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The Effects of MeHg on ROS Regulation in Neutrophil Cell Culture

By

Michael P. Rist

A Study

Presented to the Faculty

of

Wheaton College

In Partial Fulfillment of the Requirements

for

Graduation with Departmental Honors

in Biochemistry

Norton, Massachusetts

May 12, 2014

Acknowledgements

I would like to thank all of my professors at Wheaton College for providing me with an excellent education that allowed for me to develop and complete this project. I would especially like to thank Professor Jani Benoit for supporting me and allowing me to do this project. I have learned so much about independent research and science as a whole. I would also like to Professor Lanni and Professor Buthelezi for serving on my thesis committee. I would like to thank Professor Morris for providing me with chick embryo brain tissue and for using his lab space. I would also like to acknowledge my friends for my best year at Wheaton. Finally I would like to thank my entire family for supporting me in my academic endeavors. They have gone through so much and have helped me become who I am today.

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Abstract

Methylmercury (MeHg) is an environmental toxin that causes targets the nervous system but can cause an array of effects on other systems as well. The main source of exposure is through fish consumption, because MeHg bioaccumulates in fresh water and marine food chains. MeHg has a high affinity for selenium and sulfur in the body, which assists in its solubility as well as its toxicity. MeHg has been shown to inhibited redox regulatory enzymes such as glutathione peroxidase (GPx). Inhibition of this enzyme leads to toxic levels of reactive oxygen species (ROS) which can damage DNA, proteins and lipids inside of the cell. Neutrophils, cells of the innate immune response, utilize these ROS to degrade pathogens. These cells utilize GPx to regulate ROS from causing cell damage. Protocols, assays and analytical methods were developed and modified to measure GPx activity and amount of lipid peroxidation within cell cultures. Embryonic chick brain cells were used to develop and modify methods. These cells showed decreases in GPx activity but not lipid oxidation. The neutrophil cell culture did not produce enough signals to measure either suggesting a possible interference within the neutrophil matrix. The data from this study suggests that cell culture can be used to analyze direct exposure of MeHg but the methods might need to be altered for every different cell that is being tested.

Chapter 1: Introduction

1.1 Mecury as an Environmental Pollutant

Mercury is a metal that can be found in a variety of chemical forms which can be separated into two main categories. One category is inorganic mercury, which includes elemental mercury (Hg^0), mercurous ($Hg\text{-}Hg^{++}$) cation and mercuric (Hg^{++}) cation. Organic mercury is often derived from mercuric cations that have bound to a one-carbon or two-carbon chain. Organic mercury compounds that consist of short chain carbon compounds tend to be more stable than those of a larger mass. Both organic and inorganic mercury can be toxic, but the short-chain alkyl mercurials tend to cause the most damage due to their ability to be absorbed by living organisms (Clarkson et al, 2007).

An important inorganic form, elemental mercury, is in the liquid state at room temperature but it can also be present in the atmosphere in the gas state (Clarkson et al, 2007). Mercury enters the environment via natural and anthropogenic sources. The natural contributors of elementary mercury are volcanic eruptions, forest fires, and degassing of water surfaces and soil on land. A larger source of mercury pollution is caused by human activity. Mercury emission occurs during metal production, burning of wood and coal, waste handling and treatment among other sources (Morel et al, 1998). Gold mining uses mercury to extract gold from the earth, and this activity can also lead to mercury pollution (Nyland et al., 2011; Sherman et al., 2013). These are the primary routes by which mercury enters the atmosphere, where it can stay for up to one year before being deposited on both land and water (Morel et al, 1998).

Elemental mercury is not readily dissolved in water, but becomes soluble when it is oxidized to Hg^{++}, the mercuric cation, leading to its precipitation by rain. Once Hg^{++} is deposited into aquatic ecosystems, sulfate-reducing bacteria promote the methylation of mercury from dissolved Hg^{++} to an organic derivative, mainly methylmercury (MeHg). Mercury is taken into bacteria through several mechanisms. Some bacteria contain a specialized transport protein that can take mercury into the cell but the majority of mercury uptake occurs through lipid soluble mercuric complexes. In this mechanism, Hg^{++} ionically bonds to chloride in water to form $HgCl_2$. $HgCl_2$ has much higher lipid solubility than elemental or ionic mercury, allowing mercury to effectively pass through the plasma membrane. Inside sulfate-reducing bacteria, mercury methylation is mediated by enzymes and methylation is increased during fermentation; however, the reason for the mercury methylation process is still unknown (Morel et al. 1998). Nonetheless, this process is a critical step making mercury pollution a serious public health concern.

Mercury is a public health concern because bioaccumulation of MeHg in aquatic food webs leads to the buildup of toxic levels of mercury in contaminated fish, which are a main source of food for many populations around the world. (Clarkson et al, 2003; Farina et al., 2011). This means that organisms at the top of the food chain will have higher levels of MeHg than the smaller organisms upon which they feed. A food chain in which there are multiple levels results in more bioaccumulation of mercury in the long-living large predatory fish. This increase results from biomagnification, an increase in tissue Hg concentration at each trophic level. Top-predatory fish which have the highest concentrations of MeHg are often valuable to the human diet (Morel et al, 1998).

The main source of mercury exposure to humans is through the consumption of contaminated fish, while exposure from air, drink and amalgam-treated teeth is less frequent. The air contains much less MeHg than the average fish contains which contains about 0.4 μg/g (Young-Seoub Hong, et al, 2012). Mercury poisoning becomes a major problem when communities rely on fish as main part of their diet because the larger and longer living predatory fish, such as tuna, swordfish and shark, have accumulated unsafe levels of MeHg up to around 10 to 50 times greater than the average. Consistent consumption of high doses of MeHg can then become toxic to humans resulting in a wide variety of different diseases and disorders (Young-Seoub Hong, et al, 2012). Due to the high absorption rate of MeHg it can be distributed throughout the body; however, it is most damaging to the central nervous system (Farina et al., 2011).

MeHg exposure in adults can lead to lack of motor coordination, loss of sensation such as touch and vision, and in rare causing death. These clinical symptoms of mercury poisoning have been observed from instances where people were accidentally exposed to dangerous amounts of MeHg. A famous case occurred in Iraq when hundreds of people died and hundreds more developed neurological symptoms after consuming grain that was treated with MeHg as a fungicide (Young-seoub et al, 2012). MeHg's clinical manifestations are most prominent in developing nervous systems. MeHg exposure causes inhibition or neuronal migration in developing brains, synapse transmission and amino acid transport. Exposure to a developing fetus can result in cerebral palsy-like symptoms. During the 1950s a community in Minamata, Japan was exposed to a large concentration of MeHg over a period of time. While the mothers showed no signs of exposure their children developed what is now known as minamata disease. This disease

is characterized by paralysis, intelligence disorders, mental retardation, physical growth disorder, seizures and limb deformities in addition to the effects that arise in exposure in adults (Young-Seoub Hong et.al, 2012).

Nervous system disturbances, especially during development, are the main effects of MeHg; however, studies have shown that mercury affects other bodily systems as well. MeHg can affect the reproductive system with effects such as decreased sperm count, testicular atrophy, and decreased fetal survival rate and fetus deformity. MeHg exposure has also been correlated with cardiovascular diseases, coronary heart disease and cancer of the kidney (Young-Seoub Hong et al, 2012). The full suite of consequences of MeHg exposure is still being determined, especially in regards to the immune system.

The characteristics that cause MeHg to be so toxic are its liposolubility and its affinity for sulfur and selenium atoms that allow for it to be absorbed by the body. As in fish, 95 percent of MeHg consumed by humans will absorb easily and quickly into the body with a half-life of about 70 days. Upon consumption of MeHg-contaminated fish, MeHg is released from food during digestion and is subsequently absorbed in the duodenum. Absorption of the MeHg is mediated in several ways. Firstly, the pollutant binds to thiol (R-SH) and selenol (R-SeH) groups that are broken down in the digestion process. For example, MeHg will bind to the amino acid cysteine and this complex will mimic a neutral methionine amino acid allowing for easy passage across epithelial cells in the duodenum into the blood stream (Clarkson et al. 2007). MeHg also binds to other cysteine-rich proteins and enzymes that are taken up in the digestion process (Farina et al, 2011). Once in the blood stream, MeHg can pass the blood brain barrier leading to the neurological damage for which MeHg poisoning is best known.

