THE USE OF PLATELET MONOAMINE OXIDASE-B AS A BIOMARKER FOR MERCURY NEUROTOXIC EFFECTS AMONG INUIT IN ARCTIC CANADA

by

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Abstract

Mercury (Hg) is a widespread neurotoxin, therefore it is important to identify a biomarker of effect in populations that could be at risk of Hg exposure. Platelet monoamine oxidase (MAO) could be a biomarker since animal studies indicate that brain and platelet MAO activity decreases as blood Hg increases. This study examined the relationship between blood Hg and platelet MAO activities among Inuit in the Canadian Arctic. This study found an increase in platelet MAO activities as blood Hg levels increased, which could be due to the high selenium (Se) in the population. This study then examined the relationship between Hg and MAO activities in an established cell line (SH-SY5Y) and found that Hg treated cells had an increase in MAO activities, but no changes occurred in MAO-A mRNA levels. Discrepancies between animal studies and these *in vitro* results could be due to differences between cell lines and whole organisms.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BMI: Body mass index

CAT: Catalase

Cd: Cadmium

CNS: Central nervous system

DA: Dopamine

GPx: Glutathione peroxidases

GSSG: Glutathione disulfide

Hg: Mercury

ICP-MS: Inductively coupled plasma mass spectrometry

LOAEL: Lowest observed adverse effect level

MAO: Monoamine oxidase

MeHg: Methyl mercury

Mn: Magnesium

NADPH: Nicotinamide adenine dinucleotide phosphate

NE: Norepinephrine

NOAEL: No observed adverse effect level

PCB: Polychlorinated biphenyl

PEA: Phenylethylamine

Pb: Lead

PBDE 47: 2,2',4,4'-tetrabromodiphenyl ether

pp-DDE: dichlorodiphenyldichloroethylene

ROS: Reactive oxygen species

Se: Selenium

TBARS: Thiobarbituric acid reactive substances

Thx: Thioredoxin

ThxR: Thioredoxin reductases

TYR: Tyramine

SOD: Superoxide dismutase

5-HT: Serotonin

H_2O_2: Hydrogen peroxide

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Co-Authorship Statement

Chapter two used data from the 2007 Inuit Health Survey, which was led by Dr. Grace Egeland from the Centre for Indigenous Peoples' Nutrition and Environment (CINE) at McGill University. As such chapter two is a collaborative work and details of my contribution are outlined below:

- Performing the research: I measured the monoamine oxidase-B activity in all platelet samples from the 2007 Inuit Health Survey at the University of Northern British Columbia. In 2008 I was one of five laboratory technicians onboard the Canadian Coast Guard Ship (CCGS) Amundsen where I isolated the blood components collected from the research participants and helped ship them to various labs and universities across Canada.
- Data analyses: I conducted all the statistical analysis for chapter two. I used data collected from the Inuit Health Survey interviews and contaminant data that had been analyzed at the Centre de Toxicologie du Quebec, which had been compiled into an extensive database at CINE.
- Manuscript preparation: I prepared the manuscript for chapter two, my supervisor and committee members were involved in the editing process.

Chapter three was a pilot study to examine the potential use of the SH-SY5Y cell line for examining MeHg effects on MAO-A and MAO-B expression. I performed all the laboratory work, data analysis and the manuscript under the supervision of Dr. Laurie Chan.

Chapter 1.

Introduction

Mercury (Hg) is a global contaminant that it is capable of long range transportation and bio-accumulates in aquatic food webs in the form of methylmercury (MeHg). MeHg is a potent neurotoxin that can cross the blood brain barrier and causes severe neurological impairment, particularly in the developing fetus. Fish consumption is the predominant route of MeHg exposure in humans and, as such, higher risk populations are those that rely on marine foods for food subsistence. Some of these populations include coastal communities in the Seychelles, Faroe Islands and New Zealand as well as the Inuit in the Canadian Arctic. There are currently many uncertainties regarding the acceptable level of MeHg exposure in humans due to contradictory findings in epidemiological studies, which could be due to several differences among study populations such as their respective diets. It is therefore important to identify early biochemical changes from Hg exposure that could serve as a reliable biomarker in populations that could be at risk of Hg neurotoxic effects prior to the onset of clinical outcomes.

Monoamine oxidase (MAO) is a flavin-enzyme that is responsible for the oxidation of biogenic and dietary amines and thereby initiates the deactivation pathway of catecholamine neurotransmitters, noradrenaline, adrenaline and dopamine. Animal dosing studies found a decrease in MAO activity in both brain and platelets of rats exposed to MeHg (Chakrabarti, Loua, Bai et al., 1998). An epidemiological study conducted on a fish eating community in the St. Pierre region of Quebec found a significant negative correlation between Hg blood levels and platelet MAO-B activity (Stamler, Abdelouahab, Vanier et al., 2006). These

findings suggest that platelet MAO-B could serve as a biomarker of Hg exposure in communities that could be at risk of Hg neurotoxicity.

The purpose of this thesis was to examine the relationship between Hg and MAO activity and to determine if platelet MAO-B activities could be a reliable biomarker of Hg exposure in higher risk communities. The thesis contains one manuscript examining the relationship between whole blood Hg and platelet MAO-B activities in coastal Inuit communities from Nunavut and Inuvialuit, Canada. The second manuscript is a laboratory dosing study examining the relationship between MeHg and MAO activities using a cell culture model.

Literature Review

Mercury and Selenium in the Canadian Arctic

The prevalence of mercury (Hg) in the Canadian Arctic has risen dramatically over the past fifty years. The major pathway by which Hg enters the Arctic is through atmospheric transportation (MacDonald, Harner, & Fyfe, 2005). Emissions from fossil fuel combustion are currently the primary source of global atmospheric Hg (Pacyna, Pacyna, Steenhuisen et al., 2006). In its elemental form 95% of Hg is in a gaseous state, enabling its entry into the atmosphere in a stable form which is capable of long range transportation and has an atmospheric resistance time of 6-24 months (Schroeder & Munthe, 1998). At higher latitudes it is converted to divalent mercury (HgII) or reactive elemental Hg and is deposited in terrestrial or aquatic environments in the form of particulate phase Hg (Kirk, 2006). When Hg enters the marine environment abiotic and biotic processes are involved in the biotransformation of inorganic Hg into organic Hg, including methylmercury (MeHg). The methylation of Hg can occur in both sediments and the water column and several factors

including, pH, temperature and amount of available organic matter contribute to methylation rates (U.S.Environmental Protection Agency, 2001, Loseto, Siciliano, & Lean, 2004).

The increasing prevalence of MeHg in the Canadian Arctic suggests that the health of Arctic wildlife and residents could be at risk. A study on Canadian beluga whale measured Hg in liver, kidney, muscle and muktuk and found that all livers and kidneys exceeded the Hg guideline levels of 0.5 μg/g (Lockhart, Stern, Wagemann et al., 2005). A review conducted on Canadian Arctic contaminants found that 40% of fish and 32% of mammal meat samples also exceeded the Health Canada consumption guideline levels (Chan & Receveur, 2000). This poses potential adverse health effects for Inuit communities that rely on a traditional diet for food subsistence and cultural heritage, since the primary pathway of Hg bioaccumulation is through the food chain (Poissant, Zhang, Canario et al., 2008). A recent study found that more than half of the Inuit women in Arctic Canada had elevated levels of Hg in their umbilical cord blood (Walker, Houseman, Seddon et al., 2006) indicating a need to monitor the health of Canadian Arctic residents for Hg toxicity.

Selenium (Se) is an essential nutrient that is thought to mitigate the toxic effects of Hg but can also be toxic at elevated levels. Natural sources of Se include soil, volcanoes and sea salt. Anthropogenic sources include combustion of coal, oil, nonferrous metal melting, agricultural products and mining (Wen & Carignan, 2007, Presser & Ohlendorf, 1987). Se levels are historically high in the traditional diet of Inuit residing in Greenland (Hansen JC, 1986; Hansen, Deutch, & Pedersen, 2004) and likely the Canadian Inuit, since they have a similar diet of marine animals. The distribution of Se throughout the Canadian Arctic is not homogeneous. Studies on Arctic char showed a significant increase in Se levels in Amituk

Lake between 1999-2003, but no consistent trend in Se levels was found in Resolute Lake (Muir, Wang, Bright et al., 2005).

The effects of Se and its interaction on Arctic wildlife are complex and remain to be elucidated. A study on Common Eiders in the Canadian Arctic found a negative relationship between corticosterone concentrations and Se levels, which could affect species stress response levels (Wayland, Smits, Gilchrist et al., 2003). In the Yukon hepatic Se concentrations in moose were higher when compared to moose from other locations, although no indications of Se toxicity was found (Gamberg, Palmer, & Roach, 2005).

Mercury-Toxicological Effects

MeHg produces toxic effects in both humans and other animals and can bioaccumulate in the food chain. MeHg is primarily absorbed in the gastrointestinal tract. It can penetrate the blood brain barrier, permanently damage the liver and kidneys and can enter cell membranes more readily than inorganic Hg. The developing fetus is particularly sensitive to the toxic effects of MeHg since MeHg can cross the placenta where the brain is the most sensitive target organ (U.S.Environmental Protection Agency, 2001). Information on the effects of MeHg exposure on human health was first acquired from accidental high-dose exposures. One incident occurred in the 1950s when an industrial plant had been discharging mercury directly into Minamata Bay, Japan. The most common clinical signs of MeHg toxicity in adults included paresthesia, ataxia, sensory disturbances, tremors, hearing impairment and difficulty walking (U.S.Environmental Protection Agency, 2001, Murata, Djean, & Dakeishi, 2007). Several children were born with congenital cerebral palsy and symptoms included primitive reflex, cerebellar ataxia, disturbances in physical development and nutrition, deformity of the limbs, hyperkinesias and hypersalivation (U.S.Environmental

Protection Agency, 2001). In 1995 a study examined clinical symptoms in Minamata residents where 69% of subjects demonstrated sensory disturbance, ataxia, speech impediment, hearing impairment, constriction of visual fields and tremors (Harada, Nakanishi, Konuma et al., 1998). The mechanisms of MeHg toxicity are not fully understood although its involvement in increasing oxidative stress has been implicated in its mode of toxicity (do Nascimento, Oliveira, Crespo-Lopez et al., 2008, Pinheiro, Macchi, Vieira et al., 2008). A recent study on mice fed MeHg found a reduction in glutathione peroxidases (GPx) and catalase (CAT) activity, which are antioxidant enzymes, as well as an increase in thiobarbituric acid reactive substances (TBARS) (de Freitas, Funck, Rotta et al., 2009).

Longitudinal studies have been conducted to examine the relationship between chronic low dose MeHg prenatal exposure and its neurobehavioural effects in fishing communities in the Faroe Islands, New Zealand and Seychelles. In both the Faroe Islands and New Zealand high Hg hair concentrations were correlated with reduced neurodevelopment performance including motor function, visual spatial ability and verbal memory (Grandjean *et al* 1997 and Crump *et al* 1998). In the Faroe Islands, where cord blood was also examined, the same relationship was also found between Hg cord blood concentrations and neurodevelopment (Grandjean *et al* 1999). No relationship between prenatal MeHg exposure and neurodevelopment was found in Seychelles (Davidson *et al* 1995).

