Published in final edited form as:

Environ Toxicol. 2015 December ; 30(12): 1445–1458. doi:10.1002/tox.22014.

Cytotoxic, Genotoxic, and Neurotoxic Effects of Mg, Pb, and Fe on Pheochromocytoma (PC-12) Cells

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Abstract

Metals such as lead (Pb), magnesium (Mg), and iron (Fe) are ubiquitous in the environment as a result of natural occurrence and anthropogenic activities. Although Mg, Fe and others are considered essential elements, high level of exposure has been associated with severe adverse health effects including cardiovascular, hematological, nephrotoxic, hepatotoxic, and neurologic abnormalities in humans. In the present study we hypothesized that Mg, Pb, and Fe are cytotoxic, genotoxic and neurotoxic, and their toxicity is mediated through oxidative stress and alteration in protein expression. To test the hypothesis, we used the pheochromocytoma (PC-12) cell line as a neuro cell model and performed the LDH assay for cell viability, Comet assay for DNA damage, Western blot for oxidative stress, and HPLC-MS to assess the concentration levels of neurological biomarkers such as glutamate, dopamine (DA), and 3-methoxytyramine (3-MT). The results of this study clearly show that Mg, Pb, and Fe, respectively in the form of MgSO₄, Pb(NO₃)₂, FeCl₂, and FeCl₃ induce cytotoxicity, oxidative stress, and genotoxicity in PC-12 cells. In addition, exposure to these metallic compounds caused significant changes in the concentration levels of glutamate, dopamine, and 3-MT in PC-12 cells. Taken together the findings suggest that MgSO₄, Pb(NO₃)₂, FeCl₂, and FeCl₃ have the potential to induce substantial toxicity to PC-12 cells.

Keywords

heavy metals; cytotoxicity; genotoxicity; neurotoxicity; PC-12 cells

INTRODUCTION

Heavy metal toxicity is a major public health concern due to human exposure from both natural and anthropogenic sources. Although metals such as Fe and Mg are considered essential elements that function as cofactors for a number of enzymatic reactions, they are also of concern at higher level of exposure where they may induce adverse neurotoxic effects. Human exposure to large amounts of these elements is often occupational in nature,

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although exposure ingestion through food, drinking water, and medications may be substantial. The risk for acute or chronic toxicity depends on the type and chemical form, frequency, intensity, route and duration of exposure (Neustadt and Pieczenik, 2007; Calderon et al, 2003).

One of the major risks arising from environmental pollution by heavy metals is the damage to the nervous system of exposed individuals. Several studies have pointed out that heavy metals such as Hg, Pb, and Cd, cause adverse health effects and learning disabilities to millions of people in the United States each year. Metal toxicity affects all organ systems, however the brain is especially susceptible to damage. Many studies have shown that metals can induce neurotoxicity, including neurological disturbances (Ortinero et al, 2010; McGuigan, 1928; Prins, Park, and Lurie, 2010).

Glutamate and dopamine are both important neurotransmitters in the brain and are responsible for various functions. 3-Methoxytyramine (3-MT), a metabolite of the neurotransmitter dopamine formed by the introduction of a methyl group to dopamine by the enzyme catechol-O-methyl transferase (COMT) is responsible for an important part of dopamine (DA) metabolism and plays a role in DA-related activity. 3-MT can be further metabolized by the enzyme monoamine oxidase (MAO) to form homovanillic acid (HVA), which is then typically excreted in the urine. 3-MT is deaminated by MAO-A so effectively that increased formation does not increase its endogenous levels (Waldmeier et al., 1981). It has been suggested that 3-MT may not be suitable as an indicator of increased DA release, however it may have value as an assessment marker of lowered DA release. Exposure to metals can affect the neurological processes of these neurotransmitters and lead to debilitating behavioral disorders. Studies have shown that abnormalities in glutamate and dopamine levels are associated with Alzheimer's, Parkinson's, and other neurological disorders (Elseweidy and El-Baky, 2008; Jones and Miller, 2008, Fitsanakis et al, 2006).

Recent studies have shown that Mg, Pb, and Fe can induce cytotoxic effects in cells in both in vivo and in vitro (Dribben et al, 2009; Sharifi and Mousavi, 2008; Garcia-Alfonso et al, 1995). It has been suggested that the effects are dose-dependent and higher doses can induce apoptosis. Both in vivo and in vitro studies have reported the potential of Mg, Pb, and Fe to induce genotoxic damage (Wolf et al, 2008; Valverde et al, 2002; Nú ez Millacura et al, 2002). It has been reported that exposure to Pb, along with other metals (Cd, Cr, Ar), produces reactive oxygen species (ROS) that leads to the onset of oxidative stress (Tully et al, 2000; Selvin-Testa, 1997; Altura et al, 2003; Ur-Rehman, 1984; Braughler, 1986). Other studies have reported that exposure to metals such as Mg, Pb, and Fe can cause changes in dopamine and glutamate levels, which can result in significant damage in the brain and may also result in apoptosis (Santiago et al, 2000; Hallak et al, 1996; Penugonda et al, 2006; Borisova et al, 2011; Atchison and Narahashi, 1984; Kober and Cooper, 1976; Minami et al, 2001; Minnema et al, 1988).

In the present study, we investigated the in vitro mechanisms of Mg, Pb, Fe(II), and Fe(III)-induced toxicity using pheochromocytoma (PC-12) cells that have been derived from the rat adrenal medulla as a test model (Greene et al., 1998). These cells are useful as a model system for neuronal differentiation and for studying the function of neurotrophic factors

because they have the ability to stop dividing and to terminally differentiate when treated with nerve growth factor (NGF). Hence, they have become a commonly employed model system for studies of neuronal development and function. In particular, PC-12 cells have been a convenient alternative to cultured neurons for studying the trophic and differentiative actions of NGF (Levi-Montalcini and Angletti, 1968; Levi and Alema, 1991; Teng et al., 1993). Several attributes including the relatively high degree of differentiation before and after NGF treatment, homogenous response to stimuli, availability in large numbers for biochemical studies, and suitability for genetic manipulations, have led to their widespread popularity in neurobiological research.

