Methylmercury and Paraquat Induced Toxicity in the Mitochondria of Dopamine Neurons

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Abstract

Methylmercury and Paraquat are environmental toxins that affect the central nervous system. Exposure to Paraquat and methylmercury causes movement impairments similar to the symptoms of Parkinson's disease (PD). The degeneration of dopamine neurons due to mitochondrial dysfunction has been implicated in PD. This study investigates the mechanism of methylmercury and Paraquat induced mitochondrial dysfunction in dopamine neurons. Using *in vitro* assays, it was found that exposure to methylmercury (0.1-5μM) and Paraquat (300-500μM) inhibited complex I of the electron transport chain in mitochondria. This was associated with an increase in superoxide anion levels, decrease in superoxide dismutase activity, and loss of ATP. All of these factors led to the loss of mitochondrial membrane potential. Similar results were found in co-exposure treatment of 300μM of Paraquat with 0.1μM of methylmercury. These results indicate that methylmercury and Paraquat induce mitochondrial dysfunction causing the death of dopamine neurons.

Résumé

Le méthylmercure et le Paraquat sont des toxines environnementales qui affectent le système nerveux central. L'exposition au Paraquat et au méthylmercure provoque des altérations du mouvement similaires aux symptômes de la maladie de Parkinson. La dégénérescence des neurones dopaminergiques, à cause de la dysfonction mitochondriale, a été impliquée dans la maladie de Parkinson. Cette étude enquête sur un mécanisme d'induction du méthylmercure et du Paraquat sur la dysfonction mitochondriale dans les neurones dopaminergiques. Utilisant des essais *in vitro*, on a trouvé que l'exposition au méthylmercure (0,1-5μM) et au Paraquat (300 à 500μM) ont inhibé le complexe I de la chaîne respiratoire mitochondriale. Ce changement est associé à l'augmentation des anions superoxyde, à la réduction de l'activité de superoxyde dismutase et à la diminution d'ATP. Tous ces facteurs ont causé la perte du potentiel de la membrane mitochondriale. Des résultats similaires se trouvent dans le traitement d'exposition de 300μM de Paraquat avec 0,1μM de méthylmercure. Ces résultats indiquent que le méthylmercure et le Parquat provoquent un dysfonctionnement mitochondrial provoquant la mort des neurones dopaminergiques.

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Abbreviations

ATP	Adenosine triphosphate
BBB	Blood brain barrier
BSA	Bovine serum albumin
D_1	Dopamine receptor subtype 1
DAT	Dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport chain
FBS	Fetal bovine albumin
FCCP	Carbonyl cyanide o-trifluromethoxyphenylhydrazone
GI	Gastrointestinal tract
GSTTI	Glutathione S-transferase theta 1
HCl	Hydrochloric acid
HoAc	Acetic acid
HVA	Homovanillic acid
IP3	Inositol Triphosphate
LAT	Large neutral amino acid transporter
LD_{50}	Lethal dose
LOAEL	Lowest observed adverse effect level
MAO	Monoamine oxidase
MeHg	Methylmercury
Mn	Menadione
MnSOD	Manganese superoxide dismutase
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
Nrf-2	Nuclear factor (erythroid-derived factor 2)-related factor 2
PBS	Phosphate buffered solution
PCA	Perchloric acid
PI	Propidium iodide
PQ	Paraquat
PSI	Photosystem I
ROS	Reactive oxygen species
SNPc	Substantia pars nigra compacta
TMRE	Tetramethylrhodamine ethyl ester
UNEP	United Nations Environmental Programme
US EPA	United States Environmental Protection Agency
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area
WHO	World Health Organization

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1.0 Introduction

Methylmercury (MeHg) is a persistent environmental toxin that is released into the environment from natural and anthropogenic sources. Elemental mercury is converted to MeHg by sulfate reducing microorganisms in aquatic ecosystem (Sparling, 2009), where it bio-accumulates and biomagnifies in the food chain (Clarkson and Magos, 2006). The World Health Organization (WHO) considers mercury exposure to be one of the top ten public concerns (WHO, 2016). Symptoms of MeHg neurotoxicity include memory problems, attention deficits, sensory impairment, delayed motor skills and visual problems (Harada, 1995). Exposure among the general population is primarily through the consumption of predatory fish and marine mammals. Once absorbed, MeHg is able to pass through the blood brain barrier by using neutral amino acid transporters. In pregnant women, it can cross the placental barrier to reach fetal circulation. The developing brain is the most sensitive to MeHg neurotoxicity (Clarkson, 1990). In some cases there seems to be a latency period between MeHg exposure and the appearance of neurotoxicity in people. In instances of chronic low dose exposure, it seems to take years before symptoms emerge (Weiss *et al.,* 2002; Barrett, 2008). It is hypothesized that the aging brain loses neurons and this diminishes its abilities to compensate for the damage caused by MeHg. The loss of dopamine neurons is accelerated in the presence of MeHg accumulation in the brain. Thus, motor impairments emerge with an aging brain (Weiss *et al*., 2002).

The symptom of motor impairments associated with MeHg neurotoxicity is similar to the symptoms of the neurodegenerative disorder, Parkinson's disease (Beuter and Edwards, 1998). The impairments in movements are due to the loss of neurons in the

nigrastriatal pathway that are involved in the production and release of the neurotransmitter dopamine (Margenelli *et al.,* 2016). Dopamine controls a broad range of functions in the central nervous system. The control of voluntary movement is done via the nigrostriatal pathway (Chinta and Andersen, 2004). Dopamine neurons comprise less than one percent of total brain neuron population and their number declines with age (Naoi and Maruyama, 1999). Over the decades, many studies have noted links between MeHg exposure and impairments in dopamine function. However, a mechanism of MeHg induced dopamine dysfunction in the nigrostriatal pathway is not fully known.

The herbicide, Paraquat (PQ) is used to kill broad leaf weeds in plantations and orchards. It has been banned in the European Union and its use is restricted in Canada and the United States. However, it is extremely popular in many developing countries due to its fast acting properties. Farmers in developing countries are exposed to PQ as a result of lack of proper personal equipment and lenient regulations (Watts, 2011). Chronic exposure to PQ in farmers can increase the risk of developing Parkinson's disease (Tanner *et al.,* 2011; Dinis-Oliviera *et al.,* 2006). PQ targets dopamine neurons in the brain. The herbicide increases oxidative stress by disrupting functions of the mitochondria in dopamine neurons (Bus *et al.,* 1975). Populations in Asia who use PQ on a routine basis could also be at risk to MeHg exposure since seafood is a dietary staple in many of the communities.

Both of the toxins cause movement abnormalities similar to the neurodegenerative disorder, Parkinson's disease. Defective mitochondrial functions are believed to be one of the main impairments that set off various downstream effects, which ultimately manifest as motor impairments (Dreiem *et al.,* 2005; McCormack *et al.,* 2011). Mitochondria

provide energy to the cell by producing ATP through the electron transport chain. The damage to mitochondria can initiate apoptosis of the neurons because of increase in reactive oxygen species, oxidative stress and loss of mitochondrial membrane potential (Castro *et al.,* 2011).

The following literature review will focus on the sources, exposure, toxicokinetics and mechanism of neurotoxicity of both MeHg and PQ in dopamine neurons.

1.1. Literature Review

1.1.1 MeHg in the environment

In the environment, mercury exists in three states, elemental, organic and inorganic. Both natural and anthropogenic sources release elemental mercury into the atmosphere, which are carried long distances by winds due to its volatility. Sources of elemental mercury include coal, mineral deposits, and volcanic eruptions. Anthropogenic sources are small-scale gold mining, burning of coal, cement production and use of mercury in industrial settings. Since the industrial age, anthropogenic sources are the major contributor of mercury in the environment (Pacyna *et al*., 2006). Almost 35% of mercury is released from small-scale gold mining. Monitoring programs and strict regulations have led to the decrease in mercury emissions in many regions of the world however mercury emission is still increasing in Asia. In particular, East Asia accounts for 40% of man-made mercury release or about 727 tones (UNEP, 2013). Large production of mercury in eastern Asia impacts all regions of the world owing to long-range air transport of mercury (Jaffe *et al.,* 2005).

In the atmosphere, elemental mercury is converted into inorganic mercury (Hg^{2+}), which is then absorbed by precipitation and settles into terrestrial and aquatic ecosystems (Sparling, 2009). Mercury deposits disproportionately more in the Arctic compared to rest of the world. This is due to long-range transport of Hg^{2+} on air currents from Asia and prevailing air currents move in the direction of the Arctic during summer (Environment and Climate Change Canada, 2013). Large amount of halogen radicals are found in the Arctic sea and along with cold temperature, the deposition of mercury into the ground is amplified (Kirk *et al.,* 2012). Mercury can also directly enter the water system as discharge from industries (UNEP, 2013). In the aquatic environment, Hg^{2+} is transformed to MeHg, an organic state via methylation by sulfur reducing and iron reducing bacteria (Sparling, 2009). MeHg accumulates in planktons and lower trophic organisms usually through passive diffusion (Environment and Climate Change Canada, 2013). As the lower trophic organisms and planktons are eaten by higher trophic animals, MeHg bio-accumulates and biomagnifies in the food chain. The higher trophic aquatic animals such as shark and tuna have the highest concentration of MeHg in their system (Scudder *et al.,* 2009).

In order to reduce the atmospheric levels of mercury and protect population from mercury toxicity, global initiatives have been taken to implement regulations. United Nations Environment Programme (UNEP) collects data relating to mercury release from countries to monitor global levels of mercury. An international treaty called the Minamata Convention on Mercury was signed by 140 countries in 2013 in an effort to reduce anthropogenic mercury levels in the environment. The goal of the program is to implement various preventative measures and to protect population from the adverse

effects of mercury. The treaty will be fully implemented around 2018 (UNEP, 2013). Due to long persistence of mercury in the environment, it will remain a health problem for years.

1.1.2 Toxicokinetics

Human exposure to MeHg is primarily through the consumption of fish and other seafood. Once ingested, 95% of MeHg is absorbed by the gastrointestinal tract and travels to the bloodstream where it binds to thiol and cysteine residues (Clarkson and Magos, 2006). MeHg targets various tissues and organs. The brain is the most sensitive to MeHg and about 10% of it accumulates in the brain a week after exposure. In pregnant women, it can cross the placental barrier and reach fetal blood circulation (Hong *et al.,* 2009). It has a long half-life in the body between 70-80 days depending on exposure dose (Al-Shahristani, 1979). MeHg is subjected to substantial enterohepatic cycling, which could explain its long half- life in the body. Liver is responsible for demethylation of MeHg, which binds to glutathione in the bile and is then excreted in the feces (Hong *et al.,* 2009).

1.1.3 Exposures and Toxicity

Most of the information about symptoms of MeHg toxicity is known from poisoning incidents in Japan and Iraq. In Minamata Bay, Japan, a chemical company, Chisso Co. Ltd., discarded liquid waste containing mercury into the Bay from 1951 to 1968. The fish and marine life accumulated high concentration of MeHg and the local population started showing symptoms of MeHg toxicity after eating fish (Ekino *et al.,* 2007). The symptoms of toxicity included loss of sensation in limbs, uncoordinated

movement, hearing and vision impairments and tremors. These symptoms collectively became known as Minamata disease. By late 1950s, 16 people died from acute MeHg poisoning. However, individuals started to exhibit some of the symptoms of MeHg toxicity as early as the 1950s. Strange behavior in animals in the city was also observed, such as fish floating upside down on the surface and the sudden death of cats following seizures. Women who did not show any signs of toxicity gave birth to babies with neurological impairments that resulted in motor control impairments, convulsions and mental disturbances well into the 1960s (Harada, 1995). Symptoms related to chronic exposure to mercury still remains an issue. In many cases, some of the symptoms worsened or made people more vulnerable to developing other diseases such as neurological disorders. It is reported that in few cases symptoms appeared years after exposure to MeHg (Igata, 1993).

The second major incident of MeHg poisoning occurred in rural areas of Iraq in 1972. People became ill after eating bread made with mercury contaminated grains. Wheat and barley grains were treated with a fungicide containing mercury (Bakir *et al.,* 1973). The grains were imported from Mexico and the United States but were not meant for human consumption. Misunderstanding of the labels on the bags of seeds resulted in human consumption. Approximately 6500 people showed signs of MeHg toxicity similar to symptoms observed in Minamata and 459 people died (Skerfving and Copplestone, 1976). In many cases, it took weeks and months following exposure for symptoms to appear. Numerous children were born with deficits in motor skills and delayed mental development (Greenwood, 2005).

There have been number of cohort studies done on population with seafood as a dietary staple to examine the effects of chronic low dose exposure to MeHg (Mergler *et al*., 2007). Epidemiological studies focusing on the effect of MeHg in children have found that mercury was associated with reduced cognitive functions (Transende, 2005). However, no cognitive and behavioural impairments were found in teenagers who had prenatal exposure to MeHg in the Seychelles (Myers *et al*., 2009). In the Faroe Islands, seven-year-old children with prenatal exposure to MeHg exhibited decreases in cognitive and language skills, attention problems and decreases in fine motor skills (Grandjean *et al*., 2009). There is a high prevalence of Parkinson's disease among the Faroese population but no causation was found between MeHg exposure and Parkinson's disease (Petersen *et al.,* 2009). The Tohoku child development study in Japan, found motor deficits in seven years old children to be associated with prenatal MeHg exposure (Suzuki *et al.,* 2011; Tatsuta *et al*., 2012). Delays in cognitive development and language skills in a small sample of four-year old children were associated with MeHg in Granada, Spain. The Spanish population consume large amount of fish and there is deposition of mercuric sulphur, also called cinnabar along the Spanish coast (Freire *et al*., 2009).

In Canada, the Inuit population is constantly exposed to MeHg via their diet. The traditional diet of Inuit consists of fish and marine mammals. The concentration of MeHg found in some marine mammals such as the ringed seal is quite high. Individuals in these communities have mercury levels higher than accepted level (Kirk *et al.,* 2012; Muckle and Ayotte, 2001).

