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Effects of Environmental Heavy Metals on Neural Stem Cell Survival and Differentiation

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EFFECTS OF ENVIRONMENTAL HEAVY METALS ON NERUAL STEM CELL SURVIVAL AND DIFFERENTIATION

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ABSTRACT

Heavy metals are usually found in the environment (air, water, land, food chains), but their concentrations have been increasing due to increased industrialization and improper waste disposal. Many heavy metals have been characterized for their toxic effects, including developmental toxicity, mental retardation, neurological impairment such as blindness, neuromuscular weakness, altered neurobehavioral development, neurocognitive deficits and many other related disorders, in both human beings and animals. In this study, we seek to understand the specific effects of cadmium, mercury and lead on neural stem cell (NSC) survival and differentiation, to gain further insight on their toxic effects during embryogenesis. NSCs are self-renewing, multi-potent resident brain cells, which differentiate into various neural and glial lineages depending on the spatial-temporal cues and signaling molecules they receive. Here we investigated the effects of cadmium, lead and mercury on embryonic rat NSC differentiation and neurite outgrowth in a 3D environment over a 14 day culture period. Type I collagen was used to create an extracellular 3D microenvironment for NSC culture, and various concentrations (0.01 nM – 10 μM) of metal ions were added. NSC survival, differentiation and neurite outgrowth into various neural and glial lineages was assessed both qualitatively and quantitatively, at various time points. Results suggest the tremendous adverse impact

metal ions could exert, even at as low as 0.1 nM concentration, on the initiation and propagation of neurological deficits in the developing central nervous system.

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CHAPTER I

INTRODUCTION

Heavy metals are defined as the metals having density greater than 5 g/cm^3[1]. Some of them such as iron, copper, selenium, and zinc are essential for normal metabolism. But, many heavy metals such as lead, mercury and cadmium are toxic even in minute concentrations. They naturally exist in the earth's crust and are emitted during activities like volcanic eruptions, erosions, etc. Growth of industrialization in the past few decades and activities such as mining, combustion of fossil fuels, improper disposal of batteries, paints and industrial wastes containing heavy metals, usage of phosphate fertilizers and sewage sludge for cultivation of crops have led to an increase in the concentration of toxic heavy metals in the environment. These increased concentrations are posing many health issues either directly or indirectly. Federal bodies, such as the World Health Organization (WHO), The Environmental Protection Agency (EPA), and The Food and Drug Administration (FDA), are making constant efforts to minimize these emissions into the atmosphere. There are many places where concentrations of these

heavy metals are beyond allowable limits. Studies have shown that most of these affected places are located in under-developed countries. Reports from some recent studies implicate that these metals cause toxicity even at concentrations lower than the standard limits set by federal agencies.

In this study, we chose to investigate the effects of cadmium, mercury and lead, as these are some of the most hazardous heavy metals. These metals are known for their systemic toxic effects causing damage to the organs such as nervous, cardiovascular, reproductive, renal, digestive and the endocrine systems[2]. Most common occurrence of heavy metal toxicity is due to occupational exposure, contaminated food and water. As these metals are non-degradable, they bio-accumulate through food chains. A very sensitive case of toxicity is developmental toxicity which occurs due to exposure to these metals during pregnancy. Much research has been done on the causes of developmental disorders by using different kinds of animal and cellular models. Developing nervous system of fetus is reported to be the major site of action of these metals [1,4,5,6]. Our long-term goal is to study the toxic effects of cadmium, mercury and lead on the development of nervous system during embryogenesis. Rat neural stem cells were used in our study as they are the precursor cells to various neural cell lineages which constitute the nervous system.

The stem cells were exposed to various concentrations of heavy metals for different culture periods. Determining the viability of cells under given conditions was the initial aim in our study. The second aim was to observe morphology and neurite outgrowth at the chosen concentrations, and to compare them with respective control

group at different exposure times. Finally, we determined the effects of these metal ions on neural stem cell differentiation into broad neural and glial (astrocytes, oligodendrocytes) lineages using immune-fluorescence staining for specific markers.

Chapter-II discusses the sources of metal toxicity, systemic toxic effects, mechanism of toxicity of the metals, characteristics of neural stem cells, its lineages and specific markers. This chapter also has a brief description about previous toxicity studies.

Chapter-III details the materials and methods used in this study, including preparation of the 3D scaffolding, cell culture conditions, the protocols used for Immunofluorescence staining and quantification of data.

Chapter-IV provides the results obtained from our proposed studies and discusses our data in context to that reported in literature. Finally, chapter-V consolidates the conclusions from our results obtained in this study and provides a roadmap for future studies in this area of research.

CHAPTER II

BACKGROUND

Cadmium, mercury and lead are among the top 10 hazardous agents in the substance priority list of 2011, prepared by the United States Environmental Protection Agency EPA and the Agency for Toxic Substances and Disease Registry (ATSDR) [3]. All humans come across these metals in their daily lives, as they are natural constituents of the environment. There has been a rapid increase in the production, usage and emission of these heavy metals since the beginning of the 19thcentury, coinciding with the western industrial revolution [1]. The issues concerned with heavy metal pollution are continuously reviewed by federal agencies such as the EPA, the ATSDR and the World Health Organization (WHO) [1].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 exceed the allowable limits in many regions of the world [1]. Such an increase in environmental heavy metal concentration poses an alarming concern towards their negative impact on human health.

The U.S. Department of Health and Human Services (DHHS) and the International Agency for Research on Cancer (IARC) and the EPA state that cadmium, mercury and lead are carcinogenic beyond allowable limits, and cause developmental toxicity causing birth defects in newborns[4,5,6].Their systemic toxic effects have been studied extensively in animals, occupationally exposed workers and in exposed children[4, 5,6]. It has been noted that children, infants and the developing embryos are at a higher risk to toxic effects than adults, as their physiology and defense mechanisms are less developed [1,4,5,6,7]. In general, they affect most of the organs in human body, but the nervous system was found to be the most sensitive target [1, 7]. The common neuro-developmental toxic effects caused by cadmium, mercury and lead include altered neuro-behavioral development, neurocognitive deficits, mental retardation, muscular weakness, poor co-ordination of motor and sensory responses and teratogenic effects[7].These elements could easily penetrate the blood-brain-barrier (BBB) and enter the brain extracellular environment, or taken up by the cells lining the BBB. Lot of research has been done in the past to study their various toxic effects, mechanisms and factors involved. Some of the basic information about them is discussed in the following sections.

2.1. Cadmium

Cadmium is a heavy metal found associated with other metals such as copper, zinc and lead in ores [4]. It is released into soil, water and air due to mining and refining of non-ferrous metal ores, combustion of fossil fuels, manufacturing and application phosphate fertilizers, waste incineration (combustion of cadmium containing substances and batteries) and their disposal along with the domestic waste[1,3,7].Cadmium

accumulates in plants and marine organisms due to improper disposal into soil and aquatic ecosystems respectively [4,8].

Highest usage of cadmium (83%) occurs in nickel-cadmium battery production facilities followed by industries producing pigments (8%), coatings and plating (7%), stabilizers for plastics (1.2%) and others (0.8%)[4]. People working at these industries can be at risk of cadmium toxicity[4, 8]. There are strict federal regulations concerned with disposal of industrial wastes containing cadmium which require the wastes to undergo necessary treatments in order to convert toxic forms of the metal into non-toxic ones. According to the EPA, cadmium concentration for natural surface water and ground water is <1 µg/L. But, in water sources near cadmium industries both in current operation and in the past, there is a marked elevation to more than 70 µg/L[4].

The major concern with cadmium is its non-degradable nature, due to which it stays in the environment forever[4, 8]. Cadmium exists in different forms such as oxide, chloride or sulfate, gets transported long distances by wind, and is subsequently deposited in soil or water resource[4]. The heavy metal when taken up by marine organisms, plants, animals, and water resources will be carried over to human beings[4, 8, 10]. Tobacco leaves are known to accumulate the highest levels of cadmium[4]. According to ATSDR, national average blood cadmium level for adults is 0.38 µg/L, whereas for New York City smokers it is 1.58 µg/L (more than 4-fold)[4]. The amount of cadmium absorption from one pack of cigarette is nearly 1-3 µg[4,8, 10].For non-smokers, cadmium contaminated food, water, air or inhalation are the major sources of exposure and the average cadmium intake through food is in between 8 and 25 µg per day[7,8,10].

About 5-50% of inhaled cadmium is absorbed through the lungs[4]. The chance of absorption of cadmium through gastro intestinal tract increases if the diet is deficient in iron and also during pregnancy (because of iron deficiency)[4, 7, 10, 12, 13]. Absorption of cadmium through skin is not reported[4]. Cadmium toxicity mostly affects kidneys and bones[4, 8, 10]. It also affects reproductive, hepatic, immune and hematological functions[4]. Inhalation of high levels of cadmium in humans can cause severe lung damage and death[4]. Inhaling low levels of cadmium over long periods (years), and ingestion results in accumulation in kidneys and bones[4]. In later ages these long term exposures can result in end-stage renal disease, worsens the diabetic effects on renal system and de-mineralizes the bones [4, 8, 10]. It is reported to be involved in prostrate, breast, pancreatic and other type of cancers[4, 8, 10]. It also contributes to motor neuron disease and other nervous system disorders. Evidence also shows that it has a role in neuro-degenerative diseases such as Parkinson and Alzheimer's disease. Further information on the neurotoxic effects of cadmium needs to be investigated [4, 8, 10, 11].

Children absorb more amounts of cadmium than adults and therefore experience higher body burden and toxic effects[7]. Cadmium is capable of passing through the placenta, and thus could affect the fetus in early developmental stages[12, 13]. Animal studies have shown that exposure to high levels of cadmium during pregnancy results in harmful effects in the new born, such as reduced head size with unclear boundaries in brain[4]. Nervous system is a sensitive target in developing embryos and damage to neurons and glial cells have been reported in the developmental toxicity studies[4]. It might contribute to hyperactivity, attention deficits and mental disorders in children [4, 7]. It also affects the birth weight and development of skeleton in the fetus[4,14]. Under some

conditions such as pregnancy, lactation, old age and in certain diseases, there are high chances that the accumulated heavy metal may re-enter the systemic circulation [12, 13].

Cadmium increases the oxidative stress in neural cells by acting as a catalyst in the formation of reactive oxygen species, and increases lipid peroxidation [4,11]. It depletes anti-oxidant enzymes such as glutathione peroxidase and superoxide dismutase and sulfhydryl groups on various proteins[4]. It replaces the calcium, zinc, iron, copper and selenium ions which are involved in cellular metabolic pathways [4, 11].

2.2 Mercury

Mercury naturally exists as metallic mercury in coal and gold ores, mercuric sulfide (cinnabar ore), mercury chloride and methyl mercury[5,1]. It is broadly categorized as metallic mercury, and also called as elemental mercury, inorganic mercury and organic mercury[1,5]. Metallic mercury exists as a liquid at room temperature, and elevation in temperature causes an increase in the release of vapors from the liquid mercury[5]. Inorganic forms of mercury are chloride, sulfate and oxides forms of the metal[1,5]. When mercury combines with carbon it is called organic mercury[1,5]. Many micro-organisms can convert elemental and inorganic mercury into organic forms which are highly potent[1,5].

Mercury is used in the production of chlorine gas, caustic soda, thermometers, barometers, batteries, fluorescent light bulbs and electrical switches[1,5]. According to the ATSDR, 80% of mercury emissions into the air in vapor form are primarily due to fossil fuel (coal) combustion, production of cement, mining, smelting and solid waste incineration[1,5]. These vapors can condense and deposit over soil, water, and other

substances which may come in contact with it[1,5]. Fertilizers, fungicides, municipal solid waste contribute about 15% of pollution and 5% is from contamination of natural water resources by industrial waste water[5].

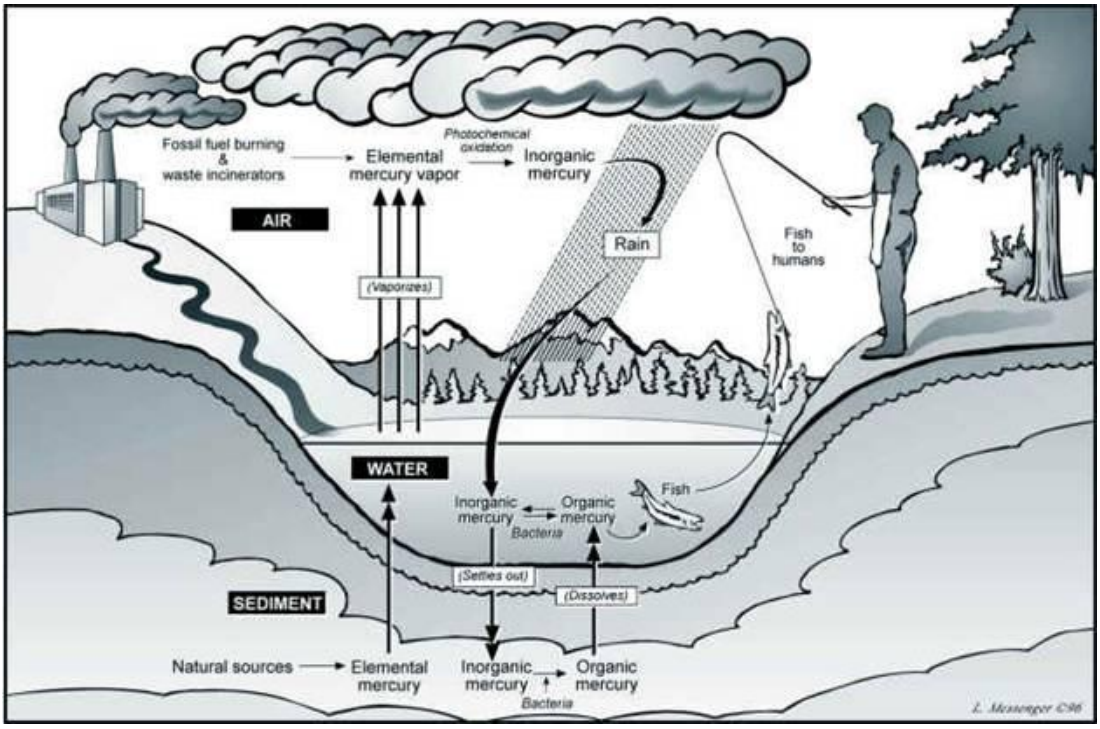


Figure 2.1 Schematic diagram showing bio-accumulation of mercury[22].

In some places of Latin America and Asia, mercury is used to treat several ailments. For example, mercury chloride is used in topical agents as an antiseptic agent[1]. Mercuric sulphide and oxide are used in paints and tattoo dyes[5]. Inorganic mercury forms have been used in cosmetic products, laxatives, teething powders and worming medications[1]. As toxic effects of mercury became more evident, the heavy metal is being refrained from use in some of these medications[1]. But Thiomersal, a phenyl mercuric nitrate, is still being used as a preservative in some medications[17]. Until recently, dental amalgams contained nearly 50% metallic mercury and release nearly 3-17 □ g mercury

per day, depending on the number of dental fillings[5, 16]. According to The FDA, most people of average weight are exposed to 3.5 µg of mercury per day[5].

Potential sources of mercury exposure are inhalation, ingestion of contaminated water and food, dermal contact, dental amalgams, accidental spillage from batteries, thermometers, switches and other appliances[1,5]. Nearly 80% of inhaled mercury is absorbed into the systemic circulation which rapidly enters the vital organs and remains there for years[1,5]. As a consequence of this accumulation, the heavy metal is reported to cause systemic toxic effects[1,5]. Acute exposure to high concentrations can be lethal and at chronic exposure level brain and kidney damage are more prominent[5].

Mercury can affect many different areas of brain and their functions resulting in impaired learning and cognitive abilities, vision, hearing, muscle coordination, memory in children[5,15]. Many children were affected by Acrodynia in the past due to usage of teething powder containing mercury[5,15]. Mercury passes to developing embryo through placenta during gestation period from affected mother and could cause birth defects[5, 12, 13, 15, 16]. Infants can be exposed to mercury during lactation if the mother has been previously exposed to the heavy metal. According to a recent study, mercury exposure through dental amalgam in pregnant women and use of vaccines containing thiomersal has increased prevalence of autism in children. [12, 13, 14]

The major mechanism of toxicity is due to binding of the heavy metal to the sulfhydryl groups of proteins. These proteins are mainly present in extra and intracellular membranes and organelles[5]. By targeting these proteins, mercury inactivates various enzymes, alters structural proteins, ceases transport processes and imposes changes in

cell permeability[5]. Binding of mercury to amino and carboxylic groups is also observed to be a mechanism of toxicity[5].Mercury increases oxidative stress, obstructs microtubule formation, interferes in cellular activities such as DNA replication, protein synthesis, calcium homoeostasis, and synaptic transmission. These effects may occur individually or in combination[5].