MATERIALS AND METHODS

Chemicals and Test Media

Magnesium sulfate (MgSO_4), lead nitrate [$\text{Pb}(\text{NO}_3)_2$], ferrous chloride (FeCl_2), and ferric chloride (FeCl_3) were purchased from Sigma Chemical Company (St. Louis, MO). Their physico-chemical characteristics are presented in Table 1 (www.chemicalbook.com- CAS DataBase List). Growth medium RPMI 1640 containing 1 mmol/L-glutamine was purchased from American Type Culture Collection- ATCC (Manassas, VA). Horse serum (HS), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were purchased from ATCC. The lactate dehydrogenase (LDH) cytotoxicity detection kit was purchased from Takara Bio Incorporated (Otsu, Shiga Japan). The comet SCGE assay kit was purchased from Enzo Life Sciences (Plymouth Meeting, PA).

Tissue Culture

The PC-12 cell line was purchased from the American Type Culture Collection-ATCC (Manassas, VA). The PC-12 cells grow as a suspension culture. In the laboratory, cells were stored in liquid nitrogen until use. They were next thawed by gentle agitation of their containers (vials) for 3 minutes in a water bath at 37°C. After thawing, the content of each vial was transferred to a 100 × 20 mm tissue culture plate, diluted with up to 10 ml of RPMI 1640 containing 1 mmol/L-glutamine (ATCC/Manassas, VA) and supplemented with 5% (v/v) fetal bovine serum, 10% (v/v) horse serum (HS), and 0.4% penicillin and streptomycin. The 100 × 200 mm culture dishes were observed under the microscope followed by incubation in a humidified 5% CO_2 incubator at 37°C. Two times a week, they were diluted under same conditions and harvested in the exponential phase of growth.

Lactate dehydrogenase (LDH) Assay

To assess cytotoxicity, the LDH assay was performed as follows: PC-12 cells were seeded in a 96-well plate with 2×10^6 cells/ml with assay medium for a period of 18 hr at 37°C in a 5% CO_2 humidified incubator. The culture medium was removed and replaced with horse serum and fetal bovine serum free medium and 1% bovine serum albumin (BSA) was added. The plates were treated with Mg, Pb, Fe(II), and Fe (III) at 5.01 and 50.01 $\mu\text{g/ml}$ for 18 hr. These two concentrations were used to reflect lower and higher levels of exposure based on our preliminary range-finding tests. After incubation, the samples were centrifuged for 10 minutes at 250g. 100 μl /well supernatant (without disturbing cell pellet) was removed and transferred into corresponding wells of a new 96-well plate. 100 μl of Solution C (reaction

mixture) was added to each well and incubated for 30 minutes at room temperature. The 96-well plate was protected from light during incubation. The absorbance of the samples was measured at 490–492 nm using the SQ-2800 Single Beam Scanning UV/Visible Spectrophotometer. Percentage cytotoxicity was calculated as follows: $(\text{Absorbance of treated cells} - \text{absorbance of low control}) / (\text{absorbance of high control} - \text{absorbance of low control}) \times 100$ (low control: 100 ml cell suspension/100 ml of medium; high control: 100 ml Triton X 100/100 ml of cell suspension).

Single Cell Gel Electrophoresis (Comet) Assay

PC-12 cells were seeded on 6-well plates at a density of 1×10^5 cells/well in complete growth medium and grown to 75–80% confluency in a humidified incubator under an atmosphere of 95% air and 5% CO₂ at 37°C. The plates were treated with 5.01 and 50.01 µg/ml of Mg, Pb, Fe(II), and Fe(III) for 18 hr. Cells incubated in complete growth medium served as a control. After chemical exposure, the single cell gel electrophoresis (Comet) assay was performed following the manufacture protocol (Enzo Life Sciences, Plymouth Meeting, PA).

Following treatment, the medium was removed and the cells were washed three times in 1X ice cold PBS. The cells were spun down at 3000 rpm for 5 min. The pellet was resuspended in PBS at a density of 1×10^5 . The cells were combined with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v), and 75 µl was immediately pipette onto the comet slide. The comet slides were placed flat in a 4°C refrigerator for 30 minutes and then immersed in prechilled lysis solution on ice for 1 hr. The lysis buffer was removed, and the slides were immersed in freshly prepared alkaline solution, (pH>13) for 30 minutes (in the dark). Slides were then transferred to a horizontal electrophoresis apparatus. The slides were placed flat onto the gel tray and aligned equidistant from the electrodes. The alkaline solution was carefully poured until samples were covered. Slides were electrophoresed at 1 volt/cm (20v) for 20 min. Slides were placed in 70% ethanol for 5 minutes. Excess ethanol was removed. Slides were air dried overnight, stained with SYBRgreen, and analyzed using the Olympus Epifluorescence Microscope with Loates Associates, Inc. (LAI) Automated Comet Analysis Scoring System (LACAAS) software (LAI, Westminster, MD). A total of 150 comets were scored per treatment. 75 comets were randomly selected from three replicated slides. The experiment was repeated three times.