The average range of Hg in whole blood in the U.S. is 1-8 μg/L (Agency for Toxic Substances and Disease Registry, 2008). The acceptable Hg blood levels set by Health Canada is 20 μg/L (Health Canada Mercury Issues Task Force, 2004). A study conducted in fish-eating communities in Canada found that residents in Innu First Nations communities in

Sheshatshiu, Labrador, had Hg hair concentration fourteen times lower than the expected value based on the MeHg concentrations in fish meat consumed (Canuel, de Grosbois, Atikesse et al., 2006). As such whole blood Hg concentrations could be a preferred measure of MeHg body burden.

Selenium-Toxicological Effects

Se exists in an elemental, organic and inorganic form and, once in the body, it is found in selenoproteins; nonspecific proteins containing Se; as well in inorganic forms such as elemental Se. Humans primarily acquire Se in food and water, which exists there as selenite, selenate and organic-Se (Yang, Chen, Gunn et al., 2008b). Se is an essential nutrient in humans, it has antioxidant effects since it is present in many antioxidant selenoenzymes including glutathione peroxidases (GPx) and thioredoxin reductases (ThxR) (Burk, Hill, & Motley, 2003). GPx activity is often measured in blood or platelets, since reduced GPx activity is an indicator of Se deficiency (Dumont, Vanhaecke, & Cornelis, 2006). At certain dosages Se might have anti-carcinogen effects and it has been suggested that Se supplements could be administered to cancer patients (Jackson & Combs, Jr., 2008, Holmgren, 2006, Rayman, 2005).

Se-deficiency occurs when consumption levels are below 0.1 μg/g a day (Dumont et al., 2006). Two Se-deficiency diseases have been identified: Keshan disease and Kashin-Beck disease. Keshan disease is a cardiovascular disease that causes multifocal necrosis of the myocardium causing cardiac enlargement, congestive heart failure, cardiogenic shock, and death (Barceloux, 1999). Mean whole blood levels of a population in China with low Se levels and Keshan disease was 21 μg/L (Yang, Wang, Zhou et al., 1983). Kashin-Beck

disease primarily affects the cartilage of young children impairing development and resulting in shortened height stature (Barceloux, 1999).

Cases of chronic Se toxicity, or selenosis, are rare and less is known about its mechanism of toxicity. Selenosis from long term chronic exposure primarily occurs through the diet. In humans symptoms of selenosis include a garlic odour of the breath, hair loss, brittle nails and neurological effects which include hyperflexia, peripheral paresthesia, anesthesia and hemiplegia. Long term exposure cases occurred from 1961-1964 in Hubei, China where residents ingested crops high in Se. Almost 50% of residents demonstrated signs of selenosis, where mean whole blood Se levels were 3200 μg/L (1300-7500) (Yang et al., 1983). The lowest observed adverse effect level (LOAEL), which was based on nail deformities, had whole blood Se at 1050 μg/L (Yang, Zhou, Yin et al., 1989). Recovering patients had a mean no observed adverse effect level (NOAEL) when whole blood Se was 968 μg/L (Yang & Zhou, 1994). The NOAEL corresponds to approximately 800 μg consumed/day and was divided by an uncertainty factor of two so that the tolerable upper intake level is 400 μg/day and corresponds to whole blood Se of 559 μg/L (Yang & Zhou, 1994; National Academy of Science, 2000). Another area of China high in Se but with no signs of selenosis had mean whole blood Se levels of 440 μg/L (350-580) (Yang et al., 1983).

More recently it has been suggested that long term Se supplementation may adversely affect glucose metabolism and increases the risk of type II diabetes (Stranges, Marshall, Natarajan et al., 2007, Bleys, Navas-Acien, & Guallar, 2007). An increase in type II diabetes was observed in the eastern U.S. when Se supplements were given at 200 μg/g per day in a low Se area (Stranges et al., 2007). Other documented cases of populations exposed to high Se levels include a population in Nawan Shahr India (Hira, Partal, & Dhillon, 2004), North

Dakota U.S.A (Longnecker, Taylor, Levander et al., 1991), Venezuela (Bratter, Debratter, Jaffe et al., 1991), Amazon (Lemire, Mergler, Huel et al., 2009), Greenland (Hansen et al., 2004) and an Inuit population in Nunavik (Muckle, Ayotte, Dewailly et al., 2001).

Selenium and Mercury Interactions

Se has long been known to exert a protective effect against Hg toxicity, due to the formation of an HgSe complex in a 1:1 molar ratio (Yang, Chen, Gunn et al., 2008a and Whanger, 2001) but causes Hg to accumulate in the brain (Whanger, 2001). Se is also essential in many antioxidant enzymes therefore it could mitigate MeHg toxicity by reducing oxidative stress (de Freitas et al., 2009). The toxicological effects of Hg could thus be dependent on both the relative and absolute amounts of Se and Hg in an organism. Epidemiological studies on fish-eating populations in the Faroe Islands reported adverse clinical outcomes from MeHg exposure (Grandjean, 1997) but no adverse outcomes in Seychelles was discovered at similar MeHg levels of exposure (Davidson, Myers, Cox et al., 1995). It has been suggested that differences between populations could be due to the availability of Se (Ralston, Blackwell, & Raymond, 2007).

Animal dosing studies have proposed using the Hg:Se molar ratio as a more accurate estimate of Hg toxicity (Ralston, Ralston, Blackwell, III et al., 2008; Yang, 2008). As the Hg:Se molar ratio approaches 1, the availability of Se is reduced and increases the concern that an inadequate supply of Se will be available for essential selenoproteins and antioxidant pathways. Some current research thus emphasizes the consumption of foods with a high Se:Hg molar ratio to reduce the potential effects of Hg toxicity (Ralston et al., 2007) .

Monoamine Oxidase as a Peripheral Biomarker for Hg Neurotoxicity

Neurotoxins cause subtle biological changes prior to the onset of nervous system impairment (Manzo, Castoldi, Coccini et al., 2001). Identifying reliable biomarkers of effects could aid in monitoring at-risk populations prior to the onset of clinical outcomes from MeHg exposure. The central nervous system (CNS) is inaccessible, to most clinical tests, therefore biomarkers of neurotoxicity include enzymes and neurotransmitter receptors that can be extracted from whole blood and serve as a surrogate measure of biochemical changes in the CNS (Manzo, Artigas, Martinez et al., 1996). One potential peripheral biomarker for Hg related neurotoxicity is platelet monoamine oxidase-B (MAO-B). Monoamine oxidase is a flavin-enzyme that is responsible for initiating the oxidative deamination of biogenic and dietary amines. There are two known isoenzymes, monoamine oxidase A (MAO-A) and MAO-B. MAO-A preferentially oxidizes serotonin (5-HT) and norepinephrine (NE), whereas MAO-B preferentially oxidizes phenylethylamine (PEA). Both dopamine (DA) and tyramine (TYR) are substrates for MAO-A and MAO-B. Platelets only contain the MAO-B isoform (Shih, 2004).

MeHg dosing studies examining MAO activity in the CNS and peripheral tissues, found a decrease in MAO activity in both brain and platelets of rats exposed to MeHg (Chakrabarti, Loua, Bai et al., 1998), suggesting that platelet MAO-B could serve as a potential biomarker for neurological health. The amino acid sequences of MAO-B from human platelets, liver and brain frontal cortex are identical (Chen, Wu, & Shih, 1993), further supporting the potential use of platelet MAO-B activity as a surrogate measure for neurological health. A study conducted on a fish eating community in the St. Pierre region of Quebec found a significant negative correlation between Hg blood levels and platelet MAO-B activity

(r=-0.193, p=0.029) (Stamler, Abdelouahab, Vanier et al., 2006), whereas no relationship was found among children in the Faroes Island at age 7 (Coccini, Manzo, Debes et al., 2009).

A recent study examined the relationship between inorganic and organic Se on brain MAO activity in rats and found a significant decrease in brain MAO-B activity for both inorganic and organic groups (Tang, Wang, & Lin, 2008). A study examining DA turnover rate in rat hippocampus when rats were fed a Se deficient diet, found a significant decrease in glutathione reductase and GPx activity and a significant increase in DA turnover rate, suggesting an increase in MAO activity when Se is limited (Castano, Ayala, Rodriguezgomez et al., 1995).

The significant effects of both Se and MeHg on brain MAO activity demonstrate that Se and MeHg can reduce MAO activity independently. Other possible confounding factors associated with reduced MAO-B activity include iron deficiency (Callender, Grahame-Smith, Woods et al., 1974), lead (Caspers & Menon, 1983), styrene (Coccini, Randine, Li et al., 1999), females having higher MAO activity than males (Coccini, Randine, Castoldi et al., 2005), alcohol consumption (Coccini, Castoldi, Gandini et al., 2002) and smoking (Fowler, Volkow, Wang et al., 1996).

Extensive research has been conducted on the relationship between MAO activity, smoking and alcohol which are crucial factors that should be considered when utilizing MAO-B activity as a surrogate measure for neurotoxicity. Human studies on smoking have found that a significant reduction in MAO-A (Fowler *et al* 1996a) and MAO-B (Fowler *et al* 1996b) activity in different brain regions exist between smokers and nonsmokers. Reduced platelet MAO-B activity in smokers has been repeatedly found and occurs in a dose dependent manner (see review Shih *et al* 1999, Snell 2002). Nicotine has not been found to

reduce MAO expression, but cyanide and formaldehyde may form adducts with the amino groups in the MAO protein (Boulton *et al* 1988) and could likely be the compounds in cigarette smoke compromising MAO expression. A study examining non-smokers, ex-smokers and current smokers supports the hypothesis that components of cigarette smoke irreversibly change platelet MAO-B substrate accessibility and normal MAO-B activity does not occur until new MAO-B proteins are generated (Snell *et al* 2002). Alcohol dependent subjects have been shown to have significantly lower platelet MAO-B activity than non-alcoholics (see Shih review 1999 and Bo *et al* 2006) and have significantly reduced MAO-B protein concentration (Snell *et al* 2002), suggesting that ethanol could interfere with MAO transcription or translation. A study examining human cell lines further suggests that ethanol interferes with MAO transcription since ethanol-treated cells caused changes in transcription factor binding to the MAO-B core promoter, although differences in transcription factor binding were found between cell types (Ekblom *et al* 1996). Therefore both cigarette smoke and alcohol contribute to reducing MAO-B platelet activity but through different stages of MAO gene expression.

Potential Mechanisms Reducing MAO Activities by Hg and Se

The exact pathways by which Se and MeHg inhibit or increase MAO activity remain to be elucidated. A previous study conducted on salmon parr found that fish fed MeHg at 5 mg/kg per day had a significant increase in superoxide dismutase (SOD) and GPx activity but no effect on MAO activity (Berntssen, Aatland, & Handy, 2003b). When the dose was 10 mg/kg per day there was a significant decrease in both SOD and GPx activity as well as MAO, suggesting the collapse of the antioxidant defense pathway and new effects on the MAO pathway (Berntssen et al., 2003b). In both dosages, clinical effects of neurotoxicity

were not observed (Berntssen, Aatland, & Handy, 2003a). It is likely that the reduction in MAO activity would be a defensive mechanism against the production of reactive oxygen species (ROS) since the antioxidant pathway had been compromised by MeHg. An epidemiological study on women residing in the Amazon found that women with elevated Hg hair levels had significantly higher glutathione and lower catalase activity, further demonstrating that Hg impairs antioxidant pathways (Pinheiro et al., 2008).