1.2 Mechanisms of MeHg Toxicity

Neurological disorders and other toxic results are due to the damage MeHg causes once inside of the cell. MeHg binds strongly with sulfur and selenium atoms, and this binding can lead to cellular damage in two ways. Thiol- and selenol-containing molecules such as glutathione are important cellular antioxidants that help maintain cellular redox status, but once they are bound to MeHg they lose their function. This type of inhibition cannot account solely for the drop in antioxidant capacity and increased production of reactive oxygen species (ROS) associated with MeHg exposure. Selenium containing biomolecules such as selenoproteins and selenoenzymes also have a high affinity for MeHg. Many of these selenoenzymes play a critical role in a cell's redox control system, specifically in the removal of ROS, and they can be inhibited by MeHg as well (Clarkson et al., 2007)

Glutathione peroxidase (GPx) is a selenoenzyme that mediates the oxidation of glutathione (GSH) and the reduction of H_2O_2 into H_2O. For example, GPx-1 reduces H_2O_2 formed by superoxide dismutase from the superoxide ions produced in the mitochondria during cell respiration. GPx consists of four subunits, all of which are identical and each contains one selenocysteine residue. The selenocysteine is a necessary part of the enzyme's active site due to redox catalysis efficiency. (Arthur, 2000). GPx mediates the reduction of H_2O_2 to H_2O by GSH. In this process, GSH is then oxidized to GSSG (oxidized glutathione) and NADPH is oxidized to $NADP^+$. The GSH oxidation process is very important for maintaining cellular homeostasis. If peroxide molecules are not reduced then they can cause damage to the cell in a variety of ways (Farina et al., 2011; Arthur, 2000).

MeHg has been shown to inhibit GPx by binding to the active site, specifically the reactive selenol group (Franco et al., 2009; Farina et al., 2011; Branco et al., 2012; Carvalho et al. 2008). When mice were exposed to MeHg, GPx activity was shown to decrease. This decrease correlated with signs of apoptotic cell death, increase in ROS formation and lipid peroxidation (Franco et al., 2009). The results of this study suggest that MeHg inhibited GPx since the activity was lowered and ROS formation and cell damage increased, suggesting that oxidative stress was a source of cell death. Decreased GPx activity has also been shown in other studies (Glaser et al., 2010; Branco et al., 2012). Glaser et al. (2010) looked at the effects of adding selenium supplements to MeHg-exposed mice to better determine the role selenium plays in MeHg toxicity. The study demonstrated observations of a slight decrease in GPx activity with MeHg-exposed and MeHg + Na_2SeO_3-exposed groups of mice. There was no significant difference in the activities of GPx between the selenium treated MeHg groups and the MeHg-exposed groups but a difference was shown in GR activity and lipid peroxidation. Some of the evidence contradicts what was concluded from the Branco et al. (2012) study. This study looked at the effects of MeHg exposure in zebra-seabreams as well as the effect of additional selenium. GPx activity was decreased in the brain but activity was recovered with additional selenium. Free selenium compounds bind to MeHg and prevent it from interacting with selenoenzymes, and it aids in excretion, and formation of selenocysteine for better enzyme activity (Branco et al., 2012)

Oxidative stress occurs when the cellular concentration of pro-oxidants exceeds the capacity of the intracellular antioxidants. This situation occurs when there is a high concentration of reactive oxygen species (ROS) inside of the cell. ROS consist of several

different molecules, such as superoxide ion radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot -}$), and organic derivatives $RO^{\cdot -}$ and $ROO^{\cdot -}$, and hydrogen peroxide (H_2O_2). These oxygen species are generated as a result of aerobic metabolism in the mitochondria. Two superoxide radicals can react with another superoxide ion to form H_2O_2, mediated by the enzyme superoxide dismutase. Furthermore, H_2O_2 can be broken down back into superoxide ion radicals which then oxidize and damage different biomolecules. In addition, H_2O_2 is also dangerous in the cell since it can damage lipid membranes (Kohen and Nyska, 2002). Oxidative stress inside of the cell can lead to deleterious effects on lipids, proteins and DNA.

Several studies have shown that MeHg causes an elevation in pro-oxidant levels resulting in oxidative stress. For example, Franco et al. (2009) compared rates of ROS formation in MeHg-treated mice versus control mice, and found that MeHg increased rates of ROS formation. Other studies have looked at MeHg exposure and oxidative stress by using several different biomarkers. The biomarkers include enzymes and biomolecules involved in the antioxidant system or byproducts of oxidation. Several studies have shown decreases in enzyme activity and increases in malondialdehyde (MDA), which is a molecule formed as a result of lipid oxidation. This latter analysis reveals that lipid peroxidation can be used to indicate oxidative stress. When oxidative stress occurs lipids are oxidized which leads to higher levels of MDA concentrations. Mice exposed to MeHg have shown significant increases in MDA concentrations, which suggest that oxidative stress caused cell damage (Huang et al., 2008; Glaser et al., 2010; Franco et al., 2009). Malagutti et al. (2009) showed increased MDA in conjunction with reduced GPx activity in male mice exposed to MeHg.

Lipid peroxidation, oxidation of lipid molecules, occurs in three stages. The first stage is initiation which occurs when the pro-oxidant removes a hydrogen atom from an unsaturated fatty acid in the lipid. The fatty acid radical that forms can undergo rearrangement in order to stabilize the lone electron, thus forming a conjugated diene. Alternatively, in the presence of enough oxygen the fatty acid can form an organic peroxide radical ($ROO^{\cdot -}$) that leads to a second stage of lipid peroxidation, called propagation. Propagation occurs when a radical fatty acid removes a hydrogen atom from a neighboring unsaturated fatty acid, setting off a radical chain reaction which can propagate throughout an entire membrane. The peroxidation enters the termination stage when the organic peroxide radical reacts with another $ROO^{\cdot -}$ or an antioxidant (Kohen and Nyska, 2002).

ROS can also affect proteins and nucleic acids. These molecules are much more sensitive to radical oxygen species than non-radical species. The radicals can damage protein in several ways; peroxidation, cleavage of peptide bonds, changes in tertiary structure, and oxidation of reduced-sulfur-containing amino acid residues such as cysteine. DNA can also be fragmented by ROS. These radicals can affect the nitrogenous bases, the deoxyribose sugar causing breakage in the DNA strand. Therefore DNA repair systems can also. The interaction of ROS with proteins and nucleic acids can lead to many cellular effects such as inhibited enzyme activity, mutation, altered cell metabolism, and disrupted homeostasis of the cell (Kohen and Nyska, 2002).

1.3 Neutrophils and the Respiratory Burst

As previous mentioned, increased levels of oxidative agents inside of a cell can be very dangerous to the life of the cell. ROS can cause damage and can contribute to initiation of apoptosis, which is programmed cell death. However, these destructive substances are also used to enhance the immune response. Neutrophils and other leukocytes develop large amounts of ROS to degrade and damage foreign matter in the blood as the first line of the innate immune response. Neutrophils are multi-nuclear phagocytic cells that express a variety of receptors that recognize and bind to common pathogens, such as different types of bacteria. Like a leukocytes, neutrophils develop in the bone marrow and enter the blood stream upon maturation, with a life span of about 48-72 hrs. While circulating in the bloodstream, neutrophils are attracted to sites of inflammation or infection by chemical signaling of cytokines released by other neutrophils, the complement system or macrophages at these sites (Altstaedt, 1996). Upon binding to their target, Neutrophils engulf bacteria and damaged or infected cells.

