in Alzheimer's disease (Leong et al., 2001) and axonal degeneration to MeHg (Castoldi et al., 2003).

1.5. Heavy metals (Pb, Cd, As, and MeHg) neurotoxicity

Exposure to these metals is associated with a variety of adverse health effects (Farina et al., 2013; Montgomery, 1995). Acute /chronic exposures to Pb, Cd, As and MeHg are known to cause neurological damage (Wright and Baccarelli, 2007; Stackelberg, 2015; Wang and Du, 2013). A common susceptibility factor for Pb, Cd, As and MeHg comprises the cognitive deficit but the risk level depends on exposure intensity (Gavazzo et al., 2008; Ceccatelli et al., 2010). Basha et al. (2005) observed that developmental exposure to Pb exhibits latent effects in brain. Cd produces neurological alterations including memory loss and mental illness (Wang and Du, 2013), that chiefly influences the repressing of neurochemical glycine and ¥-aminobutyric acid (GABA) into the extracellular space, which upset the balance between excitation–inhibition in synaptic neurotransmission (Minami et al., 2001). As exposure induces the cognitive disability in human (Tyler and Allan, 2014) by increasing the β-amyloid protein deposition and hyperphosphorylation of tau protein in brain (Giasson et al., 2002). Previous studies reported that the developmental exposure to MeHg have long-term consequences in the brain (Johansson et al., 2007), and also alter the homeostasis in

brain (Atchison and Hare, 1994) resulting in oxidative stress (LeBel et al., 1990) and cognitive disability.

1.5.1. Pb neurotoxicity

Environmental exposure to Pb is a widespread (Ruff et al., 1996), it's developmental effects are thoroughly documented and exposure of children to high levels of Pb has been greatly reduced (Neal et al., 2011). However, even low and moderate concentrations are now being recognized as capable of producing subtle neurological deficits in children, and perhaps contributing as a risk factor for later development of neurological disease (Guilarte et al., 2000). Chronic, low level exposure is considered a risk factor for Alzheimer's disease (Guilarte and Miceli, 1992). In brain, Pb mainly disrupts the hippocampus region by interacting with different receptors results cognitive dysfunction (Zhang et al., 2002). The brain damage induced by Pb depends on age (Kim et al., 2005) and level of exposure (Bradbury and Deane, 1993). Generally children absorbs significantly more Pb than adults (Goyer, 1996) due to an underdeveloped BBB (Ruff et al., 1996). There is a very little proof that Pb will harm the functions of the blood-brain barrier at a lower dose of <80 µg/dl (Bradbury and Deane, 1993)**.** The centre for disease control (CDC) limits the childhood Pb^{+2} intoxication to 10 µg/dL in blood (Landrigan, 2000). This level is thought to be the threshold for potential adverse effect of childhood cognitive deficits (Jusko et al., 2008). The exposure of Pb during early postnatal life produces a greater deficit in learning performance than in older animals (Kuhlmann et al., 1997).

1.5.2. Cd neurotoxicity

Cd plays a critical role in neurobiology (Afifi and Embaby, 2016); a growing number of clinical investigations have pointed to Cd intoxication as a possible etiological factor of neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD) (Ishitobi et al., 2007). The neurotoxic effects of Cd were associated with both biochemical changes of the cell and functional changes of CNS(Rahman et al., 2017), suggesting that neurotoxic effects may play a role in the systemic toxic effects of the exposure to Cd, particularly the long-term exposure (Andersson et al., 1997). Cd causes a wide variety of toxic effects on the central nervous system (CNS) (Pari and Murugavel, 2007). Kumar et al., (1996) reported that Cd affects the brain by disruption of specific membrane function, principally in the hippocampus region. Higher neurotoxicity is reported in new-born than an adult, and this variation may be due to lack of BBB maturation in new-born (Wong and Klaassen, 1982). In occupational and epidemiological studies with workers exposed to Cd evidence of memory loss was found (Hart et al., 1989). Chronic occupational exposure to Cd leads to slowing psychomotor functions of the brain (Viaene et al., 2000). In vivo study of Cd reported, the disruption of hippocampus region in the brain resulting long term potentiation (LTP) function blocked (Luo et al., 2009). In-vivo experimental studies of pups, it has been shown that the uterus exposure to Cd could inhibit the acetylcholine esterase (AchE),

Na^+/ K^+ - ATPase pump which reduces the neuronal activity in pups (Alka, 1991). Cd also mimics the ubiquitous intracellular ion Ca^{+2}, which acts as a signaling mediator in numerous cellular processes including cell proliferation, and differentiation (Xu et al., 2011).

1.5.3. As neurotoxicity

The inorganic As is a potent neurotoxic induces hippocampal-dependent behavioral deficits in rodent models (Martinez-Finley et al., 2011). Bellinger et al. (2013) proved that higher concentrations of As alter growth and development in children resulting in neurological deficits. As metal induced defects in nervous system include alterations in peripheral nerves, encephalopathy, depression, impaired facial recognition, and mental retardation (Naujokas et al., 2013). The inorganic form of As targets the human nervous system producing the cognitive dysfunction (Luo et al., 2009; Rodriguez et al., 2002; Tyler and Allan, 2014). As exposure could produce behavioral changes through a direct effect on the developing brain since As freely crosses the mammalian placenta and BBB (Wright and Baccarelli, 2007). Embryonic exposure has been shown to produce neural tube defects (Molly Tolins et al., 2014). Multiple mechanisms in As neurotoxicity have been suggested in experimental studies (Kumar et al., 2013). Recently, reported that As has been linked with enhanced vulnerability to the incidences of neurodegeneration diseases in humans (Rodríguez et al., 2003). Gong and O'Bryant, (2010) found a relationship between As exposure and AD. Experimental animal models have disclosed potent alterations in hippocampal function with As exposure (Cronican et al., 2013). Studies in children have found evidence of poor performance scores after long-term exposure to As (Wasserman et al., 2004). In experimental animal models, it has been observed that As causes a potent alteration in hippocampal region and dysfunctional cognitive behavior (Cronican et al., 2013). Another in vivo study showed that As primarily impact the synaptic plasticity of neurons in the hippocampus region of the brain (Krüger et al., 2006). Reported ex-vivo cell culture study provided evidence of As capacity to increases β-amyloid protein, which induces the hyperphosphorylation of tau protein resulting in neurodegeneration (Giasson et al., 2002). These findings raised concern over neurotoxicity induced by arsenic in humans (Ahmed et al., 2011; Vahter, 2009).

1.5.4. MeHg neurotoxicity

MeHg has long been documented to damage the CNS in humans (Castoldi et al., 2003).There have been many studies investigating the manner(s) by which mercury compromises neuronal health and function (Aschner et al., 2007). Organic mercury (MeHg) affect many different areas of the brain and their associated function (Davidson et al., 2008; Murata et al., 1999). Exposure of MeHg to the fetal brain is more susceptible to mercury-induced damage than the adult brain (Clarkson et al., 2003). There exists strong evidence for mercury in the etiology of several neurodegenerative diseases. Many case studies and several epidemiological studies have identified elevated

blood levels of mercury in patients with AD and PD(Ceccatelli et al., 2010; Johansson et al., 2007; Yee and Choi, 1996). In vitro studies in animals have indicated that MeHg can affect the biochemical processes believed to be involved in AD (Leong et al., 2001). Animal studies showed that the exposure to MeHg has long-term consequences in the brain (Johansson et al., 2007) and also alter the homeostasis in the brain (Atchison and Hare, 1994) resulting in oxidative stress (Morais and Garcia, 2010), and cognitive disability. Johansson et al. (2007) ascertained that developmental exposure of MeHg has long-term consequences in the brain. Neurotoxicity expressions of MeHg are based on its interaction with cellular elements such as a neurotransmitter, disruption of microtubules, and alteration of intracellular Ca^{+2} ion homeostasis (Lebel et al. 1990). Biochemically, MeHg has high affinity to sulfur (-SH) containing molecules, mainly targeting cysteine and methionine-containing proteins (Suzuki et al., 1976). The MeHg mode of action in neuronal synapsis initiated by inhibiting astrocytic glutamate (Glu) uptake process and then stimulating its efflux from cytosol vesicles, resulting in higher Glu concentration in the extracellular fluid (Brookes and Kristt, 1989; Dave et al., 1994) results in excitotoxicity in neurons.

2. Chemical mixtures exposure

Humans are exposed to a large number of chemicals on a day to day life from different sources (ATSDR, 2004). In most cases, simultaneous exposure occurs to a mixture of chemicals (Gonza et al., 2013). The risk assessment of individual chemicals provides the quantitative estimate of its possible effects on human health (Laetz et al., 2009). The standard definition of a chemical mixture is any set of multiple chemicals, regardless of source that may or may not be identifiable that contribute to joint toxicity in a target population (Rice et al., 2008; Spurgeon et al., 2010). There is a huge data gap in the toxicology of chemical mixtures, and mixtures are considered as one of the toxicology's big problem (Sarigiannis and Hansen, 2012; Sharma et al., 2016). Since in most cases, the component of the mixtures has not been known and the risk associated with the chemical mixtures also not characterised properly (Feron and Groten, 2002; Kienzler et al., 2016). The most common contaminants present in the environment comprise both organic and inorganic chemicals, including pesticides, herbicides, dioxins, poly-aromatic hydrocarbons (PAHs), polychlorinated biphenyls, volatile organic compounds, and the inorganic contaminants, Pb, Cd, As. and MeHg (Feron et al., 2002; Safe, 1998). Among the various groups of environmental contaminants, heavy metals are frequently encountered in the environment (Adam et al., 2009; Chaperon and Sauvé, 2007; Stockdale et al., 2010).

The toxicity data of chemical mixtures and predicting the possible combined effects is necessary for risk assessment toxicology (Kortenkamp, 2014). Mixtures are classified into simple and complex mixtures based on the number of components (Rider and LeBlanc, 2005). Simple mixtures contain a small number of chemicals (up to 10) and the composition is known. Complex mixtures contain more than ten chemicals

(hundreds to tens of thousands), of which the composition (dose or constituents) is not known (ATSDR, 2004). Mixed contamination of chemicals is ubiquitous in the environment from various sources, mainly from industrial sources and they can be a combination of organic-organic or organic-inorganic or inorganic-inorganic chemicals (Backhaus et al., 2000; Faust et al., 2001). The focus of this section summarizes the recent literature examining chemical mixtures and health outcomes, with an emphasis on metal mixtures.

2.1. Challenges in chemical mixtures research

Chemical exposure to humans via food, consumer products, and the environment etc., can imply to an infinite number of different combinations of chemicals in mixtures (Angelica and Fong, 2014; Mari et al., 2014; Nadal et al., 2004) Chemical\ metal mixtures toxicity is an effectively infinite problem (Orton et al., 2014); it is an ongoing challenge to integrate this issue into regulatory regimes (Sarigiannis and Hansen, 2012). Testing of all kinds of mixtures of chemicals existing in the real world or of all possible combinations of chemicals of a simple mixture at different dose levels is virtually impossible (Cassee et al., 1998; Cobbina et al., 2015). Moreover, even if toxicity data on individual compounds are available (Clarkson 1987; Snyder et al. 2005), we are still facing the immense problem of extrapolation of findings obtained at relatively high exposure concentration in laboratory animals to human population (Cobbina et al., 2015). Although research on the health effects of metal mixtures spans several decades, a number of challenges have limited progress in this field (Kortenkamp et al., 2009). First, the imprecision inherent in exposure biomarkers, coupled with an uncertainty about which biomarker most accurately represents exposure for each chemical, limits our ability to evaluate chemical mixtures (ATSDR, 2004). On the other hand, prediction of mixtures effects is a great challenge (Teuschler, 2007). Studies on exposure of metal mixtures are critical since there is a lack of information on the toxicities and associated mechanisms (Tichý et al., 2002). Recommendations for study design and evaluation of combined effects of metal mixtures are not clear (Wu et al., 2016). The regulatory frameworks such as REACH in the EU are becoming more and more critical regarding the use of animal testing (Cedergreen, 2014).

The toxicity testing strategy of mixtures is depending on the available information about mixtures and its components in the mixtures (Adam et al., 2009). There are various risk assessment methods for evaluating combined exposures in practice, these methods are derived from the dose addition concept and effect addition concept (Scholze et al., 2014). The conceptual framework of the combined action of chemicals was first provided by Bliss (Bliss 1939). Similar joint action (dose addition, Loewe additivity) is defined as the chemicals in a mixture follow the same mechanism or mode of action and cause similar biological effects. With dissimilar joint action (response additivity, Bliss independence) the chemicals in the mixture act independently and follow the different mode of actions (Berenbaum, 1978). The dose addition and response addition concepts are commonly used to evaluate the mixtures toxicity (Axelrad et al., 2002; Chou et al., 2010). The dose additivity method is used for chemicals which have the same mode of

action (Wang et al., 2015). The chemicals in the mixture behave as a dilution of other chemicals. The mixture effect is assessed by the sum of the dose of the components. When the components in the mixture have a dissimilar mode of action with different target sites, the concept of response addition is applied to evaluate the mixture effects (Foucquier and Guedj, 2015).

3. Toxicity of metal mixtures

The possibility of simultaneous exposure to heavy metals mixtures is higher compared to other toxicants due to their ubiquitous presence in the environment (Feron and Groten, 2002; Frías-Espericueta et al., 2009; Jadhav et al., 2007a; Wu et al., 2016; X. Xu et al., 2011). This co-contaminant exposure has attracted significant attention due to their known neurotoxicity potential and systemic toxicity (Stackelberg, 2015). Some reported binary mixture data As, Cd, and Pb on various biological endpoints are inconsistent for same endpoints from study to study and are less relevant in terms of risk assessment (ATSDR, 2004). Moreover, prediction of mixture effects is a great challenge, because of their synergism or antagonism in a combination of two or more chemicals may occur and no currently available mathematical model can predict and solve this problem fully (Pape-Lindstrom and Lydy, 1997). In most of the studies, the effects of mixtures were assessed based on the significant difference compared to controls, however, the contribution of individual metals to mixture toxicity was not evaluated (Feron and Groten, 2002). Thus, the contribution or effect of individual chemicals in the mixture toxicity was not known in these studies. In the in vitro studies, the observed interaction between the mixtures of metal varies depending on the components of mixtures and the cell lines (Lin et al., 2016). The toxicity studies on the interaction between Pb, Cd, As, and MeHg is relatively limited. Previously reported studies have established toxicity of metal mixtures on various organs and their functions: the immune system (Jadhav et al., 2007a), mortality (Vellinger et al., 2012), neurotoxicity (Hu et al., 2013; Rai et al., 2013), bladder cancer (Feki-Tounsi et al., 2013) cytogenicity (Jadhav et al., 2006), induction of oxidative stress (Jadhav et al., 2007b), and interactions on essential elements (Cobbina et al., 2015). Mixtures of As, Cd, and Pb increased the oxidative stress production compared to those of individual metals in rats (Fowler et al., 2004, Wang and Fowler, 2008). In another set of studies, combined exposure of As with Cd or Pb caused a synergistic effect in the brain (Ashok et al., 2015) and nephrotoxic and hepatotoxic effects in the rat (Bhattacharjee et al., 2016). The metal mixtures containing Pb, Cd, As, and MeHg was toxic to brain, liver, and kidney of mice and the toxicity was associated with changes in the oxidative stress parameters, including a decrease of antioxidant enzyme activities and increased lipid peroxidation (Cobbina et al., 2015). However, the predicted mixture effects are not consistent across the endpoints; the lack of proper experimental strategy for evaluating metal mixtures is a huge problem so that always the question arises to decide which of the two concepts (dose additivity, response additivity) are most appropriate for the mixtures toxicity. There is an ongoing discussion regarding the most appropriate method

for the evaluation of mixtures interactions (Kortenkamp and Altenburger, 1998). The current reported studies results vary from synergism, additivity or antagonism at low to higher effect levels, respectively due to the lack of mechanistic interaction.

This simultaneous heavy metals exposure has attracted significant attention due to their known neurotoxicity potential and systemic toxicity. There are three main problems in the metal mixtures toxicity analysis:

1. The mixture toxicity of heavy metals (Pb, Cd, As, and MeHg) is not studied extensively. The available mechanistic data are limited. The reported interaction effects between heavy metals are inconsistent and inconclusive.

2. The combined toxicity of heavy metals on various biological endpoints is required for better understanding and risk assessment of mixtures. Given the complexity of interaction between these heavy metals and the involvement of the huge cost and ethical issues, it is difficult to use the animal models for toxicity evaluation of all possible combinations of heavy metals.

3. Moreover, even if toxicity data of individual metals are available, we are still facing the immense problem of mixtures risk assessment. Most of the interaction studies are investigated specific endpoint, did not provide the insights into the biological system at consistent biomarker/ protein level.

Therefore, smart approaches are needed to assess the potential toxicity of mixtures by using the new tools, which rely less on in vivo testing and incorporate alternative experimental and computational tools. There has been a continuous effort to find alternative approaches which avoid testing on animals wherever possible. Whenever replacement is not possible, the development of methods which use fewer animals or cause the least harm to the animals is supported. Therefore, the scientists are trying to use alternative / in vitro methods in order to recognize the toxic chemicals and their toxic effects (Cuello et al., 2012, 2010; Heimfarth et al., 2017). These invitro methods should be ideally simpler, faster, and more robust in providing the necessary toxicological information of defined and undefined mixtures than in vivo/ animal experimental methods (Malard et al., 2005). One of the major challenges for toxicology is to find how toxicants exert their effects on living organisms (Wild, 2005). Toxicology needs to provide knowledge of the molecular targets of the toxicants, and also on how an action on a target(s) propagates in living cells to give rise to adverse effects (Vinken and Blaauboer, 2017). Progressive accumulation of biochemical and physiological knowledge at both the organism and cellular levels has allowed the toxicologists to decipher many mechanisms of toxicity (Pease and Gentry, 2015).

In the past few years, substantial improvements in toxicogenomics knowledge have led to an increase in the application of omics methods to answer mechanistically-based biological questions (Han et al., 2012; Kita et al., 2007; Lei et al., 2008; Pan et al.,

2010). The omics-technologies have already shown promising results for improving the toxicity tests, in particular, transcriptomics based screenings (Smith et al., 2005). However, studying the transcription level of a gene only gives a rather rough estimate of its corresponding protein expression level (Dos santos et al., 2016). The other type of omics has become widely applied in many fields of biology in protein level, it has been named that proteomics can be used to identify and characterize new biomarkers associated with given chemical mixtures at the protein level. Similar to transcriptomics, the main advantage of proteomic analysis can provide the meaningful mechanistic insights. In proteomics not only protein expression is monitored, but also post-translational modifications and protein interactions are estimated. These cannot be monitored by transcriptomics and are of substantial value for obtaining full insight in toxicity mechanisms.

3.1. Role of new scientific proteomics tools for toxicity assessment of chemical mixtures

In vitro assays are increasingly applied in human health risk assessment (Bhogal et al., 2005). Numerous *in vitro* tools have been developed over the last few decades, which claim to be useful either for predicting target organ toxicity or in assessing mechanistic aspects of target organ toxicity at the molecular, cellular and tissue level (Whaley et al., 2016). The majority of the *in vitro* studies are based on dispersed cell cultures, either as primary cultures or as continuous cell lines. The cells may be derived from many different species, including humans (Niu and Wang, 2015). The advancement and development of *in vitro* and omics methods make them powerful tools for evaluation of chemical/ metal exposure related early cellular changes and also the mode of action of chemicals on cell lines (Bal-Price et al., 2010). Omics technologies are increasingly applied to gain insight in the mechanism of action of compounds and mixtures in two levels, at the transcription level (transcriptomics) and the protein level (proteomics) or even the whole metabolome (metabolomics) (Sauer et al., 2017). Omics are suitable to study effects in low doses which are more relevant for environmental mixture exposure due to their high sensitivity (Healy et al., 2016). However, the effects observed at omics level need to be interpreted with care since the molecular responses do not necessarily lead to an adverse outcome at the physiological level (Borgert 2007; Beyer et al., 2014). Furthermore, mechanistic information on the mode of toxic action and affected pathways can be derived, which makes the tools valuable in the context of mixture toxicity as well as single substance toxicity investigations. Altenburger et al., (2012) reported in a review on the application of omics techniques in investigations of chemical mixtures, among the 41 studies found (2002-2011). Omics techniques can facilitate the identification of key molecular events and complex sequential events caused by toxic chemicals. They can support building a complete overview of stress-response profiles (e.g. toxicity pathways), both for single stressors and mixtures by identifying key molecular events. Omics results can useful in generating hypotheses on possible interactions between mixture components (El-Masri, 2007).

4. In vitro Proteomics in Mechanistic toxicology

The rational use of proteomics in the chemicals risk assessment will provide valuable insights into the molecular mechanisms when a cell or an organism is exposed to a given toxicant (Buesen et al., 2017). Thus, the general implementations of this approach are to submit the cell or organism either to a moderately toxic (sub-lethal) dose of the chemical of interest or to a highly toxic (lethal) dose but to examine the target cell or tissue at early time points, where the cellular death has not increased too much. In both cases, the rationale is to use a high enough dose to induce a significant cellular response that can be easily observed by proteomics, keeping at the same time the cell mortality low enough in order to protect the proteomic analysis getting polluted by events that are strictly related to cell death, and will not give interesting insights into the specific molecular mechanisms involved in toxic injury.

Currently in vitro proteomics has measurably improved its robustness, sensitivity, and usability and is now a routine part of biological inquiry workflows (Tong et al., 2015). Mass spectrometry (MS)-based proteomics is clearly a versatile tool and will become even more useful as currently, novel proteomics approaches mature. Although proteomics technologies can now deliver very high-quality data for basic biological research, their utility is most notable when the biological problem can be conceptually confined and experimentally approached in a focused fashion, with relevant biomarker discovery. It is critical to the field's success that proteomics is treated as a component of broader biological studies. As part of larger studies, there is no doubt that proteomics technology can help and answer important biological questions. For example, with the rapid pace of technological improvements, systems-wide profiling experiments are emerging as valuable additions to genomic technologies. Proteomics at the organism level, however, continues to pose significant conceptual and technical challenges. As our ability to deeply profile proteomes becomes more time and cost effective and the general understanding of biological systems is refined, biomarker candidates are likely to surface at increasing rates.

5. Proteomics on metals

Mass spectrometry-based proteomics has emerged as a powerful methodology for investigating protein expression of toxic metals exposure (Singhto et al., 2013). Generally, bottom-up techniques used for heavy metals toxicity quantification, in which proteins are first digested, and resulting peptides separated via multi-dimensional chromatography then analyzed via mass spectrometry provide a wide depth of coverage of expressed proteomes. This proteomics technique has been successfully and extensively helpful to find the metal induced protein expression (expression proteomics) and also to investigate proteins and their associated interacting partners in order to ascertain the function of unknown proteins (functional proteomics).

One of the major challenges for heavy metal toxicology is to find how toxicants exert their effects on living organisms. Toxicology needs to provide knowledge of the

molecular targets of the metals, and also the mechanism of toxicity. A review recently published on proteomics for the investigation of metal toxicity (Bridges et al., 2017) clearly indicates that in this specific area, toxicoproteomics on heavy metals plays a crucial role in human health risk assessment.

Table 1: Mechanistic based proteomics applications in heavy metals risk assessment

Metal compound	In vivo /In vitro	Cell line type	References
Arsenic trioxide	In vitro (Human)	Myeloma	(Ge et al., 2009)
Arsenite	In vitro (Human)	Myeloma	(Wang 2008)
Arsenite	In vitro (Human)	Keratinocytes	(Berglund et al., 2010)
Cadmium	In vitro (Human)	He La	(Rousselet et al., 2008)
Cadmium	In vitro (Human)	Lung cell line	(Choi et al., 2009)
Cadmium	In vitro (rat)	Leydig cells	(Zhang et al., 2011)
Methylmercury	In vitro (Human)	Hepatoma cell line	(Cuello et al., 2012)
Lead	In vivo (mouse)	Skin	(Pan et al., 2010)

Ideally, integrating the conventional invitro toxicological assays to proteomics and systems biology approach can useful for understanding the toxicity mechanism. This information will contribute to the development of alternative models for heavy metal toxicity, characterized by a better prediction of neurotoxicity and a reduction of the number of laboratory animals. Furthermore, the application of high throughput proteomics methods will lead to more cost-effective toxicity testing. Along with proteomics, increasing the system biology based pathway and functional analysis can helpful for the addressing toxicodynamic mechanisms and metal interactions through protein expression profiling (Spurgeon et al., 2010). Quantitative proteomics approaches mapping the molecular targets of toxic chemicals may lead to better understanding underlying mechanisms well as to the discovery of new biomarkers (Rousselet et al., 2008).

The overall objective of the PhD work is to develop tools for the determination of heavy metals (Pb, As, Cd, and MeHg) and their binary mixtures interaction profile by using the advanced in vitro tools. My PhD work utilized the in vitro omics and system biology tools to understand the single metals and mixtures mechanism in hippocampal based HT-22 cell line.

Hypothesis and Objectives

Hypothesis and Objectives

2. Hypothesis

The hippocampus is responsible for the memory and cognitive function; it is the main target for heavy metals (Pb, Cd, As, and MeHg) induced toxicity in the brain. Most of the reported studies focused on the effects of metals on the brain in a generalized manner (Stackelberg, 2015). However, the exact mechanisms invoked by exposure to mixtures of the metals (Pb, Cd, As, and MeHg) on the hippocampus are still unclear (Johansson et al., 2007; Sadiq et al., 2012; Tyler and Allan 2014). Therefore, the identification of potential neurotoxic mechanism by metal mixtures represents a critical step in the hippocampus damage. More sensitive and reliable endpoints are necessary to detect the hippocampal damage. Currently, several approaches to study the metal mixtures toxicity have been conducted to evaluate the interaction profile in the brain. Exploring the proteomic responses to environmental toxic metals are increasingly useful for the mechanistic-based experiments. Combining this proteomics knowledge with systems biology information can serve to broaden the perspective of cell physiology under specific stress condition. Systems biology approaches include the acquisition of proteins data, which gives a picture of what is possible for a cell, as well as elucidation of which genes are influenced a given exposure. Thus, quantitative proteomics and systems biology approach mapping the molecular targets of heavy metals lead to better understanding underlying mechanisms well as to the discovery of new biomarkers.

Recent advancement in *in vitro* techniques, with an appropriate target cell and high throughput omics mechanistic tools; allows an accurate understanding of metal mixtures underlying mechanism in the hippocampus. The potential use of omics technology can comprehensively improve the mixtures neurotoxicity knowledge. Furthermore, such approach will also facilitate the better insight into the mechanism by integrating systems biology. This indicates the value of proteomics in the metals and their mixture toxicity in hippocampal cell lines. In addition, the toxicological endpoints measured in vivo by means of proteomics, are similar to the endpoints measured in vitro. Besides the reduction of used laboratory animals, these in vitro models can contribute to the identification of potential neurotoxicity mechanism of single and mixture metals in relevant cell line/organ (Jennings, 2015). The use of mice HT-22 hippocampal cell line is recommended because of their relevance to the hippocampus (Niska et al., 2015).

3. General Objective

The main goal of this project was taking advantage of in vitro and proteomic technologies and other systems biology tools for assessing toxicity signature profiling of heavy metals (Pb, Cd, As, and MeHg) and their binary mixtures on the hippocampal HT-22 cell line.

Specific Objectives

1. Review of the state of art of the heavy metals (Pb, Cd, As, and MeHg) and their mixtures cognitive dysfunction toxicity mechanism to hippocampus region of the brain.

2. To determine the *in vitro* effects of Pb, Cd, As, and MeHg metals on HT-22 hippocampal cells, and comparison of the potency of cytotoxicity, genotoxicity and apoptosis effects.

3. To determine the interaction profile of binary metal mixtures (Pb, Cd, As, and MeHg) in HT-22 cells by using the dose addition and response addition models.

4. To determine the cellular proteome of mice HT-22 hippocampal cells to detect heavy metals (Pb, As, and MeHg) induced neurotoxicity during long exposure.

5. To determine, the underlying HT-22 hippocampal proteome damage of selective binary metal mixtures (Pb+MeHg, Pb+As, and MeHg+As) on the hippocampal HT- 22 cells during long exposure.

Materials and Methods

This work is part of a research project entitled "Health and Environment-wide Associations based on Large population Surveys (HEALS)", funded by the European Union. The overall objectives of the PhD work is to develop tools for the determination of heavy metals (Pb, Cd, As, and MeHg) and their binary mixtures interaction profile by using the advanced *in vitro* tools. This work is a collaborative effort of TecnATox, Unidad de Toxicologia (UTOX) and Plataforma de Proteòmica from Parc Científic de Barcelona (Barcelona), and data refinement and systems biology analysis was supported from the Functional Genetics and BigCAT Bioinformatics from Maastricht University (Maastricht- The Netherlands).

4. Material and Methods

In this chapter, the details of chemicals, cell culture, medium, and reagents used in this project are provided.

4.1. Chemicals

Lead chloride ($PbCl_2$ [CAS no: 7758-95-4]), Cadmium chloride ($CdCl_2$ [CAS no: 10108-64-2]), Sodium metaarsenite ($NaAsO_2$ [CAS no: 7784-46-5]) , Methyl mercury chloride ($MeHgCl_2$ [CAS no: 115-09-3]), Dimethyl sulphoxide (DMSO [D5879]), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT [M5655]), trypsin (TrypLE [Gibco: 12604013]), and proteomics reagents; Urea (GE HealthCare, Life Sciences, CAS Number: 57-13-6), Sodium Dodecyl Sulphate (SDS) (Merck, CAS Number: 151-21-3), Ammonium Hydroxide (Fluka, CAS Number: 1066-33-7), Dithiothreitol (GE HealthCare, Life Sciences, CAS Number: 3483-12-3), Iodoacetamide (GE HealthCare, Life Sciences, CAS Number: 144-48-9), Formic Acid (Merck, CAS Number: 64-18-6), Acetonitrile (HPLC grade) (Fisher Chemical, CAS Number: 75-05-8), Water (HPLC grade) (Fisher Chemical, CAS Number: 7732-18-5). All are analytical grade and purchased from Sigma-Aldrich Química, S.L-Madrid (Spain).

4.2. Cell line and culture medium

Among various research tools, neuronal cell lines are the most commonly used in vitro model for relevant mechanistic studies. With particular concerns for memory and Alzheimer's disease-related studies, hippocampal neuronal cell lines are very limited. HT-22 is one of the cell line subcloned from its parent line HT4, which are immortalized mouse hippocampal neuronal precursor cells (Niska et al., 2015).

The HT-22 cells that we used in this study were a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA). HT-22 cells were maintained in Dulbecco's modified Eagle's medium (DMEM [D6429]) containing 10% fetal bovine serum (FBS Gibco [10500-064]) and 100 U/mL penicillin (Pan-Biotech- Germany), and 100 µg/mL streptomycin (Pan-Biotech- Germany) in a humidified incubator with 5% CO_2 in air at 37^0 C. For all the experiments cells were grown at 70- 80% confluence. The cells were cultured in 75 cm^2 cell culture flasks. For experimental purpose, cells were seeded in 96 well plates and grown for 24 hours before metal treatment. Duplicates wells of cells

were treated with test samples. In subchronic and chronic case medium containing given test concentration was refreshed at every 2 days interval for maintaining exposure in a long time

4.3. Cytotoxicity

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out using a modification of the method of Mossman (1983). The HT-22 cells were seeded in 96-well plate. After 24 h, when the cells had reached a confluence of 70–80%, they were exposed to various concentrations of test samples. After the incubation period, the medium was aspirated from well and MTT working solution at 0.5 mg/mL was added to each well. The absorbance of the solubilized reduced MTT was then measured in a micro titter plate spectrophotometer reader at a wavelength of 570 nm. The measured absorbance or optical density (OD) values were converted to percent of cell viability (%) with respect to control. The cell viability results were used as a quantitative parameter for chemical toxicity.

4.4. Analysis of genotoxicity by Comet assay

To measure the DNA damage of genotoxic chemicals at low concentrations, the alkaline comet assay was conducted according to the OECD guidelines (OECD, 2016) with some modification. To determine the genotoxic potential of in HT-22 cells, cells were seeded in 96 well plates (1×10^6) and incubated at 37 °C in 5% CO_2 for 24 hours for cells attachment. To knowing the genotoxicity effect, we applied the low/chronic IC_{10} - IC_{30} concentration of test chemicals to the cells during 24 hours, also monomethyl sulfonate (MMS) 400 µM was used as positive control. After incubation, each well of cells was washed with 50 µL PBS (pH = 7.4), then added 50 µL trypsin and double amount of DMEM (100 µL). The collected cells were centrifuged at 3000 rpm for 3 min at 4 °C. The supernatant was discarded and the cell pellets were collected. Next, 160 mL of low melting point agarose (LMP – 0.5%) were added to the tube and mixed with the cells. This mixture was deposited on pre-gelatinized slides (normal melting point agarose, 1.5%) and then slides were placed in lysis solution for at least 1 hour for electrophoresis. After denaturation (20 min) and alkaline electrophoresis (25 V, 300 mA, 20 min); the slides were neutralized, fixed and kept refrigerated until the time of analysis. The slides were stained with DAPI (4′, 6-diamidino-2-phenylindole), inspected visually and analyzed using the Nikon epifluorescence microscope, comet analysis scoring system (Comet assay - IV) software (Perceptive instrument, UK). The most commonly used parameters for measuring the genotoxicity are tail length, the relative fluorescence intensity of head and tail (normally expressed as a percentage of DNA in the tail), and tail moment (Kumaravel and Jha, 2006). Collins et al., (2004) reported that tail intensity is a most rational parameter for measuring the DNA damage even in low dose exposure, and analysis of 50 comets per slide is recommended. The experiment was repeated three times.

4.5. Apoptosis assay by Annexin V-FITC/Propidium iodide staining

To evaluate the translocation of phosphatidylserine (PS) from inner leaflets to outer leaflets of the plasma membrane, annexin V- FITC apoptosis detection kit (BD Pharmingen, Poland) was utilized. In this kit, annexin V and propidium iodide (PI) were used to distinguish the apoptotic and necrotic cells from the viable cells. According to the manufacture's protocol, the exponentially proliferating cells were exposed to the test chemicals in culture plate at a density of 0.56 x 10^6/mL during 8 days; control cells were made without chemical. Cells were harvested by trypsinization, washed twice with ice-cold PBS (pH = 7.4). Thereafter, cells were centrifuged at 1200 rpm for 5 min at 4^0C, resuspended in 1mL 1X binding buffer and then transferred the 100 µL of the solution to 5 mL culture tube, and added 5 µL of both annexin-V, PI to the samples. After staining, cells were incubated for 15 minutes in the dark at room temperature. Finally, cells were re-washed with 1X binding buffer 400 µL and analyzed by flow cytometry (Beckman Coulter, Germany). Three independent experiments were performed.

4.6. Toxicity evaluation of chemical mixtures

4.6.1. Assessment of interactions using the response additivity method

This method for testing interaction between chemicals has been described by Lau et al., (2006). In this model, the combined effects of two agents are thought to be equal to the sum of the effects of the single compounds. Deviations from this are either synergistic or antagonistic. In the present study, chemicals were tested at their IC_5- IC_{20} concentrations, derived from individual concentration-response curves. For binary mixtures, cells were then exposed to pairs of the compounds in equal proportions. To assess the interaction between heavy metals, combined effects include: additivity, where metals are no more and no less effective in combination than they are separately; synergism, where the effectiveness of agents is increased when in combination; potentiation, where the increased effect of a toxic compound is acting concurrently with a non-toxic compound; and finally, antagonism, where the effectiveness of agents is decreased when in combination (Costa et al., 2007).

4.6.2. Assessment of interactions using the isobole method- dose additivity

In this method, five different mixtures ratios of each binary pair were prepared (0:100, 25:75, 50:50, 75:25, and 100:0). Each of these mixture ratios was tested in triplicate around the mixture IC_{30} according to the method (Axelrad et al., 2002). For the data analysis of these mixtures, compusyn isobole analyses were used to assess the interaction of the mixtures. The combination index (CI) - isobologram method is widely used in pharmacology to study the nature of the interaction between drugs. The interaction is analyzed by using the median-effect/combination index (CI)- isobologram equation (Chou, 2010), which is based on the median-effect principle (mass-action law). This method has been applied to predict the mixture toxicity of environmental chemicals (Wang et al., 2015). The benefit of a non- constant combination toxicity study is not simply due to the property of the metals, but could also depend on the dose ratios. As the cells do not make the difference between a single metal or a combination, two

metals combined at a given ratio could be considered as a third agent with its own dose-effect relation. The isobole model (Berenbaum, 1978) allows the construction of graphs showing curves describing various combinations of two compounds A and B, which together produce the same, specified effect. Isoeffective doses A and B of the single compounds are connected by an additivity line, which predicts the combinations of A and B required to yield the specified effect, provided the interaction between A and B is additive (zero interaction).
This relationship is expressed by the equation: $c_A/C_A + c_B/C_B = 1$

Where c_A and c_B are the concentrations of A and B in a mixture that produce a specified effect, and C_A and C_B are the concentrations of the single agents, which on their own elicit the same effect as the mixture. Synergistic agents require lower concentrations to produce a given effect when in combination, giving concave isobole; therefore the equation is expressed as $c_A/C_A + c_B/C_B < 1$. Antagonistic combinations give convex isobole resulting in $c_A/C_A + c_B/C_B > 1$. The localization of the experimental mixture point (a, b) corresponding to the doses actually needed for a combination effect mixture with respect to the line of additivity can be translated in term of synergy, additivity, and antagonism; if experimental point below the line corresponds to a combination index (CI < 1) and indicates synergy; a point on the line corresponds to a CI = 1 and indicates simple additivity; finally a point above the line corresponds to a CI > 1 and indicates antagonism. Sometimes the CI values are >3 or much greater, especially at low effect levels (i.e., low fa level). Keep in mind that the synergy scale is from 1 to 0 and the antagonism scale is from 1 to infinity (Chou, 2010). The limitations of the CI method are that it is highly sensitive to small changes in effect measurement at low and high concentrations and lack of statistical evaluation of synergy, additivity or antagonism (Zhao et al., 2010).

4.7. Proteomics methodology

Mass spectrometry-based proteomics has emerged as a powerful methodology for investigating protein expression. In this technique, proteins are first digested, and resulting peptides separated via multi-dimensional chromatography then analyzed via mass spectrometry provide a wide depth of coverage of expressed proteomes. This technique has been successfully and extensively used to survey protein expression (expression proteomics) and also to investigate proteins and their associated interacting partners in order to ascertain the function of unknown proteins (functional proteomics). Combining this proteomics knowledge with systems biology information can serve to broaden the perspective of cell physiology under specific conditions. Systems biology approaches include the acquisition of protein function, which gives a picture of what is possible for a cell, as well as elucidation of which genes are a translation in a given situation. Further, systems biology approaches include a number of techniques that investigate the average population of proteins present within a cell as well as their modifications and possible function.

4.7.1. Protein extraction and digestion

For the proteomics study, the exponentially proliferating HT-22 cells were exposed to the selected sublethal dose (IC_{10}) of each heavy metal in petri dish at a density of 0.025 x 10^6/mL during 8 days; control cells were made without chemical exposure treatment and control culture protein was extracted from HT-22 cell line with 200 µL lysis buffer (8 M urea, 0.1% SDS, 50 mM ammonium bicarbonate [ABC]) and quantified using Micro BCA™ Protein Assay Kit (Thermo Scientific). 30 µg of protein from each sample was digested in a Filter Assisted Sample Preparation (FASP) approach. The volume corresponding to 30 µg of protein was adjusted to 270 µL with 8 M Urea/ 50 mM ABC. The reduction was done with 20 mM Dithiothreitol (DTT) treatment for 1h 30 min at 32^O C. Samples were then alkylated using 30mM of Iodoacetamide (IAA) and incubated in the dark for 30 min at room temperature (RT). Afterwards, samples were loaded in 10 KDa Filters (Amicon) and centrifuged for 30 min, 12,000 g at RT followed by two washes with 300 µL 1M urea/50 mM ABC by centrifugation (30 min, 12,000 g, RT) in order to remove interferents and establish optimal pH (7.5-8.5) and urea concentration for proteins to be denatured. The filter remaining volume was resuspended in 400 µL 1M urea / 50 mM ABC. Digestion was done in two steps: an initial digestion with 1:30 (w/w) porcine trypsin 0.25 µg/µL (Sequence grade modified Trypsin, Promega) for 3hrs at 32^OC followed by a digestion with 1: 50 (w/w) trypsin 0.25 µg/µL for 16h at 32^OC. Peptides were eluted by centrifugation (12,000g for 15 min at RT) and the filters were cleaned with 200 µL 50 mM ABC by centrifugation at 12,000g for 15 min at RT. Samples were dried in the speed vacuum (Eppendorf) and passed through C18 chromatographic columns (P200 top tip, PolyLC Inc.). Briefly, peptides resuspended in 100 µL of 1% formic acid (FA) were charged in the tip columns (previously washed with 70% acetonitrile [ACN] in 0.1 % FA and equilibrated with 0.1% FA) by centrifugation (300g for 1 min 30 sec). Columns were washed twice with 100 µL 0.1% FA by centrifugation (300g for 1 min) and then peptides were eluted in 2 x 100 µL of 70% ACN / 0.1% FA by centrifugation (300g for 1 min). The peptides were dried in speed Vacuum (Eppendorf) and stored at 20 ^{O}C until LC-MS analysis.

4.7.2. LC-MS/MS analysis

The peptide mixtures were resuspended (1% FA, 2% ACN) and the corresponding volume of 500 ng of protein was analyzed by LTQ- Orbitrap Velo's mass spectrometer (Thermo Fisher Scientific). Peptide mixtures were injected into the capillary column (75 µm× 25 cm) in full loop mode and separated by a 5 µm C18 column using nano-acquity liquid chromatography system (Waters). Peptides were eluted with a linear gradient of 1-35 % buffer B (0.1% FA, 100% ACN) for 150 min, followed by 35-45 % buffer A for 20min (A: 0.1%). The mass spectrometer was operated in positive ion mode (source voltage 2000V) and data-dependent manner. The full MS scans were performed in the Orbitrap at the range of 300–1,700 m/z at a resolution of 60,000. For MS/MS scans, the 15 most abundant ions with multiple charge states were selected for collision-induced dissociation (CID) fragmentation following one MS full scan.

4.7.3. Label-free quantitative (LFQ) data analysis using Progenesis QI software

Raw MS peak intensities were analyzed using Progenesis QI data analysis software v4 (Non-Linear Dynamics, Waters, U.S.). Ion feature matching was achieved by aligning consistent ion m/z and retention times. Progenesis label-free quantification (LFQ) was done using non-conflicting unique peptides and protein grouping, and the software normalization algorithm was applied to all proteins. Abundances are calculated as the area under the MS peak for every matched ion feature. Search results were filtered based on peptide ion score≥ 40 and contaminants were removed and abundances were normalized to all proteins. A total of 428,801 spectra were obtained from the LFQ LC−MS/MS proteomic analysis of samples including a control and test sample.

4.7.4. Protein identification

Proteins identification was done using Mascot search engine (v. 2.3.01). All MS/ MS spectra passing an arbitrary cut-off in the Progenesis software were included in the list used to perform the search against SwissProt Mouse database using the Mascot search program (Matrix Science, London, UK, www.matrixscience.com). The following criteria was applied: (1) trypsin as enzyme; (2) allowed two missed cleavages; (3) variable modifications, acetyl (N-terminus, protein) and oxidation (M); Carbamidomethyl (C) fixed modification (4) Peptide tolerance, ±10 ppm (MS); MS/MS tolerance, ± 0.6 Da.

4.7.5. Statistical analysis of data

All experiments were performed three times (n=3) and each concentration tested in replicates. The results were given as mean ± standard deviation (SD). IC_5 to IC_{30} values calculated from dose-response curve fitted by using the Graph pad prism version 5.01. Compusyn software (http://www.combosyn.com) used for non-constant ratio isobologram fitting. All data were analyzed by one way ANOVA ($p^{***} < 0.05$) procedures followed by dun net's. A p-value of less than 0.05 was considered statistically significant.

Label-free quantitative data was submitted to T-test method ($p \leq 0.05$) using Microsoft Excel in order to find out significant differences among the treatments and control. Data were further curated applying the following filters: minimum 1.2-fold change and p - value<0.05. Meaboanalyst 3.0 (http://www.metaboanalyst.ca) programme was used for presenting the unsupervised data in principal Component Analysis (PCA), volcano plot and hierarchical clustering analysis.

4.7.6. Bioinformatics analysis

We used gene ontology (GO)-Elite (http://www.genmapp.org), a flexible and powerful tool for gene ontology (GO) analysis (Zambon et al., 2012). In addition to the gene ontology, GO-Elite allows the user to perform over-representation analysis (ORA) on any structured ontology annotations, or biological IDs (e.g. gene, protein or metabolite) (Huang et al., 2009). GO-Elite utilizes the structured nature of biological ontologies to report a minimal set of non-overlapping terms. The results can be visualized as

networks, GO-Elite ranks each analyzed GO term according to a Z-score along with a p-value (Ren et al., 2010). In general, three main steps are included in the framework for the quantitative functional interpretation of proteomics data. The first step is an aimed at identifying the statistically significant genes across by using the T-test method. The next step is to identify significantly altered functional GO gene categories using unsupervised GO analysis mapping tool such as Go -Elite. The last step is to extract the pruned list to interoperate the GO biological processes, molecular functions, and cellular components relevant to neurodegeneration. Significantly altered categories were identified based on the p-value <0.05, Z-score > 1.96, and a minimum of 3 genes changed within each specific GO ID. We used the Cytoscape network tool (http://www.cytoscape.org/), to quantitatively evaluate and visualize the functional changes in gene expression linked GO categories. Cytoscape was also used to indicate the absolute average fold change (FC) between each metal exposure group and to control for all significantly altered genes in each enriched GO term. In the results section, GO-elite figures are showed specifically relevant to the neurodegenerative diseases.

Pathvisio is a commonly used pathway editor, visualization, and analysis software for omics-based experiments. Here, first, the required mus musculus pathway collection was obtained from wiki Pathways (http://www.wikipathways.org).Pathway analysis was performed in Path Visio 3.2.4 (http:// www.pathvisio.org) to interpret and visualize the molecular changes on a pathway level which relates the system biology. Generally, the pathways were selected based on a standardized difference score (z-score) using the expected value and standard deviation of the number of differentially expressed genes in a pathway under a hypergeometric distribution. Here, we consider the desired pathways relevant to neuro degeneration processes with a minimum number of changed proteins are 3. Additionally, the log2FC and p-value were visualized on pathways with the visualization module in Path Visio.

Material and Methods

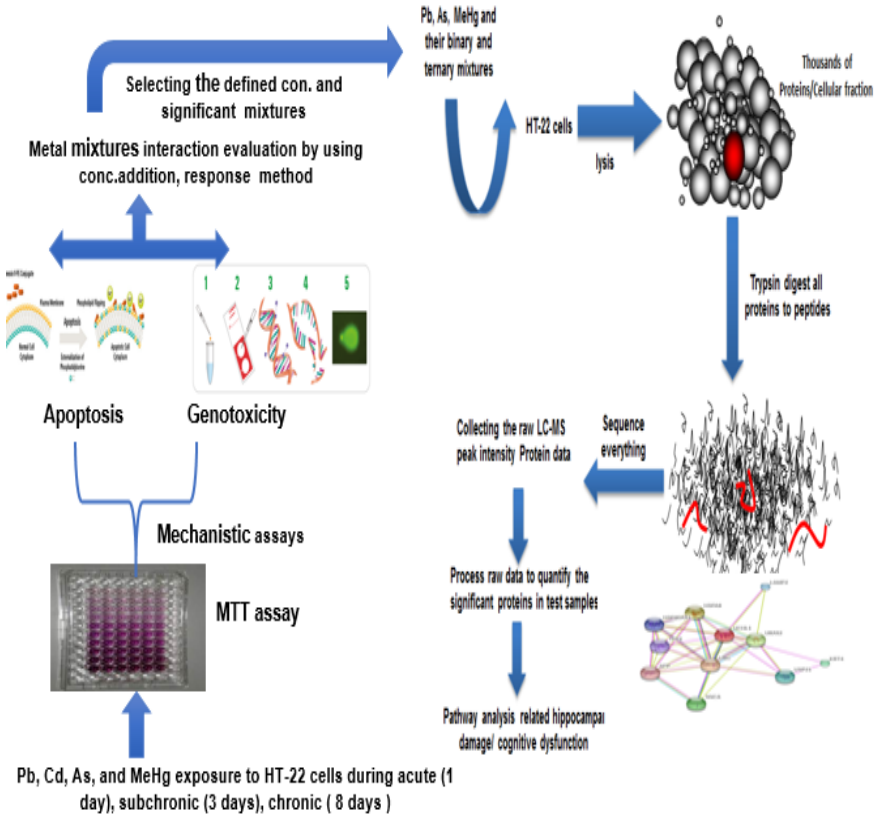

Figure 1: Brief summary of methodological workflow used in the present project

Environmental exposures are virtually always to complex metal mixtures. Current toxicological studies dictate a largely metal-by-metal evaluation of exposures and risks in brain, which fails to adequately address potential interactions of metals in hippocampus region of brain. We evaluated the similar/dissimilar neurotoxicity of each metal in brain that can give strong support to the mixtures effect in brain (Chapter 1). The scope of our study is to prove the single metal (Pb, Cd, As, and MeHg) and their mixtures chronic toxicity impact in hippocampal cells by using the *in vitro* and systems biology methodology. Initially, we performed the cytotoxicity, apoptosis, and genotoxicity characterization of single metals for assessing the potency of each metal, their mixtures interactions profile on hippocampal cells (Chapter 2, 3). The results clearly showed the potency of metals MeHg>As>Cd>Pb on different exposure conditions. Another aim of the study (Chapter 4) was the heavy metals toxicity in HT-22 cells using (Pb, Cd, As, and MeHg), as some stress proteins could be considered as biomarkers for heavy metals. Identification of these proteins and their mapping into specific pathways may help to gain deeper insight into the toxic mechanisms of heavy metals in hippocampus. Analysis of the stress response of HT-22 cells to metal mixtures was another aim of the study (Chapter 5) to identify proteins, which are involved in the neurodegeneration mechanism. This enables to gain a global understanding of the way in which heavy metal single and mixtures exposures response in the HT-22 hippocampal cells.

Chapter 1

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Heavy metals (Pb, Cd, As, and MeHg) as risk factors for cognitive dysfunction: A general review of metal mixture mechanism in brain.

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Chapter 1

Chapter 1

Heavy metals (Pb, Cd, As, and MeHg) as risk factors for cognitive dysfunction: A general review of metal mixture mechanism in Brain

Abstract

Human exposure to toxic heavy metals is a global challenge. Concurrent exposure of heavy metals, such as lead (Pb), cadmium (Cd), methylmercury (MeHg) and arsenic (As) are particularly important due to their long lasting effects on the brain. The exact toxicological mechanisms invoked by exposure to mixtures of the metals Pb, Cd, As and MeHg are still unclear and they share many common pathways for causing cognitive dysfunction. The combination of metal may produce additive/synergetic effects due to their common binding affinity with NMDA receptor (Pb, As, MeHg), Na^{+} - K^{+} ATP-ase pump (Cd, MeHg), biological Ca^{+2} (Pb, Cd, MeHg), Glu neurotransmitter (Pb, MeHg), which can lead to imbalance between the pro-oxidant elements (ROS) and the antioxidants (reducing elements). In this process, ROS dominates the antioxidants factors such as GPx, GS, GSH, MT-III, Catalase, SOD, BDNF, and CERB, and finally leads to cognitive dysfunction. The present review illustrates an account of the current knowledge about the individual metal induced cognitive dysfunction mechanisms and analyse common Mode of Actions (MOAs) of quaternary metal mixture (Pb, Cd, As, MeHg). This review aims to help advancement in mixture toxicology and development of next generation predictive model (such as PBPK/PD) combining both kinetic and dynamic interactions of metals.

Key words: Metal mixture, Cognitive dysfunction, Hippocampus, Mode of actions.

Abbreviations: ABCC = ATP-binding cassette, AchE = Acetylcholine esterase, As = Arsenic, BBB = Blood brain barrier, BDNF = Brain derived neuronal factor, CaM=Calmodulin, CAM-K= Calmodulin K, CAMKII = Calmodulin-dependent protein kinase-II, CAM-II=Calmodulin-II, Cd = Cadmium, CERB = c-AMP response element binding protein, CH_3 Hg = Methyl mercury, CNS = Central nervous system CP= Choroid plexus, CSF = Cerebrospinal fluid, DMT-I = Divalent metal ion transporter-I, GABA= ¥-amino butyric acid, Glu = Glutamate, GP_x = Glutathione peroxidase, GS = Glutathione synthase, GSH = glutathione, JNK3= c-Jun N-terminal kinase3, LTP = Long-term potentiation, MAPK = Mitogen-activated protein kinase, MDR =P-glyco protein, MT-III= Metallothionein, NMDA = N-methyl-D-aspartate, n-NOS= Neuronal nitric oxide synthase, Pb= Lead, PCB = Polychlorinated biphenyls, PKA= Protein kinase-A, PKC = Proteinkinase-C, PLC = Phospholipase-C, P^{38} MAPK= P^{38} mitogen-activated protein kinase, ROS= Reactive oxygen species, SOD = Superoxide dismutase, Tf = Transferrin.

Chapter 1

1. Introduction

Heavy metals are naturally occurring elements with high atomic weight and are released by natural events and human activities. Their multiple industrial, domestic, agricultural, medical, and technological applications have led to their wide distribution in the environment, raising concerns over their potential effects on human health and the environment. Human exposure to toxic metals has lately been decreasing in developed countries but in other parts of the world (mostly in developing countries), is increasing (Järup 2003). Several population-based surveys indicate that metal exposure is still widespread (Alves et al. 2014; CDC 2014; Esteban-Vasallo et al. 2012; Mari et al. 2014; Nadal et al. 2004; Rovira et al. 2015; Vilavert et al. 2015). Major human exposure of metals results from anthropogenic activities such as mining and smelting operations, industrial production, domestic and agricultural use metal-containing compounds (He et al. 2005). Metals are systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure (Hullmann et al. 2012). In general, lead (Pb), cadmium (Cd), methylmercury (MeHg) and arsenic (As) are some of the most toxic metals human are exposed and targets essential organs namely kidney, liver, and brain that causes nephrotoxicity, hepatotoxicity, and neurotoxicity (WHO 2007). According to world health organization (WHO) these four elements rank among the priority metals that are of great public health concerns (WHO 2010).

The Pb, Cd, As, and MeHg a very frequently occurring metal mixture in environment, have common exposure with common disease outcome such as cognitive dysfunction. The current literature of metal induced neuronal damage is primarily confined to single metal exposure (Zhu et al. 2014) and no published literature was found on quaternary mixture of Pb, Cd, As and MeHg evoked neurotoxicity. Metal mixture exposure and their mode of action relation evaluating either independent or addition and synergistic effects are not well developed (Rodríguez et al. 1998; Sasso et al. 2010). However, the evidence for these kind of interactions continues to grow (Stackelberg 2015). Predictive in-silico model developments are also constrained by lack of mixture experimental data. Growing evidence of neurotoxicity for these metals demands further research in the area of cumulative risk assessment.

Most of these metals (with the exception of Cd) are known to increase susceptibility to cognitive dysfunction and neurodegenerative outcomes (Clarkson 1987). In case of Pb, experimental evidence have shown that, children who experienced Pb exposure were found their brain volume modified (Cecil et al. 2008). The centre for disease control (CDC) limits the childhood Pb^{+2} intoxication to 10 µg/dl in blood (Landrigan 2000). This level is thought to be the threshold for childhood cognitive deficits (Jusko et al. 2008). The exposure of Pb during early postnatal life produces a greater deficit in learning performance than in older animals (Kuhlmann et al. 1997). The Arsenic, also a potent neurotoxic, induces hippocampal-dependent behavioural deficits in rodent models (Martinez-Finley et al. 2011). Bellinger et al., (2013) proved that higher concentrations of As alters growth and development in children resulting in neurological

deficits. Experimental animal models have disclosed potent alterations in hippocampal function with As exposure (Cronican et al. 2013). Gong and O'Bryant, (2010) found a relationship between As exposure and Alzheimer's disease. In vivo studies in rat showed that As exposure impacts on the synaptic activity of neurons localized in the hippocampus region of the brain (Krüger et al. 2006). In case of mercury, certain compounds have well established link to neurotoxicity, affecting the normal development of the central nervous system (Crespo-López et al. 2009). However, exposure to inorganic mercury results in brain or nerve damage is not as certain, since it does not easily pass from the blood into the brain (Debes et al. 2006). Organic mercury (MeHg) may affect many different areas of the brain and their associated function. Exposure of MeHg to fetal brain is more susceptible to mercury-induced damage than the adult brain (Clarkson et al. 2003). In vitro studies in animals have indicated that MeHg can affect the biochemical processes believed to be involved in Alzheimer's disease (Leong et al. 2001), and axonal degeneration is unique to MeHg (Castoldi et al. 2003). In some recent studies, Cd has also been shown to produce free radicals in the brain (Czarnecki et al. 2012). In occupational and epidemiological studies with workers exposed to Cd evidence of memory loss was found (Hart et al. 1989). Chronic occupational exposure to Cd leads to slowing psychomotor functions of brain (Viaene et al. 2000). In vivo study of Cd reported, the disruption of hippocampus region in brain resulting long term potentiation function(LTP) function blocked (Luo et al. 2009).

In mixture, metals have competitive interactions with macromolecule/transporter because of their functional similarities. Normally metals are transported and eliminated through many common cellular mechanisms usually termed as a molecular mimicry (Bridges and Zalups 2005). In addition, toxic metals have significant interactions with essential metals (iron, manganese, calcium) which can influence the essential metal status in the human body (Goyer 1997). The metal mixture toxic interactions could be either dose additive, interactive (synergistic or antagonistic) or independent of each other, which can generate high level biochemical changes in different regions of brain. Recently, Rai et al. (2010a) found that subchronic exposure to Pb, Cd and As ternary mixture in wistar rat caused neuronal developmental disorder by synergistic action. This result gives significant evidence of the metal mixture's neurotoxic activity and their potential interactions.

