

**TRANSGENERATIONAL EFFECTS OF PERICONCEPTION CADMIUM AND
MERCURY CO-ADMINISTRATION TO MICE ON INDICES OF CHRONIC DISEASE
IN OFFSPRING AT MATURITY**

By

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A DISSERTATION

**Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of**

Animal Science - Doctor of Philosophy

2015

ABSTRACT

TRANSGENERATIONAL EFFECTS OF PERICONCEPTION CADMIUM AND MERCURY CO-ADMINISTRATION TO MICE ON INDICES OF CHRONIC DISEASE IN OFFSPRING AT MATURITY

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The rapidly growing increase in prevalence of adult chronic diseases poses a significant long term threat to health and well being of humans. While the etiology of chronic diseases is complex, a growing body of evidence indicates the *in utero* environment can determine susceptibility of offspring to adult chronic diseases. For the last decade, much emphasis has been placed on impact of nutritional insults. Less attention has been placed on *in utero* developmental programming effects of exposure to environmental contaminants such as the heavy metals, cadmium (Cd) and mercury (Hg). Cadmium and Hg are widely used in various industries with limited understanding of their developmental programming effects. Available data suggest that individual exposure to Cd or Hg throughout pregnancy can impact birth weight, glucose homeostasis and behavior of offspring. Moreover, accumulating evidence suggests that developmental programming effects of adverse *in utero* conditions are not limited to offspring directly exposed to relevant insults and effects can persist in subsequent generations even in the absence of original insult. However, developmental programming effects of combinational exposure to Cd and Hg in the context of administration during early life, even before conception, and their persistent transgenerational effects have not been studied. Therefore, it was hypothesized that periconception co-administration of Cd and Hg increase indices of chronic diseases in offspring at adulthood in subsequent generations. This hypothesis was tested by a) subcutaneously administering Cd and Hg at 0, 0.125, 0.5 and 2 mg/kg body weight doses of each

compound to adult naïve female CD1 mice four days before and four days after conception b) determined anxiety-like behavior, glucose homeostasis, insulin sensitivity, body weight gain and abdominal adiposity, and serum and molecular markers of metabolic syndrome in offspring of control females and females treated with Cd and Hg and c) determined persistent transgenerational effects in F2-F4 generation offspring in the absence of additional heavy metal exposure. Results indicate anxiety-like behavior was increased in male offspring and glucose tolerance was impaired in male and female offspring of Cd plus Hg-treated females. Male offspring of treated females in the F1 generation displayed molecular and endocrine markers of insulin resistance, including significantly higher serum concentrations of insulin and leptin, higher glucose concentrations in response to insulin administration, as well as increased mRNA abundance for genes associated with glucose and lipid homeostasis in liver and decreased mRNA abundance in abdominal adipose tissue. Increased anxiety-like behavior persisted in the F2 generation male offspring descended from the female germ line but not in subsequent generations. Glucose homeostasis was altered in male offspring descended from the maternal germ line through four generations, including impaired glucose tolerance and increased phosphorylation of insulin receptor substrate 1 (IRS1) at serine residue 307, a biochemical indicator of insulin resistance. Increased body weight gain and abdominal adiposity was observed in male but not female offspring through the F4 generation who were descendants of periconception Cd plus Hg-treated females (F0 generation). Based on these results, it is concluded that periconception heavy metal administration to female mice impacts developmental programming of offspring susceptibility to chronic diseases later on adulthood and the effects persist transgenerationally in male offspring and are inherited through the maternal germ line.

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ACKNOWLEDGEMENTS

I am grateful for my supervisor Dr. George W. Smith for his support, guidance and for all the training I have received in his research program. I wish to thank my doctoral committee members Dr. James J. Ireland, Dr. Asgerally T. Fazleabas, Dr. Steven J. Bursian and Dr. Jason G. Knott for their valuable feedback, critical input and suggestions.

I thank Dr. Joe Folger, Dr. Sandeep K. Rajput, Devin McGee and Callie Swanepoel for the various important contributions to this study. My special thanks are also extended to Dr. Hongbing Wang, his former graduate student Dr. Ferzin Sethna and other laboratory members for their help with behavioral analyses.

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KEY TO ABBREVIATIONS

ACACA	Acetyl-coA carboxylase alpha
ATSDR	Agency for toxic substances and disease registry
ACh	Acetylcholine
AChE	Acetylcholinesterase
Akt	Protein kinase B
AUC	Area under the curve
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BPA	Bisphenol A
BTB	Blood-testis barrier
C/EBP α	CCAAT/enhancer-binding protein alpha
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CD36	CD36 molecule (Thrombospondin Receptor)
Cd	Cadmium
CdCl ₂	Cadmium chloride
CdSO ₄	Cadmium sulfate
CH ₃ HgCl	Methylmercury (II) chloride
CYP19A1	Cytochrome P450 aromatase
DBP	Dibutyl phthalate

DDT	Dichlorodiphenyltrichloroethane
DEHP	Di-(2-ethylhexyl) phthalate
DMR	Differentially methylated region
DMT1	Divalent metal transporter 1
DNMTs	DNA methyltransferases
DOHaD	Developmental origins of health and disease
ED	Embryonic day
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental protection agency
ER α	Estrogen receptor alpha
FASN	Fatty acid synthase
FATP2	Solute carrier family 27 (Fatty acid transporter), member 2
FDA	Food and drug administration
FOAD	Fetal origin of adult disease
FSH	Follicle stimulating hormone
G6PC	Glucose-6-phosphatase
GLUT4	Glucose transporter type 4
h	Hours
HbA1c	Hemoglobin A1C
HFD	High fat diet
Hg	Mercury
HgCl ₂	Mercuric chloride
HPRT	Hypoxanthine phosphoribosyltransferase

3 β -HSD	3 β -Hydroxysteroid dehydrogenase
17 β -HSD	17 β -Hydroxysteroid dehydrogenase
ip	Intraperitoneal
IGF2	Insulin-Like Growth Factor 2
IRS1	Insulin receptor substrate 1
JECFA	Joint Food and Agriculture Organization and the World Health Organization Expert Committee on Food Additives
JNK	c-Jun NH2-terminal kinase
LH	Luteinizing hormone
MAO	Monoamine oxidase
MeHg	Methylmercury
miRNA	microRNAs
MS	Metabolic syndrome
MT	Metallothionein
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NAFLD	Nonalcoholic fatty liver disease
ncRNA	Non-coding RNAs
P450scc	Cholesterol side-chain cleavage enzyme
P45017 α	17 α -hydroxylase-C17,20-lyase
Pb	Lead
PCOS	Polycystic ovary syndrome
PEG1/MEST	Paternally expressed gene 1/mesoderm specific transcript
PI3K	Phosphatidylinositol-3-kinase

PGCs	Primordial germ cells
PKC	Protein kinase C
POMC	Proopiomelanocortin
PPAR γ	Peroxisome proliferator-activator receptor gamma
PPAR γ 2	Peroxisome proliferator-activator receptor gamma-2
ppm	Parts per million
PTWI	Provisional Tolerable Weekly Intake
ROS	Reactive oxygen species
RXRA	Retinoid x receptor alpha
sc	Subcutaneous
StAR	Steroidogenic acute regulatory protein
TBT	Ttributyltin
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TNF α	Tumor necrosis factor alpha
ZT	Zeitgeber time

CHAPTER 1
INTRODUCTION

The recent increase in prevalence of adult chronic diseases (e.g. diabetes, cardiovascular disease) has significant implications for health and well being of the population and increases economic burden of health care [1, 2]. Despite the complex etiology of adult chronic diseases, the increase in prevalence might be attributed, at least in part, to inappropriate environmental conditions *in utero* (e.g., nutritional deprivations, exposure to toxic chemicals). This idea is in line with the theory of thrifty phenotype, which states that inappropriate environmental changes during early development result in adverse health effects and chronic conditions later in life, such as coronary heart disease, stroke, diabetes and hypertension [3]. The Dutch Hunger Winter during the Second World War provided key information for the understanding of developmental origins of health and diseases [4]. Examination of health records revealed that individuals born to mothers who conceived during the famine winter demonstrated higher risk of obesity later in adult life. Likewise, offspring of these individuals born to mothers experiencing the famine displayed reduced birth weight, increased adiposity and poor health quality in adulthood [5, 6].

Cadmium (Cd) and mercury (Hg) are naturally found in the environment in trace amounts with no known biological benefits [7, 8]. Human activities are the major source of Cd and Hg release into the environment. Their widespread use in industry, presence in pharmaceuticals, pesticides and cosmetics as well as combustion of fossil fuels, incineration of solid wastes and as a byproduct of mining activities pollute terrestrial and aquatic ecosystems where they bioaccumulate in the food chain [7, 9, 10]. Therefore, human exposure to Cd and Hg occurs mainly via dietary consumption of vegetables, livestock and fish [8]. Available evidence obtained from both human epidemiological studies and rodent models suggest developmental programming effects of *in utero* Cd and Hg exposure. For instance, a positive association has been demonstrated (in previous epidemiological studies) between gestational chronic low dose

exposure to Cd or Hg through diet or smoking and reduced birth weight [11], impaired glucose homeostasis and insulin resistance [12], and behavioral deficiencies in children [13]. Likewise, long term exposure to Cd or Hg during the entire gestation or after embryo implantation in rodent models at varying doses (0.5-10 mg/kg body weight) resulted in similar developmental programming effects in offspring at adulthood, including altered behavior and neurological functions [14, 15], reduced fertility [16] and cardiovascular problems [17].

Over recent decades, potential persistent transgenerational effects of adverse early life conditions on subsequent generations have gained more interest, even when the original maternal insult disappeared in the subsequent generations. Available data on transgenerational developmental programming is generally derived from maternal dietary manipulations or exposure to toxic chemicals, including BPA [18], vinclozolin [19] and pesticides [20]. Results of such studies revealed impaired fertility [21], behavioral alterations [22] and increased risk of obesity [23], type-2 diabetes [24] and cardiovascular problems [25] in the subsequent generation(s) of offspring at adulthood.

In association with increased education and public consciousness, many women change their life style upon becoming aware of their pregnancy. However, exposure to toxic chemicals via maternal life style before detection of pregnancy might have adverse effects on the developing fetus and even persist in subsequent generations. Moreover in real life, there is a simultaneous exposure to multiple toxic chemicals. However, current data in the literature are primarily derived from individual exposure to toxic chemicals throughout the majority of gestation and/or lactation. Therefore, there is a gap in understanding of combinational administration of multiple chemicals in the context of early life and even before conception. Likewise, potential transgenerational effects of *in utero* Cd and Hg exposure have not been

established. Studies reported here are focused on acute and transgenerational developmental programming effects of combinational administration of Cd and Hg at environmentally relevant doses during the periconception period of pregnancy and the implications for susceptibility to chronic diseases in adulthood, including anxiety-like behavior, obesity and type-2 diabetes.

The following sections of this literature review will discuss toxicological profiles of Cd and Hg and their developmental programming effects on indices of chronic diseases in response to early life exposure. Their effects on health and well being in response to adult exposure of humans and rodents are also highlighted. Transgenerational inheritance of developmental programming effects of adverse *in utero* environmental conditions obtained from human epidemiological studies and rodent models of nutritional manipulations and exposure to various toxic chemicals are also outlined. Potential epigenetic mechanisms mediating the effects of *in utero* insults are described, but the reader is referred to other reviews [26, 27] for a detailed discussion of epigenetic transgenerational inheritance.

CHAPTER 2
LITERATURE REVIEW

OVERVIEW OF CADMIUM CONTAMINATION: SOURCES AND FORMS

Cadmium (Cd) is a toxic metal found in the earth's crust naturally at an abundance of 0.1-0.5 parts per million (ppm) [10, 28]. It was discovered by German chemist F. Strohmeier in 1817 [29]. In the environment, it is usually found together with sulfide ores of zinc, lead and copper or with other elements, such as chloride [10, 30]. Cadmium compounds (oxide, sulfate, chloride) in the atmosphere are chemically stable and do not undergo significant transformation. Cadmium can be distributed in the air globally to distant regions and deposited into terrestrial and aquatic sources that persist for longer duration [28]. In the soil, Cd generally bonds strongly with organic compounds that immobilize it for longer periods of time. However, in surface and groundwater, Cd can either complex with organic and inorganic substances to become immobilized or it can be found as a mobilized ionic form that can be distributed to remote deep water [28].

Even though Cd is naturally found in the environment at lower concentrations, it is mainly released into the atmosphere via human activities including industrial uses, combustion of fossil fuels (coal, oil, gas and wood), and discharge of wastewater and solid wastes [10]. It is also produced as a by-product from mining, smelting and refining sulfidic ores of zinc, lead or copper [28, 31]. In addition, phosphate fertilizers contain Cd at varying concentrations from trace amounts to as high as 300 mg Cd/kg dry product weight [32]. As a result of increased application and longer periods of fertilization, Cd concentration increases in the soil resulting in greater uptake by plants [33]. Cadmium is widely used in industry worldwide [34] and most commonly during the production of nickel-cadmium batteries [35]. Moreover, it is used in pigments and stabilizers for plastics, plating on iron and steel, and in nonferrous alloys of lead, copper and tin [28]. In the United States alone, annual Cd production has been estimated at approximately 600

tons [36]. There is a worldwide demand for Cd and global industrialization increases Cd release that eventually accumulates in the ecosystem. Cadmium is also used in advanced technology products in the form of nano-particles that exhibit great promise for medical imaging, cancer treatment and drug delivery to target tissues, which further increases Cd demand. However, at the same time, Cd released from these products poses increased risk of toxicity via direct delivery to cells and biological systems [9, 37].

In conclusion, Cd is deposited in the soil and can contaminate streams and aquatic sources leading to bioaccumulation in plants, animals and fishes. As a result, humans are exposed to Cd in daily life from multiple sources. Coupled with its long biological-half-life, Cd build-up in the body over time can increase the risk of adverse health effects [38].

Route of Cadmium Exposure

There are several ways the general population is exposed to Cd, including dietary intake, inhalation of Cd containing ambient air, ingestion of contaminated water as well as primary and second-hand smoking [39]. Conversely, dermal absorption of Cd is very limited [28]. Depending on the size of the particle, 10-50% of inhaled Cd dust or 5-10% of ingested Cd is absorbed into the blood stream. There are certain conditions that increase the intestinal absorption of Cd, including nutritional deficiencies of iron, zinc or calcium. Therefore, Cd absorption can be higher in women due to their tendency for lower iron stores compared to men [30, 40].

Cadmium has a high soil-to-plant transfer rate and is absorbed by plant roots and carried to plant components above the ground [41, 42]. As a result, tobacco and many plant-derived food items contain higher amounts of Cd, including lettuce, spinach, potatoes, grains, peanuts, soybeans, sunflower seeds, cocoa and mushrooms [28, 42]. Likewise, Cd is accumulated from the soil during grazing of livestock and ingested into humans via consumption of animal

products, especially organ meats such as liver [43]. Similar to terrestrial environments, Cd also enters aquatic ecosystems mainly through anthropogenic activities and bioaccumulates in the aquatic food chain [44]. Reports indicate accumulation of Cd in several types of fish and shellfish, with high Cd concentrations detected in clams, mussels, squid, octopus and anchovies [45].

Tobacco plants are able to take up higher levels of Cd from the soil and Cd accumulates in tobacco leaves resulting in a higher body burden of Cd in smokers compared to non-smokers. [46]. Every cigarette contains approximately 1-2 μg of Cd. Approximately 10% of Cd in each cigarette is inhaled and 50% of the inhaled Cd is absorbed into the blood [36, 47]. As a result, an average of 1 μg of Cd can be absorbed into the bloodstream of a person who smokes one pack of cigarettes per day [47]. For comparison, dietary Cd absorption for an average non-smoker is estimated to be 1-3 μg per day [47]. As a result, smoking contributes similar amounts of Cd accumulation as that derived from the daily diet, and in turn significantly increases the body burden of Cd in smokers [47].

Absorption and Distribution

There are multiple transporters along the intestinal wall that are required for the absorption of ingested essential metals such as calcium, zinc, selenium, chromium and iron, into the circulation, [41, 48]. Similar to other essential nutrients, ingested Cd requires an efficient intestinal absorption mechanism for transport to systemic circulation and target organs. Divalent metal transporter 1 (DMT1) is one of the most common metal transporters and belongs to a protein-coupled metal ion transporter family. DMT1 is found at the apical membrane of the small intestine and is required for the transport of ingested essential metal nutrients. However, DMT1 also has a high affinity for other divalent cations, such as Cd, manganese, cobalt, nickel,

lead and copper [49]. Therefore, deficiency of any essential metal can result in increased absorption of Cd and other toxic metals through the digestive system [48, 50, 51].

Exposure to Cd and other divalent metals can induce metallothionein (MT) gene expression as a means to promote clearance. MT is a small, cysteine-rich protein synthesized mainly by the liver and kidney [52]. MT is not involved in gastrointestinal absorption of heavy metals, but plays a major role in tissue retention [52]. Therefore, MT bound heavy metals are accumulated primarily in liver and kidney, but especially in the case of elevated exposure, they can be stored in various organs and tissues including reproductive organs, lungs, pancreas, thyroid, heart, muscle, brain, eyes and bones [41, 53, 54]. This is a required step for preventing systemic toxicity and protecting essential cellular functions to improve survival [55]. However, MT induction and binding of heavy metals is also responsible for the enhanced biological half-life of Cd and other heavy metals that can result in increased cellular damage [55].

Absorption of Cd from the digestive system and its distribution into organs for sequestration has been shown previously in rodent studies. Mice fed an iron (Fe) deficient diet for 4 weeks had increased expression of intestinal DMT1 transporters [56]. Intestinal uptake of Cd was significantly increased in Fe-deficient rats following a single oral administration suggesting DMT1 is a non-specific metal transporter for essential and toxic metals [56]. In a similar manner, administration of Cd to mice via drinking water or food at doses of 30-300 ppm resulted in a dose-dependent accumulation in liver and kidney of wild-type and MT knockout (MT-null) mice [57]. However, following 6 months of exposure, tissue Cd levels of MT-null mice were just one-fifth of those in wild-type mice, but animals exhibited severe renal injury [57]. These results demonstrated a protective role of MT proteins even though they increase tissue retention and biological half-life of heavy metals [57].

Safety Levels and Cadmium Exposure during Pregnancy

Cd has no known biological function and bioaccumulation has increased in parallel to global industrialization. The biological half-life of Cd is estimated to be approximately 19 and 38 years in the liver and kidney, respectively [58] indicating the lack of efficient ways to eliminate it and raising potential concerns about acceptable exposure levels.

In 1989, the Joint Food and Agriculture Organization and the World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) established a Provisional Tolerable Weekly Intake (PTWI) level for Cd at 7 $\mu\text{g}/\text{kg}$ body weight corresponding to a daily Cd intake of 70 μg for a 70 kg body weight person [59]. However, a growing amount of data suggest significant human health risk associated with previously determined safety levels for Cd intake. Therefore, in 2010, JECFA updated the reference level for provisional tolerable monthly intake (PTMI) of Cd to 25 $\mu\text{g}/\text{kg}$ body weight, which corresponds to weekly Cd intake of 5.8 $\mu\text{g}/\text{kg}$ body weight [60]. On the other hand, the European Food Safety Authority (EFSA) established a lower weekly intake of Cd at 2.5 $\mu\text{g}/\text{kg}$ body weight [39]. The variation in the established tolerable levels of Cd intake among regulatory agencies suggests that there is a lack of consensus regarding safe levels of exposure to Cd and a need for further research.

A growing body of evidence from epidemiological studies suggests that dietary intake or blood levels of Cd commonly exceed recommended safety levels [61-63]. For instance, a study conducted on 67,000 individuals from 22 different countries in the European Union revealed that 14.8%-31.2% of the subjects have weekly dietary Cd intake levels ranging from 2.65-4.99 $\mu\text{g}/\text{kg}$ body weight and exceeding safety levels established by EFSA at 2.5 $\mu\text{g}/\text{kg}$ body weight per week [61]. Likewise, long term Cd uptake by Korean children up to 6 years of age was estimated to be 2.66 $\mu\text{g}/\text{kg}$ body weight per week, which exceeded reference values [62]. According to the

National Health and Nutrition Examination Survey conducted between 2003 and 2004, the average blood Cd concentrations of non-pregnant American women of childbearing age is 0.33 µg/l, and among pregnant women the average Cd concentration is 0.22 µg/l [64]. However, a recent cohort study conducted with 1854 women in North Carolina during the period of 2005-2010 revealed more than 50% of non-smoking women at childbearing age had elevated blood Cd concentrations that exceeded the average concentration of 0.33 µg/l [63].

ASSOCIATION OF CADMIUM EXPOSURE WITH CHRONIC DISEASES

In the general population, life-time Cd exposure occurs via multiple sources and it accumulates in organs over time due to its long biological half-life [7]. Several studies conducted on both humans and animals investigated long-term health effects of Cd exposure. Human epidemiological studies provide evidence of altered kidney and liver function [65], reduced fertility and accumulation of Cd in follicular fluid [53, 66]. Moreover, blood Cd concentrations of premenopausal women were positively associated with elevated testosterone and inversely correlated with estradiol concentrations [67]. A positive association between blood and urinary Cd levels through diet or smoking and high incidence of atherosclerosis [68], elevated systolic blood pressure [69], elevated fasting glucose levels and type-2 diabetes [70], and chronic kidney disease [71] have also been reported previously in adults.

Similar tissue bioaccumulation of Cd has been reported in rodent studies. Chronic cadmium chloride (CdCl₂) administration to mice for 6 months at doses of 30-300 ppm through drinking water or food resulted in a dose-dependent Cd accumulation in liver and kidney [57]. Likewise, elevated concentrations of Cd have been reported in kidney, liver, spleen, muscle, ovaries and testes of mice 48 hours (h) following intraperitoneal (ip) administration of CdCl₂ for two days at doses of 0.25 and 0.5 mg/kg body weight [72]. Previous studies demonstrated an

association between serum Cd concentrations and reduced oocyte yield in superovulated mice [73]. Likewise, chronic administration of Cd to female rats at doses of 1, 2 or 5 mg/kg body weight via gastric tube for 7 weeks resulted in significant Cd accumulation in the uterus and placenta and increased the abundance of metal transporter DMT1 in the placenta suggesting a potential route for Cd transport to the developing fetus [74]. Adverse effects of Cd on pulmonary function have also been reported in a mouse model utilizing short term inhalation exposure for 7 days (3 h/day) at a dose of 240 $\mu\text{g Cd/m}^3$ [75].

In conclusion, the above evidence substantiates the association of Cd exposure in the general population with the risk of potential effects on health and well-being and suggests potential direct effects of Cd exposure. The long biological half-life of Cd increases the risk associated with maternal exposure and subsequent impacts on offspring at adulthood, but the mechanism of maternal-fetal Cd transport and subsequent effects in the developing embryo still remains unclear.

Maternal Cadmium Exposure as a Possible Cause of Chronic Diseases

Since an association between adult Cd exposure and the onset of chronic diseases has been shown previously, Cd exposure is likely to also have health consequences for offspring exposed maternally [70, 75]. However, impacts of maternal and fetal Cd exposure on incidence of chronic diseases in offspring at adulthood are not well known.

Maternal intestinal nutrient absorption is increased during pregnancy due to nutritional demands of the developing fetus. Moreover, the higher requirement for iron and other essential nutrients during pregnancy up-regulates intestinal metal transporters, such as DMT1 [76, 77]. As a result, heavy metals that share transporters with other essential nutrients can compete and get absorbed into the maternal circulation at the expense of essential nutrients. Therefore, pregnant

women can take up more Cd and other heavy metals via such transporters relative to non-pregnant individuals [78-80]. As a result, exposure to Cd during gestation inhibits placental transfer of essential nutrients and can adversely influence fetal growth and metabolism [81]. Such a relationship has been shown in previous epidemiological studies demonstrating that maternal and cord blood Cd concentrations are inversely correlated with infant birth weight [82-84]. A similar association has been established between reduced birth weight and maternal smoking during entire gestation [11, 85]. Long term studies to examine the effects of prenatal Cd exposure on the health of children prior to puberty demonstrated significant impacts including, elevated leptin concentrations [86], neural tube defects [87], behavioral problems [88], reduced IQ scores [89], changes in adiposity [90] and body weight [84], and increased risk of upper respiratory diseases [91].

Reduced birth weight has also been reported in rodent models treated daily with varying doses of CdCl₂ (0.5-2 mg/kg body weight of Cd) during the entire gestation period [92, 93] or during the third trimester only [16]. Moreover, maternal Cd exposure might cause fetal impairment and growth restriction via increased oxidative stress and production of free radicals [94]. Embryonic lipid peroxidation was increased in response to subcutaneous (sc) administration of 4 mg/kg body weight CdCl₂ from gestational day 7 to gestational day 9 [95]. Similarly, 24 h after administering the same amount of CdCl₂ to pregnant mice on gestation day 8, significant DNA damage and upregulated apoptotic genes, including p53, p21 and Bax, were noted in embryos [96].

Maternal and fetal Cd exposure can impact numerous physiological parameters in neonatal and adult life such as, behavior, adiposity and hormonal regulation of metabolism. Such developmental programming effects may be mediated via increased fetal exposure to

glucocorticoids [97]. Intraperitoneal CdCl₂ administration at a dose of 0.5 mg/kg body weight to female rats on days 5-19 of gestation induced components of preeclampsia, including hypertension, proteinuria, placental abnormalities and fetal growth restriction. Such changes were linked to Cd-induced local production of placental and fetal corticosterone that promoted formation of preeclamptic placentas [92]. This effect on glucocorticoid production was achieved via increased expression of enzymes 21-hydroxylase (CYP21) and 11 β -hydroxylase (CYP11B1) that are essential for glucocorticoid synthesis [92]. Similarities in the ionic structure of Cd and calcium (Ca) suggest Cd could possibly mimic the stimulatory effects of Ca ions on steroidogenic pathways and induce corticosteroid synthesis via presence of Cd in the maternal circulation [92]. Likewise, exposure of pregnant rats to Cd (50 ppm in drinking water) throughout the entire gestation decreased offspring birth weight and increased maternal and fetal corticosterone concentrations without any detectable heavy metal accumulation in fetal tissues [98].

In conclusion, current data support developmental programming effects of Cd exposure during pregnancy. However, studies examining impacts of Cd exposure during gestation on incidence of chronic disease in offspring at adulthood are more limited, especially in the context of administration only during the very early stages of pregnancy around the time of conception and before the formation of a functional placenta.

Effects of Cadmium Exposure on Glucose Homeostasis

Maintenance of glucose homeostasis is a complex process that requires the interaction of several organs involved in the control of metabolism. Following a meal, insulin is released from pancreatic β -cells and binds to receptors on liver, adipose tissue or skeletal muscle to promote uptake and utilization of glucose present in the circulation [99-101]. Disruption of glucose

homeostasis leads to the onset of diabetes [102]. Substantial evidence supports a positive association between toxic chemical exposure and impaired glucose tolerance and insulin resistance leading to type-2 diabetes. Epidemiological studies in adults demonstrated a correlation between urinary Cd concentrations and altered fasting blood glucose [70] and serum hemoglobin A1c (HbA1c) concentrations[103]. Similarly, elevated fasting insulin concentrations and insulin resistance have been observed in children of smokers frequently exposed to second hand smoke [12].

Exposure to Cd in early developmental stages is also of great potential concern because of higher sensitivity of the developing fetus to chemical toxicity [104] and the potential impacts on glucose homeostasis and insulin resistance. However, there is limited information on the contribution of early life exposure to Cd to the onset of diabetes in adult life. Chronic exposure of neonatal rats to 0.1 and 1.0 $\mu\text{g/g}$ CdCl₂ for 45 days via oral gavage impaired glucose homeostasis, reduced liver glycogen stores via enhanced gluconeogenesis and suppressed glucose-induced insulin release from the pancreas at adulthood [105]. Evidence also suggests a functional impairment in pancreatic function in response to Cd exposure leading to a decline in insulin levels [106, 107]. Such association has been shown in smelter workers who are occupationally exposed to elevated concentrations of Cd and had reduced serum insulin concentrations compared to the nonoccupationally exposed individuals [108]. Incubation of primary human islet cells with CdCl₂ (0.1 and 1.0 $\mu\text{mol/L}$) corresponding to the levels detected in healthy non-diabetic human pancreas caused a dose-dependent islet Cd accumulation [109]. Accumulated Cd in human primary islet cells impaired β -cell functions without significant change in cell viability or increased oxidative stress [109]. Likewise, sc Cd administration to rats at doses of 1 or 2 mg/kg body weight resulted in Cd accumulation in the pancreas, impaired

glucose tolerance and reduced insulin mRNA expression [107]. These studies show the detrimental effects of Cd exposure on insulin producing pancreatic β -cells at environmentally relevant doses.