The inhibitory effects of Se on MAO is likely to occur via another pathway. Selenocysteine, selenite and other Se compounds react with thioredoxin (Thx) and thioredoxin reductases (ThxR), which results in NADPH oxidation and the formation of ROS (Bjornstedt, Kumar, & Holmgren, 1992a, Bjornstedt, Kumar, & Holmgren, 1992b; Whanger, 2001). Research on malignant mesothelioma cells demonstrated that the activity of ThxR is directly dependent on the concentration of selenite and elevated levels of Se increased the formation of ROS (Nilsonne, Sun, Nystrom et al., 2006). MAO activity is a significant contributor to ROS and the amount of H_2O_2 created by mitochondrial MAO has been shown to greatly exceed that formed during electron flow (Cohen & Kesler, 1999). It is likely that a reduction in MAO activity would also be an adaptive defense mechanism to the elevated levels of ROS created by the thioredoxin pathway in the presence of high Se.

The glutathione pathway involving GPx, a selenoenzyme, eliminates H_2O_2 created by MAO and forms glutathione disulfide (GSSG) (Cohen, Farooqui, & Kesler, 1997). Although this pathway removes the immediate threat from H_2O_2, GSSG reacts to thiol groups to form mixed disulfides that can suppress biological activity involving thiols (such as NADH dehydrogenase and ATPases) (Cohen et al., 1997). Therefore elevated GPx activity can also impair crucial cellular processes, suggesting that a reduction in the formation of

H_2O_2 through MAO activity rather than an increase in GPx activity would be a more optimal cellular response to increased ROS.

It has also been suggested that Hg might interfere with the MAO enzyme directly. Hg is known to interact with thiol groups and MAO-B has nine cysteine residues and seven of these residues are highly conserved (Binda, Newton-Vinson, Hubalek et al., 2002). Research conducted on bovine liver MAO found that MeHg and other sulfhydryl reagents inactivated MAO activity (Gomes, Kloepfer, Oi et al., 1976). Protection experiments with competitive inhibitors and kinetic studies, suggested that only two of the cysteine residues present in both MAO-A and MAO-B were required for normal activity (Gomes et al., 1976). The structure of MAO-A and MAO-B was later determined and no cysteine residues were found in the MAO catalytic site (Binda et al., 2002). There are four conserved amino acid residues lining the substrate cavity and they are required for MAO substrate specificities, since mutations in these amino acids result in altered substrate binding (Geha, Rebrin, Chen et al., 2001). In MAO-B, one of the amino acids lining the substrate cavity is cys-172 (Wu, Chen, & Shih, 1993), yet mutations in this cysteine does not alter MAO activity. Therefore there is little evidence demonstrating the direct inhibition of Hg on MAO activity.

Clinical Outcomes Associated with Altered MAO Activity

MAO plays a crucial role in neurological health since it catalyzes the deactivation pathway of catecholamine neurotransmitters, noradrenaline, adrenaline and DA. Therefore several clinical outcomes could result from a reduction in MAO-B activity. It has been demonstrated that platelet MAO-B activity corresponds to acquired colour vision loss (Stamler et al., 2006). Reduced platelet MAO-B activity has also been associated with schizophrenia, migraines and alcoholism (Sandler, Reveley, & Glover, 1981; Stamler et al.,

2006). Conversely elevated levels of MAO activity have been associated with Alzeimer's disease (AD), Parkinson's disease (PD) and depression and there has been renewed interest in designing selective MAO inhibitors for the treatment of neurodegenerative diseases (Cohen & Kesler, 1999; Youdim & Bakhle, 2006; Youdim, Edmondson, & Tipton, 2006).

MAO in SH-SY5Y human neuroblastoma cells

The neuroblastoma cell line, SH-SY5Y, has been used extensively to identify the effects of endogenous toxins and MAO inhibitors on oxidative stress and MAO activities, since an increase in brain MAO activity has been implicated in neurodegenerative diseases such as AD and PD (Youdim & Bakhle, 2006). Potential inhibitors include Ladostigil, which inhibits both MAO-A and MAO-B in SH-SY5Y cells (Bar-Am, Weinreb, Amit et al., 2009). The SH-SY5Y cell line has also been used to examine the toxicological effects of low dose chronic MeHg exposure on N-Methyl-D-Aspartate (NMDA) receptor binding, where MeHg was found to increase NMDA (Ndountse & Chan, 2008). The SH-SY5Y cell line has not been used to examine MeHg effects on MAO activities but could prove to be a potential model when examining neurotoxicity.

Increased MAO-A activity has also been induced in serum-deprived SH-SY5Y cells, since monoamine oxidation contributes to the production of ROS and is involved in the pre-apoptotic cell signaling pathway (Fitzgerald, Ufer, & Billett, 2007). In serum-deprived SH-SY5Y cells there is an increase in both MAO-A activities and MAO-A mRNA levels (Fitzgerald et al., 2007). When apoptosis is induced in SH-SY5Y by treatment with staurosporin (STS) there is an increase in MAO-A activities but no change in MAO-A mRNA transcript levels (Fitzgerald, Ufer, De Girolamo et al., 2007).

Chapter 2.

This chapter contains a manuscript describing the relationship between environmental contaminants including Hg, the dietary nutrient Selenium (Se) and platelet MAO-B activities from Inuit residing in coastal communities in Nunavut and Inuvialuit, Canada.

Relationship between Blood Selenium and Methylmercury and Platelet Monoamine Oxidase-B Activities Among Inuit in the Canadian Arctic

Alyssa Shaw, Grace Egeland, Hing Man Chan

Abstract

Mercury (Hg) has become increasingly more prevalent in the Canadian Arctic. Inuit who rely on traditional foods for food subsistence are at increased risk of Hg exposure. Our objective was to identify a peripheral biomarker for effects of Hg-related neurotoxicity. Concentrations of platelet monoamine oxidase-B (MAO-B) and their relationship with blood Hg and selenium (Se) were studied in 971 adult participants in the Inuit Health Survey conducted in 2007. An increase in whole blood Hg was associated with a significant increase in platelet MAO-B activities for females ($r=0.194$) and results from a forward multiple regression showed that Hg accounted for 8.5% of MAO-B activities in females and only 0.9% in males. No relationship was observed between MAO-B activity and Se concentration. The relationship between dietary Hg and Se and its effects on platelet MAO-B activity were examined using a Se:Hg molar ratio. Females from the third and fourth Se:Hg quartiles had significantly less MAO-B activity than females from the first and third quartiles. The variations of MAO-B may be affected by combinations of Se and Hg.

Introduction

Mercury is a widespread neurotoxin that has become increasingly more prevalent in the Canadian Arctic due to long range atmospheric and oceanic transportation of Hg from southern latitudes (Nguyen, Kim, Shon et al., 2009). Hg bio-accumulates in Arctic food webs primarily in the form of methylmercury (MeHg) and can reach high concentrations in meat and organs of marine mammals such as beluga whales and ringed seals (Lockhart, Stern, Wagemann et al., 2005; Riget, Muir, Kwan et al., 2005). As a result Inuit communities that rely on these marine mammal species as traditional foods for food subsistence are at increased risk of MeHg exposure. A study conducted on Canadian Arctic contaminants in Northern foods found that 40% of fish and 32% of mammal meat samples exceeded the Hg guideline levels set by Health Canada and found that the major source of Hg exposure among Inuit in the Canadian Arctic is through marine mammal and fish consumption (Chan & Receveur, 2000).

In 2004, Inuit residing in Nunavik, Canada had mean whole blood Hg levels that were higher than the general population in Quebec and 28% of individuals had Hg blood levels that exceeded the acceptable Hg blood concentration set by Health Canada (99.7 nmol/L) (Fontaine, Dewailly, Benedetti et al., 2008). A study conducted on Inuit women from the Northwest Territories and Nunavut between 1994-1999 found that 56% of umbilical cord samples had Hg levels exceeding the US EPA lower benchmark dose and Inuit participants had higher maternal Hg blood concentrations than any other ethnic group examined in Arctic Canada (Walker, Houseman, Seddon et al., 2006). In Nunavik, adverse clinical outcomes associated with chronic MeHg dietary exposure in preschool children included altered visual

information processing (Saint-Amour, Roy, Bastien et al., 2006) and an increase in tremor amplitude (Despres, Beuter, Richer et al., 2005).

Selenium (Se) is an essential nutrient but can be toxic at elevated levels. Se is naturally high in seafoods and Inuit from the Canadian Arctic have higher Se in their diet than in other populations (Kuhnlein, Receveur, Soueida et al., 2008). In 2004, Inuit from Nunavik had mean Se whole blood levels of 635.5 μg/L (Belanger 2007) which is higher than average levels in the US at 210 μg/L and the suggested safe Se whole blood level of 559 μg/L (Yang, Zhou, Yin et al., 1989b). Se has long been known to exert a protective effect against inorganic Hg toxicity, due to the formation of an Hg and Se complex in a 1:1 molar ratio, (Khan & Wang, 2009). However, Se has been reported to increase accumulation of Hg in the brain (Whanger, 2001) and the effects against MeHg is unproven (Beyrouty & Chan, 2006). Se is also essential in many antioxidant enzymes therefore it could mitigate MeHg toxicity by reducing oxidative stress (de Freitas, Funck, Rotta et al., 2009).

Neurotoxins cause subtle biological changes prior to the onset of serious nervous system damage (Manzo, Castoldi, Coccini et al., 2001). Identifying reliable biomarkers of effects could aid in monitoring at risk populations prior to the onset of clinical outcomes from MeHg exposure. The central nervous system (CNS) is inaccessible to clinical testing, therefore biomarkers of neurotoxicity include enzymes and neurotransmitter receptors that can be extracted from whole blood often serve as a surrogate measure of biochemical changes in the CNS (Manzo, Artigas, Martinez et al., 1996). One potential peripheral biomarker for Hg related neurotoxicity is platelet monoamine oxidase-B (MAO-B). Monoamine oxidase is a flavin-enzyme that is responsible for initiating the oxidative deamination of biogenic and dietary amines. There are two known isoenzymes, monoamine oxidase A (MAO-A) and

MAO-B. MAO-A preferentially oxidizes serotonin (5-HT) and norepinephrine (NE), whereas MAO-B preferentially oxidizes phenylethylamine (PEA). Both dopamine (DA) and tyramine (TYR) are substrates for MAO-A and MAO-B. Platelets only express the MAO-B isoform (Shih, 2004).

Studies have found a decrease in MAO-B activity in both brain and platelets of rats when fed MeHg (Chakrabarti, Loua, Bai et al., 1998a). Similarly, a study examining the relationship between inorganic and organic Se on brain MAO activity in rats found a decrease in brain MAO-B activity for both high inorganic and organic Se groups (Tang, Wang, & Lin, 2008). The amino acid sequence of MAO-B from human platelets and brain frontal cortex are identical (Chen, Wu, & Shih, 1993), further supporting the potential use of platelet MAO-B as a biomarker for neurotoxicity of Hg in human populations.