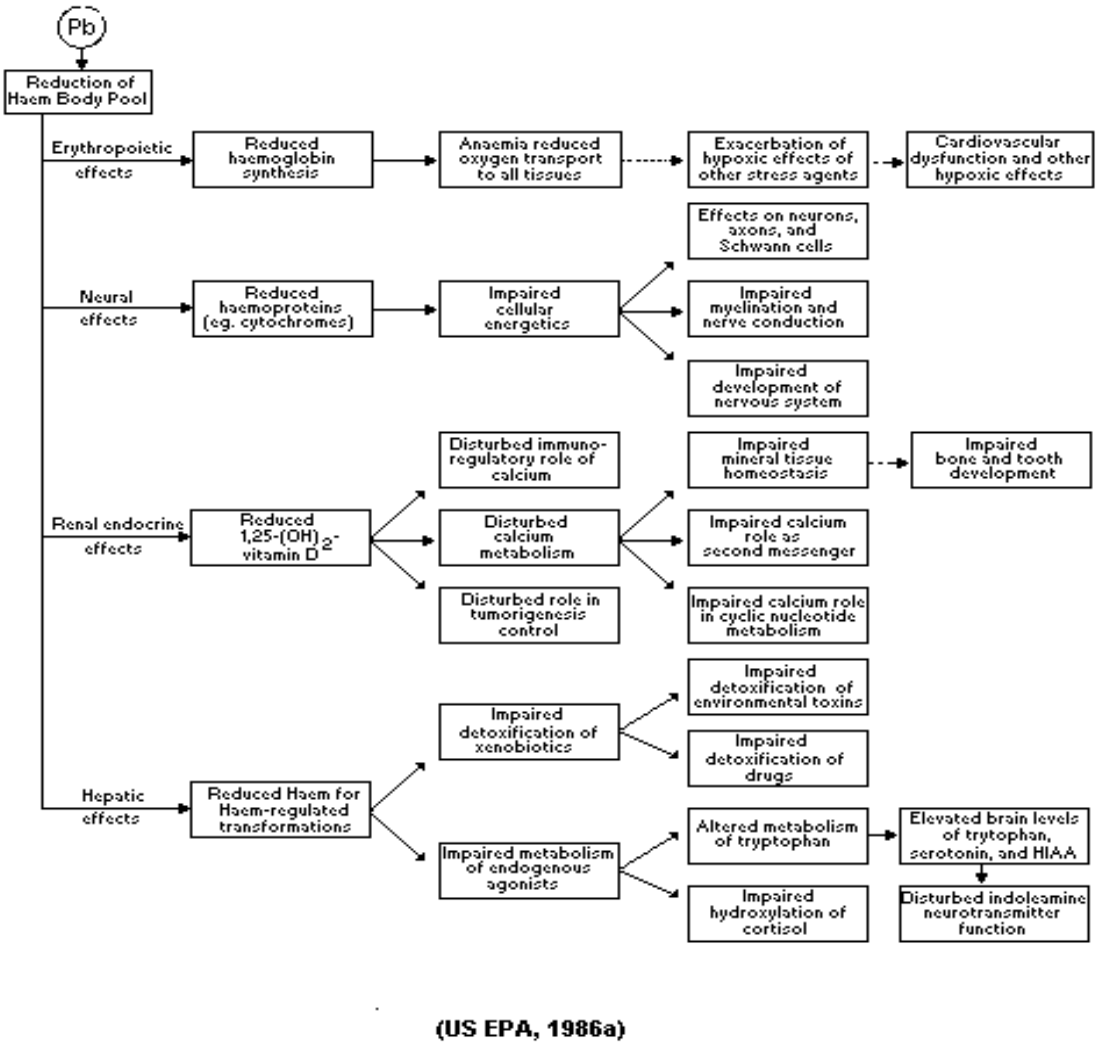
2.3 Lead

Lead is a bluish-gray metal naturally existing in the earth's crust in different ores[6]. Lead is resistant to corrosion when exposed to air and water[6]. It has been found in toxic concentrations at ~1272 NPL listed sites[6]. Many other places still need to be investigated for the occurrence of lead. It is used in manufacturing of pipes, storage batteries, weights, bullets and ammunition materials, cable covers and sheets used for protection from radiation[1,6]. The highest usage of lead is in the production of batteries for automobiles[6]. It is also used in the production of ceramics, paints and dyes[6]. For years lead was used as a constituent in gasoline before it was banned in 1996[1,6]. Lead emissions in the United States during 1979 were estimated to be 94.6 million kilograms into the air[6]. Though the use of lead has been banned in many applications, being a non-degradable metal it still exists in the atmosphere.[1,6].The secondary source for lead emissions is due to paints containing lead[1,6]. Withering and chipping of lead based paints from old houses and building is also the source of lead release to the soil[6]. Lead particles can travel in air and settle on soils, surface waters and crops[1,6].

Over the last three centuries, environmental levels of lead have increased by 1000-fold due to human activity[6]. The major way to lead exposure is due to inhalation and swallowing dust near roadways, older houses, old orchards where lead containing

pesticides were used, power plants, landfills, incinerators and hazardous waste sites[1,6]. The secondary sources are contaminated food, air and water[1,6]. Cigarette smoke can also contribute to the lead exposure[6]. Pipes made of lead or the fittings and soldering made with the metal in water lines can release toxic amounts of lead into the water[6]. Soil sediments in the water bodies may contain lead if the soil from contaminated areas is washed into them[6].

Exposure to toxic levels causes lead encephalopathy in adults and children[6, 7, 19]. Although the toxic effects are systemic, nervous system is the sensitive of all organ systems[6, 7, 18,19]. It causes elevation in blood pressure and anemia[6,7,18]. Large amounts of lead is absorbed into the systemic circulation which further gets transported to vital organs such as brain, kidneys, heart, liver and bones and accumulates[6]. Sometimes it can re-enter the blood stream under some conditions like pregnancy, breast feeding, old age, bone fracture and osteoporosis[6,12]. In pregnant woman high levels of exposure are reported to cause abortion and adverse birth defects[6, 13].



(US EPA, 1986a)

Figure 2.2 Schematic presentations of multiple toxic effects of lead. [6]

Children are more vulnerable to the lead toxicity compared to adults[6, 7,19]. No safe blood concentration has been reported in children. Lead toxicity affects the developmental stages of fetus if the mother has higher concentrations of lead in her blood stream[6,12,13]. Lead is capable of passing through placenta and is also secreted in the breast milk[6,12,13]. Exposure during gestation can result in reduced head circumference, low birth weight and other abnormalities[6]. Exposed children may suffer from kidney

damage, colitis, muscle weakness and brain damage which can be fatal[6, 7, 19]. It is reported to cause intellectual deficits, poor learning abilities and retardation[6,7,19].

Lead affects the nervous system by mimicking the action of calcium ions physiologically[6]. As calcium is involved in many signaling pathways it affects many cellular functions[6]. One of the important groups of enzymes called protein kinase C (PKC) is involved in many processes which play an important role in synaptic transmission such as synthesis of neurotransmitters, ligand-receptor interactions, conductance of ionic channels and dendritic branching[6]. The changes in PKC enzyme causes decreased expression of glial fibrillary acidic protein (GFAP) specific for astrocytes[6]. Astrocytes along with the endothelial cells form the blood brain barrier which restrains most of the materials entering the brain[20,21]. Therefore, the permeability of blood brain barrier is increased in the developed nervous system due to lead exposure[6]. The γ-isoform of PKC family is a potential target by lead[6]. Most of this enzyme is present in membrane bound vesicles in adult cells, but in developing stages these enzymes occur in cytosol which makes fetus vulnerable to toxicity[6]. Lead modulates the structure of another enzyme calmodulin which plays a key role in cAMP messenger pathways[6]. Lead also substitutes for zinc and affects the enzymes and cellular functions that depend on zinc as a co-factor[6]. This affect causes difference in the expression of myelin basic protein in the nervous system which is specific for oligodendrocytes[6]. These cells secrete myelin, which prevents the leakage of action potential, coordinate the sensory and motor impulses. Lead disrupts neurotransmitters systems especially cholinergic, dopaminergic and glutamatergicsystems[6]. Dopamine is involved in cognitive functions of brain such as learning[6]. Acetylcholine is involved in

various processes including memory[6]. Lead toxicity on these systems is suggested to be the possible reasons for mental retardation, can be a contributing factor for neuro-degenerative diseases like Parkinson's and Alzheimer's disease.[6]

2.4. Neural stem cells

Neural stem cells are a class of self-renewing, multi-potent, neural progenitor cells which have the capacity to differentiate into major types of neural and glial cells of the central nervous system (CNS) [21, 23]. Studies show that the NSCs are more prevalent at early stages of the formation of brain in embryo [21, 23]. They divide by symmetric and asymmetric divisions to give rise to new NSCs at this stage. As the development progress the NSCs decrease in number and get committed to restricted progenitor cells and differentiated cells. They decline to significant low level at the end of gestation as the formation of brain completes. Part of the remaining cells helps in further development of nervous system after the birth and during childhood. They are also present in some parts of adult brain such as neurogenic regions, sub-ventricular zone, hippocampus and spinal cord[23]. Adult neural stem cells help in repairing the damage caused by injury or disease conditions to limited extent[21].

The type of lineage they differentiate into is dependent on the growth factors and signaling molecules[23]. Different concentrations of fibroblast growth factor (FGF), bone morphogenetic proteins (BMP) and noggin regulate the cell proliferation and differentiation from neural induction through adulthood. In early embryonic neurogenesis, noggin inhibits BMP to allow the proliferation of NSCs to neurons, but in later stages BMP regulates the differentiation and favors the formation of astrocytes. FGF helps in proliferation of NSCs and formation of neurons at very low concentrations

during early development but later it inhibits the differentiation. Changes in signaling pathways can cause difference in the NSC behavior. The exact mechanisms and signaling pathways of NSCs are still under investigation.

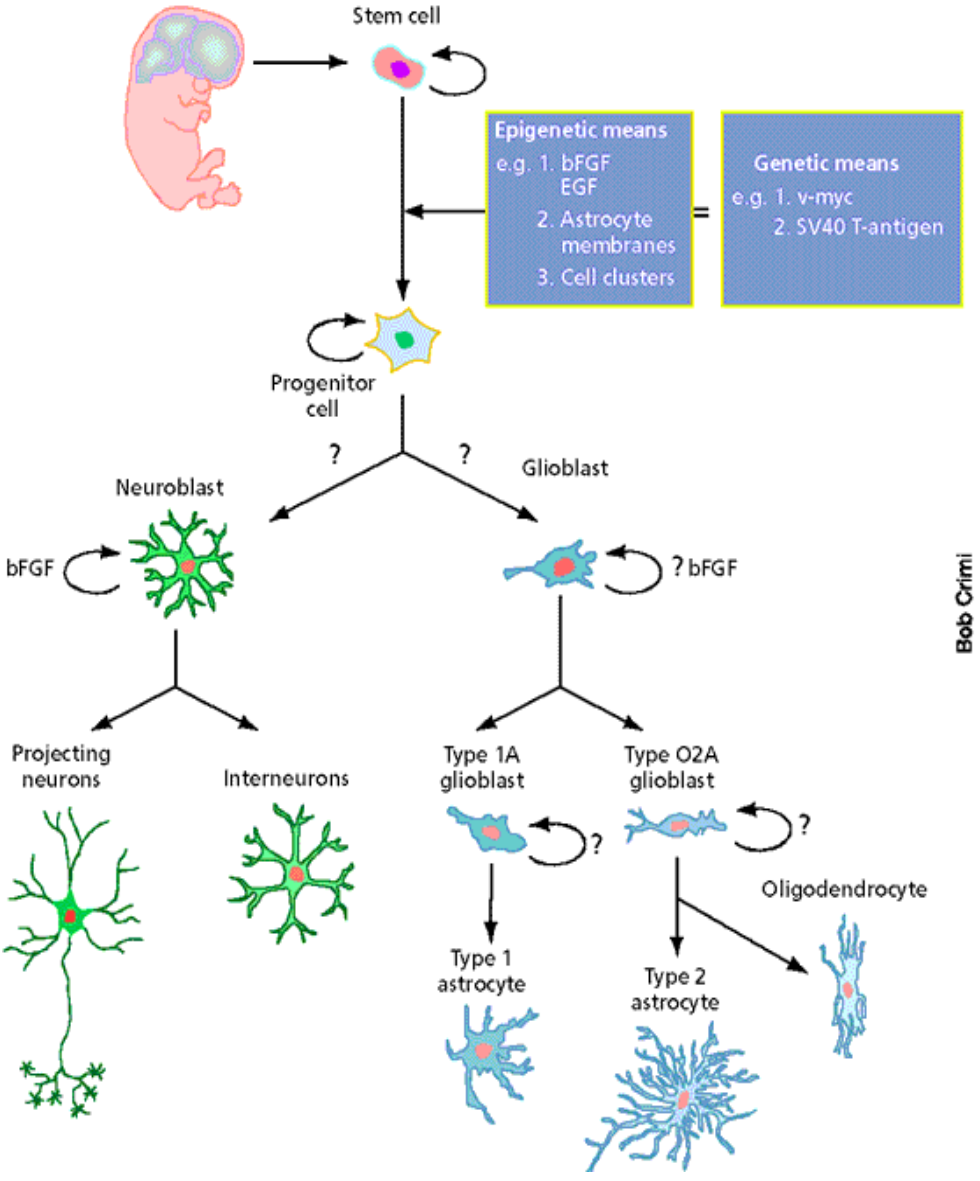


Figure 2.3 Schematic of NSC differentiation mechanisms [20].

CHAPTER III

MATERIALS AND METHODS

3.1 Preparation of Neural Stem cell (NSC) Media

Two types of media were use, one media containing b-FGF (non-differentiating NSC media) and the other without b-FGF (differentiating NSC media). To make 20 ml of differentiating media, we used

- 20 ml DMEM w/ Na-pyruvate (Gibco -cat # 10313-021)
- 20 µl 100x L-glutamate (Gibco -cat # 15039-019)
- 200µl 100x N2 Supplement (Gibco –cat#17502-048)
- 400 µl 100x B27 Supplement (Gibco -cat # 17504-044)
- 200 µl 100x NAC (N-acetyl cysteine) (Sigma A9165)

The final solution was sterile filtered using 0.02 µm filter. All the ingredients remained the same within non-differentiating media, except the addition of 20 µL of 1000× (10 µg/ml) b-FGF (Gibco -cat # 13256-029) to maintain the cells undifferentiated [31].

3.2 Preparation of type I collagen solution

The collagen solution at 2 mg/ml concentration was prepared by using collagen type-I (9.46 mg/ml; BD biosciences).To make 10 ml of collagen solution, 1 ml of PBS, 2.114 ml of collagen, 48.6 µl of 1N NaOH and 6.84 ml of DI water was used[32].

The solution was placed over ice throughout the experimental use.

3.3 Preparation of metal solutions

The 100 µM metal solutions (master batches) were prepared for each metal by dissolving calculated amount of specific metal in water and lower concentrations prepared by serial-dilution method.

3.3.1 Preparation of Cadmium Chloride ($CdCl_2$) solution

A 0.01 M cadmium chloride solution was prepared by dissolving 183 mg of cadmium chloride (anhydrous, 99.99%, Alfa aesar) in 100 ml of de-ionized sterile water. A 1ml of 0.01M cadmium chloride solution was diluted to 100 ml resulting in 100 µM solution, which was used as stock solution for further dilutions. The concentrations of 10 µM, 1 µM, 0.1 µM, 0.01 µM, 1 nM, 0.1 nM and 0.01 nM solutions were prepared by serially diluting the stock solution with culture media.

3.3.2 Preparation of mercury chloride solution

A 0.01 M mercury chloride solution was prepared by dissolving 272 mg of mercury (II) chloride (98+%, Alfa aesar) in 100 ml of de-ionized sterile water. A 1ml of 0.01 M mercury chloride solution was diluted to 100ml which gave 100 µM solution which was used as stock solution for further dilutions. The concentrations of 10 µM, 1

µM, 0.1 µM, 0.01 µM, 1 nM, 0.1 nM and 0.01 nM solutions were prepared by serially diluting the stock solution with media

3.3.3 Preparation of Lead acetate solution

A 0.01 M lead acetate solution was prepared by dissolving 379 mg of lead (II) acetate trihydrate (99%, Alfa aesar) in 100 ml of de-ionized sterile water.

Aliquot of 0.01 M lead acetate solution was diluted to 100 ml which gave 100 µM solution which was used as stock solution for further dilutions. The concentrations of 10 µM. 1 µM, 0.1 µM, 0.01 µM, 1 nM, 0.1 nM and 0.01 nM solutions were prepared by serially diluting the stock solution with media.

3.4 Culturing the rat neural stem cells

Embryonic rat neural stem cells were obtained from Neuracell, NY. The cells were shipped in a cryovial, frozen on ice. The cells were thawed by immersing the vial in 37 °C water bath and transferred to a sterile 15ml tube. Four milliliters of pre-warmed non differentiating NSC media was added drop-wise to the cell suspension, while rotating the tube to ensure proper mixing. Then the suspension was centrifuged at 10.6×*g* for five minutes[31]. The supernatant was removed and 4ml of pre-warmed non differentiating NSC media was added to the cell pellet at the bottom of the tube[31].

The pellet was re-suspended by pipetting up and down gently with a micropipette. The cell suspension was distributed equally into T-25 flasks, pre-coated with poly-L-lysine (Sigma Aldrich)[31]. The cells were cultured for 4 to 5 days or until the formation of neurospheres was noticed. Media was changed every day in order to provide a

continuous non-differentiating environment (b-FGF).After this period cells were ready for experimental use[31].

On the day of seeding, all the media from culture flasks was removed and 3 ml of trypsin-EDTA was added to detach the cells from the surface[31]. It was allowed to act for 5 minutes, and we observed in the microscope to ensure cell detachment. The cell suspension was transferred to a 15 ml tube and centrifuged at 10.6 g for 5 min. The supernatant was removed and the pellet was suspended in 2 ml of differentiating media (without b-FGF)[31].

3.5 Cell cultures

Cells were seeded within a 3D collagen matrix. In order to create a 3D matrix, collagen-I solution 2 mg/ml was used. 30 µl of collagen-I solution was added to the wells and was polymerized at room temperature for 30 minutes on an even surface. It was made sure that the layer of collagen was thin enough to view the cells while imaging. Then equal volumes of cell suspension were added on the top of collagen layer. Another 30 □ l of collagen-I solution was added on top of the cells and was allowed to polymerize for 30 minutes. The media containing different concentrations of metals solutions were added on top of the collagen layer within respective culture wells (n =3 wells per metal ion concentration).

3.6 Phalloidin staining

We stained the cells with 488 phalloidin (Life Technologies) for measuring the neurite outgrowth. The cells were cultured in 3D collagen matrix for various durations for these experiments: 1, 4, 7 and 14 days. At the end of the culture period, the cells were

fixed by removing the media from all the wells and washing 3 times with PBS[33]. Then, 3.7% Para-formaldehyde solution (PFA) was added and incubated for 10 minutes[33]. The PFA solution was removed from the wells, and washed 3 times (5 minutes per wash) with PBS. Then 0.1% triton X-100 was added and was allowed to act for 5 minutes[33].The wells were washed three times with PBS and 1% bovine serum albumin (BSA) was added to the wells and was allowed sit for 30 minutes[33]. BSA was removed, phalloidin solution was added to the wells in the dark, and the well plate was mounted on the microscope for observation after 30 minutes[33]. Images for neurite outgrowth at different concentrations of different metals and controls were captured using Zeiss Axiovert A1 fluorescent microscope with a 40× objective. From the images the neurite outgrowth was quantified using Image-J software.