Evidence suggests a direct impact of Cd exposure on cellular signaling mechanisms in metabolic tissues, such as liver and adipose tissue that impairs glucose homeostasis. The Cd-induced reduction in glucose tolerance in rats has been linked to a dose-dependent decline in glucose transporter type-4 (GLUT4) protein and a reduction in insulin-induced glucose uptake in adipocytes [110]. Moreover, adult male rats treated with 9.7 mg/L dose of Cd in drinking water for 6 weeks displayed a reduced number and density of insulin receptors in epididymal adipose tissue revealing additional diabetogenic effects of chronic Cd exposure [111].

Effects of Cadmium Exposure on Obesity and Metabolism

Obesity has reached global epidemic proportions and imposes a significant risk for health and well being of individuals. Disruption of metabolism causes a cluster of conditions including increased blood pressure, altered glucose homeostasis and increased adiposity leading to the onset of hypertension, diabetes and obesity [112, 113]. This condition is termed metabolic syndrome (MS) and there are several factors responsible for the onset of MS, including high calorie diet and sedentary life style [114-116]. Available evidence demonstrates exposure to toxic chemicals is a significant contributing factor to the onset of MS and obesity. For example, an increased incidence of MS has been observed in portions of the Korean population with elevated Cd concentrations in blood serum [117]. Maternal blood Cd concentrations can also impact indices of fetal metabolic functions, including fetal leptin and adiponectin concentrations [86]. Considering the higher Cd concentrations above the recommended safe doses in the

majority of women at childbearing age [63], results support the potential for metabolic problems in offspring mediated via maternal Cd exposure. However, direct evidence is limited.

Cadmium exposure can impact adiposity via disturbing cholesterol and fatty acid metabolism. Oral administration of 1.5 mL/kg body weight cadmium sulfate (CdSO_4) in drinking water to adult rats for 4 weeks caused increased free fatty acid, total cholesterol, triglycerides, low density lipoprotein-cholesterol, and serum albumin concentrations and reduced high density lipoprotein-cholesterol concentrations [118]. Similar Cd-induced alterations in lipid metabolism have been observed following long term administration of CdCl_2 to rats at doses of 5 or 50 mg/kg body weight in drinking water for 6 months [119].

Similarly, exposure of adults to Cd might alter expression/production of adipokine factors released from adipose tissue that are linked to obesity, insulin resistance and type-2 diabetes. Treatment of adult rats for 7 days with 0.5 or 0.75 mg/kg body weight of CdCl_2 reduced serum concentrations of leptin and decreased leptin mRNA expression in white adipose tissue [120]. Likewise, treatment of cultured adipose-tissue derived stromal cells (obtained from 5-day-old male mice) with CdCl_2 at varying doses (0, 1, 5, 10, 50 and 100 μM) resulted in increased triglyceride release into culture medium and reduced transcript abundance for genes required for fatty acid synthesis and hydrolysis of lipids, including fatty acid synthase (FASN), acetyl-coA carboxylase alpha (ACACA) and peroxisome proliferator-activated receptor gamma ($\text{PPAR}\gamma$) [120]. Results suggest Cd treatment impairs adipocyte metabolism linked to development of MS, including obesity, insulin resistance and type-2 diabetes [120]. Moreover, abnormal white adipocyte differentiation and function has been reported in response to sc administration of 5, 10 or 20 $\mu\text{mol/kg}$ body weight of Cd to adult mice. In such studies, reduced transcript abundance for genes involved in adipocyte differentiation and hypertrophy, including peroxisome receptor

gamma 2 (PPAR γ 2), and paternally expressed gene 1/mesoderm specific transcript (PEG1/MEST) was observed [121]. In this study, Cd accumulated in white adipose tissue and reduced mRNA expression for the adipose-derived hormones adiponectin and resistin was observed and linked to Cd-induced altered function of white adipocytes [121]. Moreover, treatment of 3T3-L1 preadipocyte cell lines with 3 μ M CdCl₂ suppressed mRNA expression for key transcriptional activators of adipogenesis, including CCAAT/enhancer-binding protein alpha (C/EBP α) and PPAR γ [122]. Results provide evidence for effects of Cd on cellular differentiation and signaling pathways in adipocytes linked to altered adipocyte metabolism and function.

Effects of Cadmium Exposure on Male Reproductive System

With the advent of technology and industrialization, human exposure to chemicals in daily life has increased steadily. In recent years, effects of toxic chemical exposure on male fertility have gained increased interest [123]. Likewise, data obtained from rodent models demonstrated negative effects of Cd exposure during adulthood on male reproduction manifested as reduced sperm concentration and motility, increased germ cell apoptosis and altered steroid biosynthesis [124-126].

Cadmium is an endocrine disrupting compound and can alter steroid biosynthesis. Cadmium administration to adult Wistar rats at a dose of 2.5 mg/kg body weight for 4 weeks decreased testes weights, and serum testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations [127] but its mechanism of action in regulating steroidogenesis is unclear. One of the rate limiting steps in steroid biosynthesis is cholesterol transport from the cytoplasm into the mitochondria that is mediated by steroidogenic acute regulatory protein (StAR) [128]. Mitochondrial cholesterol transport is under the control of LH

that increases the intracellular cyclic adenosine monophosphate (cAMP) concentrations via activation of adenylate cyclase [126, 129, 130]. Indeed, incubation of Leydig cells with Cd caused a decline in progesterone production and mitochondrial membrane potential with increased doses suggesting a direct impact of Cd on steroidogenic cells [126]. Moreover, incubation of Leydig cells with Cd also caused a decline in intracellular concentrations of cAMP [126]. Adult male rats treated with 2.5 mg/kg body weight of CdCl₂ for 4 weeks displayed a decline in the activities of testicular steroidogenic enzymes, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) [127]. Similar reduced enzyme activities of 3β-HSD and 17β-HSD were also observed in adult male rats treated with Cd acetate for 15 days at a dose of 0.025 mg/kg body weight [131].

A Cd-induced decline in Leydig cell testosterone production may also be linked to increased oxidative stress [132]. Wistar rats administered a single sc injection of 5 μmol/kg CdCl₂ displayed apoptosis markers in the testis, including suppression of p53 and overexpression of c-jun mRNA 48 and 96 h following Cd treatment [125, 133]. Testicular apoptosis was also detected in these Cd-treated animals [125]. In a similar manner, studies of Cd-induced cytotoxicity and genotoxicity in cultured rat Leydig cells revealed reduced cell viability via increased concentrations of malondialdehyde and activity of glutathione peroxidase resulting in higher numbers of single strand DNA breaks [132]. On the other hand, Cd exposure might induce apoptosis of germ cells linked to reduced male fertility. Germ cell survival is regulated by a glycoprotein clusterin that is produced by Sertoli cells [134]. Clusterin influx into germ cells via voltage-gated Ca channels is a cell death signal. Using an *ex vivo* cultured mouse testis model, administration of 10 μM Cd ions for 24 h to the culture medium increased clusterin production and secretion suggestive of Cd-induced germ cell damage [134]. Male rats treated

with 5, 50 or 100 mg/L of Cd for 8 weeks in drinking water beginning at puberty displayed a dose-dependent reduction in sperm motility [124]. Moreover, sperm obtained from dissecting epididymes of these Cd-treated rats exhibited altered transcript abundance for L-type voltage-dependent Ca channel isoforms present in sperm tail regulating Ca and Cd influx. Therefore these results manifest a link between reduced sperm motility and Cd exposure via voltage regulated Ca channels [124].

The blood-testis barrier (BTB) is considered one of the tightest blood-tissue barriers that divide the seminiferous epithelium into the basal and apical compartments [135]. Sertoli cells covering the walls of the seminiferous epithelium are required for the regulation of all aspects of germ cell development [134]. Therefore, disrupting the integrity of the BTB and Sertoli-germ cell adhesion might have negative impacts on spermatogenesis leading to infertility [131, 136]. Nectin-2 is a junction protein required for the formation of the BTB and Sertoli-spermatid adhesion and Nectin-2 knockdown causes infertility [136, 137]. Treatment of *in vitro* cultured mouse Sertoli cell lines with 20 μ M CdCl₂ suppressed Nectin-2 expression both at transcriptional and post-transcriptional levels [136]. Further molecular analyses by EMSA and ChIP assays demonstrated an inhibition of binding of positive regulators to the Nectin-2 promoter in response to Cd treatment [136]. Moreover, Cd treatment of a cultured mouse Sertoli cell line promoted Nectin-2 protein degradation and its endocytosis indicating Nectin-2 is a direct molecular target of Cd linked to impaired spermatogenesis [136]. Cadmium administration can also impact BTB integrity through targeting proteins required for its maintenance, such as claudin-11 [138]. These results support a change in the localization of cell adhesion proteins leading to the disruption of Sertoli cell-BTB integrity and function [139].

Impacts of Cd exposure during adult life on indices of male fertility have been well documented in the literature. However, there is less known about impacts of Cd exposure during fetal life on male reproduction. Treatment of cultured human fetal testis (recovered during the first trimester) with 1 μM CdCl_2 caused reduced germ cell numbers without any impact on testosterone production [140]. Maternal administration of CdCl_2 to pregnant mice at 0.5 mg/kg body weight dose from gestational day 13-17 caused a decline in fetal serum testosterone concentrations and downregulation of mRNA expression for StAR, P450scc, P45017 α and 17 β -HSD in fetal testis with only trace amounts of fetal Cd accumulation [16]. Moreover, decreased serum and testicular testosterone concentrations and reduced transcript abundance of testicular P450scc were persistent in offspring at adulthood exposed to Cd via maternal administration during late pregnancy [16]. Studies support developmental programming effects of maternal Cd exposure on male fertility in adult life.

Effects of Cadmium Exposure on Female Reproductive System

Available evidence suggests Cd has pronounced effects on the female reproductive system and can compromise follicular growth and differentiation, and alter steroidogenesis [141-143]. However, there is still less known about the impact of maternal Cd exposure on fertility of female offspring. Toxic effects of Cd on the adult female reproductive system have been observed previously in human and animal models [144, 145]. Significantly higher concentrations of Cd are present in follicular fluid of smokers compared to non-smokers [53]. Evidence also supports suppressive effects of Cd exposure on oocyte growth and maturation [143, 146]. Numbers of superovulated oocytes that reached metaphase II stage were significantly reduced in female rats sc administered a single dose of CdCl_2 (0.02-0.08 mmol/kg body weight) [146]. Similar results were obtained when *in vitro* cultured ovine oocytes were treated with 2.0 μM

CdCl₂ resulting in reduced maturation rate to metaphase II stage, decreased number of fertilized oocytes as well as increased polyspermy [143].

Different *in vitro* and *in vivo* models have been utilized to investigate whether changes in steroidogenesis are potentially responsible for the observed inhibitory effects of Cd on oocyte growth and maturation. Oocyte-cumulus complexes isolated from large antral porcine follicles displayed a suppression of FSH-induced cumulus expansion in response to incubation with Cd at doses of 10⁻⁶, 10⁻⁵ and 0.5x10⁻⁴ M [147]. Incubation of Cd-treated oocyte-cumulus complexes with FSH for 42 h was associated with decreased progesterone production suggesting a potential link to suppressive effects of Cd on cumulus expansion [148]. Cultured rat ovarian follicles treated with 1.6 µg/mL CdCl₂ on day 2 of culture, which corresponds to the pre-antral stage of follicle development, resulted in reduced follicle survival rate and increased morphological abnormalities [141]. Moreover, treatment of cultured rat ovarian follicles with the same dose of Cd increased the percentage of oocytes arrested at the germinal vesicle stage [141].

Current evidence demonstrates a higher incidence of chronic diseases, including diabetes and obesity in human populations [149, 150]. Therefore, diabetes and obesity in women of child bearing age is becoming more common. To determine the interaction of Cd exposure and diabetes on the female reproductive system, insulin-resistant rats were administered 0.05 mg/kg body weight of Cd acetate for 28 days, resulting in prolonged duration of estrus and reduced serum estradiol concentrations [151]. Histological examination of the ovaries revealed the absence of mature follicles in the Cd-treated insulin-resistant female rats [151]. In addition to these effects, mRNA and protein concentrations for steroidogenic enzymes, StAR, 17β-HSD and cytochrome P450 aromatase (CYP19A1) were significantly reduced in the ovaries of Cd-treated

insulin-resistant rats [151]. Results support impact of Cd exposure on the female reproductive system.

Effects of Cadmium Exposure on Behavior

Available evidence suggests that Cd exposure can cause behavioral alterations [54, 152, 153]. Long term dietary Cd treatment of rats at doses of 1, 5 or 25 mg/kg body weight for 5 months elevated anxiety-like behaviors in the elevated plus maze test [54]. Investigation of brain regions involved in the regulation of anxiety-like behavior, including cerebral cortex, hypothalamus and cerebellum in such animals revealed reduced acetylcholinesterase (AChE) and Na⁺-K⁺-ATPase activity following Cd administration [54]. AChE and Na⁺-K⁺-ATPase are involved in neurite outgrowth and synaptic neurotransmission, respectively, and impairment of their functions is linked to memory deficits and anxiety-like behavior [153]. One hour after acute ip CdCl₂ administration to adult rats at doses of 1, 2 or 3 mg/kg body weight, increased anxiety-like behavior and depression were observed in a dose-dependent manner in open field, force swimming and Morris water maze tests [152]. These behavioral alterations have been attributed to Cd-induced increase in lipid peroxidation and decreased superoxide dismutase activity in the brain of treated rats [152].

While less information is available, evidence suggests maternal and lactational Cd exposure might have significant impacts on behavior and accompanying changes in neurotransmitters in specific brain regions of offspring. For instance, long-term combined administration of 10 mg/L of Cd and 300 mg/L of lead (Pb) in drinking water to pregnant rats during pregnancy and lactation increased anxiety-like behavior of offspring in the elevated plus maze test at 75 days of age [154]. These behavioral changes were linked to deficits in dopaminergic and serotonergic systems of the hippocampus [154]. Developmental effects of

prenatal Cd exposure during lactation have also been examined in rat offspring whose dams were exposed to 5 ppm CdCl₂ via drinking water during three weeks of lactation period. [155]. In these studies, Cd treatment did not affect the concentrations of acetylcholine (ACh), dopamine and norepinephrine. However, lactational Cd exposure significantly reduced cortical concentrations of serotonin suggesting impacts of early life Cd exposure on the serotonergic system [155]. These results suggest that fetal or adult Cd exposure can cause behavioral alterations through impacts on specific neurotransmitters/brain regions.

OVERVIEW OF MERCURY CONTAMINATION: SOURCES AND FORMS

Mercury (Hg) is a non-essential element with a long half-life that has no benefits to the human body. However, its known toxicity, common use in industry and ability of small amounts to accumulate over time in the environment and human tissues poses a threat to health and well being of society. For instance, Hg emission due to human activities is estimated at approximately 2000 metric tons per year [156]. Moreover, the ability of Hg to reach distant regions through atmospheric transfer increases the risk of its accumulation even in remote areas, such as the arctic regions [157]. Even though most of the developed countries have reduced industrial use of Hg, worldwide Hg exposure is still likely to increase through its use in developing countries. For instance, China, with one of the most rapidly growing economies in the world, contributes 28% of global Hg emissions accounting for approximately 600 tons of Hg released into the atmosphere [158]. Therefore, it is critically important to determine potential health risks of Hg exposure. Moreover, developmental programming effects of Hg exposure need to be documented in greater detail. Because of its lipid solubility, Hg can easily pass through biological membranes, including the placenta and the blood-brain barrier (BBB).

Mercury exists as 3 different forms in nature;

- i. Elemental Hg
- ii. Inorganic Hg
- iii. Organic Hg

Route of Mercury Exposure

The Earth's crust contains Hg naturally in small amounts and it is released into the environment via natural or human related activities. Humans are exposed to Hg through inhaled air and food and water consumption [159]. Elemental Hg has a long atmospheric half-life of 6-12 months [156], is volatile at room temperature, and forms Hg vapors [159]. The primary source of elemental Hg emission to the atmosphere is fossil fuel burning, mining activities and incineration of solid waste [159, 160]. Elemental Hg was commonly used in thermometers, but its use in thermometers is now restricted in many developed countries [58, 159]. Elemental Hg is currently used in many types of manufacturing/industries including batteries, chlorine-alkali production, dental amalgams, electronic switches, fluorescent lamps, refining and lubrication oils [159, 161]. Although its industrial use has been reduced in the United States, 714 hazardous waste sites containing Hg have been recently detected [58] indicating the potential for release into the atmosphere and contamination of soil and water sources.

The combination of Hg with other elements, including chlorine, sulfur and oxygen, yields inorganic Hg [58]. In the past, inorganic Hg was commonly used in developed countries as a component of pharmaceutical products, including laxatives, skin-lightening creams and soaps, as well as in latex paint [161]. Even though it is not currently used in agricultural and pharmaceutical products in the United States, mercuric chloride (HgCl_2) is still commonly used as a disinfectant and pesticide [161]. Despite documented health concerns regarding exposure, inorganic Hg is still used in the cosmetic industry in several countries [160].

Organic Hg is formed upon combination with carbon-containing compounds [58]. Among the organic Hg compounds, methylmercury (MeHg) is the most commonly found in nature with high toxicity among all other organic Hg compounds. Methylmercury was first synthesized by two laboratory technicians in a chemical laboratory located in London in the mid-19th century [162]. In nature, MeHg is produced through methylation of inorganic Hg ions [161]. When Hg is released in the atmosphere as the gas form of elemental Hg, it reacts with atmospheric oxidants, such as bromine, and forms higher water-soluble divalent Hg species [156]. Then, water soluble Hg is rapidly deposited in terrestrial and aquatic ecosystems where activity of microorganisms found in wetlands, lakes, rivers and seawater methylates inorganic Hg that gives rise to MeHg [156]. Therefore, traces of MeHg can be detected in nearly all fish and shellfish [163]. Its ability to successively accumulate in each level of the food chain greatly increases its concentrations up to a million times higher in larger and long-lived predatory fishes than concentrations in seawater or in small fishes [156]. Therefore, higher MeHg concentrations can be detected in many fish and shellfish including, swordfish, shark, tuna, lobster and cod [164]. On the other hand, freshwater is expected to contain higher concentrations of MeHg compared to seawater due to the close proximity of freshwater to Hg-contaminated areas. However, recent evidence demonstrated that toxicity of MeHg is retained for longer durations in deep seawater because of slow decomposition rates [165]. Therefore, MeHg is able to contaminate almost all types of fish found in oceans, lakes and streams.

Furthermore, all types of Hg present in the soil also bioaccumulate throughout the ecological food chain and can be ingested via consumption of plants and livestock [8]. Since Hg accumulates in tissues and organs over time, long-term chronic exposure can cause significant health issues.

Absorption and Distribution

As explained above for Cd, Hg in the blood is also sequestered into several organs, such as the kidney, liver, spleen and the central nervous system via binding to MT [166-168]. Mercury exposure during early life has potentially greater health impacts compared to adulthood due to underdeveloped production and binding capacity of fetal MT proteins following maternal heavy metal exposure. Evidence from human studies revealed a positive correlation between maternal Hg exposure throughout pregnancy, mainly via fish consumption, resulting in higher total Hg concentrations in cord blood [169-171] and reduced birth weights [170, 172, 173]. Similar results were obtained in rodent studies where offspring were exposed to Hg during *in utero* development via maternal diet, oral gavage or sc injections during the entire gestation period [174, 175].

Based on chemical composition, the absorption and excretion efficiency of Hg varies among different Hg compounds. Elemental Hg has a poor intestinal absorption. The main route of elemental Hg exposure is via inhaling Hg vapors, such as those given off from combustion of fossil fuels or from broken thermometers. Once elemental Hg is taken up by tissues, it is oxidized into inorganic Hg [176]. Approximately 80% of inhaled Hg vapor is taken up by the lungs and readily distributed into the circulation and tissues [176] with a half-life of approximately 60 days [177]. Upon exposure to Hg vapor, the resultant inorganic Hg also bonds with selenium in the brain, which extends its biological half-life and accumulation [178]. The main route of Hg elimination is through urine and feces and to a small extent via breath, saliva and sweat [176].

Inorganic Hg is mainly taken up by the gastrointestinal tract with an approximately 60 day half-life [179, 180]. Due to its lower solubility, toxicity through inhalation is rare. In

addition, inorganic Hg is not very lipid soluble and cannot pass the BBB easily. It is mainly excreted through urine and feces [176].

Methylmercury uptake is mainly via the gastrointestinal tract with an absorption rate of approximately 95% [181]. Its biological half-life is approximately 50 days [160, 182]. Upon entry into the bloodstream it can be distributed to all organs, easily cross the BBB and is mainly excreted via the feces [181]. Upon entering the body, MeHg complexes with the thiol ligand in the amino acid cysteine that structurally resembles the neutral amino acid methionine. Therefore, formation of the MeHg-cysteine complex increases cellular uptake through its ability to enter into cells using neutral amino acid carriers for entry [181].

Safety Levels and Mercury Exposure during Pregnancy

The Environmental Protection Agency (EPA) updated the reference dose for daily MeHg exposure in 1997 due to extensive data in the literature about the toxicity of Hg compounds. As a result, the reference dose for MeHg has been reduced to 0.1 $\mu\text{g}/\text{kg}$ body weight/day, corresponding to 0.7 $\mu\text{g}/\text{kg}$ body weight/week [162]. However, there is still not a consensus regarding safe levels of Hg intake. For example, weekly tolerable intake levels of MeHg can be easily reached by a person of average body weight who consumes 7-oz of tuna fish a week [162]. In addition, different regulatory agencies have their own safety limits for Hg exposure. For example, the reference dose for MeHg has been set at 0.5 $\mu\text{g}/\text{kg}/\text{day}$ by the U.S. Food and Drug Administration (FDA), versus 0.3 $\mu\text{g}/\text{kg}/\text{day}$ by the Agency for Toxic Substances and Disease Registry (ATSDR) [162]. Moreover, PTWI of MeHg has been set at 1.6 $\mu\text{g}/\text{kg}$ (0.2 $\mu\text{g}/\text{kg}/\text{day}$) by the JECFA of the FAO/WHO [183].

Extensive research indicates wide Hg exposure in the form of MeHg through aquatic sources and bioaccumulation in the food chain. Maternal fish consumption is one of the major

routes of fetal MeHg exposure resulting in developmental effects, such as neurological damage and behavioral changes [14, 184]. A significant portion of pregnant women have serum concentrations of MeHg higher than the U.S. EPA reference dose indicating potential health risks for the developing fetus [185]. Data on the amount of fish consumed and MeHg concentrations in seafood suggest such consumption is a major contributor to blood Hg concentrations above the reference doses [186]. Likewise, high maternal serum and cord blood total Hg concentrations were positively associated with the frequency of fish consumption throughout pregnancy and correlated with increased neurobehavioral problems in children at 3 years of age [171]. Similarly, urinary and hair total Hg concentrations in children were positively associated with the number of fish consumed or the presence of dental fillings [187].

ASSOCIATION OF MERCURY EXPOSURE WITH CHRONIC DISEASES

Due to its long half-life, lower doses of Hg can accumulate over long periods of time with increased potential to adversely affect health and well-being. Negative effects of MeHg exposure on the human nervous system and behavior have been studied in detail [14, 184]. A growing amount of evidence indicates a positive association between MeHg exposure during early adulthood and indices of diabetes, including reduced glucose tolerance and higher plasma glucose and HbA1c concentrations [188]. Further evidence links MeHg exposure via fish consumption to pancreatic β -cell dysfunction [188]. Likewise, rodent studies also demonstrated that plasma glucose, insulin and lipid peroxidation concentrations were impacted by HgCl_2 or MeHg administration at environmentally relevant doses [189]. In addition, a correlation between total serum Hg concentrations and liver dysfunction provides a potential link between Hg exposure and the onset of metabolic diseases [190]. There is also evidence suggesting an

increased prevalence of hypertension in response to elevated total serum concentrations of Hg [191].

Maternal Mercury Exposure as a Possible Cause of Chronic Diseases

Recent toxicological studies monitoring cord blood toxicant concentrations reported higher Hg concentrations than regulatory agencies reference guidelines [17] suggesting a high potential for maternal-fetal transfer of Hg [36]. Due to its lipophilic structure, Hg can easily pass through biological membranes, including the placenta and BBB. However, the amount of Hg the fetus is exposed to depends on several factors including gestational age and dose and route of Hg exposure [192, 193]. Epidemiological studies provide a link between prenatal long-term MeHg exposure, mainly through diet, and reduced birth weight of infants [169, 194]. Likewise, rodent studies of prenatal MeHg exposure during the entire gestation period support similar detrimental effects on birth weight. However, the majority of these studies incorporated chronic gestational administration or single dose *in utero* administration of Hg at higher doses than an average person is exposed to in daily life [174, 175, 195]. In actuality, many women change their life style after recognizing their pregnancy. But maternal lifestyle prior to the detection of gestation might have negative impacts on the developing embryo. Therefore, it is difficult to draw conclusions regarding the effects of early life Hg exposure at lower doses, especially for a shorter duration of time.

Toxic metal exposure in adults triggers expression of MT proteins that bind and store toxic chemicals, mainly in the liver and kidney. Even though less effective, similar mechanisms exist for the developing fetus, where fetal MT binds heavy metals such as Hg present in the fetal circulation [193]. *In utero* Hg vapor exposure in pregnant guinea pigs at doses of 0.2-0.3 mg/m³ during late gestation resulted in higher amounts of Hg accumulation in neonatal liver than

maternal liver [192, 193]. Likewise, MT protein abundance and formation of Hg-MT complex is increased in fetal liver and brain of rodents in response to late gestational Hg vapor exposure [193, 196, 197]. This suggests a defensive mechanism is in place, albeit less effective, to protect the developing fetus from Hg that crosses the placenta.

Impact of prenatal MeHg exposure on the developing nervous system was studied by open field and locomotor activity tests in offspring as an indicator of behavioral changes [14, 15]. Offspring of pregnant mice fed food containing 0.01 mg/kg body weight of MeHg during gestational days 8-18 demonstrated defects in motor abilities and coordination problems as well as altered activities in the open field test [14]. In a similar study, 1 mg/kg body weight of MeHg administered daily to pregnant rats (via oral gavage) from gestational day 5 until parturition resulted in enhanced open field activity of offspring [15]. Furthermore, Minamata disease patients, who were prenatally exposed to higher doses of MeHg mainly through maternal diet, exhibited delayed neurotoxicity later in adult life. Effects included increased difficulty performing daily life activities with increased age compared to adults with no history of significant maternal MeHg exposure [198]. Gestational Hg exposure is also associated with higher risk of neural tube defects in children [199] and impaired immune system function in rodents [200].

There is limited number of studies examining the mechanisms responsible for the neurotoxic effects of early life Hg exposure. These studies suggest maternal Hg exposure may induce neuronal toxicity via impacting neuron development. Neurotrophins are required for neuronal survival and differentiation, and neurotrophin modulation may be linked to Hg toxicity [201]. For example, brain-derived neurotrophic factor (BDNF) is one of the most commonly studied neurotrophins and its concentration in the serum of neonates demonstrates an inverse

association with cord blood MeHg concentrations [201]. Moreover, *in utero* MeHg exposure reduced mRNA abundance for several genes required for fetal neuronal migration including Rac1, Cdc42 and RhoA [202].

Effects of Mercury Exposure on Glucose Homeostasis

Mercury exposure during adult life has pronounced effects on body homeostasis and onset of chronic diseases. However, there are limited numbers of studies focused on linking fetal Hg exposure to incidence of chronic diseases at adulthood. Conversely, more evidence exists linking Hg exposure during adulthood and its potential adverse health effects. Recent studies demonstrated that chronic HgCl₂ or MeHg exposure potentially impacts glucose homeostasis leading to the onset of diabetes [189, 203]. Furthermore, epidemiological studies provide evidence of a positive correlation between hair and blood Hg concentrations and indices of MS, including insulin resistance, fatty liver, diabetes, and hypertension [204-207].