Based on the results of the poisoning incidents and cohort studies, Health Canada has set an intake guideline of 8µg/ml for the population (Health Canada, 2008). The U.S.

Environmental Protection Agency (EPA) suggests blood Hg level of 5.8 μg L^{-1} for adults (EPA, 2000). The World Health Organization (WHO) has set a reference dose of 1.6μg/ kg body weight per week to protect developing fetus and children from MeHg toxicity. Despite confounding results from the cohort studies, it is agreed by experts that chronic low dose exposure to MeHg is associated with neurodevelopmental and neurodegenerative disorders.

1.1.4 MeHg in the Brain

The brain is the most vulnerable organ to MeHg toxicity. Once absorbed, MeHg is able to pass through the blood brain barrier (BBB) (Ashner and Ashner, 1990) using large neutral amino acid transporters (LATS) by binding to L-cysteine. By forming a complex with L-cysteine, MeHg resembles methionine, which is a substrate for LATS (Simmons-Willis *et al.,* 2002). MeHg can accumulate in the central nervous system due to its lipophilicity (Clarkson *et al*., 2007). High concentrations of MeHg are observed in cerebral cortex, basal ganglia and cerebellum. The build-up of MeHg in these three regions accounts for some of the symptoms related to its toxicity. MeHg seems to exert toxicity in the brain by generating reactive oxygen species leading to an increase in oxidative stress, hindering the activity of the antioxidant enzymes, disrupting calcium signaling and binds to thiol groups. The mechanism of MeHg induced toxicity remains unknown.

The primary mechanism of MeHg neurotoxicity is thought to be via disruption of redox cycling in neurons (Farina *et al.,* 2011). An increase in oxidative stress is observed across numerous species: salmon, mouse, *C. elegans,* following chronic sub-acute and acute exposures. MeHg causes an increase in different reactive oxygen species such as

superoxide anions, hydroxyl radicals, peroxides and nitric oxides depending on the regions of the brain. It can also block the activity of anti-oxidant enzymes such as glutathione reductase and glutathione peroxidase because of its interactions with thiol groups (Van Duyn *et al.*, 2012; Franco *et al.,* 2009). Disruptions in calcium signaling and increases in intracellular calcium level are observed in the presence of MeHg, especially in the cerebellum. MeHg binds to muscarinic calcium channels and activates inositol triphosphate (IP3) in cerebellar granule neurons (Limke *et al*., 2004). The activation of IP3 causes it to bind and open calcium channels in the endoplasmic reticulum, resulting in an increase in intracellular calcium concentration (Leybaert, 2016). The increases in oxidative stress, impairments in antioxidant enzymes and disruption of calcium homeostasis eventually activate pathways responsible for cell death.

1.1.5 MeHg, Dopamine Neurons and Movement Impairments

Results from studies indicate that the damage to dopamine neurons in the substantia nigra pars compacta (SNPc) is responsible for movement impairments associated with MeHg neurotoxicity. However, there are insufficient data to determine a mechanism of neurotoxicity. Dopamine neurons in the nigrostriatal pathway control autonomic movements (Chinta and Andersen, 2006).

The alterations in dopamine transmission in the nigrostriatal pathway is observed in several studies (Faro *et al.,* 1997; Faro *et al.,* 2002; Dreiem *et al.,* 2009; Vidal *et al*., 2007; Zhou *et al.*, 1999). MeHg causes spontaneous increase in dopamine by blocking the reuptake of dopamine from the cleft by the enzyme, dopamine transporter (DAT) (Minnema *et al.,* 1989; Zimmer *et al.,* 2011). The reuptake of dopamine by DAT

terminates dopamine signaling and allows it to be sequestered in vesicles by other enzymes or to be degraded. In the presence of MeHg, the release of dopamine from the vesicles is independent of extracellular calcium concentrations (Faro *et al*., 2002; Tiernan *et al.,* 2013) and it seems that voltage gated sodium and potassium channels do not play a role in the increase in dopamine concentrations (Minnema *et al.,* 1989). The enzyme monoamine oxidase (MAO) breaks down dopamine to its metabolites DOPAC and homovanillinic acid (HVA). MeHg prevents the breakdown of dopamine by down regulating the activity of MAO (Chakrabarti *et al*., 1998; Beyrouti *et al*., 2006). By impairing the expression of MAO and DAT, MeHg increases dopamine levels while decreasing the levels of its metabolites, DOPAC and HVA. The change in ratio between dopamine and its metabolites has been noted by several studies (Faro *et al*,. 2002; O'Kushky *et al*., 1987; Bordeneaud *et al.,* 2011; Vidal *et al.*, 2002; Zhou *et al.*, 1999).

Gestational studies in rats found altered expression of the dopamine receptor, D1. The decrease in expression of dopamine receptor subtype D1 was found only in male offspring by Coccinni *et al.* (2011). However, in five weeks old offspring, D1 receptor density was comparable to the control group. On the contrary, Castoldi *et al. (*2006*)* noted an increase in all subtypes of dopamine receptors but they administered a single high dose of MeHg during gestation and effects were observed early. Antagonism of D1 receptors showed no change in expression of dopamine (Pereira *et al.*, 2006). Genes of dopamine receptors are down regulated following exposure to low doses of MeHg (Zimmer *et al.,* 2011). The results from gestational exposure to MeHg imply that D1 receptors are affected during the neonatal period but are usually recovered at an older age. Another gestational study revealed that in the presence of MeHg, neurons do not

branch properly and are unable to make connection with other neurons (Gotz *et al.*, 2002). The branching of neurons is critical for neuronal survival, growth and signaling. MeHg decreases neuronal branching by causing the cell nucleus to condense, which eventually serves as a signal for apoptosis in the cell (Gotz *et al.*, 2002).

The production of dopamine is dependent on the enzyme tyrosine hydroxylase as it coverts tyrosine to L-Dopa, which is then transformed to dopamine. In dopamine synthesis, tyrosine hydroxylase is the rate-limiting enzyme (Daubner, 2011). The impact of MeHg on tyrosine hydroxylase is contradictory. In two studies, it was found that MeHg decreases the expression of tyrosine hydroxylase (Zimmer *et al*., 2006; Bartolome *et al.,* 1982) while another found an increase in tyrosine hydroxylase activity (Tiernan *et al.,* 2013). The difference could be due to the use of different models and doses. Bartolome *et al*. (1982) used Sprague-Dawley rats while another group used pheochromocytoma cell line and Zimmer *et al.* (2006) used embryonic cell line. There were no changes in tyrosine hydroxylase mRNA expression in response to MeHg (*Rossi et al*., 1997). The expression of tyrosine hydroxylase impacts dopamine synthesis along with the concentration of reactive species. Tyrosine hydroxylase is necessary in dopamine synthesis but the conversion of L-dopa to dopamine also creates reactive species like hydrogen peroxide, oxygen radicals and quinones (Chinta & Andersen, 2005).

The origin of the reactive species resulting in increases in oxidative stress is unclear. MeHg targets different anti-oxidant pathways like nuclear factor (erythroid-derived factor 2)-related factor 2 (nrf 2) and glutathione in dopaminergic neurons. A decrease in SKN-1 expression by MeHg was noted in *C. elegans* (Van Duyn *et al.*, 2012).

SKN-1 is an ortholog of nrf2 in *C. elegans.* Both SKN-1 and nrf 2 are involved in cellular responses to oxidative stress (Blackwell *et al.,* 2015). Another experiment performed with SKN-1 knockout strain found an increase in reactive oxygen species, which ultimately resulted in the loss of dopaminergic neurons (Martinez-Finley *et al.,* 2013). MeHg decreases the activity of glutathione in the mitochondria (Franco *et al.,* 2009). Glutathione peroxidase converts hydrogen peroxide to water, thus inhibiting the enzyme results in an increase in reactive oxygen species in the mitochondria (Handy *et al.,* 2009). Furthermore, decreased glutathione levels were observed in neuroblastoma cells after exposure to different doses of MeHg (Petroni *et al*., 2010). The depletion of glutathione is an indicator of oxidative stress in the cell (Patel and Reynolds, 2013).

MeHg increases reactive oxygen species in the synapses of the striatum (Dreiem et al 2005) similar to other regions of the brain (Castoldi *et al.,* 2011; Shanker and Ashner, 2003). MeHg exposure led to a reduction in overall mitochondrial activity (Dreiem and Seegal, 2009). Currently, no studies have specifically analyzed changes in mitochondrial function in dopamine neurons as a result of MeHg exposure. Numerous studies have focused on mitochondrial functions and properties following MeHg exposure in various regions of the brain (Nori *et al.,* 2011; Limke *et al*., 2002; Castoldi *et al*., 2000). These studies provide some insight into the possible mechanism of mitochondria mediated neuronal death. The changes in mitochondrial morphology and increases in electron density in the inner membrane of mitochondria were observed in cortical neurons (O'Kusky, 1983). MeHg increases reactive oxygen species in particular superoxide anions (Yee and Choi,1994) and hydrogen peroxide in the cerebellum (Nori *et al.,* 2011), lipid peroxidation (Yin *et al.,* 2007) and results in the loss of mitochondrial

membrane potential in astrocytes and neurons (Lee *et al.,*2012; Limke and Atchison, 2002; Yin *et al.,* 2007; Castoldi *et al.,* 2000). Furthermore, MeHg raises intracellular calcium concentration in the mitochondria of cerebellar neurons (Levesque and Achtison, 1990; Okazaki *et al.,* 1997). Cell death occurs mainly via three processes, autophagy, necrosis and apoptosis. The latter is favored in the presence of low doses of MeHg (Kunimoto, 1994). It remains to be elucidated whether MeHg activates the intrinsic or extrinsic pathway of apoptosis. The intrinsic pathway of apoptosis is initiated by the mitochondria following the loss of membrane potential and opening of the mitochondrial permeability transition pore (Elmore, 2007). Reactive oxygen species can leave the mitochondria and attack other organelles due to the opening of the mitochondrial permeability transition pore (Kowaltowski *et al*., 2001). The markers of apoptosis include DNA fragmentation, chromatin condensation, cell shrinkage, swelling of the cell membrane and activation of proteases such as calpains and caspases (Orennius *et al.,* 2003).

It is likely that MeHg initiates different pathways in distinct regions of the brain or the favoured pathway depends on the dose. One of the proteases involved in intrinsic apoptosis, caspase 3, is upregulated by low concentrations of MeHg in microglia (Nishioku *et al.,* 2000) and in neuronal stem cells (Tamm *et al.,* 2006). In addition, the appearance of DNA fragments in dorsal root ganglions (Wilke *et al*., 2003) and cerebellar neurons (Nagashima *et al*., 1995) support apoptosis caused by MeHg. A component of the electron transport chain, cytochrome c, dislocates from the mitochondria (Mori *et al.,* 2011), increases calcium concentration (Sakaue *et al*., 2005) and activates a family of proteases called calpains involved in apoptosis (Tamm *et al.,* 2006) following MeHg

exposure. The release of cytochrome c from the mitochondria is in accordance with the activation of caspase 3. In order for caspase 3 to become activated, cytochrome c must be relocated to the cytosol. Sakaue *et al.* (2005) found that MeHg impairs the activity of cyclin dependent kinase 5 (CDK5) by cleaving p35 to p25, which then binds to CDK 5. The binding of p25 to CDK5, causes overexpression of CDK5 resulting in neurodegeneration of dopamine neuron (Smith *et al.,* 2003). These findings suggest that at low doses of MeHg, the apoptotic pathway is the preferred method of cell death. (Mailloux *et al.,* 2015)

Based on the literature evidence, it can be hypothesized that MeHg decreases the activities of antioxidant enzymes in the mitochondria, resulting in increases in reactive oxygen species. Low concentrations of MeHg seem to activate apoptotic pathways.

1.1.6 PQ in the Environment

Paraquat (N, N′-dimethyl-4, 4′-bipyridinium dichloride) is a non-selective herbicide used to destroy broad leaf weeds in orchards and plantations. It is one of the most widely used herbicide and is commercially available as Gramoxone, manufactured by Syngenta. PQ desiccates the leaf without damaging roots and mature bark. The majority of PQ is adsorbed in the soil and is subsequently deactivated (Roberts *et al.,* 2002). The remaining PQ is transformed to its inactive state by microorganism in the soil. Due to its strong binding with soil particles, it does not contaminate ground water. In certain cases, PQ is also used to manage aquatic algae and weeds at concentrations between 0.25-0.5 mg/L in freshwater and 5mg/mL in saltwater. PQ does not gather in fat, thus there is no accumulation in the aquatic food chain (Eisler, 1990). .

1.1.7 Mode of Action in Plants

PQ works by dehydrating the leaves in broad leaf weeds and grasses. The toxic property of PQ is its ability to undergo redox cycling. In cells, the divalent state of Paraquat (PQ^{2+}) is reduced to its monovalent state, PQ^{+} (Figure 1). The redox potential is -0.446V similar to the redox potential of the enzyme nicotinamide adenine dinucleotide phosphate ($NADP^{+}$). Hence, it is suspected that PQ hinders cell processes by blocking the reduction of $NADP^{+}$. It interferes with photosynthesis by disrupting the electron transport of photosystem I (PS I) in the thylakoid membrane of the chloroplast (Hawkes, 2014). In particular, it blocks the reduction of $NADP^{+}$ to NADPH in PS I (Blanco-Ayala *et al*., 2014) by reducing itself to the monovalent cation, PQ^{+} (Haley, 1979). In the presence of light, a molecule of PQ^{+} takes an electron from an oxygen molecule to create a superoxide anion, O_2^{-} (Farrington *et al.,* 1973), thus increasing oxidative stress in the plant. Normally, few superoxide anions are formed during the transfer of electrons in the photosystem, which are neutralized by antioxidant enzymes within the chloroplast such as superoxide dismutase. However, PQ shifts the balance towards an increase in reactive oxygen species (ROS) and the antioxidant enzymes are unable to compensate for this increase in ROS. This leads to oxidative stress in the cells, which results in cell death via various mechanisms.

