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Effects of mercury on the dopamine transporter cell surface expression in PC12 cells

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Effects of Mercury on the Dopamine Transporter Cell Surface Expression in PC12 cells

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Abstract

Environmental factors, such as heavy metal exposures, have been suggested to have an impact not only on neurodegenerative disease, such as Parkinson's disease, but also on psychostimulants abuse and their toxicity. In this study, two questions were addressed: 1) effects of mercuric chloride on parkinsonian toxicant 1-methyl-4-phenylpyridinium (MPP^+) induced cytotoxicity and 2) effects of mercuric chloride on cell surface dopamine transporter. Pheochromocytorma cells (PC12) were treated with various concentrations of mercuric chloride (0.02~2.0 ppm) for 4 hours with and without 0.1 mM MPP^+. Significant potentiation of toxicity was observed when there was co-treatment with 0.5 ppm $HgCl_2$ and 0.1mM MPP^+ vs. $HgCl_2$ alone. The potentiation of toxicity with the co-treatment of 0.5 ppm mercuric chloride and 0.1mM MPP^+ was not observed in PC12 cells expressing DAT mutant L368Q, which has impaired uptake activity. Cell surface expression of dopamine transporter was quantitatively seen to be transiently increased via western blot analysis which was correspondingly visualized via immunocytochemical assay. In summary, mercuric chloride enhanced MPP^+ toxicity in a dopamine transporter-dependent manner and increased the surface expression of dopamine transporter in PC12 cells. This toxicological effect of mercury on dopamine transporter could trigger an unpredicted toxicological/pharmacological interaction with drugs affecting the dopaminergic system.

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1. Introduction

Mercury (Hg) is a known ubiquitous neurotoxic metal that is able to travel through the environment and food web while interconverting between its different chemical forms (Morel, Kraepiel, & Amyot, 1998). Mercury species in our environment can be in various oxidation states including mercuric (+2) and mercurous (+1). In particular, methyl mercury (MeHg) compounds are a major contaminant in the food chain, MeHg is formed by the conversion of inorganic mercury in the environment by microorganisms (Morel et al., 1998). MeHg can be bioaccumulated and reach high levels especially in organisms at the top of the food chain, which includes humans.

In humans and other animals, the body's general protective measures for eliminating mercury from the body include elimination through the intestines (MeHg) and kidneys (inorganic Hg) (Morel *et al.* 1998; Guzzi & Porta, 2008). When these measures are unable to cope with the amount of accumulated mercury, toxicity or accumulation can occur. Direct exposure to inorganic elemental mercury (Hg^0) vapors is a concern for dental professionals and patients that are chronically exposed to mercury containing dental amalgams (Mackert & Berglund, 1997). Critically, internal conversion of mercury species, such as methylmercury and elemental mercury in the body, would result in the accumulation of Hg at the brain and kidneys. Chemically, after entering the bloodstream some of the elemental mercury diffuses into erythrocytes and is converted to divalent mercuric cation (Aschner & Aschner, 1990). The elemental mercury that crosses the blood brain barrier, is then oxidized by peroxide catalase from elemental mercury to divalent mercury cation which then attaches to thiol containing ligands (Aschner & Aschner, 1990).

It was originally thought that methylmercury crosses the blood brain barrier (BBB) due to its lipophilic properties. In rat, methyl mercury was found to pass the BBB via the L (large neutral) amino acid carrier as a complex with L-cysteine (MeHg-L-cysteine), which is structurally similar to the naturally occurring amino acid methionine (Kerper, Ballatori, & Clarkson, 1992; Morkrzan, Kerper, Ballatori, & Clarkson, 1995).This complex is formed as the result of mercury's affinity for sulfhydryl group containing proteins (Morkrzan et al.,1995). A study of methylmercury ingestion in macaques demonstrated accumulation of inorganic mercury rather than methylmercury within astroglia and neurons of the brain thalamus (Charleston et al., 1996; Charleston, Body, Mottet, Vahter, & Burbacher, 1995; Nagano, Yasutake, & Miura, 2010). Inorganic mercury is not lipophilic and thus, would not be expected to cross the BBB; however, long term exposure has resulted in eventual detectable levels within the brain of monkeys (Vahter et al., 1994). The travel of inorganic mercury Hg^{2+} past the BBB has been proposed to occur as a result of an unidentified transporter (Castiglioni & Qian, 2001). Similar to what was observed in the macaque study, large amounts of inorganic mercury were found in human brain who had been exposed to high levels of methyl mercury in the Minamata mercury disaster (Minamata bay, Japan) (Takahashi, Suetomi, & Konishi, 1991). Since the mercury accumulated in the brain is in the form of the inert mercuric selenide and sulfide, the neurotoxic effect of Hg^{2+} must occur after the demethylation of methylmercury and before the conversion to the final inert Hg species. However, as far as our knowledge, the possible underling mechanism is not clear.

Investigation of environmental heavy metals and toxins is of importance due to their possible individual or combined effects on the neuronal system of the body (Andrade, Aschner, & Marreilha dos Santos, 2017). Both exposure and accumulation of environmental heavy metals

and toxins may lead to detrimental effects, which can worsen when combined with neurotoxins. The link between heavy metal exposure including lead (Pb), manganese (Mn), and Mercury (Hg) to neurodegenerative diseases including Parkinson's and Alzheimer's has been investigated (Charlet et al., 2012). Additionally, effect on the neurons can lead to change in responsiveness or adverse reactions when combined with drug use.