Both *in vivo* and *in vitro* studies in rodents provide insight into possible mechanism of Hg toxicity related to glucose homeostasis. Adult mice treated daily with 0-500 µg/kg body weight HgCl₂ or 20 µg/kg body weight of MeHg for 2-4 weeks demonstrated reduced circulating insulin concentrations and elevated blood glucose concentrations [189]. *In vitro* treatment of isolated mouse islet cells with low MeHg doses (0.2-1 µmol/l) caused pancreatic β-cell dysfunction and reduced insulin signaling via increased phosphorylation of protein kinase B (Akt). Increased oxidative stress and phosphatidylinositol-3-kinase (PI3K) activation was linked to observed regulation of Akt phosphorylation in response to MeHg treatment [189]. Further investigation of pancreatic β-cell dysfunction in MeHg-treated isolated mouse pancreatic cells showed increased production of reactive oxygen species (ROS) [208] and mitochondrial apoptosis that disturbed mitochondrial membrane potential leading to the activation of caspase 3 [209]. Similarly,

transcript abundance for apoptotic and anti-apoptotic genes was altered (in a fashion reflective of increased apoptosis) in murine pancreatic islets treated with MeHg (2 mg/kg body weight) or HgCl₂ (5 mg/kg body weight) for 2 weeks [203].

Glucose homeostasis involves complex mechanisms that require interplay between pancreas, liver, adipose tissue and skeletal muscle. Increased glucose concentration in circulation induces the pancreas to release insulin, which binds to its cognate receptors on target organs to stimulate uptake of glucose for storage and energy metabolism. 3T3-L1 adipocytes treated with 1-10 μM HgCl₂ for 2 days displayed impaired glucose homeostasis via increased phosphorylation of p38 kinase suggesting Hg-induced stress in adipocytes may disturb glucose homeostasis [210]. Moreover, higher total Hg concentrations in the serum of adults in the United States were correlated with markers of nonalcoholic fatty liver disease (NAFLD), one of the hallmarks of MS associated with insulin resistance and disturbed glucose homeostasis [211]. In a similar manner, feeding adult rats Hg-containing foods increased free fatty acid accumulation in the liver [212]. On the other hand, biological systems are complex and require interaction of more than one organ to maintain homeostasis. Therefore, evidence provided by *in vitro* studies or a single model system might not fully apply to complex biological systems. For example, *in vivo* HgCl₂ treatment of newborn rats increased hepatic alanine aminotransferase and glucose 6-phosphatase activity suggesting increased hepatic glucose production [213]. However, opposite results were obtained when rat hepatocytes were treated with HgCl₂ *in vitro* [213].

Effects of Mercury Exposure on Obesity and Metabolism

In the past decades, obesity has reached an epidemic level. Based on recent data obtained between 2011 and 2012, in the United States alone, more than 70 million people were diagnosed as clinically obese with a body mass index (BMI) of equal to or greater than 30 [214]. In general,

obesity is associated with other metabolic complications leading to the onset of diabetes and cardiovascular problems [113]. In addition, the social and financial burden of obesity and related healthcare costs has been estimated at approximately \$200 billion in the United States [215]. Therefore, understanding the underlying causes of obesity is crucially important. It was previously believed life style factors, such as intake of a calorie dense diet and/or sedentary life style were the primary causes of obesity. However since the beginning of the 20th century, there was an exponential rise in the invention and production of industrial chemicals [216] and several reports in the literature demonstrate an association between obesity and exposure to toxic chemicals [217-220]. In 2006, the "obesogen hypothesis" was postulated to explain this phenomenon [221]. This hypothesis states that exposure to xenobiotic chemicals during fetal development or adult life is linked to metabolic programming of obesity [221].

In spite of the data suggesting impact of xenobiotics on the onset of obesity, little is known about the toxicity and contribution of Hg exposure to increasing body weight and adipose accumulation in the human population. However, epidemiological studies provide a correlation between Hg concentrations in blood and components of MS, including obesity, elevated blood glucose and triglyceride and total cholesterol concentrations [222-226]. Recent epidemiological studies in Korean adults demonstrated an association of increased blood total Hg concentrations with indices of MS, which is stronger in men compared to women [223, 224] suggesting effects of Hg exposure may be sexually dimorphic. The link between Hg exposure and MS is also supported by studies in animals and *in vitro* models. Body fat gain is increased in diabetic mice treated with 5 mg/kg body weight of MeHg for 6 weeks [227]. Likewise, transcript abundance for GLUT4 and insulin-stimulated glucose uptake were significantly decreased in a dose-dependent manner in HgCl₂-treated 3T3-L1 adipocytes without any effects on cell viability

[228]. In conclusion, human and rodent studies demonstrated an association between blood Hg concentration and indices of MS, including increased adiposity, insulin resistance and altered glucose homeostasis.

Effects of Mercury Exposure on Male Reproductive System

Recent reports indicate an average of 15-20% of couples in industrialized countries are using assisted reproductive techniques for conception [229] and male factor infertility is responsible for reduced conception in approximately 50% of cases [230]. This reduction in fertility is attributed, at least partially, to reduced sperm concentration, motility and abnormal morphology [231], and/or decreased testosterone concentrations [232, 233]. There are several factors contributing to increased rates of male infertility, including life style factors, chronic diseases, and environmental, occupational and/or *in utero* toxic chemical exposures [234-237]. Recently, more attention has been focused on the negative impacts of toxic chemical exposure on male fertility [124, 238].

Fetal and adult life exposure to toxic chemicals have been shown to cause histological abnormalities in the testis tubules [239], and to negatively impact steroidogenesis [240] and spermatogenesis [241]. These effects could be mediated either via modulation of intracellular and intercellular signaling actions at the cellular level for germ cells and supporting somatic (Sertoli and Leydig) cells [239, 242], via modulation of the hypothalamic-pituitary-gonadal axis [243], or via inducing DNA damage and apoptosis [132]. Similar to other toxic chemicals, Hg has the potential to damage the male reproductive system leading to infertility. Previous studies have focused primarily on the impact of Hg exposure in adult rodents on indices of male reproductive function. However, fewer studies have investigated reproductive toxicity of Hg exposure in men. A positive correlation between Hg exposure through dietary fish consumption

and indices of male subfertility and reduced steroidogenesis has been reported [244]. Occupational Hg exposure, mainly via inhalation of HgCl₂-containing air over long periods of time, also is linked to reduced serum testosterone concentrations and male infertility [233].

It is important to determine the targets of Hg action within the reproductive system to understand its impact on fertility. Sperm production and steroid hormone synthesis are adversely affected by Hg exposure. Leydig cells are required for the production of testosterone, which is regulated by LH released from the pituitary gland [245]. On the other hand, Sertoli cells contribute to the formation of the BBB by lining the seminiferous tubule walls and support and nourish the developing germ cells during the process of spermatogenesis. Sertoli cell functions are regulated by FSH [245]. Adult rats treated with 20 mg/L of MeHg in drinking water for 12 weeks accumulated Hg in the Leydig and Sertoli cells of seminiferous tubules [246]. Isolated rat Leydig cells treated with 100 µM Hg displayed decreased cell viability and reduced testosterone production [247]. Following administration of 0.5 mg/kg body weight of MeHg for 14 days sperm motility, concentration and normal morphology were significantly decreased in adult rats [231]. In the same study MeHg treatment at higher doses (3 mg/kg body weight for 14 days) reduced serum testosterone concentrations [231]. Likewise, treatment of rats with 20 mg/l of MeHg via drinking water for eight weeks reduced intra-testicular levels of testosterone [232]. Daily exposure to 1 mg/m³ Hg vapor for six weeks decreased the number of Sertoli cells and spermatogonia, spermatocyte and spermatid numbers in rat seminiferous tubules [239]. Administration of HgCl₂ at lower doses (4 ppm) to mice in drinking water reduced epididymal sperm number and caused degenerative lesions within the testes [248]. Subcutaneous injection of MeHg at higher doses (10 mg/kg body weight) for eight days in rats suppressed sperm production, reduced testosterone concentrations, increased testicular DNA fragmentation and

caused apoptosis of spermatocytes and spermatids [241]. Administration of lower doses of HgCl₂ (0.01, 0.05 and 0.1 µg/mL) to rats via drinking water for longer duration (1 to 7 months) caused spermatogenic arrest at the spermatocyte stage [249]. These results demonstrate the potential impact of Hg exposure on male fertility with evidence of reduced sperm quality and altered steroidogenesis. The majority of the knowledge is derived from either long term Hg exposure or administration of Hg at very high doses. Potential developmental programming effects of Hg administration on male fertility, especially at lower doses, remain to be elucidated

Effects of Mercury Exposure on Female Reproductive System

In 2010, more than 45 million couples worldwide have been estimated to be infertile [250]. At the same time, human exposure to toxic chemicals has significantly increased. An emerging body of evidence now indicates female fertility can be impacted by exposure to toxic chemicals. Environmental exposure to toxic chemicals may impact the female reproductive system through many mechanisms including, but not limited to, altering menstrual/estrus cycles, maturation and growth of follicles and/or perturbations in steroidogenesis.

Negative associations exist between higher serum concentrations of total Hg and reduced fecundity of females [251] via high MeHg intake through diet [252, 253], occupational exposure such as in the case of dentists and dental assistants [254] or use of cosmetic products, such as skin lightening creams [255]. Adult female rats exposed to Hg vapor at doses of 2 or 4 mg/m³ for 2 h daily for 11 days displayed prolonged estrus cycles in response to both doses [256], but serum estradiol and progesterone concentrations were altered only in animals exposed to the highest dose. Moreover, an inverse correlation between higher total serum Hg concentrations and oocyte yield in IVF patients has been demonstrated [257].

Similar effects of Hg exposure on female steroidogenesis have been observed. Incubation of ovarian granulosa cells with HgCl₂ at the doses of 50-250 µg/mL for 18 h decreased progesterone production and increased apoptosis [258]. Likewise, reduced LH releasing hormone and plasma LH concentrations were noted in ovariectomized rats following administration of 1 mg/kg body weight of MeHg [259].

Effects of Mercury Exposure on Behavior

Neurological disorders constitute an important health concern for the well being of individuals and society in general. Recent evidence provides a potential link between toxic chemical exposure and neurological problems, including cognitive disorders [260], anxiety [261], Parkinson's disease [262] and others. Moreover, the developing nervous system is susceptible to environmental toxicity that increases the prevalence of neurobehavioral developmental disorders [263]. Lipid solubility and accompanying ability of Hg to cross the placenta and BBB increases the neurotoxicity risk during early life. Administration of MeHg to pregnant and lactating rats at moderate doses (5 ppm) increased motor coordination defects in the offspring at 6 weeks of age [264]. Additionally, offspring of female rats *in utero* administered a single high dose of MeHg in mid to late gestation displayed anxiety-like behavior [261] and impaired learning [265].

However, there is still less known about mechanism of Hg toxicity in the central nervous system and developing brain. It has been proposed that MeHg exerts toxicity through alterations in neurotrophic factors, such as BDNF, which are required for neuronal survival and differentiation [201]. Acute administration of 8 mg/kg body weight of MeHg to adult rats reduced transcript abundance for BDNF, especially in the hippocampal area of the brain [266] that is involved in generation and use of memory, creativity, language use as well as controlling

social behaviors [267]. The decline in BDNF transcript abundance was also associated with depression-like behavior in offspring of mice administered 0.5 mg/kg body weight of MeHg from gestational day 7 until postnatal day 7 through the drinking water [268]. Likewise, maternal fish consumption was positively associated with elevated cord blood MeHg and reduced circulating BDNF concentrations in infants [201]. Offspring of mice treated with MeHg during pregnancy and lactation had reduced transcript abundance for BDNF that was linked to DNA hypermethylation, increased histone H3-K27 tri-methylation and decreased H3 acetylation at the BDNF promoter [268, 269]. Therefore, reduced mRNA abundance of BDNF in response to developmental Hg exposure may be mediated epigenetically.

In addition, perinatal Hg exposure in rodent models can affect the dopaminergic and serotonergic systems in the central nervous system altering the levels of dopamine, serotonin, noradrenalin and acetylcholine [270-273]. The levels of these neurotransmitters are regulated by monoamine oxidase (MAO) that is required for proper neuronal development and drugs that target MAO are used pharmaceutically as antidepressants [274]. Exposure of developing rodents to MeHg in late gestation resulted in decrease levels of MOA activity in offspring further demonstrating the negative impact of toxic chemical exposure on the developing embryo and the central nervous system [271]. Mercury exposure can also change the levels and compositions of neurotransmitters released from presynaptic nerve terminals via altering the intracellular Ca ion concentration [275].

OVERVIEW OF TRANSGENERATIONAL INHERITANCE

Available evidence obtained from both human epidemiological studies and rodent models demonstrate the impact of adverse *in utero* conditions, such as maternal nutrition, stress and exposure to environmental chemicals, on adult onset of chronic diseases, including stroke,

coronary heart disease, hypertension, obesity and type-2 diabetes [17, 276, 277]. The impact of early life influences was first observed by David Barker and his colleagues who demonstrated a positive correlation between low birth weight and increased risk of cardiovascular problems and diabetes during adult life [3]. This condition whereby early life impacts late onset chronic diseases had long been known as the Barker hypothesis and is now known as the "developmental origins of health and disease (DOHaD)" or "fetal origin of adult disease (FOAD) [278]. Moreover, accumulating evidence demonstrates the persistent effects of adverse conditions across generations even in the absence of subsequent direct exposure to original insults (e.g. nutritional manipulation, toxic chemical exposure, maternal stress).

In transgenerational studies, where the insult originated maternally, exposure of pregnant females (F0 generation) to such insults directly affects the developing fetus (F1 generation) and its germ cells that give rise to the subsequent F2 generation [279]. Therefore, any effect observed in the F1 and F2 generations are referred to as multigenerational. However, any effects observed beyond the F2 generation are considered truly transgenerational due to absence of direct exposure of F3 offspring and beyond [280]. However, if the original insult is administered to males through the male germ line, only the F1 generation is exposed to the original insult via impacts on the F0 father's germ cells. Hence, effects seen in the F2 generation and beyond are referred to as transgenerational [281].

Evidence of Transgenerational Inheritance via Manipulating Maternal Nutrition

There are limited data from epidemiological studies demonstrating the impact of maternal or paternal environmental factors on adult phenotype with accompanying transgenerational epigenetic inheritance. For instance, historical health records obtained from an isolated community in northern Sweden during the years 1895, 1905 and 1920 suggest that food supply

of the paternal grandfather was correlated with the grandchild's diabetic mortality and longevity providing indirect transgenerational evidence in the case of paternally originated insults [4, 282].

Also, there are not conclusive data from epidemiological studies proving transgenerational inheritance linked exclusively to maternally originated insults due to the absence of data from the F3 generation. However, available evidence suggests the potential existence of such inheritance, including the thrifty phenotype hypothesis that states poor nutrition during early fetal life can elevate risk of late onset chronic diseases [3]. Likewise, the Dutch Hunger Winter provided key concepts for the understanding of the DOHaD hypothesis [4]. In the Second World War, the west part of the Netherlands, including Amsterdam, experienced famine in the winter and spring of 1944 with an average supply of less than 1,000 calories per day based on government food rations [283-285]. Analyses of birth records indicated that babies born to mothers who experienced famine during the second and third trimester of pregnancy displayed reduced birth weights [5, 284]. However, those born to mothers experiencing famine earlier in gestation had normal birth weights, but these infants displayed higher rates of obesity later in adult life [5, 284]. It should be noted that in the case of experiencing famine during early pregnancy food became available during the second and/or third trimester, but these babies still displayed delayed effects later in adult life. This supports the influence of a window of adverse *in utero* conditions in determining adult phenotype. Moreover, analyses of maternity records of mothers who had experienced famine during the first and second trimester of their fetal development revealed a shorter duration of gestation and babies with reduced birth weights compared to offspring born to mothers that did not experienced famine during their *in utero* development [286]. Surprisingly, birth weight of the F2 generation was not affected if the mothers were exposed *in utero* to famine during the third trimester of pregnancy.

This is in contrast to the direct impact of famine on birth weight of the F1 generation [286]. However, second generation babies born to mothers, whose own mothers experienced famine *in utero*, displayed normal birth weight, but had decreased birth length, elevated neonatal adiposity and poor health quality in adult life [6]. These results further establish the importance of a window of exposure to adverse maternal conditions and differing effects on phenotype of the subsequent generations.

The effects of grandparental smoking on birth weight and neonatal adiposity of infants was demonstrated in the Avon Longitudinal Study of Parents and Children (ALSPAC). According to this study, even if the mothers did not smoke, smoking by maternal grandmothers during pregnancy was correlated with increased birth weight and BMI of grandsons, but not granddaughters [287]. Likewise, another cohort study reported reduced birth weight of offspring born to females whose mothers smoked during pregnancy [288]. Moreover, asthma risk was significantly elevated in these infants during their childhood [289].

In contrast to humans, rodents make suitable models for transgenerational studies of the impact of environmental insults on adult chronic disease due to their short gestational duration, fast sexual maturation and average life span of two years [290]. Available evidence in the literature obtained from rodent models suggests the existence of transgenerational inheritance. For instance, male mice that were maintained on a high fat diet (HFD) for 10 weeks displayed increased adipose weight without any sign of diabetes (F0 generation). However, male and female offspring of these animals (F1 generation) and male offspring in F2 generation had increased obesity and insulin resistance even though they were maintained under a control diet [291]. In another study, F1 and F2 generation offspring derived from males (F0 generation)

maintained under HFD displayed reduced sperm motility and increased sperm DNA damage [292].

Similar to above paternal dietary manipulation studies, F0 generation female mice maintained under HFD for 6 weeks before mating displayed elevated body weight and reduced insulin sensitivity that persisted across F1 and F2 generations in both sexes [293]. However, only female offspring in the F3 generation descended patrilineally displayed elevated body weights [293]. Further analyses revealed higher expression of paternally imprinted genes in liver of F3 generation female offspring descended patrilineally than was observed in the F3 generation females matrilineally [293]. It is not only calories or protein content of the diet during pregnancy that have transgenerational effects, but other dietary factors, such as alcohol, caffeine or deficiency of essential nutrients and vitamins during pregnancy can affect subsequent generations. For example, daily maternal administration of 0.1 mg/ml of nicotine in drinking water starting 3 weeks prior to conception and continuing through the entire gestation induced hyperactivity of male and female mice in the F2-F3 generations that were descended through the matrilineal germline [294].

Consumption of a low-protein diet by pregnant females (F0 generation) resulted in transgenerational effects in offspring at weaning that were manifest as reduced pancreatic β -cell number and hyperinsulinemia persisting throughout F1-F3 generations in both sexes [24]. Likewise, similar persistent impacts of maternal food restriction in subsequent generations were observed in a study where female rats were fed a restricted diet (50% reduction in intake compared to controls) throughout the entire gestation leading to increased blood pressure of offspring of both sexes in F1-F3 generations [25]. Moreover, thoracic aorta extracted from these

offspring (F1-F3 generations) displayed impaired vasodilation in response to *in vitro* ACh treatment [25].

Evidence of Transgenerational Inheritance following In Utero Toxic Chemical Exposure

An accumulating amount of evidence suggests persistent transgenerational effects occur in response to prenatal toxic chemical exposure. Some of the most commonly studied chemicals that cause transgenerational effects and the resulting phenotypes are reviewed below.

Bisphenol A (BPA)

BPA is used commonly for the production of polycarbonate plastics and epoxy resins, including packaging for food and water, medical devices, coating for canned products and water pipe seals [295]. Both human and animal studies indicate reproductive, developmental and systemic toxicity of BPA [296, 297]. Therefore, there is growing interest regarding potential persistent transgenerational effects of BPA exposure. Transgenerational behavioral alterations were examined in F1 and F3 generation mice derived from females (F0 generation) daily administered a 5 mg/kg BPA containing diet beginning 7-10 days before mating and continuing throughout pregnancy [18]. Maternal BPA administration had no behavioral effects on F1 generation offspring, but increased locomotor activity was observed in F3 generation offspring in the open field test independent of offspring sex [18]. In a follow-up study with a similar experimental design, transcript abundance of the neuropeptides vasopressin and oxytocin, which influence many social interactions and behaviors, were significantly reduced at embryonic day (ED) 18.5 in both *in utero* BPA exposed embryos (F1 generation) and embryos in the F4 generation, demonstrating persistent behavioral impact of prenatal BPA exposure transgenerationally [298].

Daily treatment of pregnant rats with doses of 1.2 and 2.4 µg/kg body weight of BPA via oral gavage from gestational day 12 through postnatal day 21 resulted in altered transcript abundance and localization of testicular steroid receptor coregulators, including steroid receptor coactivator-1, and nuclear corepressor, p300/CBP/cointegrator-associated protein and G-receptor integrating protein-1 that regulate the function of steroid receptors, resulting in impaired spermatogenesis [299]. Such effects were manifest in F1-F3 generation offspring and thus transgenerational. Moreover, co-administration of BPA with other plastic compounds resulted in transgenerational effects in rat models [300]. In these studies, effects of combinational administration of a plastic compound mixture at doses of 50 mg/kg body weight of BPA, 750 mg/kg body weight of di-(2-ethylhexyl)phthalate (DEHP) and 66 mg/kg body weight of dibutyl phthalate (DBP) to pregnant rats from gestational day 8 to 14 (F0 generation) were examined in F1 and F3 generation offspring [300]. The results demonstrated decreased uterine weight, delayed female puberty onset, primary ovarian insufficiency and polycystic ovaries in F1 and F3 generation offspring [300]. However, F3 generation male offspring displayed reduced seminal vesicle and epididymal weight, azoospermic and atretic seminiferous tubules as well as obesity compared to F3 generation males descended from control females [300].

Vinclozolin

Vinclozolin is a fungicide that is commonly used on fruits, vegetables and turf grass [301]. Available data demonstrate adverse effects of exposure during pregnancy that persist transgenerationally [21, 302]. Daily administration of 100 mg/kg body weight of vinclozolin to pregnant mice (F0 generation) from gestational day 8 to 14 decreased epididymal sperm number and motility, increased germ cell apoptosis and resulted in uterine hemorrhage and severe glomerular abnormalities in F1-F3 generations [19, 21, 302]. Moreover, administration of

vinclozolin (100 mg/kg body weight) to gestating rats from gestational day 8 to 14 elevated anxiety-like behavior in F3 generation offspring of both sexes [22]. These offspring in the F3 generation derived from vinclozolin-treated females displayed altered metabolic profiles in distinct neural nuclei in different brain regions, including amygdala, stria, hippocampus, hypothalamus and cortex [22].

Nicotine

Nicotine is one of the toxic chemicals found in tobacco plants [303]. Available data demonstrates its ability to cross the placenta and impact fetal development, especially neural development, with persistent transgenerational effects [294, 304, 305]. For instance, F2 and F3 generation offspring of both sexes derived from female mice (F0 generation) administered 0.1 mg/ml nicotine in drinking water starting 3 weeks prior to mating and continuing throughout the duration of pregnancy displayed increased locomotor activity in the absence of further nicotine administration [294]. Similarly, F2 and F3 generation male offspring derived from pregnant rats (F0 generation) treated with nicotine at doses of 1 mg/kg body weight during entire gestation and lactation displayed impaired pulmonary functions suggestive of asthma phenotype as assessed by increased respiratory resistance and decreased total compliance following methacholine challenge, as well as increased tracheal constriction in response to acetylcholine treatment [304].

Other Chemicals

Adult male mice *in utero* treated with TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin, also known as dioxin), at doses of 10 µg/kg body weight starting from gestational day 15.5 and continuing throughout lactation, displayed reduced percentage of sperm with normal morphology and increased germ cell apoptosis as assessed by TUNEL test and immunolocalization of caspase-3 in the testes of their offspring in F1-F3 generation without any further TCDD

administration [306]. In a similar manner, ip administration of 0.1 µg/kg body weight of TCDD to pregnant rats (F0 generation) from gestational day 8 to 14 resulted in primordial follicle loss and polycystic ovary syndrome (PCOS) in the subsequent F2 and F3 generations as well as delayed pubertal onset in F3 generation females [307]. The above results suggest persistent transgenerational effects of TCDD administration to gestating females on reproductive parameters and increased incidence of adult onset diseases.

Transgenerational effects of the insecticide dichlorodiphenyltrichloroethane (DDT), including increased body weight and adipose accumulation, were observed in F3 generation male and female offspring derived from pregnant rats (F0 generation) administered 25 or 50 mg/kg body weight of DDT ip daily from gestational day 8 to 14 [23]. However, maternal DDT exposure did not affect body weight and adipose accumulation in F1 generation male and female offspring [23]. Other studies examined persistent effects of DEHP administration to pregnant females. F1 offspring derived from pregnant CD1 mice treated with 500 mg/kg body weight of DEHP via oral gavage from ED 7 to 14 were bred with naïve animals to obtain F2-F4 generations without any further DEHP administration. The F2-F4 generation offspring displayed a disruption of testicular germ cell association and spermatogonial stem cell function linked to reduced sperm numbers and motility [308]. Male offspring of pregnant mice administered 5.42, 54.2 or 542 nM tributyltin (TBT; an organotin compound used to prevent growth of marine organisms on the hulls of marine vessels) had increased adipose weight, adipocyte size and number and hepatic lipid accumulation in F1-F3 generations [309]. Moreover, liver samples obtained from these F1-F3 generation offspring revealed increased mRNA expression of genes required for lipid formation, storage and transport [309].

SEX SPECIFIC TRANSGENERATIONAL INHERITANCE

The above studies provide evidence of transgenerational inheritance of indices of chronic disease linked to environmental insults during pregnancy. However, evidence of sex-specific transgenerational inheritance of indices of chronic disease linked to *in utero* exposures is more limited. A cohort study from Avon Longitudinal Study of Parents and Children demonstrated sex specific transgenerational effects of gestational cigarette smoking linked to grandmothers [310]. In this study, if the paternal grandmother smoked during pregnancy, but not the mother, granddaughters displayed lean body mass. However, increased body weight of grandsons was observed with increasing age [310]. Similar sex-specific inheritance was reported in the Swedish Overkalix cohort [311]. In this study, if the paternal grandmother experienced sharp changes in food availability during the time of puberty, increased mortality rates from cardiovascular diseases were observed for female but not male offspring of her sons [311].

Rodent studies provide additional data demonstrating sexually dimorphic transgenerational effects of environmental insults *in utero*. Prenatal exposure of rats to the pesticide methoxychlor during gonadal development of the fetus (gestation days 8 to 14) increased the risk of obesity in both males and females of F1-F3 generations. However, obesity incidence was only increased in F4 generation offspring derived from the female germ line [20]. Likewise, behavioral effects of ip administration of 100 mg/kg body weight of vinclozolin to rats from gestational days 8 to 14 were analyzed only in the F3 generation and decreased anxiety-like behavior in male offspring was observed. However, anxiety-like behavior was increased in female offspring of the F3 generation [312]. Molecular studies suggest a difference in gene expression profiles in the hippocampus and amygdala of F3 male and female offspring [312].

Mechanisms responsible for sexually dimorphic transgenerational inheritance of indices of chronic disease linked to the *in utero* environment are not understood.

In conclusion, persistent effects of adverse *in utero* conditions on health and well being of subsequent generations have received growing interest as a potential contributing factor in the increased incidence of adult chronic diseases. Available data obtained from both human epidemiological studies and rodent models support the deleterious effects of unfavorable *in utero* exposures during entire gestation and/or lactation, with effects on metabolism, behavior and fertility of offspring observed that persist in subsequent generations. However, there is no information regarding the transgenerational impact of heavy metal administration, especially in the context of exposure during early life or before conception. Therefore, in the present dissertation research, it was hypothesized that maternal administration of a combination of Cd and Hg at 0, 0.125, 0.5 and 2.0 mg/kg body weight during the periconception period (four days before conception and four days after conception) would have persistent transgenerational effects on anxiety-like behavior, glucose homeostasis, body weight gain and adiposity of offspring in multiple generations, even though the original insult is not present in the subsequent generations. This research topic is the focus of studies outlined in Chapter 4 of this dissertation.

EPIGENETIC MECHANISMS OF TRANSGENERATIONAL INHERITANCE

Although the mechanisms responsible for increased incidence of chronic diseases in offspring in response to nutritional and environmental insults during pregnancy are not yet completely understood, a growing amount of evidence suggests a causative role for epigenetic modifications of the genome in mediating developmental programming. More specifically impacts of unfavorable maternal and paternal conditions on DNA methylation, histone

modifications and expression of non-coding RNAs are linked to the etiology of adult onset chronic diseases and their transgenerational inheritance [313].

DNA Methylation

Embryo development requires dynamic DNA methylation changes, including hypomethylation during preimplantation development followed by rapid DNA methylation during implantation that is crucial for proper fetal development [314]. There are both intrinsic and extrinsic factors, including diet, xenobiotics, or stress, that affect DNA methylation both during early life and adulthood [314]. In general, DNA methylation is defined as the addition of methyl groups to cytosine or adenine bases for covalent modification of DNA [315]. As a result, DNA methylation represses transcription and causes silencing of genes and DNA methyltransferases (DNMTs) are involved in the establishment and maintenance of the methylation process [316]. Altered DNA methylation can result in abrupt changes in transcript abundance of genes that are associated with disease conditions such as cancer, diabetes and obesity [314]. Due to dynamic methylation changes, the embryonic period is vulnerable to adverse conditions occurring in the maternal environment that can influence methylation state and potentially be manifested phenotypically as increased risk for the onset of chronic diseases at adulthood [317-319].