In a fish eating community from Quebec a negative correlation between Hg blood levels and MAO-B activity in adults was found (Stamler, Abdelouahab, Vanier et al., 2006e), whereas a study on children age 7 from a Hg exposed population in the Faroe Islands found no correlation between Hg and MAO-B activity (Coccini, Manzo, Debes et al., 2009a). The significant effects of both Se and MeHg on brain MAO activity in animal models suggest that Se and MeHg might reduce MAO activity independently. Different outcomes in MAO-B activity from epidemiological studies suggest that confounding factors in the population need to be taken into consideration, or that platelet MAO-B might not be a sensitive target for preclinical signs of Hg neurotoxicity (Coccini, Manzo, Debes et al., 2009b). Other possible confounding factors associated with reduced MAO-B activity include iron deficiency (Callender, Grahame-Smith, Woods et al., 1974), lead (Pb) (Caspers & Menon, 1983), styrene (Coccini, Randine, Li et al., 1999b), gender (female higher than male) (Coccini,

Randine, Castoldi et al., 2005), alcohol consumption (Coccini, Castoldi, Gandini et al., 2002a) and smoking (Fowler, Volkow, Wang et al., 1996).

This study explores the use of MAO-B as a biomarker for a population with potentially high Hg exposure by examining the relationship between blood MeHg and Se and platelet MAO-B activity among Inuit in Arctic Canada in the Kivalliq and Baffin communities in Nunavut, Canada. This study was a component of the adult 2007 Inuit Health Survey, which was a comprehensive health survey that took place in the Canadian Arctic with long term objectives of relating environmental factors to the health of the Inuit.

Methods

Participants from Inuit Health Survey

Participants were randomly selected to participate in the 2007 Inuit Health Survey which occurred in the Kivalliq and Baffin communities in Nunavut. All participants provided informed consent and ethics approval was granted by the ethical review committee of McGill University and the University of Northern British Columbia. The Inuit Health Survey visited 18 coastal communities from August 17, 2007 to September 18, 2007 which occurred on the Canadian Coast Guard Ship (CCGS) Amundsen. There were 1214 participants which represented 12% of the total population in the communities visited. There were 1178 blood samples that were collected for metals analysis, 1069 blood samples were collected for organochlorines and 1009 samples were collected for MAO activity analysis. There were 971 participants who had both MAO-B activity and all contaminants measured. Once onboard the CCGS Amundsen, participants answered in-person questionnaires. Data on age, gender, smoking levels and alcohol intake were acquired. Blood samples were collected and drawn into vacutainer tubes.

Platelet collection

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Platelets were collected as described in Stamler et al. (2004). Briefly, blood was collected in 10 mL vacutainer tubes containing EDTA-K_2 as anticoagulant. Blood was centrifuged at 4°C for 10 minutes at 200 x g and the supernatant containing platelet rich plasma was transferred to a 15 mL centrifuge tube. The platelet rich plasma was centrifuged at 4°C for 30 minutes at 3000 x g, supernatant was removed and the platelet pellet was suspended in Sodium/Potassium (Na/K) buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl from Fisher Scientific, pH 7.4). The platelet pellet was centrifuged for 30 minutes at 3000 X g, supernatant was removed and the pellet was suspended in 0.5 mL of Na/K buffer. Samples were transferred to cryovials and stored at 4°C.

MAO-B activity assays

Protein concentration was determined by the method developed by Bradford (Bradford, 1976). MAO-B activity was determined using a one step fluorometric method developed by Zhou and Panchuk-Voloshina (Zhou & Panchuk-Voloshina, 1997) and modified (Stamler, Basu, & Man, 2005a; Stamler, Basu, & Man, 2005b). Platelet samples were added to a 96-well plate in triplicates, each plate had Na/K buffer for a negative control, 10μM of H_20_2 as a positive control, and a resorufin standard curve ranging from 0.5 – 2.5 μM. The reaction was initiated in the dark with the addition of 100μL per well of the Amplex Red working solution, containing Amplex Red (20 mM, Invitrogen), horse radish peroxidase type IV (200 U/mL), tyramine (100 mM) and sodium potassium buffer. The plate was incubated in the dark for 30 minutes at room temperature. The fluorescence product was determined in a Thermo Scientific Varioskan Flash microplate fluorometer which was read

every 5 minutes for 30 minutes. Amount of resorufin product was converted to nanomoles of resorufin produced per minute per mg protein.

Metal and Organic Contaminant Analysis

Blood total-Hg concentrations were determined using cold-vapor atomic-absorption spectrometry. Selenium (Se), cadmium (Cd), manganese (Mn) and lead (Pb) blood concentrations were determined by using inductively coupled plasma-mass spectrometry (ICP-MS). All analyses were carried out at the Centre de Toxicologie du Quebec (Que.,Canada). The detection limit for total whole blood Hg was 0.1 μg/L, only one participant fell below the detection limit. That participant was assigned a value of 0.05 μg/L, which is half of the detection limit. Details of analysis and quality control have been reported elsewhere (Fontaine et al., 2008).

Statistical Analysis

Data transformations were conducted using STATA 10.0 (Texas, USA) and all statistical analyses were done with SPSS 16.0 (Illinois, USA). Descriptive statistics were conducted on contaminant and population variables, in the total, female and male population. Mann Whitney U tests were conducted comparing differences in non-transformed contaminant levels between females and males. Relationships between MAO-B activity, contaminants and population variables were assessed using Spearman correlations using non transformed data. MAO-B activity, Hg, Pb, Se, Mirex and serum ferritin were transformed using the Box-Cox transformation. To determine the effects of population variables on MAO-B activity one-way ANOVAs were conducted using Box-Cox transformed MAO-B activity.

A sequential multiple linear regression was conducted to determine the predictors of MAO-B activity. Variables included in the model were Hg, Pb, serum ferritin, Mirex, gender,

age, heavy smoking (y/n) and alcohol consumption, (none= no alcohol in past 12 months, moderate = alcohol within 7 days to 12 months, high= alcohol within 7 days of blood being drawn). Alcohol categories were based on results from (Coccini, Castoldi, Gandini et al., 2002b), which found that normal platelet MAO-B levels were reached in recovering alcoholics after eight days of not consuming alcohol. Separate sequential multiple linear regressions were also conducted for the male and female participants. Standardized residuals were plotted to test for normality, linearity and homoscedacity of residuals. A one-way ANOVA was conducted to compare Box-Cox transformed MAO-B activity and Bonferonni post hoc tests were conducted to compare Se:Hg quartiles.

Results

Population Statistics

There were 971 participants who had both MAO-B activity and contaminants measured. Of the 971 participants 791 completed questionnaires pertaining to alcohol consumption and smoking; 479 were female; and 312 were male. The average age in the sampled population was 41 (18-89). 73% of the participants that were smokers and 62.8% drank alcohol that year. Of the alcohol drinkers 64.6% had consumed alcohol within one week to a year from when blood samples were drawn, and 35.4% had consumed alcohol within seven days of having their blood drawn. There were 60 participants who had blood levels of Se indicative of selenosis (above 1054 μg/L) and 104 participants that had iron deficiency (below 12 μg/L).

Blood contaminant concentrations

No significant difference was found in Hg, Cd or Se levels between males and females (U=102802, U=108352, U=104151.5 respectively, $p>0.05$). Males had significantly higher levels of Pb and serum ferritin than females (U= 81563, U=67296.5 respectively, $p<0.001$). Males also had significantly higher levels of all organic contaminants than females, except PBDE47 where there was no significant difference. Organic contaminants that were compared included Mirex, PBDE 47, Cis-Nonachlor, Hexachor, Oxychlord, pp-DDE, B-HCH, Transnonachlor, Toxaphene-Parlor 26, Toxaphene-Parlor 50 and PCB congeners 1260, 105, 118, 138, 153, 156, 163, 170, 180, 183 and 187.

Table 1.1: Mean, median, standard deviation and ranges for MAO-B activity, Cd, Pb, Hg, Se, Mirex and serum ferritin in the total population (N=971).

	Total Population				
	Mean	Median	SD	Min	Max
MAO (nmoles/mg/min)	88.2	62.2	88.7	0.26	865
Cd (µg/L)	2.84	2.80	2.02	0.08	11.0
Pb (µg/L)	48.4	38.0	36.1	5.40	250
Hg (µg/L)	15.7	12.0	14.5	0.05	110
Se (µg/L)	476	370	331	85.0	2800
Mirex (µg/L)	0.14	0.06	0.23	0.01	2.00
Serum Ferritin (µg/L)	58.2	32.6	72.3	1.60	946

Table 1.2: Mean, median, standard deviation and ranges for MAO-B activity, Cd, Pb, Hg, Se, Mirex and serum ferritin in the male (N=378) and female (N=575) population.

	Males					Females				
	Mean	Median	SD	Min	Max	Mean	Median	SD	Min	Max
MAO(nmoles / mg/min)	75.6	56.4	73.5	0.5	585.7	96.6	70.9	96.6	0.3	865.4
Cd (µg/L)	2.9	2.9	2.1	0.1	11	2.8	2.8	2.0	0.1	11
Pb (µg/L)	55.0	45	35.3	8.8	210	44.5	33	36.3	5.4	250
Hg (µg/L)	16.9	12.5	15.9	0.4	110	14.9	12	13.5	0.05	94
Se (µg/L)	476.1	355	356.9	130	2800	477.2	380	315.2	85	2500
Mirex (µg/L)	0.2	0.04	0.27	0.01	2.00	0.11	0.04	0.18	0.01	1.80
Serum Ferritin (µg/L)	75.2	49.7	85.5	4.7	945.6	46.4	26.3	57.4	1.6	424.6

MAO-B Activity

MAO-B activity was significantly higher in females than males ($U=95264$, $p<0.01$). There was no effect of smoking and drinking on MAO-B activity. Comparisons among Box-Cox transformed MAO-B activity for non smokers, moderate smokers (1-14 cigarettes/day) and heavy smokers (over 15 cigarettes/day) were not significant in the total, male and female population (Fig 1.1). There was no significant difference between alcohol groups for Box-Cox transformed MAO-B activity (Fig 1.2).

Figure 1.1: Mean MAO-B activity for non smokers, moderate smokers (1-14 cigarettes/day) and heavy smokers (15 or more cigarettes/day), for the total population (N=791), females (N=479) and males (N=312). No significant difference in MAO activity was found between smoking groups. Error bars represent standard error.