Parkinson's disease (PD) is a neurodegenerative disease characterized by presence of α-synuclein Lewy body aggregates and degeneration of midbrain neurons in both the substantia nigra pars compacta neurons (Kasten, Chade, & Tanner, 2007; Dauer & Przedborski, 2003) and thalamus (Halliday, 2009), which belong to dopaminergic system (Grace, Floresco, Goto, & Lodge, 2007; Girault & Greengard, 2004). Physical manifestations of Parkinson's disease (PD) are tremor at rest, bradykinesia, muscular rigidity, and postural instability (Jankovic, 2008). The disease has been linked to both genetic and environmental sources (Jankovic, 2008), such as mutations in the α-synuclein gene and exposure to neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Polymeropoulos et al., 1997; Smeyne & Jackson-Lewis, 2004; Burns, LeWitt, Ebert, & Kopin, 1985; Wichmann & DeLong, 2003). MPTP crosses the blood brain barrier and is converted to 1-methyl-4-phenylpyridinium (MPP^+) within astrocytes. MPP^+ is specially taken into dopaminergic neurons through the dopamine transporter (DAT), leading the inhibition of the mitochondrial complex I and neuronal death (Smeyne & Jackson-Lewis, 2004).

The dopaminergic system regulates reward and motor movement and consists of the dopamine producing neurons in the brain. Neurotransmitter dopamine is released at the synapse by vesicles and dopamine signaling is cut off by the DAT (Grace et al., 2007; Girault & Greengard, 2004). Thus, any agent interfering dopaminergic transmission can result in disruption

and disorder of this system. Disrupting DAT function which results in dopamine persisting longer in the synapses, is widely accepted as the primary cause of the reinforcing and addictive properties of psychostimulants. Abusive drugs increase dopamine signaling by reducing the DAT uptake activity (Drug Facts: Cocaine, 2018., Drug Facts: Methamphetamine, 2019., Li, Cheng, & Reith, 2010; Verma, 2015). Specifically, cocaine is a DAT blocker, which inhibits dopamine reuptake from synapse (Verma, 2015). Methamphetamine affects dopamine level in synapses in a more complex manner as a DAT substrate to enhance internalization of DAT (Li et al., 2010; Wallace and Hood, 2018). In animal studies heavy metal exposure has been shown to result in alteration in sensitivity to the increase of dopamine at the synapse caused by psychostimulants (Wallace & Hood, 2018; Jones and Miller, 2008). Specifically, lead (Pb) and manganese (Mn) increase sensitivity of dopamine receptors leading to higher sensitivity to psychostimulants (Jones & Miller, 2008). Cadmium (Cd) however, reduces sensitivity to psychostimulants via reduction of dopamine turnover (Jones & Miller, 2008).

Dysfunction in the regulation of dopamine neurotransmission is involved in many neurological diseases such as Parkinson's and disorders including, attention hyperactivity disorder (ADHD), bipolarism, and depression (Girault & Greengard, 2004). Exposure to toxins and heavy metals has been linked with Parkinson's and other neurodegenerative disorders (Charlet et al., 2012). It has been suggested that the affinity of mercury for selenoproteins results in disruption of redoxregulation leading to oxidative stress promoting the onset of neurodegenerative and neurological disorders (Farina, Avila, Da Rocha, & Aschner, 2013). Mercury has been suggested to be a possible source of the neurological disorder autism (Blaxill, Redwood, & Bernard, 2004; Mutter, Naumann, Shneider, Walach, & Haley. 2005). It has been shown that individuals exposed to inorganic mercury exhibit memory deficits indicating

neurological impact by mercury (Mutter, Curth, Naumann, Deth, & Walach, 2010). A biphasic pattern of radioligand binding to DAT in the presence of $HgCl_2$was found in membrane and whole cell preparations (Schweri, 1994; Wu, Coffey, & Reith, 1997). It was suggested that DAT binding was increased as a result of conformational change of DAT caused by $HgCl_2$. The expression and function of DAT however, in the presence of $HgCl_2$ was not investigated in these samples. Wallace and Hood (2018) investigated the treatment of the combination of $HgCl_2$ and cocaine on the expression of human DAT on neuroblastoma cell surface and found that while density was increased, there was no correlation with increased functionality of the DAT to uptake dopamine (Wallace & Hood, 2018). The expression and function of DAT in the presence of $HgCl_2$ was not investigated. Alteration of the DAT surface expression in the dopaminergic system could result in unexpected effects regarding sensitivity to psychostimulants drugs and other toxins such as MPP^+ that also act on the same system. Thus, the effect of Hg^{2+} (introduced as mercuric chloride) on surface expression of DAT in PC12 cells was investigated in this study.