There are at least two major phases of DNA methylation that take place during development, including preimplantation development and gametogenesis (for detailed review see [26, 320]). The first round starts following fertilization and a wave of DNA demethylation occurs in the parental genome in a distinct fashion. At this stage, the paternal genome of the zygote undergoes an active and rapid demethylation while the maternal genome undergoes a slow and passive demethylation. However, this post-fertilization demethylation does not include imprinted

genes resulting in parental-allele-specific expression of imprinted genes in the early embryo [26]. During differentiation of the blastocyst stage embryo into inner cell mass (ICM) and trophoctoderm (TE) lineages, *de novo* methylation takes place and ICM becomes hypermethylated compare to TE [320]. The other round of prominent methylation changes occurs in the germ line where the lineage of mature male and female gametes and their precursors is linked back to the initial stem cells, the primordial germ cells (PGCs) [320]. Available data obtained from rodent studies indicates that PGCs arise in embryos at ED 7.5. Global DNA demethylation starts at ED11.5-12.5, including erasure of imprinted genes, coincident with PGC proliferation and migration towards the genital ridge. This stage is followed by *de novo* methylation of the germline where sex-specific imprints are acquired [320]. In males, this process of remethylation continues up to ED18.5 before birth. However, in females it continues after birth in maturing oocytes until near the time of ovulation.

Available evidence supports a pronounced effect of early life environmental conditions on DNA methylation. For instance, *in utero* exposure of mice to 200 µg/kg body weight of BPA during the entire pregnancy resulted in a reduced amount of time that male offspring spent exploring novel objects at 60 days of age as assessed by novel object recognition test [321]. Moreover, these behavioral changes were correlated with BDNF hypermethylation at CpG sites in samples obtained from the hippocampus [321]. Likewise, maternal BPA exposure during pregnancy at doses of 2-200 µg/kg body weight had sex-specific behavioral effects with anxiety-like behavior elevated in females but reduced in males [322]. The observed sex-specific behavioral phenotype was associated with differential DNA methylation of ER α at CpG sites in male and female offspring of BPA-treated females [322]. Assessment of human Hg exposure via

placental samples and infant toenail clipping suggested an association between infant neurobehavioral outcomes and differential methylation of 339 different loci [323].

Similarly, in a rodent model of single or combinational exposure to BPA (50 mg/kg body weight), DDT (25 or 50 mg/kg body weight), vinclozolin (100 mg/kg body weight), jet fuel hydrocarbon (500 mg/kg body weight) or TCDD (10 ng/kg body weight) from gestational day 8-14, transgenerational effects were observed in the F3 generation manifested as obesity, pubertal abnormalities, reduced sperm count and histopathological abnormalities in the testes, prostate, kidney and female reproductive tract [23, 300, 307]. Further analyses of F3 generation sperm DNA samples revealed differentially methylated regions in the promoters of genes required for cellular metabolism and signaling that were linked to the observed phenotypic changes [324-326].

Effects of maternal smoking on liver metabolism of offspring have been analyzed in a retrospective study where fetal human livers were obtained from elective gestation terminations between 11 to 21 weeks of pregnancy [327]. The results showed increased DNA methylation at the differentially methylated region (DMR) of IGF2 and reduced methylation of glucocorticoid receptor promoter in fetal liver of fetuses from mothers who smoked during gestation compared to non-smoking mothers [327].

Similar to toxic environmental agents, maternal diet can also have an impact on fetal metabolism via changing DNA methylation status. This has been established by gestational supply of methyl groups via diet to mice prior to conception and throughout gestation [328]. Such treatments modified DNA methylation and expression of genes in fetal liver linked to cellular growth and lipid metabolism [328]. Moreover, in a rat model of gestational diabetes, differentially methylated genes involved in endocrine function, metabolism and insulin response

in liver of offspring were observed. Such genes included ACACA, glycogen synthase kinase-3 β and PPAR- γ [319]. Likewise, in a mouse model of IUGR by nutrient restriction, male offspring displayed obesity and impaired glucose tolerance with increased age [318]. Moreover, offspring of these males in the second generation had similar phenotypic characteristics. These persistent effects in F2 generation were attributed to altered hepatic gene expression of the lipogenic gene, liver X receptor alpha associated with reduced DNA methylation at individual CpG sites in this gene [318].

Histone Modifications

DNA is encircled by histone proteins that undergo dynamic post-translational modifications [329]. Modification of histone tails, which includes lysine (K) acetylation, lysine and arginine (R) methylation, serine (S) and threonine (T) phosphorylation and lysine ubiquitination, is carried out by enzymes that contribute to silencing or inducing transcription of genes [330]. In general, acetylation of histone tails is correlated with transcriptional activation, whereas histone methylation has variable effects on the regulation of transcription [330].

Impacts of adverse *in utero* conditions on histone modifications have been previously shown in several maternal nutrition studies [331, 332]. Offspring of mice administered a HFD throughout gestation displayed increased H3K14 acetylation and H3K9 trimethylation of hepatic genes at 5 weeks of age, including PPAR α , PPAR δ and retinoid x receptor alpha (RXRA), genes involved in regulation of lipid metabolism [331]. Examination of fetal rat liver collected at day 21 of gestation from mothers fed HFD throughout gestation revealed a decrease in H3K9 trimethylation and increased H3K4 dimethylation in the phosphoenolpyruvate carboxykinase gene that regulates hepatic gluconeogenesis [333]. Offspring of gestating female mice subjected to HFD displayed components of MS, including elevated blood pressure, insulin resistance and

hyperlipidemia [334]. Lower acetylation and higher methylation of H3K9 in the adiponectin promoter and higher methylation of H4K20 in the leptin promoter and elevated leptin and reduced adiponectin levels were also observed in such animals [334]. Likewise, administration of 6.7% volume/volume ratio of ethanol in the diet of pregnant rats from gestational day 7-21 caused a significant deficit in proopiomelanocortin (POMC) neuronal functions in F1 generation offspring of both sexes as well as F2 and F3 generation male and female offspring descended from the male germline [335]. Such effects in F1-F3 generations were associated with increased methylation of POMC gene promoter, and increased H3K9 methylation and deacetylation [335].

Even though data are more limited, histone modifications have been observed in response to gestational exposure to toxic chemicals. Maternal exposure to a common plasticizer, DEHP, administered orally from gestational day 9-21 at doses of 0, 1, 10 and 100 mg/kg body weight per day, resulted in increased offspring blood glucose concentrations, impaired glucose tolerance, disrupted insulin signaling and reduced glucose uptake in the skeletal muscle independent of sex [336]. Likewise, offspring of DEHP-exposed mothers had increased serine phosphorylation of IRS1 protein involved in the insulin signaling pathway and down-regulated transcript abundance of GLUT4 [336]. Reduced mRNA abundance of GLUT4 was attributed to global DNA methylation at the GLUT4 promoter as well as increases in histone deacetylase 2 interaction with the GLUT4 gene [336].

In conclusion, available evidence supports impact of the maternal environment on DNA methylation and histone modifications in select genes in offspring linked to phenotypic changes, mainly in the F1 generation. However, there are limited data demonstrating impact of adverse *in utero* conditions on DNA methylation and histone modifications linked to transgenerational phenotypes in the F3 generation and beyond.

Non-coding RNA

Adverse *in utero* conditions may also impact epigenetic regulation of gene transcription via non-coding RNAs (ncRNA), including microRNAs (miRNA) [337]. In general, ncRNAs are transcribed from DNA but they are not translated into proteins. They can have regulatory actions both at the transcriptional and post-transcriptional level via regulating gene expression through changing chromatin configuration, inhibiting translation and causing RNA degradation [314, 315]. Among the best characterized ncRNA is miRNA, which are typically 21-23 nucleotides in length [338] and repress gene expression through base-pairing with a target mRNA [337]. In the case of fully complementary base-pairing, they cause degradation of mRNA [337]. However, partial sequence complementary is sufficient to block the translation of target mRNA sequence [337]. Over 2000 human miRNA have been detected so far and it is believed that miRNAs regulate approximately one-third of mRNA transcripts [338]. Since targeting only requires partial base-pairing, a single miRNA can target multiple mRNAs [337].

An epidemiological study of term placentas collected in the U.S. National Children's Study Vanguard Birth Cohort suggest maternal exposure to toxic chemicals, such as Cd, Hg or Pb, is associated with different expression levels of miRNA in the placenta, including mir-517a, mir-517c, mir-522, and mir-23a [339]. Urine samples obtained during the first-trimester of pregnancy in the Harvard Epigenetic Birth Cohort study demonstrated an association between gestational phenol and phthalate exposure and altered expression of 29 candidate miRNAs in the placenta that were linked to genes controlling multiple cellular pathways, including regulation of protein serine/threonine kinase activity [340]. Likewise, another pregnancy cohort study conducted in Mexico revealed a correlation between maternal exposure to arsenic via drinking water and changes in levels of miRNA in newborn blood associated with genes involved in the

onset of type-2 diabetes [341]. Amnion obtained from term placentas (at caesarean section) from obese and normal body weight women exhibited different miRNA expression profiles (miR-422b, miR-219, miR-575, miR-523, miR-579, miR-618 and miR-659) associated with genes linked to down-regulation of several signaling pathways, including neurotrophin, insulin, and adipocytokine, suggestive of a potential link between maternal obesity and the offspring's future risk of metabolic diseases [342].

Similar to human epidemiological data, rodent models of maternal nutrient restriction or exposure to toxic chemicals has also revealed alterations in levels of miRNAs linked to the onset of chronic diseases. For instance, offspring of pregnant mice orally administered 1 mg/kg body weight of vinclozolin throughout the entire gestation displayed reduced sperm count and increased testicular apoptosis in three successive generations. Further analyses of PGCs from the developing testis of F1-F3 generations revealed alterations in miRNA-23b and miRNA21d linked to deregulation of the *Blimp1* gene, a key regulator of germ cell differentiation [343]. Likewise, male mice maintained under HFD for a period of 10 weeks before mating displayed obesity and insulin resistance in offspring of both sexes in the F1 generation as well as male offspring of the F2 generation [291]. Further analyses of F0 generation males exposed to HFD demonstrated altered expression of 11 different miRNA in the testes as well as reduced methylation levels of germ cell DNA, which were suggested as potential mediators of transgenerational transmission of obesity phenotype [291].

CHAPTER 3

**EFFECTS OF PERICONCEPTION CADMIUM AND MERCURY CO-
ADMINISTRATION TO MICE ON INDICES OF CHRONIC DISEASE IN OFFSPRING
AT MATURITY¹**

¹ Submitted for review and potential publication in Toxicological Sciences

ABSTRACT

Long-term exposure to the heavy metals cadmium (Cd) and mercury (Hg) is known to increase risk of chronic diseases. However, exposure to Cd and Hg beginning at the periconception period has not been studied to date. In the present studies, the effect of co-administration of Cd and Hg during the periconception period on indices of chronic diseases at adulthood was examined. Adult female CD1 mice were subcutaneously administered a combination of cadmium chloride (CdCl₂) and methylmercury (II) chloride (CH₃HgCl; 0, 0.125, 0.5 or 2.0 mg/kg body weight each) four days before and four days after conception. Birth weight, litter size, behavioral alterations, organ weights, glucose homeostasis and endocrine and molecular markers of insulin resistance and metabolic syndrome were examined in offspring. Birth weight and litter size were similar in offspring from all groups, but increased anxiety-like behavior was observed in male offspring from treated females compared to controls. Glucose tolerance was decreased in offspring of both sexes of treated females, but body and adipose weights were increased only in male offspring. Significant elevation of serum leptin and insulin concentrations, higher glucose concentrations in response to insulin administration, and altered mRNA abundance for genes associated with glucose and lipid homeostasis (GLUT4, IRS1, FASN, ACACA, FATP2, CD36, G6PC) in liver and abdominal adipose tissues were observed, suggestive of insulin resistance. Results suggest periconception heavy metal administration to female mice programs susceptibility to chronic diseases in male offspring at adulthood including anxiety-like behavior and metabolic syndrome.

INTRODUCTION

The *in utero* environment, including maternal nutrition, stress, and exposure to chemicals, can influence susceptibility of offspring to chronic diseases at adulthood [278, 344]. The impact of environmental influences during early life on developmental programming of diseases in adulthood has been previously shown in both human and animal studies [17, 345] and is linked to numerous conditions including obesity, type-2 diabetes, and hypertension [277, 278]. For the last decade, much emphasis in developmental programming research has been placed on the impact of nutritional insults during pregnancy [346, 347]. Less attention has been placed on *in utero* developmental programming effects of exposure to environmental contaminants such as heavy metals.

Cadmium and Hg are two common heavy metals typically encountered in the environment with limited understanding of their developmental programming effects. Industrial and human activities are the major contributing factors for the Cd and Hg load to the environment. In industry, these chemicals are used for the production of batteries, nanoparticles, pigments, coatings and platings, dental amalgams, electronic switches, fluorescent lamps, cosmetics, pharmaceuticals, disinfectant and pesticides [7, 9, 159]. Human actions, such as fossil fuel combustion, mining activities and incineration of solid wastes also release them to the atmosphere [10]. They are biologically non-essential heavy metals that bioaccumulate throughout the ecological food chain [7, 8]. Therefore, human exposure to Cd and Hg mainly occurs through dietary sources via consumption of vegetables, livestock and fishes [8]. Compared to non-smokers, the body burden of Cd is doubled in smokers [7]. In addition, exposure to Cd and Hg also occurs through non-dietary sources, including inhalation of Cd and

Hg vapors from industrial sources and combustion of fossil fuels [158], dental amalgams [187], or via skin absorption of cosmetic products [176].

Previous epidemiological studies demonstrated an association between low dose, chronic Cd or Hg exposure through the diet or smoking during pregnancy and high diastolic blood pressure [348], increased insulin resistance [12], deficits in neural development [201] and attention deficiency in children [13]. Pronounced phenotypic effects at adulthood have also been seen in rodent studies in response to maternal and lactational Cd or Hg exposure. Gestational Cd exposure has effects on behavior [349], and indicators of semen quality of offspring at adulthood [350]. Likewise, chronic maternal exposure to Hg caused altered immune and neurologic functions [14, 200] and behavioral effects [351] in offspring.

Current data in the literature on developmental programming effects of Cd and Hg are generally derived from chronic maternal exposure to individual compounds throughout pregnancy and/or lactation. But, in actuality, exposure to multiple toxic chemicals may occur simultaneously from several different sources. Furthermore, due to increased public consciousness, many women change their lifestyle after becoming aware of their pregnancy. Therefore, administration of environmental contaminants during the very early stages of pregnancy, and even before conception, is relevant to understanding the long-term effects of adverse maternal conditions on subsequent offspring health. Evidence suggests the periconception period (comprising the time immediately before and after conception) is a critical time for developmental programming [352, 353]. However, the majority of data demonstrating developmental programming during the periconception period is derived from manipulation of the maternal diet [344, 354]. Studies of periconception developmental programming are of increasing importance because approximately 50% of pregnancies are unplanned in the United

States, which greatly increases the chances of exposure of developing germ cells/embryo to potential unfavorable conditions during this critical time [355].

In the present study, we hypothesized that maternal administration of a combination of Cd and Hg during the periconception period would increase indices of chronic disease in offspring at adulthood. To test this hypothesis, anxiety-like behavior, glucose homeostasis, insulin sensitivity, body weight gain, organ weights, adiposity, and serum and molecular indices of metabolic syndrome were measured in offspring of females treated with 0, 0.125, 0.5 or 2.0 mg/kg body weight Cd and Hg four days before and four days after conception. Results support pronounced sexually dimorphic developmental programming effects of periconception Cd and Hg administration.

MATERIALS AND METHODS

Animals

Eight-week-old CD1 mice were obtained from Charles River Breeding Laboratories and acclimatized to local conditions for two weeks prior to initiation of experiments. Animals were maintained in a controlled environment with 12h light: dark cycle and 22 ± 1 °C room temperature with food and water provided *ad libitum*. All experimental procedures were performed under approval by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University.

Research Design

Individually caged female CD1 mice were administered daily subcutaneous (sc) injections of a combination of cadmium chloride (CdCl_2) and methylmercury(II) chloride (CH_3HgCl) at doses of 0.125, 0.5 or 2.0 mg Cd and Hg/kg body weight or vehicle (0.9% NaCl) for four days before injected females were placed with age-matched naïve males for mating.

Each treatment group was designed to consist of four litters. Presence of a vaginal plug at embryonic day 0.5 was considered confirmation of conception, and males were separated from females. Subcutaneous heavy metal injections were resumed for individually housed, plug positive females for the next four days following mating. After completion of periconception heavy metal administration, no further Cd plus Hg treatment was performed and duration of gestation was recorded.

After delivery, birth weights were determined for all pups, and litter size was standardized to eight by randomly selecting four males and four females from each litter. Offspring were individually housed with their dams until weaning at 28 days of age. At weaning, offspring from each litter were then group-housed together based on sex. Body weights were recorded regularly once a month throughout the duration of the experiment. Assessment of phenotypic effects of periconception Cd plus Hg administration was initiated when offspring were eight weeks of age.

The above experiment was repeated (Experiment 2) as described above with the exception that individually caged female CD1 mice were administered daily sc injections of CdCl₂ and CH₃HgCl at a dose of 2.0 mg Cd and Hg/kg body weight only or vehicle (0.9% NaCl) for four days before conception and four days after conception.

Behavioral Tests

Anxiety-like behavior of male offspring was tested by performing elevated plus maze and open field tests [356, 357]. Behavioral analysis was not conducted on female offspring to avoid the effect of cyclic hormonal variation. For the behavioral analyses, two males per litter for a total of eight offspring from each treatment group were used. A week before the experiment, animals were individually caged and subjected to behavioral analyses at eight weeks of age. All

behavioral tests were performed between Zeitgeber Time (ZT) 4 and ZT10. Each animal was used only once for behavioral tests.

1. Elevated Plus Maze Test

The maze consisted of a central component, two opposing open arms and two opposing closed arms. Each session lasted 30 min and was videotaped. Lighting conditions and temperature of the test room were maintained constant during the entire experiment. Animals were situated individually at the center of the maze. Total entries in the open arms were recorded.

2. Open Field Test

Animals were located at the center of a mouse activity monitoring cage to determine open-field activity during 30 min sessions using the TruScan Photo Beam Activity System (Coulbourn Instruments, Whitehall, PA, USA), which is equipped with sensor rings that detect the movement of individually placed animals in every direction. The data were analyzed using TruScan 99 software. Total movement time in the margins and center area, locomotor activity, and number of entries to the center area were determined.

Glucose Tolerance Test (GTT)

Males and female offspring at 12 weeks of age were individually caged and fasted (water was provided *ad libitum*) for six hours in the morning before initiation of the experiment. A total of 15 male and 13-16 female offspring per treatment were used for glucose tolerance testing. Body weights were recorded before the experiment and 2 g/kg body weight of D-glucose (Cat. No. G7528; Sigma-Aldrich, St. Louis, MO, USA) in sterilized 0.9% NaCl was intraperitoneally (ip) injected. Five µl of blood was obtained from the tail tip before and at 10, 20, 30, 60, 90 and

120 min after ip injection and glucose concentrations determined with a glucometer (True Result; Nipro Diagnostics, Fort Lauderdale, FL, USA).

RNA Isolation and Real Time PCR Analysis

Total RNA was isolated from adipose and liver tissue using an RNeasy® Mini kit (Qiagen, Valencia, CA) based on the manufacturer's instructions. Following DNase treatment for removal of genomic DNA contamination, RNA samples were reverse transcribed using iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) based on instructions. After completion of cDNA synthesis, samples were diluted to a final volume of 100 µl with nuclease-free water.

Real-time quantitative polymerase chain reaction (RT-PCR) was used for the quantification of gene transcripts (CFX96™ RT-PCR System BioRad). PerlPrimer® Software (<http://perlprimer.sourceforge.net>) v1.1.21 was used to design all the PCR primers used in the present studies [358]. Transcript abundance for genes of interest was normalized using the $\Delta\Delta CT$ method with hypoxanthine phosphoribosyltransferase (HPRT) as the reference gene. Initial tests showed that transcript abundance for HPRT in adipose and liver tissue was similar between control and treatment group offspring ($P>0.05$).

Insulin Tolerance Test (ITT)

Twelve-week-old male and female offspring of control females and females treated with 2.0 mg/kg body weight of Cd and Hg during the periconception period (Experiment 2) were individually caged and fasted (water was provided *ad libitum*) for six hours in the morning before starting the experiment. Then, body weights were measured and 0.75 IU/kg body weight of human recombinant insulin (Novolin-R, Novo Nordisk, Plainsboro, NJ, USA) was administered ip. Five µl of blood was obtained from the tail tip before and at 10, 20, 30, 60, 90

and 120 min after i.p. injection and glucose concentrations determined with a glucometer (True Results).

Blood and Tissue Collection

Experiments were terminated when animals reached 24 weeks of age. Blood was collected at room temperature from the retro-orbital sinus under isoflurone anesthesia. Then, blood samples were centrifuged at 5000 g for 15 min. Serum was obtained and stored at -80 °C until hormone assays. After collecting blood, animals were euthanized by cervical dislocation. Uterine, oviduct, testes, liver, kidney and abdominal adipose tissues were collected and weighed.

Hormone Assays

Serum samples were shipped to the Endocrine Technologies Support Core at the Oregon National Primate Research Center (Beaverton, OR, USA) for determination of leptin and insulin concentrations using validated assays [359, 360]. Samples were analyzed by enzyme-linked immunosorbent assay (ELISA; Mouse Leptin, Millipore, Billerica, MA, USA; Mouse Insulin, Merck, Winston-Salem, NC, USA). Intra- and inter-assay coefficients of variation were 6.9% and 7.86%, respectively, for leptin and 1.67% and 2.25%, respectively, for insulin.

Statistical Analyses

Effects of periconception co-administration of Cd and Hg on birth weights, litter size, organ weights, area under the curve, hormone concentrations, differences in mRNA expression and open arm entries in the elevated plus maze were analyzed using a one-way ANOVA with Proc GLM in SAS version 9.2 (SAS Institute, Cary, NC, USA). When the ANOVA test was significant, differences between least square means and controls were analyzed with Dunnett's multiple comparison test. Differences in body weights, glucose tolerance, insulin resistance, locomotor activity, and time spent at the center area and the margin walls in the open field were

discerned using repeated measures ANOVA. Statistic significance was based on $P < 0.05$. Data are expressed as mean \pm standard error of the mean (SEM).

RESULTS

Effects of Periconception Cd and Hg Co-Administration on Gestation Length, Litter Size and Birth Weights

Periconception co-administration of Cd and Hg to female mice at all doses tested did not affect gestational length of dams, litter size or birth weight of offspring ($P > 0.05$; Appendix 1; Table A.1).

Periconception Cd and Hg Co-Administration Increases Anxiety-Like Behavior

Reduced entries into open arms in the elevated plus maze test and increased amount of time spent at the margin walls as well as reduced entries and amount of time spent in the center area in an open field test were used to assess anxiety-like behavior of control and Cd plus Hg-treated adult male offspring. Male offspring from all treatment groups had reduced open arm entries compared to controls ($P < 0.05$; Fig. 1A).

Male offspring from females treated with the intermediate and highest dose of Cd and Hg (0.5 and 2.0 mg/kg body weight) during the periconception period spent more time in the margin area and less time in the center area of the open field arena (Fig. 1B) compared to control offspring ($P < 0.05$). Likewise, offspring from females administered the highest dose of Cd plus Hg during the periconception period (2.0 mg/kg) exhibited lower numbers of entries to the center area ($P < 0.05$). However, locomotor activities of animals derived from Cd plus Hg treated females were similar compared to control offspring ($P > 0.05$). Results support developmental programming effects of periconception Cd and Hg co-administration on anxiety-like behavior in male offspring at adulthood.

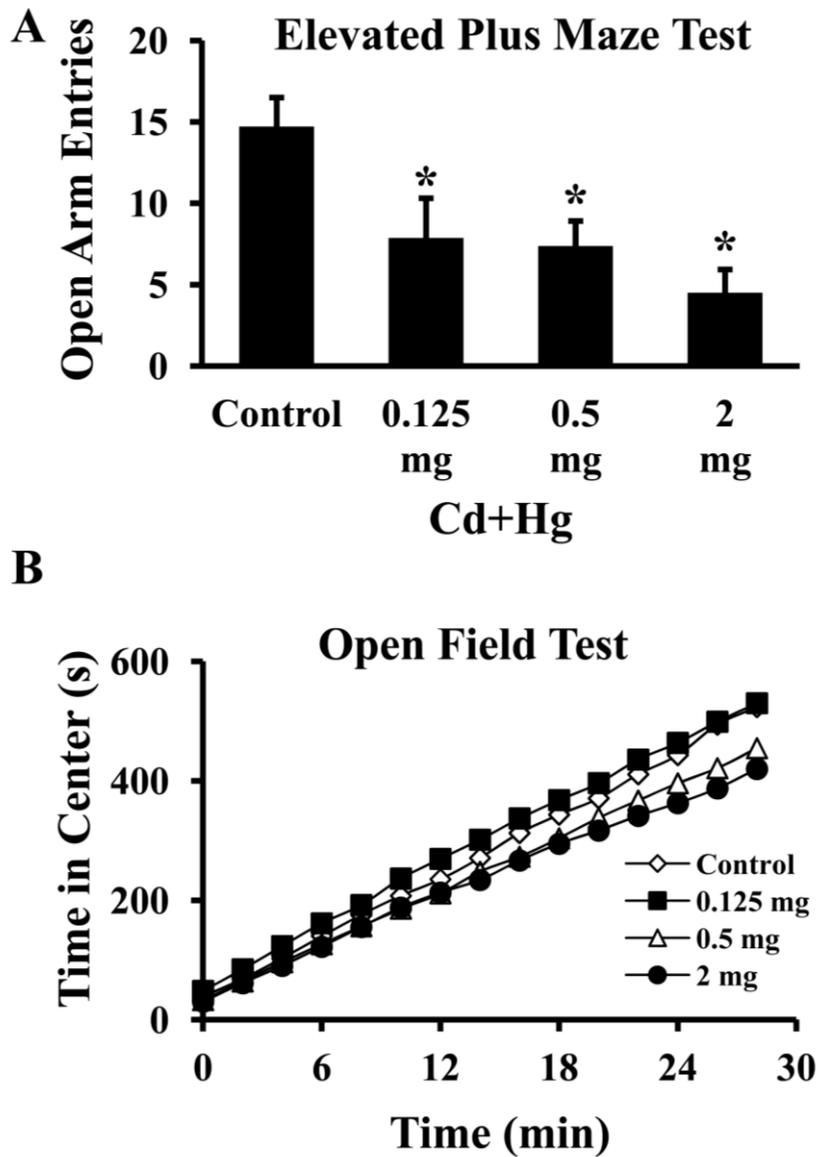


Figure 1: Anxiety-like behavior of eight-week-old male offspring of control and periconception Cd plus Hg-treated female mice. Open arm entries and cumulative amount of time spent in the center area were tested by elevated plus maze and open field tests, respectively (* $P < 0.05$ compared to controls; $n = 8$ animals per treatment). (A) Open arm entries of male offspring. X-axis represents each treatment group. (B) Cumulative amount of time spent in the center area. X-axis represents total experimental duration of open field test. Data are presented as mean \pm SEM.

Periconception Cd and Hg Co-Administration Impairs Glucose Tolerance

Glucose tolerance tests (GTT) were executed at 12 weeks of age on both male and female offspring as indices of potential developmental programming effects of periconception Cd and Hg co-administration on metabolism. In males, peak blood glucose levels were reached 20 minutes following glucose administration and remained higher until 120 minutes post-glucose challenge in Cd and Hg treatment group offspring relative to controls ($P < 0.05$; Fig 2A). Area under the curve (AUC) was also calculated, which depicts cumulative concentrations of glucose in the blood relative to baseline fasting levels. The AUCs for the glucose tolerance tests were also greater for male offspring from all three treatment groups compared to controls ($P < 0.05$; Fig. 2C).

Similar to males, female offspring of female mice injected with Cd plus Hg at all three doses tested had elevated blood glucose levels compared to controls, with peak levels 20 min after administering an ip bolus of glucose ($P < 0.05$; Fig. 2B). Likewise, AUC values were significantly higher for female offspring from all treatment groups compared to controls ($P < 0.05$; Fig. 2D). Results indicate periconception Cd and Hg co-administration negatively impacts glucose homeostasis in male and female offspring at maturity.