Western Blot Analysis

To assess the effect of MgSO₄, Pb(NO₃)₂, FeCl₂, and FeCl₃ on HSP70 protein expression, western blot was performed. PC-12 cells were washed with phosphate- buffered saline (PBS) and lysed in a cell lysis buffer [20 mM Tris, 150 mM sodium chloride (NaCl), 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin and phenylmethylsulfonyl fluoride (PMSF). The lysates were centrifuged at $13,000 \times g$ for 10 minutes. The supernatant was quantified and used for Western blot analysis. The protein concentrations were measured by folin-phenol (DC) protein reagent (Peterson et al., 1979) using bovine serum albumin (BSA) as a standard. Cell lysate containing 100 µg of the protein was fractionated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE) gel. The proteins were transferred onto a polyvinylidenedifluoride (PVDF) membrane (Millipore, Bedford, MA) at a constant current of 400 mA overnight at 4°C. The membranes were blocked with 10% non-fat milk (BioRad) in PBS containing 0.05% Tween 20 (PBST) for at least 1 hour at room temperature. The membranes were subsequently probed overnight at 4°C with anti-HSP70 (EMD Biosciences, La Jolla, CA) at a 1:1000 dilution in primary antibody dilution buffer (1% non-fat milk in PBST). After the membranes were washed three times in PBST, they were incubated for 1 hour at room temperature with horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibodies (EMD Biosciences, La Jolla, CA) at a 1:10,000 dilution in PBST. After washing the membranes with PBST, the protein bands were detected with enhanced chemiluminescence (ECL-plus) western blotting detection system (GE Biosciences, Piscataway, NJ). Membranes were exposed to blue CXposure Film (Thermo-Fisher Scientific, Houston, TX) and visualized by auto-radiography using Kodak X-OMAT Processor (Mid-South Medical Imaging, Flowood, MS).

HPLC-MS Quantification of Glutamate, Dopamine and 3-Methoxytyramine

Protein quantification of control and treated samples was done using the Modified Lowry Protein Assay (Pierce, Rockford, IL). Bovine serum albumin (BSA) standards were prepared. 40 µl of each standard and unknown sample replicate were placed into a 96-well plate. 200 µl of Modified Lowry Reagent was added to each well and immediately mixed for 30 seconds. The 96-well plate was covered and incubated at room temperature for 10 min. 20 µl of prepared 1X Folin-Ciocalteu Reagent to each well and immediately mixed for 30 sec. The 96-well plate was covered and incubated at room temperature for 30 min. The absorbance was measured at or near 750 nm on a plate reader. The average 750 nm values of the blank standard replicates were subtracted from the 750 nm value of all other individual standard and unknown sample replicates. These values were used in the quantification of glutamate and dopamine. In addition, 3-Methoxytyramine (3-MT), the major extracellular metabolite of the neurotransmitter dopamine, was also analyzed. 3-MT is formed by the introduction of a methyl group to dopamine by the enzyme catechol-O-methyl transferase (COMT). 3-MT can be further metabolized by the enzyme monoamine oxidase (MAO) to form homovanillic acid (HVA), which is then typically excreted in the urine. An important part of DA metabolism occurs through 3-MT. This amine is deaminated by MAO-A so effectively that increased formation does not increase its endogenous levels. It appears that 3-MT is an indicator of increased DA release (Waldmeier et al, 1981). It has been reported that 3-MT can induce behavioral effects in a dopamine-independent manner and these effects are partially mediated by the trace amine associated receptor 1 (TAAR1) (Sotnikova et al, 2010).

For the quantification of dopamine, glutamate, and 3-MT, 20 µl of control and treated samples were used. The samples were injected into a high performance liquid chromatography –electrospray tandem mass spectrometry (HPLC-ESI-MS) Analysis system. The system consisted of two pumps (LC-10 ADvp, Shimadzu, Tokyo, Japan), an on-line degasser (DGU-12A, Shi-madzu), and triple quadruple mass spectrometers equipped with an ESI Source (TSQ Quantum, Thermo Scientific, San Jose, CA). Both the LC and mass spectrophotometer were controlled by Xcalibur software (Thermo Finnigan). A

reversed-phase column (C₁₈, 2.1 mm × 5 cm, 3 μm) was used for separation. A switching-valve was placed after the column directing the effluent either to waste or to the MS detector. LC elution was carried out with a mobile phase of 50% methanol with 0.1% formic acid at 0.2 m/min for isocratic elution.

The MS detector was operated in the positive mode with the following parameters: evaporation temperature of 150°C, sampling capillary temperature of 250°C, ESI voltage of 3000 V, sheath gas pressure- 35 psi, and auxiliary gas flow rates of 10 (arbitrary units). Peak heights were measured for all samples. Peak height was then divided by g/protein (from protein quantification) and graphed accordingly.

Statistical Analysis

Experiments were performed in triplicates. Quantitative Data were analyzed using Graph Pad Prism 6 statistical software and presented as means ± SDs. Where appropriate, one-way ANOVA or student paired *t*-test was performed using SAS Software available in the Biostatistics Core Laboratory at Jackson State University. *p*-values less than 0.05 were considered statistically significant.

RESULTS

Effects of Mg, Pb, Fe(II), and Fe(III) on the Viability of PC-12 Cells

Figure 1 shows the effects of MgSO₄, Pb(NO₃)₂, FeCl₂, and FeCl₃ on the viability of pheochromocytoma (PC-12) cells using the LDH assay. As shown in this figure, the tested concentrations (5.01 and 50.01 μg/ml) of Mg, Pb, Fe(II), and Fe(III) have a significant impact on the viability of PC-12 cells. The percentages of cytotoxicity were 0.00±0.00% for the control, and 8.10±0.02%, 9.90±0.00%, 86.50±0.09%, and 69.40±0.09% in PC-12 cells exposed to 5.01 μg/ml of Mg, Pb, Fe(II), and Fe(III), respectively. At the 50.01 μg/ml concentration, the percentages of cytotoxicity were 10.80±0.01%, 17.10±0.00%, 96.10±0.09%, and 88.60±0.13% for Mg, Pb, Fe(II), and Fe(III), respectively. Hence, significant differences in cytotoxicity levels (*p*<0.05) were observed between control and Mg-, Pb-, Fe(II)-, and Fe(III)-treated PC-12 cells. Also, higher mortality rates were recorded at higher level of exposure (50.01 μg/ml) compared to lower exposure level (5.01 μg/ml).