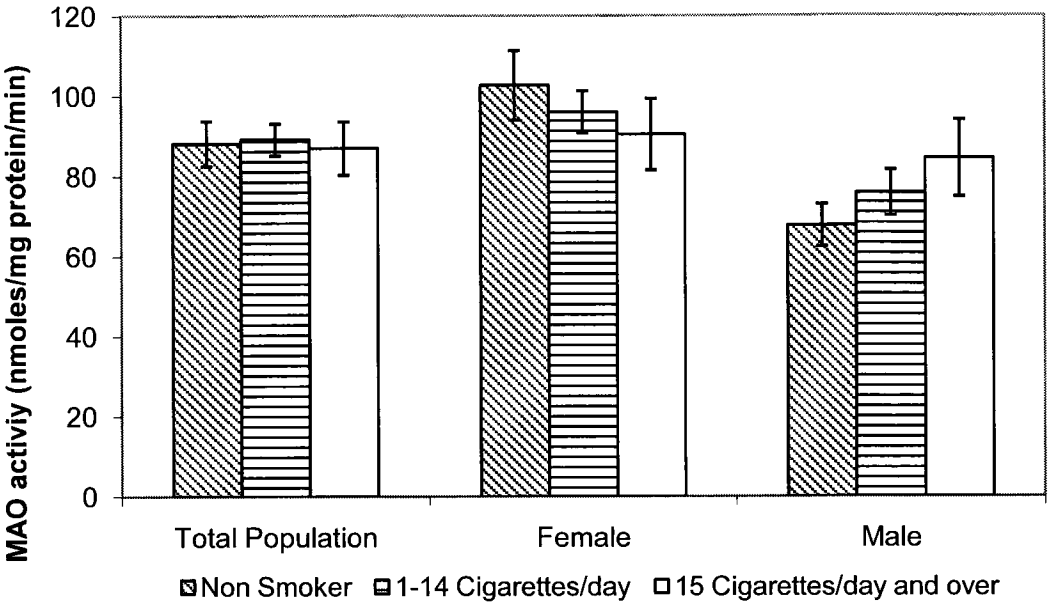
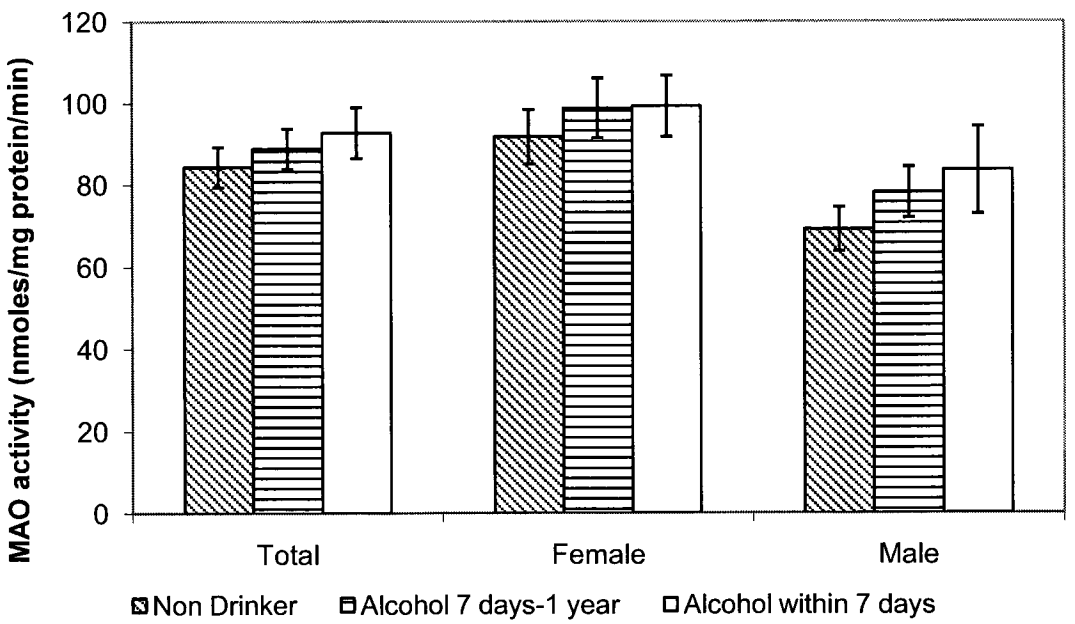


Figure 1.2: Mean MAO activity for non drinkers, participants that had consumed alcohol within 7 days - 1 year of the survey, participants that had consumed alcohol within 7 days of the survey for the total (N=791), female (N=479) and male population (N=312). No significant difference was found between groups. Error bars represent standard error.



Spearman correlations between MAO-B activity and contaminant levels demonstrated significant correlations between MAO-B activity and Pb, Hg, serum ferritin, mirex and PPDE 47 in the total population (Table 1.3). There was a positive correlation between MAO-B activity and Hg in the female population but no significance was found in the male population. Hg and Se were positively correlated ($r=0.850$, $p<0.001$, total population) and Cd and cigarettes smoked per day were positively correlated ($r=0.686$, $p<0.001$, total population). All organic contaminants were positively correlated, except for PPDE 47.

Table 1.3: Spearman correlation coefficients for MAO-B activity versus age, cigarettes smoked per day, BMI, serum ferritin as well as relevant contaminants; data was not transformed for the analysis. * = significant at p = 0.05, ** = significant at p = 0.001.

	Total population	**Males**	**Females**
Continuous Population Variables			
	r	r	r
Age	-0.036	-0.062	-0.016
Cigarettes/day	0.010	0.046	-0.004
BMI	-0.019	-0.055	-0.012
Serum ferritin	-0.087**	-0.162**	-0.010
Contaminants			
Cd	0.020	0.026	0.02
Pb	-0.096**	-0.084	-0.082*
Hg	0.111**	-0.018	0.194**
Se	0.053	-0.066	0.121**
Mirex	-0.116**	-0.126*	-0.076
PBDE 47	0.064*	0.031	0.078

The sequential multiple regression for the total population accounted for 6.9% of the variability in the MAO-B activity, Hg accounted for 4.4% of the variability and alcohol, gender, Pb and Miramex accounted for 0.5%, 0.7%, 0.6%, 0.5% of the variability respectively. The sequential multiple regression for the female population accounted for 10.3% of the variability in the MAO-B activity and was significant $F(6, 472) = 9.055$, $p<0.001$. There were two significant variables (Hg and alcohol), which accounted for 8.5% and 0.9% of the variability respectively. Both Hg and alcohol had a positive effect. The sequential multiple regression for the males accounted for 5.2% of the variability and was significant $F(6, 306)=2.78$, $p<0.05$. There were two significant variables serum ferritin and Hg, which accounted for 2.7% and 0.9% of the variability respectively. Serum ferritin had a negative effect and Hg had a positive effect. There was no significant relationship between MAO activity and blood Se.

To further examine the interactions between Se and Hg, molar ratio of Se:Hg was calculated and its relationship with MAO was investigated. Mean Se:Hg molar ratios were 138, 147 and 122 for the total population, females and males respectively. An ANOVA was conducted to determine differences in MAO-B Box-Cox transformed activity and Se:Hg quartiles followed by post hoc Bonferoni tests. One participant had an Se:Hg molar ratio of 8637 which was 80 times higher than the mean and was considered as an outliner and was not included in the analysis. Box-Cox transformed MAO-B activity was significantly higher in female participants from the first and second Se:Hg quartiles than those from the third ($p=0.044$ and $p=0.030$, respectively) and fourth quartile ($p=0.002$ and $p=0.001$ respectively) (see fig. 1.3). In males no significant difference was found between MAO-B activity and all Se:Hg molar ratios.

Figure 1.3: Mean MAO-B activity versus Se:Hg molar ratio for the female population. Error bars represent standard error. * = significant difference from first and second quartile.

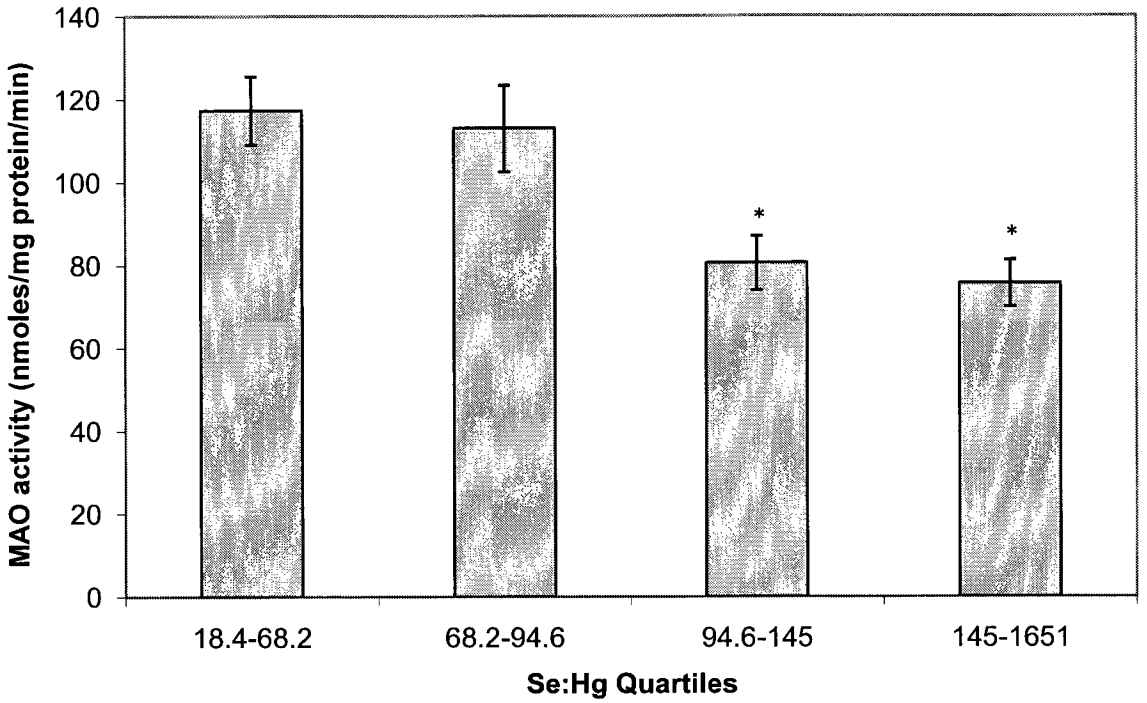


Table 1.4: Se:Hg ranges for quartiles, mean Se and Hg concentrations for quartiles and mean MAO activity for quartiles.

Quartile	Se:Hg	Mean Se (µg/L)	Mean Hg (µg/L)	Mean MAO activity (nmoles/mg protein/min)
1	18.4-68.2	562	27.8	117
2	68.2-94.7	539	17.2	113
3	94.6-145	476	10.7	80.6
4	145-1651	330	4.10	75.7

Discussion

The mean whole blood Hg concentrations in this population (15.68 μg/L) did not exceed the acceptable Hg blood concentration set by Health Canada 20 μg/L (Health Canada Mercury Issues Task Force, 2004) although 25.6% of the total population had Hg blood concentrations above that level. These levels were slightly higher than the 2004 mean whole blood Hg concentrations of Inuit communities residing in Nunavik (10.23 μg/L) (Fontaine et al., 2008) and were substantially higher than the levels in the St. Pierre Region of Quebec (2.4 μg/L) (Stamler, Abdelouahab, Vanier et al., 2006d) as well as women of child bearing age in the U.S. (1.02 μg/L) (McDowell, Dillon, Osterloh et al., 2004). Similar to the earlier report for whole blood Se among Inuit in Nunavik (635.5 μg/L) (Belanger, Dewailly, Berthiaume et al., 2006), the average whole blood Se level was high (475.95 μg/L). In comparison, the mean Se whole blood levels were 100-340 μg/L in the United States (Agency for Toxic Substances and Disease Registry, 2008), with a mean value of 210 μg/L (Barceloux, 1999). It is well known that there is a narrow window between required level and toxic dose for Se. In a seleniferous area in Hubei, China where residents ingested crops high in Se, the Lowest Observed Adverse Effect Level (LOAEL) was reported at at 1050 μg/L (Yang, Zhou, Yin et al., 1989a). Recovering patients of Se toxicity showed no observed adverse effect with whole blood Se at 968 μg/L (Yang & Zhou, 1994). The existing tolerable upper intake level established by the National Academy of Sciences in the U.S. at 400 μg/day was based on the No Observable Adversible Effect Level (NOAEL) of 800 μg/day and corresponds to 559 μg/L in blood as reported by Yan and Zhou. In this study 27.5% of the participants had Se concentrations above the NOAEL level and 60 participants exceeded 1000 μg/L.