This work is framed in the European projects, HEALS and EuroMix, and the broader objective is summarized in Figure 1, aims to review the current understanding of mode of action for several metals (Pb, Cd, As and MeHg) in brain by particularization of the metal-neurochemical interactions. Additionally, this review elaborates metal mixtures mode of actions to summarize the prevailing proofs that support the hippocampus as the major target for metal mixture for inflicting the cognitive dysfunction/loss of memory in both children and adults. In the final part of the review an integrated framework is proposed for studying the metal mixture mode of toxicity in brain. The review is concluded with a future perspective on metal mixtures study and the development of

next generation predictive model (such as PBPK/PD) combining both kinetic and dynamic interactions of heavy metals.

2. How metals enter the brain?

The microenvironment of the total brain is separated from the systemic circulation of remainder of the body by blood brain barrier (BBB) and choroid plexus (CP), that defend brain integrity from toxic chemicals/metals (Zheng et al. 2003). Metals are most frequently absorbed from the gastrointestinal tract, across the lungs, or through the skin and then enter the systemic circulation. The metal may enter into the brain from the blood by overcoming the BBB and by crossing the CP into the cerebrospinal fluid (CSF), and from CSF it will reach to a specific part of the brain (Yokel 2006). Ordinarily BBB greatly limits the diffusion of non-lipophilic substances in and out of the brain (Bhowmik et al. 2015). Saunders et al., (2012) reported that protective efflux mechanisms like as ATP-binding cassette (ABCC) and P-glyco protein (MDR) are there to forestall the entry of toxic chemicals in the brain. Despite these protective barriers, the weak integrity of blood-cerebrospinal fluid barrier allows these toxic chemicals to enter to the choroid fluxes regions of the brain (Wright and Baccarelli 2007). Zheng et al., (2001) found that metals are accumulating in the BBB and CSF and then may reach the brain. According to Bridges and Zalups (2005), there is more investigation required to know how metal exposure disrupts the BBB function during the early brain development of a fetus (Caserta et al. 2013).

The neurotoxic metals such as Pb, Cd, As, and MeHg and their role in blood-brain barrier disruption for reaching the brain have been studied individually and reported in the literature. For crossing the BBB, toxic metals mimics the behaviour of essential nutrients for utilizing the physiological ionic transporters. Manton et al., (1984) reported 100-fold increase in the Pb levels in human choroid plexus compared to the amount present within brain cortex. These Pb accumulated in choroid plexus is most probably reaching the targeted region. The other neurotoxic metal Cd has also shown high permeability to cross the BBB in rats (Shukla and Chandra 1987). In vivo studies Cd has been reported to freely penetrates and accumulate in the developing and adult rats brain (Méndez-Armenta and Ríos 2007a) and, therefore, the penetrated Cd powerfully binds with metallothionein (MT-III) (Uchida et al. 1991). MT-III is a sulphur containing macromolecule located in the cerebral cortical neurons (Xu et al. 2011). When a pregnant rat is exposed to As, fetal brain neurons initiate the apoptotic and necrosis process due to the underdevelopment of BBB influenced by the As exposure during early brain development (Wright and Baccarelli 2007). In case of mercurial compounds, they are found in two major forms specifically inorganic mercury (IHg) and organic mercury (MeHg). MeHg has shown high neurotoxic behaviour than IHg. The reason may be the limitation of brain transport mechanism or the chemical properties of the IHg, whereas MeHg freely enters to brain by binding with endothelial cysteine(AA) sulphhydral-groups(-SH) to form the neutral amino acid analog (i.e. methionine) that is

mimicking the structure of methionine for entering the brain (Aschner and Aschner 1990).

Metal mixtures exposure takes place in different life stages (embryo, fetus, new born, child, adult, old age) that is termed as windows of exposure. The amount of metal internal dose in a brain may have high inter-individual variability and highly depend on the anatomical and physiological development in the brain barrier system (Yu et al. 2011). Some experimental studies have found proof of metals transfer in fetus stage however, evidence is not clear for all four metals (Gundacker and Hengstschläger 2012). Cd accumulates in the placenta during gestation but transfer of Cd to the fetus appears to be restricted (Lin et al. 2011). Early life exposure of arsenic causes deficits in intelligence and memory by influencing brain weight and neurotransmitter system (Tolins et al. 2014). Whereas, Pb does not accumulate within the placenta and therefore, the concentration of Pb in maternal blood is almost similar as that of fetal blood (Bhattacharyya 1983) and Choi et al., (1989) reported that MeHg initiated brain damage in utero stage.

Apart from BBB, Pb, Cd, As and MeHg metals additionally influence the essential metal transporters. Toxic metals have affinity with divalent metal ion transporter-I (DMT-I), transferrin Tf-transporters that leads to toxic-essential metal interactions in BBB and brain tissues. In developing brain, essential metals (Fe, Cu, Mn) are transported from systemic circulation to brain by DMT-I, Tf (Piloni et al. 2013). Rodriguez et al., (1998) reported that Mn and As had greater accumulation in rat brains relative to the single metal exposure. McCall et al., (1996) reported that Cd, Pb and As produces synergistic action for reducing the expression of glial fibrillary acidic protein (GFAP), GFAP is crucial macromolecule in blood brain barrier. Rai et al.,(2010a) established that As, Cd, Pb ternary mixture has larger than the additive response on astrocyte toxicity by disrupting the BBB performance that culminate into neurological deficits in developing rats (Rai et al. 2010a).

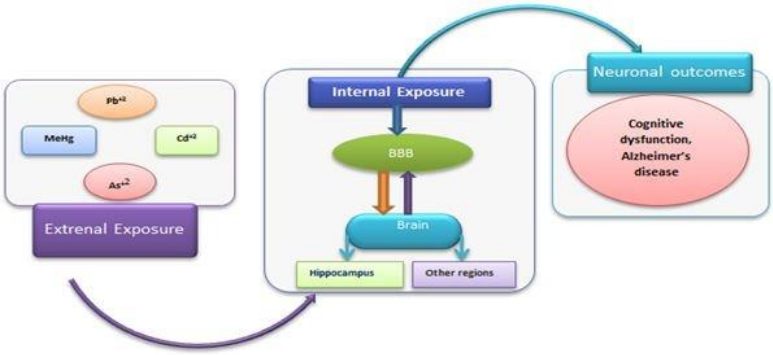

Figure 1: Conceptual diagram of metal mixture exposure- toxicology-disease outcome scenario(brain)

3. Heavy metals (Pb, Cd, As, and MeHg) risk on brain: toxicological evidence

A common susceptibility factor for Pb, Cd, As, and MeHg metal comprises the cognitive deficit but the risk level depends on exposure intensity and metal-biochemical interactions in the brain. Basha et al., (2005) observed that developmental exposure to Pb exhibits latent effects which are known to be epigenetic interaction of Pb with amyloid precursor protein gene that causes neuro degeneration in elder age. Prenatal exposure to Pb influences the long-term potentiation (LTP) machinery in the developing brain by disrupting the N-methyl-D-aspartate (NMDA) receptor expression in hippocampus region that results in cognitive deficit in children (Sadiq et al. 2012). Cd produces neurological alterations including memory loss and mental illness (Wang and Du 2013), that chiefly influences the discharging of repressing neurochemical glycine and ¥-amino butyric acid (GABA) into the extracellular space which results the degree of imbalance between excitation–inhibition in synaptic neurotransmission (Minami et al. 2001). Inorganic As have an effect on the cognitive function of brain (Tyler and Allan 2014). Ex-vivo cell culture studies showed that As exposure increases the β- amyloid protein and induces hyperphosphorylation of tau protein which results in neurodegeneration (Giasson et al. 2002). As also enhance the cellular apoptosis pathways such as caspase-3 and caspase-9 in all brain regions which raises oxidative stress and neuronal cell death (Kumar et al. 2013). Animal studies showed that the developmental exposure to MeHg have long-term consequences in the brain (Johansson et al. 2007b), also MeHg alter the homeostasis brain (Atchison and Hare 1994) resulting in oxidative stress (LeBel et al. 1990) and cognitive disability. metal mixtures exposure could be a bigger risk for cognitive dysfunction, as well as behaviour and impaired neurological (CNS) development than individual metal (Rai et al. 2010a). In vivo studies showed that As, Cd, and Pb ternary mixture -exposed rats bear the essential features of Alzheimer's-like pathology, and increased hippocampal and cortical amyloid

precursor protein(APP) gene processing causes cognitive dysfunction (Ashok et al. 2015). In another case, mixture of essential metal like Zn, Cu and Fe may influence the amyloid-β the (Aβ) aggregation (Atwood et al. 1998) and also Fe, Pb and Mn and their mixture may raise the risk of Parkinson disease (Lai et al. 2002). Limited literature has sufficient indication that Pb, Cd, As and Hg quaternary metal mixture may have high capability to cause cognitive dysfunction, however the evidence of mixture and disease relation is not clear.

4. Critical Neurobehavioral and molecular changes induced by metals

The metals Pb, Cd, As, and MeHg have toxic impact on the brain by different molecular mechanism in which metal ions interact with neurotransmitters, receptor and its subunit, biological Calcium (Ca^{+2}), ion pumps, enzymes and amino acid functional groups. Each metal has unique nature of causing neuronal damage. In developing rats, experimental studies provide evidence that metal mixtures exposure during brain development has a greater impact on the neurological deficits (Rai et al. 2010b) compare to adult rats due to the lack of barrier system and poor development of defense mechanism (antioxidants). Among various regions in brain, metal mixture majorly causes site specific damage to hippocampus (Angelica and Fong 2014). The hippocampus is an important brain region for acquisition of memory (Snyder et al. 2005). In normal physiological situation NMDA receptor plays key role for the cognitive functions and neuronal synaptic plasticity (Lasley et al. 2001). Functional sensitivity of NMDA receptor depends on glutamate (Glu) release and Glu binding at postsynaptic region. The interaction of Glu-NMDA receptor enhances the influx of Ca^{+2} into post synaptic neuronal cytosol and activates the multiple Ca^{+2}dependent enzymes (kinases, calmodulin, phospholipases) for LTP function/memory (Li et al. 2007).

To understand the potential neurotoxicity of metal mixtures and their common cellular elements involved in different molecular mechanism, we have approached the rest of the review by reviewing individual metal mode of action with hippocampus region of the brain as target site and finally finding the evidence of the mixtures interaction for this particular region of brain.

4.1. Mode of neurotoxicity of Lead (Pb)

The brain damage induced by Pb depends on age (Landrigan 2000) and level of exposure (Bradbury and Deane 1993). Generally children absorbs significantly more Pb than adults (Goyer 1996) due to an underdeveloped blood–brain barrier (Ruff et al. 1996). There is a very little proof that Pb will harm the functions of the blood-brain barrier at a lower dose of <80 µg/dl (Bradbury and Deane 1993)**.** However, Slomianka et al., (1989) found hippocampus damage at blood Pb levels of 20µg/dl. Jett et al., (1997) reported that continuous low level exposure (250ppm) of Pb causes hippocampus damage in adult rat (Jett et al. 1997). In brain, Pb mainly disrupts the hippocampus region by interacting with NMDA receptor. According with Guilarte & Miceli (1992)

Pb and NMDA receptor interaction is voltage independent, and non-competitive (Guilarte and Miceli 1992)

Pb interaction with NMDA receptor has two steps: synaptically and extra-synaptically. Pb primarily disrupts the Ca^{+2} ion signalling mechanism in neuronal synapsis by NMDA–Glu process. Pb modifies the NMDA-receptor subunit (NR2A,NR2B) expression, the forming composition leads to dysregulate the Ca^{+2}-sensitive signalling pathways in hippocampus (Toscano and Guilarte 2005). Zhang et al.(2002) ascertained that chronic exposure to Pb decreases the NR2A content and increases NR2B content within the hippocampus (Zhang et al. 2002). Hippocampus cell cultural study revealed Pb could be downregulate synaptic NR2A-NMDA receptor and concomitantly upregulate NR2B-NMDA in the extra synaptic region (Neal et al. 2011). The NR2A, NR2B part is critical for NMDA receptor expression and neuronal activity (Kim et al. 2005).

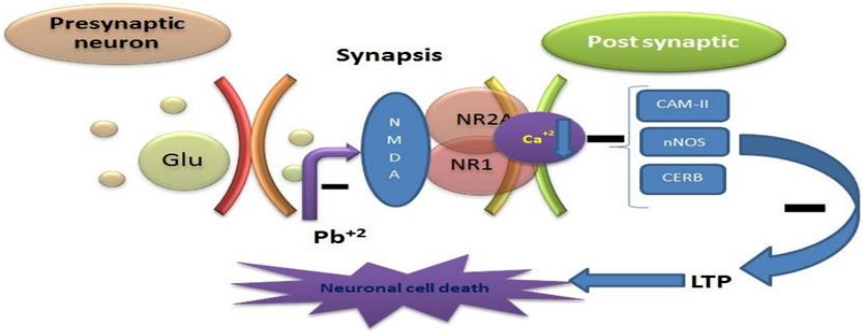

Figure 2: Schematic representation of Pb^{+2} ion entry in a hippocampal synaptic region by competition with glutamate (Glu), then binding NMDA receptor and influences the expression of NR2A subunit results release of Ca^{+2} ion decreased consequences LTP function inhibited.

In vivo rat studies showed that hippocampal expression of NR1/NR2A receptor assemblies could be altered because of Pb exposure, this is because it is linked to persistent alterations of brain derived neuronal factor (BDNF), neuronal nitric oxide synthase (n-NOS), c-AMP response element binding protein (CERB) results LTP function inhibition (Guilarte et al. 2001). The summarised molecular mechanism of Pb in hippocampal synaptic region is shown in Figure 2. Pb enters the hippocampus synaptic region and blocks the NMDA receptor function inducing the influx of NMDA ion channel dependent Ca^{+2} depletion. Therefore the effects of Ca^{+2} dependent processes like calmodulin-II (CAM-II), neuronal nitric oxide synthase (n-NOS) and cAMP response element-binding protein (CERB) are inhibition. The altered receptor

performance could influence the neuronal plasticity, because of alterations linked with long-term potentiation (LTP) dysfunction (Baranowska-Bosiacka et al. 2012).

In the extra synaptically region, concentration at levels of picomolar is enough for substituting the micromolar concentrations of Ca^{+2}, which may activate the protein kinase-C (PKC) resulting Pb induced neurotoxicity (Sanders et al. 2009). Wang et al., (2008) reported that due to the Pb exposure PKC and Calmodulin (CAM) mRNA expression influences the impairment of learning and memory. This could be another molecular mechanism of Pb evoked impairment of learning and memory. The mode of actions within the extra-synaptically neurons is represented in Figure 3 which is antagonist to synaptical regions mechanism. In extra synaptical area, the exposure to Pb increases the Ca^{+2} ion concentration because of enhanced NMDA receptor NR2B expression (Jusko et al. 2008) and producing a drastically increases of the intracellular endoplasmic reticulum calcium (Ca^{+2}), and phospholipase-C (PLC) levels (Yin et al. 1994). The enhanced PLC upregulate the protein kinase-A (PKA), the mitogen-activated protein kinase (MAPK), and the calcium/calmodulin- dependent protein kinase (CAMKII) functions leads to higher production of reactive oxygen species (ROS) and fall down of cellular protective elements such as CREB, BDNF, catalase and superoxide dismutase (SOD). The imbalance between defensive elements and ROS causes neuronal cell death (Yin et al. 1995).

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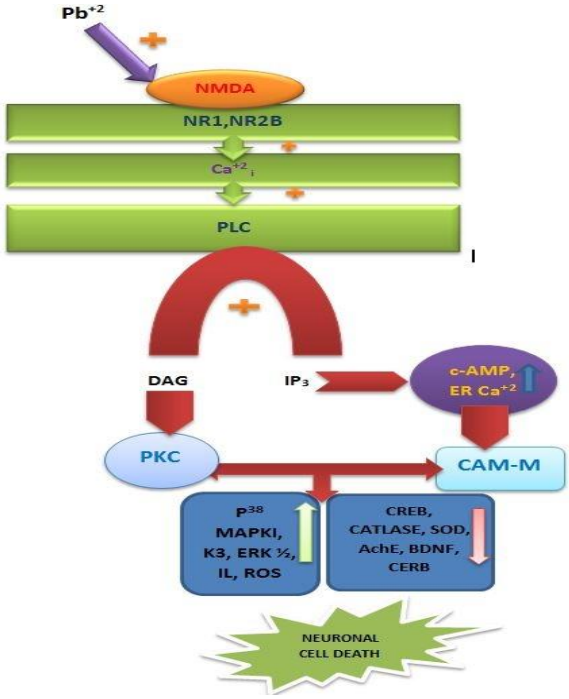

Figure 3: In extra synaptical region Pb^{+2} directly binds NMDA receptor NR2B subunit, which leads to positive enhancement of NR2B expression results Ca_i^{+2} ion flow consequences leads to imbalance between apoptosis factors and antioxidants enzymes

4.2. Mode of neurotoxicity of Cadmium (Cd)

Cd causes a wide variety of toxic effects on the central nervous system (CNS). Kumar et al., (1996) reported that Cd affects the brain by disruption of specific membrane function, principally in the hippocampus region. Higher neurotoxicity is reported in newborn than adult, and this variation may be due to lack of blood brain barrier maturation in newborn (Wong and Klaassen 1982). In new born accumulation of Cd takes place more in choroids plexus region of brain (Pal et al. 1993). In-vivo experimental studies in pups showed that the utero exposure to Cd could inhibit the acetylcholine esterase (AchE), Na^+/ K^+ -ATPase pump which reduces the neuronal activity in pups (Chandra 1991). Cd also mimics the Ca^{+2}, being this an ubiquitous intracellular ion which acts as a signalling mediator in numerous cellular processes including cell proliferation, and differentiation (Xu et al. 2011). The Cd mode of action represented in In Figure 4, Cd inhibits all of the known pathways of cellular Ca^{+2} influx and acts as a competitive ion to Ca^{+2}. Consequently, it influences the membrane action potential and neurotransmitters release (Huguenard 1996). Few studies also reported that Cd influences the Ca^{+2}-binding molecules like calmodulin K (CAM-K) (Hayat et al. 2003).

Experimental dose-dependent studies disclosed that concentration of cytoplasmic and nuclear Ca^{+2} raised in neurons as result of Cd exposure (Orrenius and Nicotera 1994). Changes in the homeostasis of cytosolic Ca^{+2} concentration affect the regulation of many cellular events (Alshuaib and Byerly 1996), and principally it causes oxidative stress in the brain cells by induction of reactive oxygen species (ROS) and consequent reduction of intracellular glutathione (GSH), catalase and SOD activity (López et al. 2006). On the other hand, Cd exposure reduces the activity of MT-III, BDNF, in the brain (Durczok et al. 2005) resulting increased ROS levels. An experimental study in cerebral cortical neurons identified as targets of Cd-mediated induced neuronal cell apoptosis (Méndez-Armenta and Ríos 2007b).

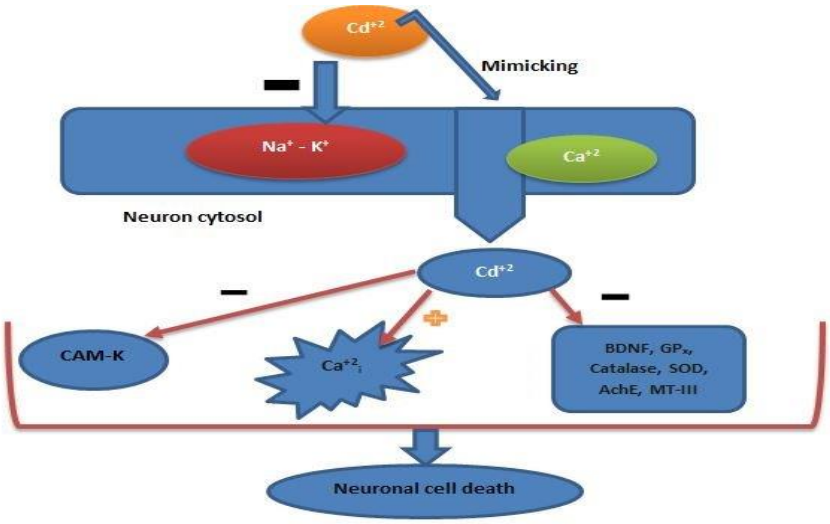

Figure 4: Cd^{+2} ion enters the inside the neuron by mimicking the voltage gated Ca^{+2} channel, once inside the cell Cd induce a decrease the BDNF, $GP_{x,}$ Catalase, SOD, AchE, MT-III, CAM-K, enhance intra cellular $Ca^{+2}{}_{i}$ producing a free radicals , this free radicals able to cause neuronal cell death.

4.3. Mode of neurotoxicity of Arsenic (As)

Low concentration exposure to As cause cognitive dysfunction (Naujokas et al. 2013), whereas growth delays and neuronal tube defects is produced at high concentration exposure (Ahmed et al. 2011; Vahter 2009). Studies in children found poor performance scores after long-term exposure to As (Wasserman et al. 2004). In experimental animal models, it has been observed potent alterations in hippocampal region and dysfunctional cognitive behavior (Cronican et al. 2013). In vivo studies showed that As primarily impacts on the synaptic plasticity of neurons in the hippocampus region of the brain (Krüger et al. 2006). Another ex-vivo cell culture study provided evidence of As capacity to increases β-amyloid protein, which induces the hyperphosphorylation of tau protein resulting neurodegeneration (Giasson et al. 2002). Cellular level experiments reported that As has property to alter the metabolism of assorted neurotransmitters like monoamines, acetylcholine, gamma amino butyric acid (GABA), and glutamate (Rodriguez et al. 2002). In a recent study, a big reduction in monoamines such as adrenaline, noradrenaline, dopamine, and serotonin was observed in corpus striatum, frontal cortex, and hippocampus areas of brain during chronic As exposure (Yadav et al. 2010). As suppresses the NMDA receptors in hippocampus, which play a pivotal role in synaptic plasticity, learning, and memory (Luo et al. 2009; Kruger et al. 2009).

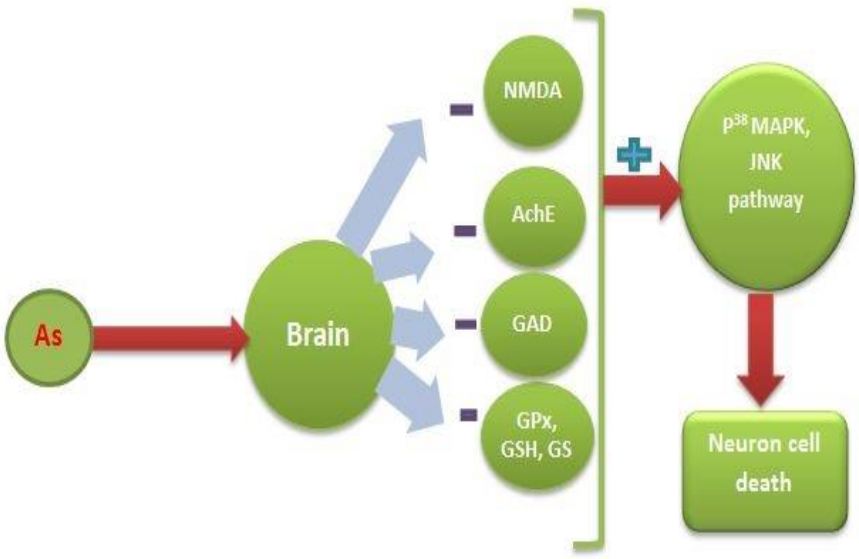

Figure 5: Schematic representation of arsenic metal neurotoxicity, -sign indicates inhibition of cellular elements and + sign shows rising apoptotic factors.

As also affects the neurotransmitter metabolism by increasing AchE activity and glutamate decarboxylase (GAD) mRNA expression. The AchE has been suggested as potential biomarker of Arsenic neurotoxicity (Patlolla and Tchounwou 2005). The toxic effects of As in the brain could be attributable not only to the change of neurotransmitter but also direct action on oxidative stress the mode of action is represented in Figure 5. In vivo study in rat from prenatal to early life stage has found that As exposure produces imbalance in defensive antioxidative mechanism and neuro transmitter metabolism in the hippocampus region of brain (Xi et al. 2010), in this region As reduces GSH, glutathione peroxide (GPx) and glutathione synthase(GS) activity and elevate the lipid peroxidation at postnatal day 0, 28, and 42 respectively (Rigon et al. 2008). In As related oxidative stress the released ROS and lipid peroxidation elements increases the activity of SOD and decreases the glutathione-related enzymes, which leads to change in the cellular redox status (Rao and Avani 2004). Biochemically, As mediated toxicity involves the induction of apopotic factors in the cerebral neurons by activating p38 mitogen-activated protein kinase (P^{38} MAPK) and c-Jun N-terminal kinase 3 (JNK3) (Namgung and Xia 2001), which enhances DNA damage and subsequently the brain cell death (Felix et al. 2005) resulting impairments of neurobehavioral function (Prakash et al. 2015).

4.4. Mode of neurotoxicity of MeHg

Generally exposure to mercury can takes place as IHg, MeHg. The distribution, toxicity and metabolism of mercury is greatly dependent on its chemical form (Aschner et al. 2007). Among all chemical forms, MeHg has the higher distribution rate than other form of mercury due to the lipophilic nature, long half-life ($t_{1/2}$ =70 days) (Clarkson et al. 2003) and MeHg freely reaches brain (Clarkson and Magos 2006). In early life stage, fetal brain is more susceptible than the adult brain, because MeHg easily crosses the placental barrier thereby easily reaching the fetus (Björnberg et al. 2003). MeHg is also found in the mother milk, exposing newborn during the breastfeeding period (Grandjean et al. 1994). Johansson et al., (2007) ascertained that developmental exposure of MeHg has long term consequences in brain. Neurotoxicity expressions of MeHg are based upon interaction with cellular elements such as neurotransmitter, disruption of microtubules, and alteration of intracellular Ca^{+2} ion homeostasis (LeBel et al. 1990). Biochemically MeHg has high affinity to sulphur (-SH) containing molecules, mainly targeting cysteine and methionine-containing proteins (Suzuki et al. 1976).

The MeHg mode of action in neuronal synapsis started by inhibiting astrocytic glutamate (Glu) uptake process and then stimulates its efflux from cytosol vesicles, resulting the Glu concentration in the extracellular fluid raised (Brookes and Kristt 1989; Dave et al. 1994). Brookes et al., (1992) reported that Hg^{+2} ion markedly inhibits the clearance of extracellular Glu in astrocyte cultures and spinal cord cultures and does not impair the sensitivity of neurons to the excitotoxic action of Glu (Brookes 1992). The enhanced Glu targets Glu based NMDA receptor in brain (Choi 1992) resulting in receptor hyperactivation and raises the Na^{+} and Ca^{+2} influx in neuronal cells (Lafon-Cazal et al. 1993). The elevated Ca^{+2} act as a second messenger causing alterations of protein phosphorylation (Sarafian 1993). On the other hand, MeHg directly disrupts the mitochondrial activity by generating the uncontrolled release of Ca^{+2} from the mitochondria and inhibiting the mitochondrial enzymes function and phosphorylation (Atchison and Hare 1994). Yee et al.,(1996) reported that MeHg inhibits the mitochondrial electron transport chain (ETC) in brain cultured cell line. In another interesting study Farina et al. 2011a found that MeHg exhibits a direct inhibitory effect on the activity of GP_x in mouse CNS resulting to increases lipid peroxidation and decreases Glu uptake into cerebrocortical slices (Farina et al. 2005). Further, it has been reported that production of NO (nitric oxide) following microglial activation causes a decline in cellular GSH levels (Moss and Bates 2001). MeHg has high affinity to bind GSH (Franco et al. 2007) resulting in weakening of antioxidant level (Johansson et al. 2007a). In the mitochondria, MeHg raises the inner membrane action potential which up-regulate the hydroxyl, superoxide, peroxide (OH, $O_2^{-\cdot}$ H_2O_2) and simultaneously down regulate defensive enzymes such as SOD, catalase, GP_x, GR functions, resulting in oxidative stress (Mori et al. 2007). Experimental evidence found that 48 hr after a single injection of MeHg (1 mg/kg, intraperitoneal) in mice and 1 week after a single injection of MeHg (5 mg/kg, intraperitoneal) in mice, increased significantly the rate of formation of ROS in both rat and mouse cerebellum (Ali et al. 1992).

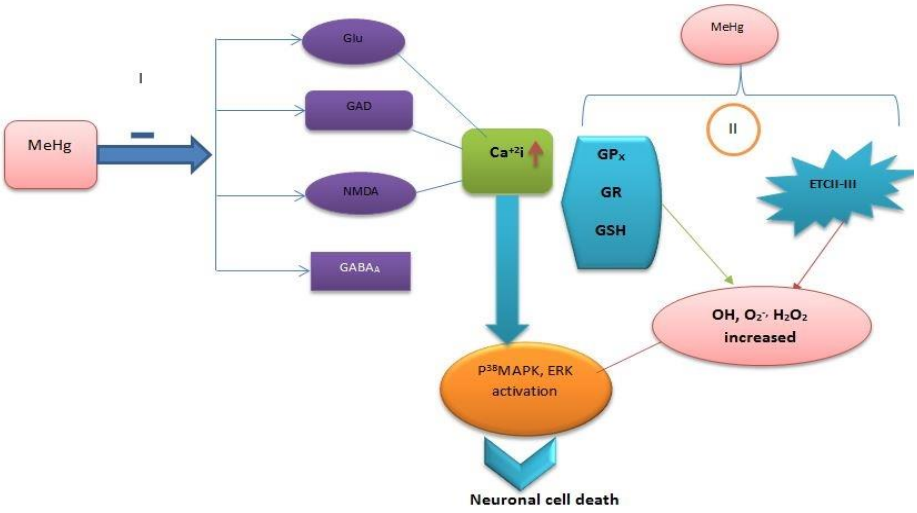

Figure 6: The mode of action methyl mercury (MeHg) by two pathway I. Neurons physiological functions and II. Reductive defensive mechanism (– sign indicates inhibition)

5. Analysing the metal mixture (Pb, Cd, MeHg & As) mode of toxicity in brain (Hippocampus)

In previous experimental studies (Wang and Fowler 2008; Wright and Baccarelli 2007; Zheng et al. 2003), individual metal neurotoxicity was observed. Some studies carried out with binary metal mixture showed that metal mixture has more strength to produce common outcome such as cognitive dysfunction. For instance, Gu et al., (2008) concluded that Pb and Cd binary mixture has greater than additive effect on divalent metal transporter (DMT1) protein synthesis in the developing rat brain, which results in enhanced metal transport rate and finally higher cognitive dysfunction. Similarly, experiment on pregnant rat showed that combined exposure of Cd and Pb have additive effect on decreasing Na^+ /K^+ -ATP ase function, in which Cd activity is potentiated by Pb for causing failure of the Na^+/K^+-ATPase pump. The Na^+/K^+-ATPase pump inhibition generates depletion of intracellular K^+, accumulation of intracellular Na^+ and increases in intracellular free Ca^{+2} resulting in intensified cognitive dysfunction (Antonio et al. 2003). In another Pb and As binary mixture study in the brain, Mejia et al.,(1997) ascertained that the binary mixture affects the hippocampus by drastically enhanced mode of action of Pb in presence of As (Mejía et al. 1997). Rai et al., (2010) observed that the Pb, As and Cd ternary mixture triggers the release of intracellular Ca^{+2}, generates the ROS, stimulates the extra cellular signal-regulated pathway (ERK), c-Jun N-terminal kinases (JNK) pathway, and mitogen-activated protein kinase3

(MAPK3), results in neuronal oxidative stress. However, it was not proved the ternary mixture and cognitive dysfunction relation.

The above evidence strengthens effects of Pb, Cd, As, and MeHg mixture, which produces common adverse outcome termed as cognitive dysfunction or loss of memory by influencing the different interactions and common interactions in hippocampus. The exact toxicological mechanisms invoked by exposure to mixtures of the metals Pb, Cd, As and MeHg are still unclear. It is also found that each metal share common cellular elements such as receptors, enzymes, neurotransmitters for causing cognitive dysfunction. Therefore, it can be safely assumed that the modes of actions of individual metal toxicity can be used as a baseline for understanding potential mechanisms associated with mixture (Pb, Cd, MeHg and As). Further we hypothesize that concurrent exposure to Pb, Cd, As. and MeHg may have greater-than additive/synergistic toxic responses to cause cognitive dysfunction. Metal mixture containing Pb, Cd, As and MeHg, have high permeability to blood-brain barrier. However, these metals have specific mode of action for example Pb interacts with NMDA receptor by two different mechanisms. Guilarte et al.,(2001) reported that hippocampal expression of NR1/NR2A receptor assemblies may alter the physiological properties of NMDA receptor during brain development, resulting in deficits in LTP. Cd mainly inhibit the Na^{+} /K^{+} -ATP ase pump and increases the intracellular Ca^{+2} ion, produces neuronal cell stress (Méndez-Armenta and Ríos 2007c). Studies in the rat showed, As impacts the synaptic activity of neurons localized in the hippocampus by inhibiting the NMDA function similar to Pb and upregulating the AchE function. MeHg inhibits the GAD, Glu transporter affecting the Glu uptake and NMDA over expression (Farina et al. 2011). MeHg also disrupts the microtubules in the brain due to high affinity to binding the sulphur containing amino acids(-SH (Farina et al. 2011).

Using this hypothesis, in the following Figure 7, we present common mode of actions of Pb, Cd, As and MeHg in the hippocampus region of brain. Figure 7 emphasizes the common links between Pb, Cd, As, and MeHg to cause the cognitive dysfunction. Pb^{+2} have been shown to interfere with the Glu transmission and may disrupt the NMDA expression in synaptic and extra synaptic region. Cd^{+2} have shown to interfere with Na^{+}/K^{+} -ATP ase and biological Ca^{+2}. As has the property to bind with AchE, and GAD, MeHg has high affinity to Na^{+} / K^{+} -ATP ase enzyme, extra synaptic NMDA receptor, Glu and biological Ca^{+2}. The functional interaction of the four metals seems to cause common adverse outcome by influencing the anti-oxidant elements status.

The dynamic interaction of metals Pb, Cd, As, and MeHg with neurochemicals may alter the essential neuronal cell integrity by down-regulation of elements such as n-NOS, MT-III, catalase, SOD, BDNF, GP_x, GSH, and GS. Therefore, common susceptibility factor of these four metals (Pb, Cd, As, and MeHg) is the imbalance between the defensive elements and reactive oxygen species which is called as oxidative stress. The oxidative stress leads to programmed neuronal cell death by cellular

signalling pathways which results in cognitive dysfunction (Ceccatelli et al. 2010; Gavazzo et al. 2008; Stackelberg 2015; Wang and Du 2013; Wright and Baccarelli 2007).

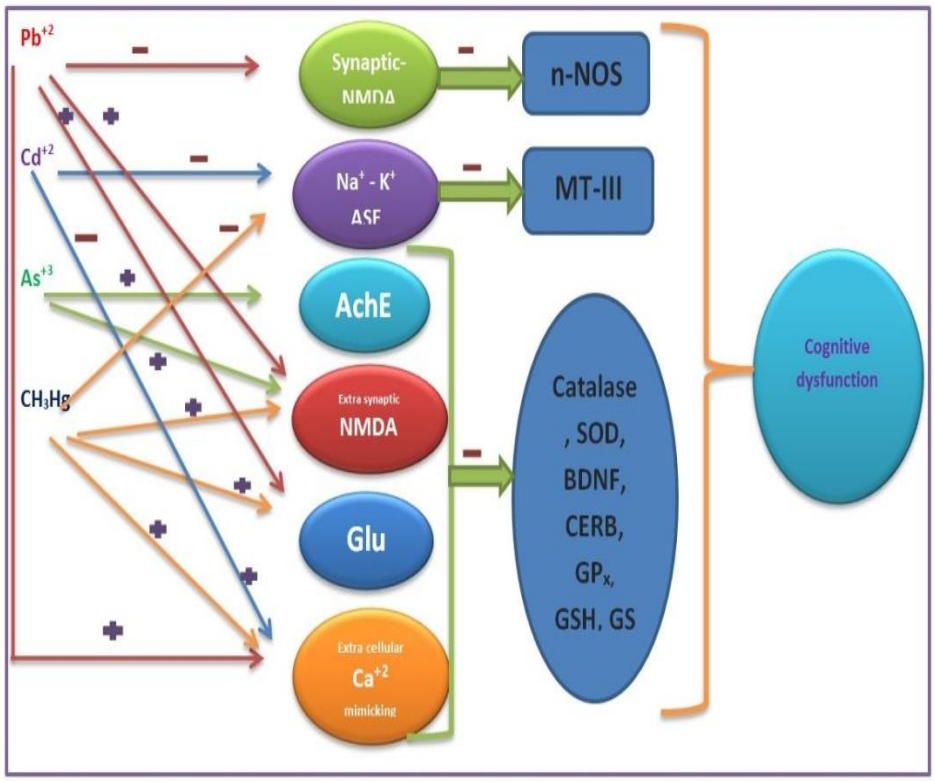

Figure 7: Metal mixture common mode of actions in brain, interactions with receptor (NMDA), enzyme (Na^+- K^+ ATP ase, AchE), ion (Ca^{+2}), alter that the n-NOS, MT-III, Catalase, SOD , BDNF, CERB, GPx , GSH, and GS level results in a cognitive dysfunction.

6. Conclusion and Future Perspective

We have reviewed the neurotoxicity of Pb, Cd, As, and MeHg extensively and found that prolonged exposure of these environmental toxicants have extreme susceptibility to the brain. During this review, we explored each of these metals modes of action on brain hippocampus region based upon disease outcome evidence. With the available information, we found clear correlation between metals and cognitive adverse effects. We also found many piece of evidences from in vivo interaction studies of metal and biochemical molecules in neurons which points to the consistent similarity with down-regulation of homeostasis of neuron functionality. .

In the individual metal mode of action studies, Pb, As, and MeHg has been found as potent neurotoxicants. However, recently some studies have proved that Cd also has neurotoxic character by mimicking the biological cellular Ca^{+2} and potentially inhibiting the Na^{+}/K^{+} -ATPase ion transporter in neuronal cell membrane. This behaviour of Cd may influence the protective elements such as BDNF, MT-III, and deprivation of protective elements leading to neuronal cell apoptosis. Pb interferes with neuronal functions by modulating the NMDA receptor subunit expression which results in alteration of neuronal plasticity by down-regulation of n-NOS and PLC upregulation causing neuronal cell apoptosis. Even in low concentrations, As produces cognitive deficit by modulating the NMDA receptor and significantly deactivate the defensive enzymes (anti-oxidants) such as GP_x, GS and GSH leads to programmed cell death. In case of mercury compounds, many chemical forms are present, but MeHg has high capacity to target hippocampus region by two possible mechanisms. First one is interacting with Glu, GAD, NMDA receptor, another is intracellular disruption, due to high affinity to sulphur containing (-SH) enzymes and microtubules, resulting in ROS upregulation which leads to cell death.

The exact toxicological mechanisms invoked by exposure to mixtures of the metals Pb, Cd, As, and MeHg are still unclear. Chemically heavy metals are polar in nature and this may be the reason for associations with common neuronal elements. However, until now, no study was found to prove mechanistic view of metal mixture external exposure and organ/brain internal exposure relation for finding the common disease end point. There is a need of more focused investigation to know the common mechanism of metal mixture causing neuronal damage.

Recommendations for study design and evaluation of combined effects of metal mixtures (binary, ternary, quartnary) are unknown due to lack of information and experimental studies on mixtures were often not well designed. It was observed that one of the disadvantages of experimental studies is the expensive large combinatorial in vivo studies involving animals. Furthermore regulatory frameworks such as REACH in the EU are becoming more and more critical regarding the use of animal testing (EC, 2013b). Therefore, better predictive tools are needed to use limited in vivo study and enable in vitro data on toxicological effects to be interpreted for wider mixtures study and comprehensive risk assessment of mixtures.

After reviewing and analysing the common neurotoxicity mechanism of the heavy metals (Pb, Cd, As, and MeHg), It is practically impossible to test all these possible metals mixtures (binary, ternary, quartnary) experimentally, especially in vivo. Therefore, rational and alternative strategies are needed to assess the metal mixtures toxicity in brain (Hippocampus). Ideally these tools should be more robust in providing the necessary neurotoxic information of defined metal mixtures (El-Masri 2007). The concentration addition (CA) approach could be helpful for finding the combination of mixtures (binary, ternary and quaternary) risk assessment in target based toxicity

(Cedergreen 2014), in our review we mentioned the biological target site of metal mixtures is similar. Adverse Outcome Pathway (AOPs) might provide insight into the relevance of combinational effects when assessing the toxicity of mixtures (Caldwell et al. 2014). Invitro mechanistic assays can be used to elucidate the mixtures mechanism of action in broader context (Ankley et al. 2010). Omics based in vitro methodology increasingly applied to gain insight in the mechanism of action of mixtures in transcription level, the protein level, and a metabolome level (Borgert 2007). In mixture study omics results help in generating the possible interactions between components. Finally user-friendly Insilco PBPK/PD, models may be helpful for studying possible interactions between metals of particular interest. PBPK/PD are built using the body as a set of interconnected compartments of differential mathematical equations describing the absorption, distribution, metabolism, elimination (ADME) of a specific chemical and/or its metabolite, and then they connect the internal dose to the dose response of the adverse dynamic effect for the metal (WHO 2010). Generally speaking, PBPK/PD models can provide a tool to estimate the internal concentration of a chemical and also useful for establishing tolerance levels for mixtures of neurotoxicity (Tan et al. 2011). In the past few decades, numerous individual PBPK or metal kinetic models have been developed for Pb, Cd, As, MeHg (Yu 1999; Carrier et al. 2001; O'Flaherty 1991). One of the early pioneer of metal research, O'Flaherty had stated in his work of 1998 that further work from mechanistic point of view and experimental data are needed to support further development and refinement of these models (O'Flaherty 1998). Generally these individual kinetic models have different set of modelling assumptions and lack interaction mechanism even at kinetic stage (such as metabolism). Integration of these individual models is difficult for mixtures toxicity estimation. However, we greatly appreciate the individual metal neurotoxicity studies, which is a key line for modelling design and development. These studies help in understanding the metal mixtures (binary, ternary, and quaternary) and disease relation by application of predictive mixture modelling framework such as PBPK/PD. Recently some attempt has been made to integrate these models (Sasso et al. 2010). However, mostly loose integration without harmonisation of modelling assumption. So far, comprehensive mixture modelling efforts have not been pursued in the field of toxic metal, despite ample evidence of interactions of these toxic metals and with other essential elements. Advance predictive model like PBPK/PD may be very useful since they provide a highly refined tool, in which it should be possible to reduce uncertainty for higher tier risk assessments of single and multiple chemicals. The availability of commonly accepted user-friendly physiological based modelling platform is crucial for toxicological study (Ashauer et al. 2011). The above all alternative novel methods contribute to the 3R (Replacement, Reduction and Refinement) principal for more human relevant approach to the risk assessment of metal mixtures.

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Chapter 2

Karri V, Kumar V, Ramos D, Oliveira E, Schuhmacher M, Comparative In Vitro Toxicity Evaluation of Heavy Metals (Lead, Cadmium, Arsenic, and Methylmercury) on HT-22 Hippocampal Cell Line.
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Chapter 2

Chapter 2

Comparative In vitro Toxicity Evaluation of Heavy metals (Lead, Cadmium, Arsenic, and Methylmercury) on HT -22 Hippocampal Cell line

Abstract: Heavy metals are considered some of the most toxic environmental pollutants. Exposure to heavy metals including lead (Pb), cadmium (Cd), arsenic (As), and methyl mercury (MeHg) have long been known to cause damage to human health. Many recent studies have supported the hippocampus as the major target for these four metals for inflicting cognitive dysfunction. In the present study, we proposed hippocampal relevant in vitro toxicity of Pb, Cd, As, and MeHg in HT- 22 cell line. This study reports, initially cytotoxic effects in acute, subchronic, chronic exposures. We further investigated the mechanistic potency of DNA damage and apoptosis damage with the observed cytotoxicity. The genotoxicity and apoptosis were measured by using the comet assay, annexin-V FTIC / propidium iodide (PI) assay respectively. The results of cytotoxicity assay clearly demonstrated significant concentration and time dependent effects on HT-22 cell line. The genotoxic and apoptosis effects also concentration dependent fashion with respect to their potency in the range of IC_{10} –$IC_{30,}$ maximal level of damage observed in MeHg. In conclusion, the obtained result suggests concentration and potency dependent response; the maximal level of toxicity was observed in MeHg. These novel findings support that Pb, Cd, As, and MeHg induces cytotoxic, genotoxic, and apoptotic effects on HT-22 cells in potency dependent manner; MeHg> As> Cd> Pb. Therefore, the toxicity of Pb, Cd, As, and MeHg could be useful for knowing the common underlying molecular mechanism, and also for estimating the mixture impacts on HT-22 cell line.

Key words: Heavy metals in vitro toxicity; Dose response; HT-22 cell line; DNA damage; Apoptosis.

Abbreviations: Ach E = Acetyl cholinesterase E, ANOVA = Analysis of variance, As = Arsenic, DAPI = 4′, 6-diamidino-2-phenylindole , DMEM = Dulbecco's modified Eagle's medium, FBS = Fetal bovine serum, GABA = γ-gamma-amino butyric acid, GAD = Glutamate decarboxylase, IC_{50} = Inhibitory concentration 50, LTP = Long- term potentiation, MeHg = Methyl mercury, MMS = Monomethyl sulfonate, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NMDA = N-methyl-D-aspartate, OD = Optical density, Pb = Lead, PI = Propidium iodide, PS = Phosphatidylserine, SD = Standard deviation, WHO = World health organization.

Chapter 2

1. Introduction

Heavy metals are environmental pollutants of great concern because of their persistent occurrence arising from increasing industrialisation, and other anthropogenic activities [1–3]. Exposure to heavy metal including lead (Pb), cadmium (Cd), arsenic (As), and mercury including methylmercury (MeHg) has long been known to be hazardous to human health [4, 5]. Lead (Pb) exposure is widespread with broad use as major components in many commercial products such as lead-based paint batteries [6–8]. Cadmium (Cd) is a widespread industrial and environmental pollutant. Fumes generated during industrial operations [9, 10] and cigarette smoking containing Cd represents a significant source of exposure which is mainly absorbed via inhalation route [11]. However, a low amount of Cd in dietary food and its gastrointestinal absorption is found to be very low [12]. Contaminated drinking water is the main source of human exposure to arsenic (As); other less common sources of As exposure include coal combustion and food particularly in U.S [13–15]. Mercury is released into the environment primarily through the burning of fossil fuels, such as coal. The released mercury contaminates the surface waters, and enters the aquatic ecosystem as MeHg. MeHg is formed from inorganic mercury by the action of microbes that live in aquatic systems and with longer half-life biomagnified in aquatic food chains [17]. Consumption of contaminated fish and other aquatic seafood is the primary source of MeHg exposure to humans [16]. MeHg has a specific concern in human because it readily absorbed from the gastrointestinal tract (GI), and is actively transported across the blood brain barrier (BBB) [17, 18]. The affected organ systems by these four metals are renal, pulmonary, hepatic, gastrointestinal and haematological systems along with peripheral and central nervous systems [17]. Our previous study showed that Pb, Cd, As, and MeHg disrupts the hippocampus by inflicting the cognitive dysfunction in both children and adults [18].

Exposure to MeHg and Pb has a significant effect on the human brain and are well known to targeting the central nervous system [19–22]. A specific population such as children are more susceptible concerning their development phase of the nervous system [23–25]. Exposure to Cd also severely affects the function of the nervous system, leading to Parkinson disease and learning disabilities [26, 27]. However, the exact mechanism and its neurotoxic effects are still ambiguous [28, 29]. The adverse effects of inorganic As on health are well known and include cancer, skin lesions, and lung diseases [30–32]. Many previous studies have been made that links As exposure with developmental neurotoxicity disorders [33–35]. The toxicity level of Pb, Cd, As, and MeHg are high in humans thus, these four elements rank among the priority metals that are of great public health significance [36]. We reported that Pb, Cd, As, and MeHg exposure has the potency to damage the hippocampus region of brain by interacting with different molecular mechanisms. For instance; Pb interacts with N-methyl D-aspartate (NMDA) receptor [37], MeHg indirectly affects NMDA receptors functioning via inhibiting glutamate decarboxylase (GAD) and glutamate (Glu) transporter [38]. Cd inhibits the Na^+ / K^+ - ATP ase [39]. Arsenic suppresses the NMDA receptors activity in

the hippocampus, where these receptors play a pivotal role in synaptic plasticity, learning and memory [34, 40].

The main objective of this study was to find the individual metal (Pb, Cd, As, and MeHg) toxicity on HT-22 hippocampal cell line in a comparative manner. In this study, we considered a well-established mammalian HT-22 hippocampal cell line to be more relevant for the model of hippocampal toxicity. For elaborating the hippocampal toxicity hypothesis, initially we performed cell viability via MTT assay of Pb, Cd, As, and MeHg in mice HT-22 hippocampal cell line to different exposure scenarios such as acute (1 day), subchronic (3 days) and chronic (8 days). Further, we extended to genotoxicity and apoptosis studies by using low toxic concentrations. These two assays were helpful for elucidating genotoxicity and apoptosis mechanism of heavy metals in HT-22 cells during chronic exposure.

2. Materials and Methods

2.1. Chemicals and Media

Lead chloride ($PbCl_2$ [CAS no: 7758-95-4]), Sodium metaarsenite ($NaAsO_2$ [CAS no: 7784-46-5]), Cadmium chloride ($CdCl_2$ [CAS no: 10108-64-2]), Methyl mercury chloride ($MeHgCl_2$ [CAS no: 115-09-3]), Dimethyl sulphoxide (DMSO [D5879]), 3- (4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT [M5655]), trypsin (TrypLE [Gibco: 12604013]), all are analytical grade and purchased from Sigma-Aldrich Química, S.L- Madrid (Spain).

2.2. Cell line and Reagents

Among various research tools, neuronal cell lines are the most commonly used in vitro model for relevant mechanistic studies. With particular concerns for memory and Alzheimer disease related studies, hippocampal neuronal cell lines are very limited. HT-22 is one of the cell line sub cloned from its parent line HT4, which are immortalized mouse hippocampal neuronal precursor cells [41].

The HT-22 cells have been used as a hippocampal neuronal cell model in numerous studies. The HT-22 cells were a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA). HT-22 cells were maintained in Dulbecco's modified Eagle's medium (DMEM [D6429]) containing 10% fetal bovine serum (FBS Gibco [10500-064]) and 100 U/mL penicillin (Pan-Biotech- Germany), and 100 µg/mL streptomycin (Pan-Biotech- Germany) in a humidified incubator with 5% CO_2 in air at 37^0 C. For all the experiments cells were grown at 70-80% confluence. We have checked the cell confluence in different time intervals (1 day, 3 days, and 8days); confluence was stable until 8 days thus considered as chronic exposure.

The cells were cultured in 75 cm^2 cell culture flasks. For experimental purpose, cells were seeded at 1 x 10^6 cells/ mL (acute), 0.57 x 10^6 cells/mL (subchronic & chronic) in 96 well plates and grown for 24 hours before metal treatment. Duplicates wells of cells

were treated with 10 exposure levels of Pb, Cd, As and, MeHg ranging from 10 to 100 µM, 0.5 to 7 µM, 0.4 to 4.6 µM, 0.6 to 12 µM, respectively, for acute (1 day), subchronic (3 days) and chronic (8 days) assays. In subchronic and chronic exposure, the medium containing given metal concentration was refreshed at every 2 days interval for maintaining metal exposure. Metal stock solutions 100X were prepared in deionized distilled water (for poorly soluble $PbCl_2$ < 0.5% DMSO added) and sterilized by filtration through 0.2 µm, and different concentrations of a working solution of each individual metal was prepared by prior dilution of the stock solution in phosphate buffer saline (PBS, pH = 7.4). The applying solution contains 10 % metal solution and 90% DMEM culture medium.

2.3. Analysis of Cell viability by MTT assay

The MTT assay was carried out using a modification of the method of Mossman (1983) [42]. MTT reagent is taken up into cells and reduced in mitochondria dependent reaction to yield a formazan product. The product accumulates within the cell, due to the fact that it cannot pass through the plasma membrane. MTT was dissolved at 5 mg/mL in phosphate buffered saline. This stock solution was filtered through a 0.2 µm filter and stored at -80°C. After the incubation period, the medium was aspirated from well and MTT working solution at 0.5 mg/ mL was added to each well. Cells were incubated at 37°C for 3 hours; after this time, the MTT was removed by aspiration. Formazan products were dissolved in 100 µL of DMSO and placed the plates on a shaker and agitated for 5 min. The absorbance of the solubilized reduced MTT was then measured in a micro titter plate spectrophotometer reader at a wavelength of 570 nm. The measured absorbance or optical density (OD) values were converted to percent of cell viability (%) with respect to control. Cell viability (%) = [Absorbance of treatment / Absorbance of Control] x 100.

2.4. Analysis of DNA damage by Comet assay

To measure the DNA damage induced by Pb, Cd, As, and MeHg at low concentrations (IC_{10} to IC_{30}), the alkaline comet assay was conducted according to the OECD guidelines with some modification. To determine the genotoxic potential of Pb, Cd, As, and MeHg in HT-22 cells, cells were seeded in 96 well plate (1×10^6) and incubated at 37 °C in 5% CO_2 for 24 hours for cells attachment. To knowing the genotoxicity effect, we applied the low / chronic IC_{10} - IC_{30} concentration of Pb, Cd, As, and MeHg to the cells during 24 hours, also Monomethyl sulfonate (MMS) 400 µM was used as positive control. After incubation, each well of cells was washed with 50 µL PBS (pH = 7.4), then added 50 µL trypsin and double the amount of DMEM (100 µL). The collected cells were centrifuged at 3000 rpm for 3 min at 4 °C. The supernatant was discarded and the cell pellets were collected. Next, 160 mL of low melting point agarose (LMP – 0.5%) was added to the tube and mixed with the cells. This mixture was deposited on pre-gelatinized slides (normal melting point agarose, 1.5%) and then slides were placed in lysis solution for at least 1 hour for electrophoresis. After denaturation (20 min) and alkaline electrophoresis (25 V, 300 mA, 20 min); the slides were neutralized, fixed and kept refrigerated until the time of analysis. The slides were stained with DAPI (4′, 6-

diamidino-2-phenylindole), inspected visually and analyzed using the Nikon epifluorescence microscope, comet analysis scoring system (Comet assay - IV) software (Perceptive instrument, UK). In DAPI staining comets showed different levels of DNA damage and found only a slight decrease in total fluorescence with an increasing fraction of DNA in the tail. The most commonly used parameters for measuring the genotoxicity are tail length, the relative fluorescence intensity of head and tail (normally expressed as a percentage of DNA in the tail), and tail moment [43]. Collins et al. reported that tail intensity is a most rational parameter for measuring the DNA damage even in low dose exposure, and analysis of 50 comets per slide is recommended [44]. The experiment was repeated three times.

Cell viability was determined along with the comet assay for each treatment. After the treatment, the cells were harvested by trypsinization, and 20 µL of the cell suspension was mixed with 20 µL of trypan blue (Gibco) in 1.5 mL micro tube. The cells were counted in a neuabauer chamber using a light microscope. The viability found was more than 80% in each exposure.

2.5. Analysis of apoptosis by Annexin V-FITC/Propidium iodide (PI) staining

To evaluate the translocation of phosphatidylserine (PS) from inner leaflets to outer leaflets of plasma membrane, Annexin V- FITC apoptosis detection kit (BD Pharmingen, Poland) was utilized. In this kit, Annexin V and Propidium iodide (PI) were used to distinguish the apoptotic and necrotic cells. According to the manufacture's protocol, the exponentially proliferating cells were exposed to the designed doses (IC_{10}, IC_{20}, IC_{25}, and IC_{30}) of heavy metals in 12 well plates at a density of 0.56 x 10^6/ mL for 8 days and control cells were made without chemical. The medium with metal concentration was refreshed every 2 days. After chronic treatment with metals, cells were harvested by trypsinization, washed twice with ice cold PBS (p^H = 7.4). Thereafter, cells were centrifuged at 1200 rpm for 5 min at 4^0C, resuspended in 1mL 1X binding buffer and then transfer the 100 µL of the solution to 5 mL culture tube, and was added 5 µL of both Annexin-V and PI to the samples. After staining, the cells were incubated for 15 minutes in the dark at room temperature. Cells were re-washed with 1X binding buffer 400 µL and analyzed by flow cytometry (Beckman coulter, Germany).

2.6. Statistical analysis

All experiments were performed three times (n=3) and each concentration was tested in a duplicate manner. The results were given as mean ± standard deviation (SD). IC_{10} to IC_{30} and IC_{50} values were calculated from dose response curve fitted by using the Graph pad prism version 5.01. The results of MTT assay were analysed by two way ANOVA and Tukey's test; P**** values < 0.0001 were considered as statistically significant. The results of genotoxicity and apoptosis were analysed using one - way ANOVA and Dunnett's test; P*** values ≤ 0.05 were considered as statistically significant.