They are also classified as granulocytes since they contain two types of granules that breakdown what they phagocytize. In neutrophils, engulfed material is fused with two types of granules. First azurphilic granules contain proteins and peptides that contribute to the digestion of foreign microbes. The second type of granule are specific granules that contain unsaturated lactoferrin bind to iron and copper containing proteins thus preventing their uptake by bacteria (Parham, 2009).

During the immune response, neutrophils undergo a process known as a respiratory burst to provide a 20-fold increase in ROS production through an enzymatic

process. Neutrophils undergo an increase in glucose utilization to produce energy in the form of the electron carrier nicotinamide phosphate dinucleotide (NADPH). NADPH-oxidase takes an electron from NADPH and forms superoxide radicals. These radicals are then released into the phagosome where the pathogen is located. The superoxide radicals are eventually converted into H_2O_2 which is converted back into water through glutathione peroxidases and catalase (Kohen and Nyska, 2002).

The oxidative burst results in a process that can kill fungi as well as both Gram-negative and Gram-positive bacteria. While this process is beneficial for getting rid of pathogens, it can also be damaging to the cell if not regulated. The toxic oxygen species can leak out of the phagolysosome and damage intracellular components as well as cells in nearby tissue (Parham, 2009). As in other cells, anti-oxidant enzymes such as catalase and glutathione peroxidase play a major role in regulating the level of ROS in neutrophils (Ito et al., 1998). If these enzymes were not present then the cell would suffer severe oxidative damage from the oxidative burst. This is evidence by a decrease in microbicidal activity in GPx deficient neutrophils (Serfass et al., 1975).

Once the oxidative burst has finished and the neutrophil has completely lost its granules, the cells undergo apoptosis. This is due to the fact that the neutrophils have completed their task of removing the pathogens. Neutrophils cannot replenish their granules; therefore, they are unable to degrade any more bacteria or fungi. The dead neutrophils are ultimately engulfed by macrophages (Parham, 2009).

In addition to attacking pathogens via the respiratory burst, neutrophils also initiate an inflammatory and immune response by releasing cytokines, small proteins that

influences other cells as a response to a stimulus such as an infection. Neutrophil cytokines can recruit other neutrophils, macrophages and adaptive immune cells to the site of infection. Neutrophils have been discovered to release interleukin-8 (IL-8, CXCL8), which assists in the recruitment process. IL-8 is classified as a type of cytokine known as a chemokine, which influences where leukocytes will flow in order to reach the infected area. IL-8 specifically affects neutrophil recruitment into the area of infection. This chemokine plays several roles. It will affect endothelial cells in the blood vessels allowing easier movement for neutrophils, and it also provides a trail for the neutrophils to follow to reach the center of the infected area (Parham, 2009). It has also been determined that IL-8 attracts T-Cells to the sites of infection to start the adaptive immune response (Meager, 1991).

1.4 The Effects of MeHg on the Immune System

MeHg's effects on the immune system are an active area of investigation. Studies have shown immunosuppression as well as the development of autoimmune-like characteristics in humans exposed to high amounts of MeHg. For example, people living in the Amazon who rely on fish as a staple of their diet or who mine gold using mercury show signs of autoimmunity. In addition, increased autoantibodies, changes in cytokine levels and increased risk of malaria infection have all been observed in these populations (Young-Seoub Hong et al, 2012).

Evidence of MeHg immunotoxicity has been observed in animal subjects as well. Mice that were exposed to 3.2 mg/kg of MeHg in their feed showed reduced thymus weight as well as a 50% reduction of thymocytes present in the body and an increase in

one cytokine, IL-1β (WHO, 2004; Young-Seoub Hong et al., 2012). Other vital cytokines measured were IFN-γ, TNF-α, IL-4 among others (Gardner et al, 2010). In another study, mice were exposed to 2 mg of MeHg/kg of body weight every three days for 14 to 30 days. The results showed a significant reduction of B- and T-cells in circulation, 47% and 9%, respectively. This indicated immunosuppression after several days, which was indicated by a lower average of IL-2, IL-10, and IL-15 mRNA expression while IFN-γ mRNA did not change. IL-4 mRNA expression was increased along with antibody production; IgE and IgG1 showed much higher concentrations in serum of experimental mice than control mice (Haggqvist et al., 2005). The results from these studies are in consistent, suggesting more research needs to be performed in order to determine whether MeHg is an immunosuppressive chemical or immune stimulating.

Given the important role of neutrophils in the immune response, several studies have focused on neutrophils in order to understand the impact of MeHg on the immune system. For example, two *in vivo* studies investigated how MeHg affected neutrophil function and longevity (Moisan et al., 2003: Kuo and Lin-Shian, 2004). Both demonstrated that MeHg can delay neutrophil apoptosis at low concentrations, while at high concentrations cell necrosis was observed. Moisan et al. (2003) demonstrated that at MeHg concentrations below 10 µM, apoptosis did not occur, while concentrations above 100 µM were harmful to the cell and caused cell necrosis. Kuo and Lin-Shian (2004) observed cell death at much lower levels; concentrations above 10 µM were toxic to the cells and resulted in early necrosis. In addition, Frietas et al. (2010) showed that heavy metals, including inorganic Hg, can induce an oxidative burst in human neutrophils. The oxidative burst was measured by determining concentrations of ROS within each cell.

This could translate to MeHg influencing the oxidative burst of the neutrophil's response to bacterial infections. These studies suggest that MeHg can be harmful to neutrophils and affect their role in the immune system.

MeHg binds to selenium in a variety of enzymes, inhibiting their functions in the cell. Therefore, studies that demonstrate the effects of selenium deficiency on neutrophils could provide some insight into how MeHg affects the immune system. Terpilowska and Siwicki (2011) point out that selenium deficiency shortens the life span of neutrophils, macrophages and lymphocytes. They suggest that this might be due to ineffective regulatory enzymes such as GPx, which would replace the selenium with a sulfur group thereby decreasing the activity of the enzyme drastically. This idea is supported in part by Arthur et al. (2003), who investigated how selenium deficiency affected the immune system in mice and showed that while neutrophils did not decrease in number, they were not functional in MeHg-exposed mice. This lack of function was associated with decreased GPx activity within the neutrophils. The researchers concluded that the respiratory burst caused harm to the neutrophil after it had engulfed a pathogen. Furthermore, it has been noted that neutrophils in selenium deficient mice are less successful in killing foreign pathogens because they die from oxidative stress associated with decreased GPx activity (Hefnawy and Perez, 2010; Terpilowska and Siwicki, 2011). This evidence suggests that selenium-containing enzymes are crucial for proper respiratory burst regulation. Since MeHg binds to selenium, MeHg exposure likely leads to similar effects on neutrophils as selenium deficiency.

The research summarized above suggests that one way in which MeHg impairs the immune system is via disruption of redox control during the respiratory burst in

neutrophils. The goal of this study is to investigate the effects of MeHg exposure on neutrophil redox status and to assess whether an unregulated oxidative burst will cause damage to the neutrophil. The specific aim is to measure the effect of MeHg on neutrophil GPx activity and lipid peroxidation when cells are exposed to a common antigen, lipopolysaccharide (LPS). I hypothesize that MeHg introduced to neutrophil cell culture will cause an unregulated respiratory burst, resulting in peroxidation of lipids within the cell inhibition of GPx.