Genotoxic Effects of Mg, Pb, Fe(II), and Fe(III) on PC-12 Cells

The profiles and percentages of DNA damage in control and metal-treated PC-12 cells are presented in Figure 2 and Figure 3, respectively. There was a concentration-dependent increase in percentage of DNA damage with chemical exposure. The mean percentage of DNA damage was 0.00±0.00% in the control cells without metal treatment. At the 5.01 μg/ml treatment, the percentages were 8.10±0.02%, 9.90±0.00%, 86.50±0.09%, and 69.40±0.09% for Mg, Pb, Fe(II), and Fe(III), respectively. At 50.01 μg/ml, the percentages of DNA damage were 10.80±0.01%, 17.10±0.00%, 96.10±0.09%, and 88.60±0.13% for Mg, Pb, Fe(II), and Fe(III), respectively. Overall, the DNA damage was significantly different (*p*<0.05) from the control as compared to the treatment groups (Figure 3).

Effects of Mg, Pb, Fe(II), and Fe(III) on HSP₇₀ Expression

HSP₇₀ expression was used as a biomarker of MgSO₄- Pb(NO₃)₂-, FeCl₂ -, and FeCl₃- induced oxidative stress in PC-12 cells. The Western blot analysis revealed a concentration-dependent response to MgSO₄, Pb(NO₃)₂, FeCl₂, and FeCl₃ exposure. As shown in Figure 4, there was an upregulation of HSP₇₀ in PC-12 cells at both 5.01 and 50.01 µg/ml.

Effects of Mg, Pb, Fe(II), and Fe(III) on Glutamate, Dopamine and 3-MT Levels

Figures 5 and 6 present the concentration levels of glutamate and dopamine in PC-12 cells respectively, after treatment with selected metallic compounds. Treatment of PC-12 cells with 5.01 and 50.01 µg/ml of Mg, Pb, Fe(II), and Fe(III) resulted in a significant increase ($p < 0.05$) in glutamate for Fe(II) at 50.01 µg/ml and Fe(III) at 5.01 µg/ml. However, there was a decrease in glutamate concentration for Mg and Pb at 5.01 and 50.01 µg/ml, Fe(II) at 5.01 µg/ml and Fe(III) at 50.01 µg/ml. For dopamine, the treatment of PC-12 cells with 5.01 and 50.01 µg/ml of Mg, Pb, Fe(II), and Fe(III) resulted in significant increases ($p < 0.05$) in protein levels. As shown in Figure 7, a similar trend was also observed for 3-MT showing a significant increase in concentration levels with the treatment of PC-12 cells at both 5.01 and 50.01 µg/ml of Mg, Pb, Fe(II), or Fe(III).

DISCUSSION

Effects of Mg, Pb, Fe(II), and Fe(III) on the Viability of PC-12 Cells

Data obtained from the present study indicates that MgSO₄, Pb(NO₃)₂, FeCl₂, and FeCl₃ are cytotoxic to pheochromocytoma (PC-12) cells. These findings are in full agreement with previous reports indicating that Mg, Pb, and Fe are cytotoxic to other cell lines (Dribben et al, 2009; Sharifi and Mousavi, 2008; Garcia-Alfonso et al, 1995). In the present study FeCl₂ and FeCl₃ appeared to be more toxic than MgSO₄ and Pb(NO₃)₂. This could be due to the higher capacity of iron to cause free radical damage. Fe is selectively absorbed because of its essential role in oxygen transfer. When Fe replaces other elements in the body, it also causes inflammation. This occurs due to the iron's direct attraction to oxygen. Although Fe is an important mineral in the body, overdose can replace other vital minerals and causes enzyme malfunction and cell damage. MgSO₄ exposure has been reported to induce apoptotic cell death in the neonatal mouse brain (Dribben et al, 2009). Recent reviews have suggested that the cytotoxic effects are dose-dependent and that higher doses of exposure may increase neonatal morbidity and mortality. For many metals including arsenic, the toxicity is dependent upon the chemical species, the route of exposure, and many other important factors (Tchounwou et al, 2002).

Genotoxic Effects of Mg, Pb, Fe(II), and Fe(III) on PC-12 Cells

Single cell gel electrophoresis (Comet) assay was used for genotoxicity assessment. It is a rapid, simple, and sensitive technique used as a quantitative assessment of toxic effects on DNA damage or apoptosis (Fairburn et al, 1995). In *in vitro* studies, the Comet assay has been shown to detect genetic damage induced by different genotoxic agents such as radiation (Tice et al, 2000), herbicides (Ribas et al, 1995), and heavy metals (Hartmann and Speit, 1999). The applications of the Comet assay include analysis of genotoxic activity,

human and environmental biomonitoring to DNA repair processes, cellular response to DNA damage, chromosomal damage, cancer risk assessment, and cancer cell resistance to treatment (Tice et al, 2000).

The present study clearly showed that MgSO_4 , $\text{Pb}(\text{NO}_3)_2$, FeCl_2 , and FeCl_3 are genotoxic to PC-12 cells, and this genotoxicity is concentration-dependent. These findings are in agreement with previous reports indicating the genotoxic potential of Mg, Pb, and Fe (Di Virgilio et al, 2011; Grover et al, 2010; Fulladosa et al, 2006). A study by Wolf et al reported that low extracellular Mg could induce oxidative damage (Wolf et al, 2008). Using the Comet assay, Di Virgilio and colleagues (2011) investigated the DNA damage potential of Mg particles. Several *in vitro* studies have also reported the genotoxic potential of Pb (Garcia-Leston et al, 2010 ; Wright, 2003), and Fe (Gurzau et al, 2003; Lima et al, 2011). Although the biochemical and molecular mechanisms of action of Pb remain still unclear, there are some studies that point out indirect mechanisms of genotoxicity, such as inhibition of DNA repair or production of free radicals (Garcia-Leston, 2010; Wright et al, 2003). Other experiments with the Comet assay have revealed a significant increase in the level of DNA damage in workers occupationally exposed to Pb (Grover et al, 2010).