Periconception Cd and Hg Co-Administration Increases Body and Adipose Weights of Male Offspring

Body weights of male offspring were similar at weaning. However, a significant increase in body weights was found for male offspring from all treatment groups compared to controls beginning at 15 weeks of age ($P < 0.05$; Appendix 1; Fig. A.6) through termination of experiments at 24 weeks of age ($P < 0.05$; Fig. 3A). Quantification of organ/tissue weights following euthanasia revealed significantly higher abdominal adipose accumulation in male

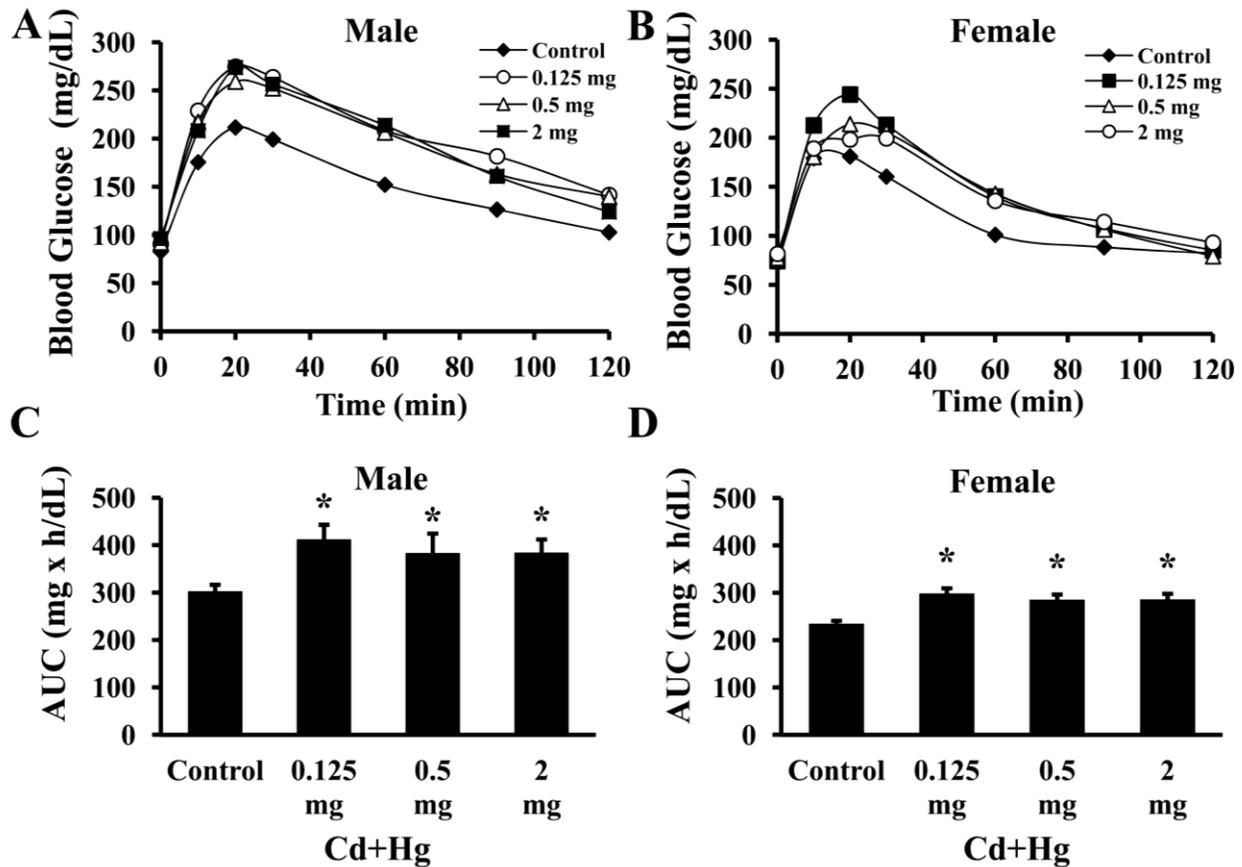


Figure 2: Glucose tolerance and area under the curve values for 12-week-old male and female offspring of control and preconception Cd plus Hg-treated female mice. Top Panel (A, B): Glucose tolerance of male and female offspring (n=15 and 13-16 per treatment in males and females, respectively). X-axis represents experimental duration in minutes and Y-axis represents blood glucose concentration in mg/dL. Bottom Panel (C, D): Area under the curve (AUC) values for male and female offspring (* P<0.05 compared to controls). X-axis represents each treatment group. Y-axis represents area under the curve values for plasma glucose in mg x h/dL of blood. Data are presented as mean \pm SEM.

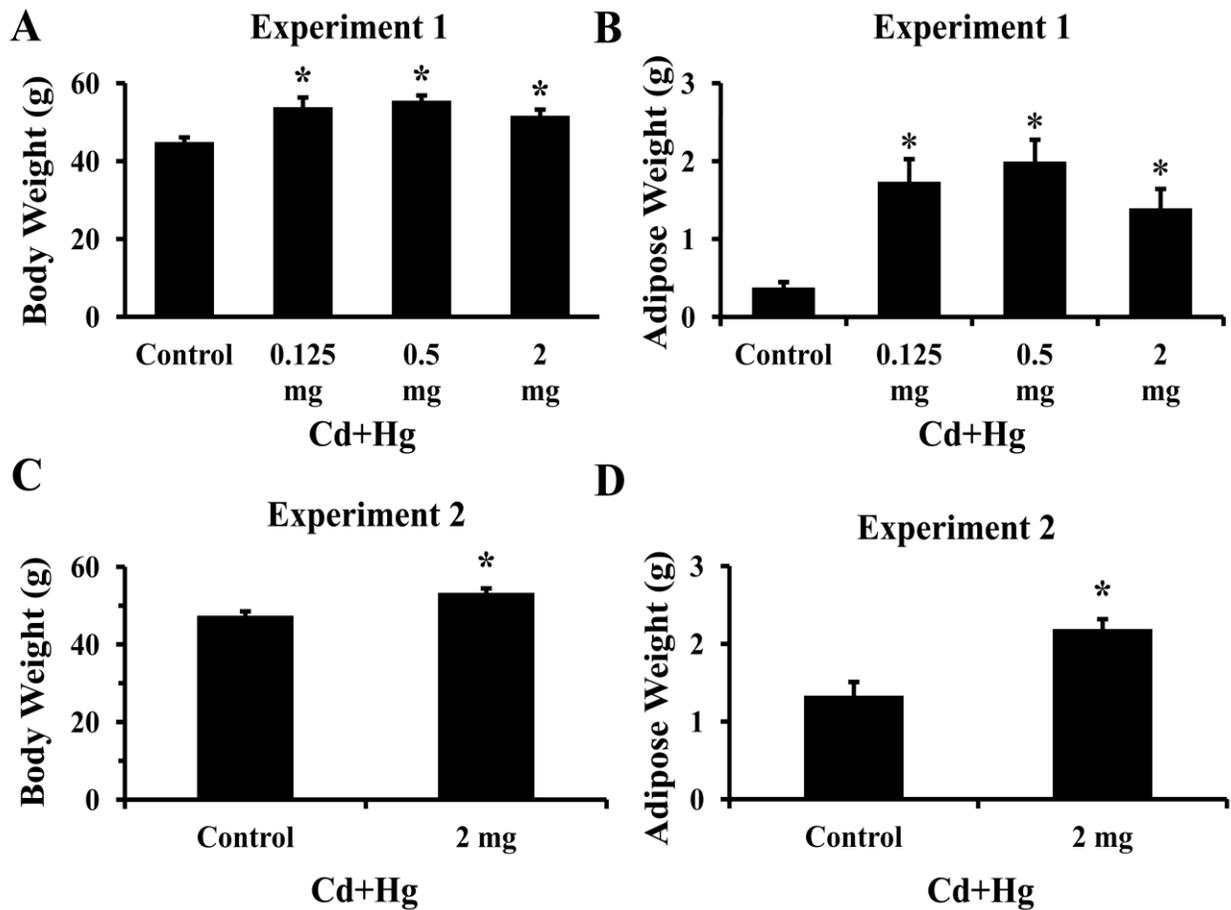


Figure 3: *Body and adipose weights of 24-week-old male offspring of control and periconception Cd plus Hg-treated female mice. [Experiment 1 (A, B) and Experiment 2 (C, D)]* (* $P < 0.05$ compared to controls; $n = 16-19$ per treatment in Experiment 1 and Experiment 2). Left Panel (A, C): Offspring body weights. Right Panel (B, D): Offspring abdominal adipose weights. X-axis represents each treatment group. Data are presented as mean \pm SEM.

offspring from all treatment groups compared to controls at 24 weeks of age ($P < 0.05$; Fig. 3B). However, liver, testes and kidney weights for male offspring from Cd plus Hg-treated females were similar to controls for all doses tested ($P > 0.05$; Appendix 1; Table A.2A; $n = 16$ male offspring per treatment). Likewise, similar effects were seen in a second experiment (Experiment 2), where male offspring of females subjected to periconception administration of 2.0 mg/kg body weight of Cd plus Hg exhibited increased body weights and abdominal adipose accumulation ($P < 0.05$; Fig. 3C and 3D). *In utero* treatment with Cd plus Hg did not affect body, adipose, kidney, liver, uterine and oviduct weights of female offspring at 24 weeks of age compared to controls ($P > 0.05$; Appendix 1; Table A.2B and Appendix 1; Fig. A.7A and A.7B; $n = 13-16$ female offspring per treatment).

Impact of Periconception Cd and Hg Co-Administration on Endocrine Parameters of Insulin Resistance

Male offspring from female mice treated during the periconception period with 0 or 2.0 mg/kg body weight of Cd and Hg (Experiment 2) were subjected to glucose and insulin tolerance tests starting at 12 weeks of age. As observed in the first experiment, male offspring of treated females had reduced glucose tolerance relative to control offspring ($P < 0.05$; Appendix 1; Fig. A.8). Insulin tolerance test was administered to the same male offspring at 13 weeks of age to elucidate whether the observed impaired glucose tolerance is linked to insulin resistance. Following insulin administration (0.75 IU/kg body weight), higher concentrations of blood glucose were observed in offspring of treated females ($n = 35$ offspring) compared to controls ($n = 34$ offspring) at all time points tested ($P < 0.05$; Fig. 4A) with the lowest levels of glucose for both groups being observed 60 min after administration ($P < 0.05$). Likewise, AUC was greater for male offspring of treated females compared to controls demonstrating that total amounts of

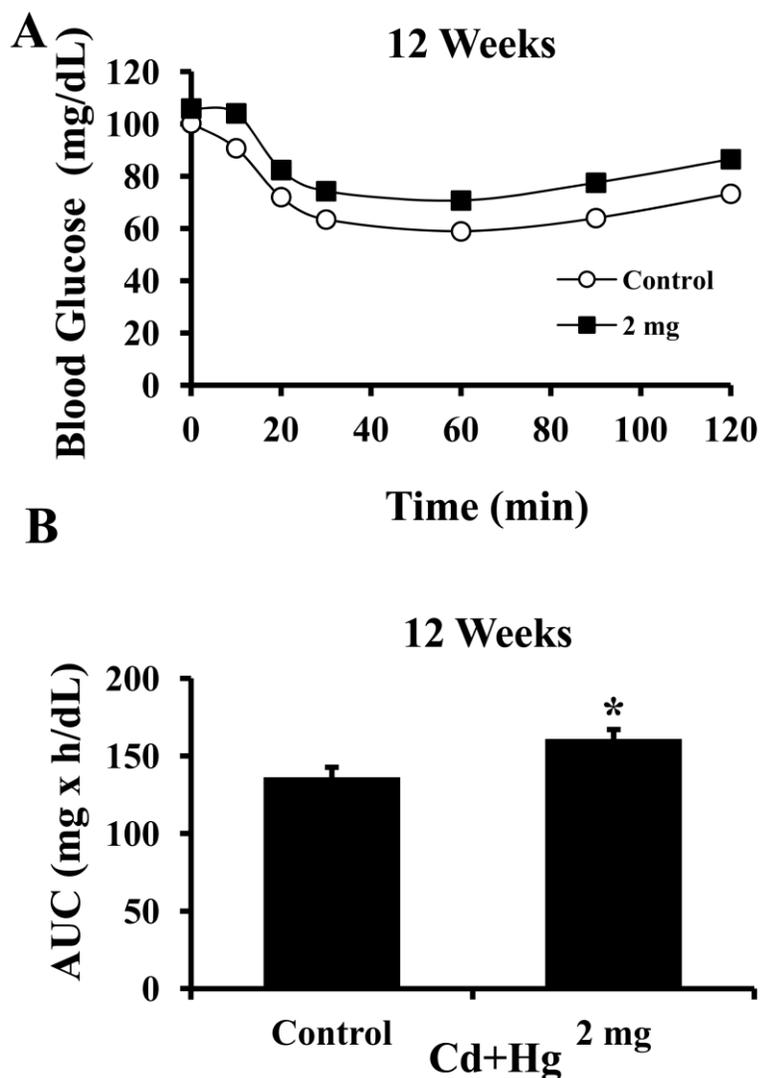


Figure 4: *Insulin tolerance and area under the curve values for 13-week-old male offspring of control and periconception Cd plus Hg-treated female mice. (A) Insulin tolerance of male offspring (n=34 for controls and n=35 for treated offspring). X-axis represents experimental duration in minutes and Y-axis represents blood glucose concentration in mg/dL. (B) Area under the curve (AUC) values for male offspring (* P<0.05 compared to controls). X-axis represents each treatment group. Y-axis represents area under the curve values for plasma glucose in mg x h/dL of blood. Data are presented as mean \pm SEM.*

glucose in circulation were significantly higher ($P < 0.05$; Fig. 4B). Following a 6 h fast, serum concentrations of insulin (Fig. 5A) and leptin (Fig. 5B) were significantly elevated in male offspring derived from treated females ($P < 0.05$; $n = 17-20$ animals per treatment used for serum leptin and insulin assays). Results indicate that periconception co-administration of Cd and Hg alters glucose homeostasis via increased insulin resistance. These results together with increased body weight and abdominal adiposity as well as elevated serum insulin and leptin concentrations suggest the onset of metabolic syndrome in male offspring of females exposed to Cd plus Hg at periconception period.

Periconception Cd and Hg Co-Administration and Associated Alterations in mRNA Abundance for Genes Linked to Metabolic Diseases

In adipose tissue, expression of genes involved in glucose uptake (GLUT4), transmission of insulin signaling (IRS1), and fatty acid synthesis (ACACA and FASN) were analyzed in male offspring of control female mice and female mice exposed periconception to Cd plus Hg. Reduced abundance of mRNA for these genes is correlated with insulin resistance and increased adiposity [361, 362]. Real time PCR analysis showed a significant reduction in mRNA abundance for all of above genes in adipose tissue of male offspring of females administered 2.0 mg/kg body weight of Cd plus Hg compared to control offspring ($P < 0.05$; Fig. 6A-D; $n = 11-17$ per treatment).

In liver, transcript abundance for genes required for fatty acid uptake (FATP2 and CD36) and glucose homeostasis (G6PC) were analyzed in male offspring of control and treated females. Aberrant mRNA expression for these genes is positively correlated with insulin resistance and impaired glucose homeostasis [363-365]. Results demonstrated increased mRNA abundance for FATP2, CD36 and G6PC in male offspring derived from female mice co-administered Cd and

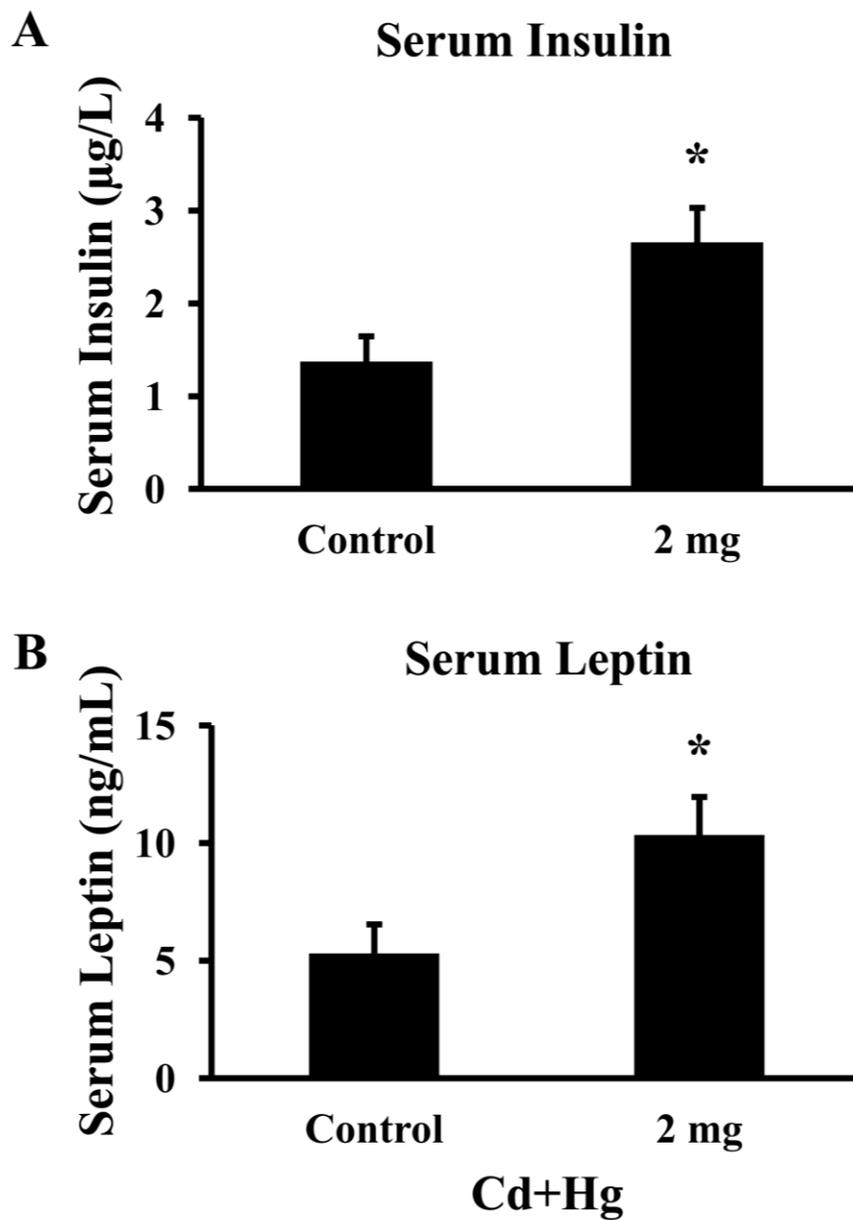


Figure 5: Serum concentrations of insulin and leptin in 24-week-old male offspring of control and periconception Cd plus Hg-treated female mice. (* $P < 0.05$ compared to controls; $n = 17-20$ per treatment). (A) Serum insulin concentration in $\mu\text{g/L}$. (B) Serum leptin concentration in ng/mL . X-axis represents each treatment group. Data are presented as mean \pm SEM.

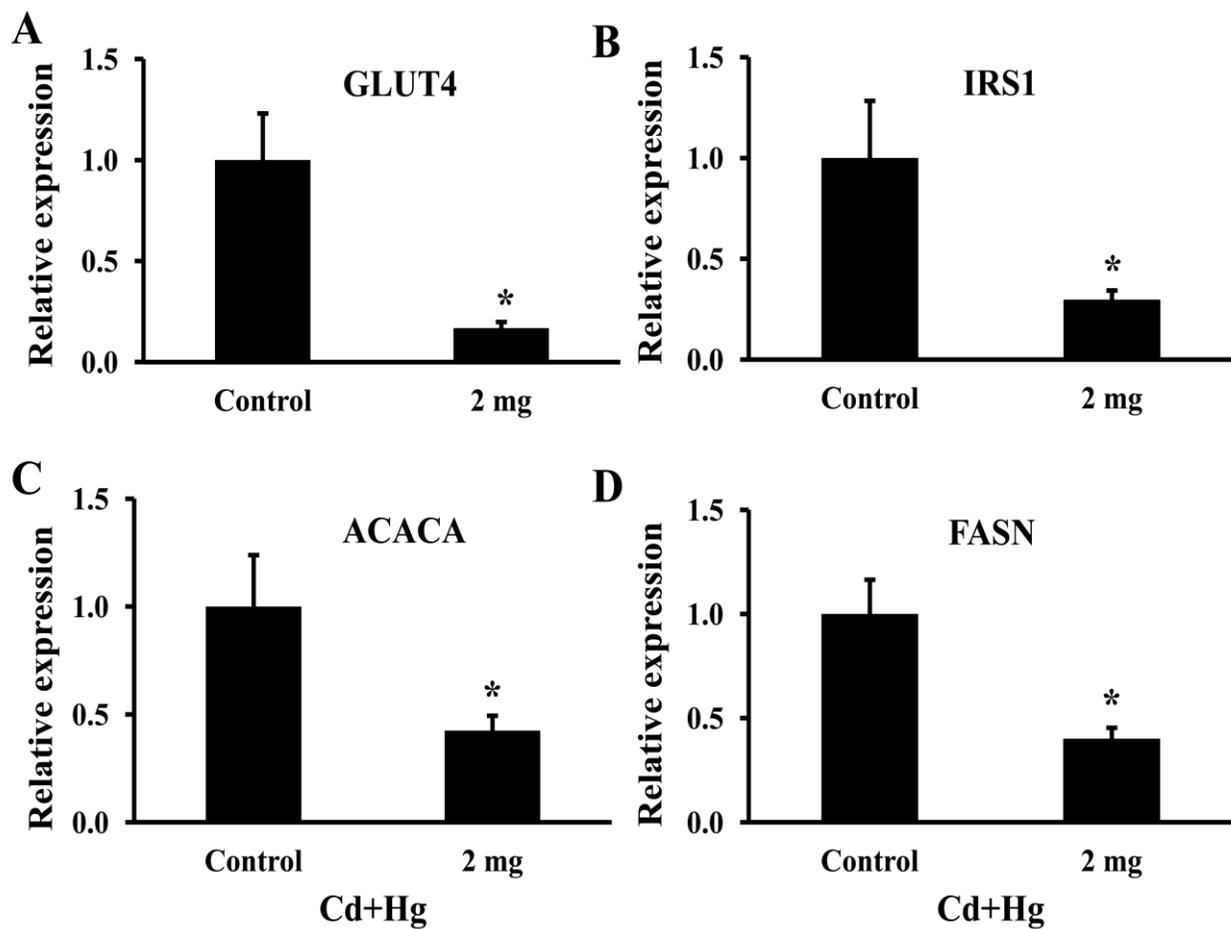


Figure 6: *Effect of periconception Cd and Hg administration on expression of mRNA in adipose tissue of adult male offspring of control and periconception Cd plus Hg-treated female mice [GLUT4 (A) , IRS1 (B), ACACA (C) and FASN (D)] (* P<0.05 compared to controls; n=11-17 per treatment). Quantitative reverse-transcription PCR analysis of male offspring adipose tissue obtained at 24 weeks of age. Data were normalized relative to abundance of endogenous control (HPRT). Data are presented as mean \pm SEM. Abbreviations: GLUT4 (Glucose Transporter Type 4), IRS1 (Insulin Receptor Substrate 1), ACACA (Acetyl-CoA Carboxylase Alpha), FASN (Fatty Acid Synthase).*

Hg compared to controls ($P < 0.05$; Fig 7A-C; $n = 11-17$ per treatment). Results further support developmental programming of metabolism and insulin resistance in offspring of females exposed to Cd plus Hg around the time of conception.

DISCUSSION

In the present studies, our aim was to investigate potential impact (using an animal model) of maternal exposure to heavy metals (initiated during the periconception period) on offspring susceptibility to adult onset chronic diseases. The present studies demonstrated the adverse effects of co-administration of Cd and Hg during the periconception window, including impaired glucose homeostasis as well as increased body weight and abdominal adipose accumulation in male but not female offspring at adulthood. To our knowledge, this is the first study in the literature demonstrating observed phenotypic changes in body and adipose weight and insulin resistance in response to Cd or Hg administration, especially in the context of co-administration of the heavy metals. Moreover, we chose our experimental doses based on previously published developmental programming effects of individual exposure to Cd or Hg in rodent models with a common lowest dose of 0.5 or 2.0 mg/kg body weight of exposure [16, 93]. Current studies in the literature with individual exposure to Cd or Hg at varying doses (0.5-10.0 mg/kg body weight) during the entire gestation or after embryo implantation often report decreased birth weights [16, 92, 93, 195]. However, body weight changes or increased adiposity at adulthood were not observed in these studies. To our knowledge, impaired glucose homeostasis and insulin resistance also has not been examined previously in response to prenatal Cd or Hg administration.

Results obtained in the elevated plus maze and open field test demonstrate anxiety-like behavioral effects without an impact on locomotor activity in response to co-administration of

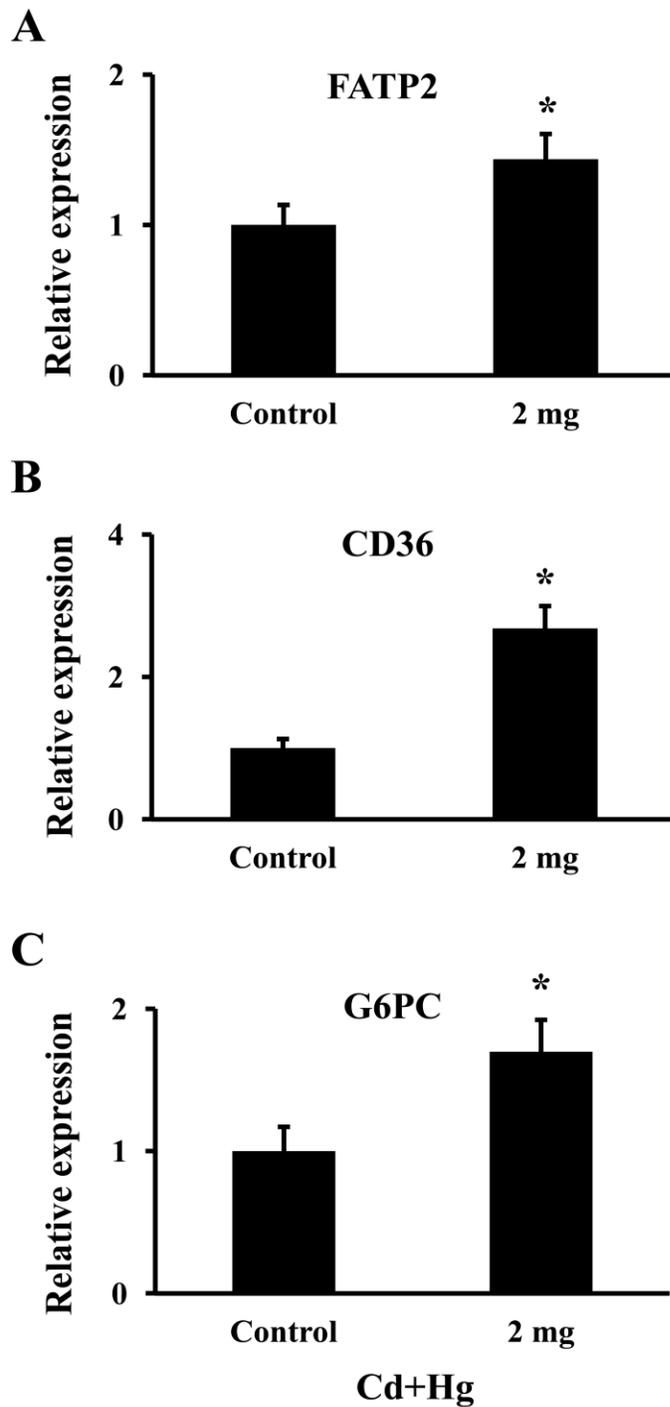


Figure 7: Effect of periconception Cd and Hg administration on expression of mRNA in liver tissue of adult male offspring of control and periconception Cd plus Hg-treated female mice.

Figure 7 (cont'd): [FATP2 (A), CD36 (B) and G6PC (C)] (* P<0.05 compared to controls; n=11-17 per treatment). Quantitative reverse-transcription PCR analysis of male offspring liver obtained at 24 weeks of age. Data were normalized relative to abundance of endogenous control (HPRT). Data are presented as mean \pm SEM. Abbreviations: FATP2 [Solute Carrier Family 27 (Fatty Acid Transporter), Member 2], CD36 [CD36 Molecule (Thrombospondin Receptor)], G6PC (Glucose-6-Phosphatase).