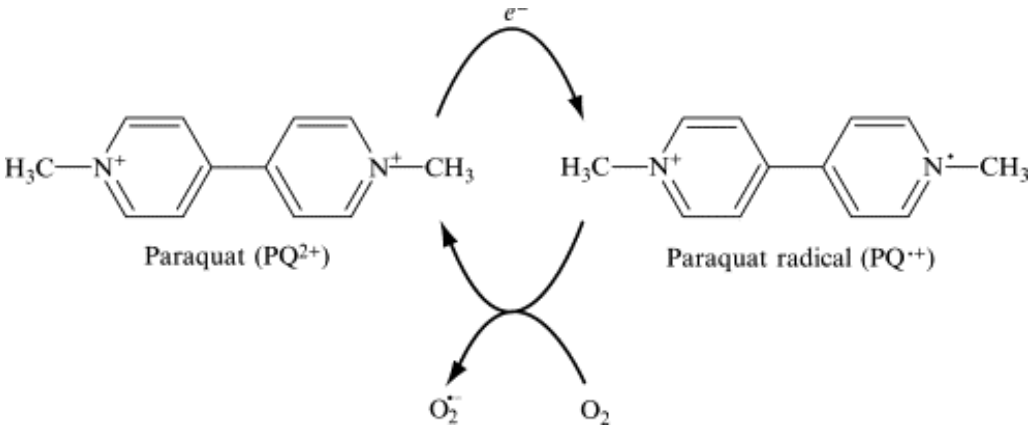


Figure1. Redox cycling of Paraquat in cells (Bus *et al.*, 1975).

1.1.8 Human Exposure and Link to Parkinson's disease

Agriculture workers and population living close to PQ spraying sites are most likely to be exposed to it. Acute high dose exposure to PQ can cause fatality, which makes it a common suicidal agent in many parts of the world. In humans, the oral lethal dose is considered to be 20mg/kg (Marrs and Adjei, 2003). The routes of exposure to PQ include oral, dermal and inhalation. The herbicide is very slowly absorbed from the skin, unless there is prolonged exposure or damaged skin. PQ is poorly but rapidly absorbed in the gastrointestinal tract following oral route of exposure (Watts, 2011). Once absorbed, it travels via the bloodstream to all organs of the body, but primarily targets the lungs. It is not metabolized by the liver and is excreted by the kidneys within 24 hours (Marrs and Adjei, 2003). PQ enters the lungs through active transport using polyamine transporter in the membrane of the Clara cells and alveolar epithelial cells (Smith, 1987). The alveolar cells permit rapid diffusion of gases by releasing surfactants and the Clara cells are responsible for removal of toxic particles from the lungs. The redox cycling properties of

PQ damages the alveolar and Clare cells in the lung. The damage to these cells causes fibrosis of the lungs (Smith, 1987).

In the last decades, there has been concern that chronic low dose exposure to PQ could be associated with Parkinson's disease. PQ is considered to worsen the emergence of Parkinson's disease in individuals with genetic predisposition. In a study by Tanner *et al.* (2011) occupational exposure to PQ increased the risk of developing Parkinson's by 2.5 times. In the Central Valley of California, an increase in incidences of Parkinson's was noted, especially in population less than 60 years old who had occupational exposure for 15 years or lived within 500metres of PQ spraying site (Costello *et al.,*2009; Kamel *et al*., 2007). Farmers who had exposure to PQ for 20 years had higher incidence of Parkinson's disease compared to non-exposure group in Taiwan (Liou *et al*., 1997). A group in France (Baldi *et al.,* 2003) found no link between PQ exposure alone and incident of Parkinson's disease in agricultural workers but PQ acted synergistically with other pesticides. PQ seems to elevate the risk of Parkinson's disease in individuals who have a genetic susceptibility to the disorder. Goldman *et al*. (2012) found that PQ increased the incidence of Parkinson's in men lacking the gene glutathione S-transferase theta 1 (GSTT1). This gene is involved in improving the activities of the enzyme glutathione (Goldman *et al*., 2012). Farmers with allelic mutations in dopamine transporter (DAT) exposed to PQ had 3-fold increase in the incidence of Parkinson's disease compared to the control group in California (Ritz *et al.,* 2007). Previous brain injury attenuates the risk of Parkinson's in population exposed to PQ (Lee *et al.,* 2012). More epidemiology studies are needed to decipher the link between PQ exposure and Parkinson's disease and to determine the interaction of PQ with other chemicals.

1.1.9 Mechanism of PQ Neurotoxicity

PQ gained attention as a possible neurotoxin due to its structural similarity to another known neurotoxin, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP). Both compounds produce symptoms similar to Parkinson's disease since both are capable of redox cycling (Sian *et al*., 1999). The two compounds act by generating reactive oxygen species and increasing oxidative stress in neurons. However, the mechanisms of the two chemicals are distinct from each other in dopamine neurons. MPTP induces damage by targeting dopamine transporters (Richardson *et al.,* 2005). Many studies have focused on PQ and its ability to increase oxidative stress in the lungs and the brain. However, there is limited data to determine potential mechanism of PQ toxicity in dopamine neurons.

It is hypothesized that PQ enters the blood brain barrier using neutral amino acid transporters (Shimizu *et al*., 2001) similar to MeHg. It deposits in various regions of the brain such as the amygdala, striatum, hypothalamus (Rudyk *et al.,* 2015) and accumulates in the substantia nigra pars compacta (SNpc) possibly through interaction with iron present in SNpc (Peng *et al.,* 2007). Paraquat (PQ^{2+}) is converted to its monovalent cation state, PQ^{+} using the enzyme NADPH oxidase (Bus and Gibson, 1984) in the microglia, which are the immune cells of the brain and are part of the blood brain barrier (da Foncesca *et al.,* 2014). Substantia nigra has a high population of microglia compared to other regions of the brain (Lawson *et al.,* 1990) and this could account for its vulnerability to PQ. It is suggested that the monovalent cation, PQ^{+} enters dopamine neurons via dopamine transporter (DAT) (Rappold *et al*., 2011). Dopamine neurons in the SNpc are vulnerable to PQ, as studies have noted a decreased the dopamine neurons

following PQ exposure (Brooks *et al*., 1999, McCormack *et al.,* 2002). PQ decreased the levels of dopamine metabolites, DOPAC and HVA (Fredriksson *et al.,* 1993) but it does not block the activity of the enzyme, monoamine oxidase (MAO) (Czerniczyniec *et al.,* 2011). Mitochondria are the ultimate target of PQ neurotoxicity but there is disputing data on the mitochondrial complexes affected by it. The accumulation of PQ^{2+} in the mitochondria is dependent on mitochondrial membrane potential (Cocheme and Murphy, 2008). Complex I of the electron transport chain in mitochondria transfer electrons to create NAD (P^{+}) by oxidizing NAD(P)H (Mailloux *et al.,* 2015). This process is interrupted by PQ^{2+} since it takes the electron from NADPH to generate PQ^{+}, which than reacts with oxygen molecules to produce superoxide anions (Zhang *et al*., 2016). The reaction with oxygen regenerates PQ^{2+}, thus continuing redox cycling of PQ in the mitochondria. However, there is contradicting evidence on the role of complex I of the mitochondria in PQ toxicity as some studies have found that PQ toxicity is independent of complex I inhibition in dopamine neurons (Choi *et al.,* 2008). Likewise, Richardson *et al.* (2005) observed no significant impairment in complex I following exposure to the herbicide. PQ appears to also interfere with complex III known as cytochrome c oxidase of the mitochondria and generate hydrogen peroxide (Castello *et al.*, 2007). However, the authors do not provide plausible explanation as connection between inhibition of complex III and production of hydrogen peroxide. Another study focusing on striatal neurons found no inhibition of complex III but complex I was inhibited alongside a mild inhibition of complex IV (Czerniczyniec *et al.*, 2011). In addition, an increase in hydrogen peroxide concentration was found following PQ treatment in isolated mitochondria of the forebrain (Dreshel and Patel, 2007) and the striatum (Czerniczyniec

et al., 2011). Another study found an increase in the activity of the antioxidant enzyme, manganese superoxide post PQ exposure in striatal neurons (Czerniczyniec *et al.,* 2013) that could explain the increase in H_2O_2 levels. The literature suggests that PQ inhibits respiratory system in the mitochondria either through complex I or III.

PQ alters the activity of endothelial nitric oxide synthase (eNOS) and NADPH diaphorase to produce superoxide anions (Day *et al.,* 1999). NADPH diaphorase and eNOS are identical enzymes in the central nervous system (Dawson et al. 1991). Nitric oxide produced by eNOS is involved in redox cycling and also act as a chemical messenger between neurons. Nitric oxide reacts with superoxide anions to generate another reactive oxygen species, peroxynitrite (Beckman and Kopennel, 1996). Mitochondrial functions and calcium signaling is disrupted by peroxynitrite, which can mediate cell death (Radi *et al.,* 2002). PQ activates glutamate activity and calcium signaling in dopamine neurons. After exposure, increases in NOS activity is observed followed by long lasting dopamine overflow (Shimizu *et al.,* 2003). The activity of the rate-limiting enzyme in the dopamine synthesis, tyrosine hydroxylase (TH) is unregulated by PQ (McCormack *et al.,* 2001). This increase in tyrosine hydroxylase activity is a plausible explanation of latency period in emergence of symptoms in chronic low dose exposure to the herbicide. PQ activates the Jun N-terminal kinase (JNK) pathway (Peng *et al.,* 2006), which is involved in neuron proliferation, apoptosis and neurodegeneration. Apoptosis mediated by JNKs is triggered by mitochondrial dysfunction (Dhanasekaran and Reddy, 2011). Furthermore, PQ results in the dislocation of cytochrome c from the mitochondria and activation of the apoptotic factors Bax/Bcl-2 (Czernicyniec *et al.,*

2013). These findings indicate that PQ results in the death of dopamine neurons through mitochondria mediated apoptosis.

Two studies have been performed to determine the possibility of interactions between PQ and MeHg. Cells exposed to both PQ and MeHg showed an increase in reactive oxygen species and oxidative stress compared to lone dose of PQ or MeHg (Mailloux *et al.,* 2015a). In another study, it was noted that there is a dose dependent interaction between PQ and MeHg and an increase of 3- fold in cytosolic levels of MeHg following PQ exposure. MeHg is demethylated to inorganic mercury in the mitochondria with PQ. A mimetic of the antioxidant enzyme, superoxide dismutase prevented the demethylation of MeHg in the mitochondria in the presence of PQ (Mailloux *et al.* 2015b).

1.2 Thesis Rationale

1.2.1 Rationale

Neurotoxic properties of MeHg have been known since the 1960s. The epidemiological evidence for PQ as neurotoxin emerged in the late 1990s. Exposure to the toxins manifest as symptoms similar to movement impairments associated with Parkinson's disease. It is the second most common neurodegenerative disorder and less than half of the people diagnosed have a genetic history of the disease (Lesage and Brice, 2009). Parkinson's disease is caused by a myriad of factors, which include gene-environment interactions and exposure to chemicals (Klein and Westenberger, 2012). Symptoms of movement deficits emerge when half of the dopamine neurons in substantia pars nigra are lost (Lesage and Brice, 2009). The deterioration in mitochondrial functions

of dopamine neurons, resulting in subsequent neuronal loss has been suggested as an etiology of Parkinson's disease (Schapira, 2007; Beal, 2003). There have been numerous studies on the induction of oxidative stress in neurons by PQ and MeHg. Recent studies have found that the mitochondria play a crucial role in the production of reactive oxygen species and regulation of neuronal death. However, the mechanism of MeHg and PQ induced mitochondrial dysfunction is unclear. To fill this gap, this thesis focuses on mitochondrial energetics and functions in dopamine neurons following MeHg and PQ treatments. In many parts of the world, especially in Eastern Asia, there is a high of probability of PQ and MeHg co-exposure in the population due to diet and lack of personal protection when handling chemicals. There have been only two studies emphasizing the increase in toxicity in cells following MeHg and PQ co-exposure. This thesis will also examine the possibility of additive toxicity of MeHg and PQ co-exposure in the mitochondria. Currently, there is no treatment for PQ or MeHg toxicity. Thus, determining the source of damage in mitochondria will provide opportunities to test compounds that can antagonize or delay the symptoms.

1.2.2 Objectives

1. To investigate the effect of the environmental toxins, MeHg and PQ on the electron transport chain, generation of superoxide anions, activity of antioxidant enzymes, oxygen consumption, ATP concentration and membrane depolarization in mitochondria of dopamine neurons.
2. To determine the toxicity of MeHg and PQ co-exposure treatments in mitochondrial energetics and antioxidant enzymes.

1.2.3 Hypotheses

1. Exposure to MeHg and PQ will block complex I of the electron transport chain (ETC) of the mitochondria resulting in an increase in superoxide anions.
2. Both PQ and MeHg will impair the antioxidant enzyme, manganese superoxide dismutase in the mitochondria, thus increasing oxidative stress.
3. There will be a decrease in ATP level due to blocking of complex I and the loss of electrons in the mitochondria
4. All of the doses of MeHg and PQ will depolarize the mitochondrial membrane however PQ will be more toxic.
5. Co-exposure of neurons with MeHg and PQ will result in an increase in oxidative stress, depolarization of mitochondrial membrane and inhibition of complex I of electron transport chain compared to neurons exposed solely to each chemical.