2. Materials and Methods

In order to investigate the effect of mercuric chloride on the DAT expression on the surface of rat pheochromocytoma cells (PC12), lab grown PC12 cells was exposed to mercuric chloride ($HgCl_2$) solutions with various concentrations range of 0.2-2.0 ppm. Mercuric chloride stock solution was prepared in phosphate buffered saline (PBS) and stock concentration was determined using a DMA 80 direct mercury analyzer (Milestone, Monroe, CT). For cell viability assessment, MTT assay was performed. Western blot analysis and biotinylation were used to assess relative change of cell surface DAT expression levels. Visualization of distribution of DAT on the surface of cells was done with immunocytochemistry.

2.1 Cell culture

Rat pheochromocytoma cells (PC12) as *in vitro* artificial nerve system tissue model is commonly used for neurodegeneration study. PC12 cell line used in this study was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 5% heat inactivated horse serum (Biowhitaker, Walkersville, MD), and 50 µg/mL gentamicin in a humidified incubator set at 37^{o}C and 5% CO_2.

For chemical treatment, cells were subcultured a day prior to the experiment. Cells were grown until about ~80% confluence on the day of chemical treatments.

2.2 Chemical treatment

Cells were treated with various concentrations of $HgCl_2$ alone (0.02-2.0ppm) or together with 0.1 mM MPP^+. Exposure time for western blot and also immunocytochemical analysis was 0.5 hour to 1 hour and for MTT assay was 4 hours. After washing, cells were analyzed with different assays.

$HgCl_2$ treatment solution was prepared via dilution of a master solution which had ~ 4-8 mg of mercury dissolved in Milli-Q (Millipore) ultra-pure water for total volume of 200 mL. Secondary testing solution was prepared by adding an appropriate volume of the master solution based on calculated concentration depending on amount of mercury used to prepare stock and diluting it to a final volume of 5 mL. Concentration of the secondary solution was analyzed for total mercury concentration using a Milestone direct mercury analyzer model # 80 (Sorisole, Italy). Concentration of the stock was then back calculated from the concentration of the

secondary solution concentration that was analyzed. Working solutions were then prepared from the secondary stock solution based on concentration calculated and ranged from 0.02-2.0 ppm.

The MPP^+ working solution was prepared fresh prior to each experiment in PBS by making a stock solution of 0.1M concentration and adding appropriate volume of stock to obtain the final concentration of 0.5mM in experimental well based on volume.

2.3 Cell Viability assay: MTT Assay

MTT assay is a colorimetric assay used as a quantitative indicator of cell viability. The cellular colorimetric assay uses a yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) which is converted to a purple formazan product through cleavage of the tetrazolium ring by active cellular mitochondria dehydrogenase enzymes (Mosmann, 1983). The amount of product formed is proportional to activity of cellular mitochondria which is indicator of mitochondrial and thus cellular health.

MTT assay was carried out by following manufacturer's protocol (Sigma-Aldrich, TOX1-1KT). In brief, following exposure (4 hours) and removal of treatment chemicals, cells were incubated (37^oC, 5% CO_2) for 2.5 hours with 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution before MTT solubilizing solution (acidified isopropanol) was added. Plate was then gently shaken for 1 hour at room temperature and absorbance at 570nm and 690nm (background) was read using the Synergy Mx microplate reader (Biotek, Winooski, Vermont).

2.4 Western Blot Analysis

To detect the expression of DAT on surface of PC12 cells, biotinylation technique was used. Biotinylation uses biotin to label cell surface proteins, which can then be isolated by NeutrAvidin gel. Surface DAT can be isolated using this method together with all other cell surface proteins. Western blot analysis will be able to detect the expression of DAT in the isolated total cell surface proteins. Therefore, chemical treated cells were first labelled with biotin and then western blot analysis was used to determine the level of biotinylated DAT in order to relatively quantitate surface DAT expression after exposure to mercury (30 minutes or 1 hour).

After removal of treatment, total cell surface proteins including DAT were labeled by biotin. In brief, cells were washed with cold phosphate buffered saline containing 1mM $CaCl_2$ and 1mM $MgCl_2$ (PBS-CM). Ice-cold sulfo-NHS-SS biotin reagent (1 mg/mL in PBS-CM) (Pierce) was then added. Cultures were gently shaken at 4^oC for 60 minutes, which was followed, by washing and incubation for 20 minutes with cold PBS-CM-100 mM glycine and PBS.

After washing with PBS, cells were then lysed and subjected for cell surface protein isolation. In brief, cells were lysed in Mammalian Protein Extraction reagent (M-PER) / Protease Inhibitor cocktail (PI) (Pierce) for 10 minutes at room temperature. Cell lysate was collected through scraping and homogenized by shaking for 1 hour at 4^oC. Lysate was then subjected to centrifugation at 14,000 xg for 15 minutes. Supernatant was collected for cell surface protein isolation. Total protein concentration was determined using the Bio-Rad *DC* (detergent compatible) Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Bovine Serum Albumin

(Sigma) was used to set up the standard curve for protein concentration calculations. Measured amount of total lysate proteins (250ug) was transferred to a centrifuge column (Pierce) and incubated with NeutrAvidin gel (Thermo Scientific) for 2 hours at room temperature to isolate biotinylated cell surface protein. NeutrAvidin gel was washed extensively with PBS, high salt buffer (500mM NaCl / 50mM Tris pH 7.5), and no salt wash buffer (50mM Tris pH 7.5). Bound biotinylated proteins were then removed from the NeutrAvidin gel by incubation with 50mM dithiothreitol (DTT)/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 10 minutes at 21^{o}C and 40minutes at 37^{o}C. Eluted biotinylated proteins were then collected through centrifugation at 8000 rpm for 2 minutes. Eluted biotinylated proteins were subjected to western blot analysis to determine the level of cell surface DAT.