Cd and Hg around conception. In previous studies, increased anxiety-like behavior was observed with administration of Hg either at a very high dose, such as 8.0 mg/kg body weight of Hg on gestational day 8 [261], or administration later in gestation corresponding to the stage of organ and nervous system development where Cd and Hg might have direct impacts on offspring behavior. For instance, offspring of pregnant mice fed a diet containing 0.01 mg/kg body weight of Hg from gestational days 8 to 18 spent less time in the open field area suggesting increased anxiety-like behavior [14]. In contrast, reduced anxiety-like behavior reflected by increased open arm entries in the elevated plus maze was reported in offspring from mothers exposed to 0.6 mg/kg body weight of Cd from gestational days 7 to 15 [366]. However, in the present studies Cd and Hg administration started four days before conception and ceased before the normal day of embryo implantation. Therefore, observed effects on anxiety-like behavior suggest the potential existence of a more complex developmental programming mechanism rather than direct impact of Cd and Hg on the organ systems of the developing fetus. On the other hand, previous studies support the likelihood of Cd and Hg accumulation in reproductive tissues or preimplantation stage embryos [53]. Therefore, direct exposure of oocytes or embryos to even very low concentrations of heavy metals should be taken into consideration as a possible mediator of observed behavioral alterations in offspring at adulthood. However, because of half-life and toxicokinetics of Cd and Hg, future experiments involving embryo transfer will be required to conclusively prove that developmental programming effects observed in the current studies are attributed, at least in part, to exposure during the periconception period.

Disruption of normal metabolism causes a cluster of conditions including altered glucose homeostasis and increases adiposity leading to the onset of type-2 diabetes and obesity [112, 113]. This situation is termed as metabolic syndrome (MS) and there are several factors

responsible for the onset of MS, including life style factors of adults or adverse *in utero* conditions including nutritional insults or toxic chemical exposure [114, 116, 334]. In the present studies, it is not clear whether disturbed glucose homeostasis is responsible for increased body weight and abdominal adiposity in males or if it is the direct effect of periconceptual co-administration of Cd and Hg as increased body weight and adipose accumulation were not observed in female offspring.

The adult phenotype of an individual is formed by a complex interaction between genotype, gonadal hormones and environmental factors. Therefore, it is possible that female gonadal steroids might mask the impact of periconception Cd plus Hg administration making males more prone to metabolic problems and chronic diseases. The potential impact of gonadal hormones is illustrated by differences in fat distribution among men and women. In general, premenopausal women are likely to accumulate fat in subcutaneous depots. On the other hand, men tend to accumulate fat in visceral depots. However, due to loss of sex hormones, menopause is associated with fat storage in visceral depots and increased risk of metabolic diseases including insulin resistance and atherosclerosis [367] and this effect can be alleviated by postmenopausal hormone replacement therapy [368, 369]. Likewise, similar effects on the control of adiposity were observed in rodent models where gonadectomized females maintained on a high fat diet exhibited rapid weight gain and increased adiposity relative to males and intact females [370]. In addition, these female mice developed fatty liver and elevated leptin and insulin levels providing evidence of sexually dimorphic gonadal influences on adiposity and metabolism [370]. Therefore, effects of periconception Cd plus Hg administration on the onset of metabolic syndrome in females could be masked by the gonadal hormones that potentially attenuate or delay the onset of MS in females.

Results provide evidence of development of metabolic syndrome in male offspring of periconception Cd plus Hg-treated females. Impaired glucose tolerance of offspring could be caused by reduced sensitivity to insulin that maintains normal blood glucose homeostasis. To examine the indices of metabolic syndrome in Cd plus Hg-exposed male offspring, a series of endocrine and molecular indices were measured. Since all doses of Cd plus Hg caused impaired glucose tolerance and increased adiposity in male offspring, female mice were treated with only the 2.0 mg/kg body weight dose of Cd plus Hg during the periconception period (Experiment 2). Insulin tolerance studies performed at 13 weeks of age indicated altered glucose homeostasis is caused by reduced sensitivity to insulin. Similar to the previous experiment, these male offspring also exhibited increased body weight and abdominal adipose accumulation at 24 weeks of age. These results suggest that periconception Cd plus Hg co-administration reduced the cellular response of metabolic tissues to insulin. This might result in reduced glucose uptake and utilization by liver, adipose tissue or skeletal muscle that could cause build up of excess glucose in the systemic circulation over time as seen in the development of type-2 diabetes [371]. At this stage of insulin resistance, reduction of insulin sensitivity could be still adequate to keep fasting blood glucose in normal range, but when challenged with a meal or a glucose upload, postprandial glucose tolerance becomes abnormal [371]. Therefore, as a compensatory mechanism, pancreatic β -cells secrete more insulin into the circulation to reduce the excess levels of glucose [372]. Consequently, increased serum insulin concentrations suggestive of insulin resistance were observed in the current studies. In addition, leptin is an adipocyte-derived hormone and its concentrations are positively associated with increased adiposity and insulin resistance [373]. Serum leptin levels were also elevated in the circulation of male offspring of periconception Cd plus Hg-exposed females. Alterations in mRNA abundance for GLUT4, IRS1,

ACACA, FASN, FATP2, CD36 and G6PC observed in adipose and liver tissues, coupled with elevated insulin and leptin in male offspring, provide further evidence for developmental programming of metabolic syndrome in this model.

In conclusion, the present studies provide novel findings regarding the effects of Cd and Hg administration on indices of chronic disease including impaired glucose homeostasis via elevated insulin resistance and increased body and abdominal adipose accumulation as well as increased anxiety-like behavior in the context of combined administration of lower doses of Cd and Hg during the periconception period. Results also suggest that developmental programming effects of periconception heavy metal administration are sexually dimorphic with elevation of body weight and abdominal adiposity observed only in male offspring. Results support further investigation of the effects of periconception exposure to heavy metals and other toxicants, on incidence of chronic disease and mechanisms involved.

CHAPTER 4

**TRANSGENERATIONAL EFFECTS OF PERICONCEPTION HEAVY METAL
ADMINISTRATION ON ADIPOSITY AND GLUCOSE HOMEOSTASIS IN MICE AT
MATURITY**

ABSTRACT

Long term exposure to heavy metals, such as cadmium (Cd) and mercury (Hg), is known to increase risk of chronic diseases. In previous studies, we demonstrated that periconception administration of Cd plus Hg increased anxiety-like behavior, impaired glucose homeostasis and increased body weights and abdominal adipose accumulation of male offspring (Chapter 3). However, transgenerational effects of early life administration of Cd plus Hg during the periconception period have not been studied to date. Therefore, the effects of periconception Cd plus Hg administration on indices of chronic diseases at adulthood in F2-F4 generations were examined. F1 offspring of male and female mice whose mothers were administered 2.0 mg/kg body weight of Cd and Hg four days prior and four days after conception were bred with naïve CD1 mice to obtain F2 offspring and offspring mated to naïve CD1 mice as above through the F4 generation. Birth weight, litter size, behavioral alterations, glucose homeostasis, molecular markers of insulin resistance and organ weights were examined. Birth weights and litter size were similar in all generations. Increased anxiety-like behavior was observed in F2 male offspring descended matrilineally. Indices of impaired glucose homeostasis were observed transgenerationally in matrilineal descended male offspring, including reduced glucose tolerance and increased phosphorylation of insulin receptor substrate 1 (IRS1) at serine 307 linked biochemically to insulin resistance. Furthermore, sexually dimorphic effects on body and adipose weights were observed through the F4 generation specifically impacting males and with the phenotype inherited through matrilineal germ line. Results of the present studies suggest periconception co-administration of heavy metals results in persistent transgenerational effects on indices of chronic disease in offspring in the absence of continued toxicant exposure, and effects are inherited specifically through the matrilineal germline.

INTRODUCTION

The early life environment during *in utero* development, impacted by maternal nutrition, stress and exposure to environmental chemicals, can influence health and well being later in adulthood [278]. The impact of early developmental interventions was initially observed by David Barker and his colleagues demonstrating a positive correlation between low birth weight and increased risk of cardiovascular problems and diabetes during adult life [3]. Further research using human epidemiological approaches and rodent models demonstrated the impact of adverse *in utero* environmental conditions in increasing the incidence of adult onset chronic diseases, including stroke, coronary heart disease, hypertension, obesity and type-2 diabetes [17, 276, 277].

There is now considerable interest in the transgenerational inheritance of chronic diseases linked to adverse *in utero* conditions [6, 23, 374]. Current studies in rodent models provide evidence of persistent changes in offspring phenotype across generations in the absence of original insult, including maternal exposure to BPA [18], vinclozolin [19], pesticides [20], nicotine [304], and nutritional manipulations [24, 293]. Several studies demonstrated impacts that persist to the F3 generation, including reduced sperm quality [21, 292], changes in body weight and adiposity [293, 300], elevated blood pressure [25] and behavioral alterations [18]. In such studies, exposure of pregnant females (F0 generation) to suboptimal environmental conditions, may directly influence the developing fetus (F1 generation) and its germ cells that give rise to the subsequent F2 generation [279]. However, any effects observed beyond the F2 generation are considered truly transgenerational due to the absence of direct exposure [280].

Cadmium (Cd) and mercury (Hg) are two commonly found heavy metals in the environment with no known biological benefits [7, 8]. They are mainly released into the

atmosphere via industrial uses and human activities, including but not limited to, production of batteries, nano-particles, dental amalgams, cosmetics, pharmaceuticals, disinfectants, pesticides, fossil fuel combustion, mining activities and incineration of solid waste [7, 9, 10, 159]. Their bioaccumulation throughout the food chain is the main route of human exposure via consumption of vegetables, livestock and fish [7, 8]. Epidemiological studies provide a positive association between prenatal maternal or cord blood Cd or Hg concentrations and reduced birth weight [83, 169], elevated leptin concentrations [86], behavioral problems [88], neural tube defects [199], increased indices of upper respiratory diseases [91], changes in adiposity [90] and body weight [84] in children suggesting developmental programming effects.

Available evidence in the literature using rodent models support developmental programming effects of individual exposure to Cd or Hg throughout gestation and/or lactation [16, 174]. Such studies of Cd exposure at doses of 0.5-5 mg/kg body weight demonstrated reduced birth weight [92], fetal growth restriction [97], reduced serum testosterone concentrations [16] and a decline in serotonin concentrations in the brain cortex [155] in offspring at adulthood. Likewise, offspring of female rodents administered Hg at doses of 1-5 mg/kg body weight throughout pregnancy and/or lactation displayed reduced birth weight [174], behavioral alterations [15] and defects in motor coordination [264] potentially linked to altered mRNA abundance for neurotrophic factors [268] as well as dopamine and serotonin concentrations in the central nervous system [270-273]. However, persistent transgenerational effects of prenatal Cd and Hg exposure in subsequent generations have not been studied to date.

There are critical times during early development, such as implantation, organogenesis and parturition, where nutritional manipulations or toxic chemical exposure can impact developmental programming leading to the onset of chronic disease later in adult life [375-377].

Accumulating evidence, mainly derived from manipulating maternal nutrition, suggests the periconception period, which comprises the time before conception and immediately after conception, is also a critical time where developmental programming effects are manifested [344, 354]. The periconception period has significance, especially in the case of unplanned pregnancies, where suboptimal preconception conditions derived from maternal life style that persist until detection of pregnancy, might have long term persistent effects on the developing fetus, offspring at adulthood and the subsequent generations. However, available data in the literature on developmental programming effects of toxic chemical exposure are mainly derived from chronic administration of individual compounds throughout the majority of gestation and/or lactation [92, 174]. However, in real life, exposure to multiple toxic chemicals occurs simultaneously. In addition, many women change their life-style after detection of the pregnancy. Therefore, it is relevant to investigate the transgenerational effects of combinational exposure to multiple chemicals in the context of early life administration and even before conception.

There is little is known about potential transgenerational effects of combinational administration of Cd and Hg on early development, especially in the context of administration during the periconception period. Our previous studies demonstrated that periconception Cd plus Hg administration resulted in impaired glucose tolerance in offspring of both sexes as well as increased anxiety-like behavior, body weight and abdominal adipose accumulation in male offspring in the first (F1) generation (Chapter 3). Therefore, in the present studies, we hypothesized that maternal administration of a combination of Cd and Hg during the periconception period (four days before conception and four days after conception) would have persistent transgenerational effects on anxiety-like behavior, glucose homeostasis, body weight

gain and abdominal adiposity of offspring in multiple generations, even in the absence of additional Cd and Hg exposure.

MATERIALS AND METHODS

Animal Housing

Mice (CD1 strain) at eight weeks of age were purchased from Charles River Laboratories and housed on a 12h light: 12h dark cycle at 22 ± 1 °C with food and water provided *ad libitum* unless indicated. Animals were subjected to an acclimatization period of two weeks prior to initiation of experiments. All experiments were approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC).

Research Design

Generation of F1 male and female offspring from females administered Cd and Hg during the periconception period was described in Chapter 3. Briefly, individually caged female CD1 mice were administered daily subcutaneous (sc) injections of a combination of cadmium chloride (CdCl_2) and methylmercury(II) chloride (CH_3HgCl) at doses of 0.125, 0.5 or 2.0 mg Cd or Hg/kg body weight of each compound or vehicle (0.9% NaCl) for four days before injected females were placed with age-matched naïve males for mating (Fig. 8A). Each treatment group was designed to consist of four litters. For the forthcoming described studies, only animals from the 2.0 mg/kg dose and vehicle control groups were utilized. Presence of a vaginal plug at embryonic day 0.5 was considered confirmation of conception, and males were separated from females. Subcutaneous heavy metal injections were resumed for individually housed, plug

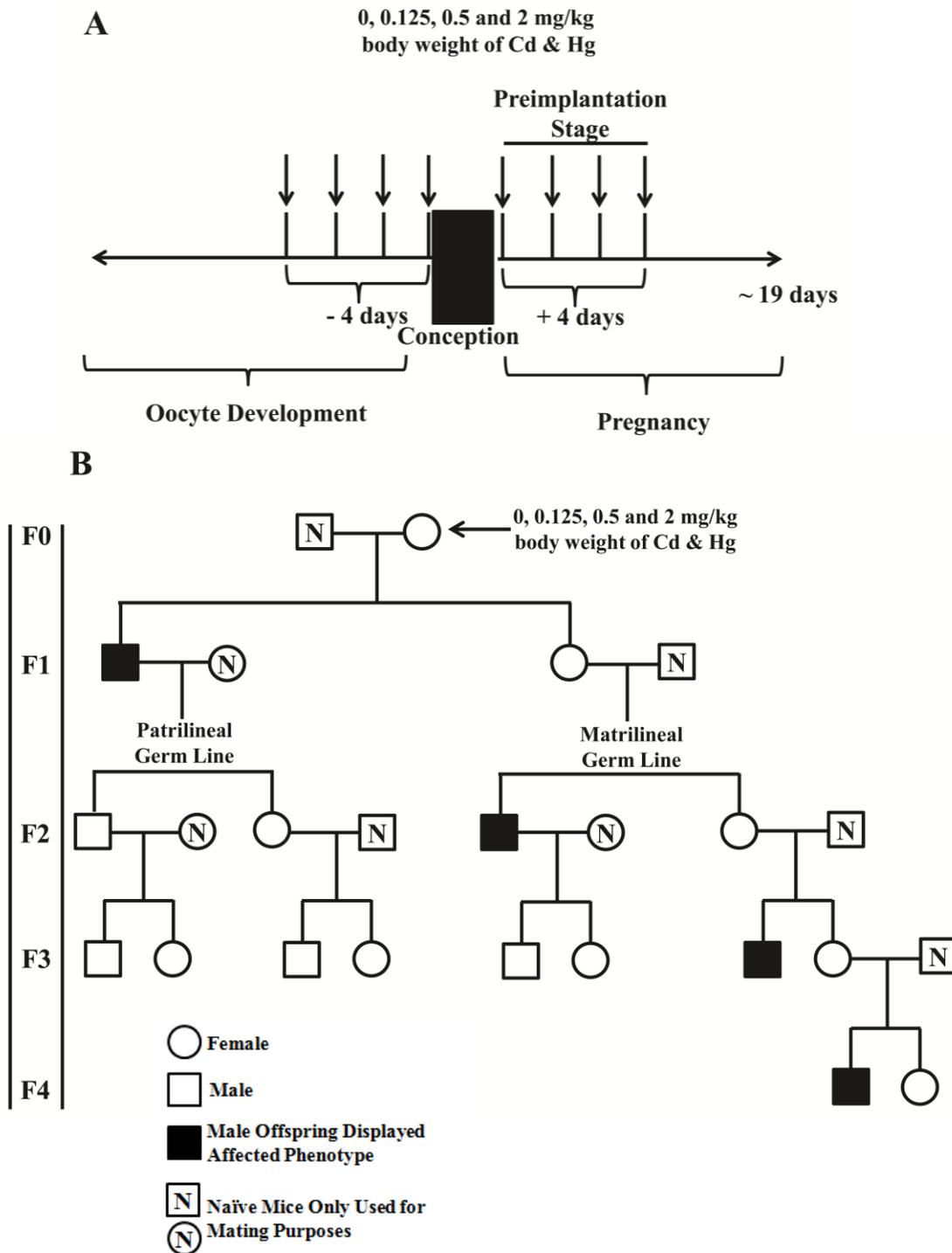


Figure 8: *Experimental design for the periconception administration of Cd and Hg to female CD1 mice and genealogy of the experiment across four generations illustrating transgenerational inheritance of chronic diseases.*

Figure 8 (cont'd): (A) Experimental design for the administration of Cd and Hg to female CD1 mice (F0 generation). (B) A genealogy of the experiment across four generations illustrating transgenerational inheritance of increased abdominal adiposity and impaired insulin signaling via increased phosphorylation of IRS1 protein at serine 307 (described in the results section) of male offspring descended through matrilineal germline. Black-filled boxes represent offspring that displayed affected phenotype (increased abdominal adiposity and impaired insulin signaling). Squares represent males and circles represent females. Naïve animals used for mating and generation of F2-F4 offspring are denoted with "N". All doses of Cd and Hg co-administration increased male adiposity in the F1 generation (black filled square; Chapter 3). However, no effects on abdominal adiposity were seen in female offspring. Subsequent F2-F4 generations were obtained from F1 male and female offspring derived from F0 females subjected to 2.0 mg/kg body weight dose of each compound during the periconception period and from offspring of control animals. In F2-F4 generations, only male offspring descended matrilineally from periconception Cd plus Hg-treated mothers (F0 generation) exhibited increased abdominal adiposity and impaired insulin signaling.

positive females for the next four days following mating. After completion of periconception heavy metal administration, no further Cd and Hg treatment was performed and duration of gestation was recorded.

Breeding of F2, F3 and F4 Generations

In the present studies, F1 male and female offspring (20 weeks old) of females administered 2.0 mg/kg body weight dose of both Cd and Hg during the periconception period and offspring of control females (20 weeks of age) were bred with 10-week-old naive CD1 mice to obtain F2 generation offspring. For the subsequent generations, the term patrilineal indicates F2 generation offspring and beyond derived from F1 males. Likewise, matrilineal indicates F2 offspring and beyond derived from F1 females. Similarly, F2 generation offspring were bred to 10-week-old naive CD1 mice to obtain F3 generation offspring and F3 offspring were mated to naïve CD1 females to obtain F4 offspring. Progeny in the F1-F4 generations were not subjected to Cd and Hg administration. At delivery, birth weights were determined and litter size was standardized to eight by randomly selecting four males and four females in each litter. After weaning at 28 days of age, offspring from each litter were group housed based on sex. Similar experimental procedures were followed and similar phenotypic parameters measured in age-matched offspring for transgenerational studies. Only altered phenotypes observed in the F1 generation (Chapter 3) that persisted across three generations were evaluated in the F4 generation.

Behavioral Tests

Elevated plus maze and open field tests were performed for 30 min duration as described in Chapter 3 to analyze anxiety-like behavior in male offspring [356, 357]. For the behavioral analyses, two males per litter for a total of eight offspring from each treatment group were used.

A week before the experiment, animals were individually caged and subjected to behavioral analyses at eight weeks of age. All behavioral tests were performed between Zeitgeber Time (ZT) 4 and ZT10. Each animal was used only once for behavioral tests.

1. Elevated Plus Maze Test

The maze consisted of a central component, two opposing open arms and two opposing closed arms. Each session lasted 30 min and was videotaped. Lighting conditions and temperature of the test room were maintained constant during the entire experiment. Animals were situated individually at the center of the maze. Total entries in the open arms were recorded.

2. Open Field Test

Animals were located at the center of a mouse activity monitoring cage to determine open-field activity during 30 min sessions using the TruScan Photo Beam Activity System (Coulbourn Instruments, Whitehall, PA, USA), which is equipped with sensor rings that detect the movement of individually placed animals in every direction. The data were analyzed using TruScan 99 software. Total movement time in the margins and center area, locomotor activity as well as the number of entries to the center area were determined.

Glucose Tolerance Test

Male and female offspring at 12 weeks of age (F2-F3 generation) or 24 weeks of age (F4 generation) were individually caged and fasted (water was provided *ad libitum*) for six hours in the morning before initiation of experiments. A total of 15 male and 13-16 female offspring per treatment were used for glucose tolerance testing. Body weights were recorded before the experiment and 2 g/kg body weight of D-glucose (Cat. No. G7528; Sigma-Aldrich, St. Louis, MO, USA) in sterilized 0.9% NaCl was injected ip. Five μ l of blood was obtained from the tail

tip before and at 10, 20, 30, 60, 90 and 120 min after ip injection and glucose concentrations determined with a glucometer (True Result; Nipro Diagnostics, Fort Lauderdale, FL, USA).

Tissue Collection

All experiments were terminated when offspring reached 24 weeks of age. For euthanasia, animals were maintained under isoflurone anesthesia and then subjected to cervical dislocation. In the F2 generation, abdominal adipose, liver, testes, kidney, uterine and oviductal tissues were collected. In F3 and F4 generations only abdominal adipose and liver tissues were collected. All tissue samples were weighed and stored at – 80 °C until analyzed.

Western Blot Analyses

For preparation of protein samples, liver tissue was subjected to homogenization for 30 sec and sonication for 90 sec duration in 30 sec intervals at 4 °C in RIPA buffer (150 mM NaCl, 1% IGEPAL®, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) that contained 1X protease and phosphate inhibitor cocktail (Roche Applied Science). Following centrifugation at 4 °C at 7000 x g for 5 min, protein content of the supernatant was determined by Bradford protein assay (BioRad) according to the manufacturer's instructions. Samples were aliquoted and stored at -80 °C until used.

For each animal/liver sample, 10 µg of protein was mixed with 5X sample buffer and denatured at 95 °C for 10 min. Then the samples were separated via SDS-polyacrylamide gel electrophoresis (4-20% gels, Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 95 V for 65 minutes. Following transfer, membranes were blocked with 5% BSA blocking buffer prepared in Tris buffered saline with Tween 20® (TBST; 137 mM sodium chloride, 20 mM Tris, 0.5% Tween 20) at room temperature for 1 hour. Membranes were incubated with respective primary antibody (listed

below) at 4 °C overnight in TBST containing 5% BSA. 1:1500, 1:1000 and 1:5000 (vol/vol) dilution was applied for primary antibodies against phosphorylated insulin receptor substrate 1 (p-IRS1; phosphorylated at Ser 307; rabbit polyclonal, Santa Cruz Biotech; sc-101709), total-IRS1 (T-IRS1; C-20; rabbit polyclonal, Santa Cruz Biotech; sc-559) and total actin (clone C4; mouse monoclonal; Millipore; MAB1501), respectively. Following overnight incubation with primary antibody, membranes were washed three times with TBST for 5 min each and incubated with HRP-conjugated anti-rabbit IgG for p-IRS1 and T-IRS1 (Anti-rabbit IgG, HRP-linked Antibody; Cell Signaling Technology; 7074) and anti-mouse IgG for total actin (Goat anti-mouse IgG (H+L) Secondary Antibody, HRP conjugate; Thermo Scientific; 31430) at a 1:5000 (vol/vol) dilution in TBST containing 5% BSA. Super Signal West Dura Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA) was used to visualize protein bands using a myECL imager (Thermo Scientific). After detection of individual protein of interest, membranes were stripped using Western blot stripping buffer (Thermo Scientific; 30 min duration at room temperature) and re-probed sequentially with subsequent primary antibodies of interest. Density of protein bands for p-IRS1, T-IRS1 and actin were determined using ImageJ software [378]. Protein abundance was determined by normalizing the data relative to abundance of total actin. Phosphorylation level of IRS1 was expressed relative to the abundance of total IRS1 (p-IRS1/T-IRS1).

Statistical Analyses

One-way ANOVA with Proc GLM in SAS 9.2 (SAS Institute, Cary, NC, USA) was used to determine differences in litter size, birth weights, organ weights, phosphorylation of IRS1 at Ser 307 and open arm entries in elevated plus maze test. Dunnett's multiple comparison test was applied to determine the difference between least square means and controls when overall

ANOVA was significant ($P < 0.05$). Repeated measures ANOVA was used to determine differences in body weight, glucose tolerance, locomotor activity, amount of time spent at the margin walls and center arena as well as number of entries to the center arena of the open field. Data were considered statistically significant if $P < 0.05$ and expressed as mean \pm standard error of the mean (SEM).

RESULTS

Effects of Periconception Cd and Hg Co-Administration on Birth Weights and Litter Size in F2-F4 generations

Differences in birth weight and litter size of offspring (F2-F4 generation) of periconception Cd plus Hg-treated females versus those of control (F0) generation females were not different ($P > 0.05$; Appendix 1; Table A.3).

Effects of Periconception Cd and Hg Co-Administration on Anxiety-Like Behavior in F2-F3 Generation Male Offspring

F2 male offspring descended through matrilineal germline, but not patrilineal germline of offspring of periconception Cd plus Hg-treated females displayed indices of increased anxiety-like behavior as indicated by a lower number of entries into the open arms in the elevated plus maze test ($P < 0.05$; Fig. 9A).

F2 male offspring from matrilineal and patrilineal germline displayed no difference in number of entries to the center area of the open field test ($P > 0.05$). However, matrilineal descended, but not patrilineal descended male offspring, spent more time at the margins and less time in the center area (Fig. 9B) compared to controls ($P < 0.05$) and displayed increased

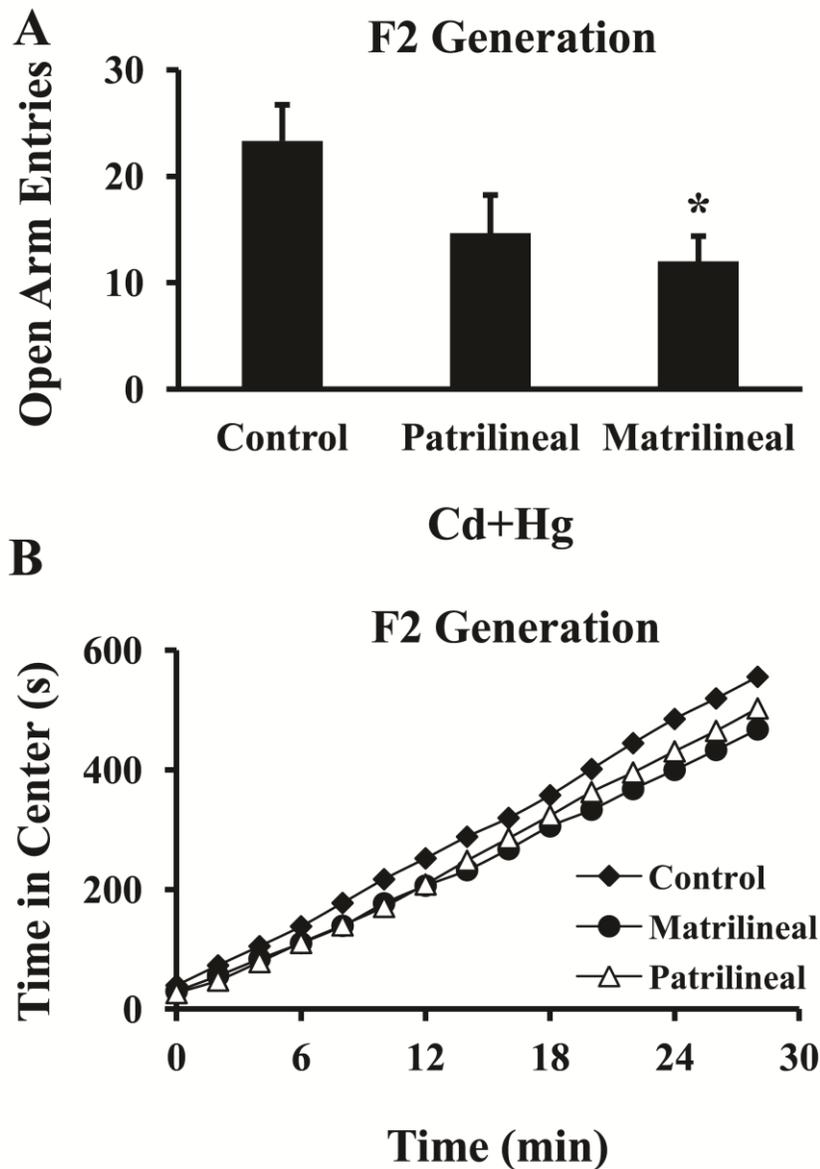


Figure 9: Anxiety-like behavior of F2 generation male offspring at 8 weeks of age. Open arm entries and cumulative amount of time spent in the center area were tested by elevated plus maze and open field tests, respectively (* $P < 0.05$ compared to controls; $n = 9-12$ per treatment). (A) Open arm entries of F2 males. X-axis represents ancestral lineage in F2 generation. (B) Cumulative amount of time spent in the center area. X-axis represents total duration of open field test. Data are presented as mean \pm SEM.

locomotor activity compared to controls ($P < 0.05$). No differences in indices of anxiety-like behavior in the elevated plus maze and open field ($P > 0.05$; Appendix 1; Figure A.9) or locomotor activity were observed for F3 generation offspring and behavioral analyses thus were not conducted for the F4 generation ($P > 0.05$; Appendix 1; Figure A.10).