Chapter 2: Materials and Methods

2.1 Experimental Design

The overall goal of this investigation was to better understand how MeHg affects aspects of the immune response, specifically the respiratory burst by neutrophils. In order to achieve this goal, the study had two objectives: 1) to develop protocols for using cell cultures in MeHg toxicity assays, and 2) to extend these protocols to test for MeHg effects on immune response using neutrophils. The first objective required adapting analytical methods previously used for measuring biomarkers of oxidative stress in an *in vivo* study using adult male mice. These methods were originally developed for analysis of homogenized; thus, changes in procedure were needed for the analysis of cell culture samples. The first experiment utilized brain cells from chick embryos. Experimental groups included controls and cells that were exposed to MeHg for 24 hrs. The second objective was to utilize the cell culture protocols and modified analytical methods to investigate changes in neutrophils that were stimulated for an immune response and exposed to MeHg. Figure 1 demonstrates the steps of the neutrophil experiment. Two of the experimental groups were exposed to an antigen, LPS, to stimulate an oxidative burst in the neutrophil. One of the LPS exposed groups was also exposed to MeHg. A third set of untreated cells served as the control. At the end of both experiments, cells were resuspended in phosphate buffered saline (PBS) solution in order to measure lipid peroxidation (MDA concentration) and GPx activity to quantify the effects of MeHg exposure. The activity of GPx was measured to test for inhibition of a key cellular peroxidase. In addition, lipid peroxidation was measured to assess cell damage due to ROS.

Steps of Experiment

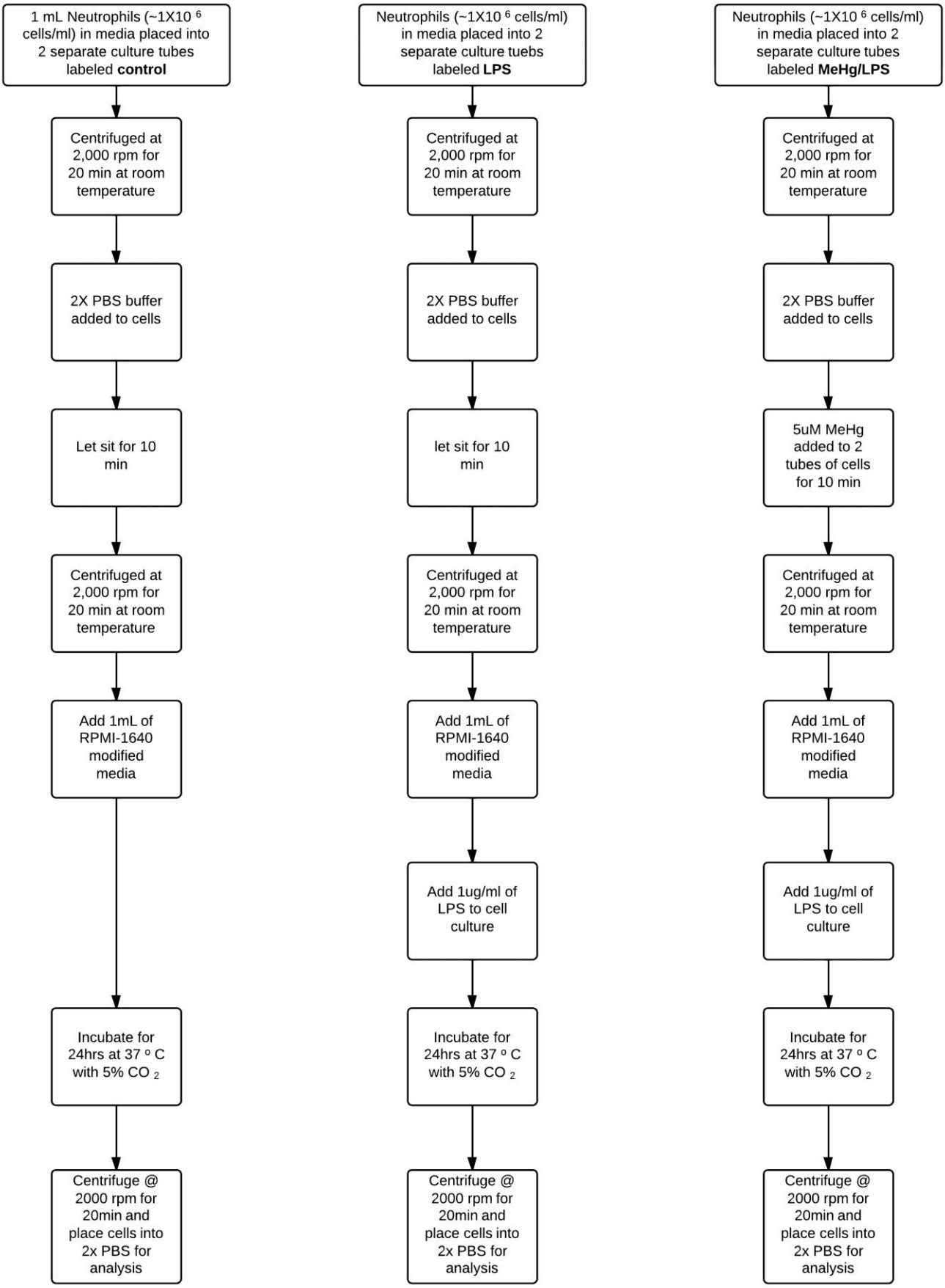

Figure 1: Above depicts the assay protocol for neutrophil culturing, incubation and preparation for analysis

10-day old chick embryos were harvested and their brains were taken out and placed into DMEM (Dulbecco's Modified Eagle Medium);Sigma-Aldrich, St. Louis, MO). The brains were minced and exposed to Hank's Balanced Salt Solution and trypsin to create a single cell-suspension. Trypsin was then removed and the cells were then placed back into DMEM and triturated to quicken the separation process. Finally, the cells were counted using a hemacytometer and cells were added to create cultures of about 6×10^6 cells/mL in 15mL cell culture tubes.

C57BL/6 mouse bone marrow neutrophils were obtained from CellBiologics, Chicago, Il. Neutrophil concentrations were 1×10^6 cells/mL in RPMI-1640 media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum, 2mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin (Altstaedt et al., 1996).

2.3 MeHg Exposure and Incubation

The experiments with chick embryo brain cells included 2 groups: no MeHg or control group and a MeHg-exposed group. Each group contained four replicates of brain cells. The neutrophil experiment consisted of three different groups; control, LPS exposed and MeHg and LPS exposed. Each group contained 2 replicates in the neutrophil experiment. The LPS exposed group allowed for the assessment of the effects of LPS on neutrophil immune response. The LPS and MeHg exposure group revealed how MeHg impacted the respiratory burst.

Prior to exposure, chick embryo brain culture contained 6×10^6 cells. They were removed from media through centrifugation and re-suspended into 2X PBS solution (Altstaedt et al., 1996). 100µl of 58µg/mL of MeHg was added to each group exposing

cells to approximately 5µM MeHg (T.C. Kuo et al. 2002) for 10 mins MeHg exposure was performed in PBS solution to avoid interactions with thiol and selenol groups that are present in the cell medium, which would reduce the amount of MeHg that actually reached the target cell culture. After 10 mins, cells were centrifuged at 2000 rpm (Kuijpers et al., 1991), placed into fresh cell growth medium and incubated for 24 hrs.

In the neutrophil experiment, 1 µg/ml of LPS was incubated with cells for 24 hrs in order to stimulate the neutrophil respiratory burst response to the antigen (Altstaedt et al., 1996). LPS is used because it has previouslybeen demonstrated to cause an immune response by neutrophils (Altstaedt et al., 1996; Tonk et al., 2010)

2.4 Protein Concentration

Cells were first centrifuged at 2000 rpm and resuspended in 2x PBS (1 in 4 dilution of 10X phosphate buffered saline solution from Fisher Scientific). Cell concentration was maintained at 6 x 10^6cells/mL. Protein concentration of cells suspensions was determined using the Bradford Assay (Bradford, 1976).On each analysis day; a calibration curve was established using bovine serum albumin standards ranging from 25.18 to 125.9 µg/mL (Appendix IV). In order to normalize these assays, total protein was measured in the cell suspensions.