For western blot analysis, both isolated biotinylated proteins and total lysates were separated on 8% acrylamide gel and then transferred to nitrocellulose membranes. Cell surface DAT (biotinylated) and total DAT were probed on membrane using rabbit anti-rat DAT polyclonal primary antibody and anti-rabbit secondary antibody conjugated with horseradish peroxidase. Blots were developed using Super Signal West Pico Chemiluminescent Substrate detection reagents from Pierce. Chemiluminescent signals were captured by Geliance 600 imaging system (Perkin Elmer, Shelton, CT) and analyzed by GeneTools software (Syngene, Frederick, MD). The integrated density values (IDV) of biotinylated DAT from western blot images were normalized with the IDV of the corresponding total DAT western blot images. The final data for all chemically treated groups were expressed as percentage of PBS control data.

2.5 Immunocytochemistry

In order to visualize the distribution of DAT on the surface of PC12 cells, immunocytochemistry was performed. Cells ($1x10^4$ cells per well) were seeded on poly-L-lysine coated 4-well chamber slides on the day before chemical treatments. Cells were then treated with mercuric chloride solution (0.2-1.0ppm) for either 30 minutes or 1 hour.

After chemical treatments, cells were washed with PBS three times and then fixed with 3.7% formaldehyde for 15 minutes at room temperature. Cells were rinsed with PBS and then incubated with Image-iTFX (Thermo Fisher Scientific™) signal enhancer for 30 minutes at room temperature. After PBS wash, the cells were blocked with 1% BSA in PBS/0.5% Tween 20 (1% BSA-PBS) for 1 hour at room temperature. Rabbit anti-DAT polyclonal primary antibody (ThermoFisherScientific™) (1:500 in 1% BSA-PBS) was applied to bind to DAT on the cells at 4^oC for overnight. Cells were washed with PBS to remove primary antibody and goat anti-rabbit secondary antibody conjugated to fluorescent dye Alexa Fluor 488(1:500 in 1% BSA-PBS) was used to visualize DAT on the cells by binding to primary antibody at room temperature for 1 hour. The cells were then washed with PBS and pre-warmed mounting buffer (ProLong® Gold antifade reagent (ThermoFisherScientific™) was applied to each well and rested for 24 hours. Then the slides were sealed with clear nail polish and analyzed under Nikon Eclipse E600 fluorescent microscope.

2.6 Transfection

PC12 cells were transiently transfected with DAT mutant L368Q (a gift from Dr. Reith, NYU School of Medicine, New York, NY). DAT mutant L368Q is known as a loss-function mutant (Kurian et *al.*, 2009). Transfection was performed using SuperFect^{TM} transfection reagent (Qiagen).

DAT mutant L368Q was mixed with SuperFect^{TM} in a ratio of 1 µg DNA to 2 µl transfection reagent according to the manufacturer's protocol. The mixtures were added to PC12 cells grown on six-well plates in 0.6 mL of serum free DMEM. The cells were incubated with the transfection mixtures for 3 hours at 37°C in humidified air containing 5% CO_2. The transfection mixtures were removed and replaced with 1 mL of DMEM containing 10% horse serum and 5% FBS after cells were washed twice with PBS. The cells were incubated for additional 24 hours at 37°C in humidified air containing 5% CO_2 prior to chemical treatments.

2.7 Statistics

All experiments were performed at least in triplicate, and results are reported as means ±SEM. Statistical significance was determined using student's t-test ($p<0.05$, with control at 100%).

3. Results

3.1 PC12 cell viability in response to $HgCl_2$ with or without MPP^+

In order to evaluate the effect of $HgCl_2$ on parkinsonian toxicant MPP^+-induced cytotoxicity, the toxicity of $HgCl_2$ with or without 0.1 mM MPP^+ co-treatment for 4 hours was evaluated via MTT assay. Toxicity was observed to be concentration dependent with higher concentrations of $HgCl_2$ resulting in lower cell viability (Figure 1). Cells treated with 0.1 mM MPP^+ alone did not show any cytotoxic effect. When cells were treated with both MPP^+ and $HgCl_2$, there was generally lower cell viability as compared with$HgCl_2$ alone. At the 0.5 ppm $HgCl_2$ concentration, a significant potentiation effect on MPP^+ toxicity was seen which resulted in a greater increase in cellular toxicity. This potentiation effect was not observed in the cells treated with MPP^+ and $HgCl_2$ at concentrations that were lower (0.02 ppm and 0.2 ppm) or higher (1.0 ppm and 2.0 ppm) than 0.5 ppm (Figure 1).