Effects of Periconception Cd and Hg Co-Administration on Glucose Tolerance in F2-F4 Generation Male Offspring

F2 Generation: Glucose tolerance tests were performed at 12 weeks of age. Ten min after glucose administration, blood glucose concentrations in male offspring in the F2 generation of matrilineal descended offspring of periconception Cd plus Hg-treated females were significantly elevated compared to controls ($P < 0.05$; Fig. 10A). In these animals, peak glucose concentrations were reached 30 min following glucose administration and stayed higher for the remainder of the experiment relative to controls ($P < 0.05$). Conversely, blood glucose concentrations of male offspring in the F2 generation of patrilineal descended offspring of periconception Cd plus Hg-treated females were not different from controls ($P > 0.05$; Fig. 10A).

In order to determine cumulative glucose concentrations in the blood during glucose tolerance test, area under the curve (AUC) was also calculated. AUC for F2 males derived from the matrilineal, but not patrilineal germline, was significantly increased compared to controls ($P < 0.05$; Fig. 10B).

F3 Generation: At 12 weeks of age, male offspring from the F3 generation had similar responses to glucose administration compared to controls ($P > 0.05$; Fig. 10C and 10D).

F4 Generation: Even though glucose homeostasis was similar in F3 generation males at 12 weeks of age, other indices measured at 24 weeks of age (described below) were indicative of

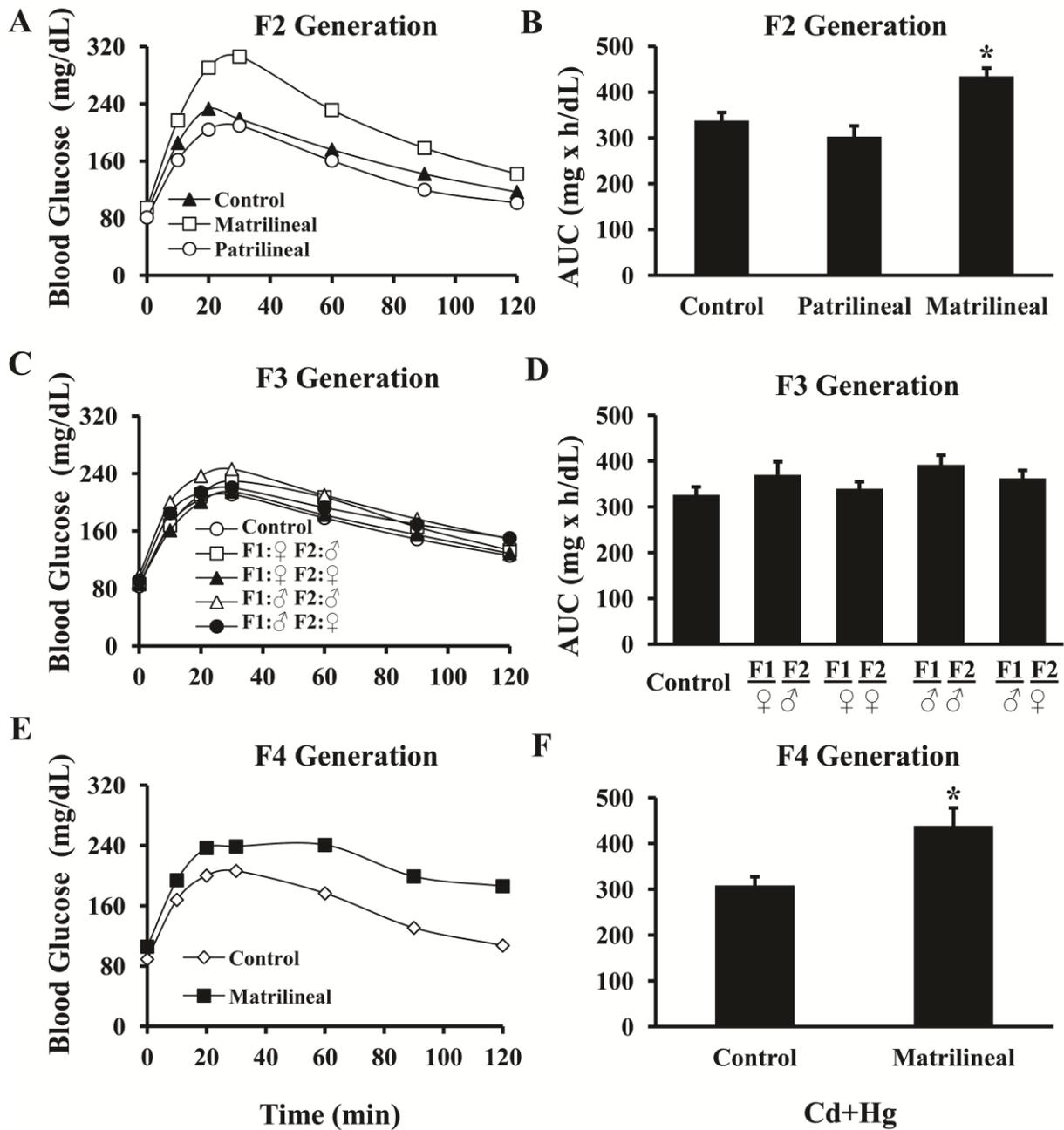


Figure 10: Glucose tolerance and area under the curve values of male offspring (n = 12-24, 15-18 and 9-20 per treatment in F2, F3 and F4 generations, respectively) Left Panel (A, C, E): X-axis represents experiment duration in minutes and Y-axis represents blood glucose concentrations in mg/dL. Right Panel (B, D, F): Area under the curve (AUC) values for male

Figure 10 (cont'd): offspring (* P<0.05 compared to controls). X-axis represents ancestral lineage of F2-F4 generations relative to F1 offspring of periconception Cd plus Hg-treated females. Y-axis represents area under the curve values for plasma glucose in mg x h/dL of blood. Data are presented as mean \pm SEM.

metabolic syndrome. Therefore, glucose tolerance tests were performed at 24 weeks of age in F4 offspring. Blood glucose concentrations in male offspring in the F4 generation descended matrilineally were significantly elevated relative to controls in response to glucose administration, with peak levels observed 60 min following ip glucose administration ($P < 0.05$; Fig. 10E). AUC analysis further demonstrated reduced glucose tolerance in F4 generation male offspring descended matrilineally ($P < 0.05$; Fig. 10F).

Effects of Periconception Cd and Hg Co-Administration on Body Weight and Abdominal Adiposity of F2-F4 Generation Male Offspring

F2 Generation: Body weights of all F2 male offspring were similar at weaning ($P > 0.05$). However, beginning at 17 weeks of age and continuing through termination of the experiment at 24 weeks of age, matrilineal descended F2 male offspring, but not patrilineal descendants of F1 animals had higher body weights compared to controls and patrilineal descended F2 male offspring ($P < 0.05$; Fig. 11A). Quantification of organ weights at euthanasia revealed increased abdominal tissue weights for male F2 offspring descended through the maternal, but not paternal germline (Fig. 11B).

F3 Generation: No difference in body weights was observed among male progeny in the F3 generations ($P > 0.05$; Fig 11C). However, male offspring in the F3 generation descended from the matrilineal germline (F1 and F2 generation) had increased abdominal adipose accumulation at 24 weeks of age compared to controls and male offspring derived from the patrilineal germline ($P < 0.05$; Fig. 11D).

F4 Generation: Beginning at 15 weeks of age and continuing through 24 weeks of age, body weights were greater for F4 male offspring that were descended matrilineally from (F1, F2 and F3 generations) periconception Cd plus Hg-treated females relative to control offspring

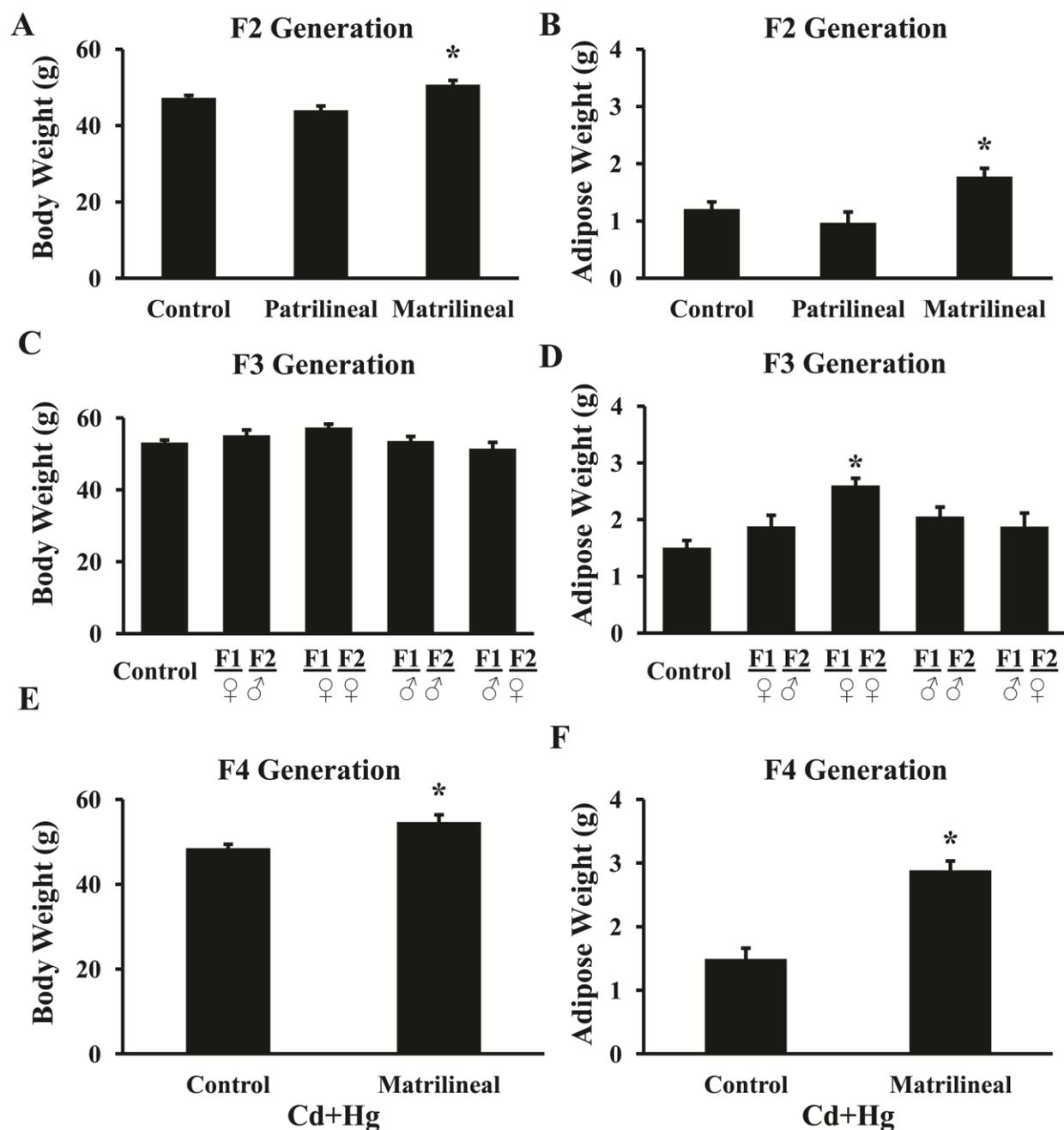


Figure 11: Body and abdominal adipose weights of male offspring at 24 weeks of age (* $P < 0.05$ compared to controls; $n = 16-23$, $19-21$ and $9-23$ per treatment in F2, F3 and F4 generations, respectively). Left Panel (A, C and E): Male offspring body weights in F2-F4 generations,

Figure 11 (cont'd): respectively. Right Panel (B, D and F): Male offspring abdominal adipose weights in F2-F4 generations, respectively. X-axis represents ancestral lineage of F2-F4 generations relative to F1 offspring of periconception Cd plus Hg treated females. Data are presented as mean \pm SEM.

($P < 0.05$; Fig. 11E). Abdominal adipose tissue weights were also greater for F4 generation offspring that were descendants through the matrilineal germline of periconception Cd plus Hg-treated females ($P < 0.05$; Fig. 11F). A genealogy to trace the lineage of male offspring displaying increased abdominal adiposity through the F4 generation is depicted in Figure 8B.

Effects of Periconception Cd and Hg Co-Administration on Molecular Markers of Impaired Glucose Tolerance in F1-F4 Generation Male Offspring

Liver tissue of F1-F4 generation males obtained from offspring of control females and of female descendants through the matrilineal germline (F1-F4 generation) of periconception Cd and Hg-treated females (F0 generation) were analyzed for changes in phosphorylation of IRS1 at Ser 307 indicating impaired insulin receptor signaling (Fig 8B). Relative abundance of IRS1 phosphorylation at Ser 307 (pIRS1/T-IRS) was significantly increased in male offspring through four generations (F1 – F4) that were descended matrilineally from periconception Cd plus Hg-treated females ($P < 0.05$; Fig. 12A-D). Results suggest impaired glucose tolerance observed transgenerationally is linked to altered insulin receptor signaling via increased phosphorylation of IRS1 protein at Ser 307.

Effects of Periconception Cd and Hg Co-Administration on Organ Weights in F2-F4 Generation Male Offspring

Liver and kidney weights were measured in the F2 and F3 generations and liver weights only in the F4 generation. No differences were observed in weights of such organs for offspring descended from control versus periconception Cd and Hg-treated females in the F0 generation ($P > 0.05$; Appendix 1; Table A.4).

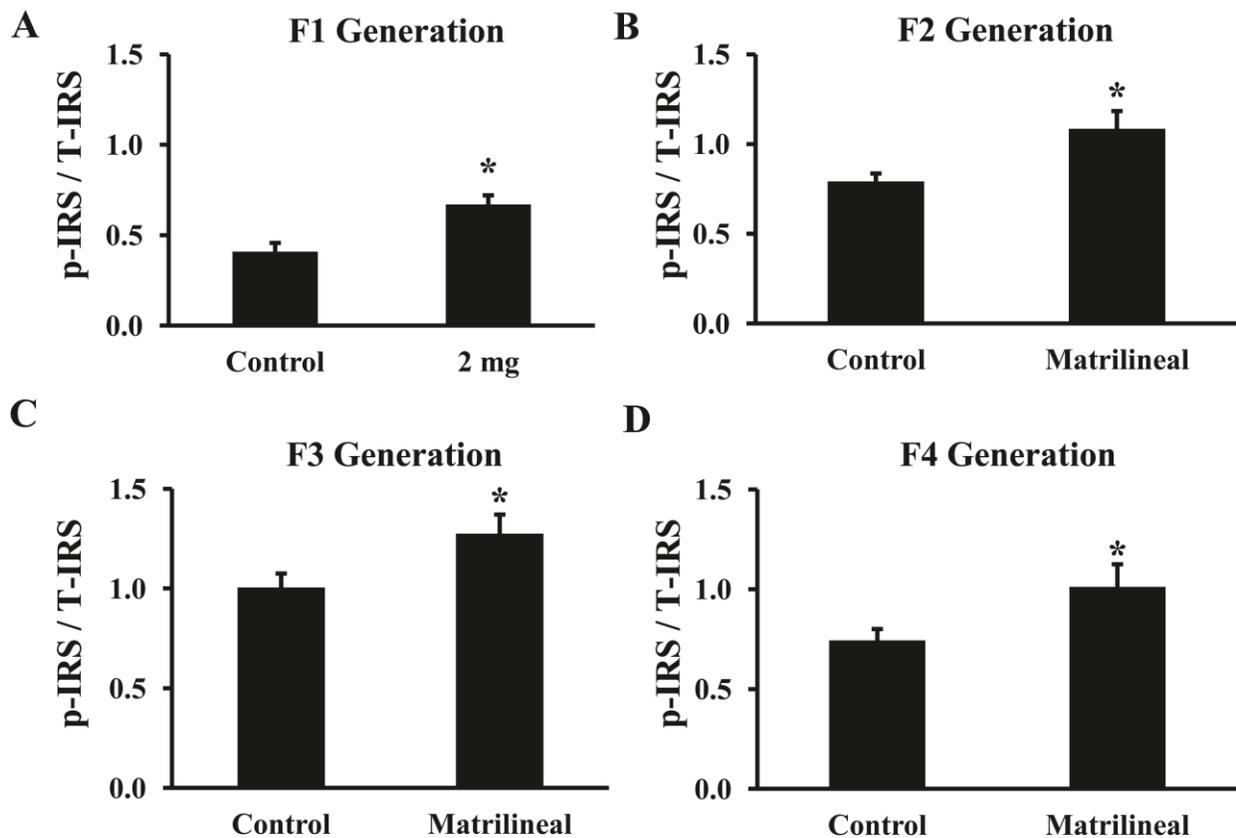


Figure 12: *Relative levels of Ser phosphorylation (residue 307) of IRS in liver tissue of male offspring at 24 weeks of age [expressed as ratio of phosphorylated (p-IRS1)/total IRS1 (T-IRS1)]* (* P<0.05 compared to controls) (A) F1 generation (n = 11-17 per treatment), (B) F2 generation (n = 22-23 per treatment), (C) F3 generation (n = 19 per treatment), (D) F4 generation (n = 21-24 per treatment). Data are presented as mean ± SEM.

Effects of Periconception Cd and Hg Co-Administration on F2-F4 Generation Female Offspring

Most parameters measured in female offspring in F2-F4 generation were not different for control offspring versus offspring descended from F0 females subjected to periconception Cd and Hg administration ($P>0.05$; Appendix 1; Table A. 5). Kidney weights of F2 female offspring descended from both the patrilineal and matrilineal germline of periconception Cd and Hg-treated females were higher relative to controls ($P<0.05$; Appendix 1; Table A. 5). For F2-F4 generation, glucose tolerance of female offspring was reduced relative to controls only for F2 animals descended patrilineally from F0 generation periconception Cd and Hg-treated females ($P<0.05$; Appendix 1; Fig A.11).

DISCUSSION

A growing body of evidence indicates environmental insults during pregnancy can increase susceptibility of offspring to chronic disease. However, less is known regarding whether effects of such environmental insults can be transmitted to subsequent generations in a multigenerational or transgenerational fashion. In the present studies, our aim was to determine whether developmental programming effects of periconception Cd plus Hg administration are transgenerational and persist through the F4 generation in the absence of further Cd and Hg exposure. Current studies in the literature examining developmental programming effects of Cd or Hg exposure during *in utero* development using murine models focused on exposure to a single compound during entire pregnancy or later stages of gestation and reported phenotypic effects in F1 offspring, including reduced birth weights [16], behavioral alterations [349], negative impacts on indices of male reproduction [350] as well as altered immune and neurologic functions [14, 200]. However, to our knowledge, there are no data in the literature reporting the transgenerational effects of *in utero* Cd or Hg exposure on indices of chronic disease. Our

previous results demonstrated F1 offspring of Cd plus Hg co-administered female mice had increased anxiety-like behavior, elevated body weight and abdominal adiposity and impaired glucose tolerance via increased insulin resistance (Chapter 3). Moreover, sexually dimorphic effects on body weight and abdominal adiposity were observed only for male offspring (Chapter 3). In the present studies, we extended our previous observations and examined phenotypes of offspring descended from the matrilineal or patrilineal germline of periconception F0 Cd plus Hg-treated females through the F4 generation. To our knowledge, this is the first study in the literature demonstrating a sexually dimorphic transgenerational phenotype through four generations in male offspring descended from the matrilineal germline of females exposed to environmental insults during pregnancy.

Results obtained from behavioral tests showed that F2 generation male offspring descended matrilineally from periconception Cd and Hg-treated females displayed increased anxiety-like behavior, but no differences were observed in the F3 generation. Results suggest direct exposure of developing fetus and its germ cells to Cd and Hg might be responsible for anxiety-like behavior in F1 and F2 generations. For each generation, offspring descended from periconception Cd and Hg-treated females were mated with naïve adults to obtain the subsequent generation without any further Cd and Hg administration that may cause a dilution of anxiety-like behavioral phenotype. In contrast to our previous results for the F1 generation, F2 male offspring descended matrilineally from periconception Cd and Hg-treated females displayed increased locomotor activity suggesting that decreased number of entries to the open arms or reduced amount of time spent at the center arena is not caused by inactivity of the animals. Similar results of increased anxiety-like behavior associated with increased locomotor activity have been reported previously [379, 380]. As a result, our findings could potentially explain the

reason for similar entries to the center arena of open field test between matrilineal descended males and controls in the F2 generation. However, this behavioral effect was transient and not observed in the F3 generation.

Our previous results demonstrated an increase in indices of metabolic syndrome, including insulin resistance and increased abdominal adiposity in F1 generation male offspring of periconception Cd plus Hg-treated females (Chapter 3). In the present studies we observed indices of metabolic syndrome throughout four generations in male offspring that were descendents through the matrilineal germline of F0 Cd plus Hg-treated females including reduced glucose tolerance, increased body and abdominal adipose weights and increased phosphorylation of IRS1 at Ser 307. In the current studies, glucose tolerance of F3 generation male offspring at 12 weeks of age was similar for animals that were descendents of control versus periconception Cd plus Hg-treated females. Since F3 generation males that were descended through the matrilineal germline displayed increased adiposity at 24 weeks of age, we hypothesized that reduced glucose tolerance may be delayed in subsequent generations. To test this hypothesis, glucose tolerance tests were performed at 24 weeks of age in F4 generation animals rather than at 12 weeks of age. Indeed, F4 generation males that were descended matrilineally from periconception Cd plus Hg-treated females displayed reduced glucose tolerance when examined at 24 weeks of age. Furthermore, increased levels of phosphorylation of IRS1 at Ser 307 were observed at 24 weeks of age in liver tissue of F1-F4 male offspring that were descended matrilineally from periconception Cd and Hg-treated females. Results cumulatively suggest impaired insulin receptor signaling and disrupted glucose homeostasis in such offspring through four generations.

Current studies in the literature examining the transgenerational impact of early life environment on glucose homeostasis and indices of metabolic syndrome primarily utilized nutritional manipulations or exposure to toxic chemicals in which the insult occurred during the entire pregnancy or after embryo implantation occurred. For instance, body weights were increased in F3 generation females that were descendants through the patrilineal germline of females subjected to high fat diet starting six weeks prior to conception and continued throughout pregnancy [293]. In contrast, administration of a low-protein diet during the entire gestation resulted in hyperinsulinemia in F3 generation offspring of both sexes [24]. Similar to nutritional manipulation studies, transgenerational effects of environmental toxicant exposure during pregnancy have been observed previously. For example, increased adiposity was observed in F3 generation offspring of both sexes that were descendants of females subjected to 25 or 50 mg/kg body weight of DDT exposure from gestational day 8-14 [23, 309]. Similarly, obesity in F1-F3 rat offspring of both sexes descended from F0 generation females subjected to 200 mg/kg body weight of methoxychlor from gestational day 8-14 has been reported [20]. In the same study, the obesity phenotype was only displayed in F4 generation females that were descended matrilineally [20]. However, studies of transgenerational effects of periconception Cd plus Hg administration are unique in the transgenerational phenotype was sexually dimorphic with only male offspring affected and mediated through the matrilineal germline.

Molecular indices of altered insulin signaling leading to impaired glucose tolerance across four generations were investigated in liver tissue of male offspring by analyzing phosphorylation of insulin receptor substrate 1 (IRS1) at Ser 307. A growing amount of evidence demonstrates impaired insulin receptor signaling via the IRS1-phosphatidylinositol 3-kinase-Akt pathway via increased phosphorylation of IRS1 at Ser 307 is responsible for insulin resistance

and diminished glucose uptake and utilization by metabolic tissues [381]. Available data regarding developmental programming of insulin resistance are limited to offspring in the first generation. These studies suggest the impairment in the insulin signaling is caused by elevated phosphorylation of IRS at serine residues that can be independent of changes in body weight and adiposity [382, 383]. In a rodent model of maternal obesity, 3-month-old male offspring of obese mice displayed hyperinsulinemia and increased phosphorylation of IRS1 at Ser 307 without any significant changes in body weight [382]. Male offspring of hyperinsulinemic and insulin resistant female mice, which was achieved via targeted disruption of IRS1, exhibited impaired glucose tolerance at one month of age even though their body weights and adiposity were similar to offspring of control females suggest a link between metabolic programming of offspring and functional IRS1 [383]. Female rat offspring over-nourished from birth until weaning via reducing litter size displayed adult-onset obesity, elevated concentrations of serum insulin, glucose and leptin, as well as increased phosphorylation of IRS1 at Ser 307 in skeletal muscle at three months of age [384]. Similar to developmental programming via nutritional manipulation models, *in utero* oral exposure to di-(2-ethylhexyl) phthalate at 100 mg/kg body weight from gestational day 9 to 21 impaired serum insulin, glucose and insulin tolerance linked to increased phosphorylation of IRS1 at Ser 307 in offspring of both sexes [336]. Such studies demonstrate the potential link between adverse prenatal conditions and impaired metabolism via IRS1 phosphorylation at Ser 307. However, to our knowledge, no studies have demonstrated transgenerational, sex-specific, developmental programming effects on impaired glucose homeostasis and insulin resistance linked to changes in phosphorylation of IRS1 at Ser 307.

Potential mechanisms responsible for increased IRS1 phosphorylation at Ser 307 in F1-F4 male offspring of periconception Cd plus Hg-treated females could potentially be mediated

via increased activation of serine kinases, including mammalian target of rapamycin (mTOR), tumor necrosis factor alpha (TNF α), c-Jun NH2-terminal kinase (JNK), protein kinase C (PKC) and stress activated protein kinases [385]. Potential stimulators of increased serine kinase activation include hyperinsulinemia, hyperlipidemia, stress and inflammation [386]. Furthermore transgenic mice lacking S6K1, which is a serine/threonine kinase, are resistant to diet induced obesity and insulin resistance suggesting importance of serine kinases leading to impaired insulin signaling and increased risk of metabolic syndrome [387].

In the present studies, increased abdominal adipose accumulation observed in male offspring through the F4 generation that were descended matrilineally from periconception Cd and Hg-treated females was not observed in female offspring. Hence the phenotype observed in the present studies might be mediated via the X chromosome. Males only receive a maternally imprinted X chromosome, whereas females receive two X chromosome imprints, one from each parent. In females, one of the parental imprinted X chromosomes is randomly inactivated in order to balance gene dosage between males and females [388]. Therefore, in the present studies, male and female offspring through four generations that were descended matrilineally inherited an X chromosome from the periconception Cd plus Hg-treated females. Hence, epigenetic modification of genes on the X chromosome could be linked to observed transgenerational phenotype in male offspring linked to metabolic syndrome and mediated via the matrilineal germline. Available evidence supports a role for genes on the X chromosome in metabolic syndrome. For example, a mutation in 5-HT2C gene increases susceptibility to type-2 diabetes and obesity [389] and mutations in NSDHL causes a disorder in lipid metabolism that impairs cholesterol biosynthesis [390]. Similarly, RSK2 knockout mice displayed impaired glucose tolerance and higher serum concentrations of insulin and glucose [391]. Available evidence

further indicates epigenetic regulation of such genes [392-395]. However, adult phenotype is determined by the complex interaction of genotype, gonadal hormones and environmental influences. A role for gonadal hormones in mediating sexually dimorphic phenotypes, including metabolic syndrome has been previously shown in both human and animal studies [396, 397]. These studies suggest that reduced concentrations of estrogen in postmenopausal women or ovariectomized rodents resulted in increased basal blood glucose and insulin concentrations, impaired glucose tolerance, increased body weight and adiposity [396, 397]. These effects are reversible as observed in estrogen/progestin hormone therapy in postmenopausal women [398], or estrogen administration in ovariectomized rodents [399]. As a result of this complex interaction, even though offspring of subsequent generations of both sexes inherited a similar maternal X chromosome, males may be more prone to develop metabolic diseases, including increased adiposity that persisted across four generations.