2.5 Glutathione Peroxidase Analytical Method

A kinetic enzyme assay for GPx was used to determine its specific activity. In this assay, NADPH is oxidized to $NADP^+$ as it transfers H^+ and e^- to oxidized glutathione (GSSG) via glutathione reductase (GR). Reduced glutathione (GSH) then transfers H+ and e- to hydrogen peroxide via GPx, producing water (see Figure 1). Since substrates

and GR are added in excess to the reaction mixture, ambient GPx activity determines the rate of NADPH consumption. NADPH absorbs strongly at 340 nm, but $NADP^+$ does not, so the decrease in A-340 can be used to quantify GPx activity. To carry out an assay, reagents were added to a microcuvette in this order: 50 mM phosphate buffer with 0.4 mM EDTA and 1mM sodium azide, 1 mM GSH, 4 µg GR, 0.2 mM H_2O_2, 0.2 mM NADPH. After one minute the 200ul of sample was added and absorbance was measured at 340nm for 3 minutes (Nakamura et al., 1974). The detailed reaction protocol is found in Appendix I. The sodium azide was added to prevent direct decomposition of peroxide by catalase. A sample assay curve is shown in Appendix II.

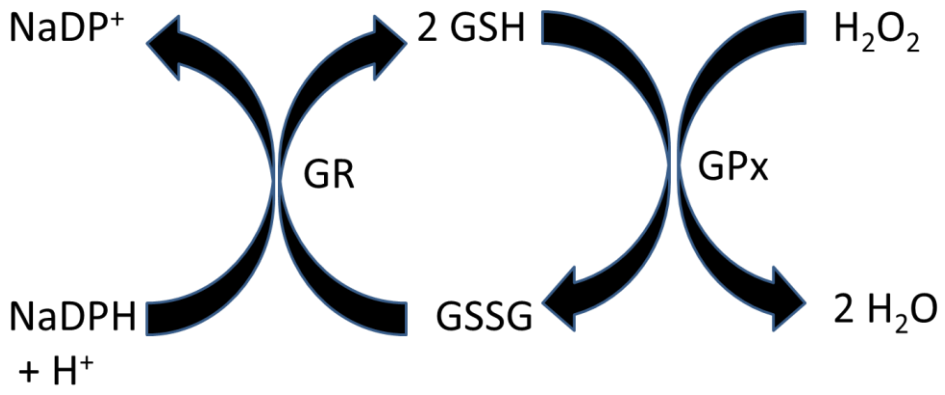

Figure 2: The reaction sequence of the GPx assay. GPx reduces H_2O_2 into water with the help of GSH, which is then oxidized and then reduced back into GSH by Glutathione Reductase using NADPH.

2.6 TBARS Analytical Method

As a means to determine cell damage and the presence of oxidative stress, concentrations of malondialdehyde (MDA) were measured using the Thiobarbituric Acid Reactive Substances (TBARS) method (Ohkawa et al., 1978). This method utilizes the chromogenic reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a

product of lipid peroxidation. These compounds react to form a 2:1 complex that absorbs strongly at 532 nm (see Fig Z;Ohkawa et al., 1978). This assay was carried out under the following reaction conditions: 0.3 g of TBA, 0.25 M acetic acid, 2.5% NaOH solution, 1.2% SDS and sample incubated at 95^{o}C for 60 mins (Malagutti et al., 2009). Samples were allowed to cool at room temperature for 10 mins, and absorption was measured at 532nm. Absorbances were related to concentrations using a standard curve that ranged in concentration from 0.15– 0.6 nmol/mL., and the cell concentrations were expressed in nmol/mg protein The detailed lab protocol for TBARS measurement is found in APPENDIX III.

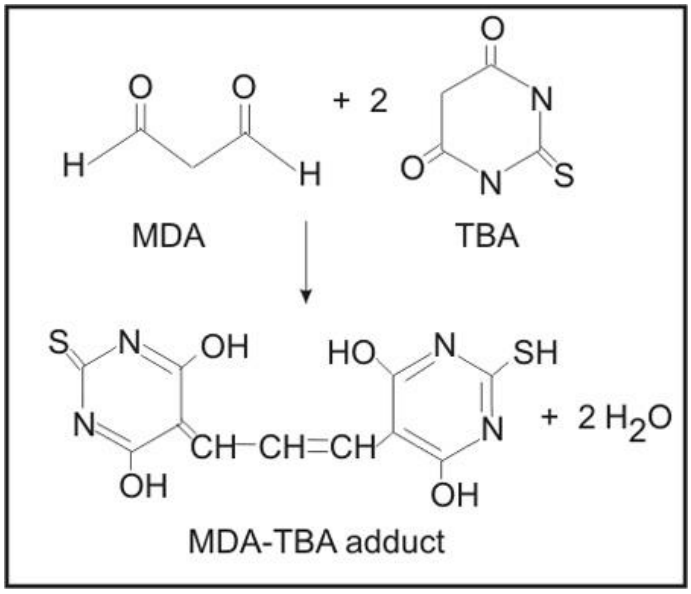

Figure 3: Reaction of malondialdehyde with two thiobarbituric acids to create a chromogenic MDA-TBA adduct molecule that absorbs light at 532 nm.

Chapter 3: Results and Discussion

3.1 Cell Culture and MeHg Toxicity Assays

The first goal of this research was to develop protocols for using cell cultures in MeHg toxicity assays. Overall, this goal required first developing procedures for harvesting and culturing cells, and then for conducting MeHg toxicity assays with the cell cultures. This work was carried out using brains from 12-day chick embryos. The following protocol for harvesting and culturing cells was established:

1. Placed excised brains in DMEM and mince into small pieces.

2. Removed the medium which the minced tissue was add a diluted trypsin solution. Incubate at 37^{o}C for 20minutes.

3. After incubation trypsin solution was removed and DMEM was added back to the separating cells. In order to further separate the cells, trituration was performed using a Pasteur pipette.

4. Dilute portions of the cell cultures with medium and cell counts were taken using a hemacytometer to establish the cell density.

5. Cells were dispensed into culturing tubes, spun down and resuspended in growth medium to obtain the required density (1×10^{6} cells/mL for the assays described here). Cells were incubated at 37^{o}C until used for assaying or analysis.

The MeHg toxicity assay was based on previous studies (Altstaedt et al., 1996; Kuo et al., 2002; Kuo et al., 2004) but the method for introducing MeHg to the cell cultures was modified. In order to expose the brain and neutrophil cell cultures to MeHg,

the growth medium was removed by centrifuging the cells out of suspension and the supernatant (media) was removed. Cells were resuspended with PBS and MeHg was added. The reason for this change was to prevent the MeHg from binding to sulfhydryl and selenol compounds that are present in the growth medium that could complex MeHg and prevent uptake by the cells. After MeHg exposure, the cell-PBS solution was then centrifuged again, and the cells were resuspended in growth medium for incubation.

After the MeHg exposure period, the assay cultures were analyzed for MDA levels using the TBARS assay and for GPx activity. The TBARS protocol (Ohkawa et al., 1978) had previously been attempted in our laboratory; however, the method had not produced reliable results in previous studies using tissue samples from mice. The main problem with this analytical method of measuring MDA concentrations was that despite a discernible color change in the samples, the absorbance measurements were very low. Using standard additions of MDA to samples, it was determined that the interference was caused by a protein precipitate that interfered with absorbance reading.

Several methods were employed to remove this precipitate, including acid precipitation prior to adding sample to the reaction mixture and centrifugation of the reaction mixture post-incubation in a water bath. The use of 10% trichloroacetic acid (TCA) to precipitate out protein prior to reaction did not correct the problem with absorbance measurements. Centrifugation did remove the precipitate from the reaction mixture, but if spun for too long the absorbance of the sample decreased, suggesting that there was some MDA-TBA complex bound to the precipitate. Spinning samples for only 2 minutes at 3400 rpm effectively removed the interference with no decrease in absorbance, so it was deemed to be the optimal centrifugation time. This short

centrifugation process allowed for reliable determination of MDA concentration in tissue and brain cell samples.