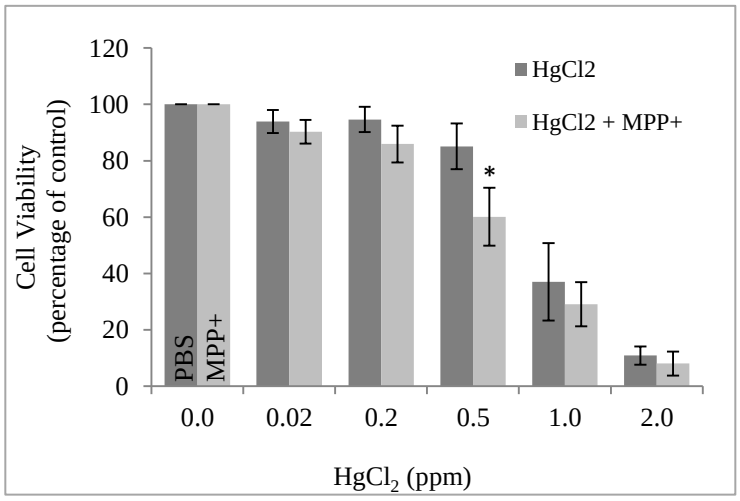

Figure 1 Effect of $HgCl_2$ on the viability of PC12 cells incubated in the presence or absence of (0.1 mM) MPP^+. Cell viability was measured by MTT assay. Following 4 hours of chemical treatment, cell viability decreased in a dose dependent manner with increasing $HgCl_2$ concentration. Co-treatment of cells with 0.5 ppm $HgCl_2$ and 0.1 mM MPP^+ resulted in a significant potentiation of cytotoxicity (*$p < 0.01$). Data were normalized to control with control as 100% cell viability. Data was obtained from 4 different experiments.

These results showed that at the 0.5 ppm $HgCl_2$ treatment concentration, some cellular changes could have occurred which have the ability to enhance the toxicity of MPP^+. The mechanism resulting in the potentiation of toxicity observed at the 0.5 ppm $HgCl_2$ treatment dose when MPP^+ was co-treated was further investigated as possibly resulting from the mechanism of toxicity of MPP^+. One of possible toxic mechanisms is via dopamine transporter. MPP^+ requires entry into the neuron via the use of a transporter, specifically the dopamine transporter prior to causing neurotoxicity (Przedborski & Vila, 2001).

3.2 Cell surface expression of dopamine transporter in response to $HgCl_2$

MPP^+ is a well-known parkinsonian toxicant and is specifically transported into dopaminergic neurons by dopamine transporter (DAT). Once inside the cells, MPP^+ is mainly accumulated in mitochondria and inhibits mitochondrial respiratory chain complex I (Nicklas, Vyas, & Heikkila , 1985; Schildknecht et al., 2015). The cause of the potentiation of toxicity of 0.1 mM MPP^+ when combined with 0.5 ppm $HgCl_2$ in PC12 cells was investigated as possibly being due to an alteration of the cell surface expression of dopamine transporter. Entry into the cell is an essential step in order for MPP^+ to exert its neurotoxic effects on the mitochondria electron transport chain leading to cell death (Przedborski & Vila, 2001). The expression of the dopamine transporter on the surface of the cells following $HgCl_2$ exposure was investigated using western blot analysis (Figure 2).