3.7. Live-dead assay

A live/dead viability/ cytotoxicity kit was purchased from Molecular Probes. The staining solution was prepared by adding 20 µl of 2mM EthD-I (component B) solution to 10 ml of warm D-PBS[34].5 µl of supplied 4mMcalcein AM in DMSO (component A) was added to the EthD-I solution and vortexed[34]. Media from all the wells was removed and the wells were washed with PBS for 3 times allowing 5 minutes for each wash[34]. Then the staining solution was directly added to the wells and allowed it to act for 30 minutes[34]. The wells were washed 3 times and then observations were made using the Zeiss Axiovert A1 fluorescent microscope. The number of live and dead cells was counted in the green and red channels respectively. Average number of cells and percentage survival was determined accordingly.

3.8. Immunofluorescence staining for TUJ1, GFAP and MBP2

3.8.1 TUJ1

It is a neuron-specific class III beta tubulin marker expressed by neurons[34].Anti-beta III tubulin [EP1331Y,Abcam)] rabbit monoclonal antibody was used as a primary antibody to stain for TUJ1[34].. Goat serum was used as a blocking agent to avoid nonspecific binding[34].Goat anti rabbit IgG-FITC (Santa Cruz Biotechnology) was used as a fluorescein secondary antibody[34].

3.8.2 GFAP

GFAP is Glial fibrillary acidic protein, specific in astrocytes[34].. Anti-GFAP rabbit polyclonal antibody (Abcam) was used as a primary antibody to stain for GFAP[34].. Goat serum was used as a blocking agent and goat anti rabbit IgG-FITC (Santa Cruz Biotechnology) was used as a fluorescein secondary antibody[34].

3.8.3 MBP

Myelin basic protein **(**MBP) is expressed by oligodendrocytes[34].. Rat anti – myelin basic protein monoclonal antibody (Abcam) was used as a primary antibody to stain for oligodendrocytes[34].. Goat serum was used as the blocking agent to avoid non-specific binding[34].. Goat anti-rat IgG –FITC (Santa-Cruz Biotechnology) was used as the fluorescein secondary antibody[34].

3.8.4 Primary staining

The cells were cultured for 7 and 14 days. At the end of the culture period, the cells were fixed by removing the media from all the wells and washing 3 times with PBS[34].. Then, 3.7% Para-formaldehyde solution (PFA) was added and incubated for 10

minutes[34]. The PFA solution was removed from the wells, and washed 3 times (5 minutes per wash) with PBS. Then 0.1% triton X-100 was added and was allowed to act for 5 minutes[34]. The wells were washed three times; blocking solution containing 0.1%triton X-100, 5% goat serum was added to the wells and incubated for 30 minutes[34]. The blocking solution was removed from the wells and the primary antibody solution containing 0.1% triton X-100, 5% goat serum and primary antibody (1:100) was added and stored at 4 °C overnight[34].

3.8.5 Secondary staining

The next day primary antibody solution was removed from the wells and Washed three times (10 minutes per was) with PBS. Secondary antibody solution was added containing 0.1% triton X-100, 5% goat serum and secondary antibody (1:400) and incubated for 30 minutes[34]. Then the wells were washed three times (10 minutes per wash) with PBS. DAPI was added and observations were made using the Zeiss axiovert A1 fluorescence microscope.

3.9 Immunofluorescence staining for HB9 and TH

3.9 .1 HB9

It is a specific marker expressed by motor neurons. Goat HB9 (H-20) antibody (SC -22542), by Santa Cruz biotechnology was used as the primary antibody to stain for motor neurons. Donkey serum was used as the blocking agent and donkey anti goat IgG-FITC, (SC-2024), by Santa-Cruz biotechnology was used as the fluorescein secondary antibody. The cells were cultured for day-7 and day-14 in different plates. At the end of culture period, all the media from the wells was removed and the wells were washed with

PBS for 2-3 times allowing 5-10 minutes for each wash. Then the cells were fixed using ice cold 3.7% Para-formaldehyde solution for 10 minutes[35]. The wells were washed with ice cold PBS 3 times for every 10 minutes to ensure all the Para- formaldehyde was washed off[35]. Then the blocking solution was added containing 0.1% triton X-100 and 5% donkey serum and was soaked in it for 20-30 minutes[35] The blocking solution was removed (washing was not required at this stage) followed by the addition of primary antibody solution containing 0.1% triton X-100, 5% donkey serum, HB9 antibody (1:100)[35]. The plate was stored at 4°C overnight. Next day the primary antibody solution was removed and the wells were washed 3 times allowing 5-10 minutes of washing time[35]. Then the secondary antibody solution was added containing 0.1% triton X-100, 5% donkey serum and donkey anti-goat IgG-FITC (1:400) (SC2024), by Santa Cruz biotechnology and was allowed to act for 30 minutes[35]. The secondary antibody solution was removed, the wells were washed 2-3 times, a drop of DAPI was added and observations were made using Zeiss axiovert A1 Fluorescence microscope.

3.9.2 Immunofluorescence staining for TH

Tyrosine Hydroxylase (TH) is a specific marker expressed by Dopamine producing neurons. Anti-TH (AB152, Millipore) was used as the primary antibody to stain for motor neurons[35]. Goat serum was used as the blocking agent and goat anti rabbit IgG-FITC (sc 2012) by Santa-Cruz biotechnology was used as the fluorescein secondary antibody[35]. The cells were cultured for day-7 and day-14 in different plates. At the end of culture period, all the media from the wells was removed and the wells were washed with PBS for 2-3 times allowing 5-10 minutes for each wash[35]. Then the cells were fixed using ice cold 3.7% para-formaldehyde solution for 10 minutes[35]. The

wells were washed with ice cold PBS 3 times for every 10 minutes to ensure all the para-formaldehyde was washed off. Then the blocking solution was added containing 0.1% triton X-100 and 5% goat serum and was soaked in it for 20-30 minutes[35]. The blocking solution was removed (washing was not required at this stage) followed by the addition of primary antibody solution containing 0.1% triton X-100, 5% goat serum, anti-TH (1:100)[35]. The plate was stored at 4°C overnight. Next day, the primary antibody solution was removed and the wells were washed 3 times allowing 5-10 minutes of washing time[35]. Then the secondary antibody solution was added containing 0.1% triton X-100, 5% goat serum and goat anti-rabbit (1:400) (SC2012), by Santa Cruz biotechnology and was allowed to act for 30 minutes[35]. The secondary antibody solution was removed, the wells were washed 2-3 times, a drop of DAPI was added and observations were made using Zeiss axiovert A1 Fluorescence microscope.

3.10 Immunofluorescence Analysis

The total number of cells was counted in DAPI channel. The number of cells expressing TUJ1, GFAP, MBP2, HB9 and TH markers was counted separately in each well. The average values and percentage differentiation of neural stem cells into neurons, astrocytes, oligodendrocytes, motor neurons and dopamine producing neurons were determined.

3.11 Statistical Analysis

All the data obtained from each experiment was analyzed statistically using sigma plot, one-way ANOVA and student's t-test in Microsoft Excel®. The variance between data was considered significant for $p < 0.05$.

Marker	Specificity	Primary antibody	Host	Serum	Secondary Antibody
Beta III tubulin	Neurons	Monoclonal beta III tubulin antibody	Rabbit	Goat	Goat anti rabbit
Glial fibrillary acidic protein	Astrocytes	polyclonal GFAP antibody	Rabbit	Goat	Goat anti rabbit
Myelin basic protein	Oligo-dendrocytes	Monoclonal MBP2 antibody	Rat	Goat	Goat anti rat
HB9	Motor neurons	HB9 antibody	Goat	Donkey	Donkey anti-goat
Tyrosine hydroxylase	Dopamine producing neurons	Anti-TH antibody	Rabbit	Goat	Goat anti rabbit

Table -I Summary of Antibodies used to stain various markers.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter discusses the results from all the experiments outlined in Chapter-III. There were two sets of heavy metal concentrations used throughout this study which are indicated as higher concentrations (0.01-10 µM) and lower concentrations (0.01-1 nM). We cultured the cells for 14 days within 3D collagen scaffolds, and quantified the viability, neurite outgrowth and differentiation of NSCs at different time intervals and compared to control cultures which did not receive any metal solution added to the media.

4.1 Live/dead assay at higher concentrations

The survival of NSCs exposed to higher concentrations of metal ions is shown in Figs. 4.1 and 4.2.From these results we found that on day-1, there was no statistical difference between the control and the three metals at concentrations 0.01 µM, 0.1 µM

and 1 µM. However, at 10 µM concentrations of three types of metals, there was a statistically significant decrease in the cell survival compared to control. The percentage of surviving cells in control was 83% and at 10 µM of cadmium, mercury and lead it was 77% (p = 0.039), 76% (p= 0.036) and 77% (p = 0.04) respectively. By day4, the survival rate was ~81% within controls, but there was an overall dramatic decline in NSC survival to less than 15% at all the higher concentrations independent of the metal type. These results suggest that cadmium, lead and mercury concentrations between 0.01 µM and 10 µM are detrimental to neural stem cell survival. We further studied the cytotoxicities of metal ions at concentrations lower than 0.01 µM. Similar cytotoxic effect has been reported in case of cadmium by Culbreth et al., where they observed that the neural stem cell viability decreased by 50% when exposed for6 h to 10 µM concentration, and similar decline at 0.001 µM-100 µM concentrations when exposed for 24 h[24,25]. In another study by Zychowicz et al., there was a significant decrease in the neural stem cell viability between the concentration 0.06 µM and 1 µM after a 48 h exposure to methyl mercury[27,26]. Their study contained a different pattern of growth factors and supplements in their experiments. Our study was not supplied with any growth promoting factors. Huang et al reported that lead is inhibiting the cell viability at concentrations 0.1 µM to 100 µM in floating neurospheres of neural stem cells at different time exposures[28].

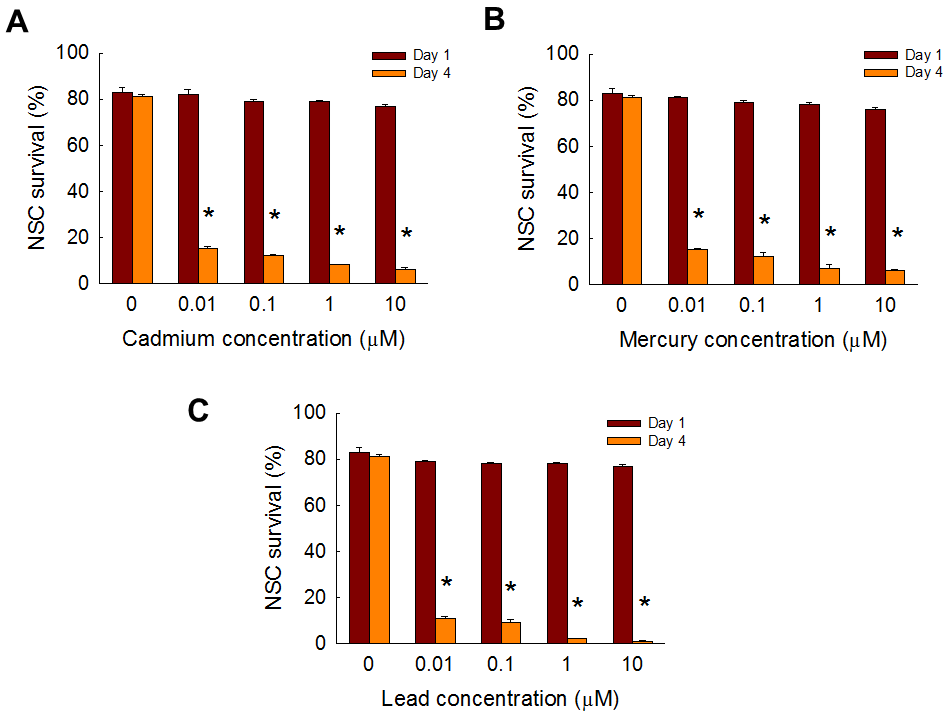


Figure 4.1 Percentage survival of NSCs at day-1 and day-4. The data shown reflects the mean ± standard error (n=3/ case).Percentage of neural stem cells surviving in the 3D cultures supplied with cadmium chloride (A), mercury chloride (B), or lead acetate (C)**.**

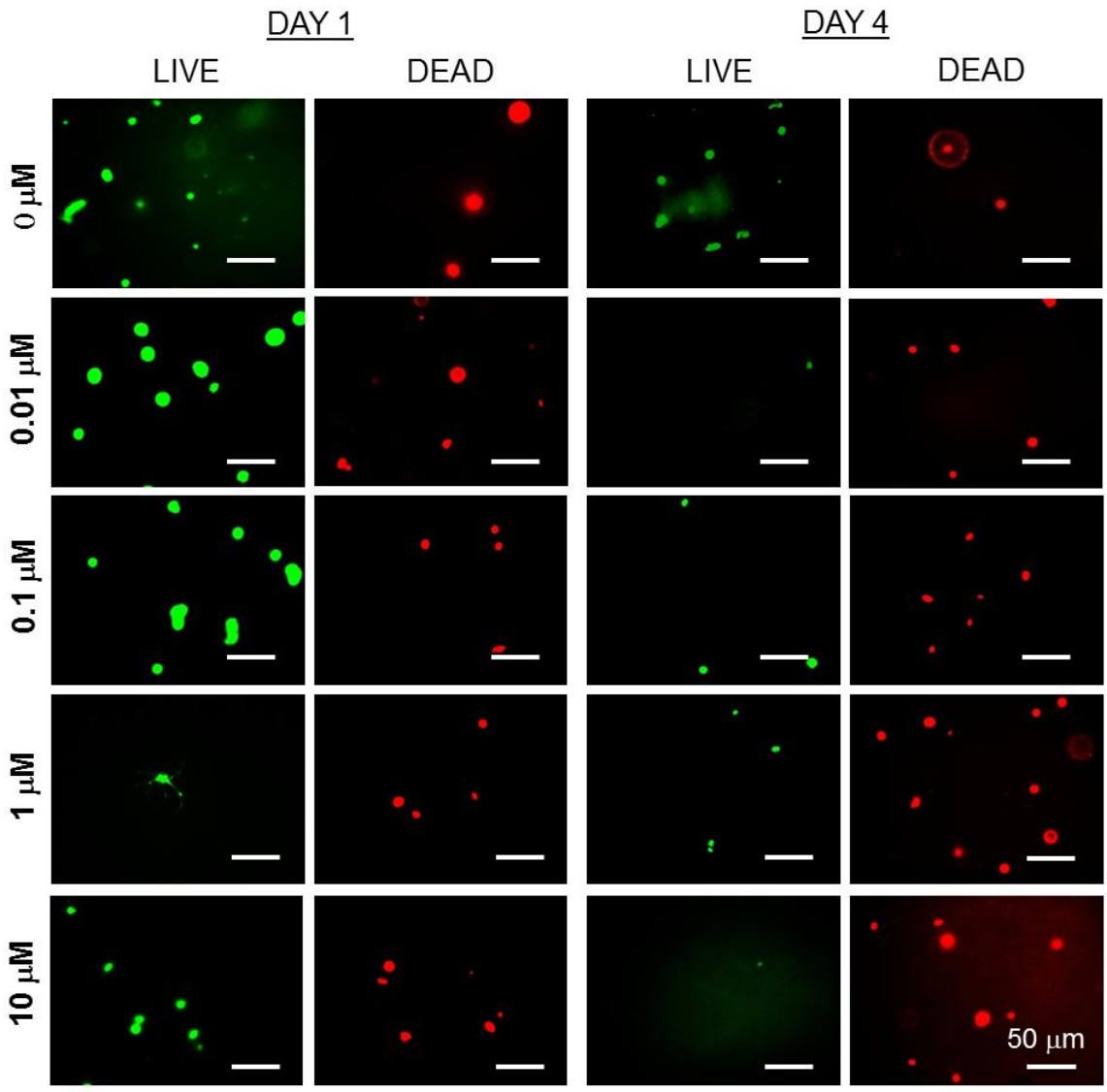


Figure 4.2 Immunofluorescence images showing live and dead cells at higher concentrations on day-1 and day-4 in presence of cadmium (n=3/case, 40× magnification, and scale bar = 50 μm). Images from Live/Dead assay in the presence of mercury and lead are shown in Appendix A

4.2 Live/ dead assay at lower metal ion concentrations:

The results from the NSC survival assays at lower metal ion concentrations are shown in Figs.4.3 and 4.4.On day-7, there was no statistically significant difference in cell survival between control and cadmium-supplied NSCs. Similar trends were noted in the presence of mercury, except at 1nM concentration where a decrease of 12% in NSC survival was noted compared to controls (p = 0.03). By day 7, lead had no significant cytotoxic effect at 0.1 nM and 1 nM compared to controls. On day-14, a decrease in cell viability with an increase in metal ion concentration was observed irrespective of the metal type studied. Cadmium showed nearly similar rate of survival at all concentrations (0.0 1nM, 0.1 nM, 1 nM), with an overall significant decrease compared to controls. In controls, the percentage of surviving cells was ~75% and the average survival rate in 0.01 nM, 0.1 nM and 1 nM was 42% (p = 0.0002), 36% (p = 0.00006) and 37% (p = 0.000015), respectively. Similarly, there was a significant decrease in the cell survival with increasing mercury concentration. The cell survival declined to 27% at 1 nM, 34% at 0.1 nM and 45% at 0.01 nM. Similar trends were noted in lead supplemented cultures with increasing ion concentration. From these results, we conclude that: (a) the toxic effects of these heavy metals increased with an increase in exposure time. On day-7 the cell survival at 0.01 nM concentrations of all metals were similar to controls, but they decreased significantly by day-14. (b) The cell viability decreased as the concentration of heavy metal increased. At 1 nM concentration, all metals induced a majority of cell death. Not much information is available in literature about the toxic effects of cadmium, mercury and lead at concentrations lower than 1 µM.