3. Results and Discussion

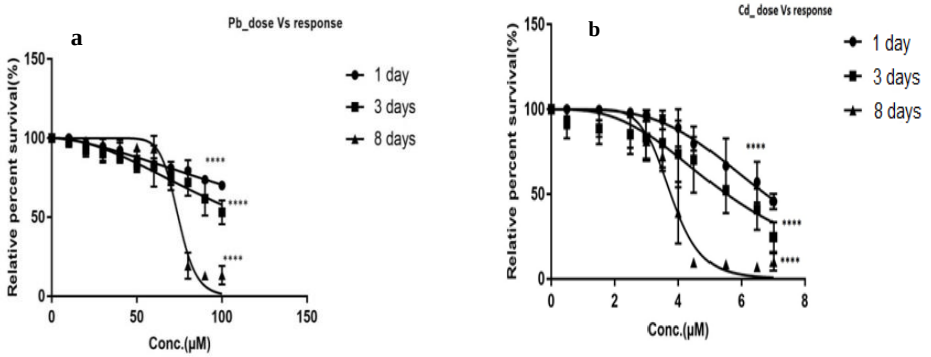
3.1. Dose response relationship of heavy metals (Pb, Cd, As, and MeHg) on HT-22 cell line

To characterize the effects of Pb, Cd, As, and MeHg individually on hippocampus during different exposures, we performed cell viability studies in mice HT-22 hippocampal cells by using the MTT assay. As expected, all 4 metals showed a concentration, time - dependent cytotoxic effect, expressed by decreased absorbance or optical density (OD). Results for Pb, Cd, As, and MeHg concentration response experiments at acute (1 day), subchronic (3 days), and chronic (8 days) exposures are presented in Figure 1 (A, B, C and D). The results showed significant concentration and time depended effects. Two-way ANOVA test showed a statistically significant effect of time and concentration in Pb, Cd, As, and MeHg toxicity ($p^{****} < 0.0001$). The mean IC_{50} values are presented in Table 1 which is defined as the concentration required for inhibiting the level of MTT by 50% compared to the non-exposed control.

Firstly Pb concentration - response curves appeared to be similar for the acute and subchronic exposure scenario and cell death was observed very low even at high concentrations. However, during chronic exposure, cell death was significantly higher at concentrations > 70 µM, indicating that sensitivity of Pb towards hippocampal cells is time depended. It can be seen from Table 1, that Pb toxicity in acute exposure was very low for which we were unable to accurately determine the IC_{50} concentration in experiment. In subchronic and chronic exposure the IC_{50} was determined to be 117.6 µM and 74.3 µM respectively. The obtained results suggest that Pb induced damage in HT - 22 cells at low conc. (10-60 µM) has no effect on MTT reduction / cells at any time point (Figure 1, a). It showed that Pb could gradually decrease the viability of HT-22 cells and this result was consistent with reported studies [45]. In acute exposure, the maximum cell death 30% was observed at 100 µM, which is in agreement with Pb induced cell death in cortical cell cultures [46]. In case of cytotoxicity of Cd in HT-22 cells, a decrease in the viability of cells with increasing exposure time and concentration (Figure 1, b) can be observed. Cells exposed to Cd concentrations from 4.5 to 7 µM exhibited statistically significant ($p^{****} < 0.0001$) decreases in the cell viability after acute exposure. Increasing incubation time to subchronic and chronic exposures, toxicity being observed between 3.5 to 7 µM is high. However, in acute exposure Cd did not affect the HT- 22 cells at 0.5 to 3.5 µM. At concentrations lower than 0.5 µM, Cd showed no effect at any of the time point. The chronic effect of Cd was more pronounced at 4 µM compared to the acute and subchronic exposures. We observed that the percentage of cell viability decreases with increasing Cd concentration reaching maximum cell death (90%) in chronic exposure than acute and subchronic. The toxic effects of Cd on HT-22 cells at different exposure times were further compared in terms of IC_{50} value at each time point as shown in in Table 1. Previously observed neurotoxic effects of Cd in various brain cell cultures are in agreement with the current HT-22 cells cytotoxic effects [47]. Cd shows inflection at 3.5 µM. (IC_{50} = 3.7 µM) for chronic

exposure. The response is clearly dose and time dependent. These results are similar to the reported dose dependent Cd cytotoxicity in neuronal glia cultures [48].

Concerning the cytotoxic effects of As on HT-22 cells, we can observe a linear concentration - response relationship (Figure 1, c). A gradual decrease in the cell viability with increasing a concentration in the acute and subchronic exposure can be observed. Increasing incubation time to chronic exposure resulted in increasing toxicity being observed between 0.8-1.2 µM. Moreover, for acute and subchronic assays the response was very low. In acute exposure, the response elevated at a concentration of 2.8 µM. Previous studies reported that As showed cytotoxicity even at a micromolar concentration [49] and similar results were observed in HT-22 cells. Other cell line studies showed that As induces cytotoxicity in human lung fibroblasts in dose dependent manner, which is consistent with current study [50]. The impact of MeHg in HT - 22 cells is provided in Figure. 1, d. In acute exposure from 0.6 to 1.8 µM cell death was very low, but for the same range of concentration in subchronic exposure, significantly greater cell death was observed. Two - way ANOVA test indicated statistically significant effect for time and concentration in MeHg toxicity (p**** < 0.0001). In chronic exposure even in the lowest concentration (0.6 µM) the cell death was significant. However, in acute exposure MeHg showed significant effects at a concentration of 2.4 µM. The obtained MeHg concentration- response curves differed with respect to their exposure duration; response curve has a similar slope in subchronic and chronic exposures than acute exposure. The curve inflection also drastically enhanced in chronic exposure when compared to the acute and sub-chronic exposures. However, the differences in the cell viability for acute and subchronic exposures are relatively narrow indicating that high potency of MeHg in hippocampal cells (HT-22). The obtained results consistent with MeHg toxicity studies in glioblastoma, neuroblastoma cultures [51] and cerebellar granule cells (CGC) [52].



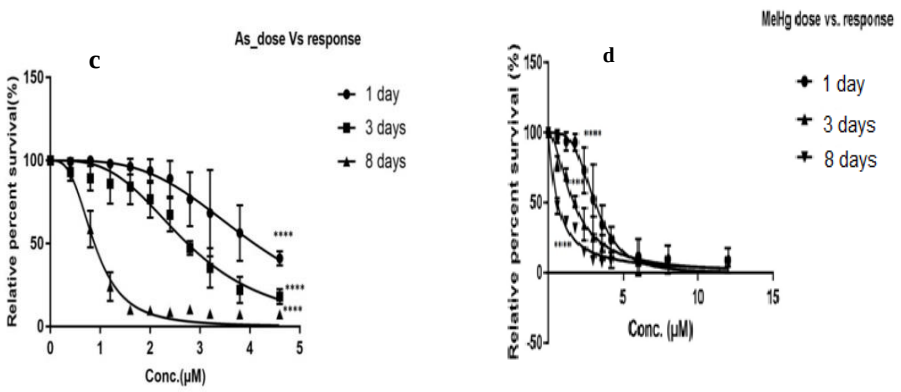


Figure 1: Cytotoxicity concentration response curve for Pb (a), Cd (b), As (c), and MeHg (d) after acute, subchronic, and chronic exposure. The two – way ANOVA followed by a Tukey's multiple comparison tests compared the control with all concentrations. Asterisks indicate significantly different in each exposure (p**** < 0.0001).

Table 1: The IC_{50} values of Pb, Cd, As, and MeHg for in vitro hippocampal HT-22 cell line after different exposure times in In vitro[a]

Exposure time	Heavy metals (IC_{50})			
	Pb (µM)	Cd (µM)	MeHg (µM)	As (µM)
Acute (1 day)	172.2	6.7	3.1	4.0
Subchronic (3 days)	117.6	5.5	1.7	2.7
Chronic (8 days)	74.3	3.7	0.6	0.8

([a]Values of IC_{50} with 95% confidence intervals from curves shown in Figure 1, Cytotoxicity was evaluated by inhibition of MTT reduction as described in the methods).

The obtained MTT assay results showed that the dose response curves for Cd, As, and MeHg are different from Pb. This difference could be due to the different uptake mechanisms of heavy metals by the HT-22 cells. Our results suggest that the sensitivity of metals is similar in all exposure scenarios, which indicate that the cell up taking concentration always depends on the type of metal. For the Pb, Cd, As metals we observed that at time dependent effect at low concentration, however at a high concentration a time independent effect. The toxicity mechanism of Pb in HT-22 cells is entirely different from Cd, As, MeHg and showing unique in dose response curve shape. In Pb, duration of exposure is a critical factor for reaching the toxic or lethal state concentration in cells. The studied four metals showed clear variation in acute and

chronic exposure rather than subchronic exposure; eventually the environmental exposure of heavy metals to humans has been decisive in continuous / chronic exposure. The obtained data showed that MeHg was the most toxic. However, the high toxicity of MeHg is due to its ability to be readily absorbed into the cells and potency [53]. Taking into account the above HT-22 cell line based dose response toxicity results and the literature of in vivo molecular pieces of evidence support that the heavy metals Pb, Cd, As and MeHg have strong affinity to damage the hippocampus [24, 26, 54–56]. The obtained chronic exposure MTT results were useful to extend the further mechanistic based assays such as DNA damage and apoptosis. So that cells were treated with a range of IC_{10} to IC_{30} concentration (µM), this concentration was calculated from the chronic dose response curve.

3.2. Genotoxic effects of Pb, Cd, As, and MeHg on HT-22 cells

For knowing the clear genotoxicity relation of heavy metals on HT-22 cells, we determined whether the heavy metal toxicity is associated with DNA damage in HT-22 cells by using comet assay. Comet assay is a rapid, simple, and sensitive technique used as a quantitative assessment of toxic effects on DNA damage. In general, effectively in vitro comet assay works after short term exposure due to the fact that cells have DNA repair mechanism used to correct DNA damages and moreover they can double themselves during long time exposure [57, 58]. To evaluate the heavy metals induced genotoxic / DNA damage in HT-22 cells; cells were treated with Pb, Cd, As, and MeHg in the range of IC_{10} to IC_{30} for 24 hours. The degree of DNA damage was quantified by the percentage of tail intensity (%). The representative comet assay images of controls and heavy metals treated HT-22 cells are presented in Figure 2. This Figure shows a significant increase in the percentage of DNA damage with respect to negative control. There was a concentration dependent increase in percentage of DNA damage. The mean percentage of DNA damage was 0.58 ± 0.02 in the negative control cells without metal/chemical treatment. For Pb, Cd, As, and MeHg at IC_{25} concentration (68.74, 3.26, 0.60, and 0.23 µM) the percentages of the DNA damage were 7.17 ± 0.05%, 6.24 ± 0.8%, 10.5 ± 1.2%, and 11.11 ± 2.5%, respectively. At IC_{30} concentration (70, 3.37, 0.66, 0.29 µM) treatment, the percentages were 11.11 ± 2.5%, 12.80 ± 1.4%, 13.46 ± 2.72 %, and 20.3 ± 2.6 for Pb, Cd, As, and MeHg, respectively. Overall, the DNA damage was significantly different ($p^{***} < 0.05$) from the control as compared to the treatment groups (Figure 3). The effect of MeHg was more pronounced than in the other three metals. The results generated from the comet assay indicated; heavy metals were able to induce the DNA fragmentation to HT-22 cell in low concentration during the 24 hrs. Quantitative histograms of the comet tail intensity (%) were represented in Figure 3; these data suggest that upon increased exposure concentration to HT-22 cells, the cellular DNA became more damaged. We found that these metals are able to produce genotoxicity during 24 hours exposure in a concentration dependent manner. Heavy metals have shown a strong comparable genotoxic potential and are able to cause DNA damage in the hippocampal cells in the range of IC_{10} - IC_{30}. Pb genotoxicity response depends on the type of cell line [59], Pb has the lowest potency to induce the DNA damage in the range of 63.5 - 70 µM on HT-22 cells. Sanders et al. also reported that

Pb induced DNA damge was very low in PC-22 cells [60]. Other studies have also indicated that Pb might cause DNA damage in vivo and in vitro and that the increased levels of DNA damage observed depends duration of exposure [61]. Robbiano et al. reported significant dose dependent increases in DNA damage in primary rat and human kidney cells by Pb[62]. The possible mechanism for the occurrence of DNA damage by the Pb might be increase the rate of free radical formation leading to DNA damage [63]. The other metal Cd has similar to Pb in 2.8 - 3.26 µM range, the significant DNA damage found at 3.25 µM. In reported studies Cd showed that DNA damage was a concentration - dependent manner in human liver carcinoma cells [64]. Other reports have shown evidence that reactive oxygen species (ROS) are involved in DNA damage induced by Cd [65]. As induced DNA damage in HT- 22 cells results showed significant increase in DNA damage which was consistent with previous studies [66]. Kumar et al. reported As induces DNA fragmentation in HL-60 cells in a dose dependent manner, similarly we propose that HT-22 cells exposed to As undergo DNA damage [67]. There is evidence that low - dose exposure (0.5 µM) to MeHg may lead to DNA damage in fibroblasts and Chinese hamster ovary cells [68]. Several studies have also observed mercury inhibiting the DNA repair system [69]. Grotto et al. demonstrated the genotoxic effects of MeHg in rats during chronic exposure [70]. We found that MeHg has high potency in HT-22; the dose dependent response was linear in range of 0.07-0.26 µM. Our results demonstrated that heavy metal induces DNA damage to HT-22 cells in a dose dependent fashion with respect to their potency, suggestive clear evidence that MeHg may be a potent DNA damaging agent against hippocampal cell line when used at even low dose. We are expecting several possible mechanisms might be involved in the induction of the DNA damages other than apoptosis due to the initiation of cell DNA damage during the 24 hrs. instead of 8 days [71]. Hartwig reported that reactive oxygen species (ROS) generation induced by heavy metals could be reason to genotoxic mechanisms in mammalian cells [72].

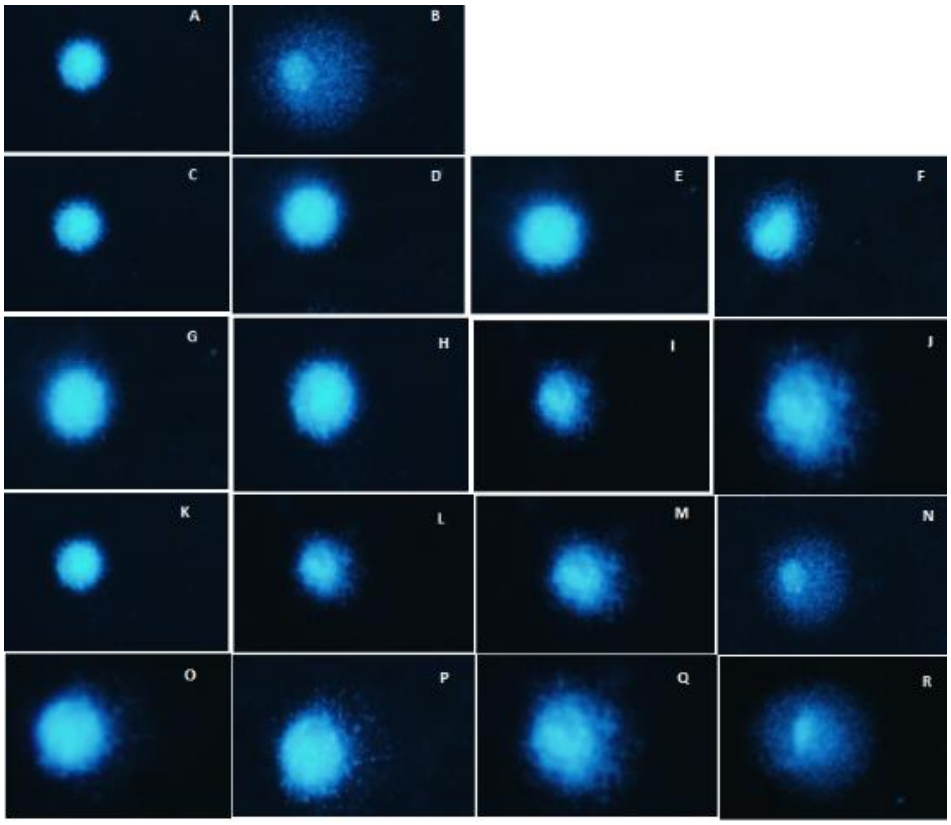


Figure 2: Representative Comet assay images of HT-22 cells. Untreated control (A), Positive control (MMS; B); Treated with: Pb (C to F) at 63.50, 67.34, 68.74, 70.00 µM; Cd (G to J) 2.80, 3.14, 3.26, 3.37 µM; As (K to N) at 0.42, 0.50, 0.60, 0.66 µM; MeHg (O to R) at 0.09, 0.18, 0.23, 0.29 µM.

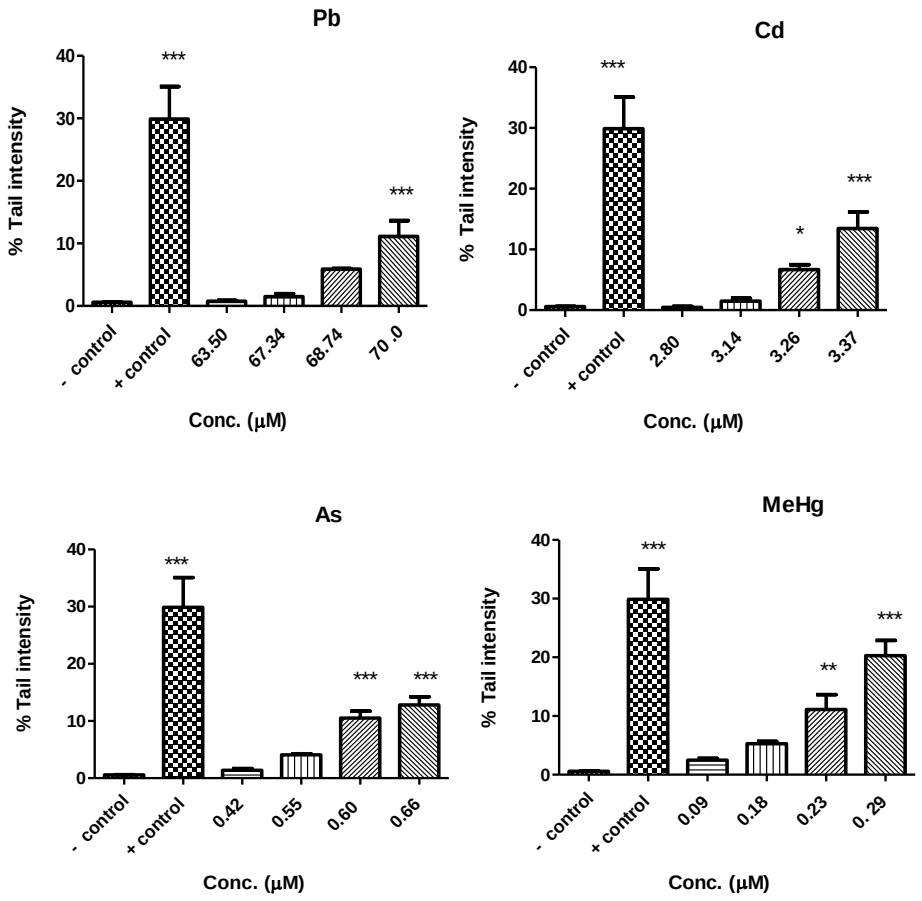


Figure 3: Effect of Pb, Cd, As, and MeHg on DNA in HT-22 cells as assessed with comet assay. Each value represents the tail intensity (% DNA damage), Mean ± SD of three independent experiments. Significantly different from the negative control (p*** < 0.05) according to the Dunnett's test.

3.3. Apoptosis effects of Pb, Cd, As, and MeHg on HT-22 cells

We carried out Pb, Cd, As, and MeHg; induced apoptosis in HT- 22 cells during chronic exposure. To accomplish this objective, we performed Annexin- V/ Propidium iodide (PI) staining that allows the discrimination of viable cell, apoptotic, and necrotic cells by binding with Ca^{+2} dependent phosphatidyl serine (PS) protein in cell membrane [73]. Apoptosis assay is helpful to gain insight into the heavy metal induced cell death mechanism in HT-22 cells. We found that heavy metals have different potency to induce apoptosis in HT-22 cells. Firstly Pb results in Figure 4.1 indicates, there was a slight trend to increased apoptotic cells (%) as concentration increases, but it was not statistically significant. However, at 70 µM response was relatively significant ($p^{***} <$ 0.05). The present study confirms the Pb could induce the apoptosis in HT-22 cells,

however the apoptosis response was very low in tested 63.5-70 µM range; it confirms that Pb shown low potency and need more concentration or exposure time for raising the response. Pb is reported, similarly like calcium to bind the internal metal binding site of the permeability transition pore and open it, which could initiate apoptosis in the retina [74]. Figure 5 shows that the Cd apoptosis effect in HT-22 cells, the apoptosis response was similar to Pb metal; the percent of apoptotic cells was a significant at 3.26, 3.37 µM ($p^{***} < 0.05$). Cd showed concentration dependent manner in HT-22 cells, effect of Cd was more pronounced at 3.37 µM. Hart et al. (1999) reported Cd induced apoptosis in rat lung epithelial cells is a time and dose dependent manner [75]. Other reports have shown that Cd induces dose dependent apoptosis in cortical neurons [47]. Cd has also been evidenced in U-937 cells from human lymphoma [76], in rat kidney epithelial cells [77], and in rat fibroblasts [78] as a dose dependent manner apoptosis. Several studies demonstrated that Cd induced apoptosis might be associated with a ROS production [79]. The other two metals As and MeHg have the significant effect even at a low concentration of exposure. As showed more percent of apoptotic cells at 0.60, 0.66 µM ($p^{***} < 0.05$); which indicates that the potency of As was high in HT-22 cells; these findings are relevant with As induced apoptosis in neuroblastoma cells [80]. The sensitivity of As in different cell lines might be due to some underlying molecular mechanism [81]. Miller et al. reported that As induces apoptosis in cell lines due to the generation of ROS [82], which enhances DNA damage [83] and subsequently the brain cell death [84] . In MeHg the apoptosis pattern was started at 0.18 µM and reached as sudden inflection at 0.29 µM ($p^{***} < 0.05$), however the percent of apoptotic cells are more than expected (47.6 ± 0.1); similar response was observed in human SH-SY 5Y neuroblastoma cells [85]. The reported MeHg studies in rat cerebellar granule cells (CGC) indicates apoptosis is cell line specific effect [86]. In cerebellar neurons treated with MeHg up to 0.3 µM showed morphological changes characteristic to apoptosis depending the concentration [87]. At IC_{30} (70, 3.37, 0.66, and 0.29 µM), the observed maximum percentage of apoptosis cells 15.4 ± 5.6, 31.8 ± 0, 41.9 ± 6.4, and 47.6 ± 0.1 of Pb, Cd, As, and MeHg respectively. Thus, the present study clearly shows that the apoptosis mechanism is potency dependent as similar to cell viability and genotoxicity. This concentration dependent pattern of apoptosis induced by heavy metals as observed in flow cytometrtic analysis of the HT-22 cells stained with PI and annexin V is summarized in Figure. 4 - 7 (dot plot). Quantitative histograms of total apoptotic cells (%) are shown in the Figure 8, these data suggest that the apoptotic cells percent was increased with respect to concentration. Among the four metals, the Pb showed very low toxicity, and MeHg showed high toxicity potency.

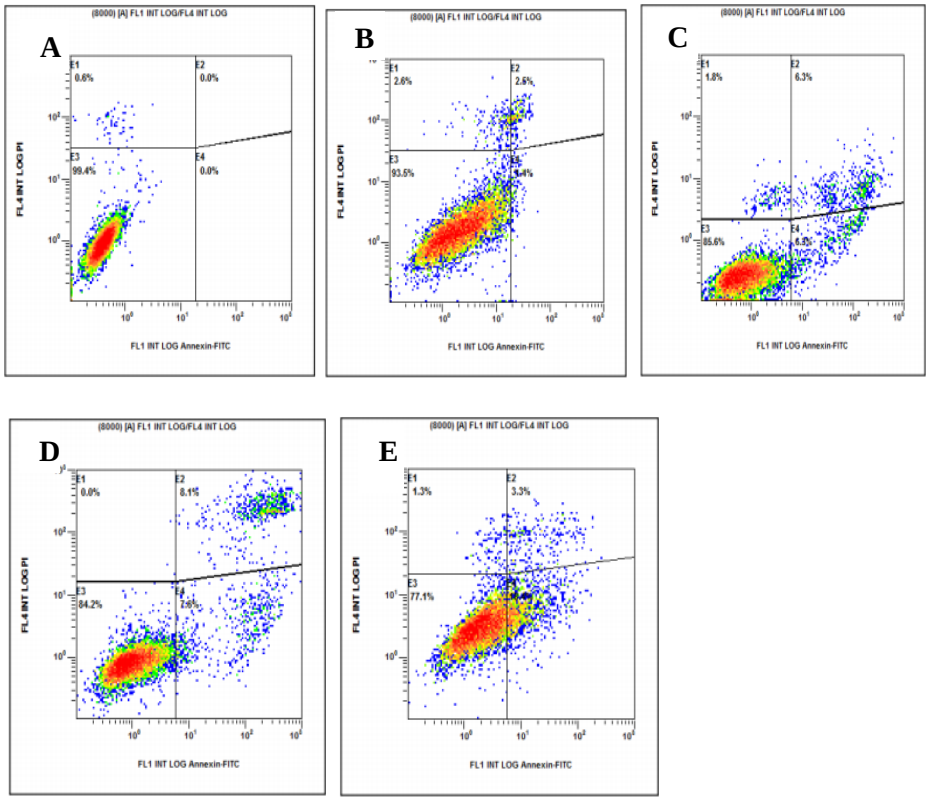


Figure 4: Representative dot plots showing the inhibitory effect of Pb to HT-22 cells upon chronic (8 days) of exposure. A= Control, B= 63.50 µM, C= 67.34 µM, D = 68.74 µM, E= 70.0 µM. Lower left (LL)/ E3 = Live cells (Annexin V− / PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+ / PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

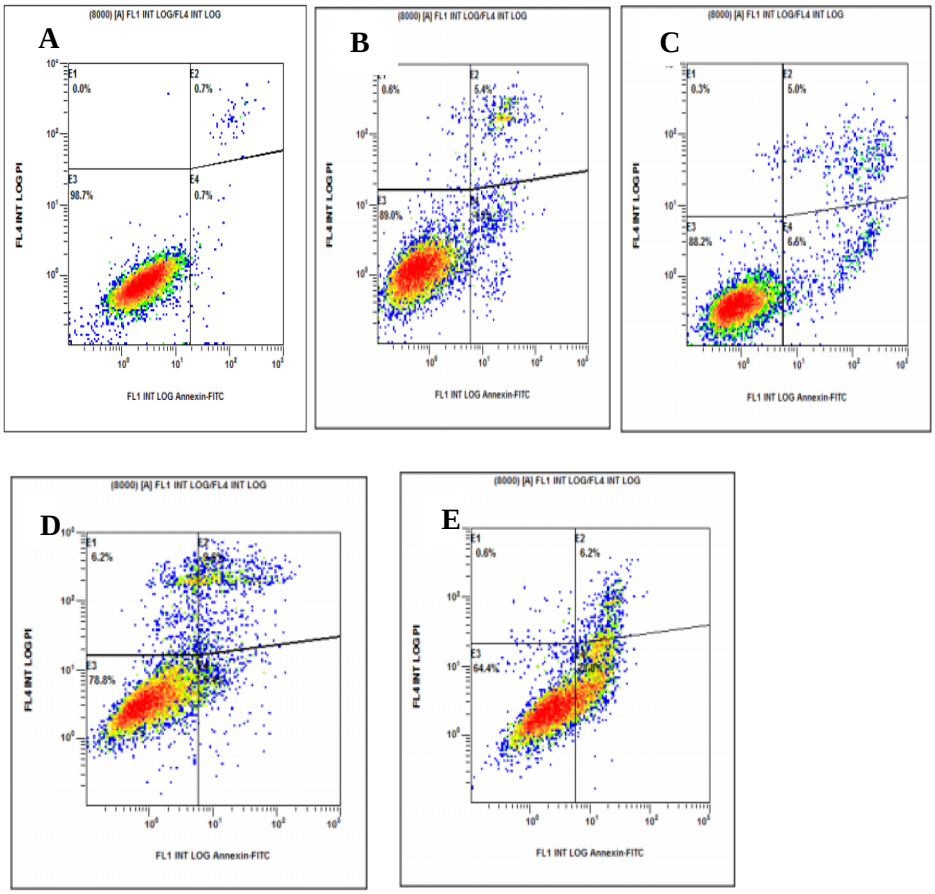


Figure 5: Representative dot plots showing the inhibitory effect of Cd to HT-22 cells upon 8 days of exposure. A= Control, B= 2.80 µM, C= 3.14 µM, D = 3.26 µM, E= 3.37 µM. Lower left (LL)/ E3 = Live cells (Annexin V− / PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+ / PI+), Upper left (UL)/ E1 = Necrotic cells (PI

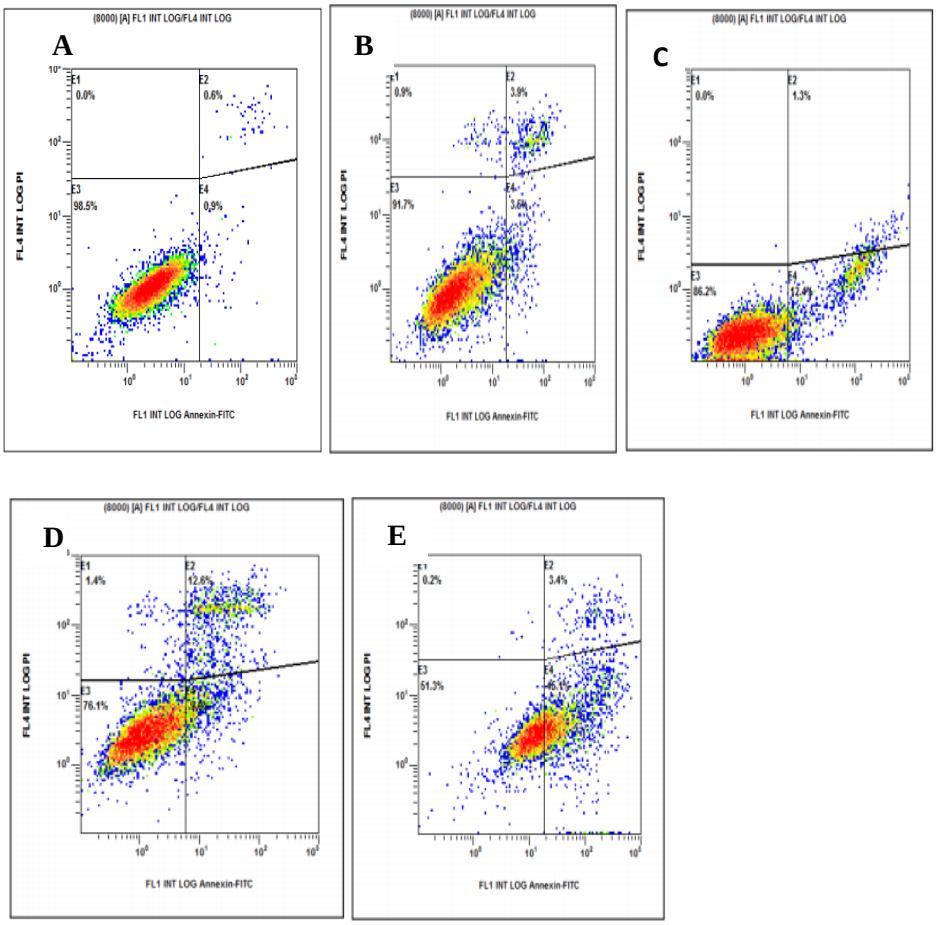


Figure 6: Representative dot plots showing the inhibitory effect of As to HT-22 cells upon 8 days of exposure. A= Control, B= 0.42 µM, C= 0.55 µM, D = 0.60 µM, E= 0.66 µM. Lower left (LL) = Live cells (Annexin V−/PI−), Lower right (LR) = Early apoptotic cells (Annexin V + / PI−), Upper right (UR) = Late apoptotic (Annexin V+ / PI+), Upper left (UL) = Necrotic cells (PI+).

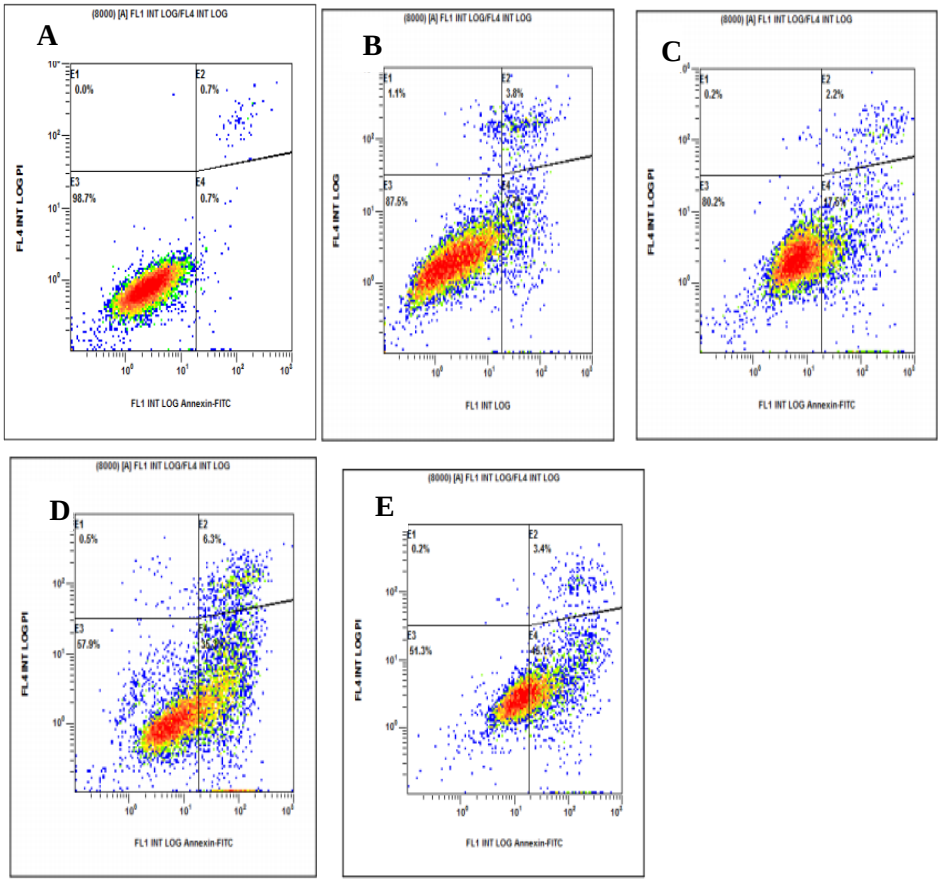


Figure 7: Representative dot plots showing the inhibitory effect of MeHg to HT-22 cells upon 8 days of exposure. A= Control, B= 0.09 µM, C= 0.18 µM, D = 0.23 µM, E= 0.29 µM. Lower left (LL)/ E3 = Live cells (Annexin V−/PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+/PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

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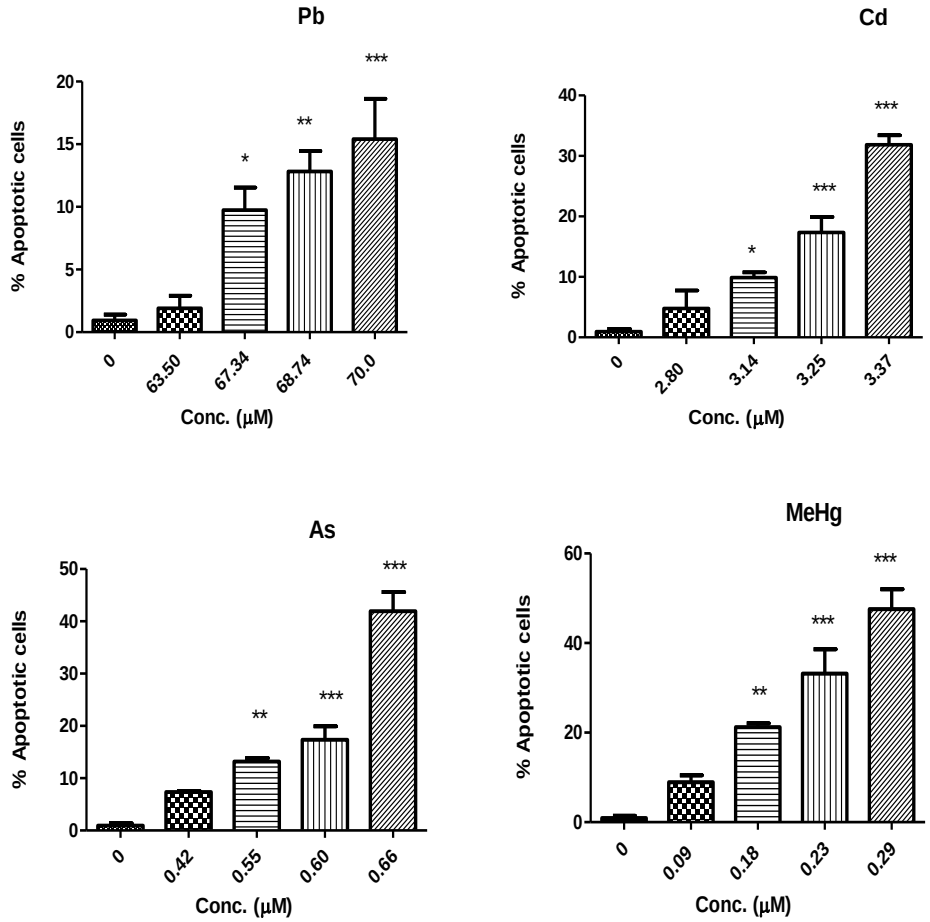

Figure 8: Quantification by flow cytometry of Pb, Cd, As, and MeHg induced apoptosis cells (%). Each point represents a mean ± SD of 3 experiments. Results are expressed as percentages of apoptosis with regard to the total cells ***significantly different (p*** < 0.05) from the control, according to the Dunnett's test

4. Conclusion

In recent years, environmental exposures to hazardous metals, such as Pb, Cd, As, and MeHg have been significant toxicological concerns in the hippocampus. We assessed the cytotoxicity of four metals in hippocampal based HT-22 cells during 1 day, 3 days, 8 days; considered as acute, subchronic, chronic exposure. For more elaborating basic toxic mechanism, chronic cytotoxicity studies were further extended to a DNA damage and apoptosis in the range of low concentration (IC_{10} – IC_{30}). Cytotoxicity, genotoxicity, and apoptosis findings are almost identical in potency. Moreover, current study

demonstrated that Pb has weak effect and MeHg has high potency in HT-22 cells. The obtained apoptotic assay results indicates heavy metal potency MeHg > As > Cd > Pb as similar to MTT assay and genotoxicity. The current study demonstrated that heavy metals differential toxicity on HT-22 cells. The parameterization of the dose response curve supporting the heavy metals toxicity was comparable among all exposure conditions. The limitation of the current study is not exploring the specific mechanism on HT-22 cell during chronic exposure. The findings from apoptosis and genotoxicity results are helpful for extending further to oxidative stress based studies to know the underlying molecular mechanism related to neurodengerative diseases. In future, the common cytotoxicity of Pb, Cd, As, and MeHg on HT-22 cell line could be useful for designing the metal mixtures (binary, ternary) interaction studies and omics based studies.

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Chapter 3

Karri V, Kumar V, Ramos D, Oliveira E, Schuhmacher M, An in vitro cytotoxic approach to assess the toxicity of Heavy metals and their Binary mixtures on hippocampal HT-22 cell line.
Toxicology letters, 282, 25-36 (2018).

Chapter 3

Chapter 3

An in vitro cytotoxic approach to assess the toxicity of heavy metals and their binary mixtures on hippocampal HT-22 cell line

Abstract

Humans are exposed to a cocktail of heavy metal toxicants in the environment. Though heavy metals are deleterious, there is a paucity of information on the toxicity of mixtures. In this study, four common neurotoxicity heavy metals lead (Pb) cadmium (Cd), arsenic (As), and methylmercury (MeHg) were exposed individually and as mixtures to HT-22 cell line for 8 days. The study established that low dose exposures induced toxicity to the HT-22 cell line during 8 days. The results indicates potency dependent response, the toxicity of single metals on the HT-22 cells; MeHg > As > Cd > Pb. The cytotoxicity data of single metals were used to determine the mixtures interaction profile by using the dose additivity and effect additivity method. Metal mixtures showed higher toxicities compared to individual metals. Synergistic, antagonistic or additive effects of the toxicity were observed in different mixtures in low dose exposure. The interactive responses of mixtures depend on the co-exposure metal and their respective concentration. We concluded that the combined effects should be considered in the risk assessment of heavy metal co-exposure and potency. In future, comprehensive mechanistic based investigations needed for understanding the real interactive mixtures effects at molecular level.

Keywords: Mixture toxicity; Metal mixtures; Cytotoxicity; Apoptosis; Isobologram analysis.

Abbreviations: Ach E = Acetyl cholinesterase E, ANOVA = Analysis of variance, As = Arsenic, CA= concentration addition, CI= combination index, DMEM = Dulbecco's modified Eagle's medium, FBS = Fetal bovine serum, GABA = ¥-gamma-amino butyric acid, GAD = Glutamate decarboxylase, LC_{50} = Lethal concentration 50, LTP = Long-term potentiation, MeHg = Methyl mercury, MTT = 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, NMDA = N-methyl D-aspartate, OD = optical density, Pb = Lead, PI = propidium iodide, SD = Standard deviation, WHO = World Health Organization

Chapter 3

1. Introduction

Heavy metals are environmental pollutants of great concern because of their persistent occurrence, arising from increasing industrialisation and other anthropogenic activities (Al-Khashman and Shawabkeh, 2006; Nadal et al., 2004; Yu et al., 2011). Exposure to heavy metal compounds including lead (Pb), cadmium (Cd), arsenic (As), and methyl mercury (MeHg) has long been known to cause damage to human health (Mari et al., 2014; Morais and Garcia, 2010). The organs affected by these metals are kidney, lung, liver, gastrointestinal and haematological systems, mainly the peripheral and central nervous systems (Angelica and Fong, 2014). Because of their high degree of toxicity, these four elements rank among the priority metals that are of great public health concern (WHO, 2010). Exposure to MeHg and Pb has a significant effect on the human brain and, are well known to target the central nervous system (Aschner et al., 2007; Clarkson, 1987; Maynard et al., 2005; Sanders et al., 2009). Exposure to Cd also severely affects the function of the nervous system, leading to parkinson like symptoms, and learning disabilities (Viaene et al., 2000; Wang and Du, 2013). The exact mechanism and its neurotoxic effects, however, unresolved (Kumar et al., 1996; Mendez-Armenta and Ríos, 2007). Recently, it has been found that As is also linked to developmental neurotoxicity (Luo et al., 2009; Rodriguez et al., 2002; Tyler and Allan, 2014).

The brain is a critical target organ for Pb, MeHg mediated cognitive dysfunction effects, and Cd, As are also highly influences the brain in continuous exposure (Giasson et al., 2002). Numerous studies have been done on the toxicity of individual metals to a brain (Wu et al., 2016). In an individual metal mode of action, Pb, As and MeHg has been found as potent neurotoxicants (Johansson et al., 2007; Sadiq et al., 2012; Tyler and Allan 2014). Experimental studies proved that Cd also influences the cognitive function of the brain (Hart et al. 1989; Luo et al. 2009; Viaene et al. 2000). Generally, humans are exposed to these metals in a simultaneous manner (Stackelberg, 2015). The simultaneous exposure may exacerbate the toxic effects, most of the heavy metals are known to increase the sensitivity to cognitive dysfunction and neurodegenerative outcomes (Clarkson 1987; Snyder et al. 2005). In a recent review, we reported that the combination of metals may produce more/ less than additive due to their common binding affinity with NMDA receptor (Pb, As, MeHg), Na^{+}- K^{+} ATP ase pump (Cd, MeHg), biological Ca^{+2} (Pb, Cd, MeHg), and glutamate neurotransmitter (Pb, MeHg) (Fig 1) (Karri et al., 2016).

Chemical mixtures toxicity is effectively an infinite problem, and it is an ongoing challenge to integrate this issue into regulatory regimes (Sarigiannis and Hansen, 2012; Sharma et al., 2016). Testing of all kinds of mixtures of chemicals existing in the real world or of all possible combinations of a simple mixture of different dose levels is virtually impossible (Orton et al., 2014). Moreover, even if toxicity data of individual chemicals are available, we are still facing the immense problem of extrapolation of findings obtained at relatively high exposure concentrations in laboratory animals to a

man being exposed to lower concentrations (Cassee et al., 1998). More than 95% of toxicological research studies are focused on single chemicals and almost completely neglect the mixtures (Kortenkamp et al., 2009). The available toxicity data for the mixtures of metals are very limited. Studies on exposure to heavy metal mixtures are critical since there is a lack of information on the toxicities and associated mechanisms. Some reported binary mixture data of As, Cd, and Pb on various biological endpoints are inconsistent for the same endpoints from study to study and are less relevant in terms of risk assessment (ATSDR, 2004). On the hand, prediction of mixtures effects is a great challenge because synergism or antagonism in a combination of two or more chemicals may occur and no currently available mathematical model can predict or fully solve this problem (PapeLindstrom and Lydy, 1997). Previously reported studies have established toxicity of metal mixtures on various organs and their functions: the immune system (Jadhav et al., 2007a), mortality (Vellinger et al., 2012), neurotoxicity (Hu et al., 2013; Rai et al., 2013), bladder cancer (Feki-Tounsi et al., 2013) cytogenicity (Jadhav et al., 2006), induction of oxidative stress (Jadhav et al., 2007b), and metal mixtures interactions on essential elements (Cobbina et al., 2015). Recommendations for study design and evaluation of combined effects of metal mixtures are not clear (Tichý et al., 2002). The regulatory frameworks such as REACH in the EU are becoming more and more critical regarding the use of animal testing (Cedergreen, 2014). There are various risk assessment methods for evaluating combined exposures in practice, these methods are derived from the dose addition concept and effect addition (Scholze et al., 2014).

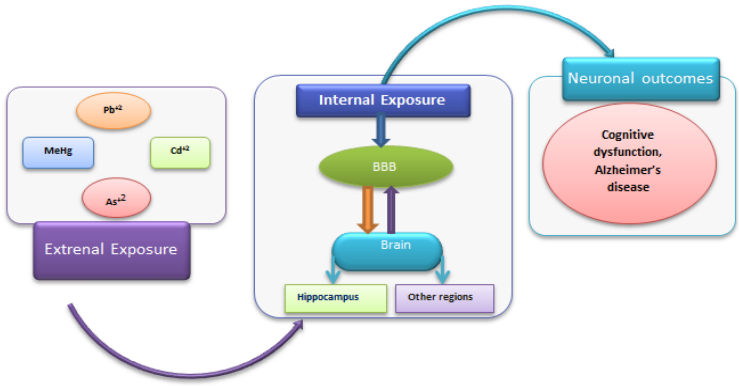


Figure 1: Conceptual diagram of metal mixtures exposure- toxicology- disease outcome scenario (hippocampus) (Karri et al., 2016)

Recent advancement in vitro techniques, with an appropriate target cell, may allow an accurate understanding of metal mixtures toxicity. However, the in vitro effects were specific to cell lines and exposure conditions. There is an ongoing discussion regarding the most appropriate method for the evaluation of mixtures interactions (Kortenkamp and Altenburger, 1998); so two methods have been employed in this study: the effect

additivity model (Axelrad et al., 2002) and the alternative dose additivity model (Berenbaum, 1978). The present study explores the toxicity of individual and binary mixtures of Pb, Cd, As, and MeHg after 8 days exposure to HT-22 hippocampal cell line. 8 days exposure used in this study is considering the maximum stability of cell confluence (80-85%). For elaborating the hypothesis, we performed cytotoxicity and apoptosis of Pb, Cd, As, and MeHg alone in the mice HT-22 hippocampal cell line. Further, we extended the binary mixtures interactions study by using the response addition and dose addition whether they interact with one another when combined.

2. Materials and methods

2.1. Chemicals and media

Lead chloride ($PbCl_2$ [CAS no: 7758-95-4]), Sodium metaarsenite ($NaAsO_2$ [CAS no: 7784-46-5]), Cadmium chloride ($CdCl_2$ [CAS no: 10108-64-2]), Methyl mercury chloride ($MeHgCl_2$ [CAS no: 115-09-3]), Dimethyl sulphoxide (DMSO [D5879]), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT [M5655]), trypsin (TrypLE [Gibco: 12604013]), all are analytical grade and purchased from Sigma-Aldrich Química, S.L- Madrid (Spain).

2.2. Cell line and reagents

Among various research tools, neuronal cell lines are the most commonly used in vitro model for relevant mechanistic studies. With particular concerns for memory and alzheimer disease related studies, hippocampal neuronal cell lines are very limited. HT-22 is one cell line sub cloned from its parent line HT4, which are immortalized mouse hippocampal neuronal precursor cells. The HT-22 cells have been used as a hippocampal neuronal cell model in numerous studies.

The HT-22 cells were a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA). HT-22 cells were maintained in Dulbecco's modified Eagle's medium (DMEM [D6429]) containing 10% fetal bovine serum (FBS Gibco [10500-064]) and 100 U/mL penicillin, and 100 µg/mL streptomycin (Pan-Biotech- Germany) in a humidified incubator with 5% CO_2 in air at 37^0 C (Niska et al., 2015). For all the experiments in 8 days duration cells were grown at 70- 80% confluence.

The cells were cultured in 75 cm^2 cell culture flasks. For experimental purpose, cells were plated at 0.57 x 10^6 cells/ mLand and grown for 24 hours before metal treatment. Duplicates wells of cells were treated with 10 exposure levels of Pb, Cd, As and, MeHg ranging from 10 to 100 µM, 0.5 to 7 µM, 0.4 to 4.2 µM, and 0.6 to 12 µM, respectively; due to the 8 days exposure medium containing given concentration was refreshed at 2 days interval for maintaining metal exposure in a long time. Metal stock solutions 100X were prepared in deionized distilled water (for poorly soluble $PbCl_2$ < 0.5% DMSO added) and sterilized by filtration through 0.2 µm and different concentrations of a working solution of each individual metal was prepared by prior

dilution of the stock solution in phosphate buffer saline (pH = 7.4) and then applying 10% working solution on DMEM culture medium.

2.3. Cytotoxicity/ MTT assay

The MTT assay was carried out using a modification of the method of Mossman (1983). The HT-22 cells were seeded in 96-well plate. After 24 h, when the cells had reached a confluence of 70–80%, they were exposed for 8 days to several concentrations of the heavy metals (Pb, Cd, As and, MeHg). After the incubation period, the medium was aspirated from well and MTT working solution at 0.5 mg/mL was added to each well. Cells were incubated at 37°C for 3 h; after this time, the MTT was removed by aspiration. Formazan crystals were dissolved in 100 µL of DMSO and placed the plates on a shaker and agitated for 5 min. The absorbance of the solubilized reduced MTT was then measured in a micro titter plate spectrophotometer reader at a wavelength of 570 nm. The measured absorbance or optical density (OD) values were converted to percent of cell viability (%) with respect to control. Cell viability (%) = Absorbance of treatment / Absorbance of Control x 100%. The cytotoxicity results were used for calculating the IC_{10} to IC_{30} of the each metal for apoptosis and mixtures interaction study.

2.4. Analysis of apoptosis by annexin V-FITC/ propidium iodide (PI)

To evaluate the translocation of phosphatidylserine (PS) from inner leaflets to outer leaflets of the plasma membrane, Annexin V- FITC apoptosis detection kit (BD Pharmingen, Poland) was utilized. In this kit, Annexin V and Propidium iodide (PI) was used to distinguish the apoptotic and necrotic cells from the alive cells. According to the manufacture's protocol, the exponentially proliferating cells were exposed to the designed doses (IC_{10}, IC_{20}, IC_{25}, and IC_{30}) of heavy metals in 12 well plates at a density of 0.56 x 10^6/mL during 8 days, control cells were made without chemical. The medium with metal concentration was refreshed every 2 days. After 8 days treatment with metals, cells were harvested by trypsinization, washed twice with ice cold PBS (p^H = 7.4). Thereafter, cells were centrifuged at 1200 rpm for 5 min at 4^0C, resuspended in 1mL of 1X binding buffer and then transfer the 100 µL of the solution to 5 mL culture tube, and added 5 µL of both annexin-V, PI to the samples. After staining, cells were incubated for 15 min in the dark at room temperature. Cells were re-washed with 1X binding buffer 400 µL and analyzed by flow cytometry (Beckman coulter, Germany).

2.5. Assessment of interactions using the response additivity method

This method for testing interaction between chemicals has been described by Lau et al., (2006). In this model, the combined effects of two agents are thought to be equal to the sum of the effects of the single compounds. Deviations from this are either synergistic or antagonistic. In the present study, chemicals were tested at their IC_5- IC_{20} concentrations, derived from individual concentration– response curves. The mixture experiments were conducted for a total of 6 mixtures in 8 days, including Pb+Cd, Pb+As, Pb+MeHg, Cd+As, Cd+MeHg, and As+MeHg, For binary mixtures, cells were

then exposed to pairs of the compounds in equal proportions. To assess the interaction between heavy metals, combined effects include: additivity, where metals are no more and no less effective in combination than they are separately; synergism, where the effectiveness of agents is increased when in combination; potentiation, where the increased effect of a toxic compound is acting concurrently with a non-toxic compound; and finally, antagonism, where the effectiveness of agents is decreased when in combination (Costa et al., 2007).

2.6. Assessment of interactions using the isobole method- dose additivity

In the current study, five different mixtures ratios of each binary pair were prepared (0:100, 25:75, 50:50, 75:25, and 100:0). Each of these mixture ratios was tested in triplicate around the mixture IC_{30} according to the method (Axelrad et al., 2002). For the data analysis of these mixtures, compusyn isobole analyses were used to assess the interaction of the metals in the mixture. The combination index (CI) - isobologram method is widely used in pharmacology to study the nature of the interaction between drugs. The interaction is analysed by using the median-effect/combination index (CI)-isobologram equation (Chou, 2010), which is based on the median-effect principle (mass-action law). This method has been applied to predict the mixture toxicity of environmental chemicals (Wang et al., 2015). The benefit of a non- constant combination toxicity study is not simply due to the property of the metals, but could also depend on the dose ratios. As the cells do not make the difference between a single metal or a combination, two metals combined at a given ratio could be considered as a third agent with its own dose effect relation. The isobole model (Berenbaum, 1978) allows the construction of graphs showing curves describing various combinations of two compounds A and B, which together produce the same, specified effect. Isoeffective doses A and B of the single compounds are connected by an additivity line, which predicts the combinations of A and B required to yield the specified effect, provided the interaction between A and B is additive (zero interaction).
This relationship is expressed by the equation: $c_A/C_A + c_B/C_B = 1$

Where c_A and c_B are the concentrations of A and B in a mixture that produce a specified effect, and C_A and C_B are the concentrations of the single agents, which on their own elicit the same effect as the mixture. Synergistic agents require lower concentrations to produce a given effect when in combination, giving concave isobole; therefore the equation is expressed as $c_A/C_A + c_B/C_B < 1$. Antagonistic combinations give convex isobole resulting in $c_A/C_A + c_B/C_B > 1$. The localization of the experimental mixture point (a, b) corresponding to the doses actually needed for a combination effect mixture with respect to the line of additivity can be translated in term of synergy, additivity, and antagonism; if experimental point below the line corresponds to a combination index (CI < 1) and indicates synergy; a point on the line corresponds to a CI = 1 and indicates simple additivity; finally a point above the line corresponds to a CI > 1 and indicates antagonism. Sometimes the CI values are >3 or much greater, especially at low effect

levels (i.e., low fa level). Keep in mind that the synergy scale is from 1 to 0 and the antagonism scale is from 1 to infinity (Chou, 2010). The limitations of the CI method are that it is highly sensitive to small changes in effect measurement at low and high concentrations and lack of statistical evaluation of synergy, additivity or antagonism (Zhao et al., 2010).

2.7. Statistical analysis of data

All experiments were performed three times (n=3) and each concentration tested in replicates. The results were given as mean ± standard deviation (SD). IC_5 to IC_{30} values calculated from dose response curve fitted by using the Graph pad prism version 5.01. Compusyn software (http://www.combosyn.com) used for non-constant ratio isobologram fitting. All data were analysed by one way ANOVA (p*** < 0.05) procedures followed by dun net's. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Cytotoxicity of individual metals (Pb, Cd, As, and MeHg) on HT-22 cell line

To characterize the effects of Pb, Cd, As, and MeHg individually on the hippocampus, we performed cytotoxicity studies in immortalized mice HT-22 hippocampal cells using the MTT assay as a measure of cell viability. As expected, all 4 metals showed a dose, dependent cytotoxic effect, expressed by decreased absorbance or optical density (OD) values of treated cells. Results for Pb, Cd, As, and MeHg dose- response curves are presented in Figure 2 and table 1. For Pb, dose- response curve clearly shows concentration dependent effect, cytotoxicity was drastically enhanced with respect to concentrations (>70 µM). The obtained results suggest that Pb induced damage in HT-22 cells is dose dependent. However, at low concentration (10-60 µM) Pb had no effect on cell viability. Exposure of HT-22 cells to different Cd concentrations (0.5–7 µM) shows a dose dependent relationship. Cells exposed to Cd concentrations from 0.5 to 2.5 µM exhibited the non-significant effect. The effect of Cd was more pronounced at 3.5 µM. We observed that the percentage of cell viability decreases with increasing Cd concentration reaching a maximum of cell death (90%) in chronic exposure. The metal As on HT-22 cells drastically decreased the viability; the toxic effect of As was high at 1.2-4.6 µM. The effects of MeHg on HT-22 cells were high, even in the lowest concentration (0.6 µM), the cell death was statistically significant. The dose response curve inflection drastically enhanced in MeHg than other three metals. The results obtained from the MTT cytotoxicity assays indicate that there are differences between heavy metals sensitivity on hippocampus cell line. The MeHg appear to be more sensitive as indicated by LC_{50} value. This difference could be due to the different uptake mechanisms of heavy metals by the HT-22 cells this difference among the cytotoxicity results of heavy metals suggests that some underlying unique intracellular mechanism is responsible for the hippocampus damage. The summary of cell viability data of heavy

metals for different exposure times (1 day, 3 days, 8 days) are presented in the supplementary material (Annex-I) and detail study can be found in Karri et al. 2017.