Fe can induce free radicals that cause DNA double-strand breaks (Gurzau et al, 2003; Reizenstein, 1991; Whysner and Wang, 2001). Iron-amplified oxidative stress may also increase DNA damage. This is supported by clinical, experimental, and epidemiological observations (Gurzau et al, 2003). Several studies have been conducted to demonstrate the potential induction of DNA aberrations by Fe and also by drugs and compounds containing this metal. However, the results are inconclusive, and the mutagenic effect of Fe has yet to be elucidated. Genotoxic effects of Fe were reported by Garry et al (2003) in rats treated with FeO (Fe_2O_3 ; .75 mg) for 24 hr. A study by Lima et al (2011) also showed that Fe in the form of FeSO_4 at 4.5, 9.0, and 18 μM concentrations induces alterations and inhibition of DNA synthesis in a dose-dependent manner. Oxidative damage resulting from Fe accumulation in N2A cells and hippocampal neurons has also been reported (Nunez-Millacura et al, 2002).

Effects of Mg, Pb, Fe(II), and Fe(III) on HSP₇₀ Expression

The present study shows that the treatment of PC-12 cells with MgSO_4 , $\text{Pb}(\text{NO}_3)_2$, FeCl_2 , and FeCl_3 induces HSP₇₀ expression. There was an upregulation of HSP₇₀ in PC-12 cells at both 5.01 and 50.01 $\mu\text{g}/\text{ml}$ for Mg, Pb, Fe(II), and Fe(III). This is indicative of the cells undergoing oxidative stress or inflammatory reaction. The HSP₇₀s are an important part of the machinery to help protect cells from stress (heat shock, heavy metal exposure, and oxidative stress). Members of the HSP₇₀ family are expressed at higher levels in times of stress, usually whenever the cell finds itself under conditions that are unfavorable for protein folding (Garrido et al., 2003). Increased expression of the chaperones guide the synthesis of new polypeptides needed to replace those irreparably damaged as well as help in the repair of proteins damaged by the particular stress event. HSP₇₀ was used as a biomarker for oxidative stress because previous studies have suggested that it is a sensitive biomarker for monitoring, not only oxidative stresses but also, cellular stresses, including inflammation and tissue injury.

Previous studies have reported that heavy metals and many other trace elements induce HSP₇₀ expression in various cell lines (Tully et al, 2000; Selvin-Testa et al, 1997; Grover et al, 2010). It has been reported that their induction of heat shock proteins is associated with the initiation of signal transduction cascades (Cavigelli et al, 1996; Daum et al, 2001). Heat, ROS, and anticancer drugs are some of the physiological and environmental insults in which stress or HSPs are expressed (Garrido et al, 2003).

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. In humans, oxidative stress is thought to be involved in the development of cancer (Halliwell, 2007), Parkinson's disease, Alzheimer's disease (Valko et al., 2007), autism (James et al., 2004) and a variety of other illnesses. Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione (Shafer, 1998). The effects of oxidative stress depends upon the size of these changes with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis.

Oxidative stress plays an important role in Pb (Sharifi and Mousavi, 2008; Penugonda et al, 2006) and Fe (Piero and Connor, 2000; Youdim et al, 2004; Gurzau et al, 2003) toxicity. Because Pb cannot readily undergo valance changes, the mechanisms that enable Pb to induce oxidative stress are not clear (Gurer and Ercal, 2000). Several mechanisms are proposed for Pb-induced oxidative stress: (1) direct effect of Pb on cell-membranes, (2) Pb-hemoglobin interactions, (3) δ -aminolevulinic acid (δ -ALA)-induced generation of ROS, and (4) effect of Pb on the antioxidant defense system of cells. Fe-amplified oxidative stress may increase DNA damage, activate precarcinogens, and support tumor cell growth. This is supported by experimental, clinical, and epidemiological observations (Puntarulo, 2005; Gurzau et al, 2003).

A study by Altura et al (2003) tested the hypothesis that the administration of low extracellular levels of Mg ions $[Mg^{2+}]_0$ to primary cultured cerebral vascular smooth muscle cells will cause lipid peroxidation, degradation of $1\kappa\beta$ - α , and activation of nuclear transcription factor kappa β (NF- $\kappa\beta$). Results from this study reported a concentration-dependent increase in malondialdehyde (MDA) production. Using electrophoretic mobility shift assay and specific antibodies, low $[Mg^{2+}]_0$ also caused two DNA-binding proteins (p50, p65) to rise in nuclear extracts in a concentration-dependent manner. These new biochemical and molecular data indicated that low $[Mg^{2+}]_0$ in concentrations found in the blood of patients, after traumatic brain injury (TBI) and diverse types of strokes, can elicit rapid lipid peroxidation and activation of NF- $\kappa\beta$ in cerebral vascular smooth muscle cells.