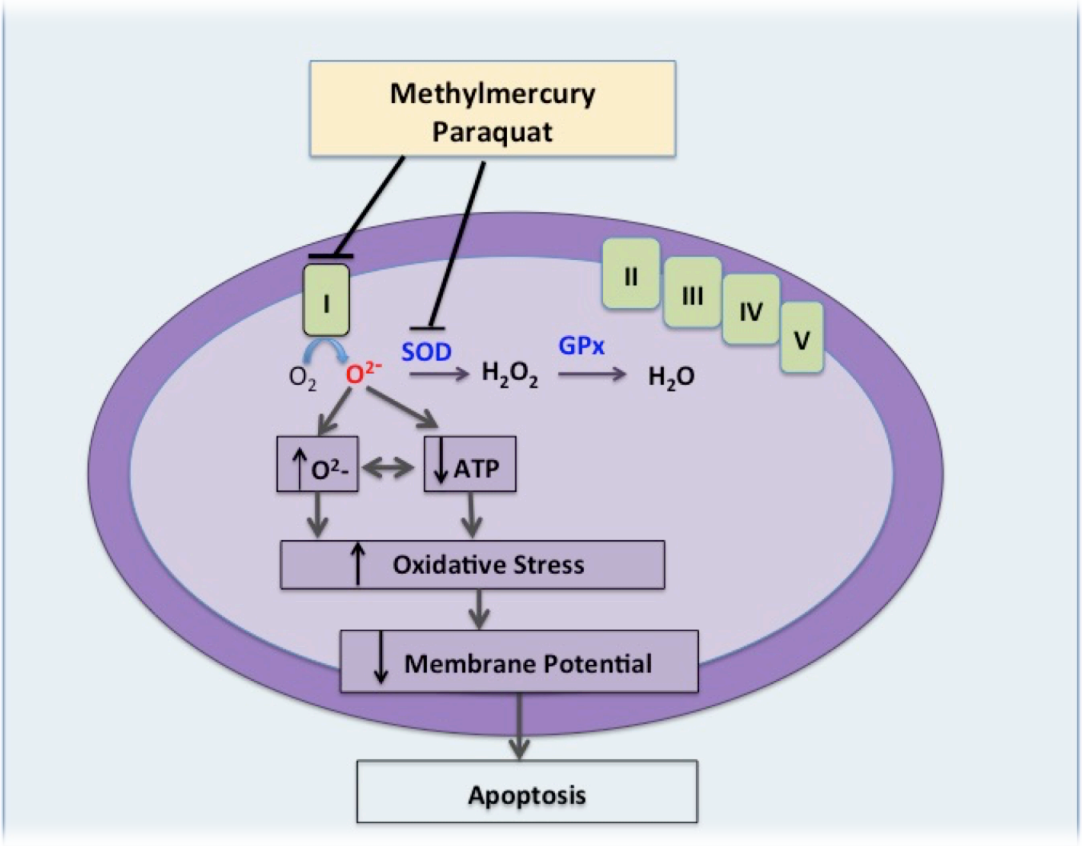


Figure1.1. Hypothesized mechanism of methylmercury and Paraquat mediated toxicity in the mitochondria of dopamine neurons.

2. Methods

2.1 Cell line

MN9D cell line is a model of dopaminergic neurons in the central nervous system. This cell line is a combination of neuroblastoma and embryonic ventral mesencephalon cells from mice (Choi *et al.,* 1991). MN9D cells were grown in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin at 37^{o}C with 5% carbon dioxide in poly-d-lysine coated flasks. Once the cells reached confluency of approximately 80% to the flasks, they were differentiated into dopamine neurons by addition of 1mM sodium butyrate in differentiation media, which consisted of DMEM with 2% (FBS) along with 1% streptomycin-penicillin for seven days. Sodium butyrate promotes neuron differentiation by inhibiting histone deacetylases (Kweon *et al.*, 2004). The differentiation and growing media were changed every 48 hours.

2.2 Tyrosine hydroxylase immunostaining

MN9D cells were differentiated to dopamine neurons with 1mM sodium butyrate in Dublecco's modified eagle medium containing 2% fetal bovine serum and 1% 1000µg streptomycin/penicillin for 7 days in 30mm dish (Corning, USA). After differentiation, the neurons were fixed with 2ml of 4% formaldehyde for 15minutes at room temperature. The neurons were washed three times with 1X PBS. Blocking buffer containing 1X PBS, 5% goat serum and 0.3% Triton X-100 was added to sample and incubated for 60 minutes. Primary rabbit tyrosine hydroxylase antibody (Santa Cruz, USA) was diluted 1:50 in 1X PBS, 1% bovine serum albumin (BSA) and 0.3% Triton X-100. Neurons

were incubated with primary antibody overnight at 4°C. The antibody solution was removed and neurons were washed three times with 1X PBS. Fluorescence conjugated secondary goat anti-rabbit antibody (Santa Cruz, USA) diluted 1:100 in BS was added to the dish. The neurons were incubated for an hour in the dark. Neurons were rinsed 3 times with PBS. On a coverslip, 40µl of Vectashield antifade mounting medium containing DAPI (Vectorlabs, USA) was added on top of the sample. Images were taken using Zeiss Axio Observer A1 fluorescent microscope (Zeiss, Germany).

2.3 Methylmercury and Paraquat Dosing

Methylmercury chloride was purchased as a powder (Alfa Aesar, USA), which was then dissolved in 0.2% acetic acid and 0.3% hydrochloric acid to create 383mg/L solution. This solution was further diluted to 50µM in DMEM (Sigma Aldrich, USA). The concentration of 50µM MeHg solution was verified by measuring the concentration in a mercury analyzer, MA-3000 (NIC, Japan) using triplicates. The 50µM solution was diluted for dosing experiments.

Paraquat chloride was purchased as a powder (Sigma-Aldrich, USA) and a concentration of 1000µM was prepared in DMEM (Sigma-Aldrich, USA). The 1000µM concentration was used for dosing experiments.

2.4 Cytotoxicity Assay

The sensitivity of MN9D neurons to PQ was determined using propidium iodide assay. In order to determine sensitivity of MN9D neurons to PQ, neurons were dosed with 100µM, 200µM, 300µM, 500µM, 600µM, 700µM, 8000µM and 900µM of PQ.

Dopamine neurons were plated in poly d-lysine coated 96 well black transparent bottom plate (Brandplate, Germany) at a density of approximately 60 000 cells / well. A period of 24 hours was allowed for neurons to adhere to wells. The spent media was removed and placed with different doses of PQ in DMEM for 24 hours at 37°C with 5% CO_2. A group of untreated neurons were used as control. Propidium iodide (PI) was purchased as 1.5mg/ml solution (Sigma-Aldrich, USA). PI binds to DNA in dead cells, thus allowing cell viability to be quantified. Stock PI solution was diluted in warmed (37°C) 1X PBS to generate 2.5μM concentrations in DMEM. Following exposure to PQ, the media was removed and neurons were dosed with 2.5μM of PI in DMEM. The neurons were incubated at 37°C for 30 minutes. After removal of PI in DMEM, 100μl of 1X PBS was added to each well. The viability of neuron was measured from PI fluorescence at 530/620nm in Cytation 3 plate reader (Biotek, USA).

The doses of MeHg were based on a previous study performed on MN9D neurons (Shao and Chan, 2015). The doses of MeHg used are 0.1μM, 1μM and 5μM. In order to examine the interaction between PQ and MeHg, neurons were also exposed to both methyl mercury (0.1μM) and PQ (300 and 500μM).

2.5 Mitochondria Complex I measurement

The activity of complex I of the electron transport chain in the mitochondria was measured using Complex I enzyme microplate (Mitosciences, USA). This is an enzyme linked immunosorbent assay (ELISA) and the complex I enzyme antibody is attached to each well of the plate. In 75cm^3 flasks (Corning, USA), about 9 million neurons were plated. The neurons were dosed for 24 hours and the mitochondria were isolated using

differential centrifugation based on the protocol developed by Frezza *et al.* (2007). Neurons were scraped of the plate, suspended in PBS and centrifuged at 600g for 10 minutes at 4°C. The pellet was re-suspended in cold mitochondria isolation buffer (0.1M Tris-MOPS, 1M sucrose, 0.1M EGTA/Tris) and the samples were homogenized on ice with 40 strokes in glass/ Teflon Potter Elvehjem homogenizer. After, the samples were centrifuged 600g for 10 minutes at 4°C. The pellets were collected and suspended in ice-cold mitochondria isolation buffer. After, the samples were centrifuged at 7000g for 10 minutes at 4°C. The pellets containing the crude mitochondria were collected and suspended in mitochondrial buffer.

The amount of mitochondria in each sample was determined using the Bradford assay. The Bradford reagent contains the dye Coomasie Brilliant Blue G-250, which binds to protein in the sample causing a shift in absorbance. Briefly, 10µl of samples were added to wells of clear 96 wells microplate, which contained 40µl of Bradford reagent and 150µl of distilled water. The absorbance was read at 595nm in Cytation 3 plate reader (Biotek, USA). The amount of mitochondria in each sample was determined from a BSA stand curve. The amount of mitochondria was adjusted to 5.5mg/ml per sample with PBS.

Protein from mitochondria was extracted by the addition of 10x detergent solution followed by incubation on ice for 30 minutes. The samples were centrifuged at 16 000g for 20 minutes at 4°C. The supernatants were collected and further diluted to 200ug/200ul in incubation buffer. Then 200µl of diluted samples were added to each well and incubated for 3 hours at room temperature. After, the liquid was removed from all wells and 300µl of mitochondria buffer was added to the wells. This was done twice.

Then 200µl of assay solution containing 1x buffer, 20X NADH and 100x dye were added to each well. The absorbance was read at 450nm in kinetic mode for 30minutes at room temperature. The rate of complex I activity was determined from the linear portion of the slope.

2.6 Measurement of Superoxide Anions

The production of superoxide anions was measured using MitoSOX red assay (ThermoFisher, USA). Neurons were plated at a density of 80 000 cells per well in a 96 well black clear bottom plate (Brandplate, Germany). Neurons were dosed with 0.1, 1 and 5µM of MeHg, 300µM and 500µM of PQ and co-exposure doses 300µM PQ + 0.1µM MeHg and 500µM PQ +0.1µM MeHg for 24 hours at 37°C and 5% CO_2. One group of neurons was used as the control. Menadione is known to generate superoxide anions in the mitochondria, thus served as the positive control. Menadione powder (Sigma-Aldrich) was dissolved in DMEM to create a concentration of 100µM and neurons were dosed with this concentration for 24 hours. A vial of MitoSOX (Molecular Probes, USA) containing 50µg was dissolved in 13µL of DMSO to generate a stock solution of 5mM. The stock solution was diluted to 5µM in DMEM to create a working solution. MeHg, PQ and menadione were removed from the wells and 1X PBS was added to wash the wells. 5µM of MitoSOX was added to the neurons and incubated for 20 minutes at 37°C and 5% CO_2. Cells were washed with 1X PBS once and fluorescence was read at 510/580nm using Cytation 3 Cell Imaging Multi Mode Reader (Biotek, USA).

Fluorescence from the assay was adjusted for protein concentration per well using the Bradford assay. After measurements, MitoSOX solution was removed and 50µM of 1N sodium hydroxide (NaOH) was added to each of the wells and incubated for 10 minutes. In clear 96 well microplates, 150µL of distilled water and 40µL of Bradford reagent was added to each well and mixed thoroughly. After neurons were incubated for 10 minutes in 1N NaOH and 10µl of neuron sample was added to each well containing the diluted Bradford reagent. A standard curve of bovine serum albumin (BSA) ranging from 0-250µg/mL was created from 2mg/ml of stock BSA solution (ThermoFisher, USA). Absorbance was read at 595nm in Cytation 3 Cell Imaging Multi Mode Reader (Biotek, USA).

2.7 Superoxide Dismutase Activity

Neurons were plated at a density of 5 million per plate in 100mm petri dish (Corning, USA). Neurons were treated with MeHg, PQ and co-exposure doses for 24 hours at 37°C and the mitochondria were isolated using centrifugal differentiation (Frezza *et al.,* 2007). Neurons were scraped of the plate, suspended in PBS and centrifuged at 600g for 10 minutes at 4°C The pellet was re-suspended in cold mitochondria isolation buffer (0.1M Tris-MOPS, 1M sucrose, 0.1M EGTA/Tris) and the samples were homogenized on ice with 40 strokes in glass/ Teflon Potter Elvehjem homogenizer. After, the samples were centrifuged 600g for 10 minutes at 4°C. The pellets were collected and suspended in ice-cold mitochondria isolation buffer. After, the samples were centrifuged at 7000g for 10 minutes at 4°C. The pellets containing crude mitochondria were collected and suspended in mitochondrial buffer.

Superoxide dismutase assay kit (colorimetric) was purchased from Abcam (USA). This kit uses tetrazolium salt; WST-1that is reduced to formazan dye by superoxide anions and xanthine oxidase activity to determine the inhibition of superoxide dismutase. In 96 well black clear-bottom flat plate (Brandplate, Germany), 200µl of WST -1 solution, 20µl xanthine oxidase and 20µl of mitochondrial samples were added. Then the plates were incubated at 37°C for 20 minutes protected from light. The absorbance was read at 450nm at room temperature with Cytation 3 Cell Imaging Multi Mode Reader (Biotek, USA). Background absorbances of WST-1, xanthine oxidase and dilution buffer were also measured. The percent inhibition of manganese superoxide dismutase was measured using the following equation:

$$\text{Inhibition rate (\%)} = \frac{(A_{blank1}-A_{blank\ 3})-(A_{sample}-A_{blank2})}{(A_{blank1}-A_{blank\ 3})} \times 100$$

where A_{blank1} = 20µl distilled water + 200µl WST-1 solution +20µl xanthine oxidase

$Ab_{lank\ 2}$= 20µl of sample+ 200µl of WST-1 solution +20µl of dilution buffer

A_{blank3}= 20µl distilled water+ 200µl WST-1 solution+ 20µl of dilution buffer

2.8 Oxygen Consumption

MitoXpress Xtra oxygen consumption assay kit (Cayman Chemicals, USA) was used to measure oxygen consumption. Neurons were plated in 96 wells black clear flat bottom microplate (Brandplate, Germany) at a density of 80 000 neurons/well in DMEM and allowed to adhere overnight at 37^{o}C. After, neurons were exposed to 0.1µM, 1µM, 5µM of MeHg, 300µM and 500µM of PQ, 300µM of PQ + 0.1µM of MeHg and 500µM of PQ + 0.1µM MeHg for 24 hours at 37°C. MitoXpress reagent was diluted in 1mL

DMEM and glucose oxidase stock solution diluted in 0.2mL of distilled water. Antimycin A was used as positive control and the concentration provided by the supplier without further dilution. After 24 hours, the media was removed and replaced with 150µl of DMEM/well. To each sample well, 10µl of MitoXpress reagent was added. Finally 100µl of mineral oil was added to each well. The plate was read kinetically for 120 minutes at 380nm excitation and 650nm emission. In another 96 well black clear bottom plate with no samples, 150µl of DMEM with 10ul of diluted glucose oxidase and 10µl of MitoXpress solution per well was added to 6 wells. After, 10µl antimycin and 10µl of MitoXpress solution was added to 150µl of DMEM per well for 6 wells. In addition 6 blanks containing only DMEM were added to the well. This plate with no samples was used to optimize the signal and determine background interferences. This plate was read kinetically for 120 minutes at 380nm excitation and 650nm emission at 37°C using Cytation 3 Cell Imaging Multi Mode Reader (Biotek, USA). From the kinetic readings, the rate of oxygen consumption for each treatment was determined using linear regression.