Table 1: The LC_{50} values of Pb, Cd, As, and MeHg for hippocampal HT-22 cell line after different exposure times in In vitro[a]

Exposure time	Heavy metals (IC_{50})			
	Pb (µM)	Cd (µM)	As (µM)	MeHg (µM)
8 days	74.3	3.7	0.8	0.6

([a]Values of LC_{50} with 95% confidence intervals from curves shown in Figure 2. Cytotoxicity was evaluated by inhibition of MTT reduction as described in the methods).

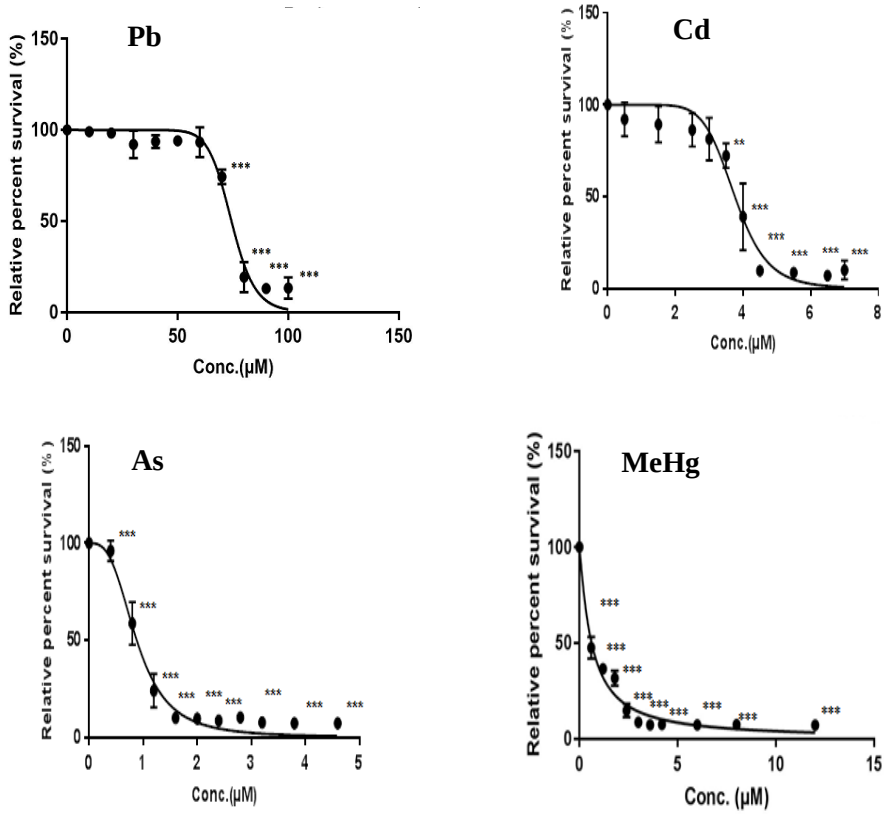
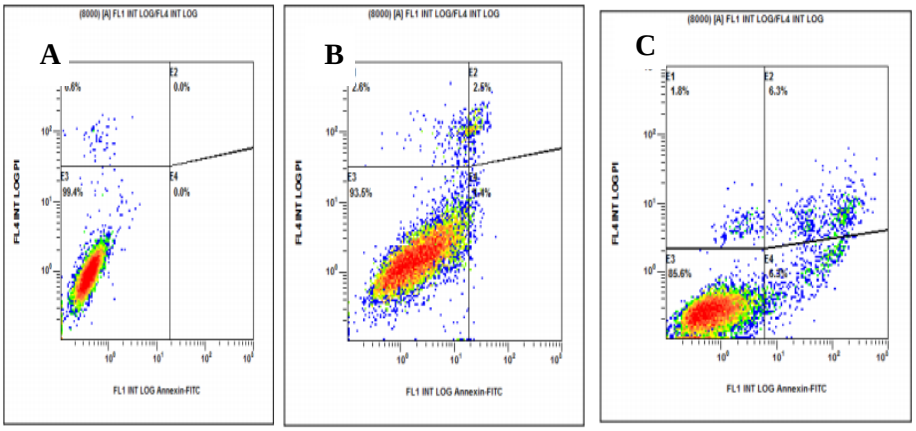


Figure 2: Dose- response curves of Pb, Cd, As, and MeHg on HT-22 cells during 8 days exposure. The one – way ANOVA followed by a dunnet's multiple comparison tests compared the control with all concentrations, asterisks indicate significantly different with control (p*** < 0.05).

3.2. Apoptosis effects of individual metals (Pb, Cd, As, and MeHg) on HT-22 cells

To gain insight into the heavy metal induced cell death in HT-22 cells, we examined the Annexin FTIC – V / Propidium iodide (PI) assay during 8 days exposure. We found that heavy metals have different potency to induce apoptosis in HT-22 cells. Firstly Pb results in Figure 3a indicates, there was a slight trend to increased apoptotic cells (%) as concentration increased, but it was not statistically significant. However, at 70 µM response was relatively significant ($p^{***} < 0.05$). Figure 3b shows that Cd apoptosis effect in HT-22 cells, the apoptosis response was similar to Pb metal; the percent of apoptotic cells was significant at 3.26, 3.37 µM ($p^{***} < 0.05$). The other two metals As and MeHg has a significant effect even at low concentration of exposure. As shows more percent of apoptotic cells at 0.60, 0.66 µM ($p^{***} < 0.05$); which indicates the potency of As was high in HT-22 cells. In MeHg the apoptosis pattern was started at 0.18 µM and reached as sudden inflection at 0.29 µM (($p^{***} < 0.05$), however, the percent of apoptotic cells are more than expected (47.6 ± 0.1). At IC_{30} (70.00, 3.37, 0.66, and 0.29 µM), the observed maximum percentage of apoptosis cells 15.4 ± 5.6, 31.8 ± 0.0, 41.9 ± 6.4, and 47.6 ± 0.1 of Pb, Cd, As, and MeHg respectively. Thus, the present study clearly shows that the apoptosis is potency dependent as similar to cell viability. This concentration dependent pattern of apoptosis induced by heavy metals as observed in flow cytometrtic analysis of the HT-22 cells stained with PI and annexin V is summarized in Figure. 3a – 3d (dot plot). Quantitative of total apoptotic cells (%) are shown in the Table 2A-2D. Overall flow cytometrtic analyses show that heavy metals induce apoptosis on HT- 22 cells dose dependent, and the potency of metals shows MeHg > As > Cd > Pb.



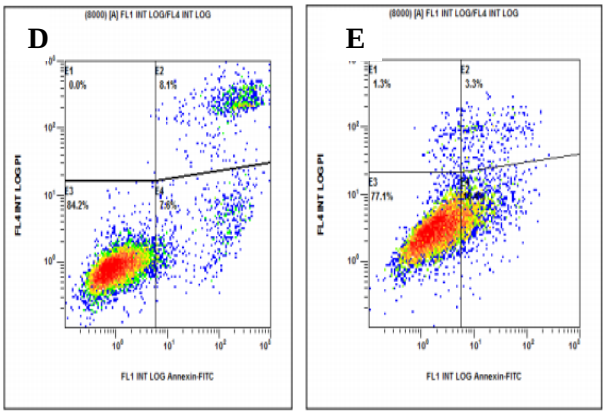


Figure 3a: Representative dot plots showing the inhibitory effect of Pb to HT-22 cells upon 8 days of exposure. A= Control, B= 63.50 µM, C= 67.34 µM, D = 68.74 µM, E= 70 µM. Lower left (LL) = Live cells (Annexin V−/ PI−), lower right (LR) = Early apoptotic cells (Annexin V +/PI−), upper left (UL) = Late apoptotic (Annexin V+/ PI+), upper right (UR) = Necrotic cells (PI+).

Table 2A: Pb Summary data of annexin – V / PI assay obtained from the flow cytometry analysis. Values are shown as means ± SD of 3 replicates per experiment.

Pb Conc. (µM)	Viable Cells (Mean ± SD)%	Apoptotic cells (Mean ± SD)%	Necrotic cells (Mean ± SD)%
0	98.8 ± 0.4	0.9 ± 0.8	0.2 ± 0.3
63.50	94.5 ± 0.9	1.9 ± 1.7	3.6 ± 0.9
67.34	88.8 ± 2.9	9.7 ± 3.1	1.5 ± 1.0
68.74	84.6 ± 2.1	12.8 ± 2.8	2.6 ± 4.1
70	81.3 ± 6.5	15.4 ± 5.6	3.3 ± 4.6

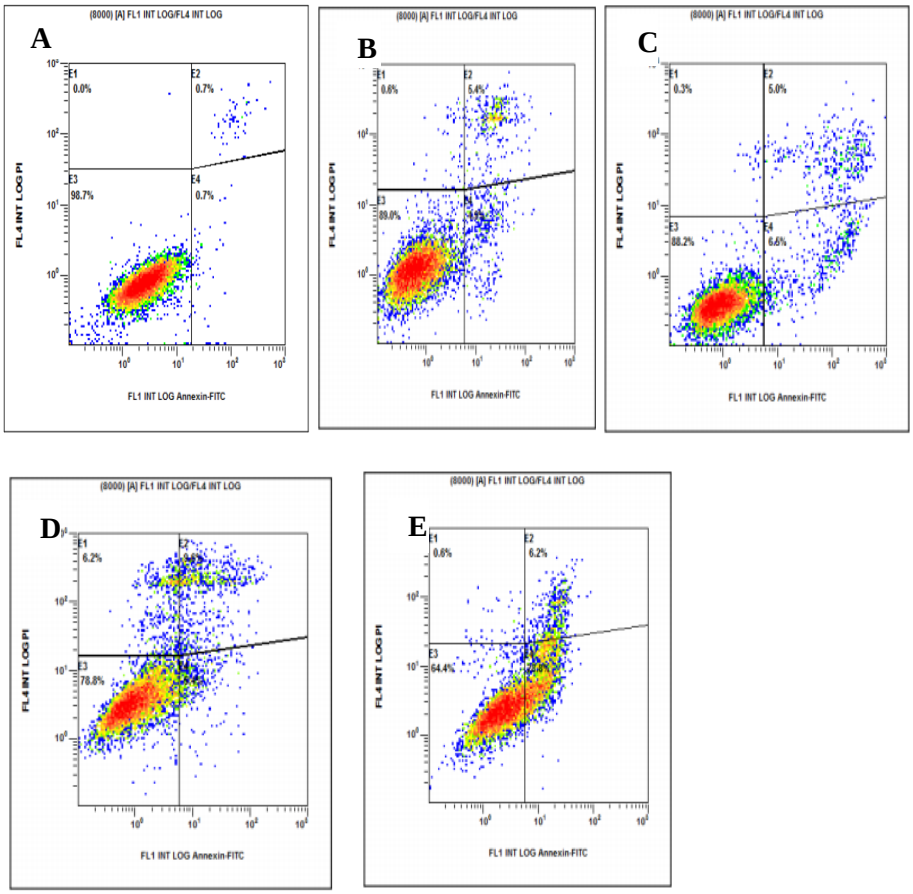


Figure 3b: Representative dot plots showing the inhibitory effect of Cd to HT-22 cells upon 8 days of exposure. A= Control, B= 2.80 µM, C= 3.14 µM, D = 3.26 µM, E= 3.37 µM. Lower left (LL)/ E3 = Live cells (Annexin V− / PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+ / PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

Table 2B: Cd Summary data of annexin – V /PI assay obtained from the flow cytometry analysis. Values are shown as means ± SD of 3 replicates per experiment.

Cd Conc. (µM)	Viable Cells (Mean ± SD)%	Apoptotic cells (Mean ± SD)%	Necrotic cells (Mean ± SD)%
0	98.9 ±0.4	0.93 ± 0.8	0.2 ± 0.3
2.80	91.5 ± 2.0	4.8 ±1.5	3.7 ± 1.0
3.14	89.0 ± 1.3	9.9 ± 0.6	0.8 ± 2.4
3.26	80.4 ± 1.8	15.7 ± 2.8	3.9 ±1.5
3.37	65 ± 1.3	31.8 ± 0.0	1.8 ± 0.0

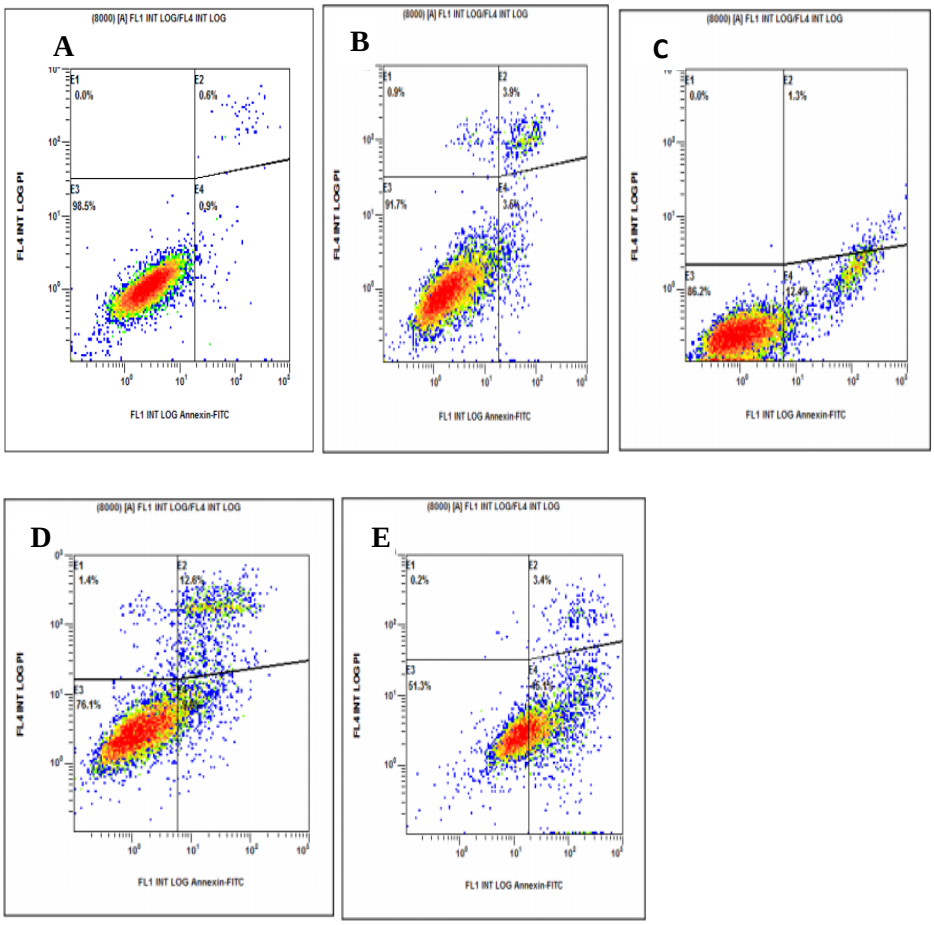


Figure 3c: Representative dot plots showing the inhibitory effect of As to HT-22 cells upon 8 days of exposure. A= Control, B= 0.42 µM, C= 0.55 µM, D = 0.60 µM, E= 0.66 µM. Lower left (LL) = Live cells (Annexin V−/PI−), Lower right (LR) = Early apoptotic cells (Annexin V + / PI−), Upper right (UR) = Late apoptotic (Annexin V+ / PI+), Upper left (UL) = Necrotic cells (PI+).

Table 2C: As Summary data of annexin – V /PI assay obtained from the flow cytometry analysis. Values are shown as means ± SD of 3 replicates per experiment.

As Conc. (µM)	Viable Cells (Mean ± SD)%	Apoptotic cells (Mean ± SD)%	Necrotic cells (Mean ± SD)%
0	98.9 ± 0.4	0.9 ± 0.8	0.2 ± 0.3
0.42	91.7 ± 0.6	7.4 ± 0.2	1 ± 0.8
0.55	86.4 ± 1.5	13.2 ± 1.2	0.4 ± 0.5
0.60	78.9 ± 3.0	17.3 ± 4.5	3.8 ± 2.4
0.66	57.8 ± 6.4	41.9 ± 6.4	0.3 ± 0.2

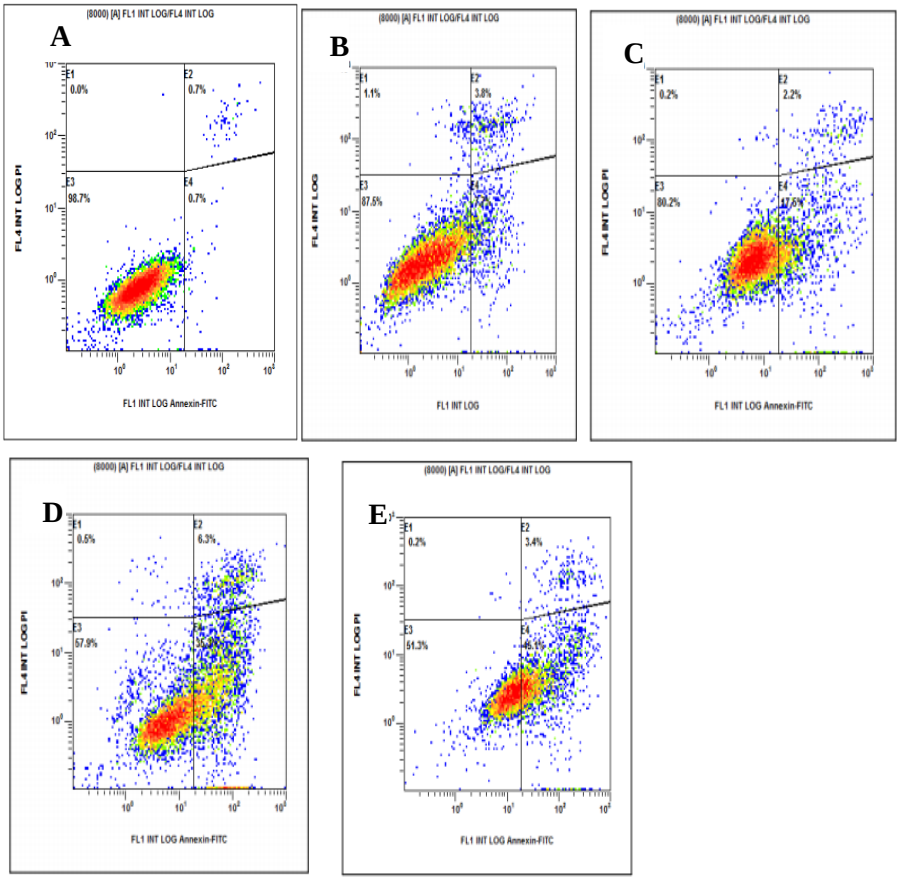


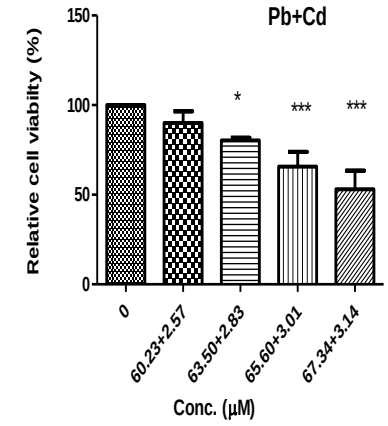
Figure 3d: Representative dot plots showing the inhibitory effect of MeHg to HT-22 cells upon 8 days of exposure. A= Control, B= 0.09 µM, C= 0.18 µM, D = 0.23 µM, E= 0.29 µM. Lower left (LL)/ E3 = Live cells (Annexin V−/ PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+/ PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

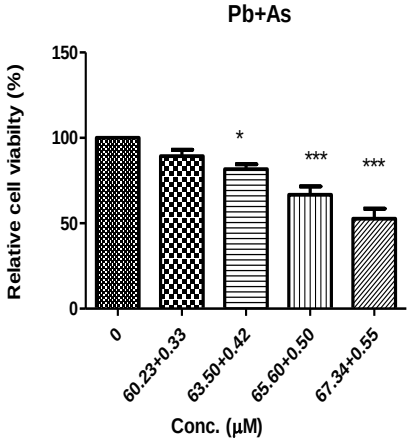
Table 2D: MeHg Summary data of annexin – V /PI assay obtained from the flow cytometry analysis. Values are shown as means ± SD of 3 replicates per experiment.

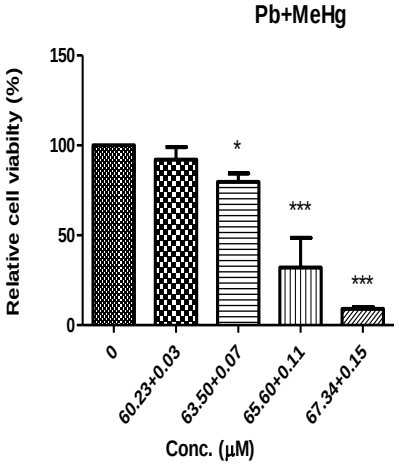
Conc. (µM)	Live Cells (Mean ± SD)%	Apoptotic cells (Mean ± SD)%	Necrotic cells (Mean ± SD)%
0	98.9 ± 0.4	0.9 ± 0.8	0.2 ± 0.3
0.09	89.8 ± 2.7	8.9 ± 1.2	1.3 ± 1.2
0.18	77.8 ± 1.5	21.2 ± 0.7	1.0 ± 0.7
0.23	68.2 ± 9.4	33.2 ± 0.3	0.7 ± 0.3
0.29	52.3 ± 7.7	47.6 ± 0.1	0.2 ± 0.1

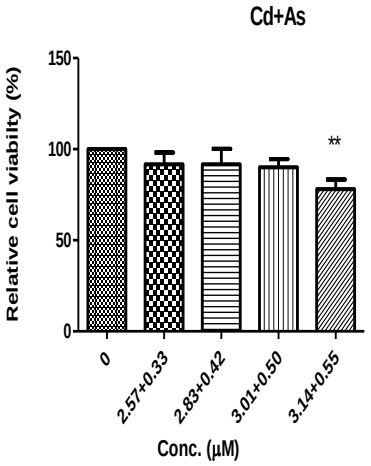
3.4. Interactions among the Pb, Cd, As and MeHg mixtures- response additivity

The concentrations of individual metals were selected for the mixture interaction based on the MTT assay, apoptosis results. The selected concentrations were expected to cover the concentration levels from non-toxic concentrations to a toxic level. The response additivity method described by Lau et al. (2006) was utilized, in which concentrations of each compound equivalent to their IC_5, IC_{10}, IC_{15}, and IC_{20} calculated from the single metal response curve. To estimate the mixtures toxicity, we performed different combinations of metal (Pb+ Cd, Pb+ As, Pb+ MeHg, Cd+ As, Cd+ MeHg, and As+ MeHg) on mice hippocampal HT-22 cell line. Generally know that MeHg and Pb have more impact on the brain rather than Cd, As. However in mixture point of view, the obtained results suggest that some interaction mechanism is there to produce the more than expected level. Several interesting findings were observed from dose response analysis; additive results were obtained in Pb+Cd, Pb+As, As+MeHg from IC_5 to IC_{20} range of mixtures, this purely additive effect is indicative of a lack of interaction. The cytotoxicity of Cd+As mixture results was less than the additive response suggesting antagonist effect, which indicates some interaction mechanism between Cd and As inhibits the potency of toxicity on HT-22 cells. The mixtures; Pb+MeHg, Cd+MeHg showed more than the additive response, for this nature of interaction considered as a synergistic effect. The obtained results suggest that there are mainly synergistic effects in MeHg containing mixtures. The Cd role in mixtures was aberrant; in the results, Cd showed additive, antagonistic, synergistic effects, depends on the co-exposure metal. A marked reduction of cell viability was found in MeHg among Pb, Cd binary mixture. This synergistic effect can be possible due to the common mechanism and it reduces the exposure dose of individual metals in a mixture (Fig 4).


Pb+Cd
Relative cell viability (%)
150
100
50
0
*
***
***
0
60.23+2.57
63.50+2.83
65.60+3.01
67.34+3.14
Conc. (µM)


Pb+As
Relative cell viability (%)
150
100
50
0
*
***
***
0
60.23+0.33
63.50+0.42
65.60+0.50
67.34+0.55
Conc. (µM)


Pb+MeHg
Relative cell viability (%)
150
100
50
0
*
***
***
0
60.23+0.03
63.50+0.07
65.60+0.11
67.34+0.15
Conc. (µM)


Cd+As
Relative cell viability (%)
150
100
50
0
**
0
2.57+0.33
2.83+0.42
3.01+0.50
3.14+0.55
Conc. (µM)

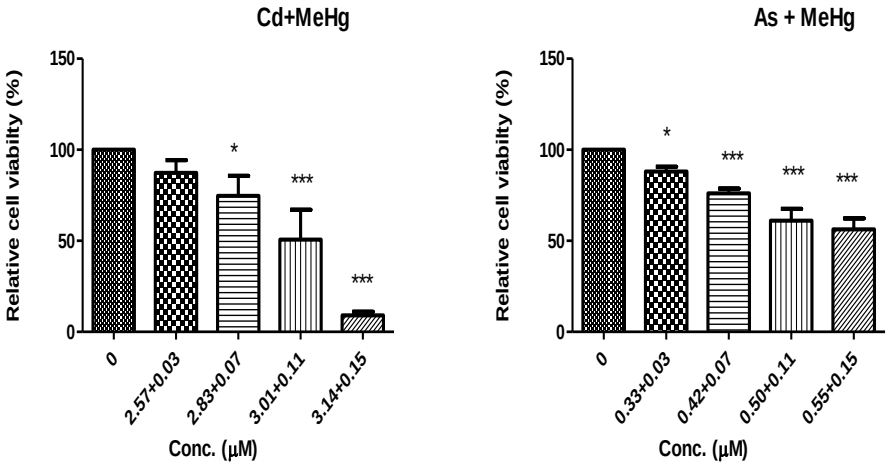
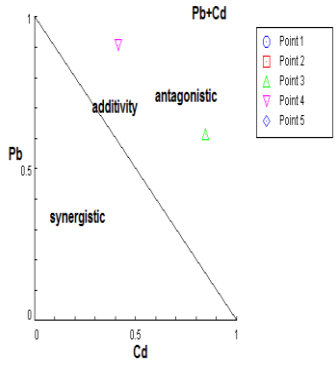


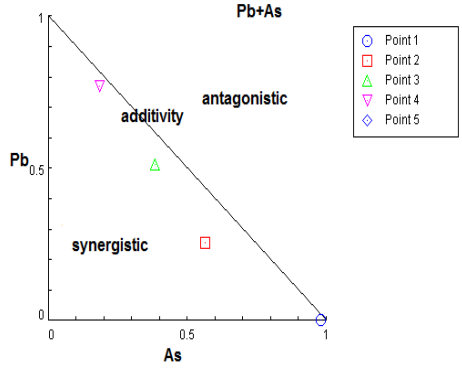
Figure 4: Effects of Pb, Cd, As, and MeHg in binary combination, on cell viability, measured by the MTT assay, in HT- 22 cell line during 8 days. In each response, concentrations of each metal corresponding to the equitoxic (in combination) were utilized. Values of IC_5, IC_{10}, IC_{15}, and IC_{20} were derived from concentration response curves reported in Figure 1. Each bar represents the mean (± SD.) of separate determinations carried out in triplicate (***$p< 0.05$).

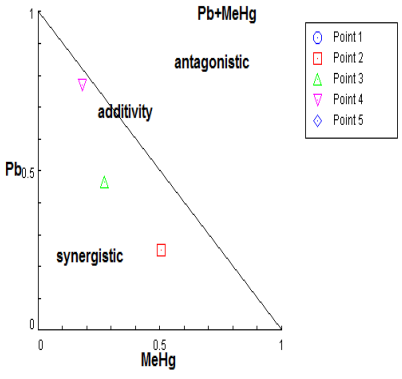
3.5. Interactions among the Pb, Cd, As and MeHg mixtures- dose additivity

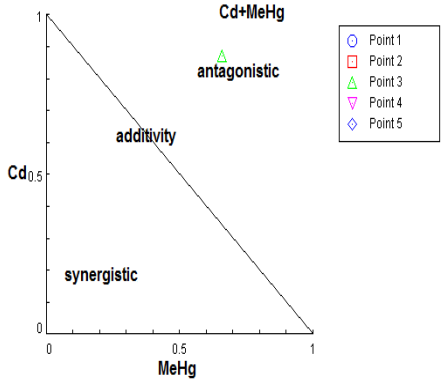
Dose additivity is an equally valid procedure for analysing interactions between metals irrespective of their mechanisms of action and aims to establish the required concentrations of individual metals within a combination that produces a specified level of effect. Individual concentration- response curves for all four metals were used to calculate the individual concentrations required to produce 30% inhibition of cell death. The effective concentration (IC_{30}) of Pb, Cd, As, and MeHg are 70.0 µM, 3.37 µM, 0.66 µM, and 0.29 µM respectively. All binary mixtures tested as non- constant ratio isobologram model and generated data points illustrated in Fig 5, the straight line between the mean single values is referred to as the additivity line and area of CI values for these two measurements the CI limits. Qualitative evaluation of curve can be in two types of descriptors; the mean value can either is within the CI belt or exist outside or inside (deviation from additivity). Mixtures interaction for Pb, Cd, As, MeHg represented in Table 3, descriptively graphic data suggest that all combinations interaction. The combination of A (X-axis) and B (Y-axis) metal using a comparison of CI values for single concentrations (100% A/ B% metal and 0% A/ 100% B, additivity line). For instance, a combination of Pb and Cd was produced CI>1 value, the significant points are shown in Fig 5, suggests that Pb and Cd were antagonistic effect (50% Pb/ 50% Cd, 75% Pb/ 25% Cd) in the mixture. The effect of Pb and As

combination clearly indicates that slightly deviation from the additive line in the different ratios, the CI < 1. The isobologram of Pb+As mixture mark slightly synergistic interaction. The Pb and MeHg mixture suggests the interaction is more deviate from the expected additivity line (25% Pb/ 75% MeHg, 50% Pb/ 50% MeHg), CI value 0.7, 0.7 respectively. The combination of Cd with MeHg, and As shows the antagonistic effect, the similar results can be seen for isobole curve; however, the antagonistic potency of interaction depends on the composition of mixtures (CI values range 1.4 to 3.8). We found highest antagonistic effect in Cd+MeHg mixture (25% Cd/ 75% MeHg, 75% Cd / 25% MeHg) CI values 3.31, 2.05 respectively that indicates MeHg concentration influencing the combination effect (except MeHg+As) (Table 4).









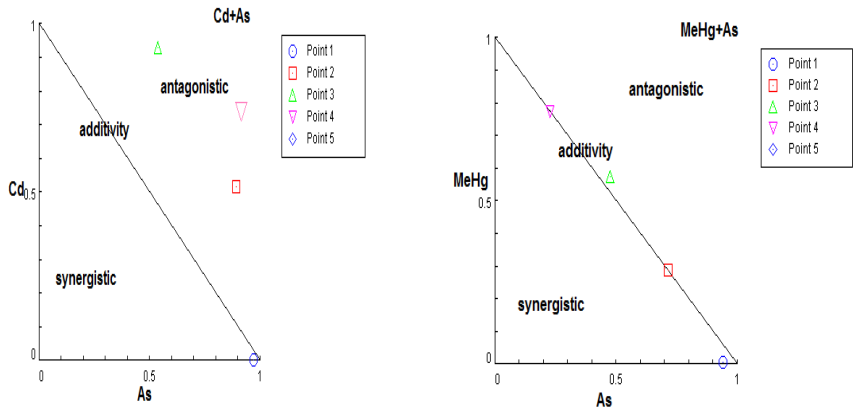


Figure 5: Normalized isobologram plot of binary mixtures of Pb, Cd, As, and MeHg on HT-22 cells. Each isobologram experiment was performed three times independently of each other; data presented for each metal combination are a mean of all three experiments. Some points are out of the limit in curve representation.

Table 3: The combination index (CI) values of each non- constant ratio of the mixture obtained from the Compusyn normalized isobole model (Note. 100% A and 100% B correspond to each metal's IC_{30} value; other metal amounts are related to a fraction of the IC_{30} as indicated).

Point	Mixture ratio		Combination Index (CI) value					
	A (%)	B (%)	Pb+Cd	Pb+As	Pb+MeHg	Cd+As	Cd+MeHg	MeHg+As
1	0	100	1.0	1.0	1.0	1.0	1.0	1.0
2	25	75	1.5	0.8	0.7	1.4	3.3	1.0
3	50	50	1.4	0.9	0.7	1.4	1.5	1.0
4	75	25	1.3	0.9	0.9	1.8	2.0	1.0
5	100	0	1.0	1.0	1.0	1.0	1.0	1.0

(*CI value =1; additive, > 1; antagonistic, <1 synergistic according to Isobologram analysis)

4. Discussion

Usually, more than one heavy metal was involved in the environmental exposure. A risk assessment of the metal mixtures would be more practical, and the combined effects are needed in future research to improve the risk evaluations. The objective of this work was to assess toxicities of heavy metals individually and as mixtures on HT-22 cell line. The results showed significant cell reduction after exposure to toxic metals. Ultimately

binary mixtures induced significant cell death compared to individual metals. The individual toxicity of these four metals to different cell lines and organisms has been widely investigated; however, mixture studies are very limited relevant to hippocampal cell line. The Pb toxicity results in the HT-22 cells suggest the potency of Pb depends on the concentration. It showed that Pb could gradually decrease the viability of HT-22 cells and the result was consistent with reported studies (Xu et al., 2006). The observed neurotoxic effects of Cd in various brain cell cultures are in agreement with the current HT-22 cells cytotoxic effects (Lopez et al., 2003). The toxicity of Cd perhaps establishes the relation with Ca^{+2} ion mechanism. Orrenius and Nicotera (1994) reported that the concentration of cytoplasmic and nuclear Ca^{+2} increased in neurons as result of Cd exposure. In experimental animal models was found that As causes a potent alteration in hippocampal region (Cronican et al., 2013). In the present study As shows significant effect on HT-22 cells; the decreasing pattern of cell viability was high. Other reported studies showed that As induces cytotoxicity in human lung fibroblasts in dose dependent manner, which is consistent with current study (Vogt and Rossman, 2001). The MeHg potency is very high in HT-22 cells rather than other three metals. The obtained results are consistent with MeHg toxicity studies in glioblastoma, neuroblastoma cultures and cerebellar granule cells (CGC) (Crespo-López et al., 2007; Fonfría et al., 2005)

To further gain insight into the heavy metal toxicity; mechanistic based assay such as apoptosis might be potential to validate the MTT assay. To know the clear cytotoxic effect on HT-22 cells, we performed Annexin- V/ PI staining that allows the discrimination of viable cell, apoptotic, and necrotic cells by binding with Ca^{+2} dependent phosphatidyl serine (PS) protein in the cell membrane (Vermes et al., 1995). The obtained results confirm Pb could induce the apoptosis in HT-22 cells, however the apoptosis response was very low in tested 63.5-70 µM range; the observed results reported similarly like calcium to bind the internal metal binding site of the permeability transition pore and open it, which could initiate apoptosis in the retina (He et al., 2000). The effect of Cd was more pronounced at 3.37 µM. Reported studies have shown that Cd induces dose- dependent apoptosis like cortical neurons (López et al., 2003). Regarding As we observed that apoptosis at a low dose (0.66 µM); these findings correlate with As induced apoptosis in neuroblastoma cells (Akao et al., 2000). The MeHg induced apoptosis in HT-22 cells was high in 0.18-0.29 µM, the similar response was observed in human SH-SY 5Y neuroblastoma cells (Ndountse and Chan, 2008). The results indicate that percentage of apoptotic cells was dependent on the metal potency like MTT assay, maximal level of apoptotic cells observed in MeHg, and potency of metals ranked MeHg > As > Cd > Pb. We are used the apoptosis to validate the MTT assay results for extending to the metal mixtures toxicity.

Based on the cytotoxicity of individual metals Pb, Cd, As, and MeHg on HT-22 cells, the response addition, dose addition methods were used to determine interaction profile of mixtures. However, there is a widespread disagreement over terminology, definitions,

and models for the analysis of mixtures (Feron and Groten, 2002; Wu et al., 2016). Several methods for calculating the expected combination effect of two or more chemicals are currently in use, the majority of which can be associated with two popular basic concepts known as response additivity (Bliss 1939) and dose additivity (Loewe 1953). Response additivity focuses on measuring the effects of mixtures at only one specified concentration for each metal, thus lacking the information on concentration-response relationships. Dose additivity is an equally valid procedure for analysing interactions between agents irrespective of their mechanisms of action. However, this method requires tedious testing with a variety of concentrations for the determination of each data point on the isobologram, There is no generally accepted agreement as to which of the two concepts is more appropriate (Teuschler, 2007); therefore we have attempted to carry out this study using both models to confirm our findings. It is noteworthy that the combined toxicity of the overall metal mixtures might be different from their individual toxicities. Thus mixture studies are important to elucidate whether these interactions to such mixtures would cause deleterious effects to hippocampal cell line. We used simple effect addition and dose addition isobologram analysis concept for mixtures interactions. Response addition implies that the effects of exposure to a mixture of such chemicals are equivalent to the effects of the sum of the potency corrected doses of each component (Ince et al., 1999). The findings from Pb+Cd results consistent with experimental studies on a pregnant rat showed that combined exposure of Cd and Pb have additive effect on decreasing Na^+ / K^+ -ATP ase function, in which Cd activity is potentiated by Pb for causing failure of the Na^+/ K^+-ATPase (Antonio et al., 2003). In the current study, significant antagonism was found in Cd+As mixture; the observed antagonistic effect supported by Vellinger et al. (2012). In addition a Pb+MeHg, Cd+MeHg mixture showed similar toxic potency level which suggests the MeHg influences the equitoxic level in both mixtures however, the combination with MeHg and other metals studies are limited. The findings mainly indicate synergistic effect among MeHg and Pb, Cd in high concentration, which is an agreement with other investigators findings of synergism (FríasEspericueta et al., 2009). The other two combinations Pb+As, As+MeHg indicates the simple additive effect on HT-22 cells. Based on the single metal results, we propose that the isobologram analysis for dose additivity method, Rodea-Palomares et al., (2010) were the first to use this method in environmental risk assessment applications. In the isobologram analysis, the metal mixture impact was different from the equitoxic exposure. The combination of Cd with Pb, As MeHg indicated the antagonist effect clearly shows that co exposure of Cd metal implies the toxicity of other metal in a reduction manner. The current Cd results disagreement with reported interactions studies such as Cd with As increases the expression of stress proteins in rat and human kidney cell lines (Madden et al., 2002). The combination of Pb with As and MeHg effects found more than the additive effect according to CI value. The other combination of MeHg & As CI value on HT-22 cells suggesting a simple additive effect. These findings give significant evidence of the metal mixtures neurotoxic activity and their potential interactions depend on the

composition of elements, cell line sensitivity. The observed interactions at dose additivity, response additivity supported by (Bae et al., 2001) interactive effect of Pb, Cd, As and Cr on keratinocytes, the results showed a trend of additivity, synergism and antagonism with increasing metal mixture concentrations. However, the current mixtures toxicity is most relevant to neurotoxicity; the literature related to metal mixtures toxicity impact on hippocampus is very limited.

Table 4: Summary of metal mixtures interaction profile on HT-22 cells during 8 days exposure.

Mixture	**Interaction profile**	
	Response addition	**Dose addition**
Pb+Cd	Additive	Antagonistic
Pb+As	Additive	Slightly synergistic
Pb+MeHg	Synergistic	Synergistic
Cd+As	Antagonistic	Antagonistic
Cd+MeHg	Synergistic	Strong antagonistic
MeHg+As	Additive	Additive

5. Conclusion

From a public health point of view, it is most relevant to answer the question whether chemicals in a mixture interact in a way that results in a reduced or increased overall response when compared with the sum of the responses to the individual chemicals in the mixture, or indeed in an effect that is simply an addition of the expected effects. We investigated the toxicity of four heavy metals and their mixtures on hippocampus relevant mice HT-22 cell line for knowing their toxicity impact. The comparison between the effect addition and dose addition was useful in the evaluation of combined effects in the mixture. The nature of interaction varies according to the effect levels and the type of components in the mixtures. This binary combination of Pb, Cd, As, and MeHg displayed synergistic and antagonistic interaction at low and higher effect levels, respectively. The mixture effects should be considered in the risk assessment of co-exposure metal potency. Toxicity order of current mixtures results on HT-22 cells, Pb+MeHg (synergistic)>MeHg+As (additive) > Cd+As (antagonistic) found in both dose addition and response addition in a similar manner. The other three mixtures (Pb+Cd, Pb+As, Cd+MeHg) interaction effects are different in an each method. We concluded from the results; dose addition isobologram analysis was a more beneficial strategy for mixtures study than response additivity. We expect this approach will be of use for a wide range of metal mixtures and indeed other types of experiments for knowing the interaction profile at each point. Moreover, improved investigations on metal combined effects are needed in future studies. The outcome of this study adds to a

new sense of urgency for research to examine the mechanisms associated with toxicities of metals mixtures. In future, comprehensive mechanistic based omics investigation will be useful for understanding the real interactive mixtures interactions at molecular level.

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Chapter 4

Karri V, Kumar V, Ramos D, Martinez J, Odena A, Oliveira E, Coorte S, Mariman E, Evelo C, Schuhmacher M,
Differential protein expression of Hippocampal cells associated with Heavy metals (Pb, As, and MeHg) Neurotoxicity: Deepening into the Molecular Mechanism of Neurodegenerative Diseases
(Under revision)

Chapter 4

Chapter 4

Differential protein expression of Hippocampal cells associated with Heavy metals (Pb, As, and MeHg) Neurotoxicity: Deepening into the Molecular Mechanism of Neurodegenerative Diseases

Abstract

An increased worldwide industrialisation has led to higher levels of pollution by potent neurotoxins such as lead (Pb), arsenic (As), and methyl mercury (MeHg). Chronic exposure to heavy metals such as Pb, As, and MeHg can be associated with an increased risk of developing neurodegenerative diseases in human since they can accumulate in the hippocampus region of the brain. Our previous in vitro bioassays results showed the potency of heavy metals Pb<As<MeHg on hippocampal cells. Examining global effects of toxic metals on protein expression can be useful for elucidating patterns of biological response, discovering underlying mechanisms of toxicity in hippocampal cells. The main objective of this study was combining the in vitro label free proteomics and systems biology for improving the neurotoxicity of Pb, As, and MeHg metals. The omics data was refined by using different filters and normalization; also different multilevel analysis tools were employed to explore the data visualization (PCA, Hierarchical clustering, Venn, and Volcano).The functional and pathway visualization was performed by using Gene ontology and Pathvisio tools. Using these all integrated approaches, we identified significant proteins across treatments within the mitochondrial dysfunction, oxidative stress, ubiquitin proteome dysfunction, and mRNA splicing relate to neurodegenerative diseases. The systems biology analysis revealed significant alterations in proteins implicated in Parkinson's disease (PD) and Alzheimer's disease (AD). The current proteomics analysis of the three metals support the insight into the proteins involved in neurodegeneration and the altered proteins can be useful for metal-specific biomarkers of exposure and its response.

Key words: Metal exposure; Proteomics; Pathway visualization; Neurodegenerative diseases.

Abbreviation: AD= Alzheimer's disease, APP= Amyloid precursor protein, Aβ =Amyloid-beta, CAM= Calmodulin, ETC= Electron transport chain, , GAD= glutamate decarboxylase, Glu= Glutamate, GO= Gene ontology, LFQ= Label free quantification, LTP= Long term potentiation, MAPK= Mitogen-activated protein kinase, MAO-A= Monoamine oxidase A, NMDA= N-methyl D-aspartate, OD= Optical density, ORA= over-representation analysis, PD= Parkinson's disease, PS= Phosphatidyl serine, PI= Propidium iodide, PKC= protein kinase-C, ROS= Reactive oxygen species ,UPS= Ubiquitin proteasome System.

Chapter 4

1. Introduction

Many environmental pollutants have been associated with human diseases (CDC, 2014). Among various pollutants, toxic heavy metals contribute to a great proportion of air, soil and water pollution and cause major health problems to human beings (Alves et al., 2014; Angelica and Fong, 2014; Nadal et al., 2004;). An increased worldwide industrialisation (Fonfría et al., 2005) has led to higher levels of pollution by potent neurotoxins such as lead (Pb), arsenic (As), and methyl mercury (MeHg) (Neal, 2015). Both environmental and occupational exposures to any of the three metals are of significant toxicological concern (Caserta et al., 2013; Yu et al., 2011). Their multiple industrial, domestic, agricultural, medical, and technological applications have led to their wide spreading in the environment (Chowdhury et al., 2016; Tóth et al., 2016). Heavy metal exposure can occur through contaminated air, food, water, and/or hazardous occupations (Ha et al., 2017; Li et al., 2017; Tang et al., 2017; Xu et al., 2016). The toxicity of heavy metals depends on several factors including dosage, route of exposure and chemical species, as well as on the age, gender, genetics, and nutritional status of exposed individuals (Bolan et al., 2017; Deshommes et al., 2016; Khan et al., 2013). Human exposure to three heavy metals (Pb, As, and MeHg) can disrupt brain function (Farina et al., 2013; Montgomery, 1995), and increase the risk of diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Basha et al., 2005; Cholanians et al., 2016; Prakash et al., 2015).

These three heavy metals (Pb, As and MeHg) are well known potent environmental toxicants, and humans is exposed to each one of them via different routes (Mauriello et al., 2017; Persoons et al., 2014). Firstly, the main routes of exposure for Pb are inhalation and ingestion (Anderson et al., 2016; Li et al., 2011). Inhalation exposure to Pb is a much more efficient route of absorption than ingestion (Chaumont et al., 2012; Li et al., 2013). Pb metal is relatively common in the environment, and its toxicity impacts the children health (Eqani et al., 2016). Pb exposure in the workplace is responsible for a wide range of adverse effects, mainly on brain (Mari et al., 2009). Secondly, humans are exposed to various forms of As mainly via oral consumption of contaminated water, food or drugs (Ng et al., 2003; Sarkar and Paul, 2016). As metal can also enter the body via inhalation, which is particularly important for certain types of occupational exposure (Jadhav et al., 2015; Mandal, 2017; Singh et al., 2015). Also As metal is rapidly absorbed, distributed and stored in different body organs such as liver, kidney, and lung. Moreover, it can easily cross the blood brain barrier (BBB) and accumulate in the brain (Tseng et al., 2005; Wang et al., 2007). Reported studies state that As metal has been directly linked to neurodegenerative diseases (Rodríguez et al., 2003). These findings raised concern over As induced neurotoxicity in humans (Luo et al., 2009; Rodriguez et al., 2002; Tyler and Allan, 2014). However, the underlying molecular mechanisms not clear. Finally, MeHg contamination is possible through consumption of fish and other seafood (Davidson et al., 2008; Murata et al., 1999). In humans, MeHg accumulates in kidneys and neurological tissues (Morais and Garcia,

2010). It is well known that ingested MeHg can interact with proteins due to its strong affinity to sulphur (-SH) containing functional groups, and causing organ dysfunction in the central nervous system (Ceccatelli et al., 2010; Johansson et al., 2007).

In the past few years, many studies have been conducted to understand the mechanisms underlying Pb, As and MeHg toxicity on the hippocampus region of brain (Stackelberg et al. 2015; Wright and Baccarelli 2007; Zheng et al. 2003). Exposure to Pb and MeHg have significant effects on human brain (Aschner et al., 2007; Sanders et al., 2009). Many reported evidences linked As exposure with developmental neurotoxicity (Tyler and Allan, 2014). Recently, Karri et al., (2016) reported that Pb, As and MeHg exposure induce damage to the hippocampus region of the brain. However the risk level depends on exposure intensity and metal nature in the brain (Luo et al. 2009; Kruger et al. 2009). Basha et al., (2005) observed that developmental exposure to Pb exhibits latent effects, through epigenetic interaction of Pb with amyloid precursor protein (APP) gene causing neurodegeneration at older age. As can disrupt the cognitive function of brain in children (Tyler and Allan, 2014). MeHg directly disrupts the mitochondrial function by generating an uncontrolled release of Ca^{+2} (Atchison and Hare, 1994), resulting in the dysregulation of the mitochondrial electron transport chain (ETC) (Farina et al., 2011) and causes the cell death. MeHg effect on mitochondrial function could be a potential cause of neurodegenerative diseases (Ho et al., 2012).

Most of the reported studies focused on the effects of metals on the brain in a generalized manner (Johansson et al., 2007; Sadiq et al., 2012; Tyler and Allan 2014). In the past few years, substantial improvements in toxicogenomics knowledge have led to an increase in the application of proteomics and systems biology knowledge to answer these mechanistic biological questions (Cuello et al., 2012; Han et al., 2012).. The main goal of this project was taking advantage of high throughput proteomic technologies and system biology tools for assessing the neurotoxicity mechanism of Pb, As, and MeHg on the hippocampal cells. We choose the mouse hippocampal HT-22 cell line due to the known sensitivity of chronic Pb, As and MeHg exposure (Karri et al., 2017a). Here, a label-free quantitative proteomics approach was used to detect the effects of Pb, As, and MeHg exposure at the protein level by using a non-cytotoxic dose (IC_{10}) of each metal to avoid secondary cytotoxic responses. The differential protein expression patterns involved in the heavy metal toxic response were quantified to deeply reveal the integrative molecular network of protein response to heavy metal stress. The proteomics data were integrated with a systems biology approach in which the analysis tools PathVisio, GO-elite, and Cytoscape were used to provide more global view of the molecular and cellular changes elicited by heavy metals in hippocampal cells.

2. Materials and Methods

2.1. Chemicals

Lead chloride ($PbCl_2$ [CAS no: 7758-95-4]), Sodium metaarsenite ($NaAsO_2$ [CAS no: 7784-46-5]), Methyl mercury chloride ($MeHgCl_2$ [CAS no: 115-09-3]), Dimethyl sulphoxide (DMSO [D5879]), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT [M5655]), trypsin (TrypLE [Gibco: 12604013]), and proteomics reagents: Urea (GE HealthCare, Life Sciences, CAS Number: 57-13-6), Sodium Dodecyl Sulphate (SDS) (Merck, CAS Number: 151-21-3), Ammonium Hydroxide (Fluka, CAS Number: 1066-33-7), Dithiothreitol (GE HealthCare, Life Sciences, CAS Number: 3483-12-3), Iodoacetamide (GE HealthCare, Life Sciences, CAS Number: 144-48-9), Formic Acid (Merck, CAS Number: 64-18-6), Acetonitrile (HPLC grade) (Fisher Chemical, CAS Number: 75-05-8), Water (HPLC grade) (Fisher Chemical, CAS Number: 7732-18-5). All are analytical grade and purchased from Sigma-Aldrich Química, S.L-Madrid (Spain).

2.2. Cell line and Reagents

The HT-22 cells have been used as a hippocampal neuronal cell model in numerous studies (Niska et al., 2015). The HT-22 cells were a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA). HT-22 cells were maintained in Dulbecco's modified eagle's medium (DMEM [D6429]) containing 10% fetal bovine serum (FBS Gibco [10500-064]) and 100 U/mL penicillin, and 100 µg/mL streptomycin (Pan-Biotech- Germany) in a humidified incubator with 5% CO_2 in air at 37^0 C. For all the experiments cells were grown at 70- 80% confluence.

The cells were cultured in 75 cm^2 cell culture flasks. For experimental purpose, cells were plated at 0.56 x 10^6 cells/ mL and grown for 24 hours before metal treatment. Duplicates wells of cells were treated with 10 exposure levels of Pb, As and, MeHg ranging from 10 to 100 µM, 0.4 to 4.2 µM, and 0.6 to 12 µM, respectively; due to the 8 days exposure-period, medium containing the given concentration was refreshed at 2 days interval in order to maintain the metal exposure along time. Metal stock solutions 100X were prepared in deionized distilled water (for poorly soluble $PbCl_2$ < 0.5% DMSO was added) and sterilized by filtration through 0.2 µm and different concentrations of a working solution for each individual metal were prepared by prior dilution of the stock solution in phosphate buffered saline (pH = 7.4) and then applying 10% working solution on DMEM culture medium.

2.3. Cytotoxicity

The MTT assay was carried out using a modification of the method of Mossman (1983). The HT-22 cells were seeded in 96-well plates. After 24 h, when the cells had reached a confluence of 70–80%, they were exposed for 8 days to various concentrations of Pb, As, and MeHg. After the incubation period, medium was aspirated from each well and MTT working solution at 0.5 mg/mL was added to each well. The absorbance of the solubilized reduced MTT was then measured in a microtiter plate spectrophotometer

reader at a wavelength of 570 nm. The measured optical density (OD) values were converted to percent of cell viability (%) with respect to control. The cell viability results were used for calculating the sub-lethal dose (IC_{10}) of each metal for apoptosis and proteomics studies.

2.4. Analysis of apoptosis by annexin V-FITC/ propidium iodide (PI)

To evaluate the translocation of phosphatidylserine (PS) from inner leaflets to outer leaflets of the plasma membrane, annexin V-FITC apoptosis detection kit (BD Pharmingen, Poland) was utilized. In this kit, annexin V and propidium iodide (PI) were used to distinguish the apoptotic and necrotic cells from the viable cells. According to the manufacture's protocol, the exponentially proliferating cells were exposed to the IC_{10} to IC_{30} of heavy metals in culture plate at a density of 0.56 x 10^6/mL during 8 days; control cells were made without chemical. Cells were harvested by trypsinization, washed twice with ice cold PBS (pH = 7.4). Thereafter, cells were centrifuged at 1200 rpm for 5 min at 4^0C, resuspended in 1mL 1X binding buffer and then 100 µL of the solution was transferred to a 5 mL culture tube, and 5 µL of both annexin-V and PI was added to the samples. After staining, cells were incubated for 15 minutes in the dark at room temperature. Finally cells were re-washed with 400 µL 1X binding buffer and analyzed by flow cytometry (Beckman coulter, Germany). Three independent experiments were performed.

2.5. Protein extraction and digestion

For the proteomics study, the exponentially proliferating HT-22 cells were exposed to the selected sub-lethal dose (IC_{10}) of each heavy metal at a density of 0.025 x 10^6/mL during 8 days; control cells were made without chemical exposure. Pb, As, and MeHg treatment (and control culture) protein was extracted from HT-22 cells with 200 µL lysis buffer (8 M urea, 0.1% SDS, 50 mM ammonium bicarbonate [ABC]) and quantified using the Micro BCA™ Protein Assay Kit (Thermo Scientific). 30 µg of protein from each sample was digested in a filter assisted sample preparation (FASP) approach. The volume corresponding to 30 µg of protein was adjusted to 270 µL with 8 M Urea/50 mM ABC. Reduction was done with 20 mM dithiothreitol (DTT) treatment for 1h 30 min at 32^O C. Samples were then alkylated using 30 mM of iodoacetamide (IAA) and incubated in the dark for 30 min at room temperature. Afterwards, samples were loaded in 10 KDa filters (Amicon) and centrifuged during 30 min, 12,000g at room temperature followed by two washes with 300 µL 1M urea/50 mM ABC by centrifugation (30 min, 12,000g, room temperature) in order to remove interferents and establish optimal pH (7.5-8.5) and urea concentration for proteins to be denatured. The filter remaining material was resuspended in 400 µL 1 M urea/50 mM ABC. Digestion was done in two steps: an initial digestion with 1:30 (w/w) porcine trypsin 0.25 µg/µL (Sequence grade modified trypsin, Promega) for 3 hrs at 32^OC followed by a digestion with 1:50 (w/w) trypsin 0.25 µg/µL for 16h at 32^OC. Peptides were eluted by centrifugation (12,000g for 15 min at room temperature) and the filters were cleaned

with 200 µL 50 mM ABC by centrifugation at 12,000g for 15 min at room temperature. Samples were prepared for chromatographic analysis. Briefly, peptides resuspended in 100 µL of 1% formic acid (FA) were charged in the tip columns (previously washed with 70% acetonitrile [ACN] in 0.1 % FA and equilibrated with 0.1% FA) by centrifugation (300 × g for 1 min 30 sec). Columns were washed twice with 100 µL 0.1% FA by centrifugation (300g for 1 min) and then peptides were eluted in 2 x 100 µL of 70% ACN / 0.1% FA by centrifugation (300g for 1 min). The peptides were dried in speed vacuum (Eppendorf) and stored at 20 ^{O}C until LC-MS analysis.

2.6. LC-MS/MS analysis

The peptide mixtures were resuspended (1% FA, 2% ACN) and the volume corresponding to 500 ng of protein was analysed by an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Peptide mixtures were injected into the capillary column (75 µm× 25 cm) in full loop mode and separated by a 5 µm C18 column using a nano-acquity liquid chromatography system (Waters). Peptides were eluted with a linear gradient of 1-35 % buffer B (0.1% FA, 100% ACN) for 150 min, followed by 35-45 % buffer A for 20 min (0.1% A). The mass spectrometer was operated in positive ion mode (source voltage 2000V) and data-dependent manner. The full MS scans were performed in the Orbitrap at the range of 300–1,700 m/z at a resolution of 60,000. For MS/MS scans, the 15 most abundant ions with multiple charge states were selected for collision induced dissociation (CID) fragmentation following one MS full scan.

2.7. Label-free quantitative (LFQ) data analysis using Progenesis QI software

Proteomics quantitative data analysis was done by using Progenesis QI data analysis software v4 (Non-Linear Dynamics, Waters, U.S.). Ion feature matching was achieved by aligning consistent ion m/z and retention times. Peptide abundances were calculated as the area under the MS peak for every matched ion feature. Progenesis label free quantification (LFQ) was done using non-conflicting unique peptides and protein grouping, and the software normalization algorithm was applied to all proteins. Mascot search results were filtered based on peptide ion score≥ 40 and contaminants were removed and abundances were normalized to all proteins. A total of 428,801 spectra were obtained from the LFQ LC−MS/MS proteomic analysis of samples including a control and test samples. After data filtering to eliminate low-scoring spectra 27,088 peptides were retained and used for protein identification. Total 3,140 proteins were quantified from 27,088 peptide ions. Only the proteins quantified with minimal 2 peptides were included in the further statistical analysis.

2.8. Protein identification

Proteins identification was done using Mascot search engine (v. 2.3.01). All MS/MS spectra passing an arbitrary cut-off in the Progenesis software were included in the list used to perform the search against SwissProt Mouse database using the Mascot search program (Matrix Science, London, UK, www.matrixscience.com). The following

criteria were applied: (1). trypsin as enzyme; (2). allowed two missed cleavages; (3). variable modifications, acetyl (N-terminus, protein) and oxidation (M); Carbamidomethyl (C) fixed modification and (4). Peptide tolerance, ±10 ppm (MS); MS/MS tolerance, ± 0.6 Da.