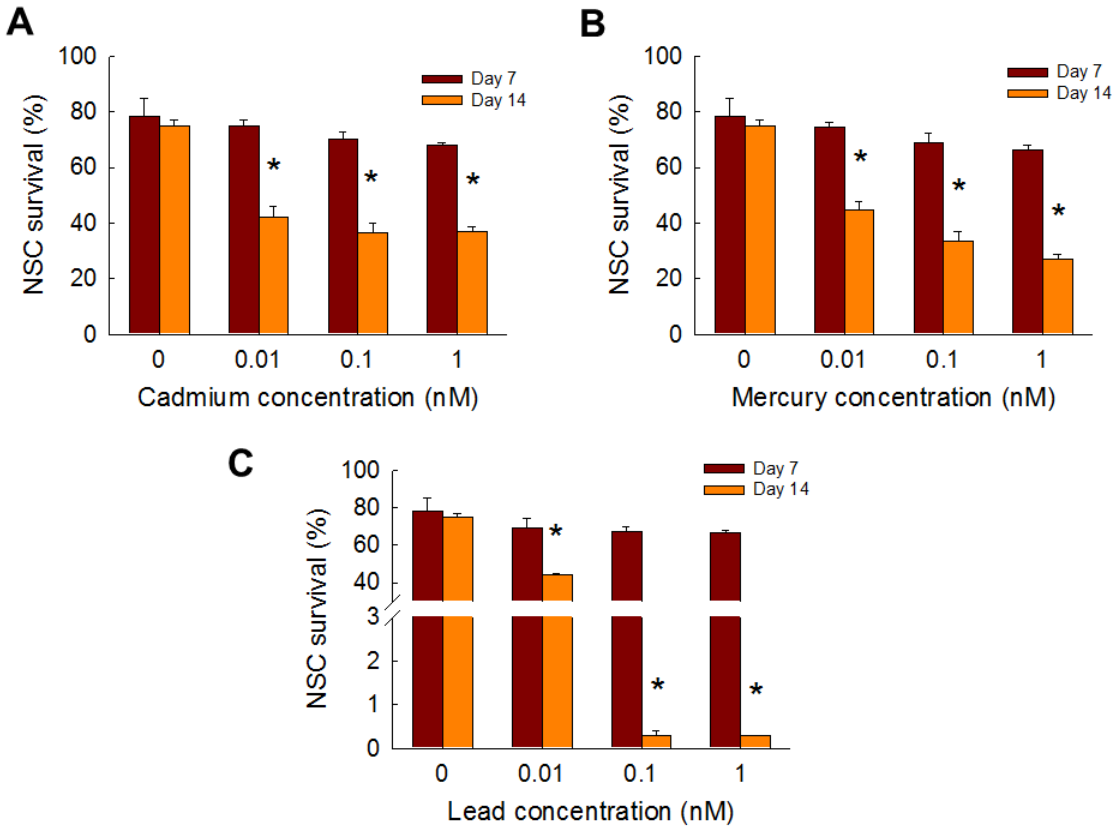


Figure 4.3 Percentage of neural stem cells surviving on day-7 and day-14 at lower concentrations. The data shown reflects the mean ± standard error (n=3/ case). Percentage survival of neural stem cells in presence of cadmium (A), mercury (B), and lead(C).

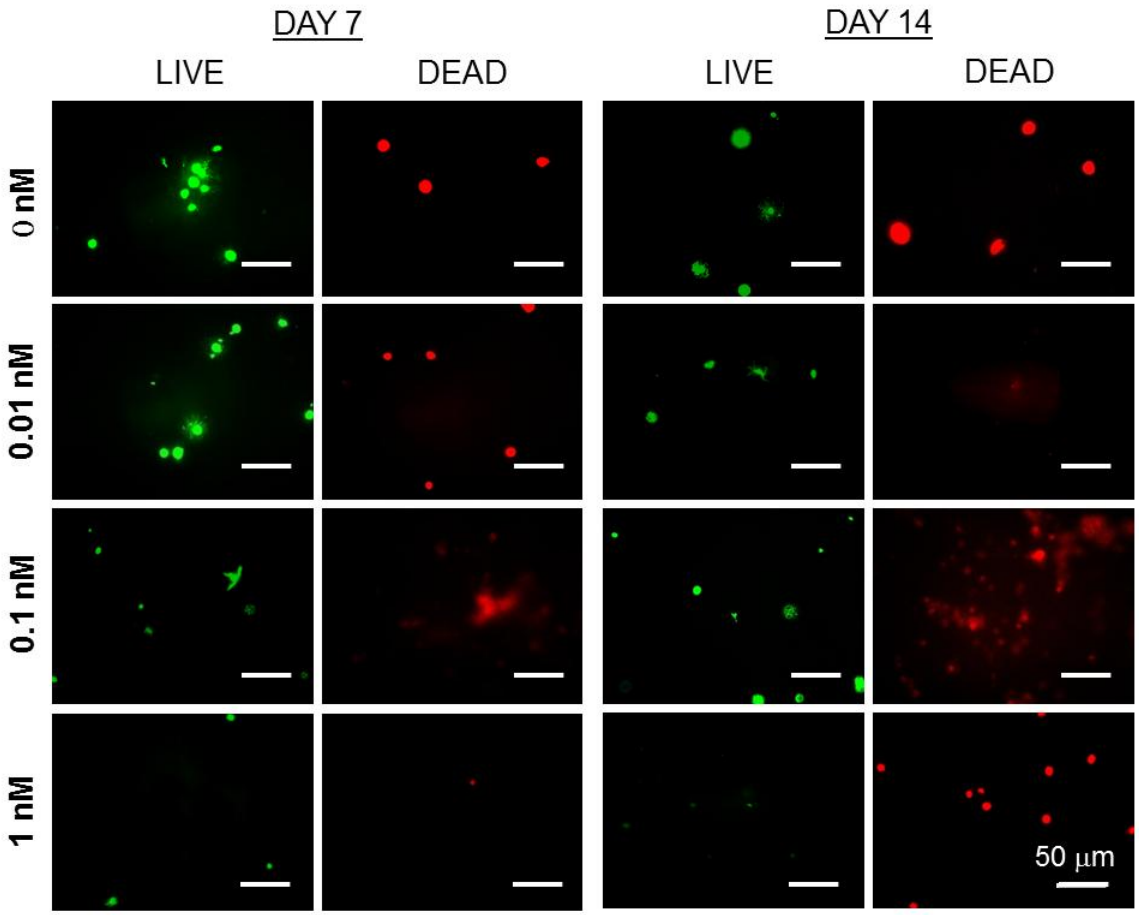


Figure 4.4 Immunofluorescence images showing live and dead cells at lower concentrations on day-7 and day-14 in presence of cadmium (n=3/case, 40× magnification, scale bar = 50 µm). Images for Live/ Dead assay in presence of mercury and lead are shown in Appendix A**.**

4.3 Neurite outgrowth at higher concentrations

The neural stem cells were cultured for day-1 and day-4 under given conditions and stained with phalloidin. It extensively stained for the actin filaments present in the cells which allowed us to measure the cellular outgrowth using Image-J. The

corresponding data is shown in Figs. 4.5 and 4.6. On day-1, no statistically significant difference in neurite outgrowth was noted between controls and metal ion receiving cultures. Although significant increase in the neurite outgrowth was observed in control cultures by day 4 ($p< 0.001$ vs. day 1), no measurable neurite outgrowth was observed in any of the heavy metal treated cell cultures on day 4. This shows that at higher concentrations, heavy metal ions (0.01 µM-10 µM) are not only contributing to cell death but also arrest the neurite outgrowth of the surviving ones. However, the molecular mechanism by which this inhibition of neurite outgrowth is modulated needs further investigation. In the previous studies it has been noted that concentrations between 0.001 µM-100 µM of cadmium caused necrosis of neural cells at higher concentrations and induced apoptosis at lower concentrations, which eventually made the neurite extensions to disappear over time[24,25].The methyl mercury was also observed to cause decrease in neurite extensions at 0.1 µM– 10 µM in previous studies [27]. The metal exposure in their study was done after culturing the neural stem cells for 24 hours. Lead is reported to cause a retraction of cell outgrowths and change in morphology of the cells to more spherical form[28,30].

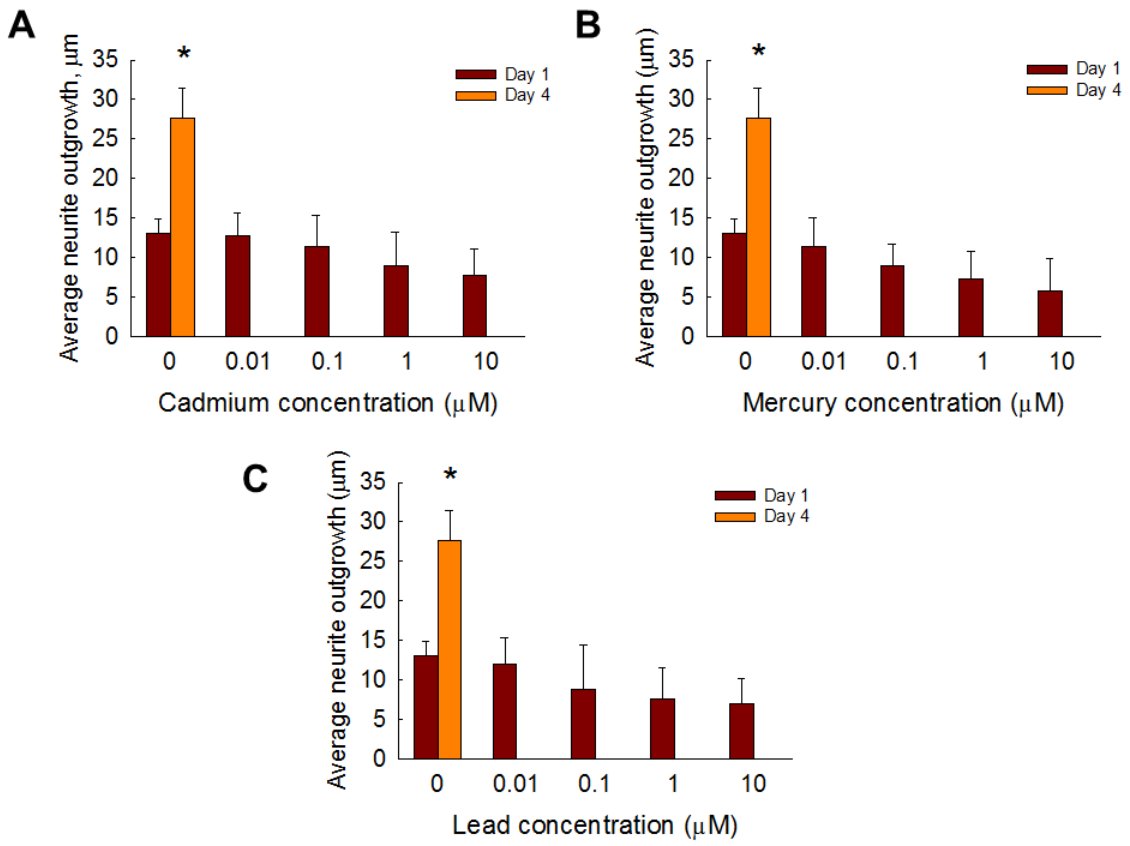
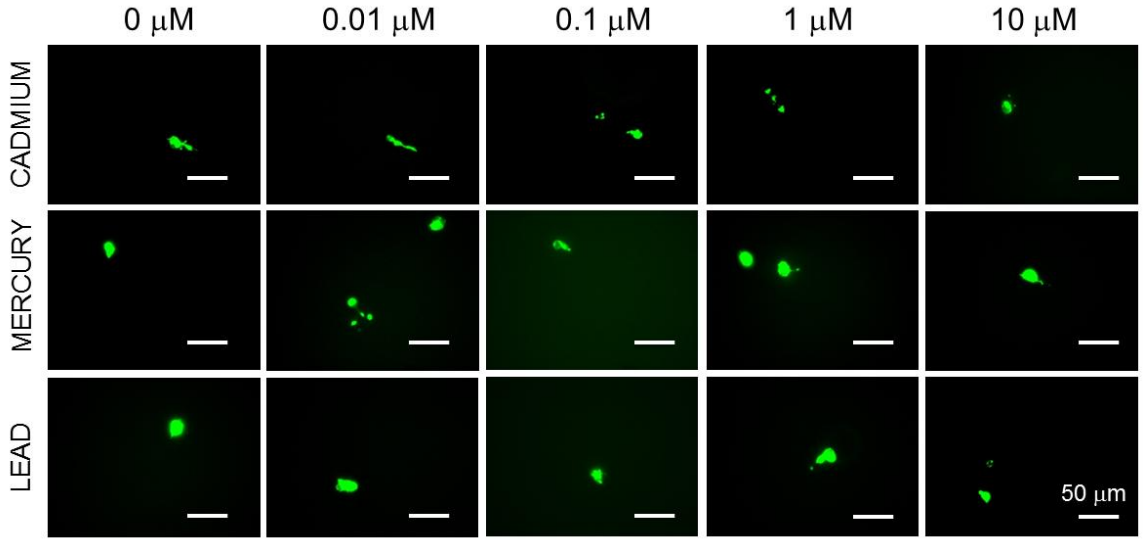


Figure 4.5 Average neurite outgrowth on day-1 and day-4. The data shown above is mean ± standard error (n=3/case). Neurite outgrowth in cadmium (A), mercury (B) and lead (C) supplemented cell cultures.

(A)



(B)

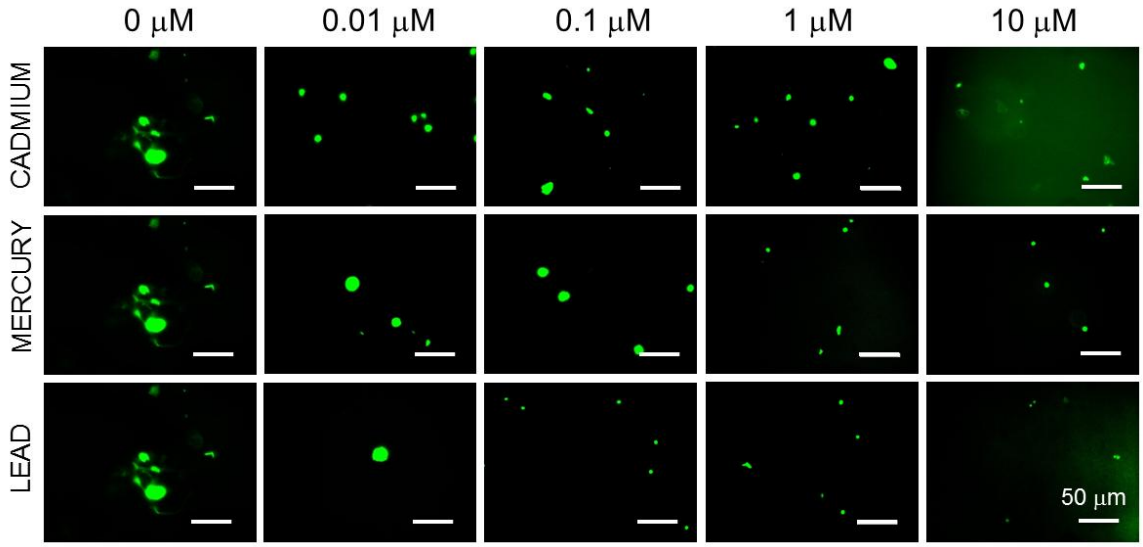


Figure 4.6 Immunofluorescence images showing neurite outgrowth in presence of cadmium on day-1(A) and day-4 (B), at higher ion concentrations (n=3/case, 40×

magnification, scale bar = 50 μm).Images for neurite growth in the presence of mercury and lead are shown in Appendix B.

4.4 Neurite outgrowth at lower concentrations

The neural stem cells were cultured in 3D collagen scaffolds for days -1, 4, 7, 10 and 14 in separate well plates. At the end of respective culture periods, the wells were stained with Alexa488® phalloidin and images were taken. The neurite outgrowth at each concentration of three metals was quantified using NIH Image-J software. The average values in each case were compared to controls. The results are shown in Figs. 4.7 and 4.8.On day-1 there was no statistically significant difference in neurite outgrowth between control and at all the metal ion concentrations tested. The neurite outgrowth in the presence of 0.01 nM concentration cadmium is similar to that in controls at all the time points. At 0.1 nM concentration, cadmium showed neurite growth similar to that in control on day-1, but neurite outgrowth significantly decreased by day-4. Surprisingly, from day-7 till day 14, the neurite outgrowth was statistically similar to controls. At 1 nM cadmium ion concentration, the neurite growth was suppressed during the entire culture period. However, on day 14 the neurite growth started to increase, albeit lower than that in control. This suggests that NSCs may be undergoing some intracellular compensatory mechanism to overcome the toxic effects over a period of time, which needs further investigation.

Compared to controls, mercury at 0.01 nM had no effect on the neurite growth of neural stem cells on any day of observation. At 0.1 nM mercury ion concentration, neurite outgrowth decreased by 48% on day 1 (p = 0.0002) and by 47% (p= 0.0053) on day4. However, from day-7 to day-14, the pace of neurite outgrowth increased and was

similar to that noted in cadmium supplemented cultures at same concentration and time points.

Lead induced a decrease in neurite length as the concentration increased, even at lower concentrations. In 0.01 nM cadmium and mercury-supplemented cultures the growth patterns showed similarity with control on day-4, but there was a decrease at higher concentrations. Lead showed similar effect as of day-1. An interesting pattern of neurite growth was observed in day-7 results. The lower concentrations 0.01 and 0.1 nM of all metals showed no significant difference compared to control, whereas all the metals at 1 nM induced lower outgrowths. On day-10 and day-14, neurite outgrowths at all cadmium concentrations showed no statistically significant difference compared to control. Mercury and lead showed a difference only at 1 nM. From these results, it is evident that initially cadmium, mercury and lead are inhibiting the neurite growth to some extent at concentrations 0.1and 1 nM. But there was regrowth of neurites at 0.1 nM, eventually nearing control values. However, 1 nM mercury concentration significantly affected the neurite growth on all the days of exposure.