2.9 ATP Content

ATP was measured using ATP colorimetric assay (Biovision, USA). MN9D neurons were plated at a density of 100 000 neurons per 60mm plate (Corning, USA) prior to each plate being dosed with 0.1, 1 and 5µM of MeHg, 300µM and 500µM of PQ, 300µM PQ+ 0.1µM MeHg and 500µM PQ +0.1µM MeHg for 24 hours. After 200µl of ATP assay buffer was added to each dish to lyse the neurons. A cell scraper was used to scrape the neurons from the plates. The neuron solution was transferred to 1.5ml tubes

and the samples were deproteinized by addition of 1M of perchloric acid (PCA) to the supernatant. The samples were incubated on ice for five minutes. The samples were centrifuged at 13 000 rpm for 2minutes at 4° C. The supernatant was collected and the samples were neutralized by adding 2M of ice-cold potassium hydroxide (KOH) at 34%. This precipitated the excess PCA present in the samples. The pH of sample was corrected to 7 by the adding of KOH or PCA as required. Then the samples were then centrifuged for 15 minutes at 13 000 rpm at 4° C. The supernatants were transferred to clean microtubes. ATP converter and ATP developer were dissolved in 220µl of ATP assay buffer provided in the kit and thoroughly mixed. 50µl of mitochondrial samples were added to each well along with 50µl of ATP reaction mix, which consisted of ATP assay buffer, ATP probe, ATP convertor and ATP developer. The samples were incubated for 30 minutes at room temperature protected from light. The absorbance was measured at 590nm in Cytation 3 Cell Imaging Multi Mode Reader (Biotek, USA).

An ATP standard curve was generated from the following concentrations; 0, 2, 4, 6, 8 and 10 from 1mM ATP stock solution. The amount of ATP in the sample was analyzed from the standard curve. The concentration of ATP was determined by using the following calculation:

$$C=Ts/Sv$$

Where Ts is the amount of ATP present in samples

Sv is sample volume added to each well

2.10 Mitochondria Membrane Potential

Mitochondria membrane potential was determined using tetramethylrhodamine ethyl ester (TMRE) assay (ThermoFisher, USA). TMRE is a negatively charged fluorophore that targets live mitochondria. It accumulates in active mitochondria that have a polarized membrane potential. Neurons were plated in a 96 well black clear flat bottom plate at density of 80 000/ well. Then neurons were treated with 0.1, 1 and 5µM of MeHg, 300 and 500µM of PQ, 300µM PQ+0.1µM MeHg and 500µM +0.1µM of MeHg for 24 hours. TMRE stock solution of 1mM was created in dimethyl sulfoxide (DMSO). This stock solution was diluted in DMEM to generate a working solution of 400nM of TMRE. The liquid was removed from the wells and replaced with 200µl of 400nM of TMRE solution per well. The plated were incubated in the dark at 37°C for 20 minutes. The TMRE solution was removed and sampled rinsed with 1X PBS. The samples were kept in 100µl of PBS and the fluorescence was measured at 549nm excitation and 575nm emission at room temperature in Cytation 3 Cell Imaging Multi Mode Reader (Biotek, USA).

Fluorescence from the assay was adjusted for protein concentration per well using the Bradford assay. After measurements, PBS solution was removed and 50µl of 1N of sodium hydroxide (NaOH) was added to each well and incubated for 10 minutes. In clear 96 well microplates, 150µl of distilled water and 40µl of Bradford reagent was added to each well and mixed thoroughly. After neurons were incubated for 10 minutes in 1N NaOH and 10µl of neuron sample was added to each well containing diluted Bradford reagent. A standard curve of bovine serum albumin (BSA) ranging from 0-250µg/mL

was created from 2mg/ml of stock BSA solution (ThermoFisher, USA). Absorbance was read at 595nm in Cytation 3 Cell Imaging Multi Mode Reader (Biotek, USA).

2.11 Statistical Analysis

The data are presented means with +SEM of independent experiments. Raw data was calculated and organized in Excel 2010 and statistical analysis was performed in Graphpad Prism 7. For all analyses, the data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test to compare with untreated neurons. In order to determine interaction between PQ doses and corresponding co-exposure doses, 1 way ANOVA was used followed by post hoc Tukey's HSD to determine differences. For all analyses, $p<0.05$ was considered significant.

3.0 Results

3.1 MN9D neurons as suitable model for dopamine neurons

MN9D neurons originate from a combination of neuroblastoma and ventral mesencephalic cells in mice. In order to determine that differentiated MN9D neurons are dopaminergic, the neurons were tested for expression of the enzyme tyrosine hydroxylase, which is the rate-limiting enzyme in dopamine synthesis. MN9D neurons were incubated with primary rabbit tyrosine hydroxylase antibody and secondary goat antibody with fluorescein dye. As shown in Figure 3.1, the differentiated MN9D neurons expressed high level of tyrosine hydroxylase in the cell body as noted by the presence of green fluorescence.

3.2 Cell viability

MN9D neurons responded in a dose dependent manner to 24 hours exposure to PQ (Figure 3.2). The viability of the neurons decreased with increasing concentration of PQ. The lowest observed effect level (LOAEL) was at 300μM ($p<0.05$) and the viability was 68%. The lethal dose (LD_{50}) was around 700μM ($F=66.21$, $df= 5$ and 18). Based on the result of this assay, the concentrations of 300μM and 500μM of PQ were chosen for the rest of the experiments.

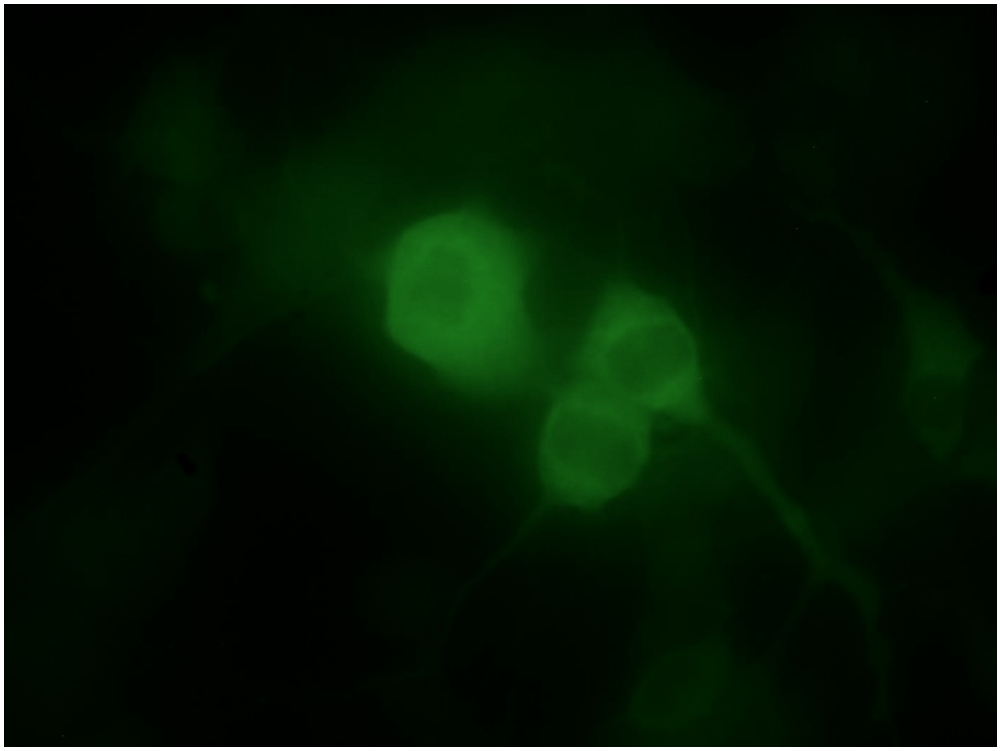


Figure 3.1. **Immunostaining of the enzyme tyrosine hydroxylase in differentiated MN9D neurons**. Image taken with Nikon Zeiss fluorescence microscope. Neurons were incubated with rabbit tyrosine hydroxylase antibody overnight and conjugated with secondary goat fluorescence antibody for two hours prior to imaging.

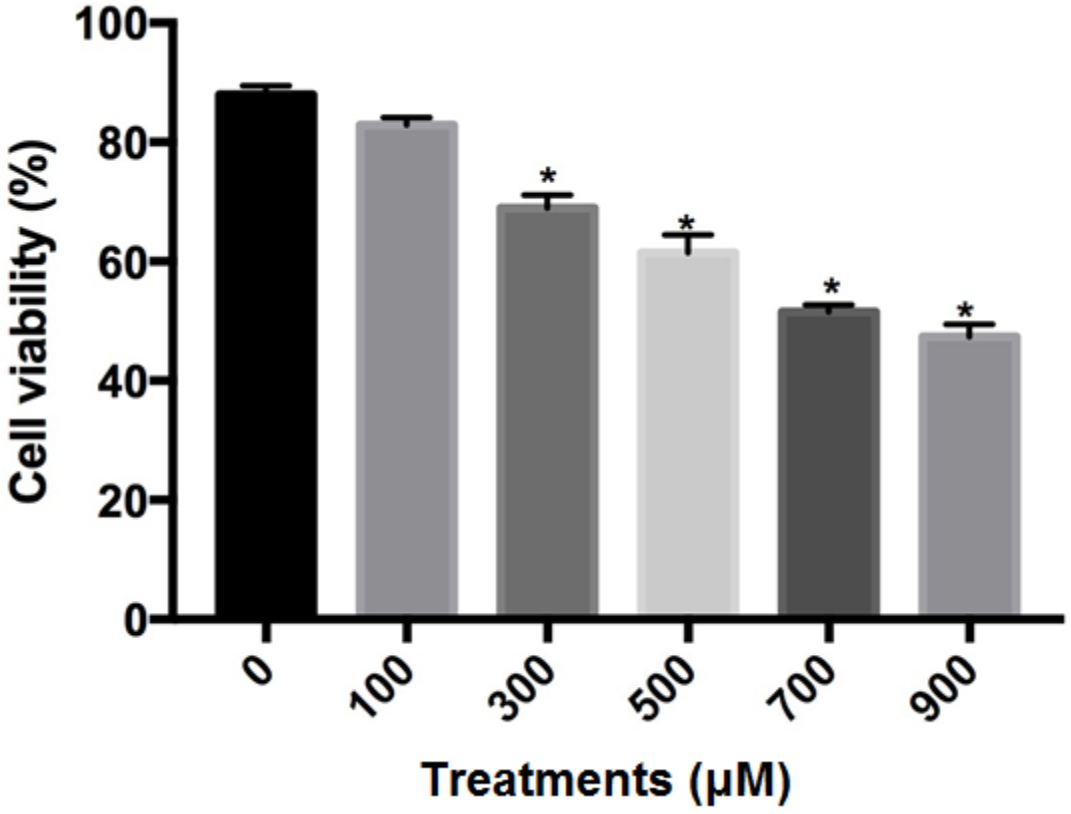


Figure 3.2. Cell viability of MN9D neurons using propidium iodide assay following 24 hours exposure to different concentrations of Paraquat. The data represented as % mean + SEM (n=4 independent experiments). Significance of the data was analyzed using 1-way ANOVA with post-hoc Dunnett's test to compare with untreated neurons. Treatments noted with '*' represent $P<0.05$.

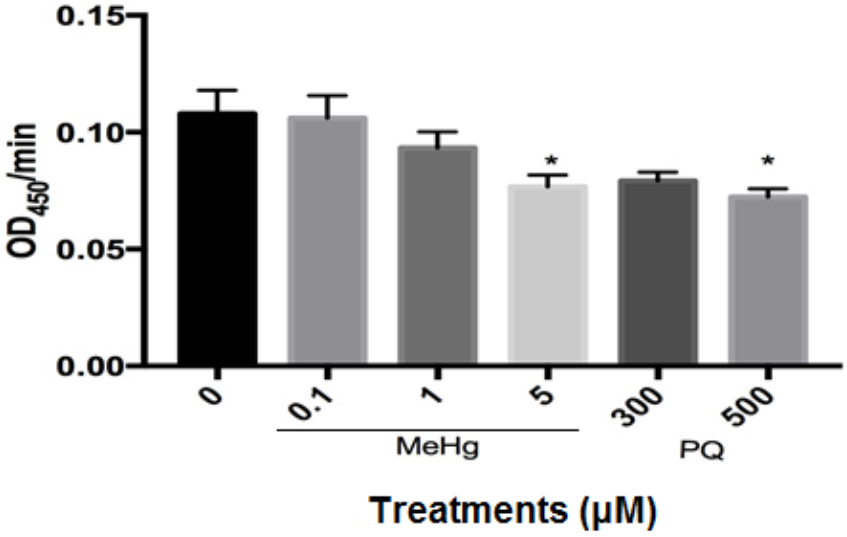
3.3 MeHg and PQ induced inhibition of complex I of mitochondria

The inhibition of complex I of the ETC following MeHg, PQ and co-exposure is shown in Figure 3. 3. A dose dependent decrease in activity was observed ($F= 34.9$, $df =7, 28$). A significant decrease in complex I activity ($p<0.05$) was found only with 5μM of MeHg. Only 500μM of PQ led to a decrease in complex I functions ($p<0.05$). There was no significant difference in complex I activity between 300μM of PQ and co-exposure dose of 300μM PQ with 0.1μM of MeHg. Similarly, no change in activity was found between 500μM and co-exposure dose of 500μM PQ with 0.1μM of MeHg.