2.9. Statistical analysis

Label free quantitative data was submitted to T-test method ($p \leq 0.05$) using Microsoft excels in order to find out significant differences among the treatments and control. Data was further curated applying the following filters: minimum 1.2-fold change and p-value< 0.05. Meaboanalyst 3.0 (http://www.metaboanalyst.ca) programme was used for presenting the unsupervised data in principal component analysis (PCA), volcano plot, and hierarchical clustering analysis.

2.10. Systems biology analysis

In our systems biology analysis three main steps were included for the quantitative functional interpretation of proteomics data. The first step was aimed to identify the statistically significant proteins by using the T-test method. The next step was to identify significantly altered functional Gene Ontology (GO) categories using GO analysis. The altered GO terms and the differentially expressed proteins in these GO terms were visualized in a network to identify the links between the terms. In the final step the changes in protein expression were visualized in selected pathways related to neurodegenerative diseases.

2.10.1. Functional interpretation of significantly altered genes using GO analysis

GO-Elite (http://www.genmapp.org/go_elite/), a flexible and powerful tool for GO functional analysis (Zambon et al., 2012) was used to find altered biological processes, molecular functions, and cellular components based on changes in proteins expression. GO-Elite performs an over-representation analysis (ORA) on any structured ontology annotations, or biological identifiers (e.g. gene, protein or metabolite) (Huang et al., 2009). The program utilizes the structured nature of biological ontologies to report a minimal set of non-overlapping terms called as pruned terms. Pruning is the process of intelligently examining the hierarchical structure of ontology, such as GO, and comparing the ORA scores of enriched terms based on their relationships to each other. Highly related terms highlighted by ORA are considered to be redundant with each other. Hence, pruning is used to select the highest scoring term among a set of related terms, while retaining any distinct related terms. GO-Elite ranks each analyzed pruned GO term according to a Z-score along with a p-value (Ren et al., 2010). Significantly altered categories were identified based on the p value <0.05, Z-score > 1.96, and a minimum of 3 proteins changed within each specific GO term. The results were visualized as networks linking the GO terms and differentially expressed proteins in the GO terms using the network analysis tool Cytoscape (Gang Su et al., 2009). In addition,

on the GO-protein network in Cytoscape the log2 fold change (log2FC) between each metal exposure group and to control were visualized.

2.10.2. Pathway visualization

PathVisio (Kutmon et al., 2015) is a commonly used pathway editor, visualization, and analysis software for omics based experiments. First the required curated mus musculus pathway collection was obtained from WikiPathways (http://www.wikipathways.org) (Kutmon et al., 2016). Pathway analysis was performed in PathVisio version 3.2.4 (http://www.pathvisio.org) to interpret and visualize the molecular changes on a pathway level. In the present study, we visualized the changes in proteins after metal exposure in biological pathways relevant to neurodegeneration processes in which a minimum of 3 proteins were changed. The log2FC and p-value were shown on pathways using the visualization module in PathVisio. A flow chart of experimental label free proteomics and system biology is shown in Figure 1.

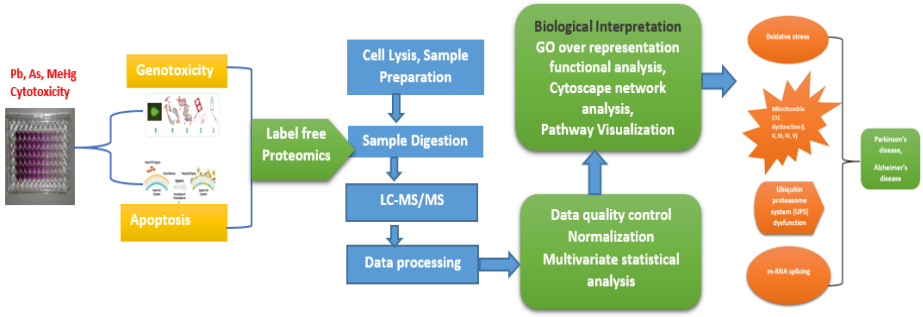


Figure 1: Flow chart of experimental label free proteomics and systems biology for evaluating the heavy metals (Pb, As, and MeHg) toxicity on HT-22 hippocampal cells.

3. Results and Discussion

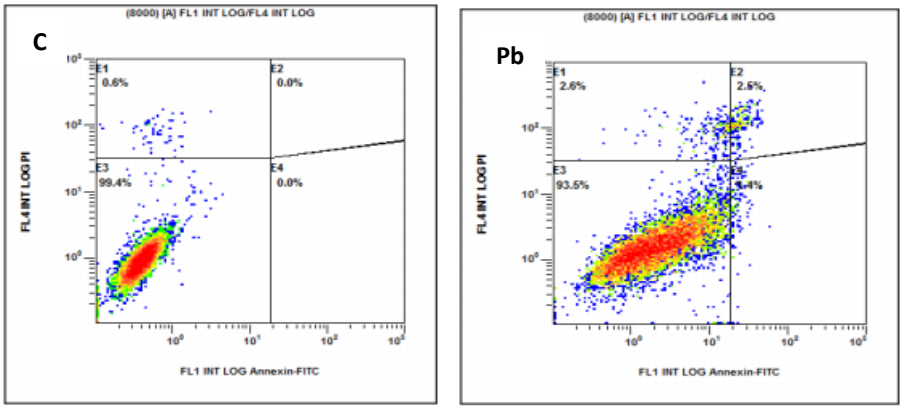
To characterize the proteomics changes of Pb, As and MeHg individually on mouse hippocampal HT-22 cells during 8 days exposure, firstly we performed cell viability studies by using the MTT assay. The cell confluence (80-85%) was stable until 8 days exposure; consequently we considered the 8 days exposure as chronic for further mechanistic studies. As expected, all three metals showed concentration and time-dependent cytotoxic effect. Results and discussion for Pb, As and MeHg cytotoxicity experiments at chronic (8 days) conditions were reported by Karri et al. (2017). Upon the Pb exposure (10-100µM)) cell death was significantly lower indicating that sensitivity of hippocampal cells towards Pb is relatively low. Previous studies reported that As showed cytotoxicity even at micromolar concentration (Hu et al., 1998), and similar results were observed in HT-22 cells treated during 8 days. However, MeHg showed high potency in hippocampal cells (HT-22) compared to Pb and As (Table 1).

The obtained MeHg results were consistent with previous studies in cerebellar granule cells (Fonfría et al., 2005; Crespo-López et al. 2007).

Table 1: The IC_{50} values of Pb, Cd, As, and MeHg for in vitro hippocampal HT-22 cell line after 8 days exposure

Exposure time	Heavy metals (IC_{50})		
	Pb (µM)	As (µM)	MeHg (µM)
8 days	74.3	0.8	0.6

The obtained 8 days exposure MTT results were useful for further mechanistic based assays such as apoptosis to know the chronic risk of heavy metals on HT-22 cells. For that purpose, in a previous study, cells were treated with a range of IC_{10} to IC_{30} concentrations (µM), and results were reported in Karri et al., (2017a) This report confirmed that Pb induced apoptosis process was low in HT-22 cells and agreement with reported studies in (Figure 2) (He et al., 2000). The other two metals, As and MeHg, showed significant effects even at low concentration of exposure (Roos and Kaina 2006; Ndountse and Chan 2008). The results of the present study (Table 2) show that non-cytotoxic (IC_{10}) apoptosis mechanism also similar to cell viability in the potency of metal. The selecting sub lethal (non-cytotoxic) concentration effects suggest that for the three metals, the necrotic process was not initiated in mild stress conditions. The MTT and apoptosis results indicate that the toxicity potency of metals on hippocampal cell follow the next way: MeHg>As>Pb. In Figure 2 showing the apoptosis effect of Pb, As, and MeHg onHT-22 cells upon 8 days of exposure.



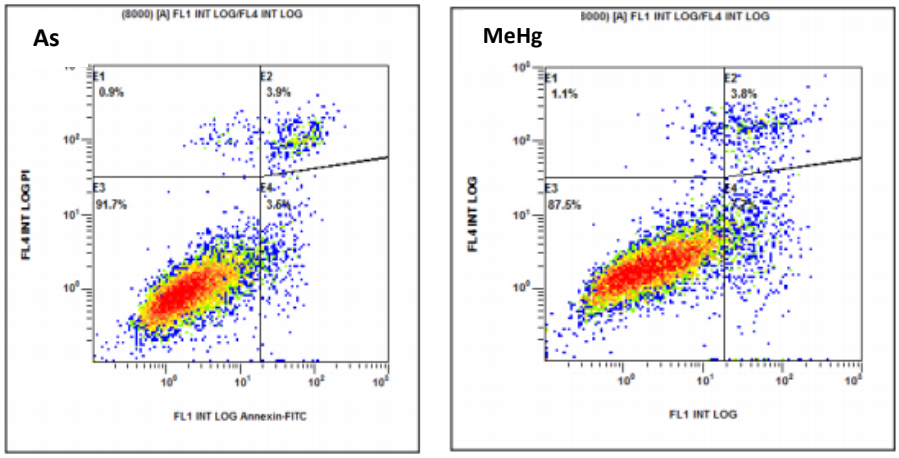


Figure 2: Representative dot plots showing the apoptosis effect of Pb, As, MeHg to HT-22 cells upon 8 days of exposure at sub lethal experimental concentration (n=3). C= Control, Pb= 63.50 µM, As= 0.42 µM, MeHg= 0.09 µM. Lower left (LL)/ E3 = Live cells (Annexin V−/ PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+/ PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

Table 2: Summary data of annexin-V/PI assay obtained from the flow cytometry analysis. Values are shown as means ± SD of 3 replicates per experiment.

Metal	IC_{10} Conc.(µM)	Viable Cells	Apoptotic cells	Necrotic cells
Control	0	98.8 ± 0.4	0.9 ± 0.8	0.2 ± 0.3
Pb	63.50	94.5 ± 0.9	1.9 ± 1.7	3.6 ± 0.9
As	0.42	91.7 ± 0.6	7.4 ± 0.2	1 ± 0.8
MeHg	0.09	89.8 ± 2.7	8.9 ± 1.2	1.3 ± 1.2

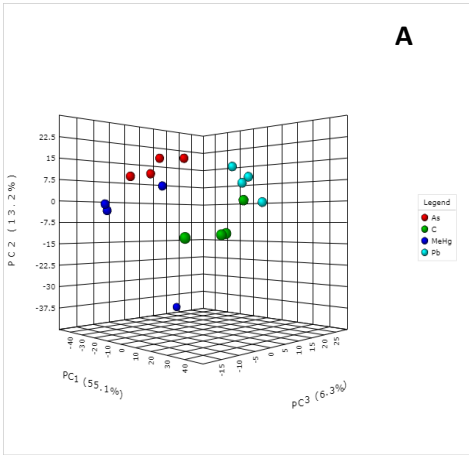
The findings from the MTT and apoptosis assay are interesting for extending further to omics experiments to find the underlying molecular mechanisms. The high throughput label free omics analysis could be helpful for understanding the complete overview of metal induced stress in hippocampal cells and also validate the potency of metals in HT-22 cells by analysing the protein expression. Here, we selected the IC_{10} /sub-lethal concentration of Pb, As, and MeHg (63.50 µM, 0.09 µM, and 0.42 µM) on HT-22 cells for omics studies.

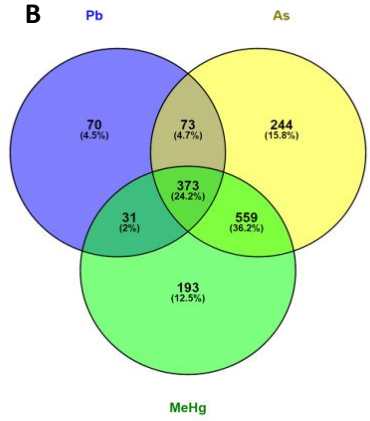
Chapter 4

3.1. Identification and quantification of proteins in HT-22 cells by using the label free proteomic analysis

In order to identify new molecular pathways related to heavy metal neurotoxicity, we have used a proteomics label-free quantitative approach with sub lethal (IC_{10}) concentration in 8 days exposure. The quantitative analysis was performed by Progenesis QI software as explained in the methods section 2.7. In total 3,140 proteins were quantified from 27,088 peptide ions. From them, 2161 proteins were identified with minimum of 2 peptides. Only the proteins with at least two unique identified peptides were retained for further quantification analysis. The unsupervised PCA was performed by using the normalized proteins (≥2 unique peptide) that were present in all four replicates (Figure 3A). The first principal component (PC1: 55.1%) describes the largest variation in the dataset in which the samples spread the most in the variable space. The second component (PC2: 13.2%) describes the next largest variation and is orthogonal to the first component. The third component describes the last largest variation (PC3: 6.3%). Figure 3A highlights clustering or pattern formations in a three-dimensional space which provides a view of the similarities and dissimilarities among the samples. For each individual metal, we extracted the proteins showing significant changes in abundance when compared to control cells (547, 1249 and 1156 proteins for Pb, As, and MeHg, respectively). The resulting proteins of the three metal treatments were compared in a Venn diagram and shown in Figure 3B. We found that 373 proteins commonly altered due to metal exposure. Interestingly, some proteins were exclusively altered due to the exposure to each metal: 70 for Pb, 244 for As and 193 for MeHg. The experimental groups (control, Pb, As and MeHg) were clustered based on the log standard abundance of the unsupervised data with the ward's hierarchical clustering algorithm. The clusters clearly separated according to expression level in each replicate as shown in Figure 3C, and the spot maps of MeHg are distinguished the most from the other spot maps. Differences were also found between the controls, MeHg and As; Pb and Control were very close in the cluster. We can notice the clear presence of group of proteins that are systematically upregulated (red) and down regulated (green) in treatment Vs control. Volcano plot presents the protein expression response to Pb, As and MeHg, respectively (Figure 4). The replicates for the four study groups were averaged and ratios for each metal treatment group to control were calculated. To ease visualization of proteome alterations, ratios (or fold change; FC) were log(2) transformed and plotted against –log(10) transformed ANOVA p-values (Figure 4, A-C) The presented pink dots (log(2)FC ≥0.26 or ≤0.26 and p-value≤ 0.05) were considered as differentially expressed among each metal treated and control comparison. A total of 547, 1249 and 1156 proteins were found to respond to Pb, As and MeHg exposure during 8 days exposure, respectively. Among them, 347, 563, 632 proteins were significantly down regulated, while 200, 686, 524 proteins were significantly upregulated by Pb, As and MeHg respectively.

Chapter 4


A
Legend
As
C
MeHg
Pb
PC1 (55.1%)
PC2 (13.2%)
PC3 (6.3%)


B
Pb
As
MeHg
70
(4.5%)
73
(4.7%)
244
(15.8%)
373
(24.2%)
31
(2%)
559
(36.2%)
193
(12.5%)

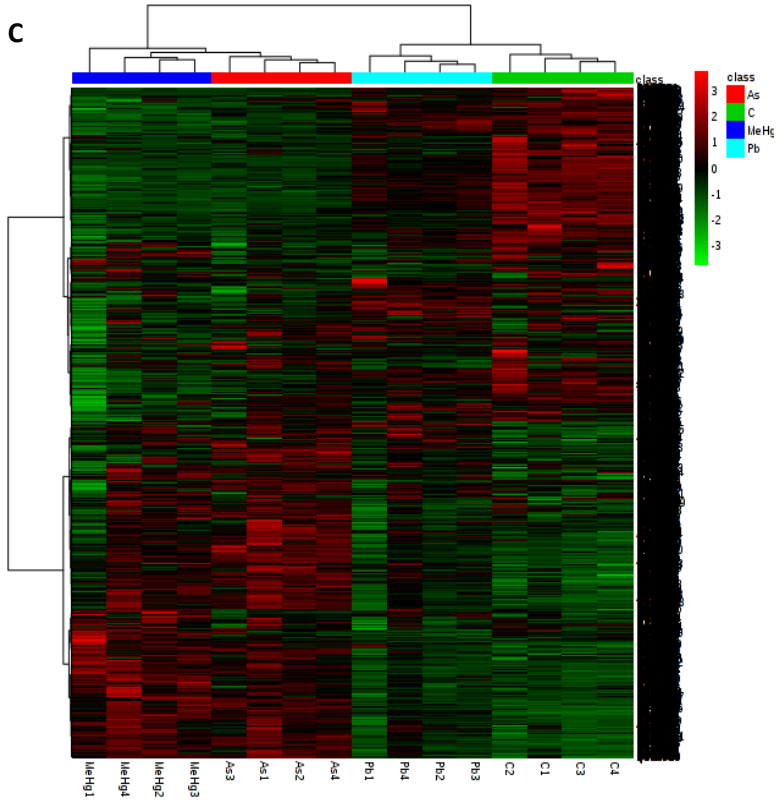

C
class
As
C
MeHg
Pb
MeHg1
MeHg4
MeHg2
MeHg3
As3
As1
As2
As4
Pb1
Pb4
Pb2
Pb3
C2
C1
C3
C4

Figure 3: A. Diagram presenting the principle component analysis (PCA) score plot (PC1, PC2, PC3) of the unsupervised proteomic profiles of HT-22 cell **B.** Venn diagram showing the overlap between significantly changed ($p< 0.05$) proteins in Pb, As, MeHg metals. **C.** Heatmap for hierarchical Euclidean clustering of normalized protein expression of the experimental groups (control, Pb, As, and MeHg).

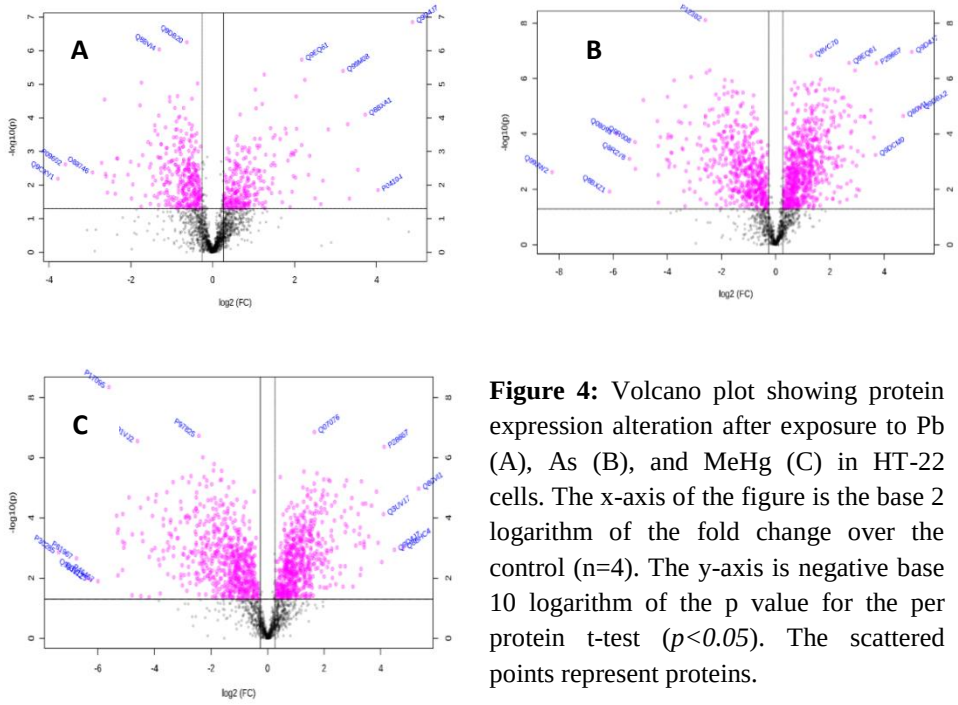


Figure 4: Volcano plot showing protein expression alteration after exposure to Pb (A), As (B), and MeHg (C) in HT-22 cells. The x-axis of the figure is the base 2 logarithm of the fold change over the control (n=4). The y-axis is negative base 10 logarithm of the p value for the per protein t-test ($p<0.05$). The scattered points represent proteins.

3.2. Functional classification and enrichment analysis of protein data by using GO –Elite

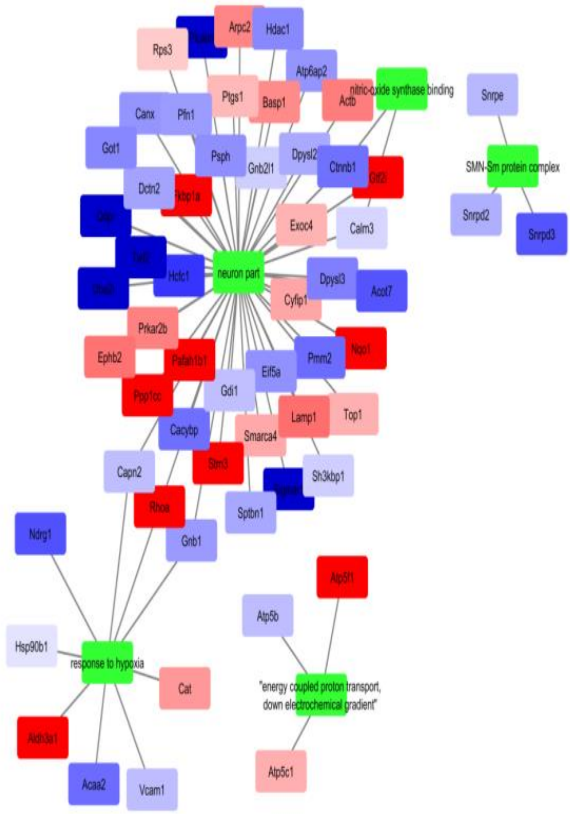
The functional interpretation and the pathway analysis of protein expression are always challenging. The application of Gene ontology (GO) mapping and pathway analysis to omics data for interpret the complex protein expression profiles in the context of the molecular pathways (Camon, 2004). This study demonstrated that GO analysis by using GO–Elite method for the functional interpretation of proteomics data. Our GO results contain multiple files, in that over-representation analysis (ORA) pruned list are most informative to understand the GO terms and proteins functional relation (supplementary file 1), which includes all summary term statistics and associated gene symbols. The ability to determine in which GO terms differentially expressed proteins are

overrepresented provides an ideal model to gain understanding of what GO classes are altered after metal exposure. The pruned ORA list contains the GO terms; i) are pruned to only include non-redundant information and ii) are significantly altered based on changes in protein expression. The GO-Elite results represent a global picture of biological processes, cellular components, and molecular functions that are significantly altered at the protein level after treatment of Pb, As and MeHg. Relationships between all regulated proteins and pruned terms can also be easily visualized as networks in Cytoscape using output the pruned sift file. We showed global networks (supplementary file 2) of the all pruned GO terms with protein expression, which gives an overview of all the significantly expressed proteins and which can be helpful to determine the heavy metal's impact on HT-22 cells proteome. For instance, by observing the GO terms and protein expression relation, we found that Pb metal impact was very low while As and MeHg showed a greater impact on HT-22 cells. A major challenge in the GO interpretation of omics data is the functional interpretation, and the linking of potentially altered proteins to most relevant disease processes. However, from the overview of the network file, it was difficult to relate the results to specific neurodegeneration processes. Using the GO hierarchical classification system, we identified enriched processes (GOIDs) within the collection of genes identified as significantly altered by Pb, As, and MeHg exposure. Generally, ORA GO terms are crucial to evaluate the significant GO processes with respect to metal exposure. We observed that the obtained GO processes were different for each metal exposure; although few of them were linked to neurodegenerative diseases. Table 3 shows the different GO processes for Pb. As and MeHg related to neurotoxicity in HT-22 cells. Regarding Pb, proteins related to cysteine-type endopeptidase inhibitor activity involved in the regulation of caspase related apoptosis are downregulated after Pb exposure, which indicates that the apoptosis process was initiated by Pb in HT-22 cells. Some studies suggested the importance of the JAK/STAT pathway for the survival of neurons (Yadav et al., 2005). This pathway has a pro-apoptotic nature in cerebellar granule neurons (Loucks et al., 2006). Thus, the Pb exposure in HT-22 cells initiated the cell death through apoptosis. In relation to As metal (Table 3), oxidative stress and transition metal ion homeostasis GO processes are critically acclaimed to metal toxicity. The regulation of oxidative stress is indicated by the upregulation of antioxidant proteins, super oxide dismutase 1 (SOD1), reduced glutathione (GSH) and the neuronal part GO term is indicated by the amyloid precursor protein (APP), and parkin7 (PARK7) protein expression that are critical for the neurodegenerative diseases. Most enriched and relevant to neurotoxicity in MeHg are the GO terms: pyruvate metabolic process, spliceosomal and transition metal ion homeostasis. In Alzheimer's disease, the process of β-amyloid (Aβ) misfolding and plaque aggregation is greatly influenced by alterations in the transition metal ion homeostasis (Kozlowski et al., 2009). Transition metal ions can influence the APP metabolism (Bellingham et al., 2004). We found in relation to the transition metal ion homeostasis GO term, that APP protein expression is differently altered by MeHg (down regulated) than by As (up regulated), which indicates that heavy metal exposure

controls the APP expression but in opposite ways best supporting the underlying mechanism of AD by As.

Our data demonstrates that GO analysis constitutes a powerful unbiased approach to define the biological pathways altered in mice HT-22 hippocampal cells in response to heavy metals exposure. The GO processes shown in Table 3 for the three metals (Pb, As, and MeHg) suggests that the involved proteins are sensitive indicators of metal induced response in association with neurotoxicity. GO based omics data analysis can provide profoundly important and novel information in toxicity mechanism. However, we are currently facing the difficult challenge of how quantitatively to interpret the differentially expressed proteins. As presented in the Table 3, the conventional GO process terms does not provide any quantitative information about current data on metal-induced neurotoxicity. Thus, we integrated the selected pruned GO processes in a Cytoscape framework to evaluate functional relations between the proteins, as illustrated in Figures 5, 6, and 7 for Pb, As and MeHg, respectively. This analysis can useful to visualize how each protein with its significant change in expression connects to the neurotoxicity GO terms. In particular, we were able to show the metals effects on the protein expression profiles in HT-22 cells as presented by Pb, As and MeHg networks. We quantitatively assessed the absolute magnitude of change in expression levels associated with significant proteins within enriched GO terms at IC_{10}. It was observed that MeHg caused the most prominent changes in protein abundance than other two metals. So that, here we will mainly focus on the MeHg induced damage in HT-22 cells which is critical to evaluate the relationship between the protein expression and Alzheimer's and Parkinson's diseases; the other two metals (Pb and As) are discussed in a comparative manner.

5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Arpc2
Hdac1
Atp6ap2
Ptgs1
Basp1
Actb
nitric-oxide synthase binding
Snrpe
Canx
Pfn1
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Gnb2l1
Dpysl2
Ctnnb1
SMN-Sm protein complex
Dctn2
Fkbp1a
Exoc4
Calm3
Snrpd2
Snrpd3
Hcfc1
neuron part
Dpysl3
Acot7
Cyfip1
Prkar2b
Nqo1
Ephb2
Pafah1b1
Pmm2
Eif5a
Ppp1cc
Gdi1
Lamp1
Top1
Cacybp
Smarca4
Capn2
Sh3kbp1
Rhoa
Sptbn1
Ndrg1
Gnb1
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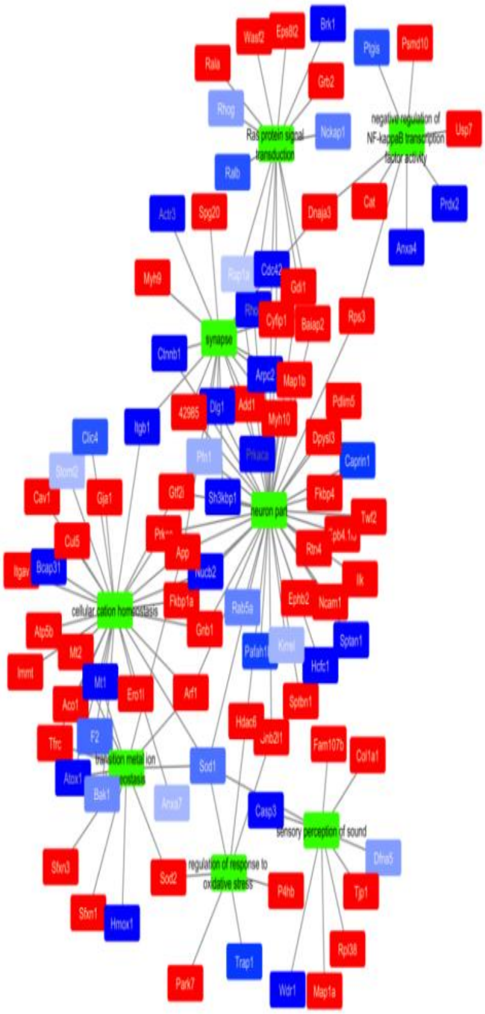
Acaa2
Vcam1
Atp5c1

6


Ras protein signal transduction
negative regulation of NF-kappaB transcription factor activity
synapse
neuron part
cellular cation homeostasis
transition metal ion homeostasis
regulation of response to oxidative stress
sensory perception of sound

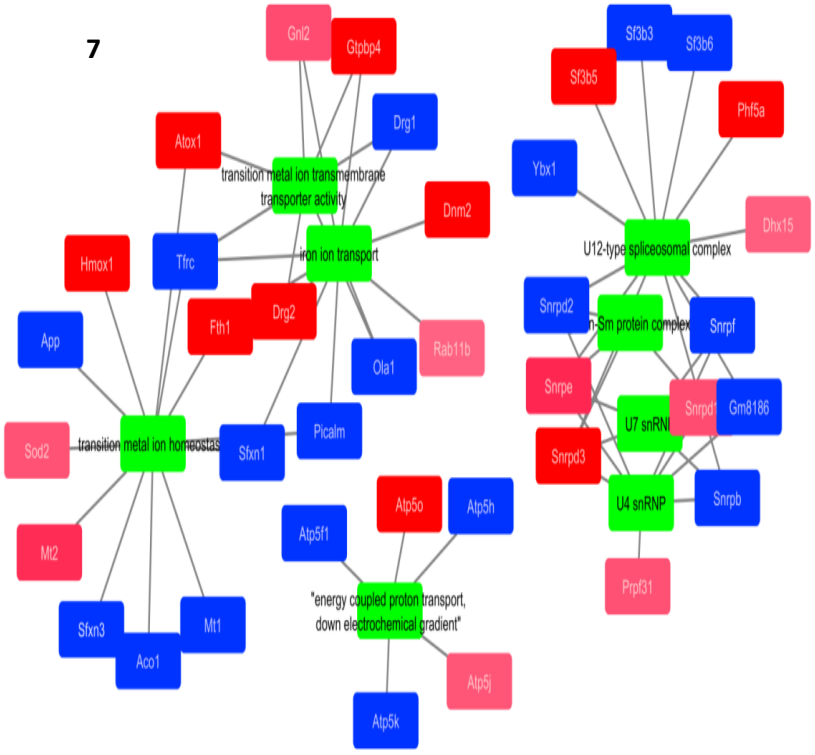


Figure 5, 6, and 7: Interaction of differentially expressed proteins and selected GO classes after Pb, As, and MeHg exposure. The network is based on a selection of the over-represented and pruned GO terms and was visualized in Cytoscape. The network includes over-represented GO terms and associated differentially expressed proteins. The changes in protein expression are coloured as a gradient going from blue (=downregulated) to red (=upregulated) and GO terms are coloured in green.

Table 3: Statistically significantly changed GO terms related to neurotoxicity in HT-22 cells

Pb						
Ontology-ID	**Ontology Name**	**Ontology Type**	**Changed genes**	**Measured**	**Ontology**	**Z Score**
GO:0043028	cysteine-type endopeptidase regulator activity involved in apoptotic process	molecular function	5	5	36	3.8

GO:0046426	negative regulation of JAK-STAT cascade	biological process	3	3	12	2.9
GO:0045738	negative regulation of DNA repair	biological process	3	4	8	2.2
GO:0034719	SMN-Sm protein complex	cellular component	6	11	17	2.2
GO:0097458	neuron part	cellular component	46	141	927	2.0

As

Ontology-ID	Ontology Name	Ontology Type	Changed	Measured	Ontology	Z Score
GO:0032088	negative regulation of NF-kappaB transcription factor activity	biological process	8	8	63	2.4
GO:1902882	regulation of response to oxidative stress	biological process	7	7	27	2.2
GO:0007265	Ras protein signal transduction	biological process	19	24	99	2.1
GO:0055076	transition metal ion homeostasis	biological process	12	14	99	2.0
GO:0097458	neuron part	cellular component	95	141	927	2.3

MeHg

Ontology-ID	Ontology Name	Ontology Type	Changed	Measured	Ontology	Z Score
GO:0006090	pyruvate metabolic process	Biological process	18	21	55	2.9
GO:0050877	neurological system process	biological process	31	43	1863	2.4
GO:0055076	transition metal ion homeostasis	biological process	12	14	99	2.4

GO:0043028	cysteine-type endopeptidase regulator activity involved in apoptotic process	molecular function	5	5	36	2.0
GO:0005687	U4 snRNP	cellular component	8	9	10	2.1
GO:0005683	U7 snRNP	cellular component	5	5	8	2.0
GO:0005689	U12-type spliceosome complex	Cellular component	13	13	25	3.3

3.3. Pathway analysis: visualization of pathways relevant to heavy metal (Pb, As, and MeHg) induced neurodegeneration in HT-22 cells

Pathway analysis was used to further explore the complex interactions in biological system and to discover the relations between differentially expressed proteins and their relevance to Alzheimer's and Parkinson's diseases (AD, PD). In general toxicological studies related to Pb, As and MeHg have proposed few common predominant mechanisms that relate to neurotoxicity. The obtained results suggests that for AD and PD the pathways: mRNA regulation/splicing, ubiquitin proteasome system (UPS), oxidative stress, electron transport chain (ETC) dysfunction, are the most affected by MeHg and As exposure; however, the Pb metal impact was low. Notable proteins found in the ETC dysfunction, oxidative stress, mRNA splicing process, and UPS degradation also relate to AD and PD. Table 4 shows the pathways that significantly changed proteins relevant to neurodegeneration process in HT-22 cells.

Table 4: Six pathways showed significantly changed proteins relevant to neurodegeneration process in HT-22 cells.

		Pb	As	MeHg
Pathway	**measured (n)**	**positive (r)**	**positive (r)**	**positive (r)**
Electron Transport Chain	31	12	17	21
mRNA processing	191	51	104	115
Proteasome Degradation	40	9	18	7
Alzheimer's Disease	17	3	9	20
Parkinson's Disease Pathway	10	1	7	6
Oxidative Stress	12	4	9	7

(*Measured (n) is the number of gene products in the pathways that are measured in the Pb, As, and MeHg data sets, and positive (r) is the number of differentially expressed proteins in the presented pathway for each metal)

3.3.1. Effect of Pb, As, and MeHg on mitochondrial electron transport chain (ETC) dysfunction

The mitochondrion is a critical regulator of neuronal cell death (Castellani et al., 2002); a failure of one or more of the mitochondrial ETC enzymes generates reactive oxygen species (ROS), and leads to neuronal death (Esteves et al., 2010). It is a tempting to speculate that the modification of ETC proteins leads to depletion of ATP production in HT-22 cells. We found protein dysregulation in all complexes of the ETC, suggesting a broad toxicity of metals on ETC of HT-22 cells. As shown in Figure 8, MeHg treatment (IC_{10}) induced significant alteration of proteins in complex I–V. The pattern of protein expression alerted by As in the ETC pathway was similar to that of MeHg. In the present study, cytochrome c oxidase (COX)/complex-IV and F_1 F_0-ATPase activities seemed highly dysregulated by the MeHg and As exposure. MeHg is known to directly disrupt the mitochondrial activity by generating the uncontrolled release of Ca^{+2} from the mitochondria and inhibiting phosphorylation (Atchison and Hare, 1994). Yee et al.(1996) reported that MeHg inhibits the mitochondrial ETC in a cultured brain cell line. The findings of mitochondrial ETC damage due to MeHg and As metal exposure is an agreement with previously reported results in AD patients from post mortem hippocampus (Bosetti et al., 2002). The reduced activity of F_1 F_0-ATPase (complex-V) could compromise the ATP production and induce the generation of ROS leading to neuronal cell death (Orth and Schapira, 2002).The ETC dysfunction after metal exposure in HT-22 cells seems to correlate to the previously reported findings of Valla et al. (2001) of AD and PD. Previous studies found a deficiency in the activity of the mitochondrial ETC NADH dehydrogenase (complex I) in PD (Lezi and Swerdlow, 2012), which is in agreement with ETC dysfunction in the HT-22 cells. Our findings confirm that the metals disrupt the ETC pathway in HT-22 cell at the protein level, and that may relate to energy deficiency, a characteristic feature of both AD and PD (Beal et al., 1993). The observed ETC changes raise further questions (Lin and Beal, 2006). Not only direct effects of ETC function, but also other proteins (next section) involved in AD and PD can also impact various mitochondrial functions (Wang et al., 2010). There is no evidence that any of the Pb, As and MeHg metals directly produce only mitochondria based neurodegeneration processes (Nakamura et al., 2011), although other pathways presents the direct or indirect relation to ETC dysfunction.

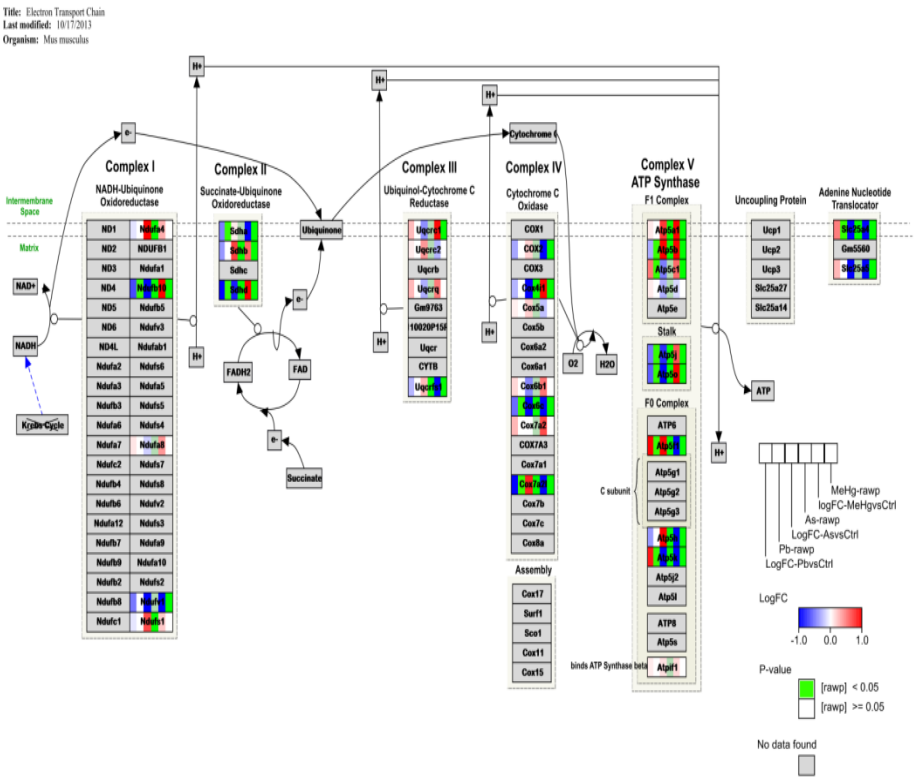


Figure 8: Changes in protein expression in electron transport chain (ETC) pathway in response to Pb, As, and MeHg in HT-22 cells. In the pathway changes in protein expression are visualized as log2foldchange comparing treatment with control expression. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p values* < 0.05 are coloured in green and *p values* ≥ 0.05 are in white. Gray indicates that the selection criteria were not met, but the protein is measured.

3.3.2. Effect of Pb, As, and MeHg on reactive oxygen species (ROS) mediated oxidative stress

The present results (Figure 9) indicate that generation of ROS can relate to ETC damage and monoamine oxidase A (MAO-A) elevation in the HT-22 cells. The presented oxidative stress pathway gives an adaptive response in HT-22 cells to maintain redox homeostasis to fight against the heavy metal induced stress. We found that MAO-A

protein expression in HT-22 cells in the all three metals, MAO-A participates in the degradation of amines and thereby controls neurotransmitter levels in the brain (Jossan et al., 1991). In the present study, MAO-A expression changed in a contradictive manner among three metals; As (downregulation) while Pb and MeHg (upregulation), which indicates that Pb and MeHg might be enhancing the monoamine metabolism in HT-22 cells. The obtained MAO-A expression in MeHg is in disagreement with reported MeHg effect in vitro embryo culture experiments (Beyrouty et al., 2006). As metal also showed that MAO-A expression was opposite to reported studies, instead of upregulation the effect was downregulated (Yadav et al., 2010). Pb altered mitogen-activated protein kinase (MAPK) functions leading to production of ROS causing neuronal cell death (Yin et al., 1995). The relationship between AD and increased enzyme activity for MAO has been known for a long time; MAO-A protein activity levels are elevated in AD (Emilsson et al., 2002). The MAO-A elevated AD brain is subjected to increased oxidative stress resulting from ROS (Butterfield et al., 1999). Thus, the MAO-A activity could be altered by Pb, As and MeHg indirectly through the changes in -SH groups (Aschner and Aschner, 1990). The results support that MAO-A may be useful neurochemical biomarker for Pb, As and MeHg on the hippocampal cells. The MAPKs plays an important role in the cascades of cellular responses evoked by extracellular stimuli such as the heavy metal exposure (Chang and Karin, 2001). Our results demonstrated a significant alteration in MAPKs mediated oxidative stress response (Figure 9). The induction of antioxidant and phase II detoxifying enzymes acts as an important defensive mechanism against heavy metal induced stress in HT-22 cells.

Pb, As and MeHg in HT-22 cell line induced significant effects in MAPKs mediated oxidative stress response such as anti-oxidant proteins, hemeoxygenase1 (HMOX1), NAD (P)-H dehydrogenasequinone1 (NQO1), glutathione disulfide reductase (GSR), superoxidedismutase1 (SOD1), superoxide dismutase2 (SOD2), catalase (CAT), thioredoxin reductase1(TXNRD1), Glutamate cysteine ligase catalytic (GCLC) subunit, and metallothionein-1(MT1). All proteins are involved in the activation of antioxidant activity in HT-22 cells supporting the heavy metals stress. The increase in the antioxidant proteins is a compensating mechanism for the decrease in the ATP producing due to ETC dysfunction in HT-22 cells. In contrast to Pb metal; MeHg and As significantly alter some forms of antioxidant and detoxification enzyme which indicates clearly the higher potency of As and MeHg. Also MeHg and As induce significant upregulation of GSR and SOD2; although CAT protein expression was observed similarly in both Pb and As. The obtained results show that expression/fold change of antioxidant proteins depends on the potency of each metal; MeHg considerably alter the normal pattern of antioxidant elements expression than other two metals. Biochemically MeHg has high affinity to sulphur (-SH) containing molecules, mainly targeting cysteine containing proteins (Suzuki et al., 1976).

Oxidative stress has been implicated in the wide variety of biological reactions such as cell death in the central nervous system (Cuello et al., 2010). ROS induced oxidative stress has been demonstrated to activate members of the MAPKs via phosphorylation

(El-Najjar et al., 2010) and it is also implicated in neurodegenerative disorders (Kim and Choi, 2010). Thus, heavy metal induced ROS can elicit oxidative stress, that can be the main cause to trigger various neurodegenerative diseases (Bush, 2000; Rana, 2008). ROS is also well known to cause mitochondrial dysfunction and induces cell death in neurons (Lu et al., 2010). Previous studies indicated that MeHg disrupts cellular redox homeostasis and the mitochondrial ETC, via excessive generation of ROS (Shanker et al., 2004; Yin et al., 2007). The disruption of ETC function can also correlate with various proteins in oxidative stress such as MT1, SOD, GSH, CAT, HMOX1 and NQ01. Oxidative stress has been shown not only to initiate the onset of disease, but also exacerbates specific diseases like AD and PD (Lu et al., 2011).

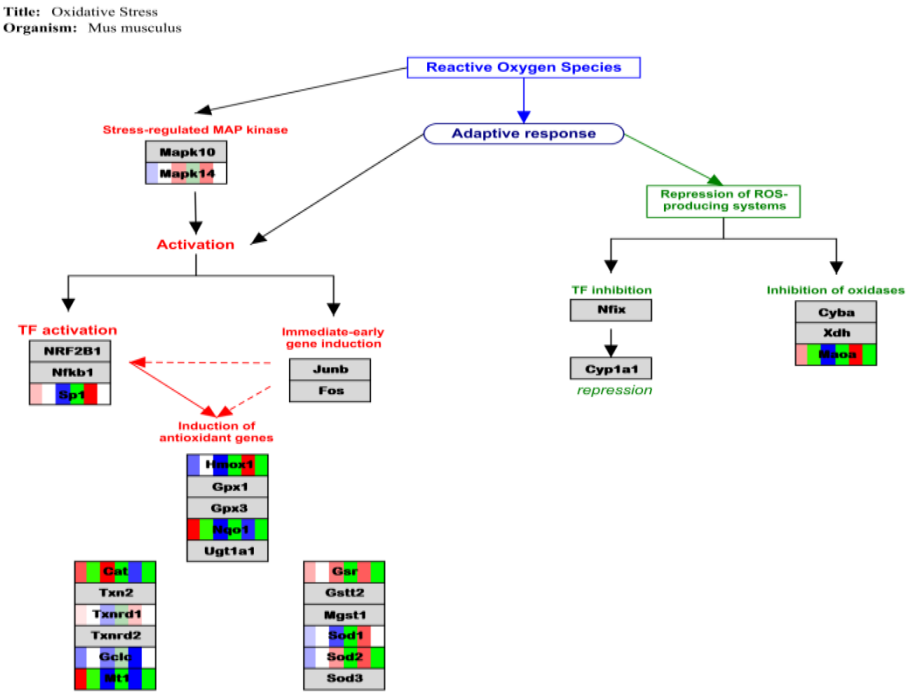


Figure 9: Changes in protein expression in oxidative stress pathway in response to Pb, As, and MeHg in HT-22 cells. In the pathway changes in protein expression are visualized as log2foldchange comparing treatment with control expression. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p values* < 0.05 are coloured in green and *p values* ≥ 0.05 are in white. Gray indicates that the selection criteria were not met, but the protein is measured.

3.3.3. Effect of Pb, As, and MeHg on Ubiquitin proteasome System (UPS)

In addition to selective damage of ETC and oxidative stress in HT-22 cells, UPS may also contribute to neurodegeneration processes (Dantuma and Bott, 2014). The UPS is essential for the non-lysosomal degradation and clearance of short-lived, misfolded, mutant, and damaged proteins in eukaryotic cells. Structural and functional deficits in the 26/20S proteasome can leads to AD, and PD (McNaught et al., 2003, 2001). The UPS is a highly conserved cellular pathway that plays an important role in the selective degradation of cellular proteins that are essential for the regulation of a variety of vital cellular functions (Joazeiro and Hunter, 2000). Disruption of this system can have significant downstream effects on critical cellular functions. In Figure 10, Pb, As and MeHg metal showed nonspecific proteasome alterations of proteasome subunits of the 20S catalytic core and the 26S (PA700) regulatory complex. The pattern of expression in UPS is metal specific with relevance for neurodegeneration. For instance, Ubiquitin Carboxyl-Terminal Esterase L3 (UCHL3) expressions was upregulated in the As exposure, although the other two metals (Pb and MeHg) had no effect. UCHL3 is a deubiquitinating enzyme that is involved in the pathogenesis of both AD and PD (Setsuie and Wada, 2007). Recently, an increased amount of oxidatively modified UCHL3 in the brains of AD and PD patients, compared to normal brains was reported (Castegna et al., 2002). Ubiquitin-like modifier activating enzyme 1 (UBA1) is a key regulator of protein homeostasis and expression of UBA1 is essential for cell survival. Our results suggest that Pb, As, and MeHg metal upregulates the UBA1 expression in the HT-22 cells. Moreover, UBA1 might be sequestered into PD disease associated protein aggregates (Groen and Gillingwater, 2015). Additionally for MeHg, we observed downregulation of the NEDD4 (ubiquitin E3 ligase/ parkin), which indicates that MeHg has significant impact on the number of protein targets in HT-22 cells. In contrast, Pb and As metal upregulates the NEDD4 protein in UPS pathway. NEDD4 plays a crucial role in ubiquitination; this enzyme enhances the cell survivability by decreasing the apoptosis process in HT-22 cells. We found that the expression was downregulated during MeHg exposure, which indicates the UPS dysfunction. The accumulation of parkin specific substrates, as a consequence of the loss of NEDD4 activity might underlie the damage of dopaminergic neurons (Dawson and Valina, 2003). Other proteins in the PD pathway (Figure 13) such as SEPT-5 and ATAXIN-2 have interaction/connection with NEDD4 function and also play a key role in PD (both proteins are upregulated in PD pathway). This upregulation process can decrease the NEDD4 activity of the proteasome after MeHg exposure in the hippocampal cell line. We found the bunch of proteins dysregulation in the proteasome 26 subunit, in which the PSMC5 protein has a critical role in degradation of unfolded proteins. The expression was downregulated in HT-22 cells after MeHg exposure, but upregulated with Pb and As.

Overall, the UPS pathway disruption shows that most of the protein expression was similar for all three metals (except NEDD4 expression). The findings with the UPS pathway clearly show that the potency and molecular mechanism related to PD is

different for the three metals. For instance, comparisons of the protein expression level between the three metals by omics data revealed the common targets in the UPS of the HT-22 cells but the way of influence/interaction on the protein is different. Pb, As and MeHg treatment in HT-22 cells induced significant alteration of UPS pathway proteins, including the significant alterations in the proteasome 26S regulatory and 20S catalytic core unit and in the ubiquitin enzyme system (UBA1, NEDD4, and UCHL3), which are highly essential for protein quality control during normal cell function. MeHg effect on E3 ligase enzyme was reported in human cell lines (Furuchi et al., 2002; Hwang et al., 2002). This report suggests that MeHg induces the cellular accumulation of certain proteins that causes neurodegeneration. Mouse neuronal HT4 cells treated with toxic metals have been reported to induce accumulation of ubiquitination with decrease in cell viability (Figueiredo-Pereira and Cohen, 1999). This report also shows the critical role of the UPS in the removal of proteins that are oxidatively modified and suggests that accumulation of ubiquitinated proteins in the HT- 22 cells contributes to an overall toxicity. In Figure 10, the comparison of alterations in protein expression between the metals Pb, As and MeHg revealed both common and unique targets in the UPS. Although the involvement of the UPS pathway might be different among various cell types, results from this omics data strongly support that the disruption of the UPS function is involved in metal-induced neurotoxic effects and denotes the potential protein targets in this pathway. Several studies have identified critical role of the UPS in the metal induced toxicity (Di and Tamás, 2007; Figueiredo-pereira et al., 1998; Kirkpatrick et al., 2003; Stanhill et al., 2006; Stewart et al., 2003). It is clear that alterations in UPS function are key factors in the final development of AD and PD; more research is needed to elucidate the exact mechanisms and order of events concerning the role of the UPS in neurodegenerative diseases.

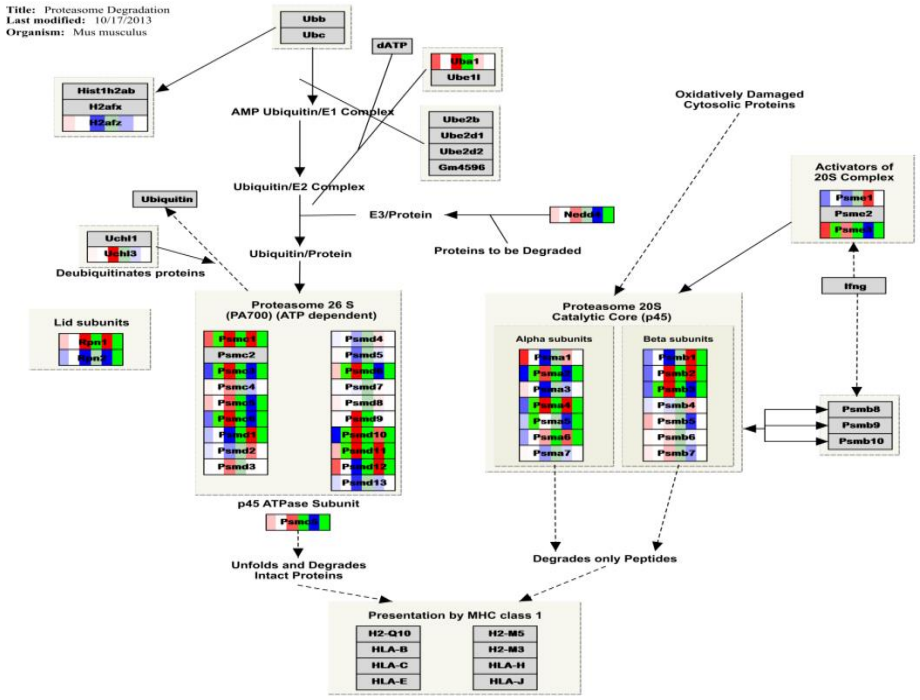


Figure 10: Changes in Ubiquitin proteasome system (UPS) in response to Pb, As, and MeHg in HT-22 cells. In the pathway changes in protein expression are visualized as log2foldchange comparing treatment with control expression. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p values* < 0.05 are coloured in green and *p values* ≥ 0.05 are in white. Gray indicates that the selection criteria were not met, but the protein is measured.

3.3.4. Effect of Pb, As, and MeHg on mRNA splicing

Alternative splicing is one of the most important mechanisms to generate a large number of mRNA and protein isoforms from the low number of human genes (Daguenet et al., 2015). It is a widespread gene regulatory process by which exons of primary transcripts (pre-mRNAs) are spliced into different arrangements to produce structurally distinct mRNA variants (Graveley, 2001). This mechanism of gene product plays a critical role in cell function (Kelemen et al., 2013). It is estimated that more than 75% of genes in the human genome are alternatively spliced in central nervous system (Johnson, 2003). Dysregulation of alternative splicing have been linked to the number of human diseases including neurodegenerative diseases (Faustino et al., 2003). The spliceosome is a complex of five small nuclear ribonucleoprotein particles (snRNP:

U1, U2, U4, U5, and U6) and numerous protein factors. In Figure 11showed snRNP bind to the pre-mRNA in a sequential manner, following the binding of U4/U6, U5 tri-snRNP, the spliceosome undergoes extensive structural remodelling leading to the release of U1, U2 subunits and activation of the spliceosome (Hales et al., 2014; Love, 2015; La Cognata et al., 2015). In Figure 11, we addressed the impact of Pb, As and MeHg on splicing and the relationship between neurological disease and the regulation of alternative splicing. Alternative splicing is particularly applicable in the present results, because of the great number of protein changes observed in the mRNA processing. In addition, we identified several components of the U1 small nuclear ribonucleoprotein (U1 snRNP) alterations in HT-22 cells. Our findings demonstrated a unique damage of splicing in HT-22 cells. The functional consequences of these observations can reflect the neurodegeneration processes (Fu et al., 2013). We found that U1snrp70K (U1 small nuclear ribonucleoprotein 70 kDa) protein expression was upregulated in As metal exposure; the other two metals have no significant effect. This protein has a critical role in the APP protein metabolism (Nuzzo et al., 2017). The alterations of U1snRNP are in agreement with discovered U1 snRNP defect in the AD brain (Bai et al., 2013). We found U2 snRNP protein dysregulation with all three metal exposures. A reported study denoted that the mutation in U2 snRNP genes causes defects in pre-mRNA splicing, leading to neurodegeneration (Jia et al., 2012). Our results showed a high number of altered proteins in the U2snRNP unit, and U2 snRNP plays an essential role in formation of the catalytically active spliceosome by base pairing with both the intron branch point and the U6 snRNA (Lee et al., 2015). The last functional tri-protein (U4, U5, and U6) system function was downregulated in HT-22 cells, which indicates the mechanism of splicing is completely altered by heavy metal exposure. Additionally, we found abnormal changes in the serine/arginine rich proteins (SR) Sfrs1, Sfrs2, Sfrs3, Sfr5, and Sfr6; SR proteins are one family of splicing factors involved in the alternative splicing of tau protein. The changes of SR proteins probably influence regulation of the alternative splicing of the tau gene (Qian and Liu, 2014). In that way, heavy metal induced protein alterations in SR proteins may contribute to AD by controlling the tau protein expression (Geuens et al., 2016). The present findings enables mechanistic insight into how aberrant changes in alternative transcript expression occur in AD and highlight the susceptibility network of splice-regulatory proteins which can potentially link the number of susceptibility pathways (Wong, 2013). Taken together, these observations imply that alterations in snRNP accumulation and consequently pre-mRNA splicing can contribute to the etiology of multiple neurodegenerative disorders (Dredge et al., 2001). Alternative splicing is tissue specific, and especially important for brain tissue, thus the metal disrupted spliceosome has a big impact in HT-22 cells.