Symptoms of selenium toxicity include a garlic odour of the breath, hair loss, brittle nails and neurological effects which include hyperflexia, peripheral paresthesia, anesthesia and hemiplegia (Agency for Toxic Substances and Disease Registry, 2008). Se levels are high in the marine environment and have historically been high among Inuit residing in Greenland (Hansen JC, 1986) and likely the Canadian Arctic since both populations consume similar traditional foods. In Greenland, muktuk or whale skin, is the primary source of Se and there is a decrease in whole blood Se as individuals consume less traditional foods (Hansen, Deutch, & Pedersen, 2004). In 1986 the range of whole blood Se among Inuit in Greenland was 803-3100 μg/L whereas in 2004 the range was 80-1890 μg/L (Hansen et al., 2004). No clinical signs of Se toxicity have been reported among Inuit in Greenland, but elevated Se levels have raised some health concerns (Hansen et al., 2004; AMAP, 2009). In this study the range of whole blood Se was 85-2800 μg/L and although no signs of Se toxicity have been reported among Inuit in Arctic Canada future studies should examine subtle signs of Se toxicity such as fingernails. Recent research has also suggested an increased risk of type II diabetes with elevated Se levels (Hansen et al., 2004; Stranges, Marshall, Natarajan et al., 2007). There has been an increasing prevalence of type II diabetes among Inuit in the Arctic (Bjerregaard, Young, Dewailly et al., 2004), therefore clinical outcomes including type II diabetes should also be examined.

The lack of reported signs of Se toxicity among Inuit could be due to the chemical form of Se found in their traditional diet. Data on the NOAEL for Se was derived during a drought from a seleniferous area in China where residents ingested plants high in Se and were primarily on a vegetarian diet. Plants and animal tissues vary in their chemical form of Se, where plants are higher in selenomethionine and animal tissues contain a wider variety of

forms including selenomethione, selenocysteine, selenotrisulfide and selenopersulfide (Young, Nahapetian, & Janghorbani, 1982); the toxicity of Se will vary based on the speciation of Se (Barceloux, 1999). The diet of the exposed population will also influence Se toxicity, where diets containing more protein and methionine reduces Se toxicity (Barceloux, 1999).

In contrast to previous studies showing a negative relationship between Hg and MAO activity in animal models (Beyrouty, Stamler, Liu et al., 2006; Chakrabarti, Loua, Bai et al., 1998b) as well as in epidemiological study (Stamler, Abdelouahab, Vanier et al., 2006c), we found a positive relationship among the female participants only. One possible factor influencing these results could be the elevated levels of Se in this population which is thought to mitigate the toxicological effects of Hg. Epidemiological studies on fish-eating populations in the Faroe Islands reported adverse clinical outcomes from MeHg exposure (Grandjean, 1997), but no adverse outcomes in the Seychelles was discovered at similar MeHg levels of exposure (Davidson, Myers, Cox et al., 1995). It has been suggested that differences between populations could be due to the availability of Se (Ralston, Blackwell, & Raymond, 2007). Se did not show any effect on MAO activity whereas Se:Hg ratio showed a negative relationship. The Se:Hg ratio has been used to infer the bioavailability of Se and in this case as the Se:Hg ratio increases there could possibly be more available Se which could also inhibit MAO activity. The range of Se is more tightly regulated than that of Hg (Table 1.4). Therefore, the influence of Se on MAO-B activities was more prominent when expressed as a Se:Hg ratio.

The effects of Hg and the Se:Hg molar ratio on MAO-B activity in males did not have a significant relationship. Several factors could account for the lack of significance in the male

population. The males had significantly higher whole blood Pb and organic contaminants than females, which have been shown to significantly reduce MAO-B activity (Caspers & Menon, 1983; Coccini, Randine, Li et al., 1999a). Males had significantly less MAO-B activity than females suggesting that MAO-B activity had already been reduced by other factors. Another factor could be one of sample size since there were fewer males in the total population.

The pathways by which elevated levels of Se and MeHg inhibit or increase MAO activity remain to be determined. High levels of dietary MeHg and Se are known to increase oxidative stress and the oxidative deamination of primary amines by MAO results in the formation of H_2O_2. MAO activity is thus a significant contributor to the formation of reactive oxygen species (ROS) and the amount of H_2O_2 created by mitochondrial MAO has been shown to greatly exceed that formed during electron flow (Cohen & Kesler, 1999). It is thus likely that the reduction in MAO activity after exposure to high levels of Se or Hg is a defensive mechanism against further production of ROS due to enhanced oxidative stress. The glutathione pathway involving GPx, a selenoenzyme, eliminates H_2O_2 created by MAO and forms glutathione disulfide (GSSG) (Cohen, Farooqui, & Kesler, 1997). Although this pathway removes the immediate threat from H_2O_2, GSSG reacts to thiol groups to form mixed disulfides that can suppress biological activity involving thiols (such as NADH dehydrogenase and ATPases) (Cohen et al., 1997). Therefore elevated GPx activity can also impair crucial cellular processes, suggesting that a reduction in the formation of H_2O_2 through MAO activity rather than an increase in GPx activity would be a more optimal cellular response to increased ROS.

MAO plays a crucial role in neurological health since it catalyzes the deactivation pathway of catecholamine neurotransmitters, noradrenaline, adrenaline and DA. Therefore several clinical outcomes could result from a reduction in MAO-B activity. It has been demonstrated that platelet MAO-B activity corresponds to acquired colour vision loss (Stamler, Abdelouahab, Vanier et al., 2006b). Reduced platelet MAO-B activity has also been associated with schizophrenia, migraines and alcoholism (Sandler, Reveley, & Glover, 1981; Stamler, Abdelouahab, Vanier et al., 2006a). Conversely elevated levels of MAO activity have been associated with Alzeimer's disease, Parkinson's disease and depression and there has been renewed interest in designing selective MAO inhibitors for the treatment of neurodegenerative diseases (Cohen & Kesler, 1999; Youdim & Bakhle, 2006; Youdim, Edmondson, & Tipton, 2006).

The purpose of this study was to identify whether MAO-B could serve as a potential biomarker for Hg related neurotoxicity. The conflicting observation from this study as well as the recently reported lack of relationship in the Faroe Island study among seven-year old children (Coccini, Manzo, Debes et al., 2009c), suggest that the relationship between Hg and platelet MAO-B activity is not consistent among different populations and is subject to multiple confounding factors. The confounding factors such as Se levels reported here need to be addressed further before MAO can be used as a meaningful biomarker for neurotoxicity in population studies. Other confounding factors that have been identified at decreasing MAO-B activity such as smoking, alcohol and other contaminants contributed to a small amount of variability in the multiple regressions, demonstrating that whole blood Hg could be a reliable predictor of MAO-B activity, particularly in the female population.

Connecting Statement

The previous chapter examined the association between platelet MAO-B activities and environmental contaminants including Hg in an epidemiological context. The next chapter will explore the cause-effect relationship between Hg exposure and MAO activity using a cell culture model. The hypothesis is that the results of the population study will be supported by laboratory-based experimental results.

Chapter 3.

This chapter includes a manuscript describing the results of an *in vitro* MeHg dosing experiment using an established cell culture model (SH-SY5Y). The study examines the relationship between MeHg and MAO activities and examines MeHg effects on MAO-A mRNA.

The activation of monoamine oxidase in methylmercury dosed SH-SY5Y cells

Alyssa Shaw and Hing Man Chan

Abstract

Monoamine oxidase (MAO) is an enzyme that is responsible for initiating the oxidative deamination of biogenic and dietary amines and is involved in maintaining a balance of neurotransmitters in the brain. Methylmercury (MeHg) is a widespread neurotoxin that decreases MAO activity in the brain and platelets of rats. This study examined the effects of chronic MeHg exposure on MAO activity in a neuroblastoma cell line (SH-SY5Y). I found that MAO-A activity was significantly higher in MeHg-treated cells and MAO-B activity was higher in MeHg-treated cells although, the latter was not statistically significant. Development of DNA primers for MAO-B using SH-SY5Y DNA yielded no results, suggesting that MAO-B might not be significantly expressed in this cell line. Quantification of MAO-A mRNA showed no difference from control indicating that changes in MAO-A occur during post transcriptional events.

Introduction

Monoamine oxidase (MAO) is a flavin-enzyme that is responsible for initiating the oxidative deamination of biogenic and dietary amines. There are two known isoenzymes, monoamine oxidase A (MAO-A) and monoamine oxidase B (MAO-B). MAO-A preferentially oxidizes

serotonin (5-HT) and norepinephrine (NE), whereas MAO-B preferentially oxidizes phenylethylamine (PEA). Both dopamine (DA) and tyramine (TYR) are substrates for MAO-A and MAO-B. Each isoform has distinct inhibitors; MAO-A is inhibited by clorygyline whereas MAO-B is inhibited by deprenyl. H_2O_2 and ammonia are byproducts of the oxidative deamination of amines by MAO, therefore MAO activity contributes to the formation of reactive oxygen species (ROS) and has been implicated in the apoptotic cell signaling pathway (Fitzgerald, Ufer, & Billett, 2007a; Fitzgerald, Ufer, De Girolamo et al., 2007b). Both isoforms of MAO are involved in maintaining a balance of neurotransmitters in the brain. Reduced platelet MAO-B activity has been associated with schizophrenia, migraine headaches and alcoholism (Sandler, Reveley, & Glover, 1981; Stamler, Abdelouahab, Vanier et al., 2006). Elevated MAO activity has been associated with Alzeimer's disease (AD), Parkinson's disease (PD) and depression (Cohen & Kesler, 1999; Youdim & Bakhle, 2006; Youdim, Edmondson, & Tipton, 2006).

Mercury (Hg) is a widespread neurotoxin, and previous studies have found that rats fed methylmercury (MeHg) have reduced platelet and brain MAO activities (Chakrabarti, Loua, Bai et al., 1998). An epidemiology study conducted in the St. Pierre Region of Quebec found that elevated levels of blood Hg resulted in a decrease in platelet MAO activity , therefore platelet MAO activity could serve as a potential biomarker of Hg exposure (Stamler et al., 2006). This study examined the effects of MeHg using a human neuroblastoma cell line (SH-SY5Y) to determine its effects in the absence of other confounding factors. The mRNA of MAO-A was also quantified to determine if changes in MAO expression occurred during transcriptional events. Changes in MAO activity were previously examined in a SH-SY5Y cells to determine its involvement in the pre-apoptotic cell signaling pathway

(Fitzgerald et al., 2007a; Fitzgerald et al., 2007b) and the use of MAO inhibitors as potential treatments for PD and AD (Bar-Am, Weinreb, Amit et al., 2009; Tazik, Johnson, Lu et al., 2009).