Effects of Mg, Pb, Fe(II), and Fe(III) on Glutamate, and Dopamine Levels

Glutamate is the most common neurotransmitter in the brain. It plays a key role in long-nerve function and is important for learning and memory (Sapolsky, 2005). It operates

through a series of specific receptors, either ionotropic such as NMDA (N-methyl-4-isoxazole-propionic acid), and kainite or G-protein operated metabotropic receptors. When these receptors are overstimulated, a series of intracellular signaling events occurs that can lead to cell death, a process called excitotoxicity (Blaylock, 2007). Excitotoxicity due to glutamic acid is a major destructive process seen in strokes and other forms of brain ischemia (Best, 1999). The interaction between activation of the excitotoxic cascade and between chronic microglial activation has been demonstrated in a growing number of neurological disorders, including neurodegenerative diseases such as Alzheimer's, dementia, Parkinson's disease, Lou Gehrig's disease (amyotrophic lateral sclerosis, ALS), Huntington's disease, and olivopontocerebral degeneration. These activations are also found in ischemia/hypoxia, strokes, hypoglycemic brain injury, glaucoma, brain trauma, meningitis, viral encephalitis, multiple sclerosis, and autism. This same immune/excitotoxic reaction has been found to play a dominant role in a number of metal-related neurotoxic reactions, including those involving Hg, Pb, Cd, Al, and Sn (Blaylock, 2007).

Dopamine (DA) is a neurotransmitter involved in a variety of CNS processes, including cognition, motor activity, motivation, and reward, mood, attention, and learning. Consequently, any changes in DA transmission may adversely affect a variety of neurological processes and lead to debilitating behavioral disorders. Parkinson's disease, a neurodegenerative movement disorder, is characterized by the loss of DA neurons and subsequent decrease in DA activity (Davie, 2008; Jones and Miller, 2008). Conversely, overstimulation of dopaminergic neurotransmission can lead to hyperactivity and is associated attention deficits, or attention deficit hyperactivity disorder (ADHD) (Staller and Faraone, 2007; Jones and Miller, 2008).

Any changes in glutamate and dopamine levels may not only cause cell death but also adversely affect a variety of neurological processes and lead to debilitating behavioral disorders such as Alzheimer's, Parkinson's, and other diseases (Jones and Miller, 2008; Tsapkis and Travis, 2002). We evaluated the concentration levels of glutamate, dopamine, and 3-MT in control and Mg-, Pb-, and Fe-treated PC-12 cells. Study data showed that PC-12 cells exposed to MgSO_4 , $\text{Pb}(\text{NO}_3)_2$, FeCl_2 , and FeCl_3 had significant alterations in glutamate and dopamine levels, compared to the control. In general, the changes in neurotransmitters' levels were concentration-dependent. Previous studies have reported that metals such as Pb and Fe, can cause protein dysregulation of functional activities in many cell lines (Elseweidy and El-Baky, 2008; Jones and Miller, 2008; Fitsanakis et al, 2006; Dribben et al, 2009; Sharifi and Mousavi, 2008; Garcia-Alfonso et al, 1995; Hallak et al, 1996; Penugonda et al, 2006; Borisova et al, 2011; Santiago et al, 2000; Lozac, 2007; Lafuente et al, 2001). In our study, there was a decrease in some levels of glutamate compared to the control. This decrease could result from the blockage of calcium influx through voltage-gated Ca^{2+} channels, and with chronic toxicity cause dopamine and glutamate depletion. Some metals are well-known specific blockers of voltage-dependent Ca channels (VDCC), but the mechanism of block may be different. Some metals may bind to the high affinity site inside the VDCC pore (Yang et al, 1993; Wakamori et al, 1998), and some metals compete with Ca at a site external to the VDCC, and the binding is not voltage-dependent (Peng et al, 2002).

The dopaminergic system appears to be a major target for environmental neurotoxins, although a variety of neurological processes can be adversely affected. The adverse neurological consequences of human exposure to heavy metals are well documented (White et al, 2007; Aschner et al, 2007; Jones and Miller, 2008). Among the trace elements, manganese (Mn) has been reported as a potent dopaminergic neurotoxicant (Fitsanakis et al., 2006; Stredrick et al., 2004; Jones and Miller, 2008) which ultimately leads to impairments in DA-associated behaviors (Diaz-Veliz et al., 2004; Jones and Miller, 2008). Recent evidence implicates cellular oxidative stress (Jones and Miller, 2008; Erikson et al., 2004) and alterations in DAT function (Jones and Miller, 2008; Fitsanakis et al., 2006) as mechanisms underlying Mn-induced neurotoxicity. MgSO₄ is often used as a treatment for preeclampsia/eclampsia and preterm labor, resulting in the exposure of a significant number of neonates to this drug despite a lack of evidence suggesting that it is safe, or effective as a tocolytic. While there is evidence that MgSO₄ may be neuroprotective in perinatal brain injury, recent reviews have suggested that the effects are dependent upon dose, and that neonatal morbidity and mortality may be actually increased by higher doses. An *in vivo* study with neonatal mice pointed out that Mg treatment caused significant brain damage in animals treated on P3 and P7, but not P14. Administration of high doses of Mg may be detrimental to the fetal brain, particularly if exposure occurs during critical periods of neurodevelopment (Dribben et al 2009).

Lead (Pb), which does not have any known physiological function, penetrates the brain blood barrier and interferes with the normal functioning of the central nervous system resulting in a variety of behavioral impairments. Developmental Pb exposure leads to alterations in multiple aspects of the DA system (Szczerbak et al, 2007; Zuch, et al, 1998; Jones and Miller, 2008) and has been linked to hyperactivity and attention deficit disorders, anti-social behaviors, and senile dementia. A large internationally-pooled analysis of Pb²⁺-exposed children estimated that children with blood Pb levels (BLLs) of 10 µg/dL experience a deficit of about 6.2 IQ points relative to children with estimated BLLs of 1 µg/dL (Neil and Guilarte, 2010, 2012; Lanphear et al., 2005). This was comparable to the deficit of 7.4 IQ points observed in children with BLLs of 10 µg/dL in another large study (Neil and Guilarte, 2010, 2012; Canfield et al, 2003). A decrease in IQ of 6–7 points would be difficult to detect on an individual level. However, the effect of a population decrease in IQ of this magnitude is significant. The number of children with impaired intelligence would increase significantly while the number of exceptionally gifted children would decrease by shifting the normal distribution of IQ scores lower (Neil and Guilarte, 2010, 2012; Gilbert and Weiss, 2006). This effect has been studied by several researchers from an economical standpoint and suggest that the monetary cost of such an effect may total over 40 billion dollars for one age group alone. Over a 20-year period, this loss may amount to nearly 800 billion dollars (Neil and Guilarte, 2010, 2012; Gilbert and Weiss, 2006; Landrigan et al., 2002).