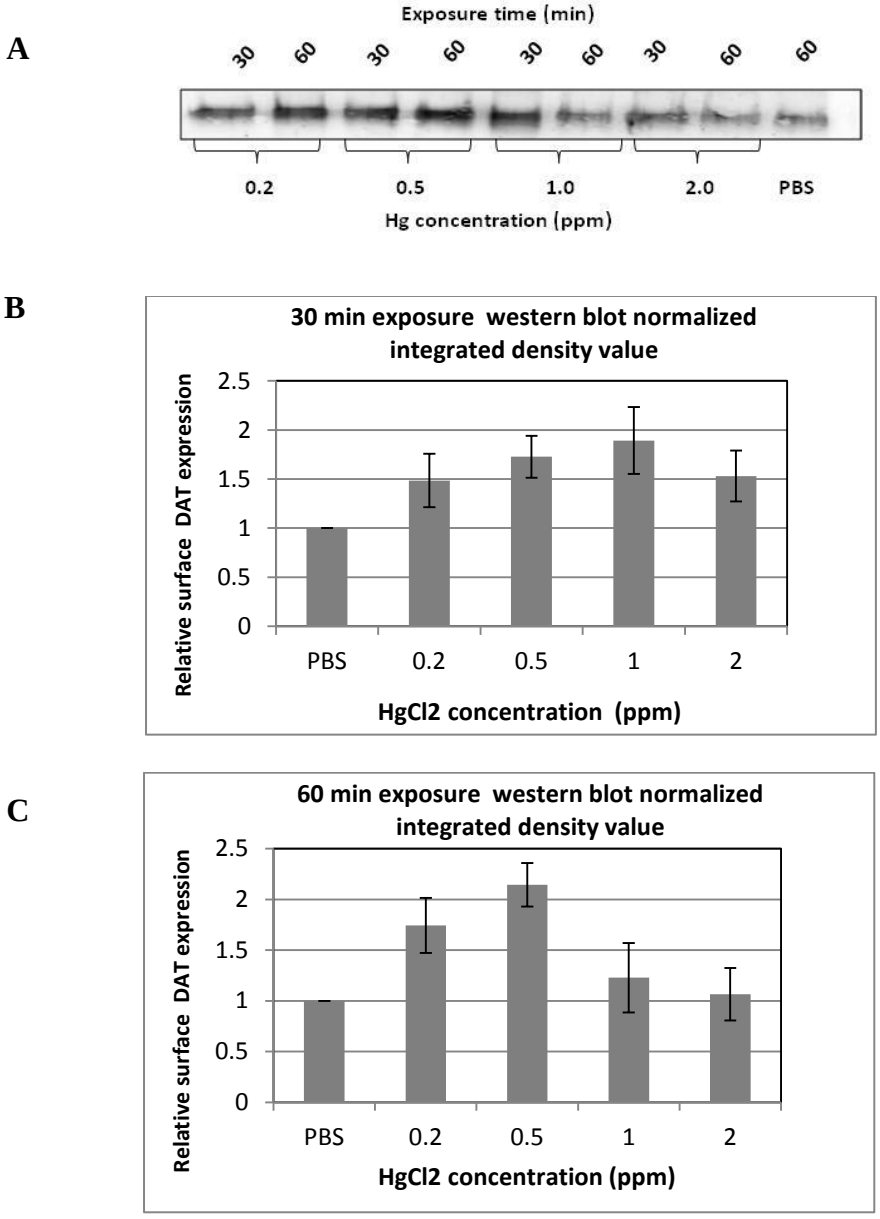

Figure 2 Western blot analysis of the effect of $HgCl_2$ on surface DAT expression following 30 or 60 minutes exposure. (A) Western blot representative image; (B) Densitometric quantification of the DAT expression following 30 min $HgCl_2$ exposure (C) Densitometric quantification of DAT expression following 60 min exposure. Integrated density values of surface DAT from western blot analysis was normalized to PBS control. The relative expression of surface DAT in the PBS control group was taken as 1.

Cells were exposed to $HgCl_2$ for 0.2-2.0 ppm for 30 or 60 minutes. Control cells were treated with PBS only. After chemical treatments, cell surface proteins were labeled with biotin and isolated for western blot analysis. The results obtained from western blot analysis showed that $HgCl_2$ has a transient effect on the expression of cell surface dopamine transporter in PC12 cells (Figure 2). This trend was observed for both the 30 and 60 minutes $HgCl_2$ exposure times. When PC12 cells were exposed to $HgCl_2$ for 30 minutes, the maximum expression of cell surface dopamine transporter was seen in cells treated with 1.0 ppm $HgCl_2$. When PC12 cells exposed to $HgCl_2$ for 60 minutes, the maximum expression of cell surface dopamine transporter was observed in cells treated with 0.5 ppm $HgCl_2$.

3.3 Localization of dopamine transporter in response to $HgCl_2$ exposure

The distribution of DAT on the surface of the cell following mercury exposure was observed via cell surface DAT immunocytochemistry. The DAT distribution on the cell membrane was seen to be denser for the 0.5ppm $HgCl_2$ (Figure 3C) and 1.0 ppm (Figure 3D) exposed cell groups than the control PBS group (Figure 3A). Slight scattering of fluorescent DAT signal was seen in cells treated with 1.0 ppm $HgCl_2$ (Figure 3D) when compared to control PBS group (Figure 3A). No obvious change in the DAT expression was observed in the cells treated with 0.2 ppm $HgCl_2$ (Figure 3B) as compared with cells treated with PBS (Figure 3A).