In the previous study investigating MAO-activities I found that Hg levels resulted in an increase in platelet MAO-B activities, which could likely be due to the high levels of Se in the study population. The following study was conducted to verify whether there is an *in vitro* cause-effect relationship between MAO-activities and Hg exposure. We also hypothesized that Hg induced the expression of MAO-A and B at the mRNA level.

Methods

Materials and Chemicals

Unless otherwise stated all chemicals were supplied by Sigma Aldrich (Oakville, ON, Canada). All cell culture media and supplements were obtained from Gibco Laboratories (Burlington, ON, Canada). The methylmercury (II) chloride was supplied by Alfa Aesar (Ward Hill, MA, U.S.A) and was of 95% + purity. The MTT and Amplex Red was supplied by Invitrogen (Burlington, ON, Canada). The Quick Start Bradford dye reagent and Quick Start bovine serum albumin (BSA) standards were supplied by Bio-Rad laboratories (Missisauga, ON, Canada). Sterile nonpyrogenic and polystyrene flasks were supplied by Corning and 96 well plates were supplied by Costar (Lowell, MA, U.S.A).

Cell Lines

SH-SY5Y cells were obtained from American Type Culture Collection (Manassas, VA, U.S.A). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4,500 mg/L L-glutamine, 1% antibiotic solution (10,000 U/mL penicillin, 10,000 ug/mL streptomycin) and 10% Fetal Bovine Serum (FBS). Cells were grown at 37° C with

5% CO_2. Cell culture medium was changed every three days until the cells were confluent. MTT cytotoxicity assays were conducted at 0-0.5 μM MeHg for 48 hours (Fig. 2.1). For the experiment, cells were seeded 24 hours before exposure to 0.1 μM of MeHg. Both treatment and control cells were in media lacking FBS during the 48 hours of MeHg exposure. After 48 hours of exposure medium was removed, cells were washed in PBS and detached by trypsinization. Cells were re-suspended in complete medium containing 5% DMSO, counted and slowly frozen at -80° C and stored in liquid nitrogen for future use. The experiment was conducted three times.

MAO Enzyme Activity Assay

Cells were thawed on ice and separated from the medium by centrifugation. Cells were washed and suspended in sodium phosphate buffer (50 mM, pH=7.4) sonicated, centrifuged and supernatant removed for analysis. Protein concentration was determined by the method developed by Bradford (Bradford, 1976). MAO-B activity was determined using a one step fluorometric method developed by Zhou and Panchuk-Voloshina (Zhou & Panchuk-Voloshina, 1997) and modified (Stamler, Basu, & Man, 2005; Stamler et al., 2005). Cell samples were added to a 96 well plate in triplicates, each plate had Na/K buffer for a negative control, 10μM of H_20_2 as a positive control, and a resorufin standard curve ranging from 0.5 – 2.5 μM. The MAO-A inhibitor clorygyline was incubated for 30 minutes at room temperature with the appropriate samples at 10 μM. The reaction was initiated in the dark with the addition of 100μL per well of the Amplex Red working solution, containing Amplex Red (20 mM), horse radish peroxidase type IV (200 U/mL), tyramine (100 mM) and sodium phosphate buffer (50mM). The plate was incubated in the dark for 30 minutes at room temperature. The fluorescence product was determined in a Thermo Scientific Varioskan

Flash microplate fluorometer which was read every 5 minutes for 30 minutes. Amount of resorufin product was converted to nanomoles of resorufin produced per minute per mg protein.

Quantitative Real Time PCR

RNA was extracted using the Qiagen RNA extraction kit (Missisauga, ON, Canada). RNA integrity was examined using the Bio-Rad Experion and RNA quantification was conducted using Nanodrop (Wilmington, DE, U.S.A). RNA was reverse transcribed into corresponding cDNA using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Streetsville, ON, Canada). Quantitative Real Time PCR (qRT-PCR) was conducted using the Applied Biosystems Relative Standard Curve Method for Quantification using the Taqman Universal PCR Master Mix No AMP Erase UNG. The following MAO-A primers were used: 5'-GCCAGGAACGGAAGTTTGTAGG-3' and 5'-AATGTTCATGGTTCAGCGTCTC-3' as described in (Nadia A et al, Personal Communications). Taqman dual labeled probe was designed using Beacon Design 7.0 (Palo Alto, CA, U.S.A): 5'-FAM-TTGACCTCCTCGGAGACCAAGTG-TAM-3'. Primers and probes were made by Integrated DNA Technologies (San Diego, CA, U.S.A). Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as an endogenous control and pre-designed primers and probe for GAPDH were from Applied Biosystems. The following PCR protocol was applied using the 7300 Applied Biosystems real time PCR instrument: 10 minutes of exposure at 95°C to initiate AmpliTaq Gold DNA Polymerase followed by 40 cycles of 15 seconds at 95°C for denaturation and 1 minute at 60°C annealing and elongation. For relative quantification standard curves were generated for both MAO-A and GAPDH using stock cDNA from SH-SY5Y. The control cells were used as the calibrator

and the relative amounts of mRNA in treated cells was determined in relation to the calibrator. All samples had an RNA template as well as a no template control during the PCR reaction, to verify that there was no DNA carryover in extracted RNA and no DNA contamination in the PCR reagents. All samples were run in duplicate and results are the average of three separate experiments. PCR products were visualized in a 1% agarose gel that was run in 0.5X TBE buffer (44.5 mM Tris base, 2.75 mM Boric Acid and 1 mM EDTA, pH 8.3) in the presence of ethidium bromide (0.5 μg/mL).

A number of primer pairs were developed for MAO-B using Primer Express V. 2.0. Applied Biosystems. These primer pairs included: 5'-CTTTTGTGGGCCAGGAAGC-3' and 5'-CAATCCAATCAGCCTGAGCAG-3', 5'-TGAATGAGGTTGAGCGTCTG-3' and 5'-GAACCCAGAACCTTGGCATA-3', 5'-TCTCGGTGCCTGCAAAGTAAA-3' and 5'-CAAAGCAGCTTGCCACTCTCT-3'. These primer pairs did not result in the MAO-B product. A panel PCR was conducted with a varying range of temperature and magnesium in attempts to target MAO-B, but the desired product was not detected after manipulating these variables.

Results

In all three experiments MAO-A and MAO-B activities were visibly higher in the cells treated with 0.1 μM MeHg. The MAO-A activity was significantly higher using a student T test ($p=0.006$) while the MAO-B activity was not ($p= 0.11$) Fig. 2.2. qRT-PCR was conducted to determine if MAO-A mRNA expression differed between control and treated cells and results demonstrated that the mRNA expression did not change between treatments. The MAO-A mRNA from the treatment group normalized to GAPDH was 0.76 relative to the calibrator, suggesting no difference between treatment and control groups.

Figure 2.1: MTT after 48 hours of exposure to MeHg (0-0.5 μM). Error bars represent standard error.

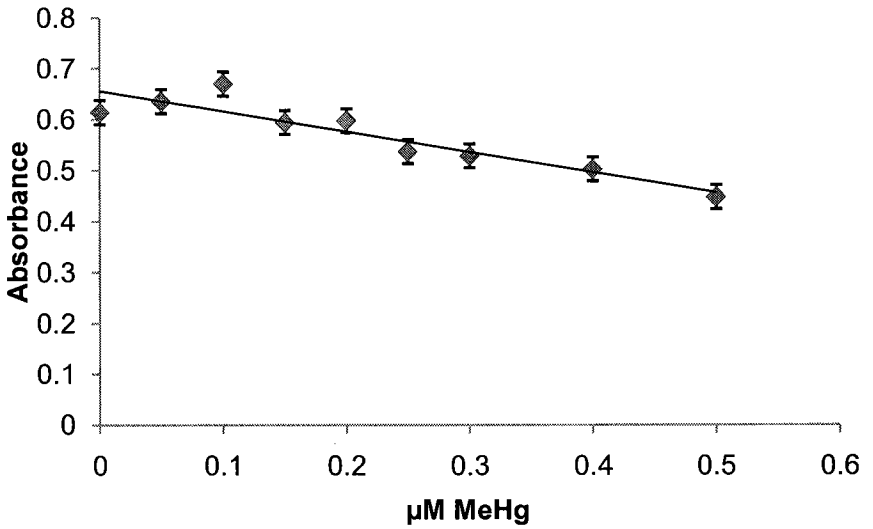


Figure 2.2: Total MAO, MAO-B and MAO-A activity in SH-SY5Y cells after 48 hours of exposure to MeHg and untreated cells. Error bars represent standard error and was an average from three independent experiments. * = significant at p=0.05.

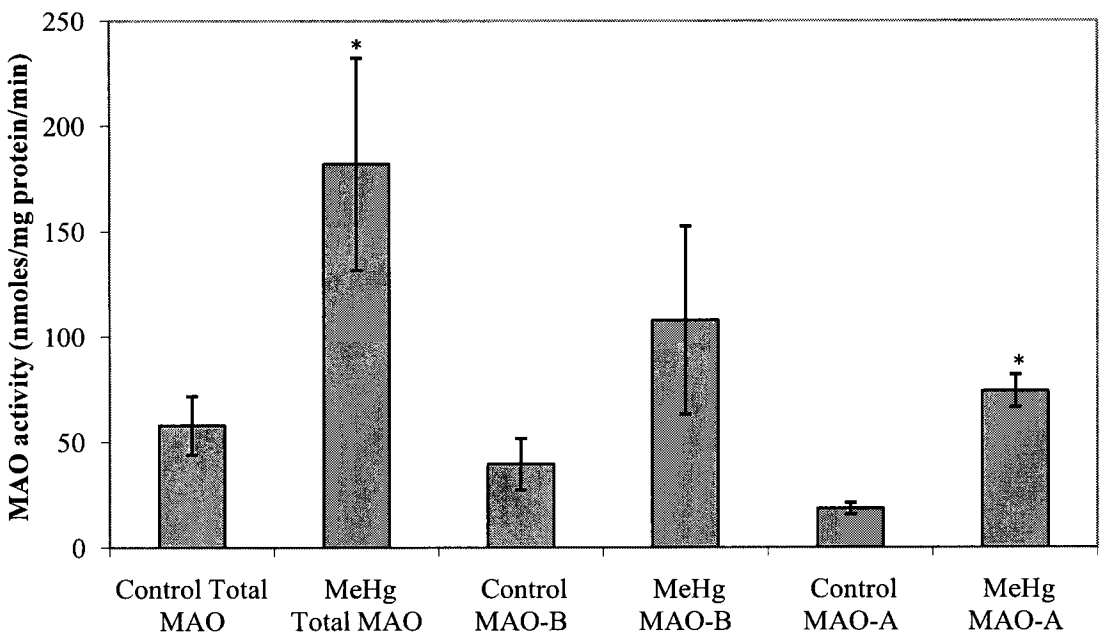
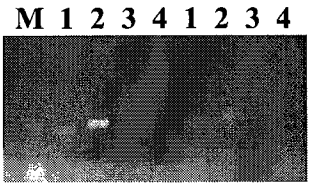


Figure 2.3 qRT-PCR agarose gel showing treatment and control results, 1: MAO-A gene expression, amplicon size: 150 bp, 2: GAPDH gene expression, amplicon size: , 3: RNA template, 4: No template control, M: DNA 100 bp ladder.