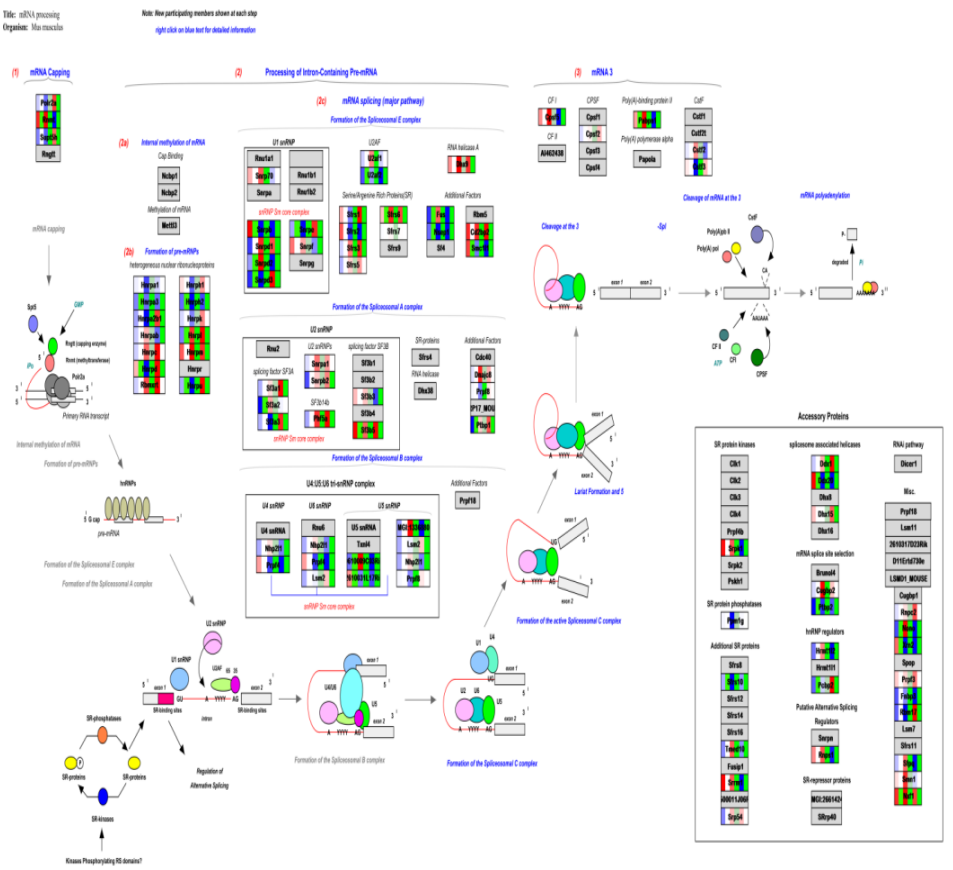


Figure 11: Changes in mRNA splicing in response to Pb, As, and MeHg in HT-22 cells. In the pathway changes in protein expression are visualized as log2foldchange comparing treatment with control expression. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p values* < 0.05 are coloured in green and *p values* ≥ 0.05 are whtie. Gray indicates that the selection criteria were not met, but the protein is measured.

3.4.5. Effect of Pb, As, and MeHg on Alzheimer's disease (AD) pathway

AD is a complex, irreversible neurodegenerative disease characterized by the impairment of cognitive function. In the present study, it is conceivable that the observed alterations in the AD specific proteome of HT-22 cells could cause the alterations in the APP. Our findings in Pb and As comply with a previous study that showed an increased hippocampal and cortical APP protein processing causing cognitive dysfunction (Ashok et al., 2015). Also we observed significant changes in

CAPN1 and CAPN2 (calcium-dependent, and non-lysosomal cysteine proteases).It's known that calpains is implicated in neuronal cell death and long term potentiation (LTP) in the hippocampus (Grammer et al., 2005). In our study, CAPN2 expression was upregulated by MeHg and As, which clearly suggests the AD relationship with heavy metals exposure. Previously it was reported that CAPN2 can improve survival of hippocampal neurons (Bevers et al., 2009). The other protein, low-density lipoprotein receptor-related protein1 (LRP1 protein) was upregulated with As and MeHg. LRP1 is implicated in the effective clearance of Aβ from the brain. It indicates that HT-22 cells compensate the Aβ clearance through the LRP1 protein (Deane et al., 2009). NEDD8-activating enzyme E1 regulatory subunit (NAE1/APPBP1) expression was found in As exposure, that binds to the APP protein carboxyl-terminal domain (Chen et al., 2000), results neuronal cell death (Chen et al., 2015). AD is also characterized by the activation of apoptotic pathways. However, the hypothesis that apoptosis plays a role in AD neurodegeneration remains controversial. Caspase activation can occur independent of cell death and may be neuroprotective (Mattson et al., 1998; McLaughlin et al., 2003). CASP8 expression was found altered after the As exposure, which leads to the receptor mediated apoptotic pathway to activate CASP3 within neurons of the AD brain (Rohn et al., 2001). We found that expression of CASP3 that indicates that HT-22 cell death may be initiated in an apoptosis-dependent manner. Generally, Pb, As and MeHg predominantly induce CASP3 like protease activity in brain cell culture (Nishioku et al., 2000; Akao et al., 1999; Xu et al., 2006). The activation of CASP3 is known to enhanced generation of alternative presenilin cleavage fragments in AD, but exactly not clear (Kim, 1997). Here, we found CASP3 expression in relation to tau protein in the AD pathway (Figure 12) which is entirely different from the metal induced apoptosis assumption (Chung et al., 2003) and thus, may contribute to cellular demise in a different manner (Rissman et al., 2004).We also found that heavy metals induced AD relation to other pathways. For instance, APP dysregulation can be possibly due to mitochondrial ETC, oxidative stress, transition metal ion homeostasis, and mRNA splicing. These AD related proteins have been previously discussed in other pathways sections 3.3.1, 2, 3, and 4. For instance, the impairment of mitochondrial function has been well known in AD patients (Hirai et al., 2001). ETC dysfunction (Figure 8) and reduced ATP have an immense impact on brain metabolism (Onyango et al., 2016). PCR analysis revealed that the downregulation of mitochondrial proteins of complex- I in AD brain, suggesting energy deficits in AD brains (Manczak et al., 2004). In addition, several in vitro studies of APP and mitochondrial function have reported that APP expression affects the mitochondrial DNA and proteins, leading to impairments of the ETC (Manczak et al., 2006; Reddy, 2009). With As and MeHg we found that the transition metal ion homeostasis GO term represents a process necessary for the correct functioning of antioxidant systems in the cell (Liu et al., 2006). Biometals such as iron (Fe), zinc (Zn) and copper (Cu) play an important role in several neurodegenerative disorders (White et al., 1999). As and MeHg are such toxic metal which influence the essential metal transportation that relates to Aβ aggregation (Atwood et al., 1998), and

also Fe, Pb and Mn and their mixture may raise the risk of PD (Lai et al., 2002). The expression of APP in the brain suggests these metals could have a direct or indirect role in neuronal metal homeostasis (Ding et al., 2009), for instance Cu deficiency downregulates APP transcription (Atwood et al., 2008; Bellingham et al., 2004). The association between APP and transition metals such as Fe and Cu lead to the generation of hydrogen peroxide, exacerbating the oxidative damage (Huang et al., 1999). Few studies have investigated the link between alternative splicing dysregulation, and AD progression (Wong, 2013). Evidence of abnormal mitochondrial ETC gene expressions from our studies suggests that APP in AD pathway may affect mitochondrial function, which generates ROS, ultimately leading to oxidative damage (Caspersen, 2005; Gibson et al., 1998). However, the precise mechanistic link between mitochondrial oxidative damage and abnormal APP processing by heavy metals has not been elucidated. Further studies are needed to support the mechanistic link between abnormal mitochondrial gene expression and oxidative damage in AD progression by heavy metal exposure.

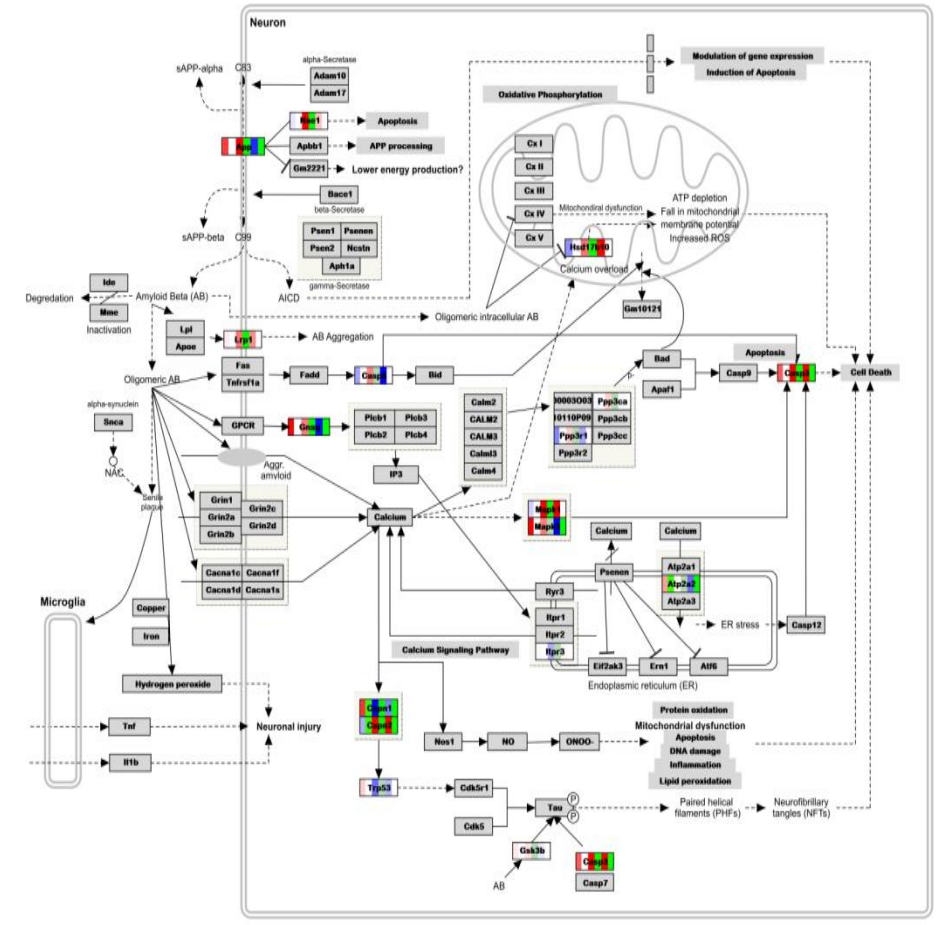


Figure 12: Changes in AD in response to Pb, As, and MeHg in HT-22 cells. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and p values < 0.05 are coloured in green and p values ≥ 0.05 are in white. Gray indicates that the selection criteria were not met, but the protein is measured.

3.4.6. Effect of Pb, As, and MeHg on PD pathway

PD is the second most prevalent progressive neurodegenerative disease (Moore et al., 2003). In this study several proteins are showed differential expression relates to PD. The pathway analysis of metal exposure on HT-22 cells proved particularly valuable for PD, because we found significantly changed proteins belonging to the PD pathway. These PD related proteins also appeared in other pathways for instance, ETC dysfunction, oxidative stress, UPS pathway, and mRNA splicing. A detailed study of the PD pathway (Figure 13) made it plausible that the observed alterations at the protein level in HT-22 cells could be related to PD such as PARK7, CYCS (cytochrome c, somatic), and CASP3 as key regulators of proteins for cell homeostasis. We found that the expression was upregulated by the three metals treatment, which clearly shows that HT-22 cells are under stress. Also, other proteins such as UBE2L3 (Ubiquitin-conjugating enzyme E2 L3), SEPT-5 (septin), and ATAXIN-2 protein expression was altered in the PD pathway. SEPT-5 protein interacts with NEED4 in PD. Indeed, we found that As and MeHg altered UBA1 and NEDD4 (Figure 10) expression, respectively, in the UPS pathway. This SEPT-5 upregulation in MeHg can decrease the activity of NEDD4 resulting in lower ubiquitin proteasome degradation in the hippocampal cell line. In As exposure, the interaction mechanism between NEDD4 and SEPT-5 proteins is entirely opposite to MeHg. NEDD4 has been intimately linked to mitochondrial quality control, which evidence suggests that PARK7, ATP13A2, and FBXO7 may also be involved (Blackinton et al., 2009). In our data, the expression of CYCS and CASP3 were upregulated by the three metals. The loss of mitochondrial membrane potential and translocation of CYCS into the cytosol lead to cell death (Tofighi et al., 2011). In parallel, PARK7 protein is upregulated, which will protect the cells from the apoptosis stress. PARK7 has been thought as an endogenous redox sensor, which prevents the cell from stress-induced apoptosis (Shadrach et al., 2013). Thus, PARK7 is believed to be protective against oxidative stress in PD brains (Fitzgerald and Plun-Favreau, 2008). PARK7 expression in HT-22 cells was suggested to be related to the oxidative stress and dopaminergic neurodegeneration (Aguilar et al., 2016).

The expression pattern of PARK7, CYCS and CASP3 supports that the three metals affect mitochondrial function which is relevant to PD, with the potency showing Pb<As<MeHg. The pattern of expression of interlinked proteins in the PD and UPS pathways of the MeHg and As metal exposures is somehow different. As metal

significantly upregulates the UBA1 and NEDD4, and downregulates SEPT-5 in HT-22 cells. Thus, UBA1 might be sequestered into disease associated protein aggregates, also shown for lewy bodies in As exposure models of PD (Groen and Gillingwater, 2015). For MeHg, we observed downregulation of NEDD4 (Figure 10). NEED4 has a role of E3 ubiquitin ligase, participating in the proteasome degradation system (Tanaka et al., 2000), of which the loss of function allows the formation of these toxic aggregates in neurons. Target proteins of MeHg treatment in HT-22 cells related to PD suggest a direct relation to the UPS pathway. The downregulated NEDD4 proteins during MeHg exposure is critical for inducing the indirect way of PD by affecting the UPS function. The results of our proteomics analysis using hippocampal HT-22 cells strongly suggest that the disruption of UPS function is an important mechanism in metal induced neurotoxicity. Moreover, additional evidence supports not only the important role of NEED4 as an E3 ligase in PD, but also this protein can lead to mitochondrial impairment in brain (Park et al., 2006). Our findings comply with the previous report that NEDD4 protein expression can correlate to alternative splicing defects, which is one of the reason for PD (Tan et al., 2005).

The heavy metal induced PD is likely to be multifactorial; ETC dysfunction, oxidative stress, and the impairment of UPS dependent protein degradation have been identified as the main processes associated with PD (Moore et al., 2003). Our findings confirm that the metals disrupt the ETC pathway in HT-22 cell and influence the PARK7, and NEDD4 expression (Beal et al., 1993). We found that NEED4 expression was downregulated in the MeHg exposure, which correlates with previously reported alternative splicing defects and PD (Tan et al., 2005). We also found that the heavy metal induces oxidative stress and mitochondrial dysfunction, which is directly or indirectly related to PD (Kalinderi et al., 2016; Scott et al., 2017). Our results also support that As and MeHg treatments highly influence the ETC, oxidative stress and UPS pathway which are relevant to the PD pathway (van der Merwe et al., 2015). Finally, PD is associated with a number of biochemical abnormalities, in which the UPS and ETC plays a major role. Indeed, inhibition of proteasomal function has been shown to cause oxidative stress and mitochondrial dysfunction (Höglinger et al., 2003; Jha et al., 2002; Keller et al., 2004; Lee et al., 2002; Shang and Taylor, 2011).

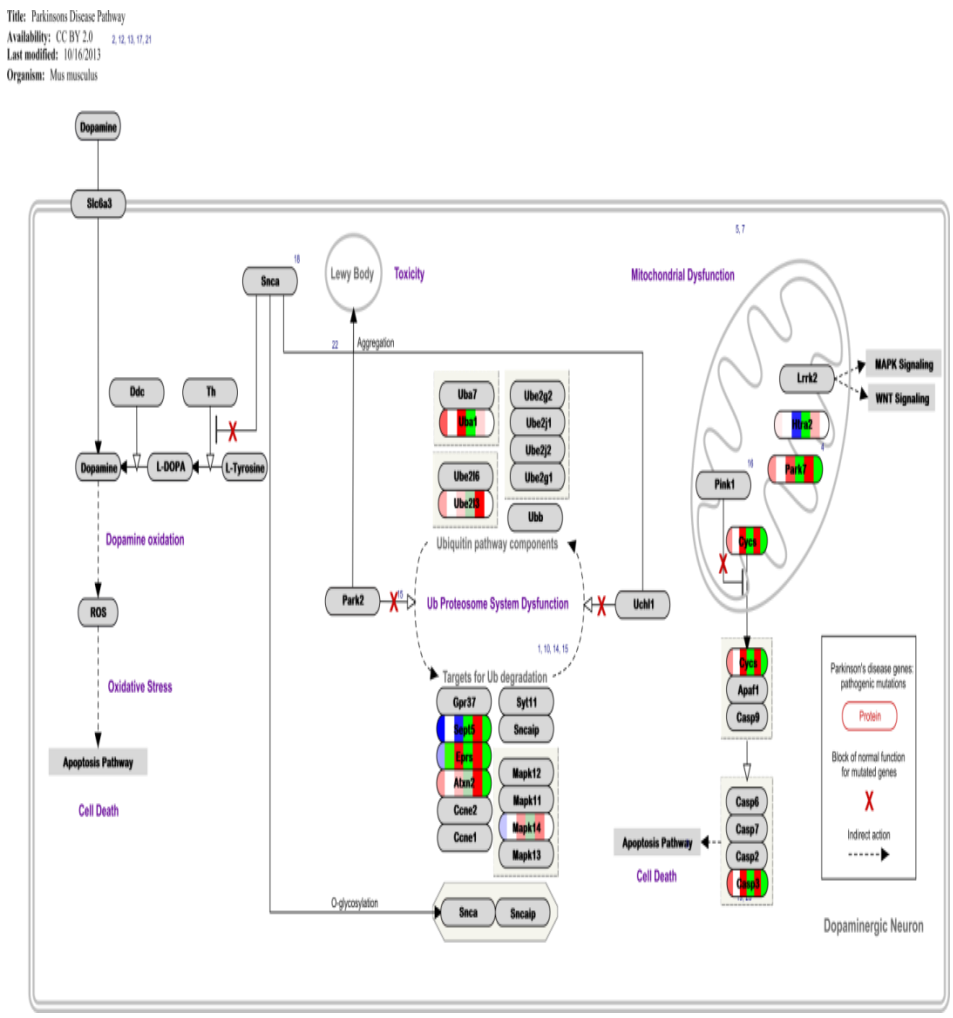


Figure 13: Changes in Parkinson's disease in response to Pb, As, and MeHg in HT-22 cells. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p values* < 0.05 are coloured in green and *p values* ≥ 0.05. Gray indicates that the selection criteria were not met, but the protein is measured.

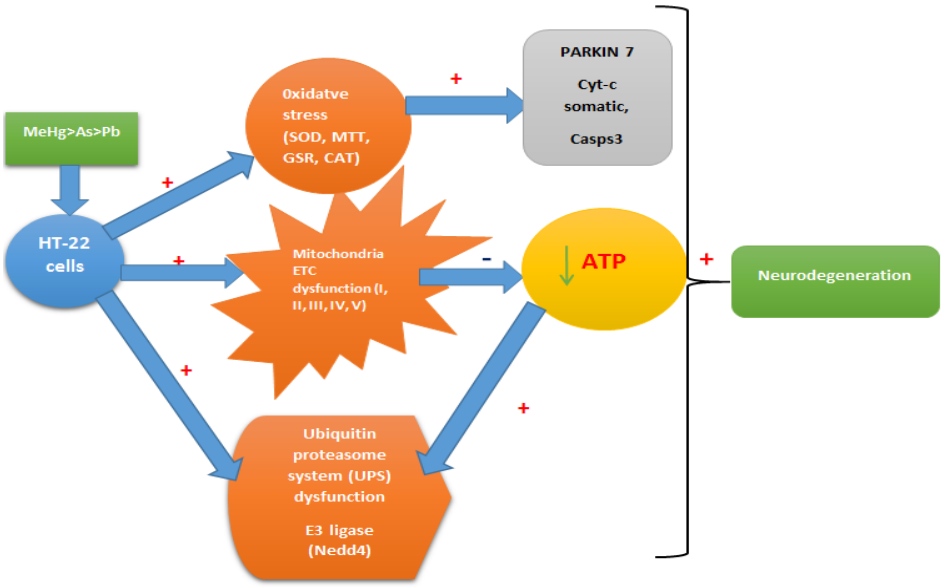


Figure 14: Proposed potential underlying neurotoxicity mechanisms of Pb, As, and MeHg. Metal exposure caused proteomic alterations in hippocampal cells including the proteins related to energy metabolism, oxidative stress, UPS, PD, AD (+ sign enhance the damage, - sign ATP depletion)

4. Conclusions and Future Directions

Chronic exposure to the toxic metals Pb, As, and MeHg have been associated with the neurological degeneration. Nowadays, knowledge of neurodegenerative diseases has advanced rapidly, and the field holds great promise for improving the understanding and the eventual treatment of disease. To date, this study is the first report of comparative heavy metals neurotoxicity assessment by using the omics and systems biology tools. For the proteomics analyses, we selected sub lethal toxic concentrations (IC_{10}) of each metal on HT-22 cells on 8 days exposure as a chronic. Omics based analysis concentration of testing chemical has had a critical role for the success of this study. If the chemical concentration is too high, the secondary effects could mask the primary response. Thus, preliminary cytotoxicity, genotoxicity, and apoptosis observations were used to select the defined concentration. The functional interpretation of protein expressions is always challenging. In this study, we employed both GO analysis and PathVisio approaches for systems biology analysis. Using these integrated tools, we identified significant protein expression changes across treatments related to AD, PD pathways. Our results confirmed that GO and PathVisio analysis is a powerful approach

to generate an unbiased view of the functional protein alterations by heavy metals. The application of such quantitative interpretation of toxicogenomics data is likely to become increasingly useful for evaluating the mechanistic similarity of novel chemicals. The current study describes toxicity profile in the form of protein expression that is observed in response to toxic metals exposure. These changes may provide further insight into mechanisms underlying the development of metal-induced diseases. These proteins are also candidate biomarkers of metal exposure that could potentially be used diagnostically in molecular and epidemiologic studies. The findings from our results have greatly expanded understanding of the role of mitochondria in the pathogenesis of neurodegenerative diseases. Mitochondrial ETC dysfunction, oxidative stress, UPS dysfunction and mRNA splicing changes occur early in all major neurodegenerative diseases, and there is strong evidence that this dysfunction has a causal role in disease pathogenesis. Greater improvement of these pathways will be required to understand fully the pathogenesis of neurodegenerative disorders. Both PathVisio and GO interpretation integrated systems biology supports that the potency of heavy metals is as follow: Pb<As<MeHg. In summary, the comparison of protein expression among Pb, As and MeHg revealed both common and unique protein targets that relate to neurodegenerative diseases. The involvement of mRNA splicing and the UPS pathway are new findings. The results of our proteomics analysis strongly suggest that the disruption of mRNA splicing, UPS, ETC and oxidative stress plays a crucial role in metal induced neurodegenerative diseases. Obviously, the utility of toxicogenomics has promising advantages on assistance and refinement of the risk assessment process, particularly the dose response analysis.

Future work will focus on using these data to explore basic neurodegeneration mechanisms of metals and the toxicity of their mixtures to generate new hypotheses. Although relatively less toxic doses (IC_{10}) were used in the current analysis, a wide dose range study including an environment relevant dose is needed to further examine the association of metal exposure and its neurodegeneration. These heavy metal response patterns may shed new light on the mechanisms of toxic metal induced neurodegeneration, and may also be useful for development of molecular biomarkers of exposure in mechanistic studies and risk assessment

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Chapter 5

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A systems-based Comparative Proteomic approach to investigate heavy metals (Pb, As, and MeHg) binary mixtures induced toxicity relates to the neurodegeneration mechanism on HT-22 hippocampal cell
(Under revision).

Chapter 5

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A system-based comparative proteomics approach to investigate heavy metals (Pb, As, MeHg) binary mixtures induced mechanism relates to the neurodegeneration in HT-22 hippocampal cell line

Abstract

Exposure to toxic metals lead (Pb), arsenic (As), and methyl mercury (MeHg) occurs to mixtures and not to single contaminants These metal mixtures in the environment, creating the potential interactive biological effects different from those observed in single exposure in brain. Metal mixtures studies have posed a big challenge in toxicity characterization and risk assessment. Thus, potential toxicity of metal mixtures has gained special scientific attention. To characterize the impacts of metal mixtures on protein and cytotoxicity responses to hippocampal cells, we established a systems biology approach integrating proteomics, bioinformatics to measure protein expression. Multivariate statistical analysis showed the correlation of protein response between mixtures. Gene functional and pathway analyses of proteomic data confirms and substantiates previous findings on single metal (Pb, As, and MeHg) induced alterations related to mitochondrial dysfunction, oxidative stress, m-RNA splicing, and ubiquitin system dysfunction related neurodegeneration. Bioinformatics analysis of mixtures protein data are presented in comparative manner. Our results showed that, the significant differences of affected proteins in the mixtures corresponded with the findings in individual metals.. This study provides a novel proteomic approach for characterization toxicities of metal mixtures in hippocampal cells.

Key words: Metal mixtures, Cytotoxicity, Proteomics analysis, Systems biology

Abbreviations: AD= Alzheimer's disease, APP= Amyloid precursor protein, Aβ =Amyloid-beta, CAM= Calmodulin, ETC= Electron transport chain, GO= Gene ontology, Glu= Glutamate, GAD= glutamate decarboxylase, LFQ= Label free quantification, LTP= Long term potentiation MAPK= Mitogen-activated protein kinase, MAO-A= Monoamine oxidase A, NMDA= N-methyl D-aspartate, OD= Optical density ORA= over represent analysis, PD= Parkinson's disease, PS= Phosphatidyl serine, PI= Propidium iodide, PKC= protein kinase-C, ROS= Reactive oxygen species ,UPS= Ubiquitin proteasome System

Chapter 5

1. Introduction

Humans are exposed daily to multiple chemicals, including complex chemical mixtures released into the environment and to combinations of chemicals that already co-exist in the environment (Henn, 2009; Valeri et al., 2016). An increased worldwide industrialisation has led to higher levels of pollution by potent toxic metals such as lead (Pb), arsenic (As), and methylmercury (MeHg) (Parajuli et al., 2014). Their multiple industrial, domestic, agricultural, medical, and technological applications have led to their wide distribution in the environment (Ha et al., 2017; Li et al., 2017). One of the most widely studied categories of neurotoxicants are metals; among them, Pb, As, and MeHg are prevalent in the environment (Parajuli et al., 2015). According to world health organization (WHO) these three heavy metals (Pb, As, and MeHg) rank among the priority metals that are of great public health concerns (WHO, 2010). Pb, MeHg neurotoxicity is well established, also recent evidences suggested that As also cause the cognitive dysfunction (Rodrigues et al., 2016). Growing evidence from animal research indicates that the central nervous system is the most vulnerable to Pb, As, and MeHg (Rodier, 2004). . While the toxicity of each of these metals has been well-described, real-world exposures involve simultaneous exposure to more than one metal (Wu et al., 2016; Karri et al., 2016) Thus, the three metals are consider as the ideal candidates to test the neurotoxicity for mixtures. The simultaneous exposure could exacerbate the toxic effects of heavy metals in brain (Spurgeon et al., 2010). In addition, toxic metals have also significant interactions with essential metals (iron, copper, manganese) that can influence the essential metals functions (Goyer, 1997). Several reported binary mixture effects on various organs have been reported: the immune system (Jadhav et al., 2007a), neurotoxicity (Hu et al., 2013; Rai et al., 2013), bladder cancer (Feki-Tounsi et al., 2013), cytogenicity (Jadhav et al., 2006), and induction of oxidative stress (Jadhav et al., 2007b).

Although research on health effects of metal mixtures spans several decades, a number of challenges have limited progress in this field (Kortenkamp et al., 2009). First, the imprecision inherent in exposure biomarkers, coupled with an uncertainty about which biomarker most accurately represents exposure for each chemical, limits our ability to evaluate chemical mixtures (Sarigiannis and Hansen, 2012; Sharma et al., 2016). In addition, even toxicity data of individual metals are available, we are still facing the immense problem for mixtures risk assessment (ATSDR, 2004). On the other hand, prediction of mixtures effects is a great challenge (Orton et al., 2014). One of the major challenges for mixtures toxicology is to find how mixtures exert their effects on living organisms (Claudio et al., 2000). However, the lack of scientific information and research tools, to date, very few biology systems and technologies are available and suitable to address metal/chemical mixtures (Scholze et al., 2014). Recommendations for experimental design and evaluation of combined effects of mixtures are not well developed (Tichý et al., 2002). The regulatory frameworks such as REACH in the EU are becoming more critical to using animals for mixtures risk assessment (Cedergreen,

2014). Therefore, there is an important need to develop novel and biology-focused methodologies and approaches for efficient analysis of environment toxicity pathways, biomarkers, and toxic mechanisms associated with exposure to metal mixtures High-throughput proteomic methods especially applied to toxicology provide opportunities for addressing these challenges. These non-animal / alternative approaches, which place an emphasis on common mechanistic studies, are more reliable.

In this study proteomics and systems biology were used to investigate the metal mixtures toxicity interaction on hippocampal HT-22 cells. The potential use of omics technique and systems biology approach will comprehensively improve the mixtures toxicity mechanism. The purpose of our systematic study is to evaluate the evidence for potential exposures to sub lethal concentrations of mixtures of metals (Pb+MeHg (M1), Pb+As (M2), and MeHg+As (M3)) on HT-22 cells during 8 days. In Mixtures, experimental design cannot proceed without addressing which metal combination (Pb, As, and MeHg) should be considered together, and which criteria (dose, exposure duration) should be used to build common assessment in in vitro system. . For elaborating the metal mixtures toxicity interaction on HT-22 cells, initially we performed individual metal (Pb, As, and MeHg) cytotoxicity, apoptosis, and genotoxicity (Karri et al., 2017a), and proteomics analysis of single metals on HT-22 hippocampal cells (Draft Karri et al., 2018). The interpreted results supported that the toxic metals impact on neurodegenerative diseases. This single metal protein data gives immense scope to measure the protein expression levels in critical pathways treated with a mixtures The integration of proteomics data with knowledge based pathway mapping facilitates the elucidation of the underlying molecular mechanisms of these three metal mixtures as similar as single metals. Comparisons based on proteomics analysis across the three mixtures (M1, M2, and M3), has allowed us to find the critical mechanism of metal mixtures toxicity in hippocampal cells. However, these results are very context-sensitive; a comparison between different mixtures would be of interest to highlight the specific pathways, as well as compared with single metals and their binary mixtures. Finally, we evaluated involvement of biological pathways related to Parkinson's disease (PD), Alzheimer's disease (AD). Overall, current study facilitates quantitative interpretation of mixtures impact in HT-22 hippocampal cells

2. Materials and Methods

2.1. Chemicals

Lead chloride ($PbCl_2$ [CAS no: 7758-95-4]), Sodium metaarsenite ($NaAsO_2$ [CAS no: 7784-46-5]) , Methyl mercury chloride ($MeHgCl_2$ [CAS no: 115-09-3]), Dimethyl sulphoxide (DMSO [D5879]), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT [M5655]), trypsin (TrypLE [Gibco: 12604013]), and proteomics reagents; Urea (GE HealthCare, Life Sciences, CAS Number: 57-13-6), Sodium Dodecyl Sulphate (SDS) (Merck, CAS Number: 151-21-3), Ammonium Hydroxide

(Fluka, CAS Number: 1066-33-7), Dithiothreitol (GE HealthCare, Life Sciences, CAS Number: 3483-12-3), Iodoacetamide (GE HealthCare, Life Sciences, CAS Number: 144-48-9), Formic Acid (Merck, CAS Number: 64-18-6), Acetonitrile (HPLC grade) (Fisher Chemical, CAS Number: 75-05-8), Water (HPLC grade) (Fisher Chemical, CAS Number: 7732-18-5). All are analytical grade and purchased from Sigma-Aldrich Química, S.L-Madrid (Spain)

2.2. Cell line and Reagents

The HT-22 cells have been used as a hippocampal neuronal cell model in numerous studies (Niska et al., 2015). The HT-22 cells were a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA). HT-22 cells were maintained in Dulbecco's modified eagle's medium (DMEM [D6429]) containing 10% fetal bovine serum (FBS Gibco [10500-064]) and 100 U/mL penicillin, and 100 µg/mL streptomycin (Pan-Biotech- Germany) in a humidified incubator with 5% CO_2 in air at 37^0 C. For all the experiments cells were grown at 70- 80% confluence.

The cells were cultured in 75 cm^2 cell culture flasks. For experimental purpose, cells were plated at 0.57 x 10^6 cells/ mL and grown for 24 hours before metal treatment. Duplicates wells of cells were treated with Pb+MeHg (M1), Pb+As (M2), and MeHg+As (M3) respectively; due to the 8 days exposure medium containing given concentration was refreshed at 2 days interval for maintaining metal exposure in a long time. Metal stock solutions 100X were prepared in deionized distilled water (for poorly soluble $PbCl_2$ < 0.5% DMSO added) and sterilized by filtration through 0.2 µm and different concentrations of a working solution of each individual metal was prepared by prior dilution of the stock solution in phosphate buffer saline (pH = 7.4) and then applying 10% working solution on DMEM culture medium.

2.3. Assessment of mixtures cytotoxicity using the response additivity method

This method for testing interaction between chemicals has been described previously by Lau et al., (2006). In this model, the combined effects of two agents are thought to be equal to the sum of the effects of the single compounds. Deviations from this are either synergistic or antagonistic. In the present study, metals (Pb, As, and MeHg) were tested at their IC_5 – IC_{20} range concentrations, derived from individual concentration–response curves (Karri et al., 2017a). To assess the interaction between metals, combined effects include: additivity where metals are no more and no less effective in combination than they are separately; synergism where the effectiveness of agents is increased when in combination; and antagonism where the effectiveness of agents is decreased when in combination (Costa et al., 2007).

The MTT assay was carried out using a modification of the method of Mossman (1983). The HT-22 cells were seeded in 96-well plate. After 24 h, when the cells had reached a confluence of 70–80%, they were exposed for 8 days to various concentrations of M1, M2, and M3 mixtures by toxic equal range doses of each metal in mixture (IC_5 to IC_{20}). After the incubation period, medium was aspirated from well and MTT working solution at 0.5 mg/mL was added to each well. The absorbance of the solubilized

reduced MTT was then measured in a micro titter plate spectrophotometer reader at a wavelength of 570 nm. The measured absorbance or optical density (OD) values were converted to percent of cell viability (%) with respect to control. The cell viability results at the sub-lethal dose of each mixture were used for apoptosis and proteomics studies.

2.4. Analysis of apoptosis by annexin V-FITC/ propidium iodide (PI)

To evaluate the translocation of phosphatidylserine (PS) from inner leaflets to outer leaflets of plasma membrane, annexin V- FITC apoptosis detection kit (BD Pharmingen, Poland) was utilized. In this kit, annexin V and propidium iodide (PI) were used to distinguish the apoptotic and necrotic cells from the viable cells. According to the manufacture's protocol, the exponentially proliferating cells were exposed to sub lethal toxic Pb+MeHg (M1), Pb+As (M2), MeHg +As (M3) mixtures in culture plate at a density of 0.56 x 10^6/mL during 8 days; control cells were made without chemical. The medium with metal concentration was refreshed every 2 days. After 8 days treatment with metals, cells were harvested by trypsinization, washed twice with ice cold PBS (pH = 7.4). Thereafter, cells were centrifuged at 1200 rpm for 5 min at 40C, resuspended in 1mL 1X binding buffer and then transferred the 100 µL of the solution to 5 mL culture tube, and added 5 µL of both annexin-V, PI to the samples. After staining, cells were incubated for 15 minutes in the dark at room temperature. Finally cells were re-washed with 1X binding buffer 400 µL and analyzed by flow cytometry (Beckman coulter, Germany). Three independent experiments were performed.

2.5. Protein extraction and digestion

For label free proteomics study, the exponentially proliferating HT-22 cells were exposed to the selected sub lethal dose (IC_5 + IC_5) of each M1, M2, and M3 mixture in petri dish at a density of 0.025 x 10^6/mL during 8 days; control cells were made without chemical. Heavy metal mixtures treatment (and control culture), protein was extracted from HT-22 cell line with 200 µL (8 M urea, 0.1% SDS, 50 mM ammonium bicarbonate[ABC] cocktail) lysis buffer and quantified using Micro BCA™ protein assay kit (Thermo Scientific). 30 µg of protein from each sample was digested in a Filter Assisted Sample Preparation (FASP) approach. The volume corresponding to 30 µg of protein was adjusted to 270 µL with 8 M Urea/50 mM ABC. Reduction was done with 20 mM dithiothreitol (DTT) treatment for 1h 30 min at 32^O C. Samples were then alkylated using 30mM of iodoacetamide (IAA) and incubated in the dark for 30 min at room temperature (RT). Afterwards, samples were loaded in 10 KDa Filters (Amicon) and centrifuged during 30 min, 12,000 g at RT followed by two washes with 300 µL 1M urea/50 mM ABC by centrifugation (30 min, 12,000 g, RT) in order to remove interferents and establish optimal pH (7.5-8.5) and urea concentration for proteins to be denatured. The filter remaining material was resuspended in 400 µL 1M urea / 50 mM ABC. Digestion was done in two steps: an initial digestion with 1:30 (w/w) porcine trypsin 0.25 µg/µL (Sequence grade modified Trypsin, Promega) for 3hrs at 32^OC

followed by a digestion with 1:50 (w/w) trypsin 0.25 µg/µL for 16h at 32^{O}C. Peptides were eluted by centrifugation (12,000g for 15 min at RT) and the filters were cleaned with 200 µL 50 mM ABC by centrifugation at 12,000g for 15 min at RT. Samples were prepared for chromatographic analysis. Briefly, peptides resuspended in 100 µL of 1% formic acid (FA) were charged in the tip columns (previously washed with 70% acetonitrile [ACN] in 0.1 % FA and equilibrated with 0.1% FA) by centrifugation (300g for 1 min 30 sec). Columns were washed twice with 100 µL 0.1% FA by centrifugation (300g for 1 min) and then peptides were eluted in 2 x 100 µL of 70% ACN / 0.1% FA by centrifugation (300g for 1 min). The peptides were dried in speed Vacuum (Eppendorf) and stored at 20 ^{O}C until LC-MS analysis.

2.6. LC-MS/MS analysis

The peptide mixtures were resuspended (1% FA, 2% ACN) and the volume corresponding to 500 ng of protein was analysed by an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Peptide mixtures were injected into the capillary column (75 µm× 25 cm) in full loop mode and separated by a 5 µm C18 column using a nano-acquity liquid chromatography system (Waters). Peptides were eluted with a linear gradient of 1-35 % buffer B (0.1% FA, 100% ACN) for 150 min, followed by 35-45 % buffer A for 20min (A: 0.1%). The mass spectrometer was operated in positive ion mode (source voltage 2000V) and data-dependent manner. The full MS scans were performed in the Orbitrap at the range of 300–1,700 m/z at a resolution of 60,000. For MS/MS scans, the 15 most abundant ions with multiple charge states were selected for collision induced dissociation (CID) fragmentation following one MS full scan.

2.7. Label-free quantitative (LFQ) data analysis using Progenesis QI software

Raw MS peak intensities were analysed using Progenesis QI data analysis software v4 (Non-Linear Dynamics, Waters, U.S.). Ion feature matching was achieved by aligning consistent ion m/z and retention times. Progenesis label free quantification (LFQ) was done using non-conflicting unique peptides and protein grouping, and the software normalization algorithm was applied to all proteins. Abundances are calculated as the area under the MS peak for every matched ion feature. Search results were filtered based on peptide ion score≥ 40 and contaminants were removed and abundances were normalized to all proteins. A total of 428,801 spectra were obtained from the LFQ LC−MS/MS proteomic analysis of samples including a control and test sample. After data filtering to eliminate low-scoring spectra 27,088 peptides were retained and used for protein identification. Total 3,140 proteins were quantified from 27,088 peptide ions. Only the proteins quantified with minimal 2 peptides were included in the further statistical analysis.

2.8. Protein identification

Proteins identification was done using Mascot search engine (v. 2.3.01). All MS/ MS spectra passing an arbitrary cut-off in the Progenesis software were included in the list used to perform the search against SwissProt Mouse database using the Mascot search program (Matrix Science, London, UK, www.matrixscience.com). The following criteria was applied: (1) trypsin as enzyme; (2) allowed two missed cleavages; (3) variable modifications, acetyl (N-terminus, protein) and oxidation (M); Carbamidomethyl (C) fixed modification (4) Peptide tolerance, ±10 ppm (MS); MS/MS tolerance, ± 0.6 Da.

2.9. Statistical analysis

Label free quantitative data was submitted to t-test method ($p \leq 0.05$) using Microsoft excels in order to find out significant differences among the treatments and control. Data was further curated applying the following filters: minimum1.2-fold change, p-value< 0.05. Meaboanalyst 3.0 (http://www.metaboanalyst.ca) programme was used for presenting the unsupervised data in PCA analysis, volcano plot and hierarchical clustering analysis.

2.10. Systems Biology analysis

In our systems biology analysis three main steps are included for the quantitative functional interpretation of proteomics data. The first step is aimed to identify the statistically significant proteins by using the T-test method. The next step is to identify significantly altered functional Gene Ontology (GO) categories using unsupervised GO analysis. The altered GO terms and the differentially expressed proteins in these GO terms are visualized in a network to identify the links between the terms. In the final step the changes in protein expression were visualized in selected pathways related to neurodegenerative diseases.

2.10.1. Functional interpretation of significantly altered genes using GO analysis

GO-Elite (http://www.genmapp.org/go_elite/) is a flexible for GO functional analysis (Zambon et al., 2012). It was used for to find the altered biological processes, molecular functions, and cellular components based on changes in proteins. GO-Elite performs an over-representation analysis (ORA) on any structured ontology annotations, or biological identifiers (e.g. gene, protein or metabolite) (Huang et al., 2009). The program utilizes the structured nature of biological ontologies to report a minimal set of non-overlapping terms called as pruned terms. Pruning is the process of intelligently examining the hierarchical structure of ontology, such as GO, and comparing the ORA scores of enriched terms based on their relationships to each other. Highly related terms highlighted by ORA are considered to be redundant with each other, hence, pruning is used to select the highest scoring term among a set of related terms, while retaining any distinct related terms. GO-Elite ranks each analyzed pruned GO term according to a Z-score along with a p-value (Ren et al., 2010). Significantly altered categories were identified based on the p value <0.05, Z-score > 1.96, and a minimum of 3 proteins

changed within each specific GO term. The results were visualized as networks linking the GO terms and differentially expressed proteins in the GO terms using the network analysis tool Cytoscape (Gang Su et al., 2009) . In addition, on the GO-protein network in Cystoscope displayed the log2 fold change (log2FC) between each metal exposure group and to control were visualized

2.10.2. Pathway visualization

PathVisio (Kutmon et al., 2015) is a commonly used pathway editor, visualization and analysis software for omics based experiments. Here, first the required curated mus musculus pathway collection was obtained from WikiPathways (http://www.wikipathways.org) (Kutmon et al., 2016). Pathway analysis was performed in PathVisio version 3.2.4 (http://www.pathvisio.org) to interpret and visualize the molecular changes on a pathway level. In present study we visualized the changes in proteins after metal exposure in biological pathways relevant to neurodegeneration processes in which a minimum of 3 proteins were changed. The log2FC and p-value were shown on pathways using the visualization module in PathVisio.

3. Results

3.1. Over view of cytotoxicity and Apoptosis response to metal mixtures

The concentrations of individual metals were selected for the mixtures interaction based on the cell viability results. The selected concentrations were expected to cover the non-toxic to a toxic concentration level. We designed the binary mixtures interactions study by using the response addition and dose addition whether they interact with one another when combined exposure. This binary combination of Pb, Cd, As, and MeHg displayed synergistic and antagonistic interaction at low and higher effect levels in HT-22 cells. Several interesting findings were observed from dose response analysis; we reported in the Karri et al., (2017b). From the all results, Pb, As, and MeHg neurotoxicity established toxic interactions rather than Cd metal. The mixture M1showed more than the additive response in cytotoxicity. The other two M2, M3mixtures results indicates the additive effect on HT-22 cells. Hence, we decided to extend the molecular based studies to assess the interaction profile in molecular level. Further, comprehensive mechanistic based omics investigation can useful for understanding the real interactive mixtures interactions in molecular level. Before performing the proteomics experiment, we estimated the selected (M1, M2, and M3) mixtures apoptosis effect with sub lethal concentration for validating the cytotoxicity results, and also quantifying the cell population as an early, late apoptosis, necrotic cells, and normal cells in respected exposure.

Here, cells were treated with M1, M2, and M3 sub lethal treatment is equal to IC_{10} of single metals (Pb, As, and MeHg) in HT-22 cells (Karri et al., 2017a). Also, we performed annexin- V/ PI staining that allows the discrimination of viable cell, apoptotic, and necrotic cells by binding with Ca^{+2} dependent phosphatidyl serine (PS) protein in cell membrane. The obtained apoptosis assay results showed in Figure 1. The apoptosis study clearly shows that the apoptosis mechanism also similar to cell viability.

Chapter 5

The present study confirms M1showed more than the additive response, for this nature of interaction considered as a synergistic effect. The other two combinations M2, M3indicates the simple additive effect on HT-22 cells. A marked reduction of cell viability was found in MeHg among Pb binary mixture. This synergistic effect can be possible due to the common mechanism and it reduces the exposure dose of individual metals in a mixture. These all findings give significant evidence of the metal mixtures neurotoxic activity depend on the composition of elements, cell line sensitivity. The findings from cytotoxicity, apoptosis assays results are highly appreciated to extending omics studies to understand the critical molecular mechanisms in HT-22 cells. The high throughput label free omics analysis could be helpful for understanding the complete over view of metal mixtures induced stress in the hippocampal cells and also validate the interaction of metals in HT-22 cells by using the proteins expression.

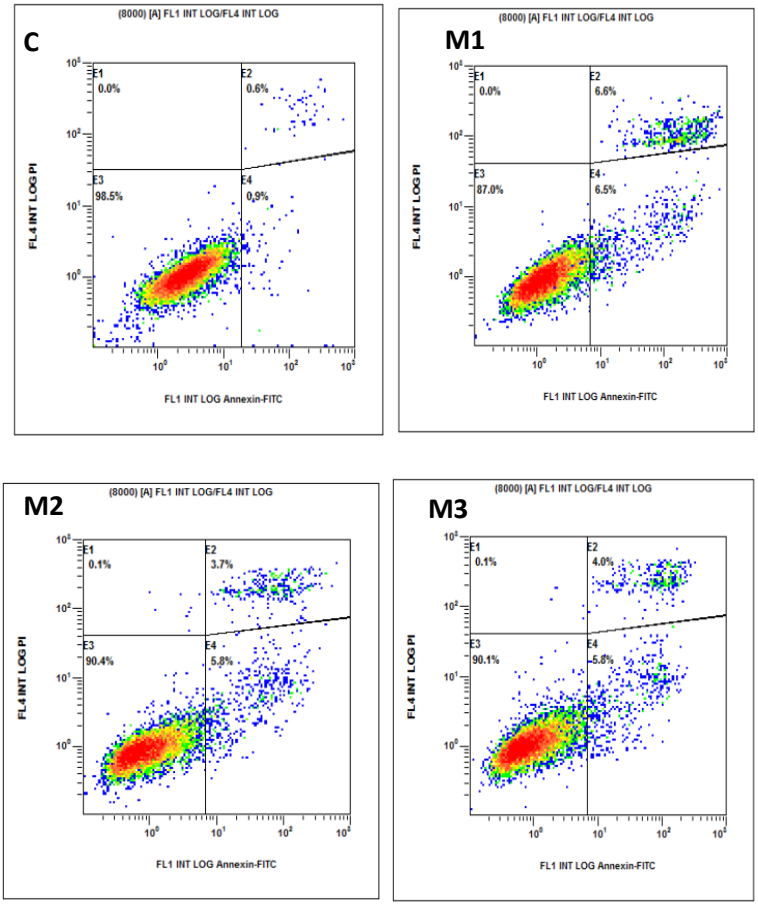


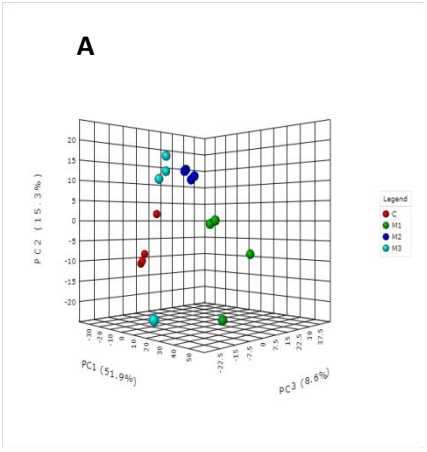
Figure 1: Representative dot plots showing the apoptosis effect of M1, M2, M3 mixtures to HT-22 cells upon 8 days of exposure (n=3). C= Control, M1= 60.23+0.03 µM, M2= 60.23+0.33 µM, M3= 0.33+0.03 µM. Lower left (LL)/ E3 = Live cells (Annexin V−/ PI−), Lower right (LR)/ E4 = Early apoptotic cells (Annexin V +/ PI−), Upper right (UR)/ E2 = Late apoptotic (Annexin V+/ PI+), Upper left (UL)/ E1 = Necrotic cells (PI+).

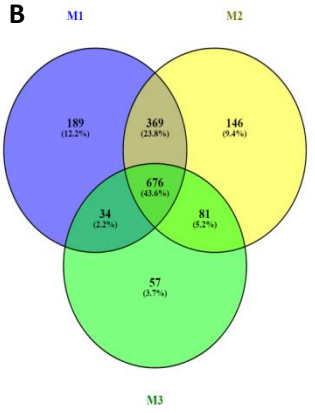
3.2. Identification and quantification of key proteins and pathways in HT-22 cells by using the label free proteomic analysis

In order to identify novel molecular pathways related to metal mixtures neurotoxicity, we have used a label-free quantitative proteomics approach. The quantitative analysis was performed by Progenesis QI software as explained in the methods section 2.7. Total 3,140 proteins were quantified from 27,088 peptide ions. From them, 2160 proteins were identified with a minimum of 2 peptides. Only the proteins with at least two unique identified peptides were retained for further quantification analysis. The unsupervised principal component analysis (PCA) was performed by using the normalized proteins (≥2 unique peptide) that were present in all replicates (n=4) (Figure 3A). The first principal component (PC1: 51.8%) describes the largest variation in the dataset in which the samples spread the most in the variable space. The second component (PC2: 15.3%) describes the next largest variation and is orthogonal to the first component. The third component describes the last largest variation (PC3: 8.6%). Figure 3 (A) highlights clustering or pattern formations in a three-dimensional space which provides a view of the similarities and dissimilarities among the samples. For each metal mixture we extracted the proteins showing significant changes in abundance when compared to control cells (1268, 1272 and 848 proteins respectively for M1, M2, and M3). The resulting proteins of the three mixtures treatments compared in a venn diagram and results are shown in Figure 3B. We found 676 proteins commonly altered in three mixtures. Interestingly, some proteins were exclusively altered due to the exposure to each mixture: 189 for M1, 146 for M2 and 57 for M3. The experimental groups (control, M1, M2, and M3) were clustered based on the log standard abundance of the un-supervised data with a hierarchical clustering ward algorithm. The clusters clearly separated according to expression level in each replicate as shown in Figure 3C, the spot maps of M1 are distinguished the most from the other spot maps. Differences were also found between the controls, and mixtures. We can notice the clear presence of group of proteins that are systematically upregulated (red) and down regulated (green) in treatment Vs control.

Volcano plot presents (Figure 4) the protein expression response to M1, M2, and M3 respectively (Figure 4). The replicates for the four study groups were averaged and ratios for each metal treatment group to control were calculated. To ease visualization of proteome alterations, ratios (or Fold Change, FC) were Log(2) transformed and plotted against –log(10) transformed t-test-values (Figure 4, A-C) The presented pink dots (Log(2)FC ≥0.26 or ≤0.26 and p-value≤ 0.05) were considered as differentially expressed among each mixture treated and control cell comparisons. A total of 1268,

1272, and 848, proteins were found to be response to M1, M2, and M3 mixtures. Among them, 429 398, and 353, proteins were down regulated; remaining 839, 874, and 495 proteins were significantly up regulated in M1, M2, and M3mixture respectively.





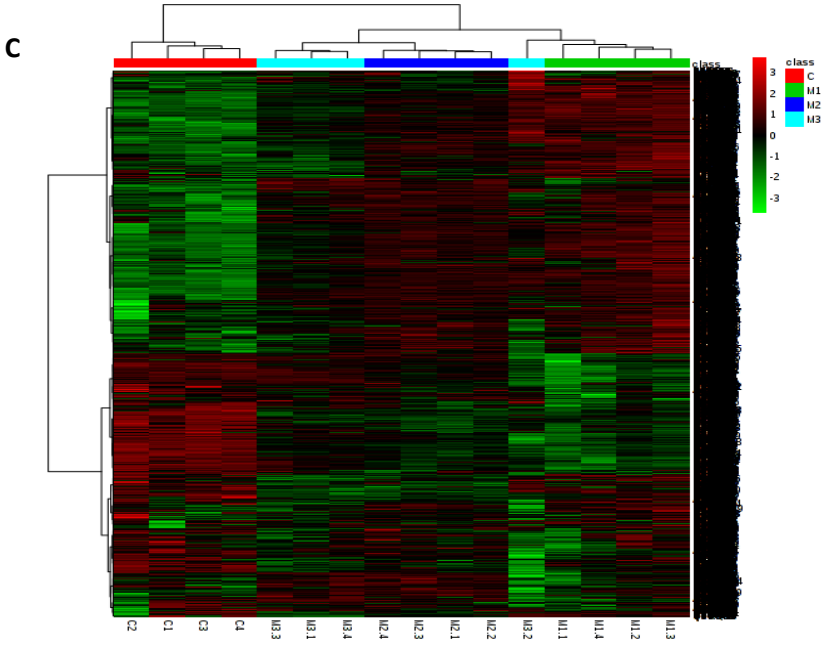


Figure 2: A. diagram presenting the principle component analysis (PCA) score plot (PC1, PC2, PC3) of the unsupervised proteomic profiles of HT-22 cell **B.** Venn diagrams showing the overlap between significantly changed ($p < 0.05$) proteins in M1, M2, M3 mixtures. **C.** Heat Map for hierarchical Euclidian clustering of differential protein expression of the experimental groups (control, Pb, As, MeHg).

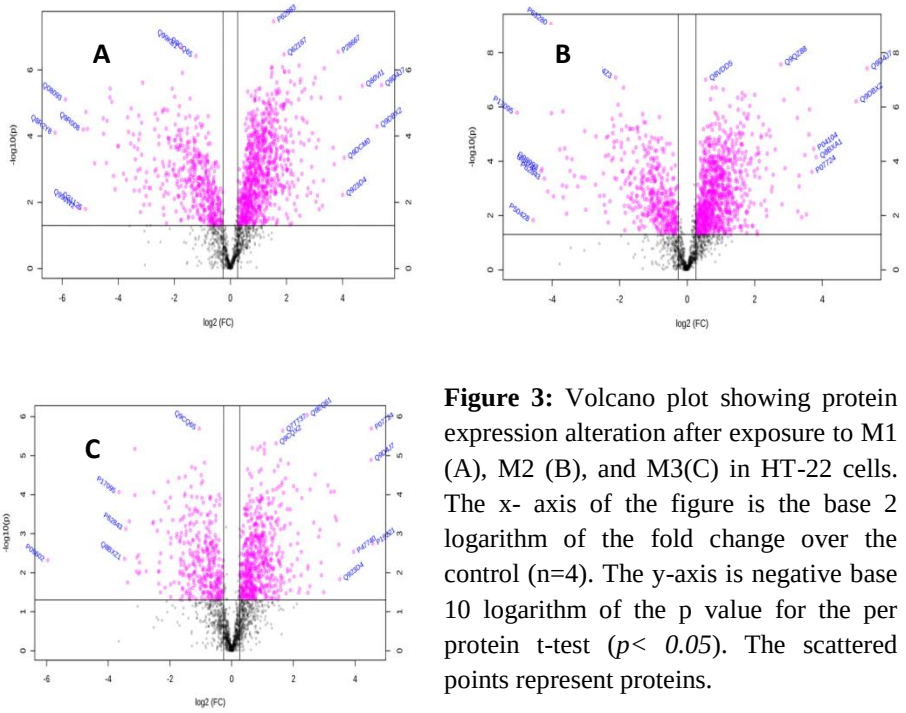


Figure 3: Volcano plot showing protein expression alteration after exposure to M1 (A), M2 (B), and M3(C) in HT-22 cells. The x- axis of the figure is the base 2 logarithm of the fold change over the control (n=4). The y-axis is negative base 10 logarithm of the p value for the per protein t-test ($p < 0.05$). The scattered points represent proteins.