The GPx analysis (Nakamura et al., 1974) had been used successfully on mouse tissue samples in our laboratory, but it had to be modified slightly for cell culture assays. The modification was to compensate for the reduced amount of protein in the cell cultures as compared to the tissue samples previously used for this analytical method. The volume of sample used in the analysis was added at a 10-fold increase to compensate for the 10-fold lower protein concentration in the cell culture. The rest of the protocol remained the same except that the amount of buffer added was decreased to compensate for the increased sample volume

3.2 Chick Embryo Brain Cell Assay Results

Although cell counts were not made at the end of the assay, there was no significant difference between the protein concentrations of the two experimental groups (control and MeHg-exposed, Figure 4), which suggests that similar cell densities were present in the two groups. The protein concentration measurements allowed for the GPx and TBARS analysis results to be normalized to the amount of protein, so that valid comparisons could be made between the treatments and controls

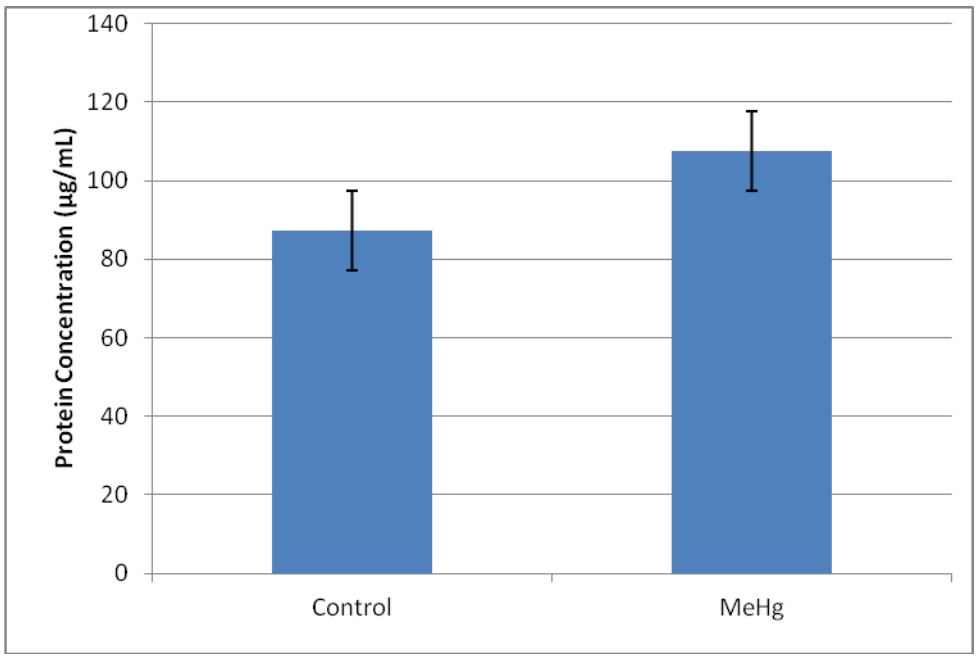

Figure 4: Protein concentration in the brain cell cultures at the end of the chick brain cell assay. There was no significant difference between the control and the MeHg-exposed groups in protein concentration (Student's t-test, 95% confidence level).

As shown in Figure 5, GPx activity showed a significant difference between the control group and MeHg-exposed group, based on a Student's t-test at the 95% confidence level (p-value < 0.005). The average specific activity of the control group was 1.4 µmol/min*mg ± 0.3 µmol/min*mg and the average specific activity of the MeHg-exposed group was 0.63 µmol/min*mg ± 0.09 µmol/min*mg. The significant difference between the MeHg-exposed group and the control suggests that MeHg had an inhibitory effect on GPx activity in the cell culture. This finding supports our hypothesis that when MeHg is added to cell culture it will bind to the active site of GPx and inhibit its activity within the cell. These results demonstrate that cell culture assays can be used to observe biochemical changes within brain cells when they are directly exposed to MeHg. This *in vitro* approach can complement *in vivo* assays while saving time and sparing

animals.

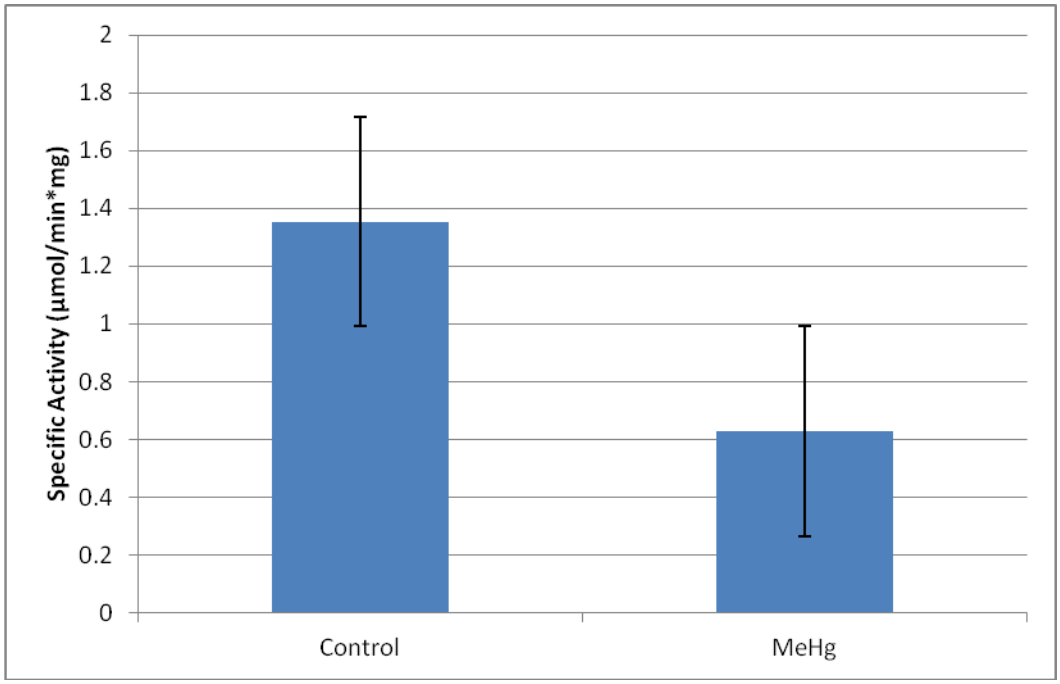

Figure 5: Specific activity of glutathione peroxidase in the control (n=3) and MeHg-exposed (n=3) chick brain cells. There is a significant difference between the two groups. The control group has a smaller size because one of the sample assays was rejected as an outlier.

Our finding of GPx inhibition in brain cell culture is consistent with previous *in vivo* studies that show that MeHg decreases GPx activity in mice (Franco et al., 2009; Malagutti et al., 2009). Franco et al. (2009) exposed male mice to 40 mg/L of MeHg in their drinking water for 21 days. This experiment resulted in a significant decrease in levels of GPx in brain tissue of exposed mice compared to controls. Furthermore, they found a significant increase in poly-ADP-ribose polymerase cleavage, which indicates increased apoptosis. In another study (Malagutti et al., 2009), possible gender-specific effects of MeHg exposure were determined by observing locomotive behavior, enzyme

activity and lipid peroxidation in mice exposed to 40 mg/L of MeHg in their drinking water for 14 days. Results showed decreased GPx activity and increased lipid peroxidation in tissue samples from MeHg-exposed male mice as compared to all other experimentgroups. This study provides further evidence for MeHg inhibition of GPx, which is consistent with the chick embryo brain cell results.

While a significant difference was observed, the GPx kinetic enzyme analysis did not produce reliable replicate runs in all cases. For future research, it is recommended to usea standard control for the kinetic enzyme analysis. Ideally, the GPx assay should be tested routinely using purified GPx enzyme and/or using homogenized liver samples, to ensure that the reagents in the analysis (especially NADPH, GSH, and GR) are functioning properly. Since the analysis has shown high reproducibility in homogenized liver tissue, this could be a useful control to indicate that the analytical method is working. With the use of a standard control, the troubleshooting process can be focused on other problems that are not directly related to the analytical method, but might reflect difficulties with the cell culture methods.