Lead exhibited higher toxicity than the other two metals, with regard to NSC cultures. Even on day-1, it affected the neurite growth at all the concentrations tested. From day4 to day-14, neurite extensions were not statistically different within0.01 nM and 0.1 nM supplemented cultures, compared to controls. The cell cultures supplied with 1 nM lead experienced significant decline in neurite growth at all the time intervals of exposure. The average neurite lengths on day-1, 4,7,10 and 14 were measured to be 15.05 µm, 22.74 µm,29.26 µm, 37.16 µm and49.1 µm respectively in controls, and8.13 µm, 12.97 µm, 13.45 µm, 13.7 µm and16.71 µm, respectively, at 1 nM dosage.

From the above results it could be deduced that 0.01 nM cadmium and mercury were not significantly affecting the neurite growth. At 0.1 nM concentration of these two metals, decrease in neurite growth was observed initially from days1-4, after which they were restored to the values noted in controls. The similar observation was made in case of 0.01 nM and 0.1 nM of lead. All the three metals are hindering the intercellular communication and development of neural stem cells at 1nM.In case of cell cultures with heavy metal ions in media, the degree of toxicity was in the order: lead>mercury>cadmium.

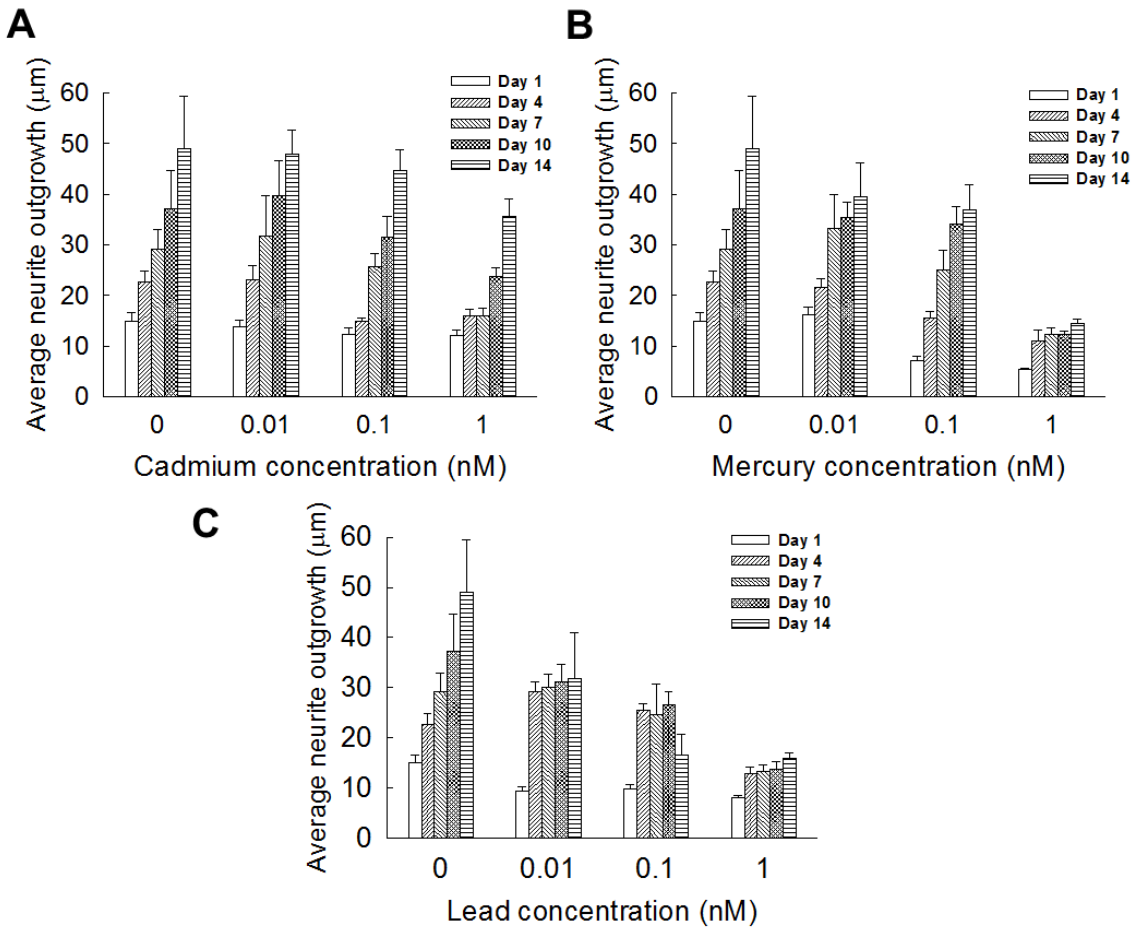


Figure 4.7 Average neurite growth at different metal ion concentrations and culture duration. The data shown reflects the mean ± standard error (n=3/case).Average neurite outgrowth in the presence of cadmium (A), mercury (B), and lead (C), over the 14 day culture period.

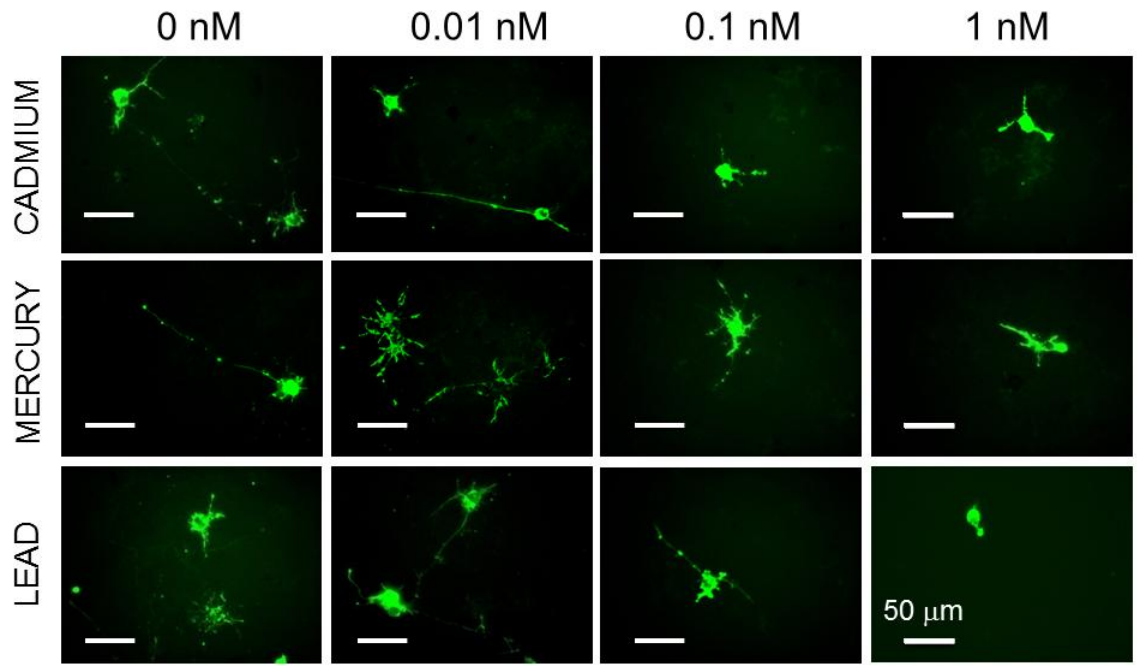


Figure 4.8 Immunofluorescence images showing neurite outgrowth at lower metal ion concentrations on day-14. (n=3/case, 40× magnification, scale bar = 50 µm). Images for day-1 and day-7 are listed in Appendix B.

4.5 Quantification of differentiated neural cells on day-7

The neural stem cells were cultured for 7- days and stained for the markers TUJ1, GFAP and MBP2. The differentiation at all conditions is shown in Figs.4.9 and 4.10. The expression of TUJ1 marker in cadmium was not statistically different from control at 0.01 and 0.1 nM, but it significantly decreased at 1 nM. Similar patterns were noted with the expression of GFAP and MBP at 1 nM, but there was no statistically significant difference between expression level of GFAP and MBP at 0.01 and 0.1 nM. In mercury-supplemented cultures, TUJ1 expression was significantly affected at all concentrations, and GFAP and MBP were affected only at 1 nM. Lead also showed similar behavior as that of mercury. All the three metal ions decreased the expression of TUJ1, GFAP and

MBP at 1 nM, compared to controls. On day-7 cadmium was noted to be ineffective at 0.01 and 1 nM. Mercury and lead did not affect the GFAP and MBP until their concentration reached 1 nM.

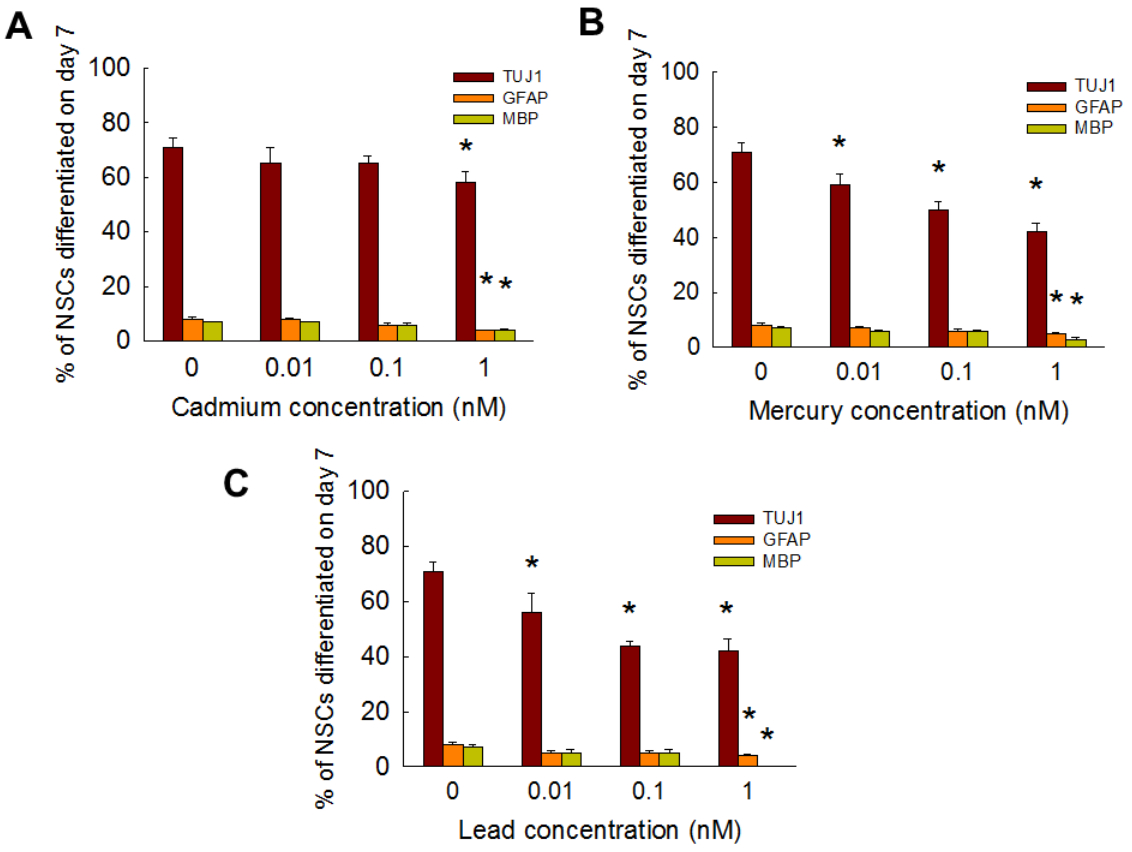


Figure 4.9 Percentage of NSCs differentiating into neural and glial lineagesby day-7. The data shown reflects the mean ± standard error (n=3/case).Percentage expression of TUJ1, GFAP and MBP in presence of cadmium(A), mercury(B), and lead(C).

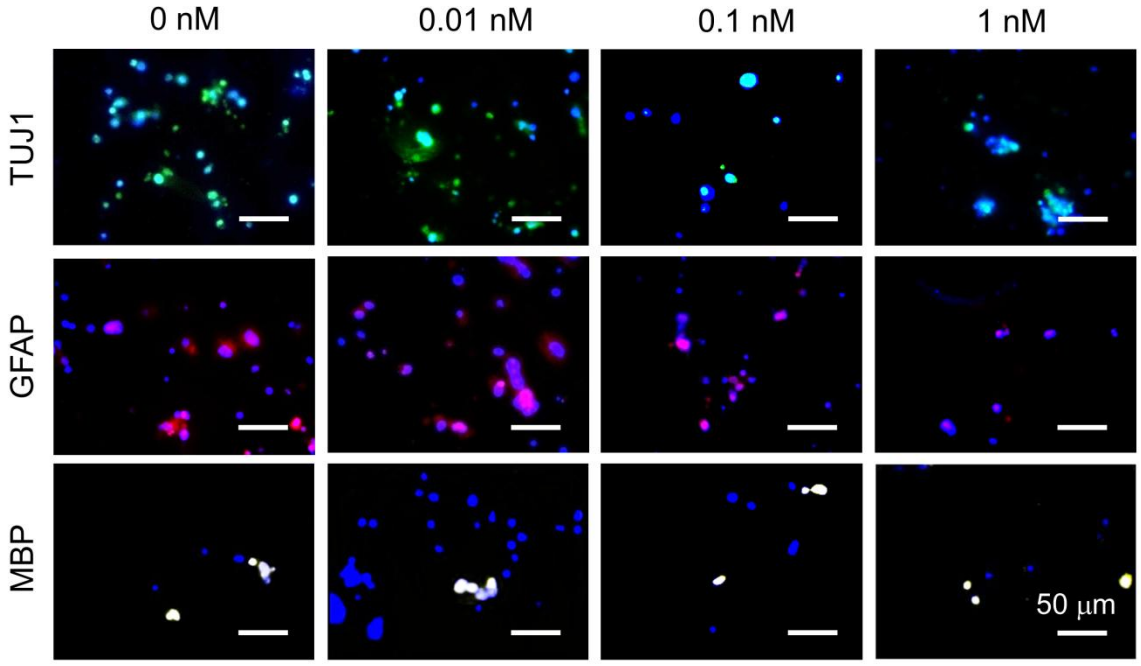


Figure 4.10 Immunofluorescence images showing differentiation of NSCs in presence of cadmium on day 7 (n=3/each case, 40× objective, scale bar = 50 µm). Images for differentiation in presence of mercury and lead are listed in Appendix C.

4.6 Quantification of differentiated neural cells on day-14

The neural stem cells were cultured for 14 days and stained for the markers TUJ1, GFAP and MBP2. The percentage differentiation is shown in Fig.4.11and fluorescence images in Fig. 4.12.Compared to that in controls, cadmium did not affect the expression of TUJ1, GFAP and MBP at 0.01 nM. At 0.1 nM, the differentiation of neurons and oligodendrocytes from neural stem cells was found to be significantly less than control. Cadmium did not affect the differentiation of astrocytes at any concentration. Mercury addition resulted in behavior similar to cadmium at 0.01 nM, by not showing any difference in expression of TUJ1, GFAP and MBP when compared to control. At 0.1 nM, it inhibited the differentiation of astrocytes and neurons, but had no effect on

oligodendrocytes differentiation. At 1nM mercury addition, the differentiation into all the three lineages was significantly reduced. In controls, the average percentage of TUJ1, GFAP and MBP expression was 57%, 8% and 6% respectively, and it reduced to 26%, 3% and 2% respectively, at 1nM. Lead was toxic than other two metals as it induced a significant decrease in differentiation even at0.01 nM. The differentiation of neurons decreased as the concentration of lead increased. Similar pattern was observed in case of GFAP expression. The oligodendrocytes differentiation went from 2% at 0.01 nM to 1% at 0.1 nM and to 0% 1 nM.

Buzanska et al. reported that after culturing the neural stem cells for 7 and 14 days, spontaneous differentiation in presence of cadmium and mercury for 48 hours induced a decrease in the expression of neuronal, astrocytes and oligodendrocytes cell specific markers[26]. Zychowicz et al. reported a considerable decrease in the expression of neural and glial cell markers at concentrations 0.05 µM– 10 µM after 48 h and 72 h exposure[27]. Lead has been noted to cause a decrease in differentiation of NSCs at concentrations between 0.1-100 µM, in floating neurospheres in a 2D model[28, 30].Not much information is available in literature at concentrations lower than 10µM for any of the metals studied here.