3.4 Impact of MeHg and PQ on superoxide anion levels

Both MeHg and PQ have been known to increase oxidative stress by increasing the formation of superoxide anions in various types of cells. There was a dose dependent increase in superoxide anions ($F=43.5$, $df= 8, 28$, $p<0.05$). Concentration of superoxide anions in the mitochondria was significantly elevated after 24 hours exposure to 1μM and 5μM of MeHg compared to the control (Figure 3.4A). PQ increased the levels of superoxide anion only at the high concentration of 500μM ($p<0.05$). The co-exposure dose of 300μM of PQ with 0.1μM of MeHg resulted in significant increase in superoxide anions compared to 300μM of PQ alone ($p<0.05$) (Figure 3.4B).

A)



B)

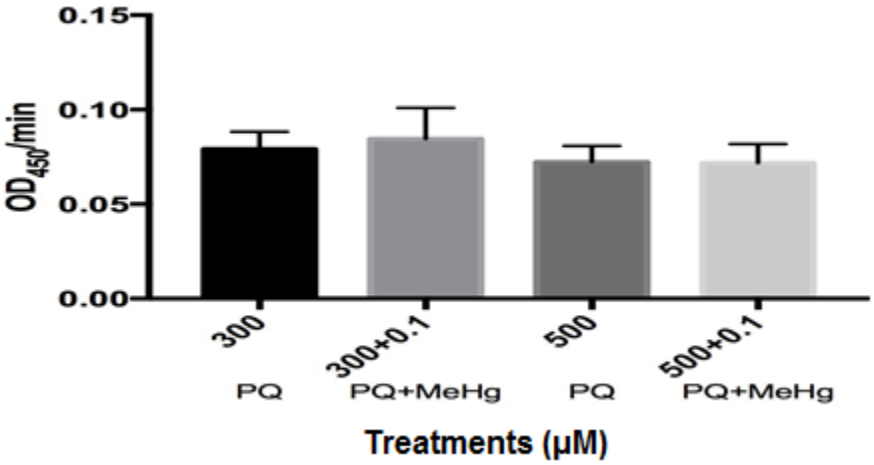
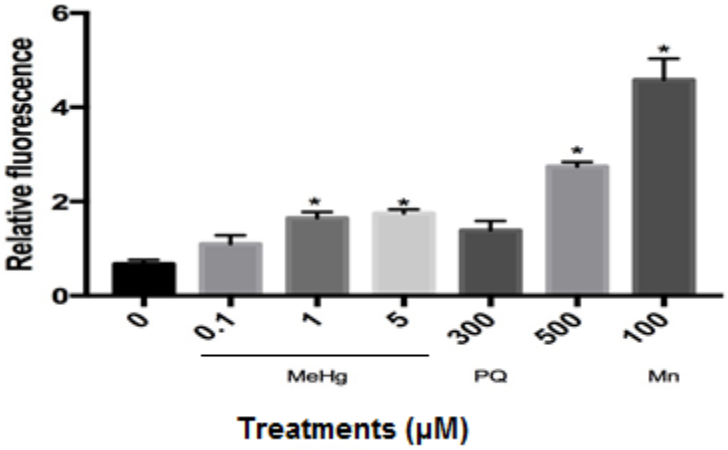


Figure 3.3 Activity of complex I of the mitochondrial electron chain following 24 hours exposure to methylmercury, Paraquat and co-exposure of methyl mercury and Paraquat treatments in MN9D neurons. A) Changes in complex I activity following methyl mercury and Paraquat exposure. B) Differences in complex I response to Paraquat and corresponding co-exposure doses. The data represent mean + SEM (n=4 individual experiments). The data was analyzed using 1-way ANOVA with post hoc Dunnett's test and Tukey's HSD. * $P<0.05$

A)



B)

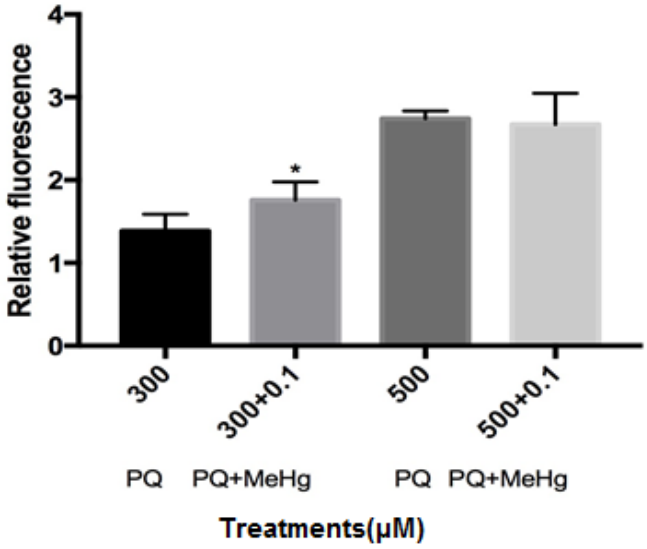


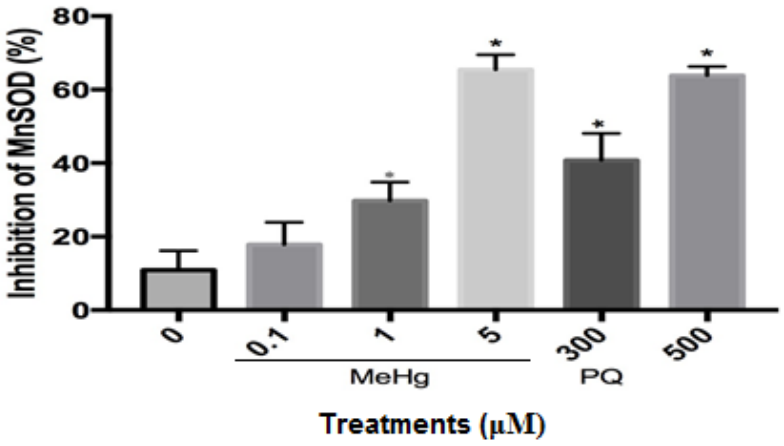
Figure 3.4. Measurement of superoxide anions levels in the mitochondria of MN9D neurons using MitoSOX assay following 24 hours exposure to methyl mercury, Paraquat and co-exposure treatments. Relative fluorescence units were normalized to protein content. A) Level of superoxide anions after methyl mercury and Paraquat exposure. Menadione (Mn) is the positive control. B) Differences between Paraquat and co-exposure treatments. Data represent mean + SEM (n= 4 individual experiments). Data analyzed with1-way ANOVA and post-hoc Dunnett's test and Tukey's HSD test. A) *P<0.05 compared to untreated neurons B) *P<0.05 compared to 300µM of Paraquat.

3.5 Manganese superoxide dismutase activity

Both MeHg and PQ induced a dose dependent decrease in the activity of manganese superoxide dismutase compared to untreated neurons ($F=24.8$, $df=7, 28$). In comparison to untreated cells, 1 and 5μM of MeHg significantly inhibited the functioning of MnSOD (Figure 5A). Both doses of PQ 300μM and 500μM decreased MnSOD functions by around 50%.

There was an increase in inhibition between co-exposure dose of 300μM of PQ with 0.1μM of MeHg compared to 300μM of PQ alone (Figure 5B). No interaction was observed between 500μM with 0.1μM of MeHg.

A)



B)

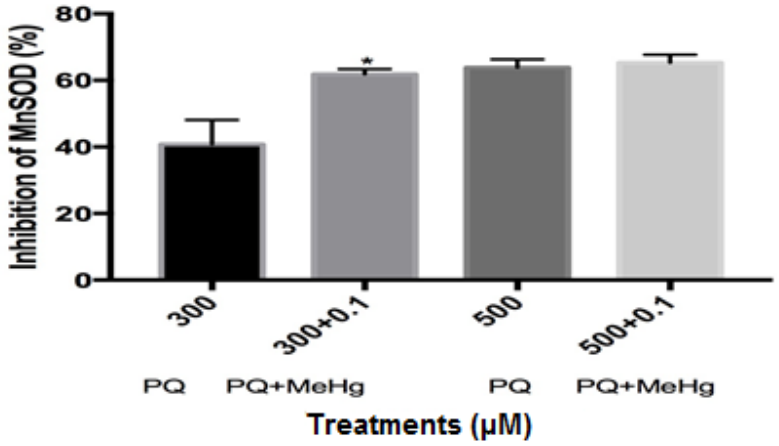


Figure 3.5. Inhibition of manganese superoxide dismutase (MnSOD) in MN9D neurons following exposure to methyl mercury, Paraquat and co-exposure treatments for 24 hours. A) Activity of MnSOD after methyl mercury and Paraquat exposure. B) Differences between Paraquat and co-exposure treatments. Data represent mean + SEM (n= 4 individual experiments). Data analyzed with1-way ANOVA and post-hoc Dunnett's test and Tukey's HSD test. A) *P<0.05 compared to untreated neurons B) *P<0.05 indicate difference between 300µM of Paraquat and 300µM Paraquat with 0.1µM methyl mercury.

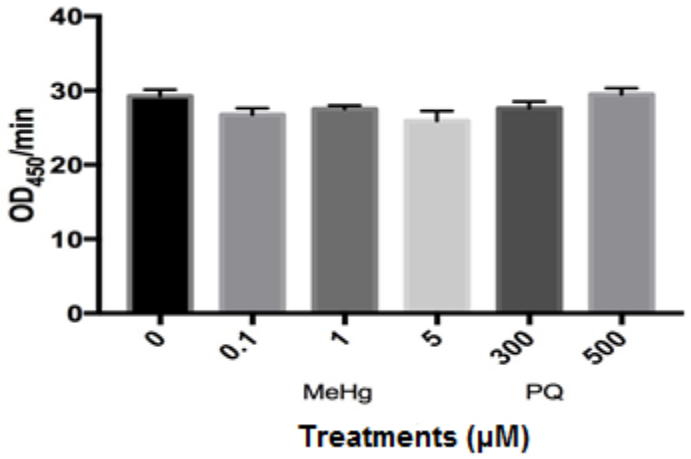
3.6 Cellular Respiration

The rate of oxygen consumption is used to determine cellular respiration in the mitochondria. There was no significant change in oxygen consumption by the doses of MeHg (0.1, 1 and 5μM) and 300 and 500μM of PQ ($F=2.14$, $DF=7, 25$, $p<0.05$)) (Figure 3.6). There were no significant differences between PQ and co-exposure treatments for both doses ($p<0.05$).

3.7 ATP Concentration

The ATP content in the mitochondria serves as an indicator of electron transport chain and oxidative phosphorylation. Exposure MeHg and PQ resulted in a dose-dependent decrease in ATP concentration in the mitochondria of MN9D neurons ($F=53$, $df=7, 25$) (Figure 3.7). There were significant differences between PQ and co-exposure treatments for both doses ($P<0.05$).

A)



B)

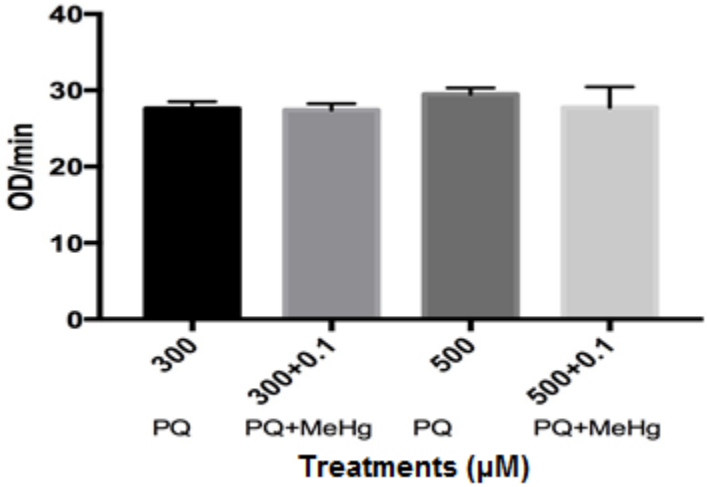
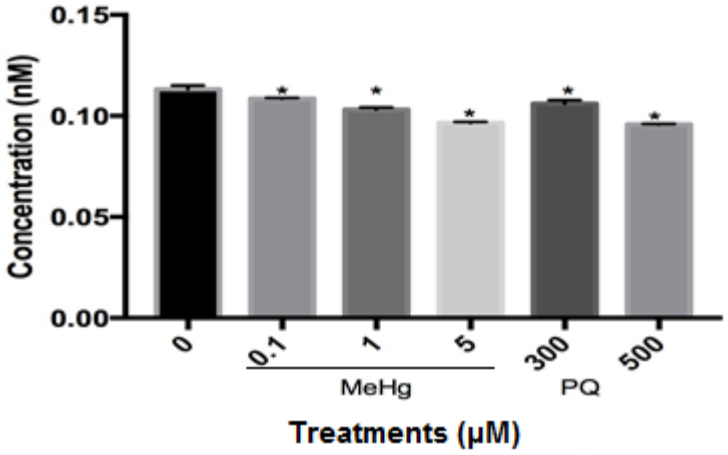


Figure 3.6. The rate of oxygen consumption following 24 hours exposure to methyl mercury, Paraquat and co-exposure doses in the mitochondria of MN9D neurons. A) Oxygen consumption after methyl mercury and Paraquat exposure. B) Differences between Paraquat and co-exposure treatments. Data represent mean + SEM (n= 4 individual experiments). Data analyzed with1-way ANOVA and post-hoc Dunnett's test and Tukey's HSD test.