Pb has been shown to disrupt cellular energy metabolism, which may underlie the learning deficits and cognitive dysfunctions associated with environmental Pb exposure. The voltage-dependent anion channel (VDAC) plays a central role in regulating energy metabolism in neurons by maintaining cellular ATP levels and regulating calcium buffering, and studies

have shown that VDAC expression is associated with learning in mice. In a study by Prins, Park, and Lurie (2010), the effect of 5 and 10 μM Pb on VDAC expression *in vitro* was examined, to determine whether Pb alters VDAC expression levels in neuronal cell lines. PC-12 and SH-SY5Y cells were used since they differentiate to resemble primary neuronal cells. VDAC expression levels were significantly decreased 48 hr after exposure to Pb in both cell lines.

Pb exposure can also cause deficits in neurotransmission. Rats chronically exposed to low levels of Pb have reduced Ca^{2+} -dependent glutamate and γ -aminobutyric acid (GABA) release in the hippocampus (Neil and Guilarte, 2010, 2012; Lasley and Gilbert, 1996, 2002; Xiao et al., 2006), which indicates presynaptic neuron dysfunction during Pb^{2+} exposure. In cultured hippocampal neurons (Neil and Guilarte, 2010, 2012; Braga, Pereira, and Albuquerque, 1999) and in brain slices (Neil and Guilarte, 2010, 2012; Xiao et al, 2006), Pb^{2+} exposure impairs excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs). EPSCs and IPSCs are dependent upon neurotransmitter release from the presynaptic neuron, thus, reductions in EPSCs and IPSCs indicate a deficit in neurotransmission in both the glutamatergic and GABAergic systems as a result of Pb^{2+} exposure. A recent study from the laboratory showed that Pb^{2+} exposure in cultured hippocampal neurons during synaptic development resulted in altered presynaptic protein expression and deficits in vesicular neurotransmitter release (Neil and Guilarte, 2010, 2012; Neal et al, 2010). Expression of key presynaptic proteins involved in vesicular release, such as synaptophysin and synaptobrevin, were reduced by Pb^{2+} . Reductions of vesicular release proteins were associated with both glutamatergic and GABAergic synapses, consistent with electrophysiological observations regarding EPSC and IPSC generation during Pb^{2+} exposure (Neil and Guilarte, 2010, 2012; Xiao et al, 2006; Braga, Pereira, and Albuquerque, 1999). Vesicular release in Pb^{2+} exposed neurons was significantly impaired relative to control conditions as determined by live-imaging studies using the synaptic vesicle dye FM 1–43 (Neil and Guilarte, 2010, 2012; Neal et al, 2010). Together, animal and cell culture studies indicate a role for Pb^{2+} in presynaptic dysfunction which results in reduced neurotransmission (Neil and Guilarte, 2010, 2012).

Excess Fe in the brain has been implicated in the pathogenesis of several human neurodegenerative disorders, i.e. Parkinson's and Alzheimer's disease. The neonatal period is critical for the establishment of normal Fe content in the adult brain. Fredrikson et al (1999) studied the long-term neurobehavioral effects of Fe exposure during this period by treating NMRI mice orally with 0, 3.7, or 37 $\text{mg/Fe}^{2+}/\text{kg}$ body weight on PND 10–12. Mice treated with the higher dose of Fe^{2+} , 37 mg/kg body weight, were hypoactive during the first 20 min of testing but hyperactive during the final 20 min, showing an almost complete lack of habituation of spontaneous activity in the test chambers. Analysis of brain Fe content indicated significantly more total Fe ($\mu\text{g/g}$) in the basal ganglia, but not frontal cortex, of the higher 37 mg/kg dose group. Increased amounts of Fe in the brain, especially in the basal ganglia, may contribute to neurodegenerative processes.

Intracellular reactive Fe is a source of free radicals and a possible cause of cell damage. Núñez-Millacura et al (2002) analyzed the changes in Fe homeostasis generated by Fe accumulation in neuroblastoma (N2A) cells and hippocampal neurons. Increasing amounts

of intracellular iron and of the reactive iron pool were elicited by increasing concentrations of Fe in the culture medium. The cells had both Fe regulatory protein 1 (IRP1) and iron regulatory protein 2 (IRP2) activities, being IRP1 activity quantitatively predominant. The increase in IRP1 activity was mediated by oxidative-stress as it was largely abolished by N-acetyl-L-cysteine protein and DNA modifications. This behavior is possibly a consequence of the paradoxical activation of IRP1 at high Fe concentrations, a condition that may underlie some processes associated with neuronal degeneration and death.

Fe imbalance in the brain, including excess accumulation and deficiency are associated with neurological disease and dysfunction (Ali, Maryam, and Ali, 2010). Maaroufi et al (2009) found a significant accumulation of Fe in the brain of adult rats subjected to Fe overload (i.e. 3 mg kg⁻¹ ip for 5 days), and pointed out that the observed learning deficits could first be explained by impairments of the hippocampus. It has been reported that oxidative stress and monoamine dysfunctions can occur in the adult brain after administration of high amount of Fe for only a few days (Maaroufi et al, 2009; Ali, Maryam, and Ali, 2010).