Discussion

This study found that MeHg exposed SH-SY5Y cells had an increase in MAO activity. These results are contrary to previous *in vivo* studies where MeHg fed rats had a decrease in brain MAO activity (Chakrabarti et al., 1998; Beyrouty, Stamler, Liu et al., 2006). These differences could be due to several possible factors. *In vitro* studies using the SH-SY5Y cell line have demonstrated that MAO-A activity increases during apoptosis (Fitzgerald et al., 2007a; Fitzgerald et al., 2007b), therefore the MeHg exposed cells might have induced the apoptosis cell signaling pathway, so that an increase in MAO-A activity was observed. Another possibility is that there are fundamental differences between cell lines and whole organisms therefore SH-SY5Y cells might not prove to be a suitable model when examining the effects of contaminants on MAO activity. Both MAO activity and MeHg induce oxidative stress, therefore the decrease in MAO activity after MeHg exposure could be a defensive mechanism against the formation of ROS. Cell line models might not reflect this defensive mechanism.

MAO-A is the predominant isoform in SH-SY5Y cells and previous studies have suggested that the MAO-B isoform is not present in SH-SY5Y cells (Yi, Akao, Maruyama et

al., 2006; Fitzgerald et al., 2007b) whereas others have suggested that both isoforms are present (Song & Ehrich, 1998). This study found that at 10 μM of clorygyline MAO activity was detected, so that 68% and 59% of MAO activity remained in the control and treated cells respectively. To examine potential MAO-B expression at the mRNA level, multiple primer pairs were designed targeting MAO-B in SH-SY5Y extracted DNA, but the desired MAO-B product was not detected. A previous study suggested that full MAO-A inhibition occurred at 1 μM (Yi et al., 2006) whereas other studies have suggested that full MAO-A inhibition occurs at 0.01 μM (Fitzgerald et al., 2007b). This study found that MAO activity still occurred when inhibitor levels were at 10 μM.

The MAO-A mRNA was quantified and there was no difference between treated and control cells, indicating that changes in MAO-A activity were due to post transcriptional events. The same observation occurred in staurosporine (STS) treated SH-SY5Y cells, where an increase in MAO-A activity occurred without a change in MAO-A mRNA (Fitzgerald et al., 2007b). Similarly, rats fed aluminum had an increase in brain MAO activity, but no changes in MAO expression was found in Western blots, suggesting changes in MAO activity after translational events (Huh, Choi, Lee et al., 2005). These findings differ from a serum withdrawal study where MAO-A mRNA expression and enzyme activity was increased in SH-SY5Y (Fitzgerald et al., 2007a). Results from this study indicate that the effects of MeHg on MAO-A activity is regulated after transcription.

These findings suggest that the SH-SY5Y cell line might not be a suitable model when examining the effects of environmental contaminants on MAO activities. Both isoforms are not present in equal amounts and their activities after MeHg treatment do not reflect those from animal models. Future studies should examine MAO activities using

animal models since they are whole organisms that might adequately reflect an organism's defensive pathways once exposed to MeHg.

Chapter 4

Conclusion

MeHg is a widespread neurotoxin and as such it is important to identify reliable pre-clinical biochemical changes in populations that could be at risk of Hg exposure. Previous studies have found that platelet MAO-B activities decrease with increasing levels of Hg exposure, whereas this study found that platelet MAO-B activities increased with increasing levels of blood Hg. I suggest that this relationship could likely be due to the elevated levels of Se in the population. Se is known to mitigate the toxicological effects of Hg and was highly correlated with blood Hg concentrations. Individuals with elevated Hg had a tendency to have higher Se levels, which could potentially be protecting individuals from the toxicological effect of Hg. A Se:Hg molar ratio was used to examine the effects of high biologically available Se on MAO-B activities and a decrease in MAO-B activity was found.

I examined the relationship between MeHg exposure and MAO-A and MAO-B activities in a human neuroblastoma (SH-SY5Y) cell line, in the absence of potential confounding factors such as Se. I found an increase in both MAO-A and MAO-B activities after MeHg exposure, but no changes in MAO-A mRNA expression was found. These results are contrary to *in vivo* animal models and suggest that cell lines might not be an adequate model for examining MeHg effects since they are fundamentally different from whole organisms. Future work should consider using primary cell culture rather than an established cell line to determine the effects of MeHg on MAO activities and possibly its interaction with other relevant compounds such as Se.

Identifying a reliable biomarker of Hg-related neurotoxicity would aid in monitoring populations that could be at risk of Hg exposure. Previous studies have found that platelet MAO-B could be a potential peripheral biomarker. Results from the epidemiological component of this study suggest that more research is required in order to determine how other potentially confounding factors affect platelet MAO-B. Future research should thus examine the individual and combined effects of Se and Hg on MAO activities, since both Se and Hg are generally high in seafood and are likely to co-occur in at-risk populations. To do this, researchers should also examine the predominant chemical forms that Se occurs in the study population since different chemical forms have varying toxicological effects and interactions with Hg.

It is also important to identify how MAO-A and MAO-B rates of monoamine catalysis are affected mechanistically by the presence of Hg. One aspect of these changes can be determined by examining which levels of MAO-A and MAO-B gene expression are affected by Hg exposure. I found that in a MeHg exposed cell line MAO-A mRNA levels were unaffected, indicating that changes in MAO expression might occur as a post-transcriptional event. Future research should thus examine MeHg effects at the post transcription level. Monoamine oxidation is a major contributor to the production of ROS therefore it is also necessary to examine how the activities of antioxidant enzymes are affected by changes in MAO activities from Hg exposure and to measure indicators of oxidative stress such as TBARS. Potential enzymes to be examined could include crucial selenoenzymes such as GPx and ThxR. These biochemical changes are likely to occur prior to the onset of clinical symptoms of Hg related neurotoxicity.

This interdisciplinary thesis examines the effects of a widespread contaminant on an enzyme that plays a crucial role in neurological health. A range of research methodologies was applied so that the effects of Hg on MAO-B activities could be examined in both a susceptible human population as well as a laboratory model. The advantage of using both research models is that the shortcomings from one methodology can be overcome by another. Epidemiologists in environmental health are faced with the challenge of identifying cause effect relationships in a world with multiple contaminants where independent, interactions and synergistic effects are likely to occur. The laboratory model provides the researcher with the capacity to control for extraneous factors but can lack real world applications. I used both approaches to satisfy our research objectives and found that bridging the gap between epidemiology and laboratory research provided deeper insight into current and potentially future research in Hg-related biomarker studies.

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Appendix: SH-SY5Y Neuroblastoma Product Description

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's Material Transfer Agreement or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, Hong Kong, India, Japan, Korea, New Zealand, Singapore and Taiwan, R.O.C. must contact a local distributor for pricing information and to place an order for ATCC cultures and products.

Cell Lines

ATCC® Number: CRL-2266 ™ CRL-2266 203.0

cellBiology~Homc Order this item

Price: **$203.00**

Designations:	SH-SY5Y	Depositors:	JL Biedler
Biosafety Level: 1		Shipped:	frozen
Medium & Serum:	See Propagation	Growth Properties:	mixed, adherent and suspension
Organism:	*Homo sapiens* (human)	Morphology:	epithelial
Source:	**Organ:** brain **Disease:** neuroblastoma **Derived from metastatic site:** bone marrow		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

	Related Cell Culture Products
Restrictions:	NOTE: SH-SY5Y was deposited at the ATCC by June L. Biedler, Memorial Sloan-Kettering Cancer Center. SH-SY5Y is distributed for academic research purposes only. Memorial Sloan-Kettering releases the line subject to the following: 1.) SH-SY5Y or its products must not be distributed to third parties. Commercial interests are the exclusive property of Memorial Sloan-Kettering Cancer Center. 2.) Any proposed commercial use of SH-SY5Y including any use by a for-profit entity must first be negotiated with Director, Office of Industrial Affairs, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; phone (212) 639-6181; FAX (212) 717-3439.
Isolation:	**Isolation date:** 1970 (SH-SY5Y is a thrice cloned (SK-N-SH -> SH-SY -> SH-SY5 -> SH-SY5Y) subline of the neuroblastoma cell line SK-N-SH (see ATCC HTB-11) which was established in 1970 from a metastatic bone tumor.) [23032]
Antigen Expression:	Blood Type A; Rh+
DNA Profile (STR):	Amelogenin: X CSF1PO: 11 D13S317: 11 D16S539: 8,13 D5S818: 12 D7S820: 7,10 THO1: 7,10 TPOX: 8,11 vWA: 14,18
Cytogenetic Analysis:	modal number = 47; the cells possess a unique marker comprised of a chromosome 1 with a complex insertion of an additional copy of a 1q segment into the long arm, resulting in trisomy of 1q
Age:	4 years
Gender:	female
Comments:	SH-SY5Y cells have a reported saturation density greater than 1 X 10(6) cells/sq cm. They are reported to exhibit moderate levels of dopamine beta hydroxylase activity [PubMed ID: 29704].
Propagation:	**ATCC complete growth medium:** A 1:1 mixture of Eagle's Minimum Essential Medium (EMEM, ATCC 30-2003, see below) and Ham's F12 Medium (F12K, ATCC 30-2004, see below), 90%; fetal bovine serum, 10% The EMEM formulation is modified to contain 1.5 g/L sodium

	bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM nonessential amino acids. The Ham's F12K formulation is modified to contain 1.5 g/L sodium bicarbonate and 2 mM L-glutamine. **Temperature:** 37.0C **Atmosphere:** air, 95%; carbon dioxide (CO2), 5%
Subculturing:	**Protocol:** These cells grow as a mixture of floating and adherent cells. The cells grow as clusters of neuroblastic cells with multiple, short, fine cell processes (neurites). Cells will aggregate, form clumps and float. Remove the medium with the floating cells, and recover the cells by centrifugation. Rinse the adherent cells with fresh 0.25% trypsin, 0.53 mM EDTA solution, add an additional 1 to 2 ml of trypsin solution, and let the culture sit at room temperature (or at 37C) until the cells detach. Add fresh medium, aspirate, combine with the floating cells recovered above and dispense into new flasks. **Subcultivation ratio:** A subcultivation ratio of 1:20 to 1:50 is recommended **Medium renewal:** Every 4 to 7 days
Preservation:	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Doubling Time:	48 hrs
Related Products:	recommended serum: ATCC 30-2020 parental cell line: ATCC HTB-11
References:	22554: Ross RA , et al. Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J. Natl. Cancer Inst. 71: 741-749, 1983. PubMed: 6137586 23032: Biedler JL , et al. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res. 38: 3751-3757, 1978. PubMed: 29704