3.2. Functional classification and enrichment analysis of protein data by using GO –Elite

One of the major potential advantages of proteomics technologies are that will enable researchers to look at the complete complement of protein and their expression. The applications of GO mapping and pathway analysis to omics data give strong interpretation in the context of the system biology. Our GO results contain multiple files, in that ORA, pruned list are most informative to understand the GO terms and proteins biological relation (supplementary file 1). The ability to determine in which GO terms are linked to differentially expressed proteins in ORA provides an ideal model to gain understanding of what GO classes are altered after mixtures exposure. The pruned ORA list contains the GO terms that: i) are pruned to only include non-redundant information, and ii) are significantly altered based on changes in protein expression. Relationships between all regulated proteins and ORA/pruned terms can also be easily

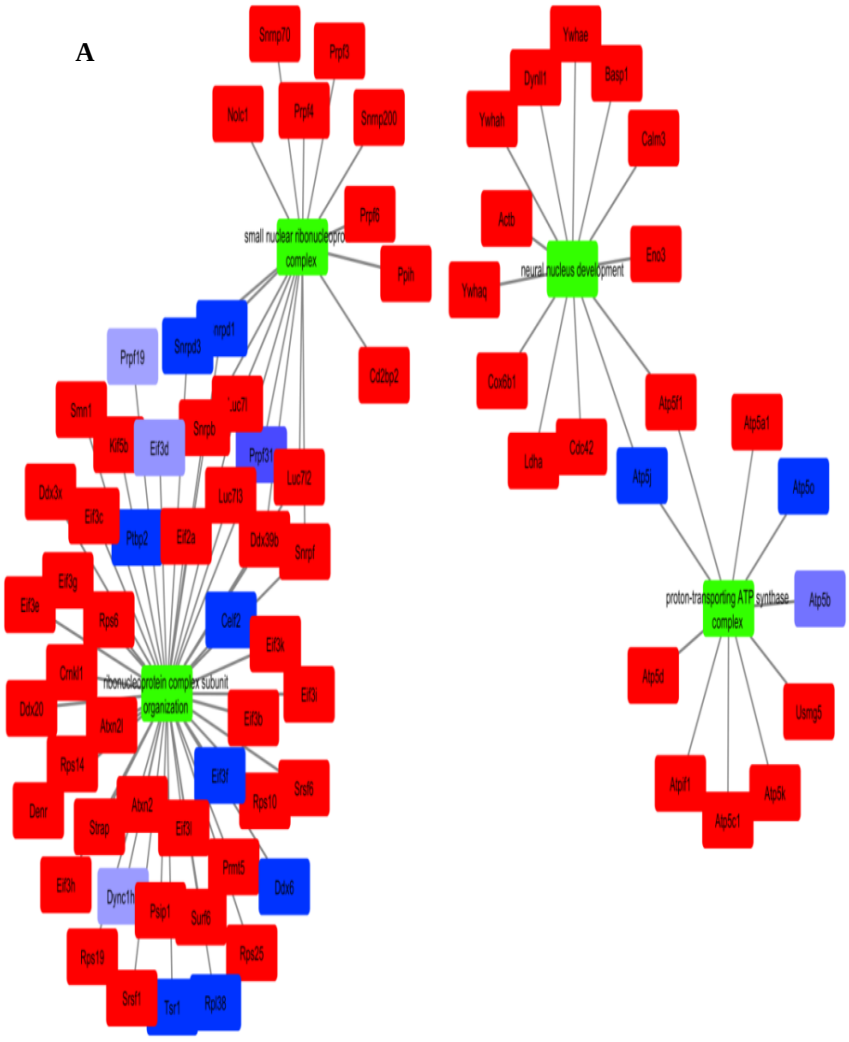
visualized as networks in Cytoscape using output the pruned sift file. We showed Cytoscape global networks (supplementary file 2) of the all pruned GO terms with protein expression, which gives an overview of all the significantly expressed proteins and which can be helpful to determine the mixtures impact on HT-22 cells. For instance, we found by observing the GO terms and protein expression relation that M3 mixture impact was very low while the M1 and M2 showed greater impact on HT-22 cells.

A major challenge in the GO of omics data is the functional interpretation, and the linking to potentially altered proteins that are the most relevant to disease processes. Using the GO hierarchical classification system, we identified enriched processes within the collection of genes identified as significantly altered with M1, M2, and M3. The obtained GO processes are unique in each metal mixture; however, few of them are linked to neurodegenerative diseases. For instance, M1 exposure showed different GO processes, which the most significantly relevant to neurotoxicity are shown in Table 1 and presented as network in Figures 4 (A, B, and C). In the M1 mixture GO functional processes are related: to neural nucleus development (GO: 0048857), proton-transporting ATP synthase complex (GO:0045259), small nuclear ribonucleoprotein complex (GO:0030532), energy coupled proton transport, and down electrochemical gradient (GO:0015985). The expression pattern of these functional processes is upregulated in synergistic manner and related to neurotoxicity (Figure 4A). In M2 mixture, the upregulation of DNA repair (GO:0006282), developmental growth involved in morphogenesis (GO:0060560), energy coupled proton transport, down electrochemical gradient (GO:0015985), and negative regulation of cell development (GO:0010721) proteins has relation to neurotoxicity. However, In M2 the protein expression changes were less than that the M1 mixture (Figure 4B). In relation to mixture M3, the proteome changes were very poor related to the neurotoxicity, being found very few proteins with GO terms with low fold change (Figure 4C). According to functional GO categories of protein expression in the three mixtures, the M1 mixture response showed synergistic, M2 mixture showed additive response, while M3 mixture response was lower than the reported cytotoxicity (additive) results. The above pruned processes are the more relevant to neurotoxicity. The functional interaction of proteins in network analysis showed proteins such as APP, PARK7, CASP3, SOD, GSH, and Snrp70. These proteins have crucial role in AD, PD. The overall identified proteins and GO functional analysis results in mixtures are entirely different to their individual metal (presented in supplementary file) and most of the proteins are upregulated in metal mixtures (except M3) than single metal.

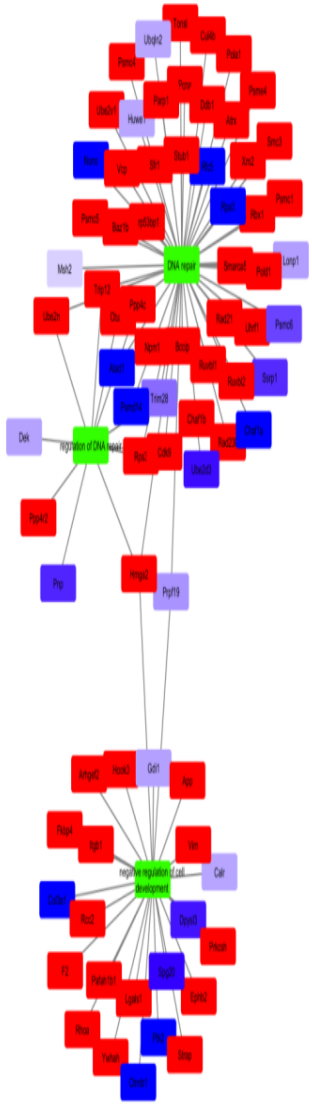
Our data demonstrates that GO analysis constitutes a powerful unbiased approach to define the biological pathways altered in rat hippocampal HT-22 cells in response to metal mixture exposure. The GO processes shown in Table 1 for the three mixtures (M1, M2, and M3) suggests that these GO term related proteins may be one of sensitive indicators of mixtures response in association with neurodegeneration processes.

Table 3: Statistically significantly changed GO terms related to neurotoxicity in HT-22 cells during mixtures exposure.

Pb+MeHg mixture						
Ontology-ID	**Ontology Name**	**Ontology Type**	**Changed**	**Measured**	**Ontology**	**Z score**
GO:0048857	neural nucleus development	biological process	13	14	61	2.6
GO:0045259	proton-transporting ATP synthase complex	Cellular component	10	11	20	2.1
GO:0030532	small nuclear ribonucleoprotein complex	cellular component	17	21	31	2.0
GO:0015985	energy coupled proton transport, down electrochemical gradient	biological process	9	10	24	2.0
Pb+As mixture						
GO:0010721	negative regulation of cell development	biological process	24	29	238	2.6
GO:0006282	regulation of DNA repair	biological process	11	12	55	2.3
GO:0060560	developmental growth involved in morphogenesis	biological process	7	7	82	2.2
GO:0015985	energy coupled proton transport, down electrochemical gradient	biological process	9	10	24	2.0
As+MeHg mixture						
GO:0048588	developmental cell growth	biological process	6	6	51	3.0
GO:0045738	negative regulation of DNA repair	biological process	4	4	8	2.4
GO:0007270	neuron-neuron synaptic transmission	biological process	4	4	59	2.4


A
Snrnp70
Prpf3
Nolc1
Prpf4
Snrnp200
Prpf6
small nuclear ribonucleoprotein complex
Ppih
Cd2bp2
Prpf19
Snrpd3
nrpd1
Smn1
Luc7l
Snrpb
Klf5b
Eif3d
Prpf31
Luc7l2
Ddx3x
Luc7l3
Eif3c
Ptbp2
Eif2a
Ddx39b
Snrnp
Eif3g
Eif3e
Rps6
Celf2
Eif3k
Cmkl1
ribonucleoprotein complex subunit organization
Eif3i
Ddx20
Abxn2l
Eif3b
Rps14
Eif3f
Srsf6
Denr
Abxn2
Rps10
Strap
Eif3j
Prmt5
Eif3h
Dync1h
Ddx6
Psip1
Surf6
Rps19
Rps25
Srsf1
Tsr1
Rpl38
Ywhae
Dynll1
Basp1
Ywhah
Calm3
Actb
Eno3
neural nucleus development
Ywhaq
Cox6b1
Atp5f1
Atp5a1
Ldha
Cdc42
Atp5j
Atp5o
proton-transporting ATP synthase complex
Atp5b
Atp5d
Usmg5
Atpif1
Atp5k
Atp5c1

B



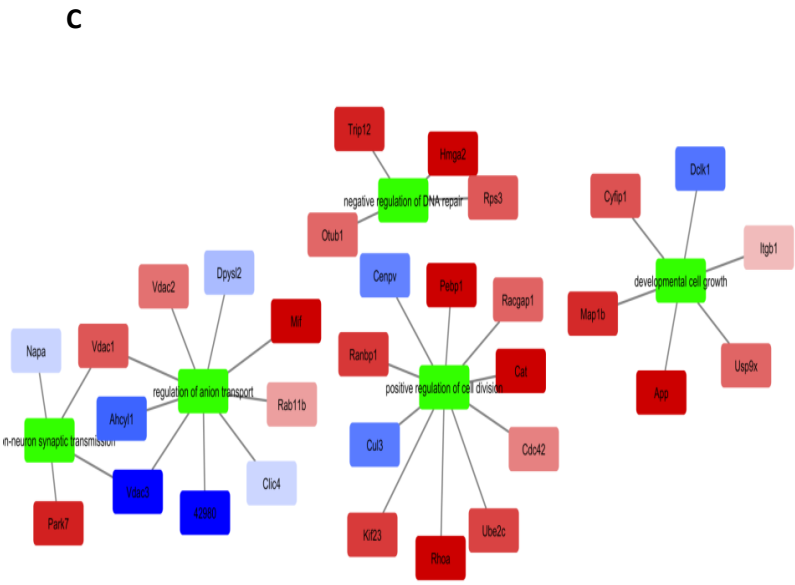


Figure 4: Interaction of differentially expressed proteins and selected GO classes after M1 (A), M2 (B), and M3 (C) mixtures exposure. The network is based on a selection of the over-represented and pruned GO terms and was visualized in Cystoscope. The network includes over-represented GO terms and associated differentially expressed proteins. The changes in protein expression are coloured as a gradient going from blue (=downregulated) to red (=upregulated) and GO terms are coloured in green.

3.3. Pathway analysis: visualization of pathways relevant to metal mixtures (M1, M2, and M3) induced neurodegeneration in HT-22 cells

Pathway analysis was used to further explore the complex interactions in biological system and to discover the relations between differentially expressed proteins and their relevance to neurodegenerative diseases. In mixtures pathway analysis, few common predominant pathways are visualized that relate to single metal neurotoxicity. The obtained results in the present study suggest that the pathways: mRNA splicing, ubiquitin proteasome system (UPS), oxidative stress, electron transport chain (ETC) dysfunction, Alzheimer's disease (AD), Parkinson's disease (PD), are most affected by mixtures as similar to their single metals.

Table 2: Six pathways showed significant changes in HT-22 cells relevant to single metals neurotoxicity HT-22 cells

		M1	M2	M3
Pathway	**measured (n)**	**positive (r)**	**Positive (r)**	**Positive (r)**
Electron Transport Chain	31	19	18	16
Oxidative Stress	12	4	9	5
Proteasome Degradation	40	29	24	15
mRNA processing	82	52	45	38
Parkinson's Disease Pathway	9	7	6	4
Alzheimer's Disease	17	6	4	5

(*Measured (n) is the number of proteins in the pathway that are measured in the M1, M2, and M3 data set, and positive (r) is the number of differentially expressed proteins in the presented pathway in each metal mixture).

3.3.1. Impacts of M1, M2, and M3 mixtures on mitochondrial electron transport chain (ETC) dysfunction

A failure of one or more of the mitochondrial electron transport chain enzymes can compromise the brain energy stores and generate reactive oxygen species (ROS) in neurons. Here, the modification of ETC proteins in HT-22 cells can influence the energy production. .Usually, omics analysis are complicated to explain the individual protein expression role in ETC complex I-V However, we found protein dysregulations in all complexes, which indicates the effect of metal mixtures in ETC.. M1, M2, and M3 sub lethal treatment in HT-22 cells, showed significant alteration of complex I –V proteins (Figure 5). The pattern of protein expression alerted by some of the mixtures in the ETC pathway was more improved (fold change) than with single metals. In the present study, cytochrome c oxidase (COX)/ complex-IV and complex –V/ F_1 F_0-ATPase activities were highly dysregulated by the three mixtures. The findings of mitochondrial ETC damage to metal mixture exposure due to an interaction mechanism. The reduced activity of F_1 F_0-ATPase (complex-V) could compromise the ATP production and induce the reactive oxygen species (ROS), which can lead to HT-22 cell death. Our findings confirm that the metal mixtures disrupt the ETC pathway in HT-22 cell similar to single metals (except that adenine translocator functional unit), which may lead to energy deficiency in HT-22 cells. The observed ETC damage of three mixtures in HT-22 cells is almost similar expression manner that is contrast to the cytotoxicity profile.

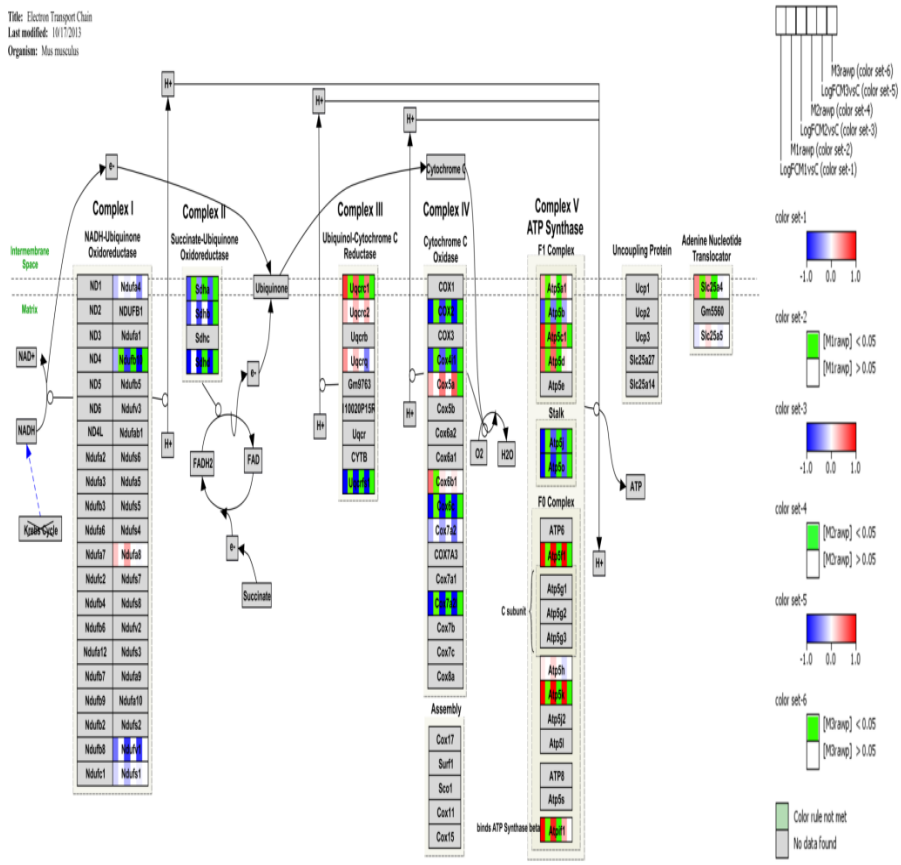


Figure 5: Changes in electron transport chain (ETC) pathway in response to M1, M2, and M3 in HT-22 cells. In the pathway changes in protein expression are visualized as log2foldchange comparing treatment with control expression. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p values* < 0.05 are coloured in green and *p values* ≥ 0.05. Gray indicates that the selection criteria were not met, but the protein is measured.

3.3.2. Impact of M1, M2, and M3 mixtures on reactive oxygen species (ROS) mediated oxidative stress

The present results indicate generation of ROS related to energy depletion and monoamine oxidase A (MAO-A) elevation in the HT- cells. The presented oxidative stress pathway in Figure 6 gives an adaptive response in HT-22 cells to maintain redox homeostasis due to mixtures induced stress. We found MAO-A protein expression upregulated only in M2 mixture (Pb+As). The other two mixtures didn´t have any response. In our previous study with individual metals, we found that the three metals

controlled the MAO-A expression. This result suggests that the M2 mixture only regulates the monoamines metabolism by controlling the MAO-A function. In anti-oxidant elements results showed a significant alteration in mitogen-activated protein kinase (MAP) mediated oxidative stress; however the response was higher in the M2 mixture (Figure 6). MAPKs play an important role in the cascades of cellular responses to external stress. The induction of antioxidant and phase II detoxifying enzymes acts as an important defence mechanism against metal mixtures induced stress in HT-22 cells.

M2 and M3 induced significant effects in MAP kinases mediated oxidative stress response such as anti-oxidant proteins heme oxygenase1 (HMOX1), NAD (P) H dehydrogenasequinone1 (NQO11), glutathione disulfide reductase (GSR) (Figure 6). These proteins are upregulated in the M2 and M3 mixtures, which give clear evidence that these two mixtures have specific targets in the oxidative stress mechanism. The other anti-oxidant proteins (metallothionein-1(MT1), catalase (CAT), specificity protein 1 (SP1)) were upregulated in the three mixtures. Additionally we observed that superoxide dismutase1 (SOD1) and thioredoxin reductase1 (TXNRD1) expressions were present in M1 and M2 mixtures. The pattern of expression in mixtures varies with single metal exposure that supports a higher stress in mixtures than single metal exposure. The expressed proteins are involved in the activation of antioxidant activity; the increasing antioxidant proteins in HT-22 cells could compensate mixtures induced stress. In contrast to single metals, mixtures significantly upregulates the antioxidant and detoxification enzyme, which may explain that the metals combination exposure show higher potency than single exposure. The obtained results showed that expression/ fold change of antioxidant elements depends on the combination of the mixture. The present results suggest that exposure to M2 mixture could considerably alter the normal pattern of antioxidant elements expression compared to the other two mixtures.

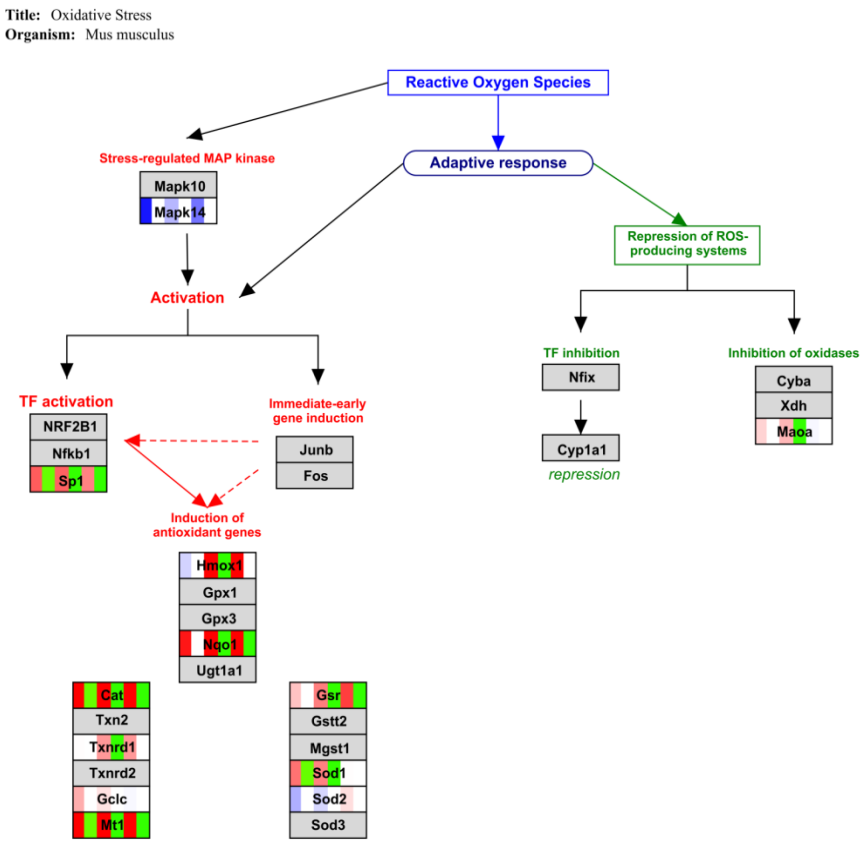


Figure 6: Changes in oxidative stress in response to M1, M2, and M3 in HT-22 cells. In the pathway changes in protein expression are visualized as log2foldchange comparing treatment with control expression. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) *and p values* < 0.05 are coloured in green and *p values* ≥ 0.05. Gray indicates that the selection criteria were not met, but the protein is measured.

3.4.3. Impacts of M1, M2, and M3 on Ubiquitin proteasome System (UPS)

The UPS is a highly conserved cellular pathway that plays an important role in the selective degradation of cellular proteins that are essential for the regulation of a variety of vital cellular functions. The UPS function is to eliminate the short-lived, misfolded, mutant and damaged proteins in eukaryotic cells. Generally, structural and functional deficits in the 26/20S proteasome can leads to neurodegeneration. Disruption of this system can have significant downstream effects on critical cellular functions. As shown

in Figure 7, M1, M2, and M3 mixtures showed a nonspecific proteasome alterations of proteasomes subunits of the 20S catalytic core and the 26S (PA700) regulatory complex. The expression pattern of UCHL3 (Ubiquitin Carboxyl-Terminal Esterase L3) was upregulated in the three mixtures exposure. UCHL3 is a deubiquitinating enzyme that is involved in the pathogenesis of neurodegenerative diseases. UBA1 is a key regulator of protein homeostasis, and the expression of UBA1 is essential for cell survival. Our results showed that M1 and M2 mixtures upregulated the UBA1 (Ubiquitin-like modifier activating enzyme 1) expression in the HT-22 cells, UBA1 is an essential for cell survival. Additionally, we observed downregulation of the NEDD4 (ubiquitin E3 ligase/ parkin) enzyme in the three mixtures. This suggests that mixtures treatment has a higher number of protein targets than single metal exposure in HT-22 cells. NEDD4 plays a critical role in ubiquitination; this enzyme enhances the cell survivability in HT-22 cells. Our results showed that the expression was down regulated, which crucial for damaging the UPS function. Other proteins in PD pathway (Figure 10) such as SEPT-5, ATAXIN -2 interact with NEDD4 function, and play a significant role in neurodegeneration (both proteins are up regulated in PD pathway). In the structural unit of UPS, lot of proteins dysregulation in 26 subunit, and PSMC5 protein has a critical role in the protein degradation process; the expression was up regulated in HT-22 cells in mixtures exposure. Additionally UBE2L3 (Ubiquitin-conjugating enzyme E2 L3) enzyme expression was found only in mixtures, which is also involved the UPS function.

Over view of the UPS pathway disruption shows that most of the proteins expression was similar in the all three mixtures. Over all in the UPS pathway, M1, M2, and M3 exposure in HT-22 cells causes significant alteration of UPS pathway proteins, including the proteasome 26S, 20S catalytic core unit and the ubiquitin enzyme system (UBA1,NEDD4,UCHL3) that are highly essential for protein quality control process in normal cell function. Results from this omics data strongly support that the disruption of the UPS proteins are similar to single metals, however the expression is higher in the mixtures exposure due to interaction.

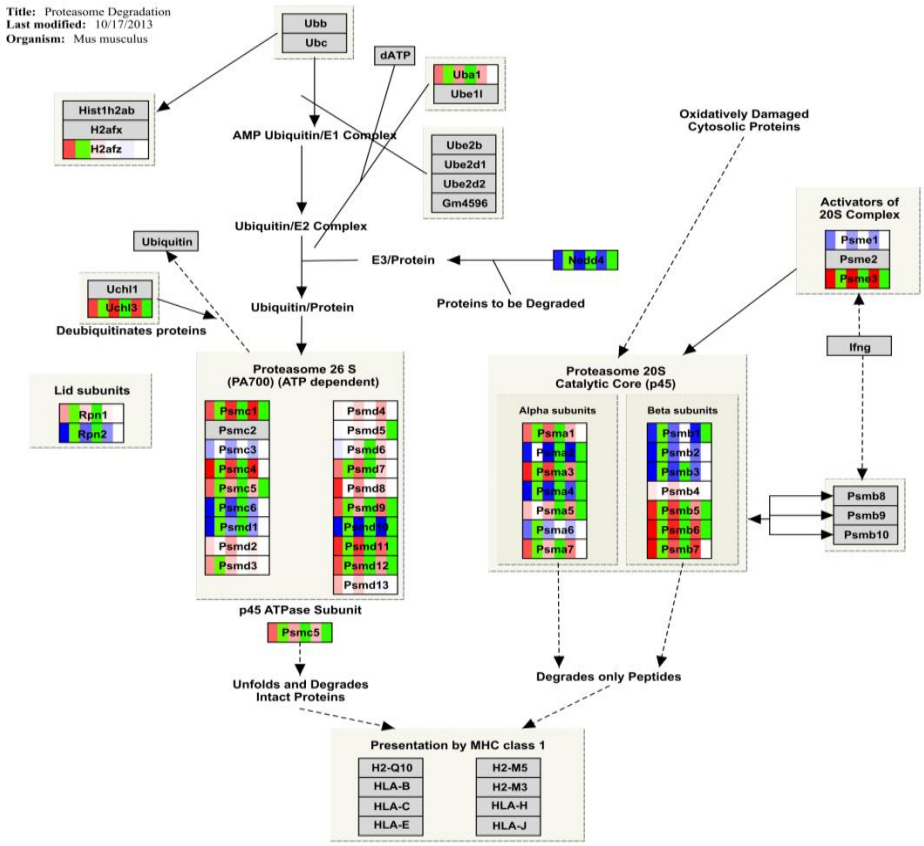


Figure 7: Changes in Ubiquitin proteasome system (UPS) in response to M1, M2, and M3 in HT-22 cells. In the pathway changes in protein expression are visualized as log2foldchange comparing treatment with control expression. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p values* < 0.05 are coloured in green and *p values* ≥ 0.05. Gray indicates that the selection criteria were not met, but the protein is measured.

3.3.4. Impacts of M1, M2, and M3 on m-RNA splicing

Splicing process in cells can be used to produce correct proteins through a translation mechanism. This mechanism plays a critical role in controlling cellular response to external stress. Major splicing is catalysed by the spliceosome, a complex of five small nuclear ribonucleoprotein particles (snRNP: U1, U2, U4, U5, and U6) and numerous protein factors (Figure 8). Dysregulation in alternative splicing has been linked to a number of human diseases including neurodegenerative diseases. As shown in the

Figure 8, U1 and U2 snRNP functional units expression are altered similar to single metals (draft Karri et al., 2018) that indicates the functional targets of single and its mixtures are similar in the U1and U2 units. We found that U1snrp70K (U1 small nuclear ribonucleoprotein 70 kDa) protein expression was upregulated in M1 and M2 exposure; however. M3 mixture has no significant effect. This protein has critical role in the neurotoxicity. The U1snrp70K protein expression supports the mixtures interaction in in m-RNA splicing process, because of Pb, MeHg single metal exposure has no response. The other three proteins (U4, U5, and U6) expression was down regulated in HT-22 cells in single metals; but in mixtures the expression was not quantified.

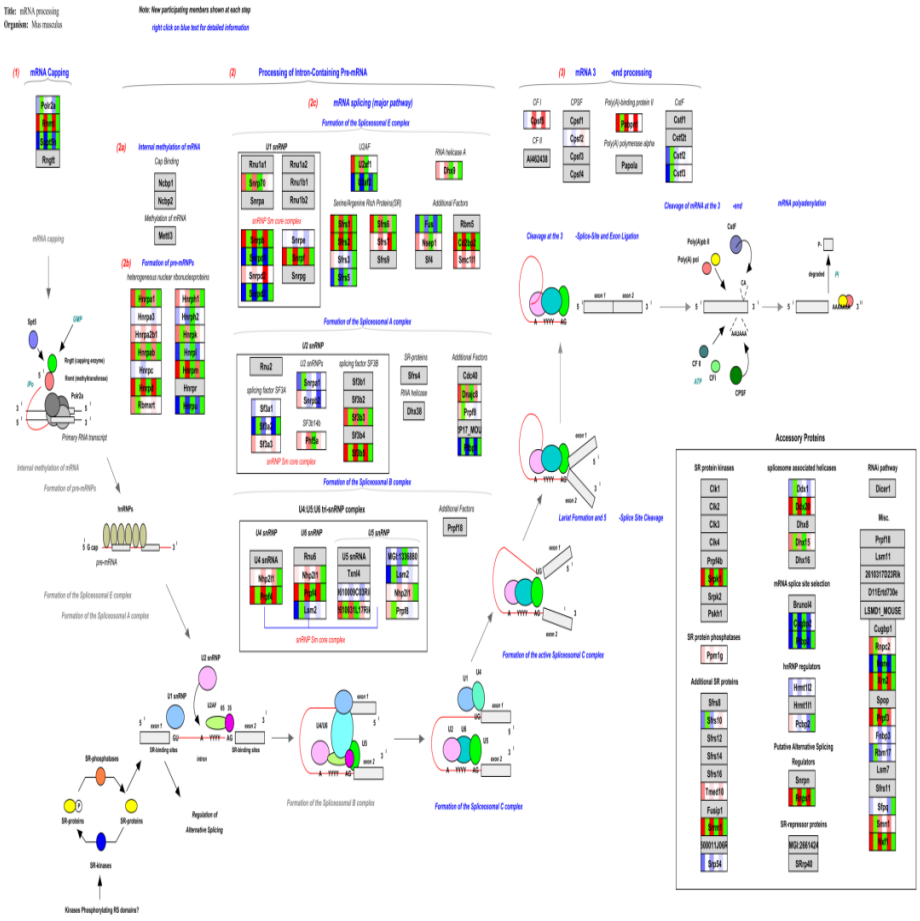


Figure 8: Changes in m-RNA splicing in response to M1, M2 and M3 in HT-22 cells. In the pathway changes in protein expression are visualized as log2foldchange comparing treatment with control expression. In addition, the significance level of these

changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p values* < 0.05 are coloured in green and *p values* ≥ 0.05. Gray indicates that the selection criteria were not met, but the protein is measured.

3.3.5. Impacts of M1, M2, and M3 on AD pathway

In this study, we identified several proteins that showed a differential expression that relates to AD in direct pathway. Also these AD related proteins expressed in other pathways allow to organelles, for instance ETC dysfunction, oxidative stress, UPS and, m-RNA splicing (discussed in 3.3.1, 2, 3, 4 sections). These proteins can combine together on their functional basis and visualized and have a direct or indirect relation to AD. It is conceivable that the observed alterations in the protein levels in HT-22 cells could be related to AD such as APP (amyloid precursor protein). The pattern of APP expression alerted by the three mixtures in the AD was similar to that of As metal (upregulated), that is entirely opposite to MeHg and Pb metal (Figure 9). The APP expression showing evidence mixtures interaction profile is different from their individual combination. Additionally, we observed significant changes in CAPN1 (calcium-dependent, non-lysosomal cysteine proteases); capains have been implicated in neuronal cell death. The CAPN1 expression is upregulated in the three mixtures, which is suggesting metal mixture interaction in HT-22 cells. For instance in single metals (As, MeHg) the CAPN1 expression was downregulated, while in the mixtures the expression was upregulated. The other protein, CAPN2 has no significant expression in mixtures, however in single metals CAPN2 was upregulated (Draft Karri et al., 2018). Also we found that CASP-3 expression was upregulated in all three mixtures similar to both As, and MeHg. However, the CASP-3 expression (fold change) value was higher than single metal exposure. All findings together related to AD, showed that APP expression was up regulated in all mixtures. This APP dysregulation can be possible due to mitochondrial damage, oxidative stress, and m-RNA splicing.. Evidences of abnormal mitochondrial ETC protein expressions from our studies suggest that APP may affect mitochondrial function, which may generate ROS results HT-22 cells death. However, the precise mechanistic link between mitochondrial oxidative damage and abnormal APP processing has not been elucidated.

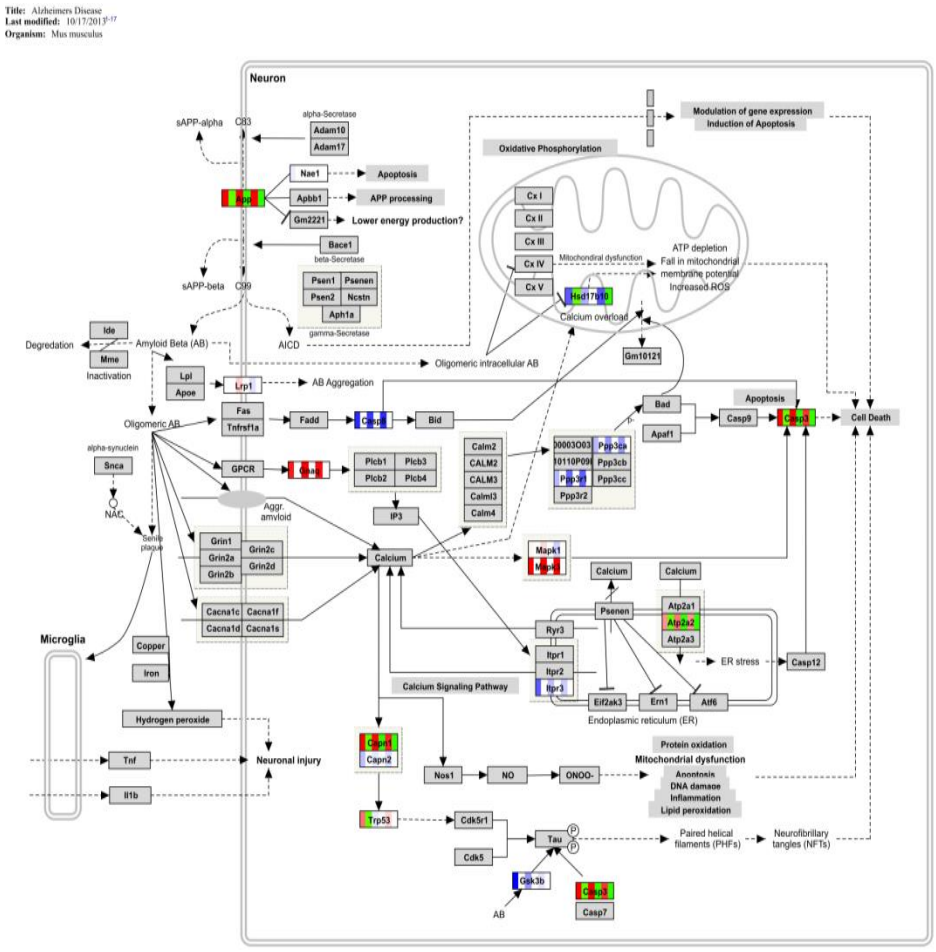


Figure 9: Changes in Alzheimer's disease in response to M1, M2, and M3 in HT-22 cells. In the pathway changes in protein expression are visualized as log2foldchange comparing treatment with control expression. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p values* < 0.05 are coloured in green and *p values* ≥ 0.05. Gray indicates that the selection criteria were not met, but the protein is measured.

3.3.5. Impacts of M1, M2, and M3 on PD pathway

In PD pathway, several proteins showed that relates to PD in HT-22 cells. These proteins were also showed in other pathways according to specificity of organelles, for instance ETC dysfunction, oxidative stress, UPS pathway and, m-RNA splicing. In the

present study PD pathway (Figure 10), it is plausible that the observed alterations in the protein levels in HT-22 cells could be related to PD such as PARK7 (Parkinson disease protein 7) and CASP-3 as a key regulators of proteins for cell homeostasis. Also, it was found that the expression was up regulated in HT-22 cells during the three individual metals treatment that indicates clearly HT-22 cells under stress condition. Other proteins such as UBE2L3 (Ubiquitin-conjugating enzyme E2 L3), SEPT-5 (Septin) and ATAXIN -2, protein expression was altered in PD pathway. SEPT-5 protein showed interaction with NEDD4 (E3 ligase) in PD. We can correlate the SEPT-4 downregulation in M1 can decrease the activity of NEDD4 expression in HT-22 cells. In the other two mixtures (M2 and M3), the process of NEDD4 and SEPT-4 protein interactions entirely opposite.. In parallel the PARK7 expression (protective protein) upregulated in the mitochondria due to mixtures stress in HT-22 cells. PARK7 is an anti-apoptotic agent, as it protects cells against oxidative stress and cell death. Mixtures exposure also influences the CASP-3 expression in mitochondria. The expression of PARK7, CASP-3 pattern supports that the three mixtures role in mitochondrial function is more relevant to single metal exposure, however the fold change/expression will not be similar. The key findings from the present study are NEDD4, UBA1 with SEPT-5 protein interaction; however, these processes are entirely different in single metal exposure (draft Karri., 2018). Over all in PD pathway,M1 mixture significantly upregulates the UBA1, and down regulates the NEDD4, SEPT-5 expression in the HT-22 cells. The other two mixtures (M2 and M3) showed the UBA1 and NEDD4 expression similar to M1 but the SEPT-5 expression was not significant.

The outcomes of PD are likely to be multifactorial; ETC dysfunction, oxidative stress and the impairment of UPS dependent protein degradation processing have been identified as the main toxicity associated with mixtures. Our findings confirm that metal mixtures also disrupt the ETC pathway in HT-22 cell, similar to single metal (As and MeHg) exposure. Our findings are important related to PD because the protein changes are significant at their nontoxic dose level. Also interestingly the combination of Pb with other metals has some significant response.

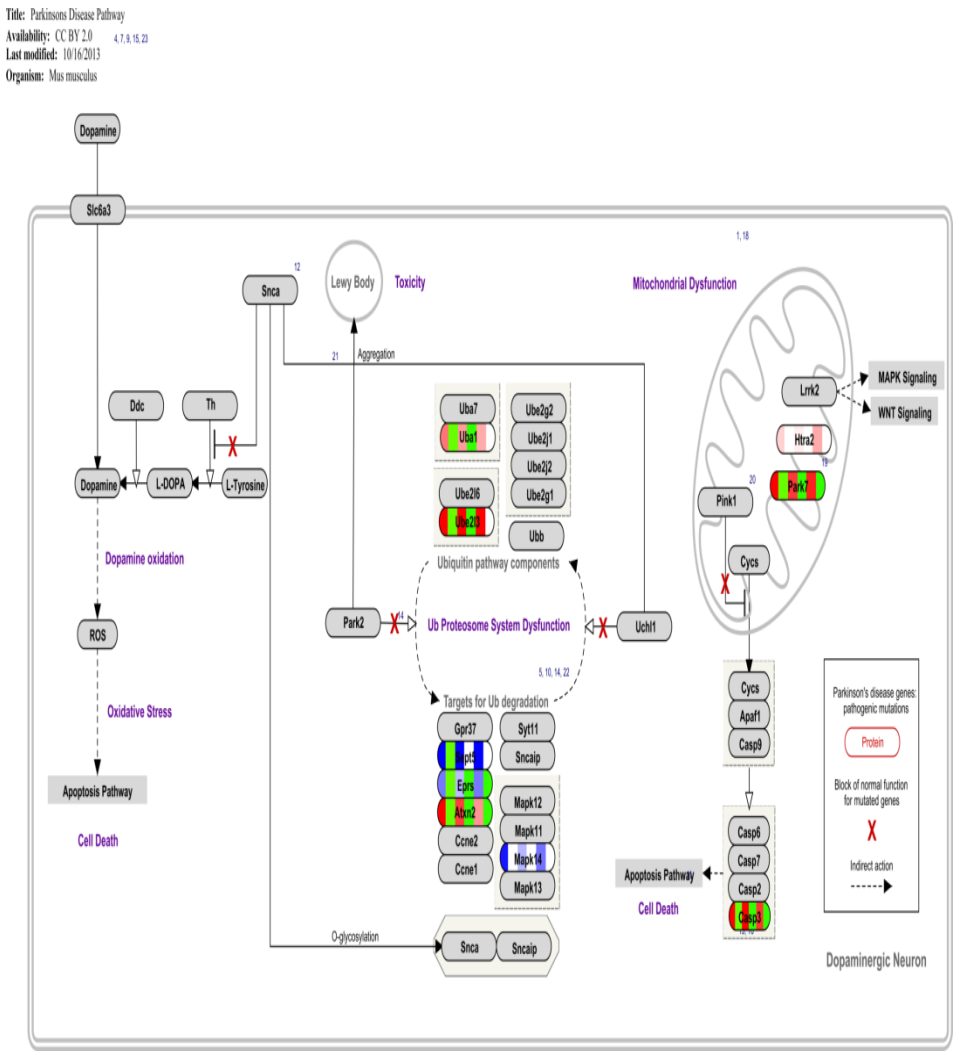


Figure 9: Changes in Parkinson's disease in response to M1, M2, and M3 in HT-22 cells. In the pathway changes in protein expression are visualized as log2foldchange comparing treatment with control expression. In addition, the significance level of these changes is indicated with p values. Red indicates a higher level of expression in the treatment. The changes in protein expression are coloured as a gradient going from blue (=downregulated) over white (unchanged) to red (=upregulated) and *p values* < *0.05* are coloured in green and *p values* ≥ *0.05*. Gray indicates that the selection criteria were not met, but the protein is measured.

Chapter 5

4. Discussion

Humans are routinely exposed to multiple chemicals/metals simultaneously or sequentially. There is evidence that the toxicity of individual chemicals may depend on the presence of other chemicals. (Rice et al., 2008). In the present study Pb, As, and MeHg binary mixtures were used to characterize the neurotoxicity interactions on protein and cellular toxic responses and the biological mechanisms behind the interactions on HT-22 hippocampal cells. The three metals mixtures were selected because of their molecular mechanism and the high possibility of co-exposure to human beings (Karri et al., 2016, 2018). The experimental design integrates the in vitro and omics methodology, a new alternative method to capture the complexity of mixtures interactions in protein level. One of the major potential advantages of proteomics technologies is that it will enable researchers to look at the complete complement of genes/protein in their expression and regulation. Recently, several system biology databases are available to perform more integrated analyses of biological functions and their changes as a result of chemical exposure (Løkke et al., 2013). Thus, incorporating systems biology information to obtained proteomics data can useful for the visualizing the proteins expression more relevant to biology (Weis, 2005).

The omics analysis could be helpful for understanding the complete over view of metal mixtures neurotoxicity in the hippocampal cells and also validate the interaction of metals in HT-22 cells by using the proteins expression. In single metal proteomics analysis we found that the disruption of m-RNA splicing, UPS, oxidative stress, and ETC dysfunction have a crucial role in metal-induced neurodegeneration disease (draft Karri et al., 2018). The involvement of the m-RNA splicing, UPS pathway might be novel in hippocampal based studies. So that, improving the mixtures mechanism in molecular level as same like single metals, we chose sub lethal toxic concentrations (equivalent to single metal IC_{10}) of the each metal on HT-22 cells during 8 days exposure.. If the metal concentration were higher, the secondary effects could mask the primary response (Zhang et al., 2005). In the present study,, we used GO pathway analysis, to analyse the proteins sets that are identified in label free omics experiments. As a result, we were able to gather comprehensive pictures of pathways and GO networks affected by the mixtures and also we compared critical protein changes with those induced by single metals (Draft Karri et al., 2018). The obtained omics data analysed with GO, network -and pathway tools to find the relationship between the proteins and neurotoxicity. This GO functional analysis could be useful to see each protein expression with significant change to connect the neurotoxicity GO terms. From the results, the three mixtures impacted different GO terms in HT-22 cells, and few of the over representation processes reflected the neurodegeneration related toxicity (Table 1) Further, the functional interpretation of omics data related to pathway visualization is essential to know that disrupted pathways by mixtures exposure. In this study, we identified significant protein expression changes across treatments within ETC

dysfunction, oxidative stress, UPS pathway, and m-RNA splicing related to AD, PD. . The interpretation of pathways are discussed below

4.1. ETC dysfunction pathway

Mitochondria have a central role in neurodegenerative diseases (Chaturvedi and Beal, 2013). It is known that the metal induced modification of ETC leading to depletion of ATP production (Atchison and Hare, 1994). We found the protein dysregulations in all complexes due to mixtures toxicity in HT-22 cells. The findings of mitochondrial ETC damage to M1, M2, and M3 exposure is in agreement to reported AD patients from post mortem hippocampus (Bosetti et al., 2002). The obtained mixtures results demonstrated a significant downregulation in complex I, II compared to single metals (Figure 5). Mixtures containing Pb (M1, M2) showed higher significant impact than single Pb exposure on ETC complex III, IV, V. The other metal mixture (M3) seems to be additive protein expression in complex III, IV, and V. The reduced activity of ETC can leads to neuronal cell death (Orth and Schapira, 2002). Our findings confirms that metals disrupt the ETC pathway in HT-22 cell, leading to energy deficiency that is a characteristic feature of both AD, PD brains (Beal et al., 1993). The current results in HT-22 cells can correlates to reported AD and PD (Dupuis, 2014). Previous studies from deficiency in mitochondrial electron transport chain NADH dehydrogenase (complex I) activity in PD (Lezi and Swerdlow, 2012) are in agreement with the HT-22 cells ETC dysfunction. Not only direct effects of ETC function but the other proteins can also impact the AD (Wang et al., 2010). These data strengthen the hypothesis that mixtures exposure may be associated with common binding affinity with ETC proteins (Rowley and Patel, 2013). The ETC dysfunction also possible with many other factors in neurodegeneration (Nakamura et al., 2011). For instance, ETC damage also causes most impressively direct interactions of disease related proteins APP, NEDD4, PARK7 discussed in the next sections (Dodson and Guo, 2007). The reduced expression of transcripts involved in mitochondrial respiration provided further evidence; mixtures can enter the hippocampal mitochondria and inhibit respiratory chain complexes resulting in the generation of ROS in the HT-22 cells.

4.2. Oxidative stress pathway

The disruption of ETC function can implicate various proteins in oxidative stress such as MT1, SOD, GSH, CAT, HMOX1, and NQO1 due to the energy depletion. However the other type of interconnected molecular mechanism might possibly cause PD, AD(Ischiropoulos and Beckman, 2003). Mixtures showed a significant alteration in ROS mediated oxidative stress response. The induction of antioxidant and phase II detoxifying enzymes acts as an important defence mechanism against environmental stressors (Chang and Karin, 2001). HT-22 cells have well developed antioxidant systems that help to protect them from mixtures stress (Lu et al., 2011). We also found differences between single and mixtures in MAO-A expression. The response of MAO-A was not significant in mixtures (except M2). This indicates that Pb and As interactions keep up the upregulation of MAO-A expression that enhance the

monoamines metabolism in neurons (Yadav et al., 2010). MAO-A elevated in AD brain is subjected to increased oxidative stress (Butterfield et al., 1999). As shown in Figure 6, M1, M2, and M3 in HT-22 cell line induced significant effects in MAP kinases mediated oxidative stress response such as HMOX1, NQO1, GSR, SOD1, CAT, TXNRD1, and MT1. The increase of MT1 protein levels is often used as a biomarker of metal exposure. However, the magnitude of the response varies depending on exposure of mixture and combination (Papouli et al., 2002). CAT is an important enzyme of the anti-oxidative element, which can modulate mixtures toxicity by metabolizing the free radical hydrogen peroxide. Also GSR is another key antioxidant that was measured in our study. Comparatively the GSR, CAT expression was higher in mixtures exposure than in single metals, that suggests the oxidative stress initiation was high in the mixture exposure (Jadhav et al., 2007b). The fold change in the antioxidant proteins in HT-22 cells was high than in single metals. Oxidative stress has been implicated in a wide variety of biological reactions such as cell death in brain (Krantic et al., 2005). Several pieces of evidence suggest that oxidative stress role in neurodegenerative disease is broadly recognized (Canet-Aviles et al., 2004).

4.3. UPS pathway

UPS has received particular attention in the study of neurodegenerative disorders due to its role as a critical regulator of protein homeostasis in neurons (Deng et al., 2017). In addition to selective damage in ETC, oxidative stress in HT-22 cells, we observed impairment in UPS function, which may also contribute to neurodegeneration processes. UPS keeps the cellular environment free of misfolded, defective proteins (Lim and Tan, 2007). Association of several proteins involved in the UPS degradation pathway have critical role in the PD. For instance, decreased abundance of several proteins involved in mitochondria complex -I function, and elevated oxidative stress revealed that UPS pathway has link to PD (Valente et al., 2004). These observations clearly suggest that UPS mediated proteolysis is essential for maintaining normal mitochondria function in brain (Palacino et al., 2004). In support of this view, we found that three mixtures significantly downregulates the NEDD4 protein, which is a critical element in protein clearance mechanism (Giasson and Lee, 2001). Also, the reduction in proteasome activity in this region may be attributed to the profound loss of 20S proteasome core components, and proteasome activators PA700 and PA28 due to mixtures exposure. Other proteins such as SEPT-5 interact with NEDD4 and ATAXIN -2 playing key role in neurodegeneration. Expression of UBA1 is essential for cell survival (Joazeiro and Hunter, 2000). Our results suggest that M1, M2 mixtures upregulates the UBA1 expression in the HT-22 cells. Additionally, UBE2L3 enzyme expression was showed only in mixtures exposure. Several recent studies have identified critical role of the UPS in the metal induced toxicity (Stanhill et al., 2006; Stewart et al., 2003). Mixtures effect on NEDD4, suggests that the cellular accumulation of certain proteins can causes neurodegeneration (Furuchi et al., 2002; Hwang et al., 2002). Very little attention has

been given to the degradative and catabolic pathways (Dawson and Valina, 2003). In UPS pathway mixtures showed higher significant effect than their individual exposure.

4.4. m- RNA splicing

Alternative splicing occupies a central position in the development and functions of the nervous system (Daguenet et al., 2015). This mechanism of gene product diversification plays a critical role in controlling cellular response to external stress (Kelemen et al., 2013). It is estimated that more than 75% of genes in the human genome are alternatively spliced (Johnson, 2003). Metal mixtures interaction evidence was showed in Snrp70 protein. We found that M1, M2 significantly upregulated the Snrp70, like As exposure. This protein showed a critical role in the APP protein metabolism (Nuzzo et al., 2017). Thus, M1and M2 mixtures have significant role in the AD. Regarding spliceosome unit, U1snRNP function was altered similarly to single metals, The alterations of U1snRNP results are in agreement with discovered U1 snRNP dysfunction, and a role for U1snrp70K in AD (Bai et al., 2013). Comparatively single metal showed the the high number of altered genes in U2snRNP unit than the mixtures in HT-22 cells. . Reported study denote that a mutation in U2 snRNP genes can causes neurodegeneration (Jia et al., 2012). The other tri protein (U4, U5, and U6) system function was poorly affected in mixture exposure which indicates the mechanism of splicing processes is completely different with single metal exposure. The abnormal changes in SR proteins have roles in regulating the alternative splicing of the Tau gene (Qian and Liu, 2014). In mixtures the SR proteins are poorly expressed. These observations imply that mixtures induced alterations in m-RNA splicing process was more poor than their individual exposure and consequently pre-mRNA splicing can contribute to neurodegenerative disorders (Dredge et al., 2001).

4.5. AD pathway

In the present study, it is conceivable that the observed alterations in the protein levels in HT-22 cells could be related to AD such as APP (Zhang et al., 2011). Our findings was confirmed with a previously described APP expression in hippocampal and cortical area of brain (Ashok et al., 2015). The three mixtures, Pb, As individually could induce APP expression, the effect being most prominent in M1 that suggests the synergistic interaction in APP expression. We observed higher significant changes in CAPN1 than single metals. CAPNs has been implicated in neuronal cell death in hippocampus (Grammer et al., 2005). We found that expression of CASP-3 was higher in the single metals As and MeHg than in mixtures. However, exactly how the activation of CASP-3, the enhanced AD will be important mechanism (Kim, 1997), accumulation of activated CASP-3 amyloid plaques have relation that found in the mice (Yang et al., 2008).

All results together relate to AD; we found that APP upregulation can possible due to mitochondrial damage, oxidative stress, and m-RNA splicing. The pathvisio and GO analysis outcomes provide critical information; for instance, the impairment of mitochondrial function in AD patients has been well known (Hirai et al., 2001). In

addition, several in vitro studies of APP and mitochondrial function have reported that APP expression affects the mitochondrial DNA and proteins, leading to impairments of the ETC (Manczak et al., 2006; Reddy, 2009). Evidences of abnormal mitochondrial ETC gene expressions from our studies suggests that APP expression may affect the mitochondrial function (Caspersen, 2005; Gibson et al., 1998). However, the precise mechanistic link between mitochondrial oxidative damage and abnormal APP processing has not been elucidated. These findings support that APP derivatives enter the mitochondria in early stages of disease progression and induce free radicals, leading to mitochondrial oxidative damage (Onyango et al., 2016). The mechanistic link between abnormal mitochondrial gene expression and oxidative damage in AD progression by heavy metals and its mixtures exposure need to be improved. Overall in the AD pathway, single metals and its mixtures showed some significant difference in few proteins expression (APP, CASP3, and CAPN1).

4.6. PD pathway

In the present study PD related proteins also showed in other pathways according to specificity of organelles, for instance ETC dysfunction, oxidative stress and UPS pathway. Mechanistically, mixtures tend to target mitochondria especially at the level of complex I of the ETC (Jackson-lewis et al., 2012). The ETC damage induces the oxidative stress that has link to increases PARK7 protein in mixtures, associated with an early-onset form of parkinsonism in human patients (Kahle et al., 2009). It is important to note that mitochondrial dysfunction and oxidative stress also affect parkin function and exacerbate the expression level in mitochondria (Chung et al., 2003). We found that the heavy metal induced oxidative stress, mitochondrial dysfunction which is directly related to PD (Kalinderi et al., 2016; Scott et al., 2017). PD pathway (Figure 9), showed alterations in the protein levels in HT-22 cells, which could be related to PD such as PARK7, CASP-3 as key regulators of proteins for cell homeostasis. A number of studies have shown that PARK7 is induced during oxidative stress and acts against cytotoxicity (Björkblom et al., 2013). PARK7 neuroprotective role has been extensively observed in a variety of oxidative stress conditions (Charan et al., 2014). Other proteins such as UBE2L3, ATAXIN -2 and SEPT-5 protein expression were down regulated in mixtures exposure more than in single metals. The down regulation (fold change) value was higher than in single metals. The accumulation of parkin specific substrates, as a consequence of the loss of NEDD4 activity might underlie the damage of dopaminergic neurons (Dawson and Valina, 2003). These findings indicated that the cell fighting against the metal mixtures induced toxicity was poorer than in single metal exposure. These all proteins are combined together to elaborate the direct or indirect relation to PD (Dawson and Dawson, 2003). ETC dysfunction, oxidative stress and the impairment of UPS dependent protein degradation processing have been identified as the main factors inPD (Moore et al., 2003) as showed in the single metal. Our findings are in agreement with previously reported studies (Tan et al., 2005). Our results also support that all three mixtures treatments influence the ETC, oxidative stress, UPS pathway significantly,

which are more relevant to PD pathway (van der Merwe et al., 2015). However the expression pattern of individual proteins in each pathway has unique nature (fold change) in single metals and mixtures (Table 3), it doesn't match exactly the cell viability quantification (interaction profile).

Table 3. Here presented major critical proteins related PD, AD with respect to fold change (Log (2) FC ≥0.26 or ≤0.26 and p-value≤ 0.05).

Expressed protein	Description	Pb	As	MeHg	M1	M2	M3
APP	Amyloid precursor protein	0.75	1.46	-0.85	2.5	1.8	1.29
CAPN1	calcium-dependent, non-lysosomal cysteine proteases	0.8	-4.3	-0.5	1.3	0.9	0.8
CASP3		0.6	1.36	1.46	1.23	1.05	0.98
NEED4	A ubiquitin E3 ligase	-	0.5	-2.54	-1.72	-1.86	-2.9
PARK7	Parkinson 7 protein	-	0.7	1.6	1.18	0.77	0.87
UBA1	Ubiquitin-like modifier activating enzyme 1	0.64	1.3	-	0.53	0.38	-
UB2L3	Ubiquitin-conjugating enzyme E2 L3	-	-	-	2.4	1.5	-
UCHL3	Ubiquitin Carboxyl-Terminal Esterase L3	-	1.03	-	0.75	0.87	0.76
SEPT5	septin	-1.3	-0.86	1.2	-1.9	-1.7	-1.5
ATAXN2	Neurodegenerative ataxn2 protein	0.37	-	3.0	1.06	0.75	0.41
U1 Snrp70K	U1 small nuclear ribonucleoprotein 70 kDa	-	0.64	-	0.61	0.54	-

CAT	Catalase	0.67	1.2	-0.78	1.6	1.33	1.08
MTT1	Metallothionein-1	1.5	-2.2	-1.61	2.96	2.25	1.29
GSR	Glutathione disulfide reductase	0.3	0.5	0.6	0.23	0.50	0.75
SOD1	Superoxide dismutase1	-	-0.7	-	0.55	0.49	-
SOD2	Superoxide dismutase2	-	0.4	0.67	-	-	-
MAO-A	monoamine oxidase A	0.39	-2.0	1.16	-	0.27	-
COX2	ETC complex -IV	-	-	-1	-1.09	-0.8	-1.26
COX4	ETC complex-IV	-	-2.2	0.98	-0.8	-0.6	-0.86

5. Conclusion

Research is needed to develop the toxicity of complex mixtures risk assessment in humans. The most difficult issues for testing the risk of the chemical mixtures are risk method, dose, and composition. Thus, risk assessors need a strong scientific support for chemical mixtures risk assessment in the form of toxicological data. Here we provide a comprehensive picture of how metal mixtures mediate protein and pathway changes and affect the hippocampal cell proteome and thereby cause the selective neurodegeneration. Additionally, we have demonstrated the systematic comparison between the single metals and mixtures on critical proteins changes during the chronic exposure. The current proteomic approach to be a unique and rapid way to distinguish single and mixtures effect on hippocampal cells, and also predicted the biomarkers for mixtures on the hippocampal cells. The multi-faceted comparison of the cellular responses to M1 (Pb+MeHg), M2 (Pb+As), and M3 (MeHg+As) allowed us to get better insights into the responses of the interaction profile in protein level. In our results, M1 induce detectable perturbation of the cellular functions than other two mixtures M2, M3. We confirm that by analysing complete cellular proteome, associated with a number of biochemical abnormalities suggested that mixtures exposure is more hazardous than single metal on hippocampal cells.