Despite the GPx inhibition by MeHg seen in this study, no significant difference in lipid peroxidation was observed between the control and MeHg-exposed cell brain samples (Figure 6). The TBARS analysis showed an average MDA concentration of the control group of 1.5 nmol/mg ± 0.2 nmol/mg while the MeHg-exposed group had an average MDA concentration of 0.95 nmol/mg ± 0.5nmol/mg. Although the MDA concentration in the control group was somewhat higher, it was not significantly different at the 95% confidence level according to a Student's t-test. Some oxidation of cellular

lipids was evident in both groups but MeHg exposure did not elevate MDA in the exposed group.

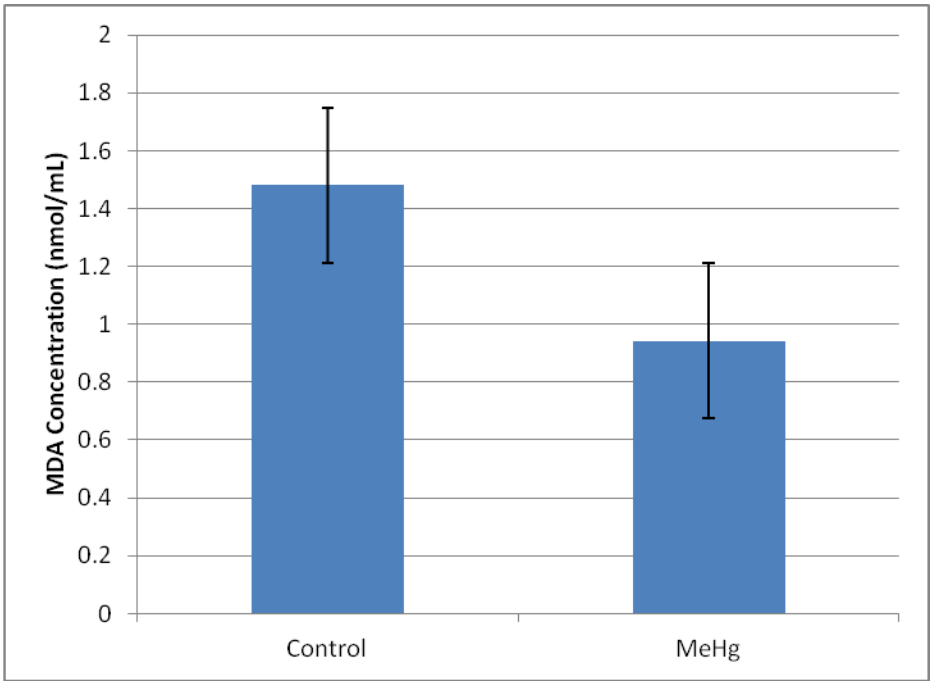

Figure 6: There was no significant difference in MDA concentrations between two chick embryo brain cell groups. In both groups n=4. The concentrations we based on a standard curve of a concentration range of 0.15µM to 0.6µM.

Our TBARS results are contrary to the literature, which has frequently shown concurrent GPx inhibition and increased lipid peroxidation from MeHg exposure. For example, in mice exposed to MeHg for 21 days (Franco et al., 2009), both inhibition of GPx and lipid peroxidation were observed in brain cells. In this same experiment, addition of GPx to mouse tissues prior to analysis resulted in lower lipid peroxidation in the MeHg-exposed group (Franco et al., 2009). The lack of elevated lipid peroxidation in the chick brain cell assay could indicate that the observed decrease in GPx activity was not substantial enough to affect regulation of ROS within the brain cells. Alternatively, if

an increase in peroxide did occur during the assay, it may have been too subtle or too short in duration to promote the lipid oxidation chain reaction. These results suggest that different MeHg doses and incubations times should be examined in future studies to determine whether they produce measurable lipid peroxidation.

3.3 Neutrophil Culture Analytical Results

With the relative success of the chick embryo brain cell analysis in identifying an inhibition in GPx activity, the same analytical procedures were performed on neutrophil cell culture. The results from this assay did not show any significant GPx activity or MDA concentrations, despite having an average cell count of 1.5×10^6 cells/mL ± 0.3 cells/mL (Table 1) and measurable protein concentrations (Figure 7). Cells were present in culture at the end of the assay, but we were not able to detect GPx activity or MDA concentration in either the treated or control samples.The inability to detect GPx in the neutrophil cultures could be a result of two separate problems. The first possible explanation for lack of detection is that cells were dead or unhealthy enough to produce detectable levels of GPx. The other possibility is that the cells were alive but something was interfering with the analytical methods.

Sample	Number of Neutrophils (cells/mL)
Control 1	1.45E+06
Control 2	1.61E+06
LPS 1	9.20E+05
LPS 2	1.22E+06
MeHg/LPS 1	1.54E+06
MeHg/LPS 2	2.01E+06

Table 1: Cell counts for the neutrophil assays. The average count of neutrophils in a sample was 1.46×10^6 cells/mL.

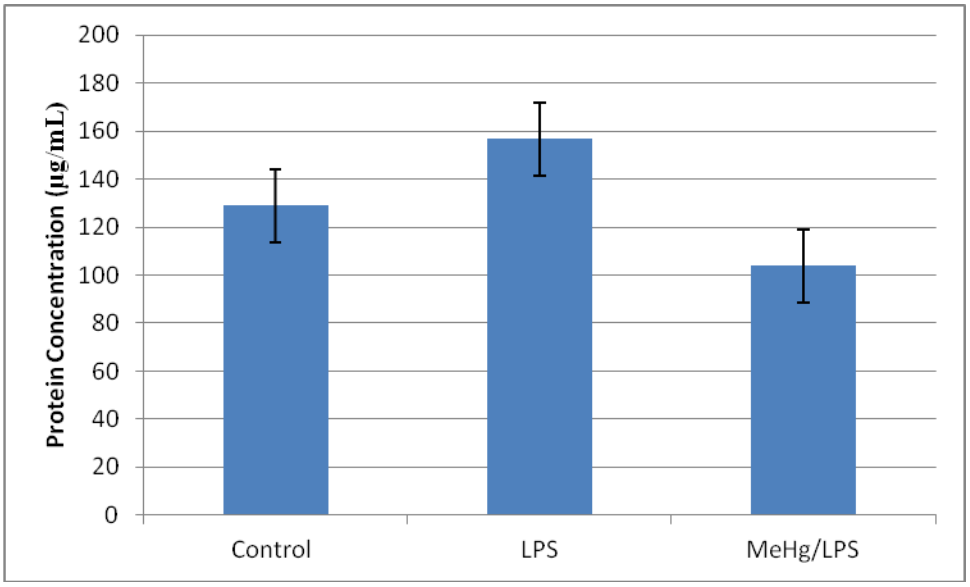

Figure 7: Average protein concentrations in neutrophil cultures. These levels were similar to those measured the chick embryo brain samples. There was no significant difference between the control, LPS and MeHg/LPS exposed groups in protein concentration (Student's t-test, 95% confidence level).

The first possibility can be addressed by considering the condition of the cells based on visible inspection. The cell culture when counted showed a variety of neutrophil states. Many cells looked to be intact, some looked dead, and some looked as if they were in the process of being lysed, with cytoplasm leaking from the cell. The dead and dying cells could have released their inner components, and when cells were isolated through centrifugation intracellular enzymes could have stayed in the supernatant. After the supernatant was removed and the cells resuspended, only cell membranes from the dead cells would have remained in the culture. This would mean that the protein measured in the samples was cell membrane associated only, which might explain the undetectable GPx activity observed in analysis. GPx is a cytoplasmic enzyme and would have been lost when lysed.

For future experiments, methods to check for cell viability anda standard enzymatic activity should be used. First, Trypan blue staining is a popular method used to determine cell viability by showing that the cell membrane is intact. Using a method such as this could allow for determining whether cells were alive. Additionally, a simple catalase kinetic analysis could be used to see if there is some enzyme activity within the culture. Catalase is another enzyme known to exist in neutrophils as well as almost every other primary cell. The catalase analytical method requires only three components for the reaction mixture. Utilizing this this relatively "foolproof" assay would show that there is the possibility for enzyme activity available in the sample solution.