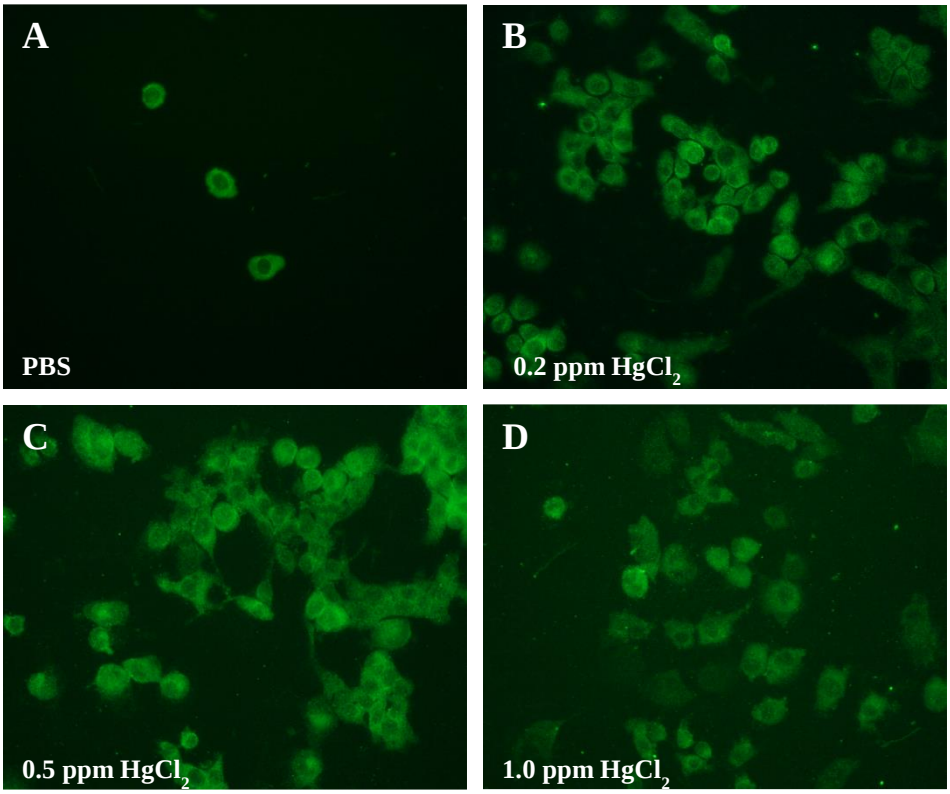

Figure 3 Immunocytochemical images of surface DAT in PC12 cells exposed to different concentrations of $HgCl_2$ for 30 minutes. (A) PBS control; (B) 0.2 ppm $HgCl_2$; (C) 0.5 ppm $HgCl_2$; (D) 1.0 ppm $HgCl_2$. No increase in surface DAT expression was observed in cells treated with 0.2 ppm $HgCl_2$ when compared to the cells treated with PBS. Increase in cell surface DAT expression was observed in cells treated with 0.5 ppm and 1.0 ppm $HgCl_2$.

3.4 The potentiation effect of $HgCl_2$ on MPP^+ triggered cytotoxicity was diminished in PC12 with dysfunctional DAT.

To elucidate whether the potentiation effect trigged by $HgCl_2$ on MPP^+ triggered cytotoxicity involves DAT, PC12 cells were transiently transfected to express null DAT mutation (L368Q). The DAT L368Q cells were then treated with $HgCl_2$ alone and 0.1mM MPP^+ combined with $HgCl_2$. The potentiation of toxicity previously observed with PC12 cells containing functional DAT when treated with 0.5 ppm $HgCl_2$ and 0.1mM MPP^+ (Figure 1) was not observed with same treatment for the DAT L368Q cells (Figure 4). At all $HgCl_2$ exposure

concentrations, when treatment included MPP^+, toxicity in the DAT L368Q cells was similar to that of treatment of cells with $HgCl_2$ alone (Figure 4). The significant potentiation effect of $HgCl_2$ at 0.5 ppm on MPP^+ induced cytotoxicity was diminished when there was no functional DAT expressed.

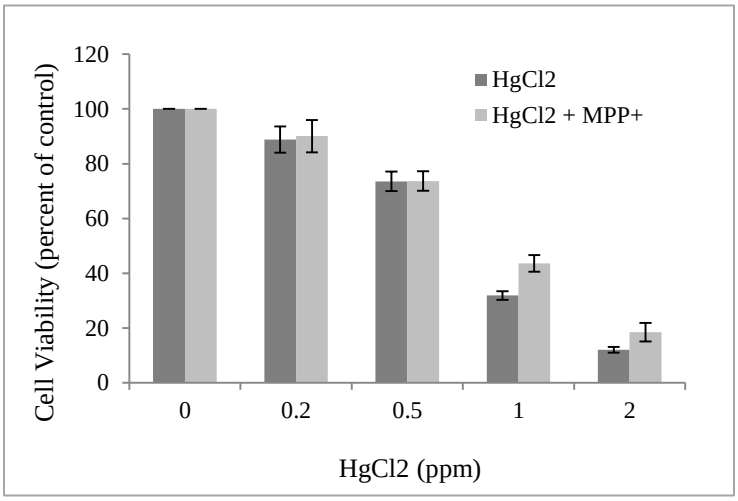

Figure 4 Effect of $HgCl_2$ on cell viability incubated in the presence or absence of (0.1 mM) MPP^+ in PC12 cells transiently transfected to express null DAT mutant (L368Q). Cell viability was measured by MTT assay. Following 4hours of chemical treatment, cell viability decreased in a dose dependent manner with increasing $HgCl_2$ concentration. With DAT L368Q mutant, the potentiation effect of 0.5 ppm $HgCl_2$ on MPP^+ triggered cytotoxicity observed in Figure 1 was diminished. Data were normalized to percent of control. Data was obtained from 4 different experiments.

4. Discussion

The dopamine transporter (DAT) is important to limit the neurotransmission of dopamine in the dopaminergic neuronal cells. Thus, drugs and toxins that target DAT can result in dysfunction in the dopamine-signaling pathway. MPP^+ is a known parkinsonian toxicant that uses DAT to enter into the neuron to cause cell death. In this case, changes in the expression or function DAT would result in an alteration of toxicity from agents such as MPP^+ that use it as a

means of entry into the cell. Studies (Bourdineaud, Redwood, & Bernard, 2011; Lin, Liou, Hsiech, Ku, & Tsai, 2011; Caudle, Guillot, Lazo, & Miller, 2012) suggest that exposure to mercury can have substantial impact on the normal functioning of the nigrostriatal dopaminergic system. Mercuric chloride has been shown to increase binding of radioligands to DAT in membrane and whole cell suspensions (Schweri, 1994; Wu et al., 1997). While binding of radioligands was increased, the effect on the expression and activity of DAT on the cell surfaces was not explored. Other researchers have found that exposure to mercuric chloride did not significant change in surface DAT density (Wallace & Hood, 2018).