A)



B)

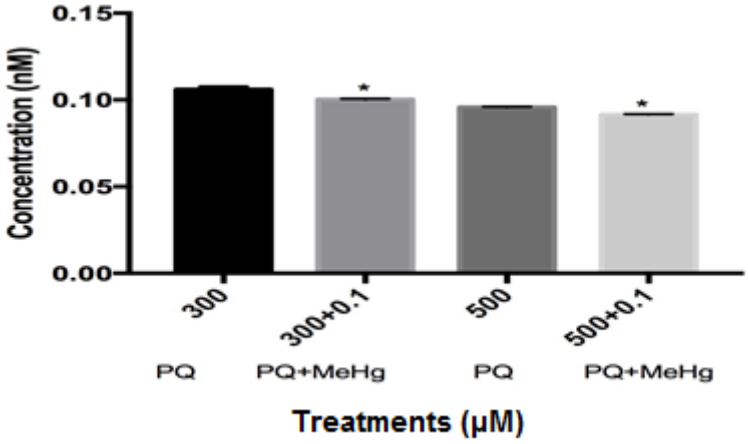


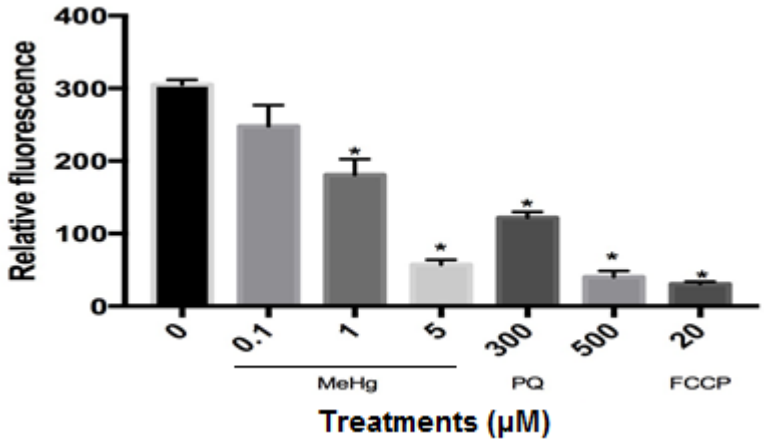
Figure 3.7. Concentration of ATP in the mitochondria of MN9D neurons following 24 hours exposure to methyl mercury, Paraquat and co-exposure doses. A) Concentration of ATP after methyl mercury and Paraquat exposure. B) Differences between Paraquat and co-exposure treatments. Data represent mean + SEM (n= 4 individual experiments). Data analyzed with1-way ANOVA and post-hoc Dunnett's test and Tukey's HSD test. A) *P<0.05 compared to untreated neurons.
B) *P<0.05 indicates difference between 300µM of Paraquat and 300µM Paraquat with 0.1uM methyl mercury; difference between 500µM of Paraquat and 500µM Paraquat with 0.1µM methylmercury.

3.1.8 Mitochondrial Membrane Potential

Measurement of the membrane potential of the mitochondria is important for analyzing the proton gradient in mitochondrial matrix and ATP synthesis. Membrane potential is also a marker of neuronal survival. All of the doses of MeHg and PQ depolarized the mitochondrial membrane in a dose dependent manner ($F=51.4$, $df=8$ and 27, $p<0.05$). However, MeHg dose of 0.1μM was not statistically significant compared to the control ($p<0.05$) (Figure 3.8a).

There were significant differences between PQ and co-exposure treatments for both doses ($P<0.05$). Both co-exposure doses depolarized the mitochondrial membrane compared to corresponding PQ doses.

A)



B)

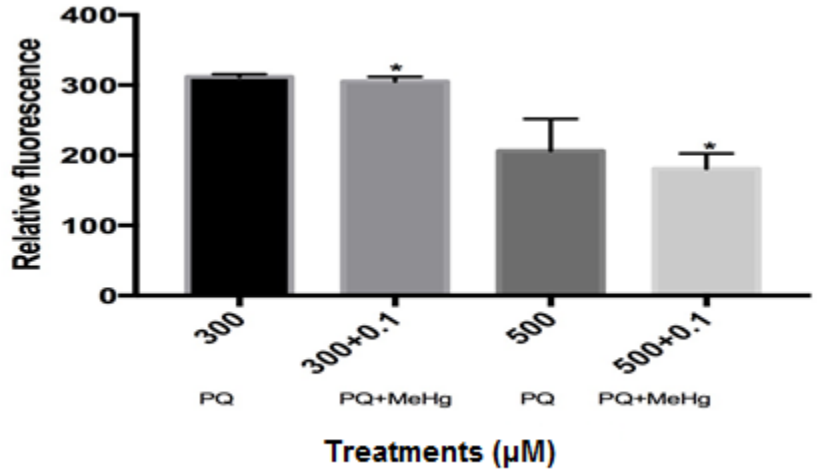


Figure 3.8. Measurement of mitochondrial membrane potential by accumulation of tetramethylrhodamine ethyl ester (TMRE) dye in MN9D neurons after treatment with methylmercury, Paraquat and co-exposure doses for 24 hours. Relative fluorescence units are normalized to protein content. A) Mitochondrial membrane potential after methyl mercury and Paraquat exposure. 20nM of FFCP is the positive control. B) Differences between Paraquat and co-exposure treatments. Data represent mean + SEM (n= 4 individual experiments). Data analyzed with1-way ANOVA and post-hoc Dunnett's test and Tukey's HSD test. A) *P<0.05 compared to untreated neurons. B) *P<0.05 indicates difference between 300µM of Paraquat and 300µM Paraquat with 0.1µM methyl mercury, difference between 500µM of Paraquat and 500µM Paraquat with 0.1µM methyl mercury.

4. Discussion

4.1 MN9D neurons

The loss of dopamine neurons in the substantia pars nigra, which is part of the midbrain, is associated with fine motor problems in Parkinson's disease (Schapira, 2011). MN9D neurons used in this study, originate from a fusion of neuroblastoma and midbrain dopamine neurons in mice (Choi *et al.,* 1991). Previously, it has been noted that only differentiated MN9D neurons have phenotypic similarities with dopamine neurons from substantia pars nigra in mice. The differentiated MN9D neurons exhibit flattened body with multiple branches similar to a neuron (Dong *et al.,* 2008).These neurons use vesicular monoamine transporter 2 (VMAT2) to transport and release dopamine, which is a marker of dopamine neurons (Chen *et al.,* 2005). Differentiated MN9D neurons used are an appropriate model of dopamine neurons since they express tyrosine hydroxylase (Figure 3.1). In the 1991 study by Choi *et al*., it was noted that about 25% of the neurons are adrenergic. In addition, these neurons have voltage sensitive sodium and calcium channels and undergo normal action potential (Choi *et al*., 1991). The potassium type A channels are missing in MN9D neurons (Rick *et al.,* 2006), however, Dong *et al.* (2008) found that MN9D neurons have electrophysiological properties similar to midbrain dopamine neurons using amperometric measurements.

4.2 MeHg and PQ doses

The sensitivity of MN9D neurons to MeHg was determined by a previous study (Shao and Chan, 2015). MN9D cells were dosed with MeHg ranging from 0.1 to 10μM and the LD_{50} was around 8μM. Thus, doses of 0.1μM, 1μM and 5μM were chosen for this study. The concentration of MeHg in brain tissue was estimated between 0.2-1.5μM in fish consuming population (Korybas *et al*., 2011). The dose of 0.1μM of MeHg is applicable to low dose chronic exposure in population and 1μM is relevant to sub-acute exposure. The concentration of 5μM of MeHg represents an extreme acute exposure dose.

MN9D neurons responded to PQ in a dose-dependent manner (Figure 3.2). As the concentration of PQ increased, the viability of the neurons decreased. The effect of PQ exposure was observed from concentration of 300μM onwards and LD_{50} was at 700μM. Currently, there are no other studies looking at PQ toxicity in MN9D neurons. One study used 300μM of PQ in an engineered form of MN9D cells with synaptophysin markers. Concentrations of 100μM, 500μM and 1000μM of PQ are applicable to another dopamine cell line, differentiated SH-SY5Y neurons (Martins *et al.,* 2013). Apoptosis induced by PQ was evaluated using doses ranging from100μM to 500μM in SH-SY5Y neurons (Garcia *et al*., 2013). The authors found changes in apoptotic markers with 500μM of PQ. Change in mitochondrial membrane potential in SH-SY5Y neurons is observed with 300μM of PQ (Chau *et al.,* 2009). The doses of PQ used in SH-SY5Y neurons are similar to the results of PQ cytotoxicity in MN9D neurons.

4.3 Complex I functions

The disruption of mitochondrial complex I functions have been implicated in many diseases including neurodegenerative disorders (Schapira et al 1990). Complex I (NADH Coenzyme Q oxidoreductase) is located in the inner mitochondrial membrane (Li and Shah, 2003). It is the one of the starting point of the electron transport chain (ETC) as electrons generated in tricarboxylic acid cycle (TCA) use Complex I to enter the mitochondrial matrix (Fernie *et al.,* 2004). Complex I reduces coenzyme Q molecules by accepting electrons from NADH and generates the proton gradient (Mimaki, 2012). In addition, Complex I is also known as one of the two sites that produce reactive oxygen species (ROS), especially superoxide anions in the ETC (Lenaz *et al.,* 2006). Thus, the integrity of Complex I function is of interest in regards to compounds known to generate reactive species and increase oxidative stress.

Both PQ and MeHg are known to produce reactive oxygen species in various types of neurons and cells. Literature evidence on the effect of PQ exposure on complex I activity is contradictory. In this study, complex I activity is reduced by 500μM of PQ and exposure to 300μM did not cause any changes in activity (Figure 3.3A). Chronic exposure to PQ in mice has been known to decrease the function of complex I in striatal neurons (Czerniczyniec *et al.,* 2011). PQ sequesters electrons from Complex I to reduce its monovalent state, PQ^{+} and blocks malate dehydrogenase, which is part of complex I in cortical neurons (Fukushima *et al*., 1993). Richardson *et al.* (2005) did not observe any inhibition of complex I in a neuroblastoma cell line with 10mM of PQ. Similarly, 250μM of PQ was unable to alter the activity of complex I in mesencephalic neurons (Choi *et al.,* 2008). In comparison to this study, Choi *et al*. (2008) used an extremely low dose of PQ,

which would not affect the functions of MN9D neurons. The neuroblastoma cell line used by Richardson *et al*. (2005) expresses both cholinergic and dopaminergic properties and this could account for the differences (Roomi *et al.,* 2013). The effect seen in this study is similar to the findings of Czerniczyniec *et al*. (2011).

In this study, complex I activity is reduced by a high concentration of MeHg, 5μM, and the lower doses do not decrease activity of the enzyme in MN9D neurons (Figure 3A). Chronic low dose exposure to MeHg for a week did not change complex I activity (Mori *et al*., 2011). However, the study compared the difference in complex I activity with mitochondria from liver. Zebrafish (*Danio rerio)* exposed to low concentrations of MeHg via diet for 50 days showed no inhibition of mitochondrial complexes in their muscles (Cambier, 2009). These findings are consistent with our results that MeHg alters complex I activity only at a high concentration.

There are no differences in inhibition of complex I activity between co-exposure doses of PQ with MeHg and their corresponding PQ doses (Figure 3.3B). The lack of additive effects seems plausible considering that PQ and MeHg only effect complex I at high concentrations.

4.4 Superoxide anion levels

Both PQ and MeHg are known to induce toxicity by increasing the levels of reactive oxygen species (ROS) (Mailloux *et al.,* 2015a). Superoxide anions are a form of reactive oxygen species generated as a byproduct in many biological reactions (Fridovich, 1997). It is considered highly toxic since it can activate other ROS pathways. Mitochondria are the primary site of superoxide anion production (Han, 2011). This

study analyzed superoxide anion levels with MitoSOX dye, which is widely used for this purpose. Exposure for 24 hours showed an increase in superoxide anions in 1μM and 5μM MeHg and 500μM PQ treated neurons. Miyamoto *et al.* (2001) also found a similar increase in superoxide anions levels with MeHg exposure in cortical neurons of rats. Similar results were also observed in astrocytes exposed to MeHg (Shankar and Aschner, 2001).

The main mechanism of PQ toxicity is increasing oxidative stress through the production of superoxide anions (Cocheme and Murphy, 2008). PQ dose of 500μM elevated superoxide anions level (Figure 3.4A). In another study, 400uM of PQ was found to increase superoxide anion concentration in dopaminergic neurons (Kwon *et al.,* 2011). Likewise 500μM of Paraquat caused increases in superoxide anions in another dopaminergic cell line (Rodriguez-Rocha *et al*., 2013). The concentration of PQ as low as 200μM increases the level of superoxide anions in neuroblastoma cells (Mailloux *et al.,* 2015a).

There was an increase in the production of superoxide anions in the co-exposure treatment of 300μM of PQ with 0.1μM of MeHg in comparison to 300μM of PQ alone (Figure 3.4B). Previous study in our lab has shown such additive interactions in astrocytoma cells, combination of PQ and MeHg increases reactive oxygen species in the mitochondria (Mailloux *et al.,* 2015b). In this study, 0.1μM of MeHg was used for co-exposure whereas Mailloux *et al*. (2015b) used 1μM of MeHg with 500μM of PQ. The increase in superoxide anions was also observed with a lower co-exposure dose than Mailloux *et al.* (2015b). These results suggest that the dopaminergic neurons are more susceptive to the combined oxidative effects of MeHg and PQ than astrocytes.