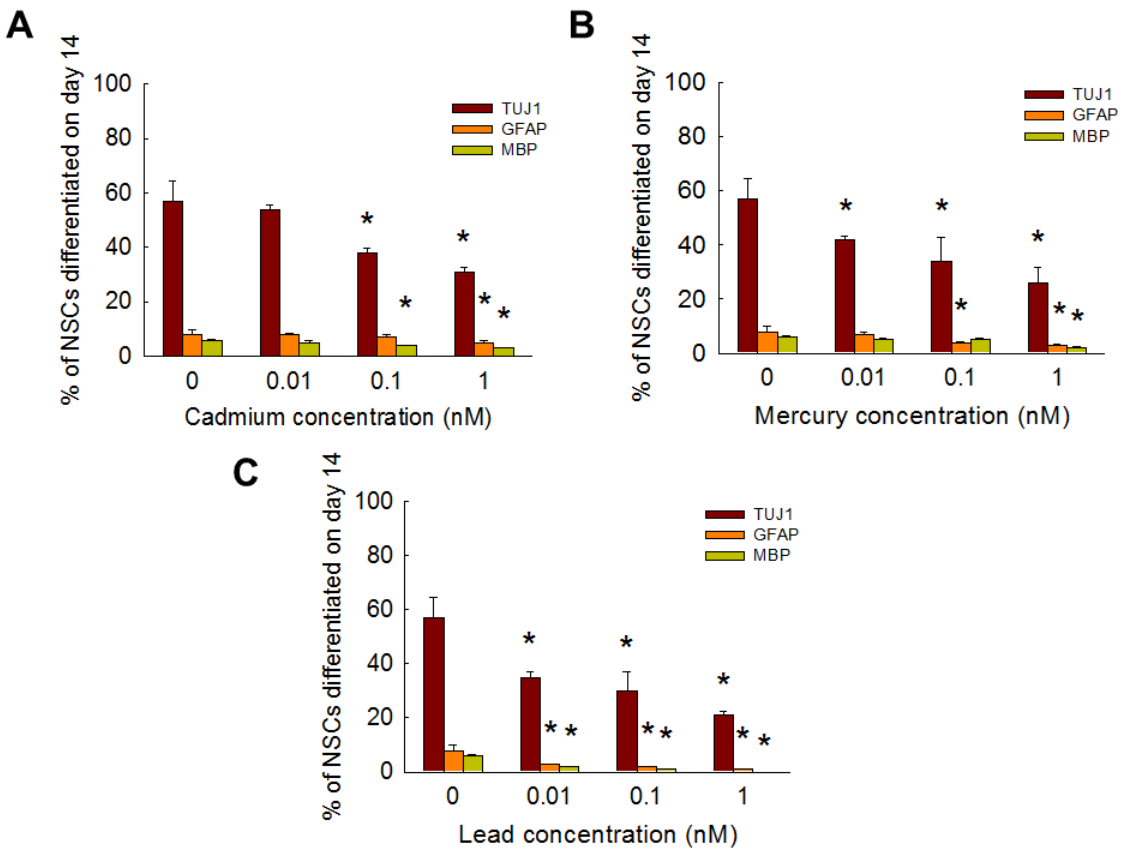


Figure 4.11 Percentage differentiations of neural stem cells on day 14 in presence of (A) cadmium, (B) mercury, (C) lead. The data shown reflects the mean ± standard error (n=3).

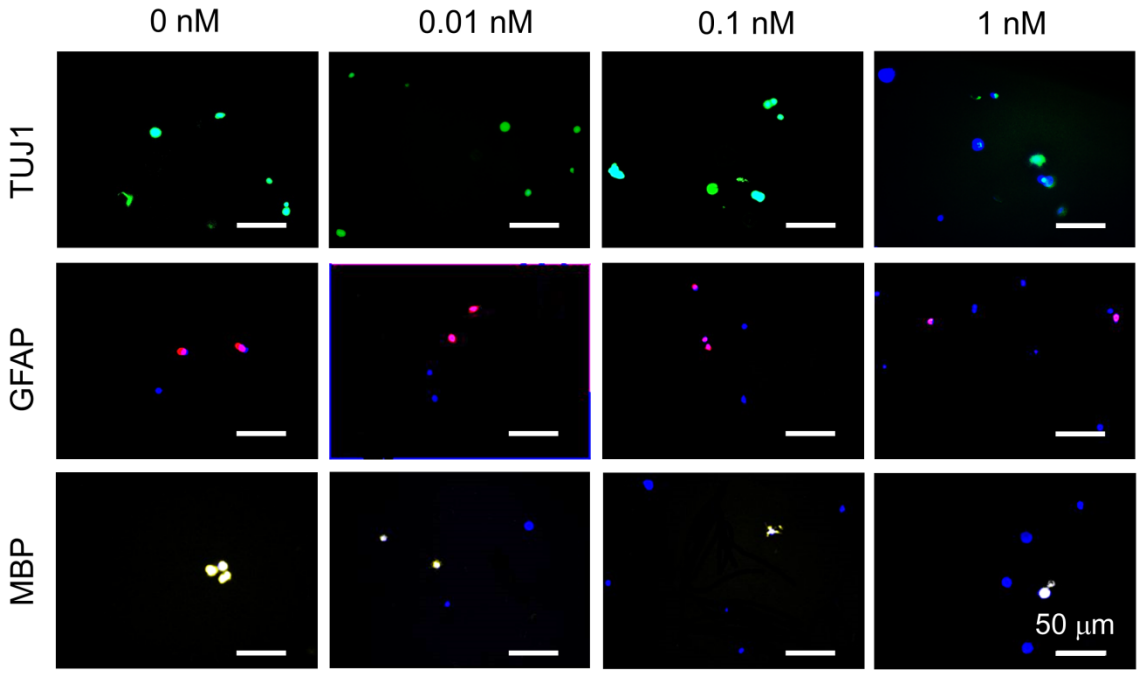


Figure 4.12 Immunofluorescence images showing differentiation of neural stem cells in presence of cadmium on day-14 (n=3/case, 40× magnification, scale bar = 50 µm).Images for differentiation of NSCs in the presence of mercury and lead are listed in Appendix C.

4.7. Differentiation of neural stem cells into motor neurons

NSCs were cultured for 7 or14 days and stained for HB9 marker. HB9 is specific for identifying the presence of motor neurons in the neural cell population. The results for this experiment is shown in Figs.4.13 and 4.14. On day 7, at 0.01 nM concentration for all metals tested, there was no significant effect on NSC differentiation compared to controls. NSC differentiation into motor neuron significantly decreased at 0.1 nM concentration of these metals in general (day 7).At 1 nM concentration, no staining for HB9 marker was noted.

When 0.01 nM concentration of cadmium or mercury was supplemented to NSC cultures, no significant difference in motor neuron differentiation was noted compared to control. However, there was a significant decrease in lead supplemented cultures, even at concentrations as low as 0.01 nM. At 0.1 nM and 1 nM dosages, all the three metal ions significantly suppressed NSC differentiation into motor neuron lineage, compared to controls. However, motor neuron formation at day 14 is significantly higher than that at day 7, for each of the metals tested at 0.1 and 1 nM dosages. It could be concluded that the decrease in motor neuron differentiation is dependent on increase in the concentration of heavy metal ions. These results are comparable to previous literature where they reported a decrease in formation of motor neurons in exposed zebra fish embryo cultures at concentrations 0.1-100 µM of cadmium[29]. Exposure to mercury at 1-10 µM of organic and inorganic mercury has been reported in many studies to cause in malformations of motor neurons[26]. Kermani et al. reported a decrease in differentiation of motor neurons at similar concentrations of lead in case of bone marrow mesenchymal derived NSCs[30]. It is also noted in their study that the differentiation is affected by the source of stem cells and other culture conditions[30].

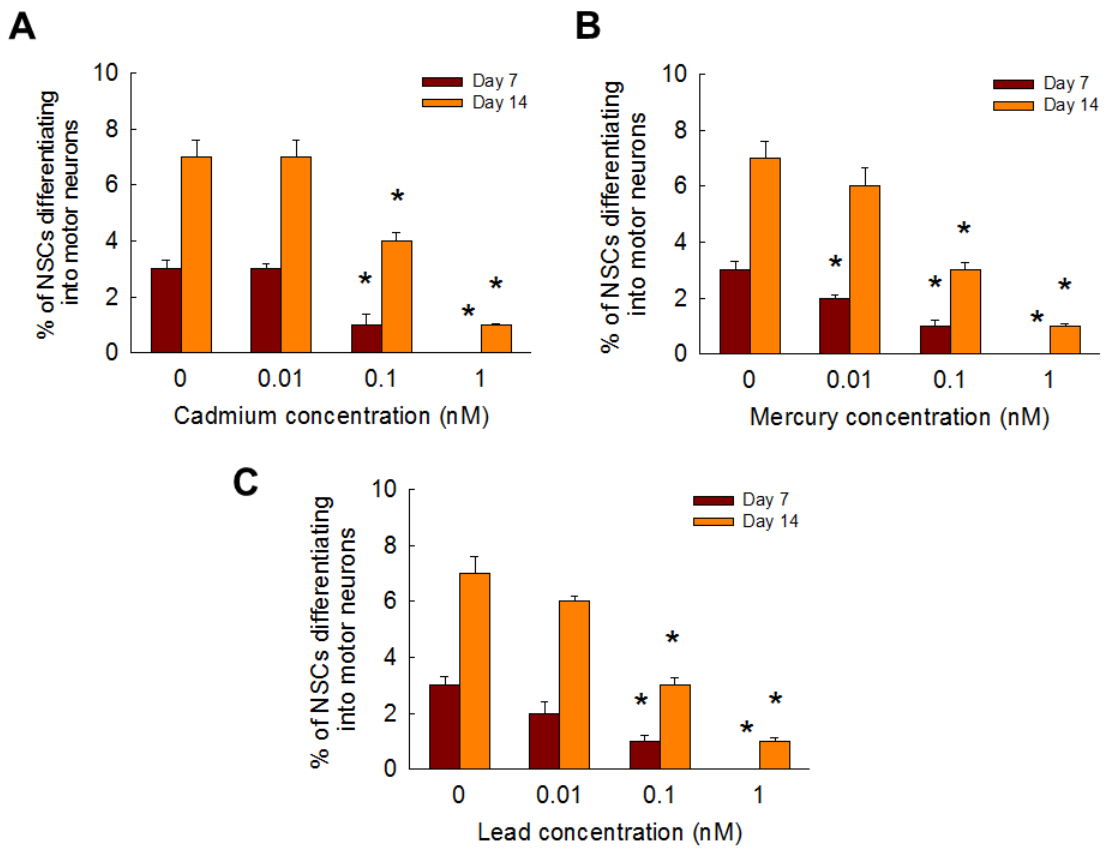


Figure 4.13 Differentiation of NSCs into motor neurons on day 7 and day 14 in presence of various concentrations of cadmium (A), mercury(B), and lead(C).The data shown reflects the mean ± standard error (n=3/case).

4.8. Differentiation of NSCsinto dopaminergic neurons

NSCs were cultured for 7 or14 days and stained for Tyrosine Hydroxylase (TH) marker. TH is specific for identifying the presence of dopaminergic neurons in the neural cell population. The results for this experiment is shown in Fig.4.14 and 4.15. On day 7, at 0.01 nM concentration for all metals tested, there was no significant effect on NSC differentiation compared to controls. NSC differentiation into dopaminergic neuron significantly decreased at 0.1 nM and 1 nM concentration of these metals in general, at all types of tested metals, on day-7 and day 14, compared to control. However there was

an increase in dopaminergic neuron marker from day-7 to day-14 in case of all metals. Previous studies have shown a decrease in dopamine formation, release or neurotransmission of dopamine in presence of these metals, but there is not much information available addressing the effects of various metal ions and their concentrations on NSC differentiation[4,5, 6,7,9].

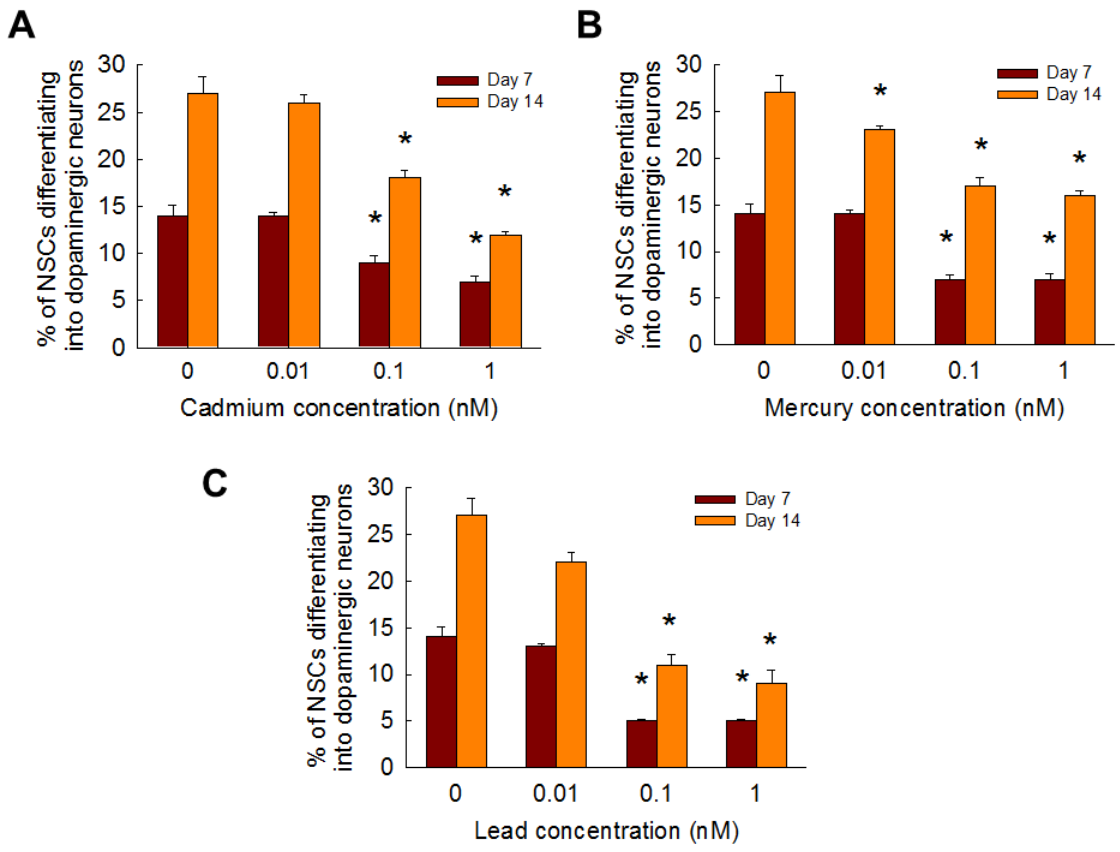


Figure 4.14 Percentage differentiation of NSCs into dopaminergic neurons in presence of cadmium (A), mercury (B), and lead (C). Data represents mean ± standard error of the results, with n = 3/case.

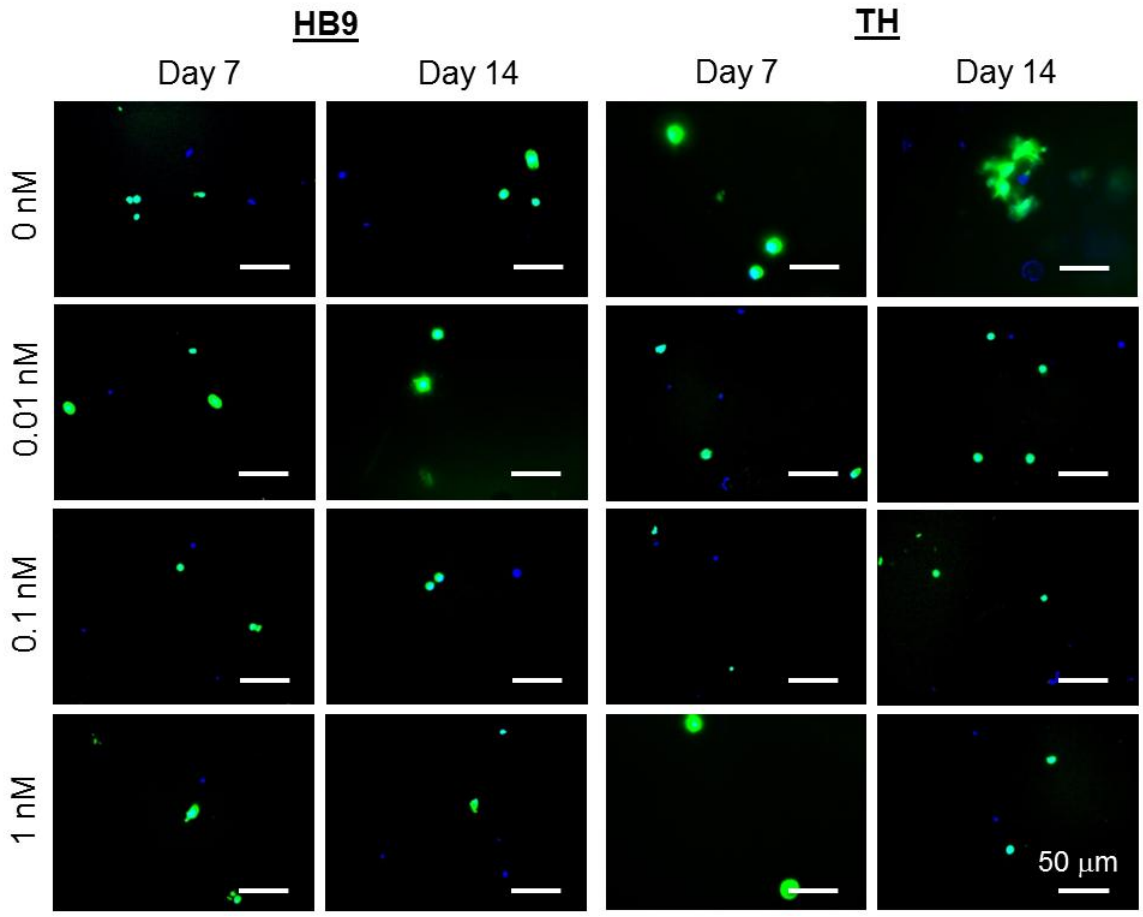


Figure 4.15 Immunofluorescence images showing differentiation of NSCs into motor neurons and dopaminergic neurons in presence of cadmium on day-7 and day-14 (n=3/case, 40× magnification, scale bar = 50 μm). Images for differnetiation of NSCs in presence of mercury and lead are listed in Appendix C.

CHAPTER V

CONCLUSIONS AND FUTURE RECOMMENDATIONS

In this work, we have studied the toxic effects of the most commonly found heavy metal pollutants-cadmium, mercury and lead, on the neural stem cell proliferation, viability, differentiation into various neural and glial lineages, and neurite outgrowth. The metal concentrations ranged from 0.01 nM to 10 μM, with cultures no supplements acting as controls. We performed the cultures for 14 days, with experimental outcomes measured at various intermediate time points. Results were compared intra-group for each metal, and inter-group across the metals, and finally with regard to controls.

5.1 Conclusions

- Culturing NSCs within 3D collagen scaffolds might be more physiological representation of in vivo pathology, compared to conventional 2D cultures.