CONCLUSIONS

The present study demonstrated that Mg, Pb, Fe(II), and Fe(III) induce cytotoxicity, oxidative stress, and genotoxicity in PC-12 cells; as characterized by significant increases in cell death, HSP₇₀ expression, and DNA damage in metal-treated cells as compared to control cells. Findings from this research also pointed that Mg, Pb, Fe(II), and Fe(III) exposure resulted in significant alterations in the concentration levels of glutamate, and dopamine and its metabolite 3-MT. These changes in the activity of neurotransmitters play a key role in metal-induced neurotoxicity and neurodegenerative disorders such as Alzheimer and Parkinson diseases.

Acknowledgments

The research described in this publication was made possible by a grant from the National Institutes of Health (Grant No. G12MD007581) through the RCMI Center for Environmental Health at Jackson State University.

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Figure 1.

Cytotoxic effect of Mg, Pb, Fe(II), and Fe(III) on PC-12 cells. The data is represented as mean $\pm$ SD of three experiments performed in triplicates. The differences were considered statistically significant with a p value < 0.05. The significance of the value is indicated by asterisk (*).

Figure 2.

Representative Comet assay images of PC-12 cells. Untreated control (A); Treated with: Mg at 5.01 $\mu\text{g/ml}$ (B); Mg at 50.01 $\mu\text{g/ml}$ (C); Pb at 5.01 $\mu\text{g/ml}$ (D); Pb at 50.01 $\mu\text{g/ml}$ (E); Fe(II) at 5.01 $\mu\text{g/ml}$ (F); Fe(II) at 50.01 $\mu\text{g/ml}$ (G); Fe(III) at 5.01 $\mu\text{g/ml}$ (H); and Fe (III) at 50.01 $\mu\text{g/ml}$ (I).

Figure 3.

DNA damage of PC-12 cells treated with Mg, Pb, Fe(II), and Fe(III) at 5.01 µg/ml and 50.01 µg/ml by comet assay. The figure presents the percentages of DNA damage. The data is represented as mean $\pm$ SD of three experiments performed in triplicates. The differences were considered statistically significant with a p value < 0.05. The significance of the value is indicated by asterisk (*).

Figure 4.
Expression of 70kd heat shock protein in Mg, Pb, Fe(II), and Fe(III)-treated PC-12 cell at 5.01 µg/ml and 50.01 µg/ml.

Figure 5. Intracellular levels of glutamate (µg/g protein) in PC-12 cells exposed to 5.01 and 50.01 µg/ml of Mg, Pb, Fe(II), and Fe(III). The data is represented as mean $\pm$ SD of three experiments performed in triplicates. The differences were considered statistically significant with a p value < 0.05. The significance of the value is indicated by asterisk (*).

Figure 6. Intracellular levels of dopamine (µg/g protein) PC-12 cells exposed to 5.01 and 50.01 µg/ml of Mg, Pb, Fe(II), and Fe(III). The data is represented as mean $\pm$ SD of three experiments performed in triplicates. The differences were considered statistically significant with a p value <0.05 . The significance of the value is indicated by asterisk (*).

Figure 7. Intracellular levels of 3-methoxytyramine (µg/g protein) in PC-12 cells exposed to 5.01 and 50.01 µg/ml of Mg, Pb, Fe(II), and Fe(III). The data is represented as mean $\pm$ SD of three experiments performed in triplicates. The differences were considered statistically significant with a p value <0.05. The significance of the value is indicated by asterisk (*).

TABLE I

Physical and chemical properties of magnesium sulfate (MgSO_4), lead nitrate [$\text{Pb}(\text{NO}_3)_2$], ferrous chloride (FeCl_2), and ferric chloride (FeCl_3) (www.chemicalbook.com-CAS Data Base List)

Characteristics	MgSO_4	$\text{Pb}(\text{NO}_3)_2$	FeCl_2	FeCl_3
Molar mass	120.366 g/mol (anhydrous) 246.47 g/mol (heptahydrate)	331.2 g/mol	126.751 g/mol (anhydrous) 198.8102 g/mol (tetrahydrate)	162.2 g/mol (anhydrous) 270.3 g/mol (hexahydrate)
Appearance	White crystalline solid	White colorless crystals	Tan solid (anhydrous) Pale green solid (ditetrahydrate)	Green-black by reflected light; purple-red by transmitted light Hexahydrate: yellow solid Aq. Solutions: brown
Density	2.66 g/cm ³ (anhydrous) 1.68 g/cm ³ (heptahydrate)	4.53 g/cm ³ (20°C)	3.16 g/cm ³ (anhydrous) 2.30 g/cm ³ (dehydrate) 1.39 g/cm ³ (tetrahydrate)	2.898 g/cm ³ (anhydrous) 1.82 g/cm ³ (hexahydrate)
Melting point	1124°C (anhydrous, decomposes) 150°C (heptahydrate, decomposes)	270°C, decomposes	677°C (anhydrous)	306°C (anhydrous) 37°C (hexahydrate)
Boiling point			1023°C (anhydrous)	315°C (anhydrous, decomposes) 280°C (hexahydrate, decomposes) (partial decomposition to $\text{FeCl}_2 + \text{Cl}_2$)
Solubility in water	26.9 g/100 ml (0°C) anhydrous 25.5 g/100 ml (20°C) anhydrous 71 g/100 ml (20°C) heptahydrate	37.65 g/100 ml (0°C) 52 g/100 ml (20°C) 127 g/100 ml (100°C)	64.4 g/100 ml (10°C) 68.5 g/100 ml (20°C) 105.7 g/100 ml (100°C)	74.4 g/100 ml (0°C) 92 g/100 ml (hexahydrate, 20°C)