4.5 Manganese superoxide dismutase activity

The main function of manganese superoxide dismutase (MnSOD) is to convert superoxide anions to hydrogen peroxide in the mitochondria. Later, the enzyme glutathione peroxidase converts hydrogen peroxide to water (Shinyasiki, 1996). Impairments in the functions of MnSOD cause the accumulation of superoxide anions. This accumulation of superoxide anions activates other ROS pathways, lipid peroxidation (Miriyala *et al.,* 2012) and initiate apoptotic signaling in neurons (Keller *et al.,* 1998). MeHg decreased MnSOD functions at1 and 5μM and both doses of Paraquat 300μM and 500μM (Figure 3.5A). The 5μM of MeHg and 500μM of PQ decreased MnSOD functions almost by half. Mimetics of MnSOD such as MnTBAP are able to reduce superoxide anion levels following MeHg and Paraquat exposures in astrocytoma cells (Mailloux *et al.,* 2015b). MeHg exposure decreased activity of MnSOD in various neuronal tissues in rats (Shinyasiki, 1996). Expression of MnSOD mRNA genes is up regulated by 500μM of PQ in primary cortical neurons (Schmuck *et al.,* 2002). MnSOD transcription is activated in astrocytes following exposure to 2mM of PQ (Rohrdanz *et al.,* 2001). Both of the studies did not directly measure the activity of the enzyme. Furthermore, in another dopaminergic cell line, 400μM of Paraquat did not change the expression of MnSOD protein (Kwon *et al.,* 2015). The activity of MnSOD was decreased almost 20% by co-exposure dose of 300μM of PQ with 0.1μM of MeHg when compared to 300μM of PQ alone. The discrepancy in the effect of PQ on MnSOD functions could be due to differences in the models used or in the isolation of the mitochondria. There is possibility of damaging MnSOD during mitochondrial isolation

and Kwon *et al*. (2015) did not provide information on the activity of MnSOD in untreated neurons. In our study, there was an inhibition of 12% in untreated neurons. Astrocytes do not express dopamine and cortical neurons have many neurotransmitters primarily glutamate and GABA. The decrease in MnSOD caused by PQ could be specific to dopamine neurons and again the dopaminergic neurons are sensitive to the combined effects of MeHg and PQ.

4.6 Cellular respiration

As shown in Figure 3.6, no change in oxygen consumption is observed with any of the doses of PQ and MeHg. These finding are consistent with the literature as no changes were found in oxygen consumption by low doses of MeHg in zebrafish neurons (Cambier *et al.,* 2012) and in worms (Caito and Ashner, 2016). Low dose exposure to MeHg decreased oxygen consumption in neurons from the cerebrum (Mori *et al.,* 2007). In the mitochondria of the lungs, 500μM of PQ did not alter oxygen concentrations but increasing the concentration to 1mM caused significant decline in oxygen levels (Rossouw and Engelbrecht, 1978). Information on changes in cellular respiration caused by PQ is scarce. Pesticides that are known to impede mitochondrial functions such as rotenone and maneb decrease cellular respiration in dopamine neurons (Radad *et al.,* 2006, Barlow *et al.,* 2011).

Cellular respiration in mitochondria is a marker of the quality of electron transport chain and overall mitochondrial functions (Brown, 1992). Changes in oxygen consumption indicate loss of electrons that could result in break-down of the electrochemical gradient and uncoupling of oxidative phosphorylation (Brand and

Nicholls, 2011). This study only looked at one time 24 hours exposure to MeHg and PQ. The biochemical changes observed may not be significant enough to cause the physiological changes in oxygen consumption. Chronic exposure to the toxicants could amplify the damage to the electron transfer chain and eventually cause decrease in oxygen concentration.

In addition, secondary messengers such as calcium and nitric oxide are regulators of mitochondrial cellular respiration (Cooper and Giulivi, 2007; Rizutto *et al.,* 2012). The regulations of the two messengers are tightly linked in the mitochondria. Nitric oxide (NO) binds to Complex IV (cytochrome c oxidase) (Traaseth *et al*., 2004), the site of oxygen consumption. In certain cases, when oxygen levels decline, the binding of NO to a subunit of Complex IV maintains oxygen concentration constant for a period of time (Taylor and Moncada, 2010). Eventually, the increase in NO leads to activation of cell death pathways (Haynes *et al.,* 2003). Calcium mediates the production of NO in the mitochondria (Haynes *et al*., 2003). Calcium monitors oxygen levels in the mitochondria (Traaseth *et al.,* 2004). Calcium signaling is vital for many mitochondrial functions such as oxidative phosphorylation and apoptosis (Gunter and Gunter, 2001). In a neuronal cell line, activation of the ion exchange transporter, Na^{+}/Ca^{2+} channel in the mitochondria resulted in a decrease in calcium concentration, which increased oxygen consumption. However, the ATP content was unaffected (Zhdanov *et al.,* 2010).

Oxyegn consumption is regulated by a complex feedback mechanism in the mitochondria. It is beyond the scope of this study to delineate all the mechanisms related to the linkage between biochemical and physiological changes in the mitochondria.

4.7 ATP concentration

One of the most important functions of mitochondria is to produce ATP using an electrochemical gradient (St. Pierre *et al.,* 2000). The enzyme ATP synthase creates ATP by using energy from the electron transport chain (Ricci *et al.,* 2004). A decrease in ATP levels was observed with all doses of MeHg and PQ. Both of the co-exposure doses reduced ATP content more than their corresponding PQ doses only (Figure 3.7). MeHg in nanomolar concentrations decreases ATP levels in worms (Wang *et al.,* 2016). Low dose chronic PQ exposure in mice leads to decrease in ATP concentration in striatal neurons (Wang *et al*., 2007). This loss of ATP caused by MeHg and PQ could be due to the loss of inner mitochondrial membrane potential and inhibition of complex I. Since ATP levels decline at low concentrations of MeHg and PQ, it suggests that these two toxins could also impede the functions of Complex III and ATP synthase. It has been suggested that PQ blocks the functions of Complex III (Fukushima *et al.,* 1994).

In addition to complex I, the other source of electron leakage in ETC is Complex III. Oxidative phosphorylation is carried out by complex IV of ETC, which produces energy that can be used to form ATP. The results from the ATP assay indicate the possibility of impaired functions of complex IV. Moreover, complexes I, II and IV are involved in the production of the electrochemical gradient which drives the formation of ATP by reducing ADP.

The decline in ATP levels in the mitochondria implies insufficient production of ATP to meet the metabolic demands of the neurons. The decreases in ATP levels in neurons have widespread negative effects. The enzyme Na^+/K^+-ATPase maintains membrane potential by shuttling sodium and potassium ions in and out of the membrane.

This enzyme requires ATP to function properly, thus the lack of ATP will impair Na^+/K^+-ATPases (Howarth *et al*., 2012). The pathways of neuronal death, apoptosis and necrosis, are regulated by the concentration of ATP. A decrease in ATP leads to the activation of apoptotic signaling whereas complete exhaustion of ATP triggers necrosis (Nikoletopoulou *et al.,* 2013). In accordance with the decrease of ATP levels in this study, these neurons will be more vulnerable to apoptosis.

4.8 Mitochondrial Membrane Potential

Finally, the effects on membrane potential of mitochondria after PQ and MeHg exposures were evaluated using the TMRE dye. MeHg depolarized the mitochondrial membrane with increasing concentration and both doses of PQ depolarized the membrane (Figure 3.8A). The loss of membrane potential by MeHg is consistent with previous studies in neurons (Qu *et al.,* 2013; Wang *et al.,* 2016). There was significant loss of mitochondrial membrane potential in synaptosomes of rats with 0.1μM of MeHg (Dreiem *et al.,* 2005) whereas in this study no difference in potential was observed. In this study, mitochondria from the entire neuron were used instead of isolating mitochondria from specific parts of a neuron. PQ caused loss of mitochondrial membrane potential with concentration of 400 μM (Rodriguez-Rocha, 2013). The loss of membrane potential could be related to the inhibition of MnSOD as Rodriguez-Rocha *et al*. (2013) observed that overexpression of MnSOD can prevent membrane depolarization. There was an increased loss of membrane potential by 300μM PQ with 0.1μM of MeHg compared to 300μM of PQ and 500μM PQ with 0.1μM of MeHg compared to 500μM of PQ (Figure 3.8B).

The loss of mitochondrial membrane potential has serious consequences for the survival of neurons. The production of ATP is disrupted due to the loss of proton gradient in the mitochondria. It can also lead to uncoupling of ATP synthase. The effect of the loss of mitochondrial potential is observed in this study is related with the decrease in ATP levels in the mitochondria (Figure 3.7).

The mitochondrial permeability transition pore (MPTP) opens as a result of loss of membrane potential. The opening of MPTP allows cytochrome c, which is a part of the ETC to move out of the mitochondria and activate apoptotic factors. The dislocation of cytochrome c in cytosol acts a marker for cell death. Once in the cytoplasm of the cell, cytochrome c activates a family of proteases, caspases which carry out apoptosis (Gogvadge *et al*., 2006). Furthermore, antioxidant molecules can exit from the mitochondria resulting in an increase in reactive oxygen species (Kowaltowski *et al.,* 2001). This could be one of the possible reasons for increase in reactive oxygen species following MeHg and PQ exposures.

The genes PINK1 and PARKIN are associated with Parkinson's disease. In mitochondria with intact membrane potential, PINK1 and PARKIN are degraded in the mitochondrial membrane. The loss of potential results in the accumulation of PINK1 and PARKIN, which causes further damage to the mitochondria by activating various pathways (Koyano *et al.,* 2013). These activations ultimately can lead to neuronal death via apoptosis.

4.9 Proposed Mechanism of Neurotoxicity

High doses of PQ and MeHg inhibited complex I, resulting in an increase in superoxide anions. In addition, the toxins blocked manganese superoxide dismutase, thus causing further increase of superoxide anions. We proposed that MeHg and PQ would decrease oxygen consumption but no change in oxygen consumption was observed. Both PQ and MeHg decreased ATP content and lead to the loss of membrane potential in the mitochondria.

Overall, the findings are consistent with the proposed mechanism of MeHg and PQ toxicity in the mitochondria (Figure 4.9). The mechanism could be improved by examining calcium signaling and ATP synthase activity. The increase in oxidative stress, decline in ATP and loss of membrane potential will trigger the activation of factors such as caspases involved in apoptosis mediated by the mitochondria.

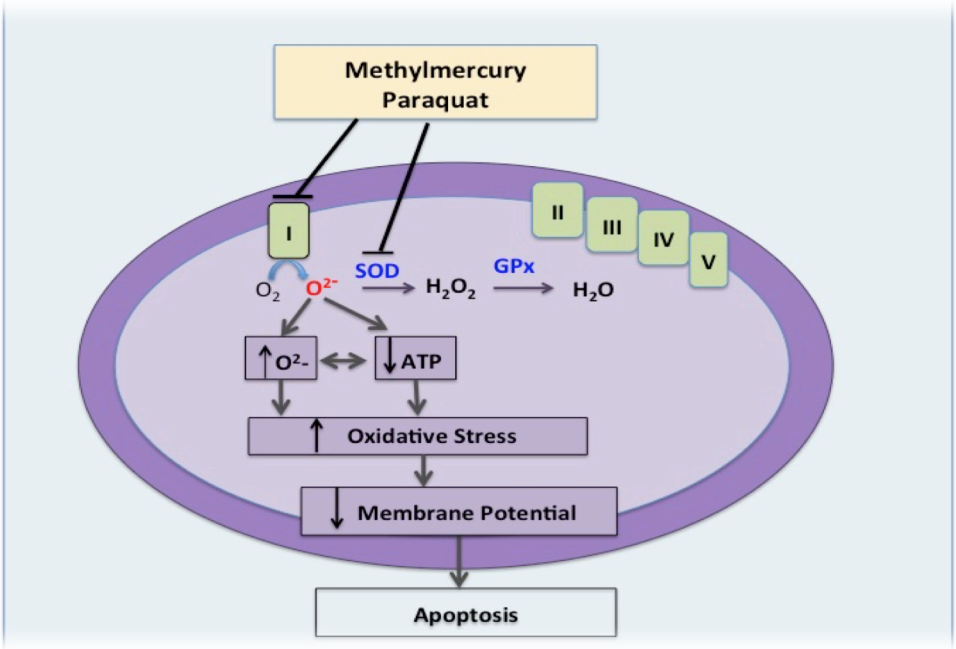


Figure 4.9. Mechanism of MeHg and PQ mediated toxicity in the mitochondria of dopamine neurons

5. Conclusion

This study aimed to investigate the effect of different doses of MeHg, PQ and co-exposure doses of MeHg and PQ on mitochondrial functions and properties in dopaminergic neurons. We found that high doses of MeHg and PQ impaired mitochondrial complex I functions. All environmental relevant doses of MeHg and PQ increased oxidative stress in the mitochondria by increasing superoxide anions and inhibiting superoxide dismutase. In addition, ATP concentration declined in neurons following MeHg and PQ exposure. There was a loss of membrane potential in the mitochondria by MeHg and PQ. There is an additive effect between low level PQ and MeHg co-exposure when analyzing ATP content, level of superoxide anions, inhibition of manganese superoxide dismutase and membrane potential. Our result suggests that both of these toxicants block other complexes of the electron transport chain, in addition to complex I.

5.1 Future Directions

Additional experiments need to be conducted to study the electron transport, especially complex IV and ATP synthase to explain the results found in this study. The decrease in ATP levels suggests an inhibition of ATP synthase by MeHg and PQ. ATP synthase in the mitochondria generates ATP by using energy from the electron gradient created by the four complexes (Hong and Pedersen, 2008). Complex IV of the ETC is the site of oxidative phosphorylation as it converts oxygen to water. The inhibition of complex IV can also decrease the membrane potential and activate apoptosis (Kalbacova

et al., 2003). Also, calcium concentration needs to be examined following PQ and MeHg exposure. Mitochondrial functions are also regulated by calcium since it accumulates inside the mitochondria. An increase in calcium inside the mitochondria can cause the mitochondrial transition pore to open resulting in bioenergetics failure (Contreras *et al.,* 2012). Furthermore, cytochrome c functions, opening of the mitochondrial transition pore, activation of the caspase signaling pathway need to be studied. The results from these experiments will provide information on mitochondria mediated apoptosis.

The effects observed in this study are relevant to human exposure concentrations of MeHg and PQ. This warrants further epidemiological studies examining the interaction between MeHg and PQ in the population.

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