- When cadmium, mercury and lead were compared to each other the order of toxicity was found to be Lead > Mercury > Cadmium with regard to viability, differentiation rate and neurite outgrowth of the neural stem cells.
- The cytotoxic effects of these metals on NSC cultures increased with an increase in concentration and time of exposure.
- By day 4, cell death was significant at concentrations of 0.01 µM, 0.1 µM, 1 µM and 10 µM, independent of the type of metal.
- Similarly, significant reduction in neurite growth at these higher concentrations was noted by day 4. This could either be due to significant release of inflammatory markers by NSCs into the surrounding media in response to heavy metal ion exposure, or due to retraction of neurite in surviving neurons due to hitherto unknown intracellular signaling pathways. Further studies are required to understand this phenomenon in greater detail.
- Investigations at concentrations lower than 0.01 µM helped us pinpoint the lowest concentration which compromises NSC phenotype.
- In general, heavy metal exposure at concentrations ranging from 0.01 nM–10 µM did not induce any significant toxic effects in NSCs within the first 24 h, except for lead.
- At 0.1 nM dosage, cadmium and mercury induced slightly different behavior in NSCs. Although neurite outgrowth was affected during the first 4 days of exposure, it recovered from day-7 and reached values noted within respective controls. This suggests that there could be a compensatory mechanism occurring to overcome the inhibition by metals or some other factor involved which needs to be further studied.

- Dramatic changes in NSC behavior and phenotype were noted at concentrations higher than 0.1 nM, irrespective of the type of metal, and 1nMinducedthe toxic effects which were clearly evident in all experiments.
- The differentiation of NSCs into neural and glial lineages was significantly reduced at 1nM concentration of cadmium, mercury and lead. The expression of MBP declined to 0% in 0.1 nM lead exposed cultures, suggesting that lead exposure might be inhibiting myelin production important for insulates neural cells to help proper signal conduction of neural communication.
- The overall differentiation of NSCs into neurons and glial cells decreased with an increase in heavy metal concentration and time of exposure. But, the opposite effect was observed within the types of neural cells differentiating at that time. The percentage of motor neurons increased with time of exposure, but less in quantity than controls. Motor neuron formation decreased with an increase in metal ion concentration.
- Similarly the expression of dopaminergic marker increased with time but decreased with an increase in concentration. The expression of TH was higher than HB9 in all cases which suggests that the motor neurons are being the sensitive targets.
- The comparison between the average mean blood concentrations provided by ATSDR as safe concentration and the tested concentration which was detrimental for NSC survival and differentiation is shown in table II.

Type of metal	National mean safe blood concentrations provided by ATSDR	Concentration found to be detrimental for NSCs
Cadmium	0.038µg/dL (3.4nM)	0.1 nM
Mercury	5 µg/dL (0.24 µM)	0.1 nM
Lead	5µg/dL (0.25 µM)	0.01 nM

Table –II Comparison of the tested concentrations with national geometric mean blood levels[4,5,6]

5.2 Future Recommendations

- Study the toxic effects of metal ions at the molecular level; investigate the biochemical changes within the cells and extracellular matrix.
- Determine the metal ion effects at the genetic level, which could help understand the cause-effect relationship between genetic level changes and cellular behavior in affected subjects.
- Investigate the effect of heavy metals on neurodegenerative diseases like Parkinson's and Alzheimer's disease.

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32. Protocol for preparation of collagen solution by BD biotechnologies.

33. Protocol for phalloidin staining by Life Technologies.

34. Protocol for Immunofluorescence staining by Abcam Biotechnology Company.

35. Protocol for Immunofluorescence staining by Santa Cruz Biotechnology Company.

APPENDIX A

Figure A-1 Immunofluorescence images showing live and dead cells in presence of mercury at high concentrations on day-1 and day- 4 (n=3/ each concentration, 40×objective, scale bar = 50 µm).

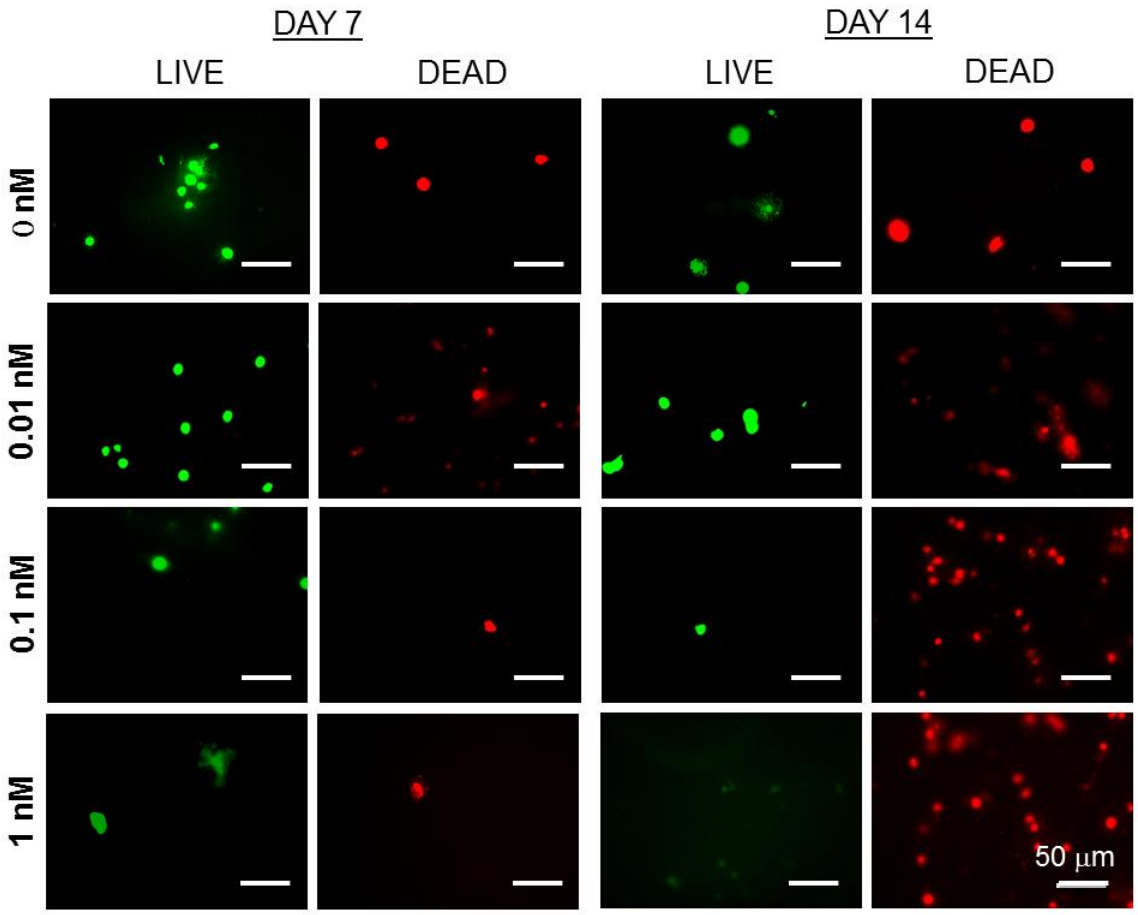


Figure A-2 Immunofluorescence images showing live and dead cells in presence of lead at high concentrations on day-1 and day- 4 (n=3/ each concentration, 40×objective, scale bar = 50 µm).

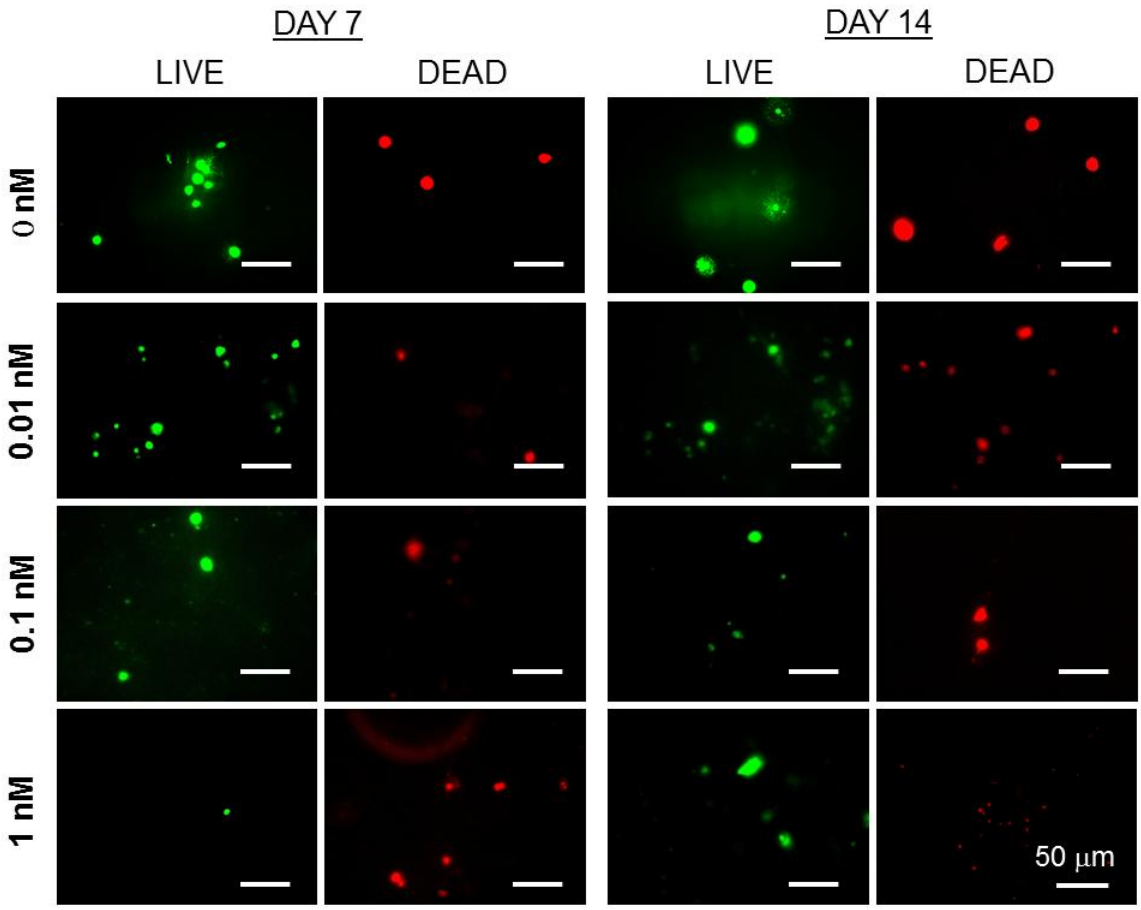


Figure A-3 Immunofluorescence images showing live and dead cells in presence of mercury at low concentrations on day-7 and day-14 (n=3/ each concentration, 40×objective, scale bar = 50 µm).

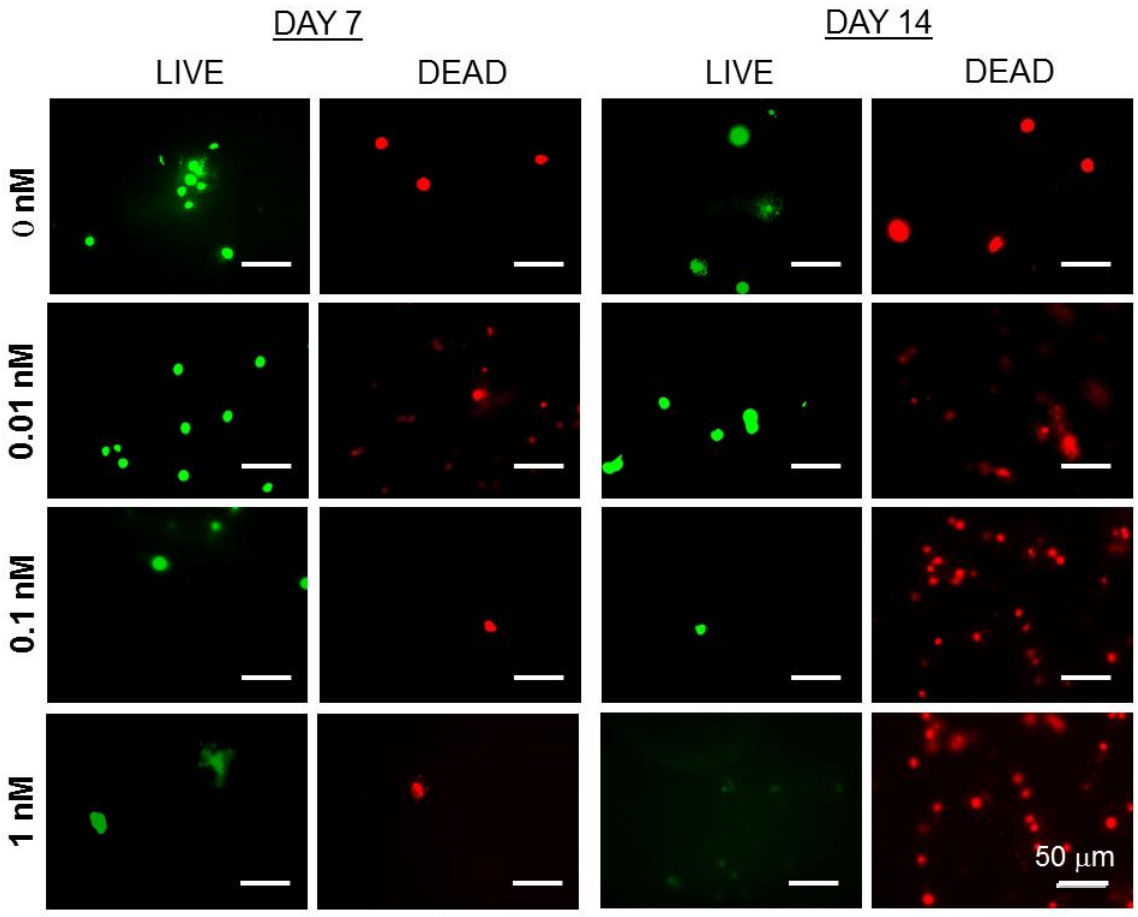
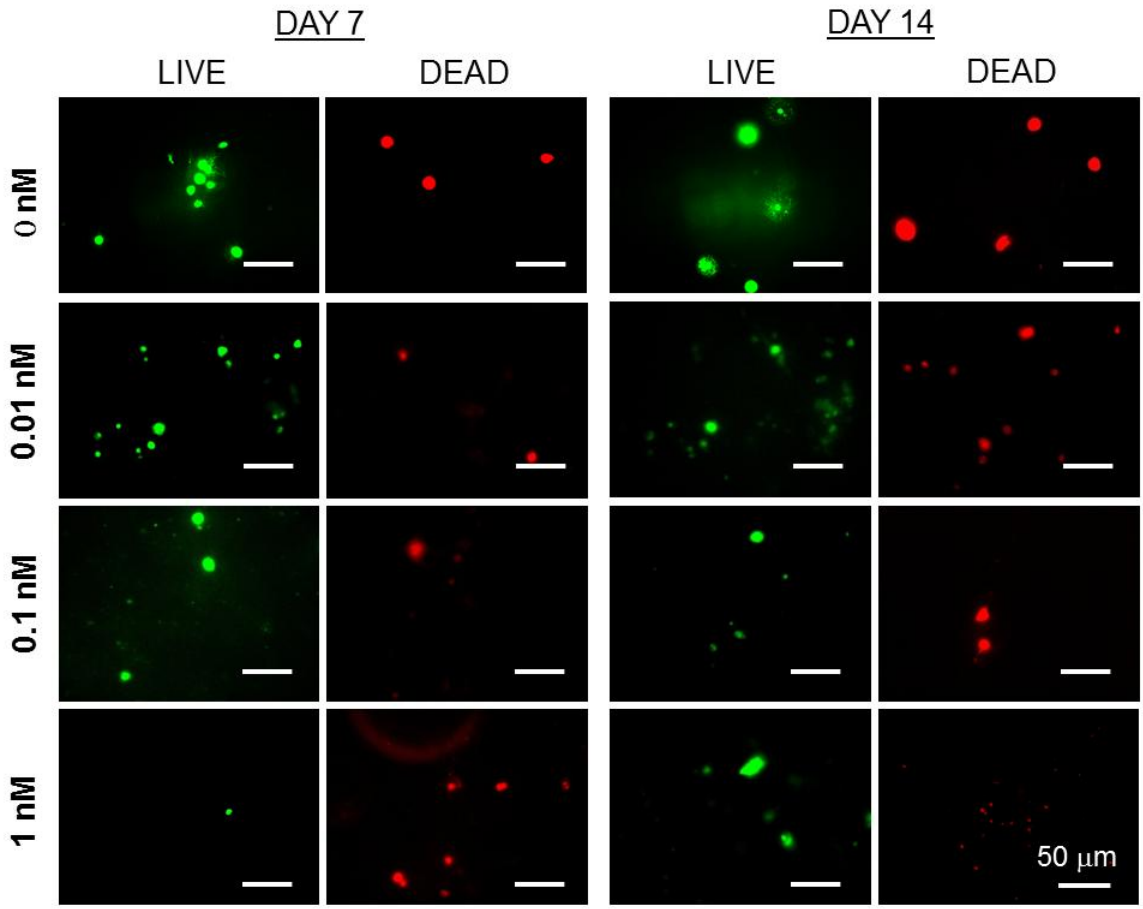


Figure A-4 Immunofluorescence images showing live and dead cells in presence of lead at low concentrations on day-7 and day-14 (n=3/ each concentration, 40×objective, scale bar = 50 µm).



APPENDIX B

Figure B-1 Immunofluorescence images showing neurite growth on day-1 at lower concentrations (n=3/ each concentration, 40×objective, scale bar = 50 µm).