In this study cell viability in response to mercury exposure was analyzed via MTT assay. As expected mercuric chloride was observed to result in increasing cell death with increase in concentration (Figure 1). Wallace & Hood (2018) also observed the decreased cell viability following $HgCl_2$ treatment. However, they found the significant decrease in cell viability only after 48-hour exposure with 10 µM $HgCl_2$ (2 ppm) in N2A cells. In this study 4 hours exposure to 2 ppm $HgCl_2$ caused cell viability decrease by 15% (Figure 1). The discrepancy in toxicity could be due to several different reasons. First, the cytotoxicity assay Wallace & Hood (2018) conduced was the lactate dehydrogenase leakage assay (LDH) assay. Principle of the LDH assay is that when the cell membranes are damaged, LDH from the cytosol is released into the cell culture media indicating plasma membrane damage (Kumar, Nagarajan, & Uchil, 2018). In comparison, in this study, the MTT cytotoxicity assay conducted monitors mitochondrial activity. As such, the MTT assay is much more sensitive in detecting cytotoxicity than the LDH assay (Fotakis & Timbrell, 2006). Secondly, the basal p53 tumor suppressor expression in PC12 cells is lower than in N2A cells (Kuenzi, Kiefer, Koryakina, & Hamburger, 2008). The cytotoxic signaling pathway triggered by $HgCl_2$ could be different between two cell lines due to the

differences in the basal p53 expressions. When the cells were additionally treated with MPP^+, it was observed that with 0.5 ppm $HgCl_2$ there was a further decrease in cell viability compared to when the cells were treated with $HgCl_2$ alone (Figure 1). This potentiation effect of $HgCl_2$ in MPP^+ induced cytotoxicity could be due to the changes in DAT expression due to the toxin's use of the transporter as a means of entry into the cell. The surface expression of DAT in response to $HgCl_2$ was explored using western blot analysis and visualized by using immunocytochemistry. Data obtained from western blot analysis indicated that the expression of DAT increased transiently with increase of $HgCl_2$ treatment concentration (Figure 2B/C). DAT had the maxima expression at 0.5 and 1.0 ppm $HgCl_2$ concentration for the 30 and 60 minutes exposures respectively (Figure 2B/C).This increase was also observed visually on the surface of the cell via immunocytochemical analysis. Agreeing with the increase in expression of the surface DAT seen with western blot, there was a maximal intensity of DAT fluorescence signal observed at 0.5 ppm $HgCl_2$ exposure concentration that decreased at higher mercury concentration (Figure 3). Wallace & Hood (2018) showed the density of DAT on cell surface was not significantly changed after 10 µM of $HgCl_2$ for 72 hours exposure, but the functionality of DAT in N2A cells treated with 10 µM of $HgCl_2$ for 72 hours was increased. The N2A cells co-treated with heavy metals and cocaine or methamphetamine caused further increases in the density of DAT on cell surface. These authors suggested that low doses of heavy metals, such as Hg, may increase the risk of exaggerated response to low doses of psychostimulants via altered DA neurotransmission/turnover. In this study, we demonstrated that lower $HgCl_2$ dosages and shorter exposure times increased the density of DAT on PC12 cells. Previous research has suggested that possible increase in DAT may be due to homeostatic response of the cell to maintain stable synaptic DA levels as a result of reduction in function of DAT following Hg toxicity (Wu et al.

1997; Wallace & Hood, 2018). Hg can attack thiol groups in the DAT disulfide bridge resulting in abnormal function (Wallace & Hood, 2018). This may explain the increase of DAT expression as the cell's response to the compromised function of the DAT as a result of Hg attack. Increase in DAT expression and increase in functionality may result in similar increase of sensitivity to psychostimulants such as that seen with heavy metal Pb and Mn exposure (Jones & Miller, 2008).

The involvement of the dopamine transporter in the potentiation of MPP^+ induced cytotoxicity was confirmed via identical chemical treatment of transfected PC12 cells containing nonfunctional DAT mutant, DAT L368Q. DAT L368Q is known as a loss-of-function DAT associated with infantile parkinsonism dystonia in humans (Kurian et al., 2009) with mutation at L368 of the DAT. Cytotoxicity MTT assay showed that the potentiated decrease in cell viability seen when $HgCl_2$ was combined with MPP^+ in the normal functional DAT cells was not observed in DAT L368Q transfected cells at the 0.5 ppm $HgCl_2$ treatment concentration. This result indicated that functional DAT is necessary for the observed $HgCl_2$ triggered potentiation on MPP^+ induced cytotoxicity.

In summary, mercury chloride enhanced the toxicity of parkinsonian toxicant MPP^+ by increasing the cell surface expression of DAT. The potentiation effect was seen to be dose-dependent. The increase in cell surface expression of DAT as a result of mercury exposure was transient and also correlated to dosage and time of exposure. This was a novel finding about the expression of cell surface DAT in mercury neurotoxicity. Further implication regarding the increase of expression of DAT and DA turnover would be possible increase of sensitivity to psychostimulants similar to that seen with Pb and Mn exposure (Jones & Miller, 2008). As a

result, low dose mercury exposure may result in altered sensitivity to drugs that affect the dopaminergic system.

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