The lack of detectable MDA in the analysis cannot readily be explained by the same mechanisms proposed for the GPx anomaly. Even cells that lost their contents would likely show some evidence of lipid peroxidation, given the high lipid content of

the cell membrane. Both the control and MeHg-exposed group's measurements in the chick brain cells demonstrated levels of lipid oxidation, suggesting that even unexposed cells experienced enough oxidative stress to show measurable MDA (Figure 6). Therefore, it seems most likely that some compound could have interfered with the MDA analysis of the neutrophil cell culture. For example, an ion or compound in the neutrophil samples could have inhibited complex formation between MDA and TBA, so that no absorbance was measured.

Chapter 4: Conclusions

The results from this study allow for several conclusions for oxidative stress response to be made. First, protocols for culturing, assaying and analyzing embryonic chick brain cells were effectively developed. The protocols could potentially applied to primary cell cultures from other tissue types. The analytical methods detecting for cellular changes in GPx and lipid oxidation can also be used for other cell types, but care must be taken to assure reliable results in new matrices.

The second conclusion that can be made is that GPx activity in embryonic chick brain cells decreased when exposed to MeHg; however, this decrease in activity did not bring about an increase in lipid peroxidation. The 5 µM MeHg added to the cell culture and incubated for 24 hours was enough to inhibit GPx activity but not enough to cause lipid peroxidation. A longer incubation or higher concentration of MeHg may be needed to see evidence of lipid peroxidation.

Assays on the neutrophil culture identified a need for further modifications, as no detectable signal from was found for enzyme activity and lipid peroxidation. First, the use of cell viability tests could establish the presence of living cells in the analytical samples. A trypan blue test or a simple catalytic enzyme analysis is two possibilities for testing viability. Further trouble-shooting is also needed to rule out (or overcome) matrix inhibition of the analyses by neutrophil cultures, for example, by using standard addition. Given the sensitivity of the GPx assay, it is desirable to use a "positive control" sample that is known to reliably demonstrate activity. Using a liver sample or chick embryo cell

culture to test for GPx activity alongside the desired experimental group would assure active reagents.

Applications of the protocols developed here can be used to better understand how direct MeHg exposure influences other cell types. Exposed cells can be analyzed for the cellular changes beyond GPx activity. For example, other biomarkers of MeHg toxicity such as glutathione reductase, thioredoxin reductase, or ROS could be measured. In addition, cell culture assay could be used to determine if specific antioxidants such as N-acetylcysteine can ameliorate the toxic effects of MeHg. These protocols could also allow for a comparison of an *in vitro* study with an *in vivo* study, looking how cellular change relates to organismal response. This tandem approach could then provide a better understanding of the mechanisms of MeHg toxicity on both the cellular and the organismal.

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Appendix

Appendix I

Protocol for glutathione peroxidase

1. Turn on UV/Vis and wait 10min for the machine to warm up.
2. Set wavelength to 340nm and time for 180s. Rate should be set at initial rate from 0 to 180s.
3. Blank machine with 1mL of buffer in a 1.5mL cuvette.
4. Add 720µL of buffer to cuvette
5. Add 20µL of 50mM reduced Glutathione
6. Add 20µL of 10X diluted Glutathione Reductase
7. Add 20µL of 10mM H_2O_2
8. Add 20µL of 10mM NADPH
9. Mix solution by rocking cuvette back and forth. Wait one minute for mixing to finish.
10. Add 200µL of sample
11. Press start and record initial rate after 180s.

Appendix II

Spectrum of NADPH absorption over the course of 3 minutes. The spectrum is from a chick embryo brain cell sample.

```
Kinetics Results Report                          Date 4/21/2014 Time 10:00:54  Page   1 of 1

Method file : KINETICS1.M ( modified )     Last update: Date 11/19/2013  Time 2:20:52 PM
Information : Default Method of Kinetics Mode

Data File   : GPX-APRIL4BRAIN1C_1.KD     Created : 4/5/14 9:28:44

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Background correction : none

Run Time    :     180.0 s
Start Time  :       0.0 s
Cycle Time  :       5.0 s

Time Trace:
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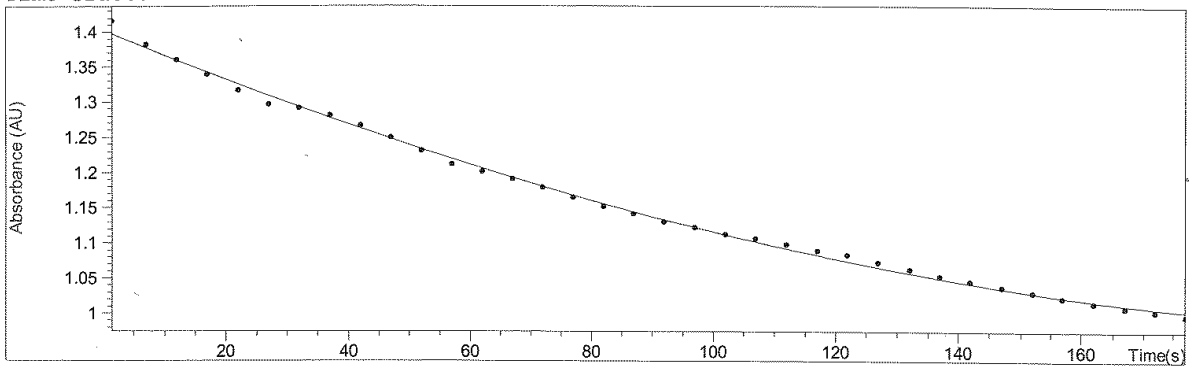


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Rate Calculation Type    :    Initial rate
Calculation Time Range   :    0 s to 180 s

Sample Name      :
Sample Comment   :

WL(nm)    Rate(AU/s)    Std.Dev
---------------------------------
340        -3.6416E-3     7.497E-5


Report generated by : Agilent                              Signature: .....

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                          *** End Kinetics Results Report ***
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Appendix III

Protocol for Lipid Peroxidation (Thiobarbituric Acid Reactive Substances Assay)

1. Turn on the water bath and set for 95^{o}C.
2. Prepare color reagent: weight out 0.3g of TBA and transfer to a 150mL beaker add 20.0mL of 20% (v/v) acetic acid and 20.0mL of 2.5% (w/v) NaOH solution. Stir until the TBA is dissolved. This provides enough color reagent for the standard curve and up to eight samples.
3. Place 0 (blank), 10, 20, 40, 60, 80uL of 300nM malondialdehyde in separate 15mL tubes. Add enough water to each tube to bring the volume up to 100uL.
4. Place 100uL of each sample into separate tubes.
5. Add 200uL of 8% SDS to each tube (blank, standards and samples).
6. Add3.00mL of color reagent to each tube; close the tubes and mix well.
7. Place tubes in the 95^{o} water bath for 60min.
8. After 60min, take the tubes out and cool for 10 min.
9. Centrifuge tubes for 2 min. There should be a white precipitate at the bottom of sample tubes.
10. Pour or decant each assay mixture into separate 3mL cuvettes. For spun down samples pipette 3mL out of tubes and try to avoid disturbing the pellet. All solutions should be at room temperature for absorbance reading.
11. Blank the UV/Vis spectrometer with the reagent blank, and then measure A-532 for the standards and samples.

Appendix IV

Standard Curve used for Protein Concentration

volume sample (ul)	conc in 100 ul (ug/ml)	abs	abs-blank
0	0	0.49254	
20	25.18	0.7077	0.21516
40	50.36	0.83998	0.34744
60	75.54	0.99519	0.50265
80	100.72	1.0501	0.55756
100	125.9	1.1812	0.68866

slope:	0.004595393
intercept:	0.115158