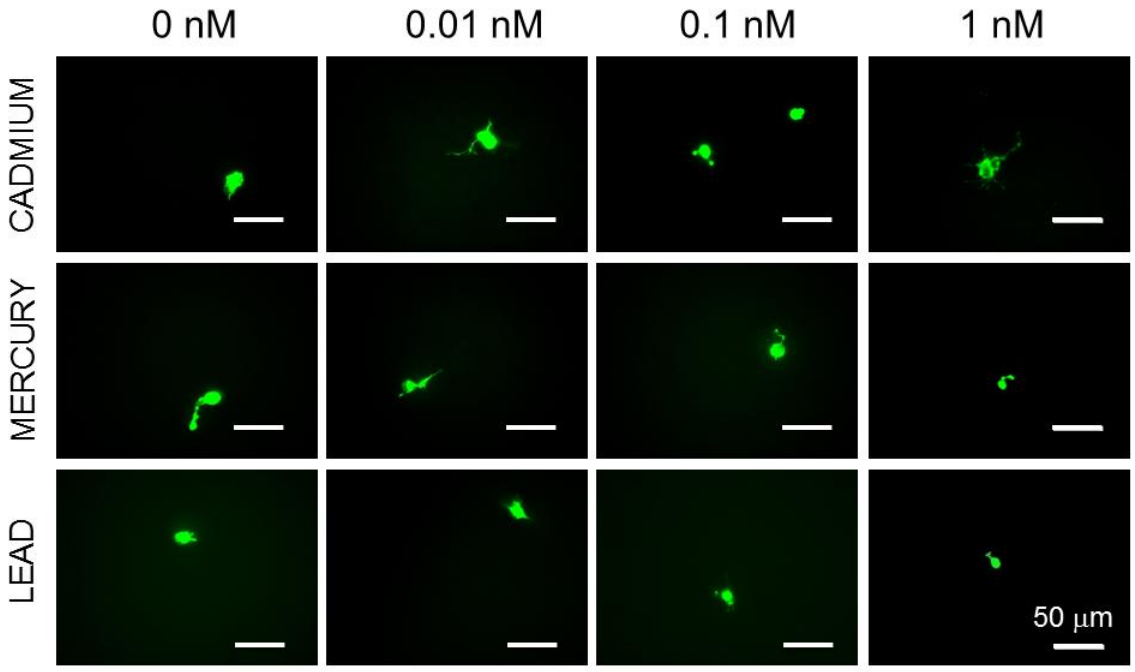
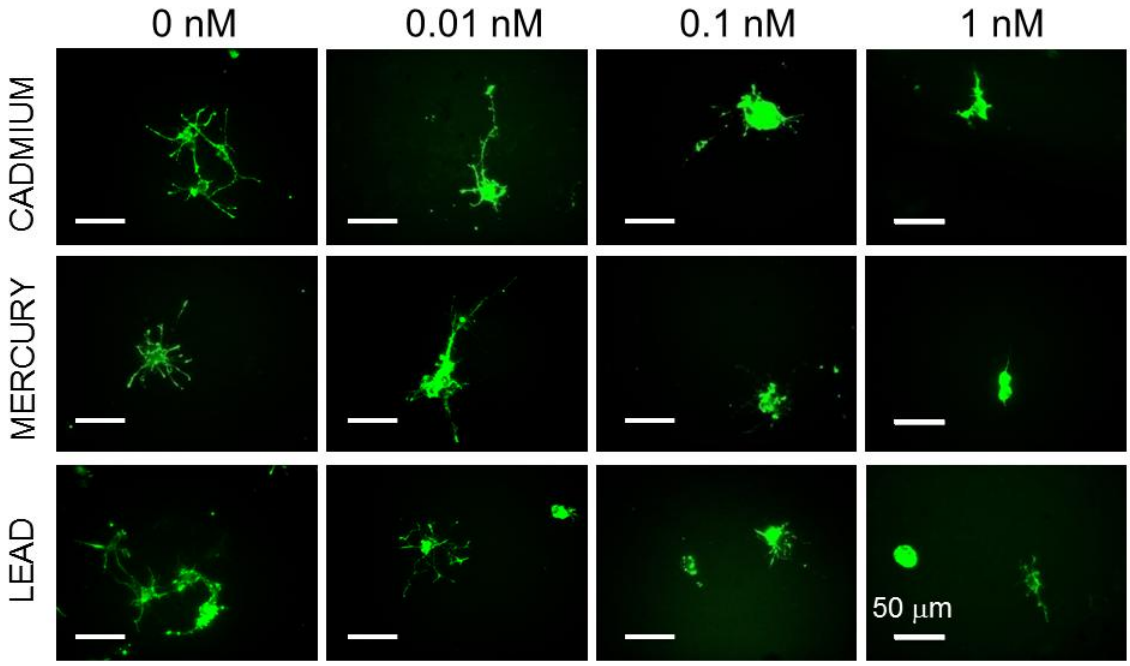


Figure B-2 Immunofluorescence images showing neurite growth on day-7 at lower concentrations (n=3/ each concentration, 40×objective, scale bar = 50 µm).



APPENDIX C

Figure C-1 Immunofluorescence images showing differentiation of NSCs in presence of mercury ions on day-7

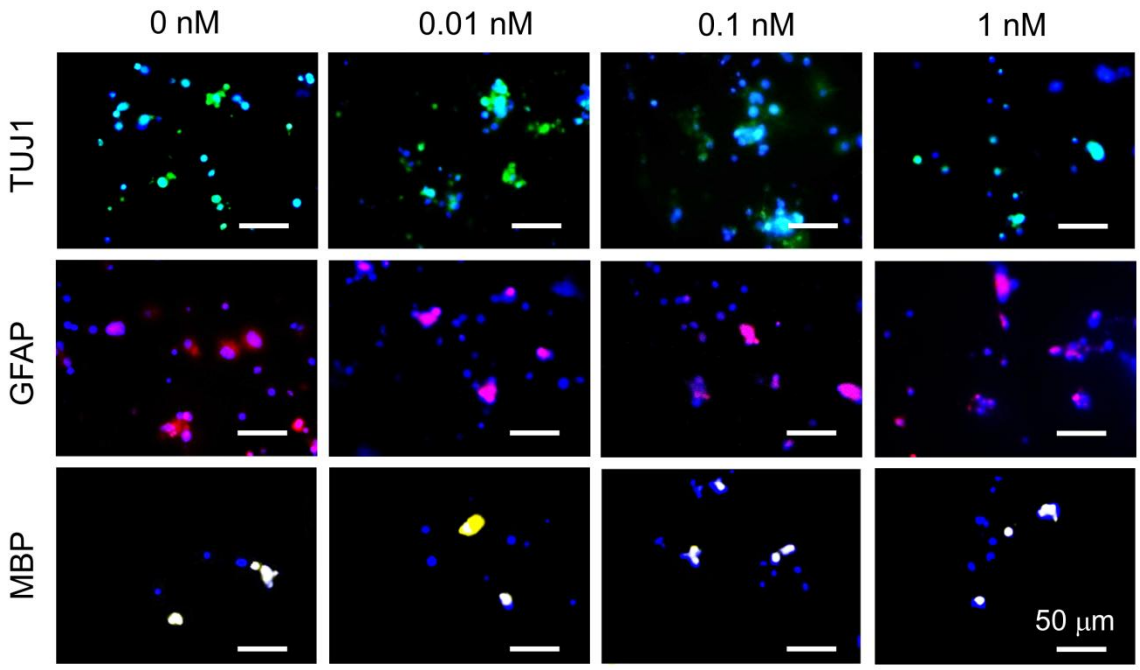


Figure C-2 Immunofluorescence images showing differentiation of NSCs in presence of lead ions on day-7.

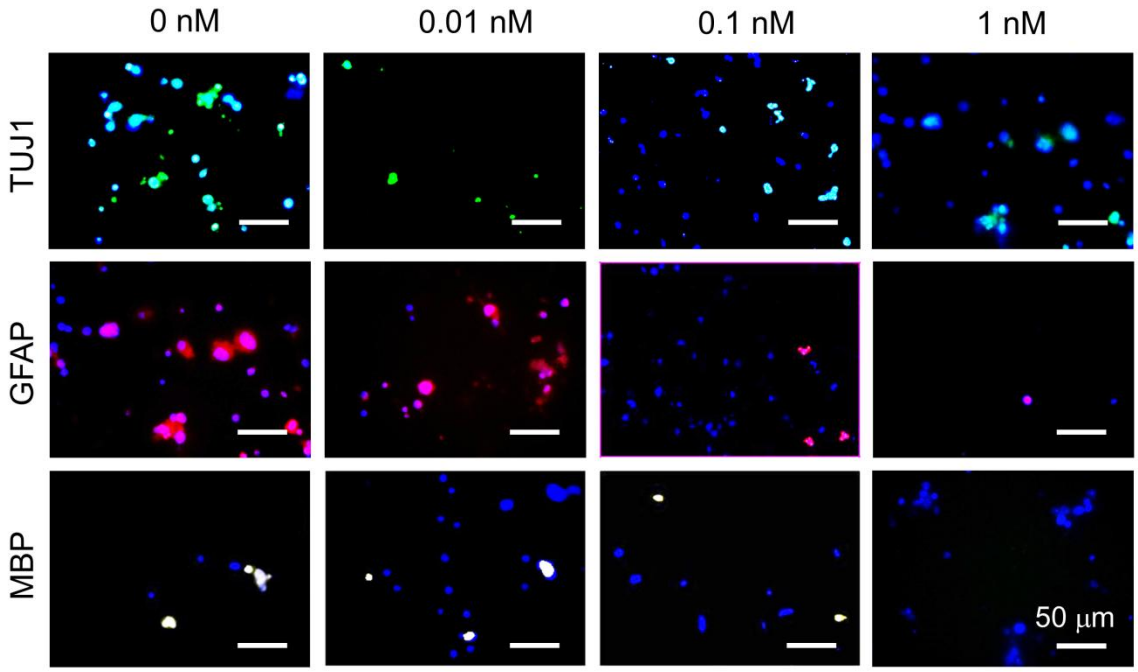


Figure C-3 Immunofluorescence images showing differentiation of NSCs in presence of mercury ions on day-14

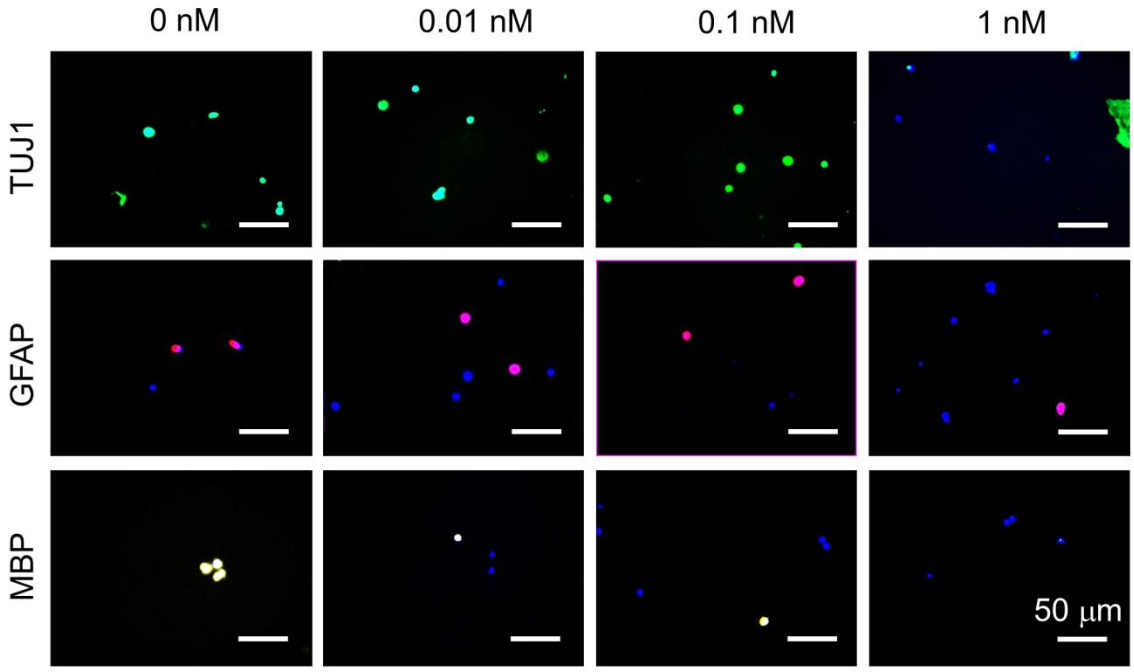


Figure C-4 Immunofluorescence images showing differentiation of NSCs in presence of lead ions on day-14.

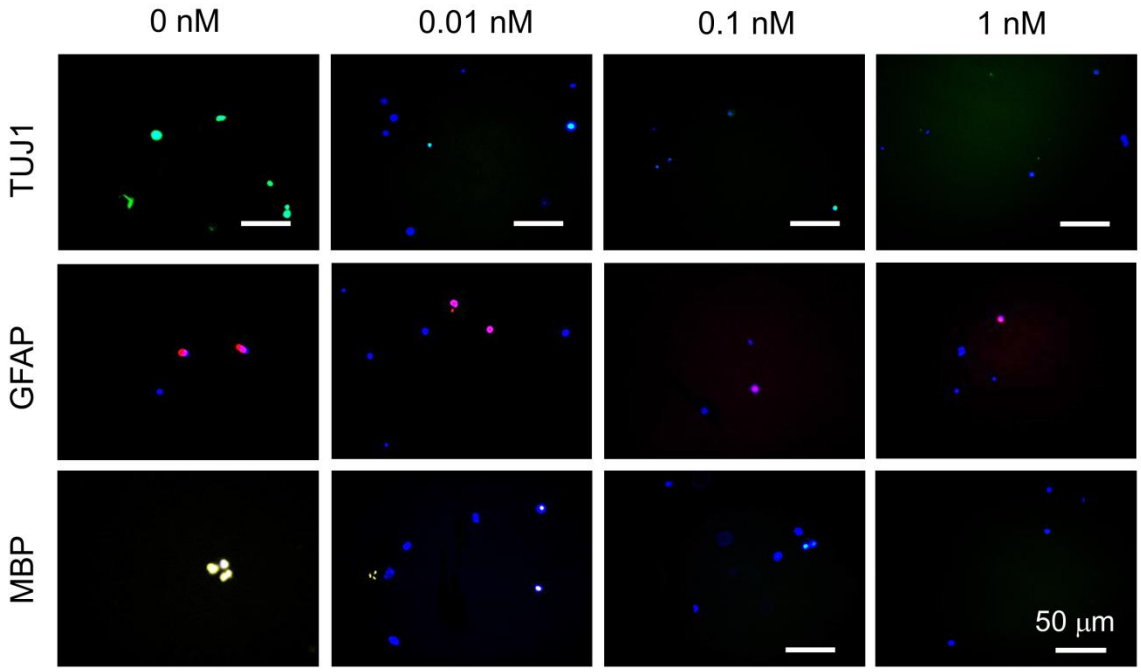


Figure C-5 Immunofluorescence images showing differentiation of NSCs into motor neurons and dopaminergic neurons in presence of mercury ions on day-7 and day-14.

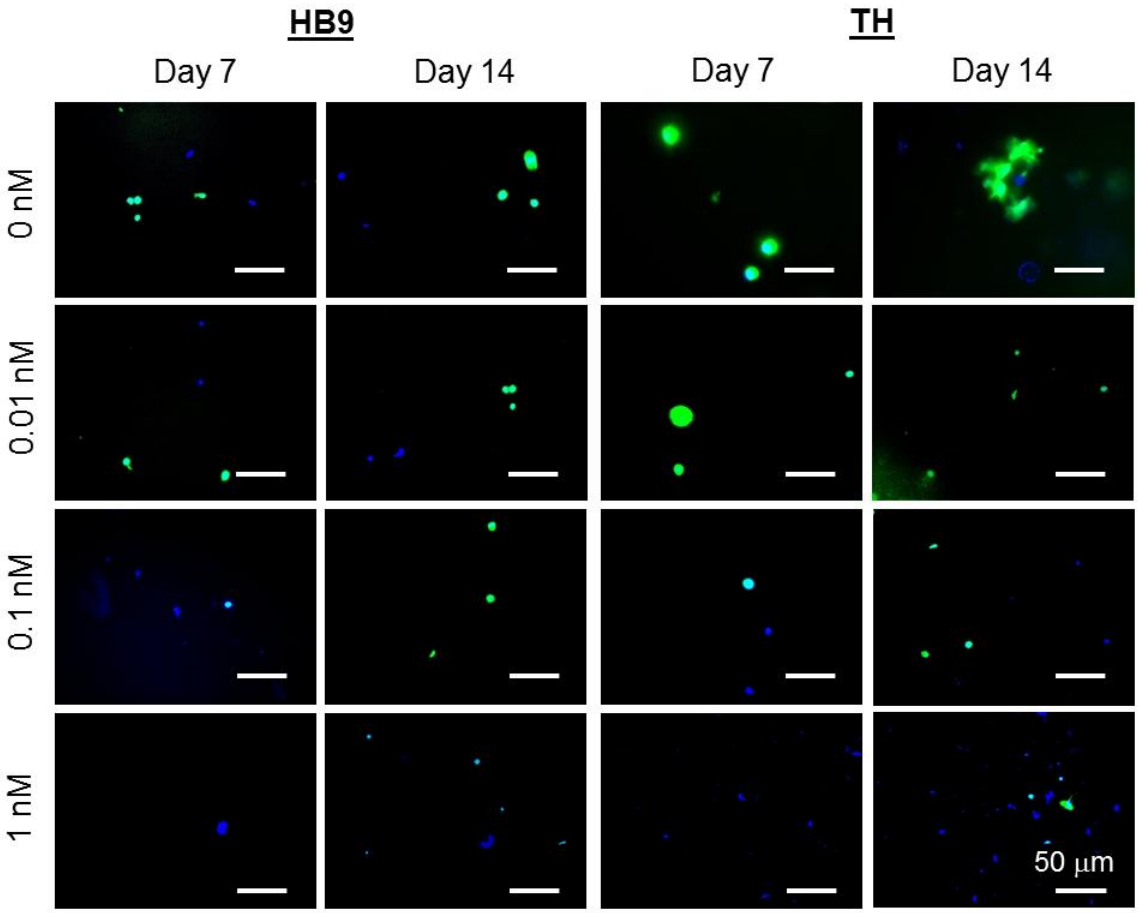


Figure C-6 Immunofluorescence images showing differentiation of NSCs into motor and dopaminergic neurons in presence of lead ions on day-7 and day-14.