The identified proteins could serve as potential biomarkers and in the future included in biomonitoring of people exposed to heavy metals. Further studies are required to prove the results obtained so far and also to increase the panel of potential protein markers. While still large gaps in the knowledge of biological pathways exist, each new study will contribute to build a base of knowledge necessary for these types of analyses. Our study contributes to the scientific literature by providing new evidence of metal mixtures effect on hippocampal cells. Laboratory studies are needed to provide toxicological evaluations of real world mixtures at environmentally relevant

concentrations. This study raises hope for that how the components interaction in the mixtures exposure, which can in turn to new from to the single chemical exposure. Refinement is needed in high throughput omics research to validate quantitative risk assessment and to support the assumptions; these may include testing for some specific proteins dose–response curves, determining whether additivity assumptions are applicable or not for describing mixture risk.

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General Discussion

General Discussions

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In recent years, environmental exposures to hazardous metals, such as Pb, Cd, As, and MeHg are shown significant toxicological concerns in the brain (hippocampus). More than 95% of toxicological research studies are focused on single metals and almost completely neglect mixtures. One of the major challenges for metals toxicology is to find how mixtures exert their effects on living organisms. The regulatory frameworks such as REACH in the EU are becoming more critical regarding the use of animal testing. Given the complexity of mixtures studies, it is difficult to use the animal models for toxicity evaluation of all possible combinations of heavy metals. Recent advancement *in vitro* assays, systems biology; with an appropriate target cell line, may allow an accurate understanding of metal mixtures toxicity mechanism. This approach can integrate the conventional in vitro assays, proteomics findings to bioinformatics analysis, for improving the toxicity mechanism. This information will contribute to the development of alternative for mixtures evaluation. Here, mice HT-22 cell lines are used as hippocampal relevant cells. The main goal of the thesis was taking advantage of in vitro, label free proteomic technologies and systems biology for assessing comparative toxicity signature profiling of heavy metals (Pb, Cd, As, and MeHg) and their binary mixtures (Pb+MeHg, Pb+As, and MeHg+As) on the mice HT-22 hippocampal cell line. Identification of proteins whose expression is specifically modified by toxic metal exposure would provide a better understanding of their mechanisms of action and allow development of sensitive and specific biomarker.

In the process of metal mixtures toxicity in hippocampal based advanced *in vitro* experiment, first we develop the state of the art from the extensive individual metals neurotoxicity mechanism from the available literature in chapter 1, especially related cognitive dysfunction, aims to review the current understanding of mode of action for several metals (Pb, Cd, As and MeHg) in hippocampus region of brain. The findings strengthen the effects of Pb, Cd, As, and MeHg metals in hippocampus. Then exact toxicological mechanisms invoked by exposure to metal mixtures were proposed. It is found that each metal share common cellular elements such as receptors, enzymes, neurotransmitters for causing the cognitive dysfunction. Using this hypothesis, we present common mode of actions of Pb, Cd, As, and MeHg in the hippocampus region of brain. The functional interaction of the four metals seems to cause common adverse outcome in brain. Further, we hypothesize that concurrent exposure may cause the greater than additive responses in brain. Therefore, common susceptibility factor of these four metals causes the imbalance between the defensive elements and reactive oxygen species results cell death. In the final part of the review an integrated framework is proposed for studying the metal mixture mode of toxicity in brain.

In a chapter 2, for elaborating the hippocampal toxicity hypothesis, initially we performed cell viability via MTT assay of Pb, Cd, As, and MeHg in mice HT-22

hippocampal cell line to different exposure scenarios such as acute (1 day), subchronic (3 days) and chronic (8 days). Further, we extended to genotoxicity and apoptosis studies by using low toxic concentrations. These two assays were helpful for elucidating genotoxicity and apoptosis mechanism of heavy metals in HT-22 cells during chronic exposure. The obtained MTT assay results in the HT-22 cells suggest the potency of Pb depends on the exposure duration. The toxicity of Pb is entirely different from other metals and unique in dose response curve shape and IC_{50} in HT-22 cells. We observed that As, MeHg exposure on cells showed in high response. Further studies are needed to establish the genotoxic, apoptosis potency in HT-22 cells. Thus, we showed that each metal has dose dependent manner of DNA damage, apoptosis; MeHg>As>Cd>Pb as similar to MTT assay.

Based on the chronic cytotoxicity of individual metals Pb, Cd, As, and MeHg on HT-22 cells, In chapter 3, we extended the binary mixtures interactions study by using the response addition and dose addition. There is no generally accepted agreement as to which of the two concepts is more appropriate therefore we have attempted to carry out this study using both models. The response addition findings mainly indicate synergistic effect among MeHg and Pb, Cd in high concentration, The other three combinations Pb+Cd, Pb+As, and MeHg+As indicates the simple additive effect, Cd+As showed antagonist effect on HT-22 cells. The other method isobologram analysis, the metal mixture impact was different from the equitoxic response exposure. The combination of Cd with Pb, As, and MeHg indicated the antagonist effect. Overall these binary combination of Pb, Cd, As, and MeHg displayed synergistic and antagonistic interaction at low and higher effect levels in HT-22 cells, respectively. The observed interactions in dose additivity, response additivity showed a trend of additivity, synergism and antagonism with increasing metal mixture concentrations. Moreover, improved investigations on metal combined effects are needed in future studies.

For the proteomics analyses (Chapter 4), sub-lethal toxic concentrations (IC_{10}) of Pb, As, and MeHg applied on HT-22 cells during 8 days exposure. The concentrations were selected based on our cytotoxicity, genotoxicity, and apoptosis observations. The omics analysis reflects the critical changes of the molecular mechanism in a protein level. A total of 547, 1249 and 1156 proteins were found to respond to Pb, As, and MeHg exposure respectively. Among them, 347, 563, 632 proteins were significantly down regulated, while 200, 686, 524 proteins were significantly upregulated by Pb, As, and MeHg respectively. For elaborating the molecular mechanisms; proteomics data was systematically analyzed by using the bioinformatics tools such as gene ontology (GO) and pathway mapping. We quantitatively assessed the protein expression associated with enriched GO terms and neurotoxicity. We found the MeHg effect was higher than other two metals (Pb, As). Also this single metal protein data gives immense scope to measure the protein expressions in critical pathways, altered proteins annotated in mRNA regulation/splicing, ubiquitin proteasome system (UPS), oxidative stress, and electron transport chain (ETC) dysfunction, relates to Alzheimer's disease (AD),

Parkinson's disease (PD). The disrupted pathways discussed here, firstly ETC dysfunction in complex I-IV that may causes the energy deficiency in HT-22 cells. In oxidative stress mono amino oxidase –A (MAO-A) protein expression considered as neurochemical biomarker for Pb, As, and MeHg in HT-22 cells, and antioxidant protein expression indicating the HT-22 cells stress condition. The present results suggest that exposure to MeHg could considerably alter the normal pattern of antioxidant elements expression. The disruption of ETC function can also implicate various proteins in oxidative stress such as MT1, SOD, GSH, CAT, HMOX1, and NQ01.

In addition to selective damage of ETC and oxidative stress in HT-22 cells, impairment in the UPS also causes the neurodegeneration. The pattern of protein expressions in UPS pathway in HT-22 cells is potency dependent. For instance, UCHL3 (Ubiquitin Carboxyl-Terminal Esterase L3) expressions was upregulated in As exposure, although the other two metals (Pb, MeHg) no effect. Also in UPS pathway three metals upregulates the UBA1 expression; additionally in MeHg downregulate the NEDD4 (ubiquitin E3 ligase), this indicates that MeHg treatment has a significant impact on a number of protein targets in UPS pathway than other two metals. Overall the UPS pathway disruption shows that most of the protein expression was similar for all three metals (except NEDD4 expression) that relates to PD. In the m-RNA splicing here, addressed the impact of the three metals on m- RNA splicing process. We identified several components of the small nuclear ribonucleoprotein (U1 snRNP, U2 snRNP, U4snRNP, U5snRNP, and U6snRNP) alterations in HT-22 cells. Also U1snrp70K (U1 small nuclear ribonucleoprotein 70 kDa) protein expression was upregulated in As metal exposure; the other two metals have no significant effect. Taken together, these observations imply that alterations in snRNP accumulation and consequently pre-mRNA splicing can contribute the neurodegenerative process in HT-22 cells.

AD, PD is irreversible neurodegenerative diseases. In this study AD, PD related proteins also appeared in other pathways for instance, ETC dysfunction, oxidative stress, UPS pathway, and mRNA splicing. Although, some specify proteins are showed in AD, PD pathways. Our findings in Pb and As showed that an increased hippocampal APP (amyloid precursor protein) expression, also significant changes in CAPN1 and CAPN2 (calcium-dependent, non-lysosomal cysteine proteases). Calpains have been implicated in neuronal cell death. AD have relation with apoptosis mechanism, CASP3 protein upregulated in three metals. In PD pathway we observed that alterations in PARK7 (Parkinson protein 7), CYCS (cytochrome c, somatic), and CASP3. Also, other proteins, UBE2L3 (Ubiquitin-conjugating enzyme E2 L3), SEPT-5 (septin), and ATAXIN-2 (ataxin) proteins changed in HT-22 cells. Indeed, specifically quantified UBA1 (As), and NEDD4 (MeHg) proteins have relation to PD. The heavy metal induced AD, PD is likely to be multifactorial; ETC dysfunction, oxidative stress, and the impairment of UPS dependent protein degradation have been identified as the main processes associated with PD. Finally, PD, AD is associated with a number of proteins in HT-22

cells; in that the UPS, ETC, oxidative stress plays a major role. The expression pattern of proteins supports that the three metals potency in HT-22 cells Pb<As<MeHg.

There are obvious limitations to studying the joint effects of metal mixtures in molecular level. The potential use of omics technique and system biology approach can comprehensively improve the mixtures toxicity mechanism. Before designing the metal mixtures omics study, we performed individual metal (Pb, As, and MeHg) proteomics analysis and binary mixtures interactions by using the response addition and dose addition. Hence, we decided to extend the omics studies to assess the interaction profile in molecular level in Chapter 5. Here, the three metals mixtures (Pb+MeHg (M1), Pb+As (M2), and MeHg+As (M3)) were selected because of their neurotoxicity and the high possibility of co-exposure in human beings.

Single metal protein data gives immense scope to measure the protein expression levels in critical pathways treated with Pb+MeHg Pb+As, and MeHg+As mixtures during 8 days exposure. In metal mixtures, significant proteins were 1268, 1272 and 848 respectively for Pb+MeHg, Pb+As, and MeHg+As. Among them, 429 398, and 353, proteins were down regulated; remaining 839, 874, and 495 proteins were significantly up regulated. Functional classification and enrichment analysis of protein data by using GO analysis indicating MeHg+As mixture was very low impact while the Pb+MeHg, Pb+As have greater impact on HT-22 cells. In pathway analysis, metal mixtures also disrupts the ETC pathway, oxidative stress, m-RNA splicing, ubiquitin proteasome dysfunction, related to AD, PD. The mixtures effect in ETC pathway was more than their single metals. Also, in Pb containing Pb+MeHg, Pb+As have showed significant impact than single Pb exposure on HT-22 cells. The disruption of ETC function can implicate various other proteins such as MT1, SOD, GSH, CAT, HMOX1, and NQO1 due to oxidative stress. Comparatively oxidative stress effect more in mixtures exposure than single metals, which suggests the mixtures toxic potency, may be high due to interaction. The findings showed that expression of antioxidant proteins depends on the combination of the mixture; here M2 mixture could considerably alter the normal pattern of antioxidant elements expression compared to other two mixtures. Also the fold change in the antioxidant proteins in HT-22 cells was high than single metals.

Over view of the UPS pathway disruption shows that most of the proteins expression was similar in the all three mixtures. In support of this view, we found that all mixtures significantly downregulates the NEDD4 protein, which is a critical protein in PD. Other proteins such as SEPT-5 have the interaction with NEDD4 and ATAXIN -2 also play key role in neurodegeneration. Additionally, UBE2L3 enzyme expression showed only in mixtures exposure. In m-RNA splicing, metal mixtures evidence was showed in Snrp70 protein, M1, M2 significantly upregulated the Snrp70 similar to As exposure. This protein has a critical role in the APP protein metabolism. The other spliceosome (U1, U2,U4, U5, and U6) system function was poorly affected in mixtures than single metals that indicates the mechanism of splicing processes completely different with

single metal exposure. Taken together, these observations imply that mixtures induced alterations in m-RNA splicing process was poor than their individual exposure.

In AD pathway three mixtures induce APP expression, the effect being most prominent Pb+MeHg, which suggests that synergistic metal interaction in APP expression. We found that expression of CASP3 was high in single metals As, MeHg than mixtures in HT-22 cells. Overall in the AD pathway, single metals and its mixtures showed some significant difference in few proteins expression (APP, CASP3, and CAPN1). In PD pathway showed alterations could be related to PARK7, CASP-3 as key regulators of proteins for cell homeostasis. Other proteins such as UBE2L3, ATAXIN -2, SEPT-5 protein expression were down regulated in mixtures exposure than single metals. We observed downregulation of the NEDD4 (ubiquitin E3 ligase/ parkin) enzyme in three mixtures. This suggests that mixtures treatment has significant targets than single metal exposure in UPS pathway.

All results together relate to AD, PD; APP upregulation can possible in higher level in mixtures than single metals. The pattern of APP expression alerted by three mixtures in the AD was similar to that of As metal (upregulated), that is entirely opposite to MeHg. However, the precise mechanistic link between mitochondrial oxidative damage and abnormal APP processing has not been elucidated. The U1snrp70K protein expression clearly indicates the metal interaction in different in m-RNA splicing, because of Pb, MeHg single metal exposure no response. Here we found that as a single metal Pb has no effect in PD related proteins, when it is combination in As, MeHg binary mixtures, significant down regulation of NEED4 protein. Overall our results support that all three mixtures treatments influence the ETC, oxidative stress, UPS pathway significantly which are more relevant to AD, PD.

Conclusions

Conclusions

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General conclusion

In this project, a unique approach of using a single test system (HT-22 hippocampal cells) based bioassays and high throughput label free proteomics were used to evaluate the effect of single metals and their mixtures relevant to neurotoxicity. The individual and mixtures toxicity profile of heavy metals revealed that heavy metals cause the neurodegenerative diseases in hippocampal cells. There are many similarities in their mechanisms of action such as electron transport dysfunction (ETC), oxidative stress, mRNA splicing, Ubiquitin proteasome degradation (UPS) related to Alzheimer's disease (AD) and Parkinson's disease (PD). We confirm that by analysing complete cellular proteome and pathways, all of the evidence suggests that metal mixtures exposure is more hazardous than single metal exposure in HT-22 hippocampal cells. Hence, it is predictable to see that there is an interaction among these heavy metals, whether additivity applies to these mixtures across a wide range of exposure regimes remains to be seen. In future, refinement is needed in this area of research to validate quantitative risk assessment and to support the assumptions; these may include testing for some specific proteins dose–response curves, determining whether additivity assumptions are applicable or not for describing mixture risk.

Chapter 1

Firstly the neurotoxicity of Pb, Cd, As and MeHg metals on hippocampus region of brain was reported. The single metal literature findings were elaborated mainly from hippocampus-based toxicity on their functional affinity to proteins, enzymes, receptors, that support inflicting the cognitive dysfunction. The metal mixtures toxicity was well correlated by their individual mode of action. We concluded that alternative strategies are needed to assess the metal mixtures toxicity in brain (hippocampus). The above alternative novel methods should follow the 3R (Replacement, Reduction and Refinement) principal in the risk assessment of metal mixtures. *In vitro* and omics based methodologies can be applied to gain insight into the mixtures interaction at the protein level.

Chapter 2

The in vitro results for four heavy metals (Pb, Cd, As and MeHg) showed clear variation in acute and chronic exposure rather than subchronic exposure. The individual metals, (Pb, Cd, As, and MeHg) were toxic to HT-22 cells. Among these four metals, MeHg was the most potent, followed by As, Cd and Pb. The parameterization of the dose-response curves provided the proof of evidence supporting the heavy metals potency that is comparable among all exposure conditions.

Chapter 3

Based on the cytotoxicity, genotoxicity, and apoptosis results of single metals (chapter 2), binary mixtures cytotoxicity were evaluated by using dose addition, response addition methods. The study of mixtures of metals on HT22 cells indicated, mixtures potency and toxicity is different from their individual metals to hippocampus. Mixtures

toxicity interaction could be initiated by apoptosis or oxidative stress factors of the observed toxic effects. The toxicity of mixtures in HT-22 cells was the following: Pb+MeHg (synergistic), MeHg+As (additive), and Cd+As (antagonist). These effects were found in both, dose addition and response addition. The other three mixtures (Pb+Cd, Pb+As, and Cd+MeHg) interaction profiles were different in each method. We can conclude from the results that dose addition isobologram analysis is more beneficial strategy for mixtures study than response additivity.

These findings give significant evidence of the metal mixtures neurotoxic activity and their potential interactions depending on the composition of elements, and cell line sensitivity. To understand the role of oxidative stress in the toxicity and effects of metal mixtures, a mechanistic proteomics approach was applied to the defined binary mixtures.

Chapter 4

The individual heavy metals toxicity in HT-22 hippocampal cells (chapter 3) gave us strong support to elaborate a mechanistic omics study. The comparative proteomic analysis of the three neurotoxic metals (Pb, As, MeHg) provided insight into the specific proteins that are related to neurodegenerative disease. The findings of our study have greatly expanded understanding of the role of mitochondria in the pathogenesis of neurodegenerative diseases. The comparison of protein expression among Pb, As, and MeHg revealed both, common and unique protein targets that are related to neurodegenerative diseases. The involvement of mRNA splicing and the UPS pathway might be novel in hippocampal based studies. The results of our proteomics analysis strongly suggest that the disruption of mRNA splicing, UPS, ETC and oxidative stress play a crucial mechanistic role in metal induced neurodegenerative diseases. Greater improvement of these pathways will be required to fully understand the pathogenesis of neurodegenerative disorders by heavy metals.

Chapter 5

The interaction between mixtures becomes complex and it is difficult to explain the mixture toxicity based on the cytotoxicity profile. Therefore, a high throughput of the omics is extremely useful for determine the effects of mixtures on the toxic pathway and the associated outcome of toxicity. The mixtures Pb+MeHg, Pb+As, and MeHg+As allowed us to get better insights into the responses of the interaction profile at the protein level. In our study, Pb+MeHg induced a higher perturbation of the cellular functions than the other two mixtures (Pb+As, and MeHg+As). In addition, our proteomic analyses were able to detect critical responses of HT-22 hippocampal to mixtures exposure, relevant to AD and PD. The identified proteins and pathways could serve as potential markers of mixtures. Further studies are required to prove the results obtained so far and also to increase the panel of potential protein markers in neurodegenerative diseases.

Conclusions

In summary, in this study, in vitro bioassays are combined with mechanistic omics tools to compare the toxicity profiles of single metals and their binary mixtures on HT-22 hippocampal cells. In future the toxicity evaluation of mixtures can be extended to other endpoints for a more comprehensive understanding of adverse effects. The in vitro results are useful to understand the effects of metal mixtures on various cellular responses and pathways. But the translation to human health assessment needs further refinement.

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Annex 1

Annex 1

Heavy metals (Pb, Cd, As, and MeHg) cell viability data on HT-22 cells in 1 day, 3 days, 8 days exposure

The summary of cell viability in different exposure times (1 day, 3 days, 8 days) are presented in following manner

I. Table 1# Pb cell viability in different exposures

Conc.(µM)	1 day	3 days	8 days
	Mean±SD	Mean±SD	Mean±SD
0	100 ± 0	100 ± 0	100 ± 0
10	100 ± 0	97.7 ± 3	99.0 ± 2
20	96.7 ± 3	92.3 ± 4	98.3 ± 3
30	94.7 ± 5	90.3 ± 4	92.0 ± 8
40	92.3 ± 7	87.7 ± 3	93.7 ± 4
50	86.0 ± 6	83.7 ± 5	94.0 ± 1
60	83.3 ± 4	82.3 ± 13	93.3 ± 8
70	81.0 ± 2	76.0 ± 9	74.3 ± 4
80	79.3 ± 7	72.0 ± 9	19.3 ± 8
90	73.7 ± 2	61.7 ± 11	13.0± 3
100	70.0 ± 2	53.0 ± 8	13.3 ± 6

II. Table 2# Cd cell viability in different exposures

conc.(µM)	1day	3days	8 days
	Mean± SD	Mean±SD	Mean±SD
0	100± 0	100.0± 0	100± 0
0.5	100± 0	92.3± 3.5	92± 9
1.5	100± 1	88.7± 4.9	89± 10
2.5	98± 2	85.3± 8.9	86± 9
3	96± 3	83.3±12.4	81± 12
3.5	94± 5.5	80.0± 13.2	72± 7
4	89± 7.5	73.7± 9.6	39± 9.5
4.5	80± 4	70.3± 5.1	10± 1
5.5	67± 11.1	52.3± 13.6	9± 1

6.5	57± 12	41.0± 12.2	7± 0
7	46± 5	24.7± 8.7	10± 5

III. Table 3# As cell viability in different exposures

Conc.(µM)	1 day	3 days	8 days
	Mean±SD	Mean	Mean
0	100± 0	100.0± 0	100.0± 0
0.4	99.3± 1.2	93.0± 5.3	96.0± 5.3
0.8	100.0± 0	89.3± 7.4	58.7± 11
1.2	98.0± 1.7	86.0± 12.1	24.0± 8.7
1.6	96.3± 4.7	84.3± 10.7	10.0± 1
2	93.7± 7.1	76.7± 11.2	9.7± 0.6
2.4	89.0±10.8	67.3± 10	8.7± 1.2
2.8	76.7± 16.3	47.3± 4.2	10.3±1.5
3.2	68.3± 6.0	35.3± 11.9	7.7± 0.6
3.8	56.3± 6.8	22.0± 7.9	7.3± 0.6
4.6	41.0± 4.4	18.0± 4.6	7.3± 1.2

IV. Table 4# MeHg cell viability in different exposures

Conc.(µM)	1 day	3 days	8 days
	Mean±SD	Mean±SD	Mean±SD
0	100± 0	100± 0	100± 0
0.6	97.3± 4.6	78.0± 5.3	47.6± 5.7
1.2	93.7± 6.5	70.0± 4.4	36.6± 0.4
1.8	93± 6.1	50.7± 4	31.7± 3.9
2.4	73± 16.5	34.7± 11.6	14.8± 3.5
3	52.3± 9.0	25.7± 14.4	8.7± 1.9
3.6	34.3± 13.8	18.0± 8.9	7.4± 0.5
4.2	23.7± 9.1	13.3± 10.1	7.5± 0.9
6	11± 13.0	8.3± 3.2	7.3± 0.4
8	9.3± 10.1	7.3±1.5	7.4± 0.9
12	8.7± 9	7.7±2.1	7.1± 0.2

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V. Table 5# The IC_{50} values of Pb, Cd, As, and MeHg ON hippocampal HT-22 cell line after different exposure times in In vitro[a]

Exposure time	Heavy metals (IC_{50})			
	Pb (µM)	**Cd (µM)**	**As (µM)**	**MeHg (µM)**
Acute (1 day)	172.2	6.7	4.0	3.1
Subchronic (3 days)	117.6	5.5	2.7	1.7
Chronic (8 days)	74.3	3.7	0.8	0.6

([a]Values of IC_{50} with 95% confidence intervals from dose response curves, Cytotoxicity was evaluated by inhibition of MTT reduction as described in the methods).

Annex 1

Annex 2

Chapter 4, Supplymentery file : A. Pb Metal pruned list, and compelete cellular proteome overview with GO functional terms

Ontology-ID	Ontology Name	Ontology Type	Number Changed	Number Measured	Number in Ontology	Z Score	P value
GO:0050998	nitric-oxide synthase binding	Molecular function	5	5	12	3.828789	0.001
GO:0043028	cysteine-type endopeptidase regulator activity involved in apoptotic process	Molecular function	5	5	36	3.828789	0
GO:0004869	cysteine-type endopeptidase inhibitor activity	Molecular function	5	5	60	3.828789	0.0005
GO:0045806	negative regulation of endocytosis	Biological process	6	7	31	3.663707	0.002
GO:0070008	serine-type exopeptidase activity	Molecular function	4	4	11	3.423747	0.0045
GO:0044421	extracellular region part	Cellular component	209	706	3094	3.10233	0.003
GO:0022625	cytosolic large ribosomal subunit	Cellular component	13	25	37	3.06242	0.0025
GO:0048713	regulation of oligodendrocyte differentiation	Biological process	3	3	29	2.964337	0.0135
GO:0046426	negative regulation of JAK-STAT cascade	Biological process	3	3	12	2.964337	0.018
GO:0032239	regulation of nucleobase-containing compound transport	Biological process	3	3	12	2.964337	0.016

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GO:0006265	DNA topological change	Biological process	3	3	7	2.964337	0.017
GO:0004930	G-protein coupled receptor activity	Molecular function	3	3	1913	2.964337	0.0125
GO:0003916	DNA topoisomerase activity	Molecular function	3	3	6	2.964337	0.017
GO:0071709	membrane assembly	Biological process	4	5	17	2.801348	0.021
GO:0045263	proton-transporting ATP synthase complex, coupling factor F(o)	Cellular component	4	5	20	2.801348	0.019
GO:0007062	sister chromatid cohesion	Biological process	4	5	22	2.801348	0.0145
GO:0048278	vesicle docking	Biological process	5	7	26	2.794941	0.0175
GO:0045814	negative regulation of gene expression, epigenetic	Biological process	5	7	24	2.794941	0.015
GO:0001666	response to hypoxia	Biological process	13	27	232	2.721826	0.0085
GO:0006475	internal protein amino acid acetylation	Biological process	9	17	101	2.609841	0.0135
GO:1901575	organic substance catabolic process	Biological process	102	328	1523	2.548844	0.0075
GO:0015985	energy coupled proton transport, down electrochemical gradient	Biological process	6	10	24	2.511715	0.024
GO:0007160	cell-matrix adhesion	Biological process	6	10	67	2.511715	0.019
GO:0051301	cell division	Biological process	12	26	123	2.435477	0.0255
GO:0000122	negative regulation of transcription from RNA polymerase II promoter	Biological process	24	62	613	2.428798	0.015

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GO:1902745	positive regulation of lamellipodium organization	Biological process	5	8	13	2.408031	0.025
GO:0004386	helicase activity	Molecular function	21	53	173	2.395105	0.0115
GO:0090595	acetyl-CoA	Molecular function	4	6	50	2.318946	0.0355
GO:0070688	MLL5-L complex	Cellular component	4	6	9	2.318946	0.0415
GO:0046933	proton-transporting ATP synthase activity, rotational mechanism	Molecular function	4	6	9	2.318946	0.031
GO:0006107	oxaloacetate metabolic process	Biological process	4	6	11	2.318946	0.0395
GO:0006002	fructose 6-phosphate metabolic process	Biological process	4	6	7	2.318946	0.0385
GO:0051491	positive regulation of filopodium assembly	Biological process	3	4	23	2.27531	0.0485
GO:0045738	negative regulation of DNA repair	Biological process	3	4	8	2.27531	0.047
GO:0032233	positive regulation of actin filament bundle assembly	Biological process	3	4	38	2.27531	0.0465
GO:0016742	hydroxymethyl-, formyl- and related transferase activity	Molecular function	3	4	8	2.27531	0.047
GO:0005671	Ada2/Gcn5/Ada3 transcription activator complex	Cellular component	3	4	15	2.27531	0.049
GO:0005092	GDP-dissociation inhibitor activity	Molecular function	3	4	11	2.27531	0.048
GO:0045259	proton-transporting ATP synthase complex	Cellular component	6	11	20	2.218723	0.034
GO:0034719	SMN-Sm protein complex	Cellular component	6	11	17	2.218723	0.032

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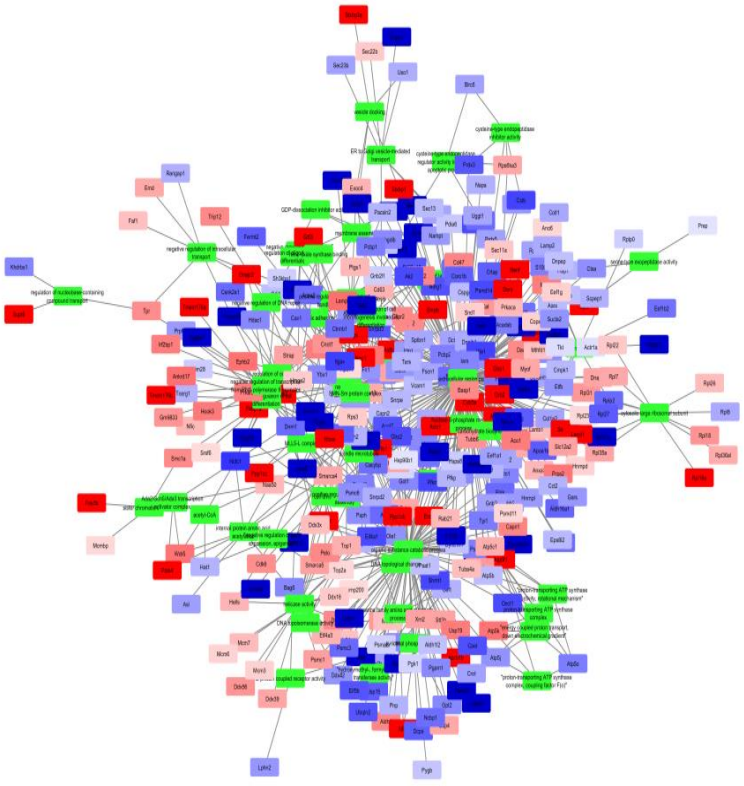
GO:0009069	serine family amino acid metabolic process	Biological process	6	11	35	2.218723	0.0305
GO:0005876	spindle microtubule	Cellular component	6	11	43	2.218723	0.042
GO:0006414	translational elongation	Biological process	9	19	33	2.200468	0.036
GO:0045596	negative regulation of cell differentiation	Biological process	21	55	564	2.192577	0.0405
GO:0001558	regulation of cell growth	Biological process	12	28	282	2.125629	0.041
GO:0032387	negative regulation of intracellular transport	Biological process	7	14	87	2.113289	0.0375
GO:0030170	pyridoxal phosphate binding	Molecular function	7	14	55	2.113289	0.0485
GO:0006888	ER to Golgi vesicle-mediated transport	Biological process	7	14	42	2.113289	0.0415
GO:0010770	positive regulation of cell morphogenesis involved in differentiation	Biological process	8	17	117	2.051012	0.039
GO:0097458	neuron part	Cellular component	46	141	927	2.019223	0.035
GO:0016051	carbohydrate biosynthetic process	Biological process	12	29	156	1.979875	0.0385

Annex 2



Annex 2

Chapter 4, Supplymentery file 1 : B. As Metal pruned list, compelete cellular proteome overview with GO functional terms

Ontology-ID	Ontology Name	Ontology Type	Number Changed	Number Measured	Number in Ontology	Z Score	P value
GO:0016051	carbohydrate biosynthetic process	Biological process	25	29	156	3.087366083	0.001
GO:0031346	positive regulation of cell projection organization	Biological process	23	27	198	2.869264518	0.004
GO:1901575	organic substance catabolic process	Biological process	213	328	1523	2.730035129	0.0085
GO:0022603	regulation of anatomical structure morphogenesis	Biological process	64	89	685	2.696016512	0.006
GO:0044712	single-organism catabolic process	Biological process	158	239	1060	2.662825047	0.0085
GO:0044421	extracellular region part	Cellular component	438	706	3094	2.602442631	0.0075
GO:0050821	protein stabilization	Biological process	18	21	79	2.576140974	0.0155
GO:0044281	small molecule metabolic process	Biological process	263	413	1802	2.561610204	0.012
GO:0050764	regulation of phagocytosis	Biological process	9	9	51	2.55191176	0.012
GO:0007605	sensory perception of sound	Biological process	9	9	117	2.55191176	0.0105
GO:0044087	regulation of cellular component biogenesis	Biological process	61	86	454	2.460119512	0.0125
GO:0061041	regulation of wound healing	Biological process	8	8	96	2.405383979	0.0255
GO:0032088	negative regulation of NF-kappaB transcription factor activity	Biological process	8	8	63	2.405383979	0.0235

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GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	Biological process	19	23	258	2.393846889	0.0165
GO:0001525	angiogenesis	Biological process	19	23	229	2.393846889	0.022
GO:0045202	synapse	Cellular component	25	32	270	2.311868147	0.035
GO:0097458	neuron part	Cellular component	95	141	927	2.308638624	0.026
GO:0030496	midbody	Cellular component	27	35	108	2.300839335	0.021
GO:1902882	regulation of response to oxidative stress	Biological process	7	7	27	2.249487132	0.043
GO:0061387	regulation of extent of cell growth	Biological process	7	7	65	2.249487132	0.0425
GO:0030017	sarcomere	Cellular component	7	7	31	2.249487132	0.046
GO:0010634	positive regulation of epithelial cell migration	Biological process	7	7	71	2.249487132	0.0425
GO:1901342	regulation of vasculature development	Biological process	13	15	185	2.249081677	0.0285
GO:0016407	acetyltransferase activity	Molecular function	13	15	99	2.249081677	0.0295
GO:0001568	blood vessel development	Biological process	13	15	107	2.249081677	0.0305
GO:0022607	cellular component assembly	Biological process	185	290	1378	2.113273338	0.0425
GO:0016853	isomerase activity	molecular_function	35	48	144	2.102789035	0.0405
GO:0005102	receptor binding	Molecular function	87	130	1254	2.102431949	0.0445

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GO:0007265	Ras protein signal transduction	Biological process	19	24	99	2.102242252	0.044
GO:0055076	transition metal ion homeostasis	Biological process	12	14	99	2.099838706	0.048
GO:0003727	single-stranded RNA binding	Molecular function	12	14	51	2.099838706	0.0485
GO:1900046	regulation of hemostasis	Biological process	6	6	67	2.082117535	0.036
GO:0031258	lamellipodium membrane	Cellular component	6	6	16	2.082117535	0.044
GO:0017025	TBP-class protein binding	Molecular function	6	6	12	2.082117535	0.0345
GO:0006002	fructose 6-phosphate metabolic process	Biological process	6	6	7	2.082117535	0.0425
GO:0030003	cellular cation homeostasis	Biological process	27	36	329	2.071227678	0.043
GO:0046906	tetrapyrrole binding	Molecular function	14	17	189	2.033559923	0.048
GO:0019439	aromatic compound catabolic process	Biological process	115	176	783	2.031117957	0.047

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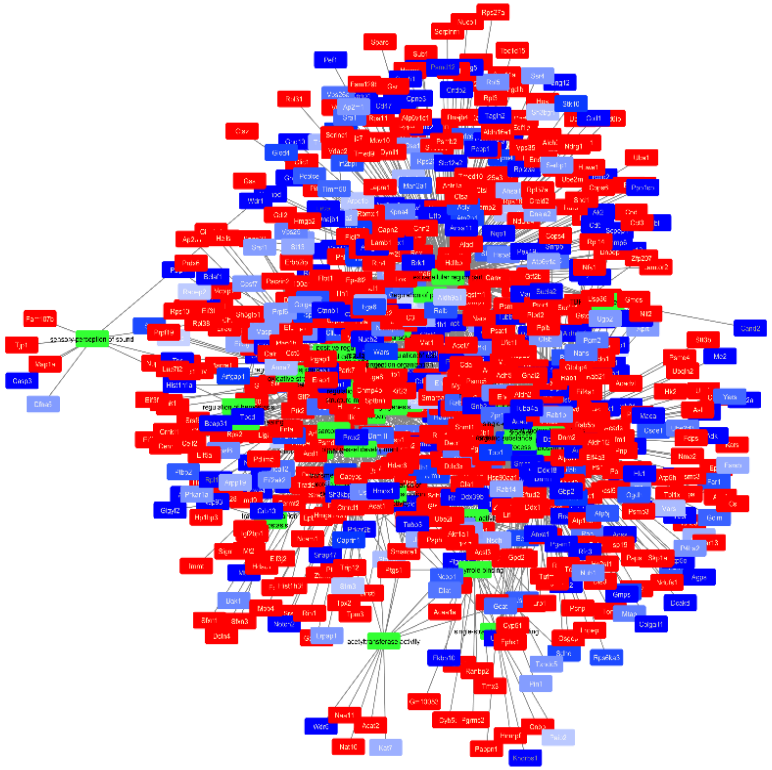

Annex 2

Chapter 4, Supplymentery file 1 : C. MeHg Metal pruned list, compelete cellular proteome overview with GO functional terms.

Ontology-ID	Ontology Name	Ontology Type	Number Changed	Number Measured	Number in Ontology	Z Score	P value
GO:0005689	U12-type spliceosomal complex	Cellular component	13	13	25	3.368599012	0
GO:0006090	pyruvate metabolic process	Biological process	18	21	55	2.970745698	0.003
GO:0005929	cilium	Cellular component	17	20	248	2.834117397	0.0045
GO:0006826	iron ion transport	Biological process	11	12	57	2.655355873	0.0075
GO:0048278	vesicle docking	Biological process	7	7	26	2.468342109	0.016
GO:0046915	transition metal ion transmembrane transporter activity	Molecular function	7	7	45	2.468342109	0.0155
GO:0045806	negative regulation of endocytosis	Biological process	7	7	31	2.468342109	0.0145
GO:0050877	neurological system process	Biological process	31	43	1863	2.464560175	0.018
GO:0055076	transition metal ion homeostasis	Biological process	12	14	99	2.421544115	0.0135
GO:0016903	oxidoreductase activity, acting on the aldehyde or oxo group of donors	Molecular function	14	17	54	2.391385209	0.03
GO:0050821	protein stabilization	Biological process	18	23	79	2.390010603	0.024
GO:0015985	energy coupled proton transport, down electrochemical gradient	Biological process	9	10	24	2.316940631	0.0225
GO:0007160	cell-matrix adhesion	Biological process	9	10	67	2.316940631	0.0225

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GO:0006002	fructose 6-phosphate metabolic process	Biological process	6	6	7	2.284696827	0.0235
GO:0005201	extracellular matrix structural constituent	Molecular function	6	6	32	2.284696827	0.022
GO:0000038	very long-chain fatty acid metabolic process	Biological process	6	6	21	2.284696827	0.0235
GO:0022625	cytosolic large ribosomal subunit	Cellular component	19	25	37	2.264997804	0.0195
GO:0070085	glycosylation	Biological process	11	13	227	2.253231979	0.0295
GO:0005200	structural constituent of cytoskeleton	Molecular function	11	13	49	2.253231979	0.029
GO:0020037	heme binding	Molecular function	13	16	181	2.230656144	0.044
GO:0016758	transferase activity, transferring hexosyl groups	Molecular function	13	16	182	2.230656144	0.0425
GO:0044270	cellular nitrogen compound catabolic process	Biological process	110	179	776	2.219545085	0.0325
GO:0044421	extracellular region part	Cellular component	405	712	3094	2.199638198	0.028
GO:0019439	aromatic compound catabolic process	Biological process	109	178	783	2.152233185	0.037
GO:0007179	transforming growth factor beta receptor signaling pathway	Biological process	8	9	67	2.130557682	0.0425
GO:0005687	U4 snRNP	Cellular component	8	9	10	2.130557682	0.0425
GO:1901361	organic cyclic compound catabolic process	Biological process	110	180	811	2.130305084	0.0375
GO:0031224	intrinsic component of membrane	Cellular component	133	221	5860	2.093045763	0.0355

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GO:0071709	membrane assembly	Biological process	5	5	17	2.085137149	0.0435
GO:0070828	heterochromatin organization	Biological process	5	5	8	2.085137149	0.0435
GO:0061035	regulation of cartilage development	Biological process	5	5	51	2.085137149	0.036
GO:0051705	multi-organism behavior	Biological process	5	5	82	2.085137149	0.046
GO:0050998	nitric-oxide synthase binding	Molecular function	5	5	12	2.085137149	0.0435
GO:0050771	negative regulation of axonogenesis	Biological process	5	5	40	2.085137149	0.0455
GO:0045263	proton-transporting ATP synthase complex, coupling factor F(o)	Cellular component	5	5	20	2.085137149	0.0435
GO:0044548	S100 protein binding	Molecular function	5	5	11	2.085137149	0.047
GO:0043028	cysteine-type endopeptidase regulator activity involved in apoptotic process	Molecular function	5	5	36	2.085137149	0.034
GO:0034715	pICln-Sm protein complex	Cellular component	5	5	6	2.085137149	0.0415
GO:0034399	nuclear periphery	Cellular component	5	5	10	2.085137149	0.0465
GO:0008250	oligosaccharyltransferase complex	Cellular component	5	5	9	2.085137149	0.047
GO:0005683	U7 snRNP	Cellular component	5	5	8	2.085137149	0.0375
GO:0004869	cysteine-type endopeptidase inhibitor activity	Molecular function	5	5	60	2.085137149	0.0335
GO:0046700	heterocycle catabolic process	Biological process	108	177	777	2.084597455	0.0415

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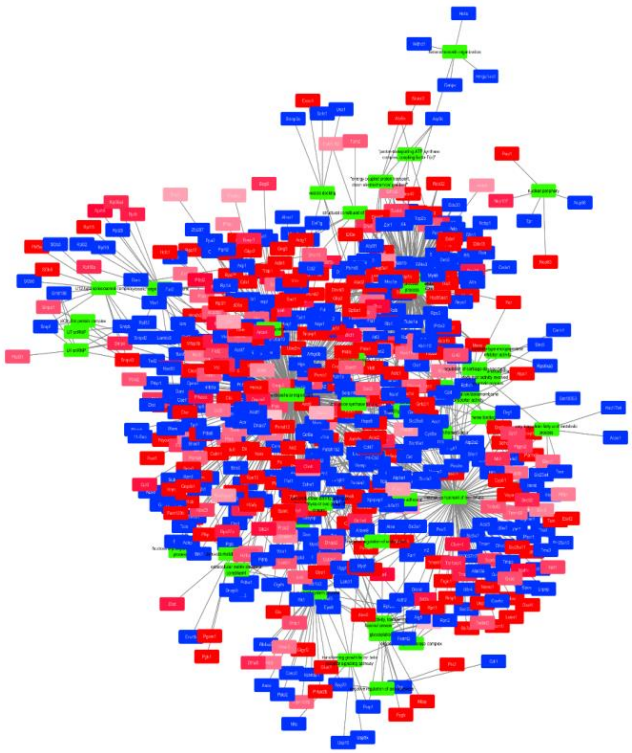

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Chapter 5 Supplementary file: A. Pb+MeHg mixture pruned list, indicating the whole cellular proteome and their GO functional terms association overview

Ontology-ID	Ontology Name	Ontology Type	Number Changed	Number Measured	Number in Ontology	Z Score	P value
GO:0044451	nucleoplasm part	Cellular component	103	138	628	3.9212267	0.0005
GO:0031328	positive regulation of cellular biosynthetic process	biological process	110	156	1393	3.1010223	0.0015
GO:0071826	ribonucleoprotein complex subunit organization	biological process	46	60	119	2.8597072	0.005
GO:0010628	positive regulation of gene expression	biological process	110	159	1297	2.7787897	0.0045
GO:0000792	heterochromatin	cellular component	17	19	66	2.732239	0.008
GO:0045595	regulation of cell differentiation	biological process	89	127	1237	2.6749546	0.012
GO:0033144	negative regulation of intracellular steroid hormone receptor signaling pathway	biological process	10	10	31	2.6555413	0.005
GO:0030218	erythrocyte differentiation	biological process	10	10	49	2.6555413	0.005
GO:0048857	neural nucleus development	biological process	13	14	61	2.6004984	0.0115
GO:0004386	helicase activity	molecular function	41	54	173	2.5970044	0.0105
GO:004593	positive regulation of nucleobase-containing	biological	103	150	1308	2.559697	0.010

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5	compound metabolic process	process				2	5
GO:0005667	transcription factor complex	cellular component	18	21	328	2.522194	0.014
GO:0016407	acetyltransferase activity	molecular function	15	17	99	2.479087	0.016
GO:0010629	negative regulation of gene expression	biological process	100	146	1063	2.478629	0.018
GO:0003713	transcription coactivator activity	molecular function	27	34	195	2.466901	0.01
GO:0070085	glycosylation	biological process	12	13	227	2.464947	0.018
GO:0016757	transferase activity, transferring glycosyl groups	molecular function	20	24	262	2.460035	0.020
GO:0010557	positive regulation of macromolecule biosynthetic process	biological process	103	151	1290	2.451092	0.013
GO:0051241	negative regulation of multicellular organismal process	biological process	52	72	829	2.362565	0.025
GO:1902493	acetyltransferase complex	cellular component	11	12	83	2.322455	0.031
GO:0045934	negative regulation of nucleobase-containing compound metabolic process	biological process	90	132	1012	2.273408	0.027
GO:0051098	regulation of binding	biological process	27	35	202	2.228627	0.039
GO:0048806	genitalia development	biological process	7	7	35	2.220199	0.044
GO:0046835	carbohydrate phosphorylation	biological process	7	7	24	2.220199	0.045
GO:0019200	carbohydrate kinase activity	molecular function	7	7	21	2.220199	0.045

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GO:0045111	intermediate filament cytoskeleton	cellular component	13	15	65	2.2037642	0.036
GO:0009125	nucleoside monophosphate catabolic process	biological process	50	70	272	2.1912314	0.0335
GO:0031327	negative regulation of cellular biosynthetic process	biological process	92	136	1066	2.1789926	0.0325
GO:0045259	proton-transporting ATP synthase complex	cellular component	10	11	20	2.1718908	0.0255
GO:0032784	regulation of DNA-templated transcription, elongation	biological process	10	11	34	2.1718908	0.031
GO:0005844	polysome	cellular component	10	11	25	2.1718908	0.035
GO:0051130	positive regulation of cellular component organization	biological process	78	114	661	2.1565085	0.034
GO:0030532	small nuclear ribonucleoprotein complex	cellular component	17	21	31	2.0768031	0.048
GO:0007275	multicellular organismal development	biological process	17	21	389	2.0768031	0.046
GO:0048524	positive regulation of viral process	biological process	9	10	36	2.0118014	0.0495
GO:0019838	growth factor binding	molecular function	9	10	107	2.0118014	0.0445
GO:0015985	energy coupled proton transport, down electrochemical gradient	biological process	9	10	24	2.0118014	0.0465

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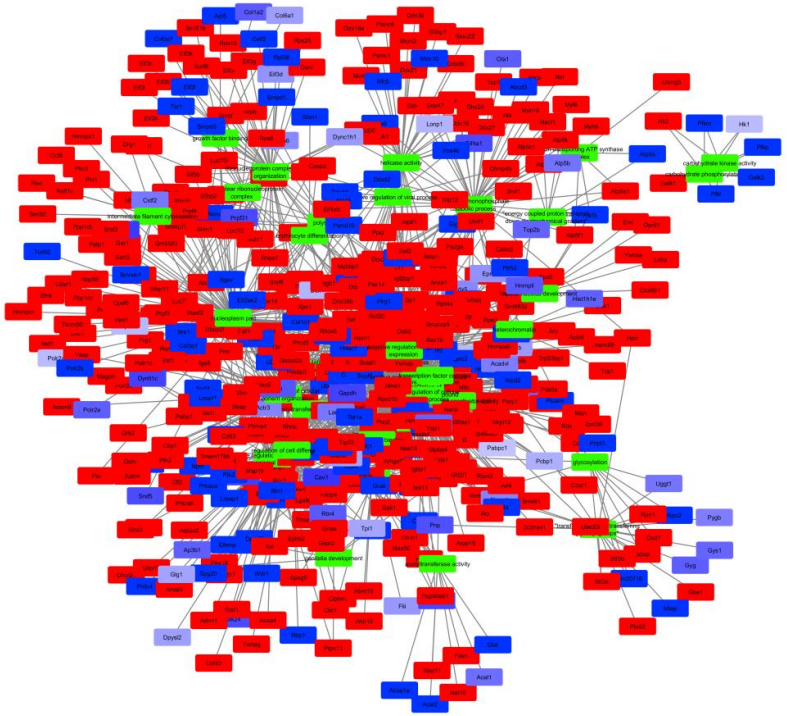

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Chapter 5 Supplementary file 1: B. Pb+As mixture pruned list, indicating the whole cellular proteome and their functional GO terms association overview

Ontology-ID	Ontology Name	Ontology Type	Number Changed	Number Measured	Number in Ontology	Z Score	p value
GO:0016072	rRNA metabolic process	biological process	42	51	124	3.478334	0.001
GO:0016234	inclusion body	cellular component	17	18	54	3.096011	0.004
GO:0015631	tubulin binding	molecular function	33	40	225	3.091329	0.002
GO:0051129	negative regulation of cellular component organization	biological process	59	79	422	2.948302	0.005 5
GO:0030529	ribonucleoprotein complex	cellular component	185	278	667	2.867541	0.005 5
GO:0044822	poly(A) RNA binding	molecular function	320	500	1001	2.778781	0.002 5
GO:0009125	nucleoside monophosphate catabolic process	biological process	52	70	272	2.700429	0.007 5
GO:0051983	regulation of chromosome segregation	biological process	10	10	48	2.66074	0.008
GO:0007160	cell-matrix adhesion	biological process	10	10	67	2.66074	0.005 5
GO:0010721	negative regulation of cell development	biological process	24	29	238	2.653673	0.011
GO:0071826	ribonucleoprotein complex subunit organization	biological process	45	60	119	2.607961	0.014 5

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GO:0005871	kinesin complex	cellular component	9	9	52	2.523599	0.0115
GO:0010770	positive regulation of cell morphogenesis involved in differentiation	biological process	15	17	117	2.486214	0.0115
GO:0016469	proton-transporting two-sector ATPase complex	cellular component	12	13	31	2.471072	0.0212
GO:0030099	myeloid cell differentiation	biological process	20	24	156	2.468684	0.0195
GO:0044445	cytosolic part	cellular component	59	83	173	2.345807	0.0195
GO:0004386	helicase activity	molecular function	40	54	173	2.330577	0.0212
GO:0051781	positive regulation of cell division	biological process	11	12	109	2.328353	0.0373
GO:0006282	regulation of DNA repair	biological process	11	12	55	2.328353	0.0363
GO:0003735	structural constituent of ribosome	molecular function	55	77	208	2.318566	0.0225
GO:0042451	purine nucleoside biosynthetic process	biological process	25	32	89	2.253343	0.0293
GO:0006413	translational initiation	biological process	25	32	66	2.253343	0.0305
GO:0045321	leukocyte activation	biological process	27	35	352	2.239354	0.0345
GO:0060560	developmental growth involved in morphogenesis	biological process	7	7	82	2.224546	0.0415
GO:0010631	epithelial cell migration	biological process	7	7	56	2.224546	0.0415
GO:0045111	intermediate filament cytoskeleton	cellular component	13	15	65	2.210497	0.0385

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GO:0035097	histone methyltransferase complex	cellular component	13	15	69	2.210497	0.036
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	biological process	13	15	53	2.210497	0.0295
GO:0006412	translation	biological process	57	81	223	2.183197	0.035
GO:0042393	histone binding	molecular function	22	28	117	2.154063	0.0335
GO:0051082	unfolded protein binding	molecular function	26	34	72	2.126369	0.041
GO:1903507	negative regulation of nucleic acid-templated transcription	biological process	70	102	882	2.096366	0.04
GO:0016607	nuclear speck	cellular component	45	63	174	2.090034	0.039
GO:0005874	microtubule	cellular component	54	77	336	2.082864	0.042
GO:0090305	nucleic acid phosphodiester bond hydrolysis	biological process	21	27	194	2.030476	0.043
GO:0005622	intracellular	cellular component	114	173	1351	2.018513	0.0405
GO:0015985	energy coupled proton transport, down electrochemical gradient	biological process	9	10	24	2.017219	0.049
GO:0016407	acetyltransferase activity	molecular function	14	17	99	1.991831	0.0435
GO:0006281	DNA repair	biological process	51	73	354	1.978939	0.0425

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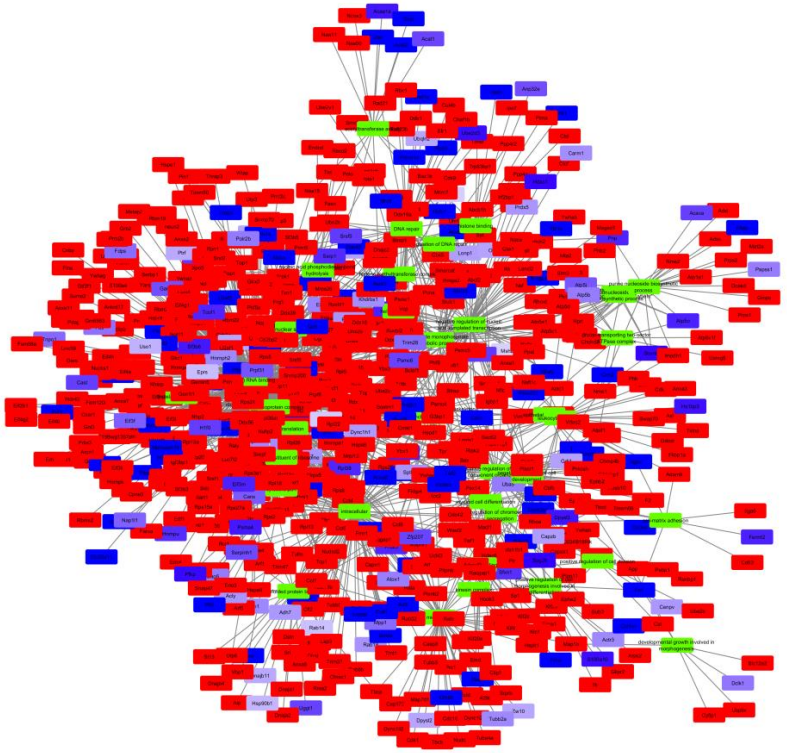

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Chapter 5 Supplementary file: C. MeHg+As mixture pruned list, indicating the whole proteome and their GO terms association overview

Ontology -ID	Ontology Name	Ontology Type	Number Changed	Number Measured	Number in Ontology	Z Score	p value
GO:0030490	maturation of SSU-rRNA	biological process	7	7	8	3.294262	0.001
GO:0019838	growth factor binding	molecular function	9	10	107	3.291306	0.001
GO:0007160	cell-matrix adhesion	biological process	9	10	67	3.291306	0.002
GO:0051781	positive regulation of cell division	biological process	10	12	109	3.133047	0.0045
GO:0048588	developmental cell growth	biological process	6	6	51	3.049168	0.0045
GO:0003824	catalytic activity	molecular function	371	866	5657	2.79537	0.0065
GO:0071709	membrane assembly	biological process	5	5	17	2.782835	0.009
GO:0061035	regulation of cartilage development	biological process	5	5	51	2.782835	0.014
GO:0043542	endothelial cell migration	biological process	5	5	43	2.782835	0.009
GO:0044420	extracellular matrix part	cellular component	11	15	119	2.709673	0.008
GO:0001568	blood vessel development	biological process	11	15	107	2.709673	0.0055

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GO:0004888	transmembrane signaling receptor activity	molecular function	8	10	2202	2.642403	0.01
GO:0000910	cytokinesis	biological process	13	19	63	2.61223	0.011
GO:0001525	angiogenesis	biological process	15	23	229	2.560576	0.0165
GO:0044070	regulation of anion transport	biological process	9	12	109	2.5404	0.018
GO:0008213	protein alkylation	biological process	9	12	104	2.5404	0.0095
GO:0050864	regulation of B cell activation	biological process	6	7	91	2.519228	0.012
GO:0045806	negative regulation of endocytosis	biological process	6	7	31	2.519228	0.0135
GO:0045738	negative regulation of DNA repair	biological process	4	4	8	2.488452	0.023
GO:0043628	ncRNA 3'-end processing	biological process	4	4	9	2.488452	0.024
GO:0034063	stress granule assembly	biological process	4	4	10	2.488452	0.018
GO:0033628	regulation of cell adhesion mediated by integrin	biological process	4	4	34	2.488452	0.026
GO:0032964	collagen biosynthetic process	biological process	4	4	7	2.488452	0.02
GO:0031519	PcG protein complex	cellular component	4	4	42	2.488452	0.023
GO:0031507	heterochromatin assembly	biological process	4	4	7	2.488452	0.025
GO:0030506	ankyrin binding	molecular function	4	4	17	2.488452	0.02

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GO:0007270	neuron-neuron synaptic transmission	biological process	4	4	59	2.488452	0.0275
GO:0005671	Ada2/Gcn5/Ada3 transcription activator complex	cellular component	4	4	15	2.488452	0.0285
GO:0005125	cytokine activity	molecular function	4	4	223	2.488452	0.019
GO:0002274	myeloid leukocyte activation	biological process	4	4	81	2.488452	0.0305
GO:0030154	cell differentiation	biological process	88	185	1651	2.41636	0.02
GO:0016569	covalent chromatin modification	biological process	24	42	285	2.393971	0.019
GO:0051156	glucose 6-phosphate metabolic process	biological process	7	9	20	2.369438	0.022
GO:0006909	phagocytosis	biological process	7	9	46	2.369438	0.026
GO:0001501	skeletal system development	biological process	9	13	133	2.217613	0.037
GO:1902106	negative regulation of leukocyte differentiation	biological process	5	6	73	2.212235	0.035
GO:0006002	fructose 6-phosphate metabolic process	biological process	5	6	7	2.212235	0.0345
GO:0019752	carboxylic acid metabolic process	biological process	84	179	676	2.189857	0.0255
GO:0050661	NADP binding	molecular function	10	15	47	2.179213	0.0415
GO:0008144	drug binding	molecular function	11	17	85	2.154886	0.0425
GO:0061448	connective tissue development	biological process	6	8	50	2.072251	0.043

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GO:0009566	fertilization	biological process	6	8	79	2.072251	0.0435
GO:0006084	acetyl-CoA metabolic process	biological process	6	8	21	2.072251	0.046
GO:0051983	regulation of chromosome segregation	biological process	7	10	48	1.9935	0.0465
GO:0009986	cell surface	cellular component	25	47	409	1.974692	0.047

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