In conclusion, the present studies provide novel findings regarding the persistent transgenerational effects of periconception Cd plus Hg administration manifested via increased body weight and abdominal adiposity and impaired glucose tolerance. Results also suggest the sexually dimorphic effects of periconception Cd plus Hg co-administration that selectively affect male offspring throughout four generations are transmitted specifically through the matrilineal germline. From a broader perspective, our results further suggest environmental toxicant exposure during pregnancy in prior generations (e.g. grandmother and beyond) could be linked to increased incidence of chronic disease in the human population, and with differences in sensitivity and transmission across male versus female offspring. However, further work will be required to establish the potential clinical significance and relevance in the human population of

transgenerational effects of environmental toxicant exposure and mechanisms responsible for transgenerational and gender specific phenotypes observed in the present studies.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Evidence obtained both from epidemiological studies and animal experiments demonstrate adverse maternal conditions, including nutritional manipulations, *in utero* toxic chemical exposure and maternal stress, can have long lasting effects on offspring at adulthood [17, 276, 277]. There is also now growing interest in the persistent transgenerational effects of adverse maternal conditions on health and vitality of subsequent generations even if the original insult is no longer present [6, 23, 374]. Cadmium and Hg are two commonly found heavy metals in the environment with no known biological benefits. They are commonly used in different industries and their persistent transgenerational developmental programming effects have not been studied previously. Results of present studies established developmental programming effects of periconception Cd and Hg co-administration on offspring susceptibility to chronic diseases that persists transgenerationally. Moreover, the results also demonstrated sexually dimorphic effects of periconception Cd plus Hg administration that impact male but not female offspring through four generations and are inherited specifically through the matrilineal germline. Results greatly enhance understanding of developmental programming effects of periconception Cd and Hg administration and provide a model system for further examination of mechanisms associated with transgenerational epigenetic inheritance of chronic disease. In a broader term, our results suggest that periconception exposure to toxic chemicals and heavy metals in previous generations in the human population, in this case, even by one's great grandmother, could potentially be linked to the increase indices of chronic diseases experienced today.

An important question that is relevant to the present studies is whether or not the observed phenotypic effects on offspring and subsequent generations are mediated via direct effects of Cd and Hg on the oocyte and or preimplantation stage embryo. The current knowledge

of the importance of the periconception period in developmental programming is derived from studies manipulating maternal diet [344, 354]. But, to my knowledge, there are no studies in the literature demonstrating effects of periconception exposure to toxic chemicals on offspring susceptibility to chronic diseases. Present studies clearly demonstrate for the first time the effects of periconception Cd plus Hg administration on the transgenerational developmental programming of chronic diseases in offspring at adulthood. However, due to the long half life of Cd and Hg, they may persist in the maternal circulation/target tissues during later stages of gestation, which could contribute to the observed offspring phenotype. Hence, these toxicokinetics properties of Cd and Hg pose a limitation to conclusively proving that the observed phenotypic effects are attributed, at least in part, to the periconception window of exposure. Therefore, embryo transfer studies, where transfer of embryos from Cd and Hg co-administered females to the naïve pseudo-pregnant recipients, and vice versa, will be required to demonstrate the observed phenotypic effects are attributed in part or entirely to the periconception window of exposure.

One of the most significant findings of the present studies is the increased body weight and abdominal adiposity observed in the male offspring descended matrilineally through four generations which was not observed in female offspring. The mechanism responsible for this sexual dimorphic phenotype that selectively affected male offspring and was inherited from the matrilineal germline is an important focus for future studies. Transgenerational transmission of this sexually dimorphic phenotype through four generations in male offspring could be attributed to inheritance of the X chromosome from periconception Cd plus Hg treated females. The only X chromosome of males is descended maternally, whereas females have two X chromosomes, one from each parent [400]. In order to have a balance in gene dosage between different sexes,

females randomly inactivate one of the parentally descended X chromosomes [388]. Therefore, in contrast to female offspring, the X chromosome derived from Cd plus Hg-treated females is inherited throughout four generations by male offspring that are descended matrilineally. Furthermore, available evidence (discussed below) clearly demonstrates the impact of genes present on the X chromosome in regulation of metabolism [389-391].

A phenotype is determined by a complex interaction between genotype and the environment and gonadal hormones can influence phenotypic differences attributed to gender. Evidence indicates onset of metabolic diseases, including obesity and type-2 diabetes, are masked or delayed by female gonadal steroids. The potential impact of gonadal hormones is illustrated by increased body weight, abdominal adiposity and insulin resistance in both menopausal women and gonadectomized animal models which are mitigated by gonadal steroid hormone replacement therapy [367-369]. To address the question of whether the observed sexually dimorphic phenotype is masked in females by sex hormones, gonadal steroid production can be ablated via ovariectomy in female offspring from each generation inherited from periconception Cd and Hg treated and control females and steroid hormone replacement performed to determine if effects are reversible. In such studies, gonadectomized females are expected to display similar increases in body weight gain and abdominal adiposity as seen in male offspring derived from Cd plus Hg treated females, and estrogen replacement should reverse the effects on body weight gain and adipose accumulation.

Another important question for the future is the potential mechanism(s) responsible for transgenerational inheritance of increased indices of chronic diseases. It would be intriguing to perform additional analyses to determine if periconception Cd plus Hg administration will impact epigenetic regulation of genes present on the X chromosome linked to the control of

metabolism leading to increased offspring susceptibility to chronic diseases transgenerationally. In the present studies, doses of Cd and Hg were chosen to reflect the common lowest doses used in previously published studies on developmental programming effects of Cd and Hg [16, 93]. In the present studies periconception administration of such doses of Cd plus Hg did not affect gestation length, litter size or sex ratio of offspring throughout four generations suggesting that experimental doses did not display maternal or embryonic toxicity. Therefore, it is unlikely the phenotype is linked to DNA mutations. Moreover, available evidence suggests that several genes present on the X chromosome, such as 5-HT2C, NSDHL and RSK2, are linked to the control of lipid metabolism, glucose tolerance and susceptibility to obesity and type-2 diabetes [389-391] and such genes are also regulated by epigenetic modifications [392-395]. Therefore, examination of expression and epigenetic modifications of X-linked genes obtained from liver and adipose tissue from offspring across four generations, including DNA methylation, histone modifications and/or post-transcriptional regulation by miRNAs will reveal potential epigenetic regulation of such genes linked to development of metabolic syndrome and the relevance to transgenerational developmental programming effects of periconception Cd plus Hg administration.

An important question that remains to be answered is the potential intracellular mechanism(s) of action whereby heavy metals elicit developmental programming effects that persist transgenerationally. Relevant to this is the mechanism of Cd and Hg uptake into cells. In the present studies, it is unclear whether or not Cd and Hg are directly transported into the cell nucleus or interact with intercellular transporters to affect gene expression and epigenetic modifications. Due to their structural similarities with essential nutrients, heavy metals can often enter cells in a similar fashion as micronutrients. To do this, they utilize similar transport pathways that essential metals use, such as protein transporters and ion channels [401]. For

example, divalent cation transporter-1 is involved in the cellular uptake of essential divalent metals, such as Fe^{+2} . However, this transporter is not specific to the essential metals, but also allows cellular uptake of Cd and other toxic divalent metals [401]. Likewise ion channels, including voltage-gated calcium channels, are involved in the facilitated transport of Cd and other heavy metals into cells [402]. In contrast, lipid solubility of Hg facilitates its transport across the cellular membranes. Available evidence suggests that Hg has a high affinity for reduced sulfhydryl groups, including cysteine and glutathione, and formation of a complex between Hg and these groups structurally mimics the neutral amino acid methionine. This complex can easily pass through cellular membranes using neutral amino acid carriers [181].

Mechanisms of action of heavy metals inside the cell are also relevant to understanding potential developmental programming effects. Upon entry into the cell, heavy metals can form complexes with endogenous proteins and reach the nucleus [401]. In the nucleus, heavy metals directly interact with DNA through opposite electrical charges and can cause conformational changes in the DNA double helix structure [403]. Such changes inhibit interaction between DNA and nuclear proteins and impair availability of DNA for transcription factors [404]. More importantly, heavy metals also induce epigenetic changes within the genome. For example, it has been shown both *in vitro* (liver cells) and *ex vivo* systems that Cd is able to inhibit DNA methyltransferase activity via interacting with its DNA binding domain leading to DNA hypomethylation [405]. Heavy metals can also affect the activity of chromatin remodelling enzymes, including histone acetyltransferases, deacetylases, methyltransferases, and kinases, via directly interacting with enzyme sulfhydryl groups or utilizing metal cofactors or indirectly via modulation of specific signal transduction pathways [406, 407]. As a result, changes in activity of these enzymes regulate gene transcription via modifications of histone tails, including

acetylation, methylation, phosphorylation and ubiquitination [330]. For example, mouse Hepa-1 hepatoma cells treated with 50 μ M chromium for one hour caused histone deacetylation leading to histone methylation as well as DNA hypermethylation resulting in silencing of CYP1A1 gene expression [408]. Similar epigenetic modifications may occur in the oocyte or developing preimplantation stage embryo or developing fetus in response to periconception Cd and Hg treatment. It will be of interest to link persistent changes in gene expression in developing embryos or adult tissues linked to phenotype observed in response to periconception Cd plus Hg administration to accompanying alterations in epigenetic signatures at affected loci in future studies.

In summary, studies completed for my dissertation research provide novel evidence that periconception Cd and Hg co-administration has transgenerational developmental programming effects on offspring susceptibility to adult onset chronic diseases. Male but not female offspring were affected through four generations and the phenotype inherited through the matrilineal germline. My research provides a novel model for study of mechanisms involved in transgenerational epigenetic regulation of susceptibility to chronic disease and support the periconception window as a new paradigm for testing effects of administration of other heavy metals and toxic chemicals on susceptibility to adult-onset chronic diseases. However, further research is required to determine clinical significance and mechanisms whereby developmental programming effects of periconception Cd plus Hg administration are potentially mediated on oocytes and (or) preimplantation embryos.

APPENDIX

Treatment	Birth Weight
Control	1.58 ± 0.03
0.125 mg	1.61 ± 0.02
0.5 mg	1.57 ± 0.02
2 mg	1.63 ± 0.02

	Litter Size			
	Control	0.125 mg	0.5 mg	2 mg
Litter 1	18	14	14	14
Litter 2	12	15	14	12
Litter 3	14	12	13	16
Litter 4	11	10	14	11

	Gestational Length (Days)			
	Control	0.125 mg	0.5 mg	2 mg
Litter 1	18	18	18	19
Litter 2	18	18	18	19
Litter 3	19	19	19	19
Litter 4	19	19	18	19

Table A.1: Birth weights and litter size of offspring of control and preconception Cd plus Hg-treated female mice and impact on gestational length. No difference was found in any parameters in any group tested compared to controls ($P > 0.05$). Data are presented as mean ± SEM.

A

Treatment	Liver	Testes	Kidney
Control	2605.41 ± 112.40	271.52 ± 9.33	902.36 ± 37.39
0.125 mg	2723.38 ± 196.19	251.62 ± 6.45	863.47 ± 35.20
0.5 mg	2828.71 ± 110.28	303.91 ± 11.63	939.79 ± 40.38
2 mg	2469.88 ± 90.53	253.46 ± 12.11	958.48 ± 22.73

B

Treatment	Liver	Uterine	Oviduct	Kidney
Control	1767.06 ± 95.77	277.66 ± 60.54	36.59 ± 2.22	499.20 ± 22.94
0.125 mg	1684.41 ± 105.45	205.48 ± 18.06	38.54 ± 1.68	505.76 ± 13.36
0.5 mg	1841.12 ± 57.01	215.55 ± 22.11	39.62 ± 3.33	515.02 ± 9.60
2 mg	2002.25 ± 53.32	285.64 ± 36.81	47.77 ± 5.82	567.13 ± 42.62

Table A.2: Organ weights of male and female offspring of control and preconception Cd plus Hg-treated female mice. (A) Male organ weights. No difference was found in liver, testes and kidney ($P > 0.05$). (B) Female organ weights. No difference was found in liver, uterine, oviduct and kidney ($P > 0.05$). Data are presented as mean ± SEM.

Generation	Treatment	Birth Weight	Litter Size
F2 Generation	Control	1.62 ± 0.02	12.83 ± 1.25
	Matrilineal	1.66 ± 0.02	12.00 ± 0.95
	Patrilineal	1.62 ± 0.02	13.67 ± 0.33
F3 Generation	Control	1.62 ± 0.01	13.00 ± 1.47
	Paternal Grandmother	1.66 ± 0.02	13.33 ± 1.28
	Maternal Grandmother	1.59 ± 0.01	14.67 ± 1.09
	Paternal Grandfather	1.66 ± 0.01	13.50 ± 0.67
	Maternal Grandfather	1.65 ± 0.02	12.83 ± 0.70
F4 Generation	Control	1.73 ± 0.02	11.33 ± 1.33
	Matrilineal	1.70 ± 0.03	10.00 ± 1.53

Table A.3: Birth weights and litter size of offspring of F2-F4 generations derived from F1 offspring of periconception Cd and Hg treated females. No difference in birth weights or litter size were found compared to controls (P>0.05). Data are presented as mean ± SEMs.

Generation	Treatment	Liver	Testes	Kidney
F2 Generation	Control	2308.76 ± 63.43	275.20 ± 5.94	799.70 ± 28.91
	Patrilineal	2066.85 ± 37.91	263.68 ± 5.14	813.48 ± 35.32
	Matrilineal	2306.58 ± 81.84	258.47 ± 4.73	804.19 ± 24.26
F3 Generation	Control	2732.71 ± 77.97		
	Paternal Grandmother	2834.41 ± 157.04		
	Maternal Grandmother	3044.66 ± 149.27		
	Paternal Grandfather	2548.16 ± 62.70		
	Maternal Grandfather	2519.77 ± 70.51		
F4 Generation	Control	2335.25 ± 63.11		
	Matrilineal	2432.95 ± 139.11		

Table A.4: *Male organ weights of F2-F4 generation offspring derived from F1 offspring of control and periconception Cd plus Hg treated females. No difference relative to controls was found in organ weights across 4 generations (P>0.05). Data are presented as mean ± SEMs.*

Generation	Treatment	Adipose	Liver	Uterine	Oviduct	Kidney
F2 Generation	Control	841.53 ± 141.82	1966.92 ± 54.90	203.59 ± 13.61	43.95 ± 2.28	465.01 ± 9.38
	Patrilineal	724.30 ± 130.85	2008.95 ± 48.32	261.68 ± 27.11	43.86 ± 3.64	532.60 ± 12.95*
	Matrilineal	943.15 ± 222.71	2095.14 ± 92.73	252.20 ± 15.12	42.22 ± 2.53	533.64 ± 14.25*
F4 Generation	Control	1908.28 ± 262.88	1917.25 ± 66.86			
	Matrilineal	2257.84 ± 223.81	2159.41 ± 96.09			

Table A.5: *Female organ weights of F2 and F4 generation offspring derived from F1 offspring of control and periconception Cd plus Hg treated females. No difference was found in weights of any organs measured (P>0.05), except kidney weights of maternally and paternally derived F2 females compared to controls (* P<0.05 compared to controls). Data are presented as mean ± SEM.*

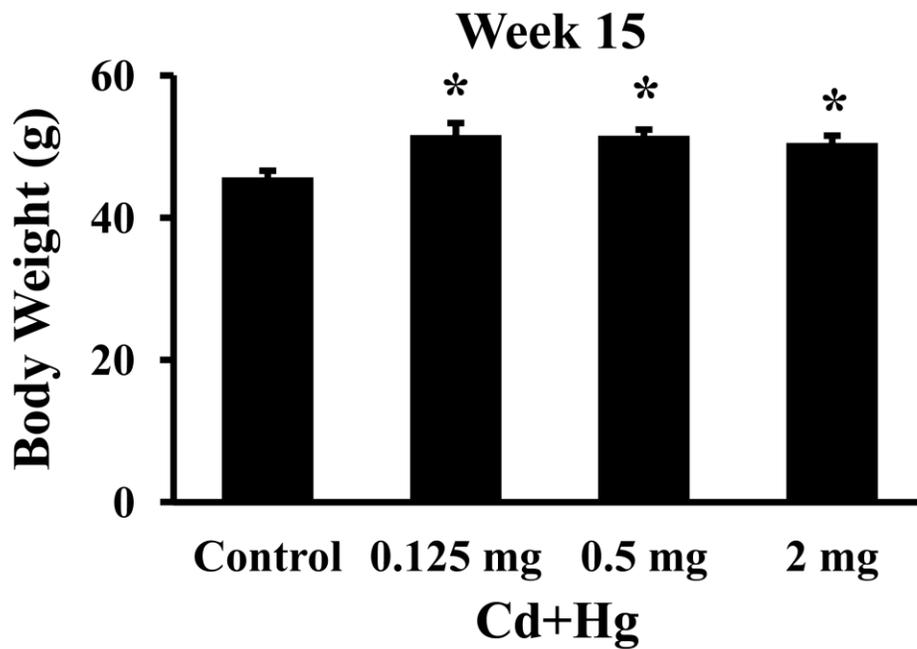


Figure A.6: Body weights of 15-week-old male offspring of control and periconception Cd plus Hg-treated female mice. ($P < 0.05$ compared to controls; $n = 16$ per treatment). X-axis represents each treatment group. Data are presented as mean \pm SEM.

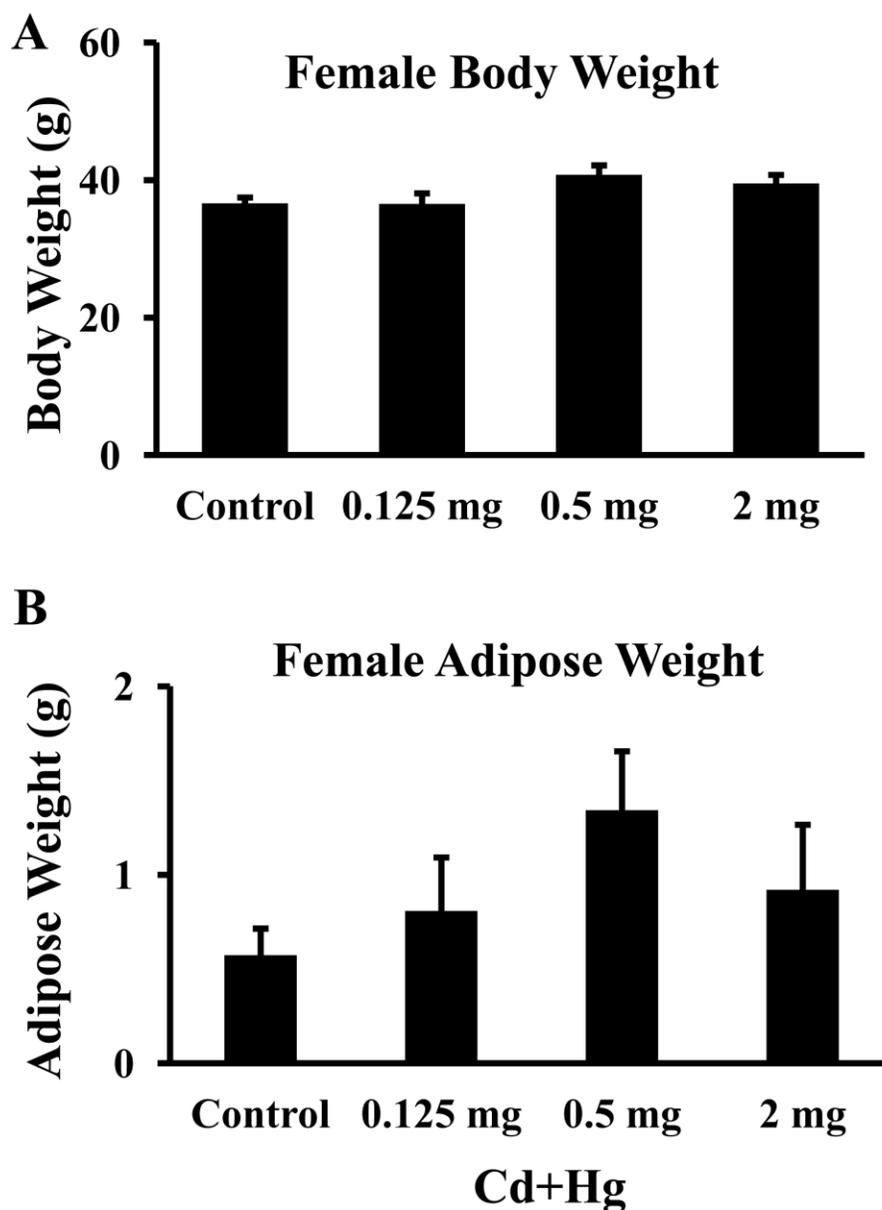


Figure A.7: *Body and adipose weights of 24-week-old female offspring of control and periconception Cd plus Hg-treated female mice. (P>0.05 compared to controls; n=13-16 per treatment). (A): Female offspring body weights. (B): Female offspring abdominal adipose weights. X-axis represents each treatment group. Data are presented as mean ± SEM.*

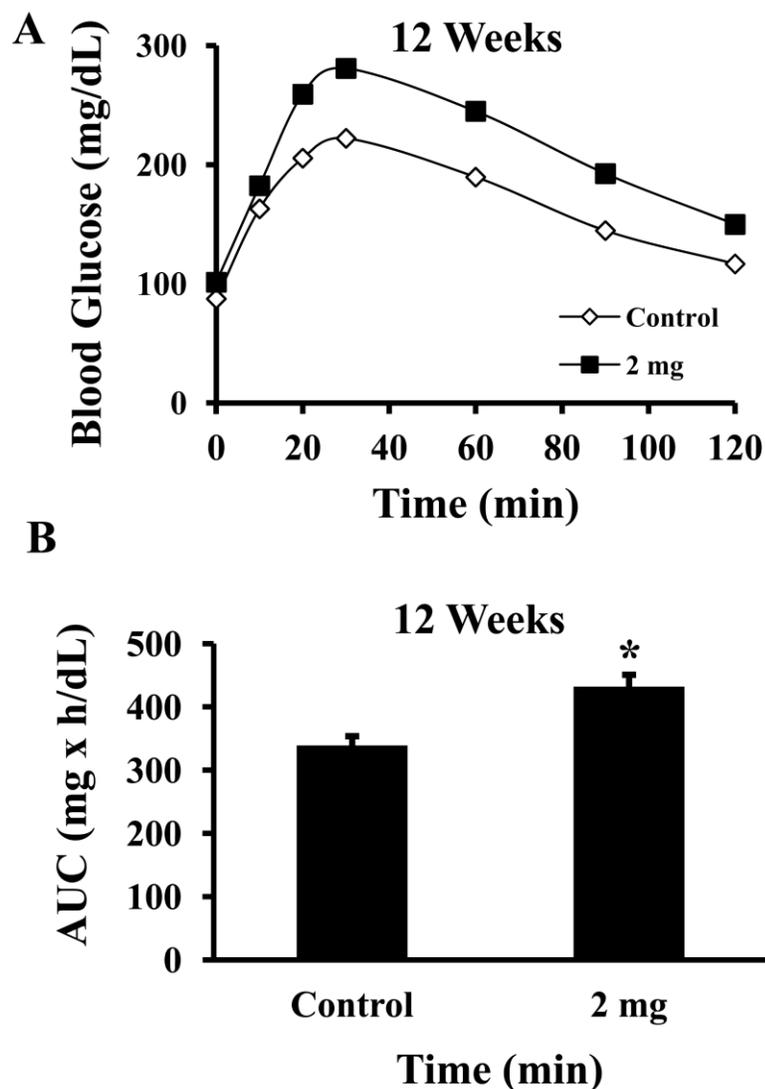


Figure A.8: Glucose tolerance and area under the curve values of 12-week-old male offspring of control and periconception Cd plus Hg-treated female mice in Experiment 2. (A) Glucose tolerance of male offspring (n=34 for controls and 35 for treated offspring). X-axis represents experimental duration in minutes and Y-axis represents blood glucose concentration in mg/dL. (B) Area under the curve (AUC) values of male offspring (* P<0.05 compared to controls). X-axis represents each treatment group. Y-axis represents area under the curve values of plasma glucose in mg x h/dL of blood. Data are presented as mean \pm SEM.

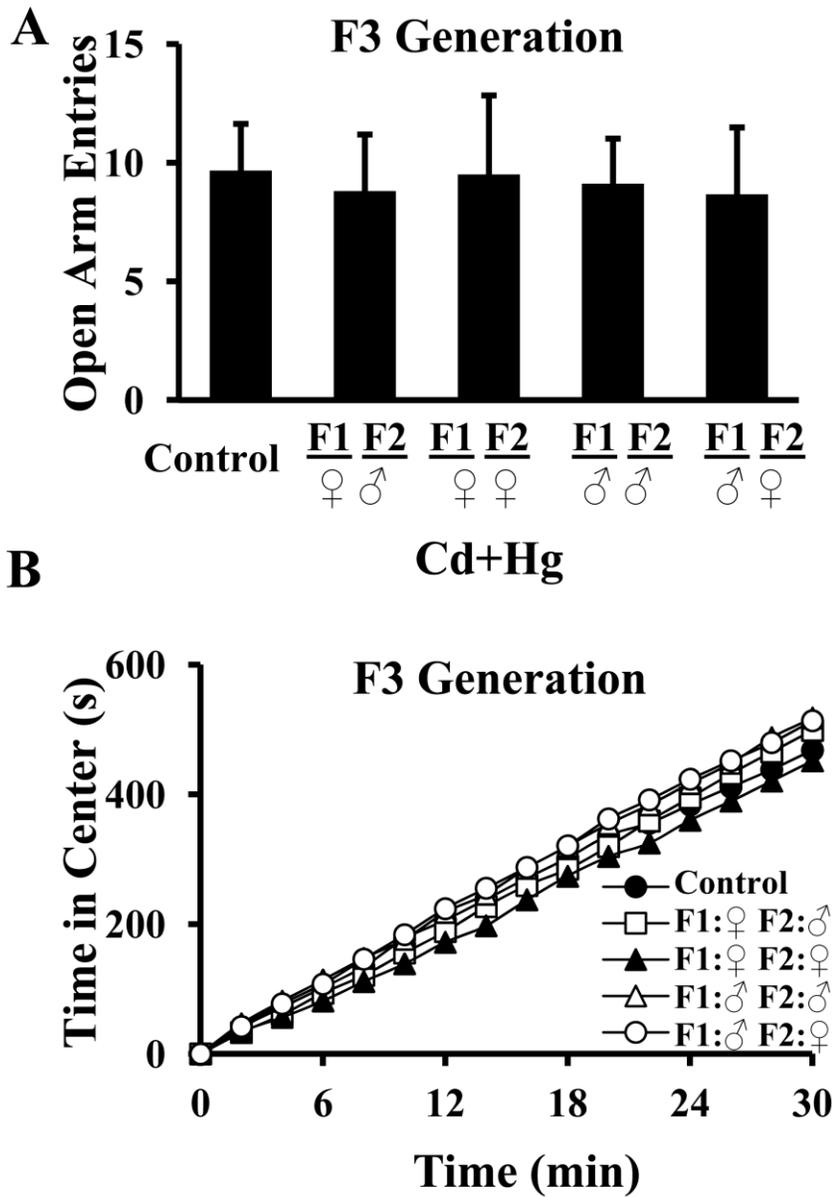


Figure A.9: Anxiety-like behavior of F3 generation male offspring at 8 weeks of age. Open arm entries and cumulative amount of time spent in the center area were tested by elevated plus maze and open field tests, respectively (* $P < 0.05$ compared to controls; $n = 8$ per treatment). (A) Open arm entries of F3 males. X-axis represents ancestral lineage in F3 generation. (B) Cumulative amount of time spent in the center area. X-axis represents total duration of open field test. Data are presented as means \pm SEMs.

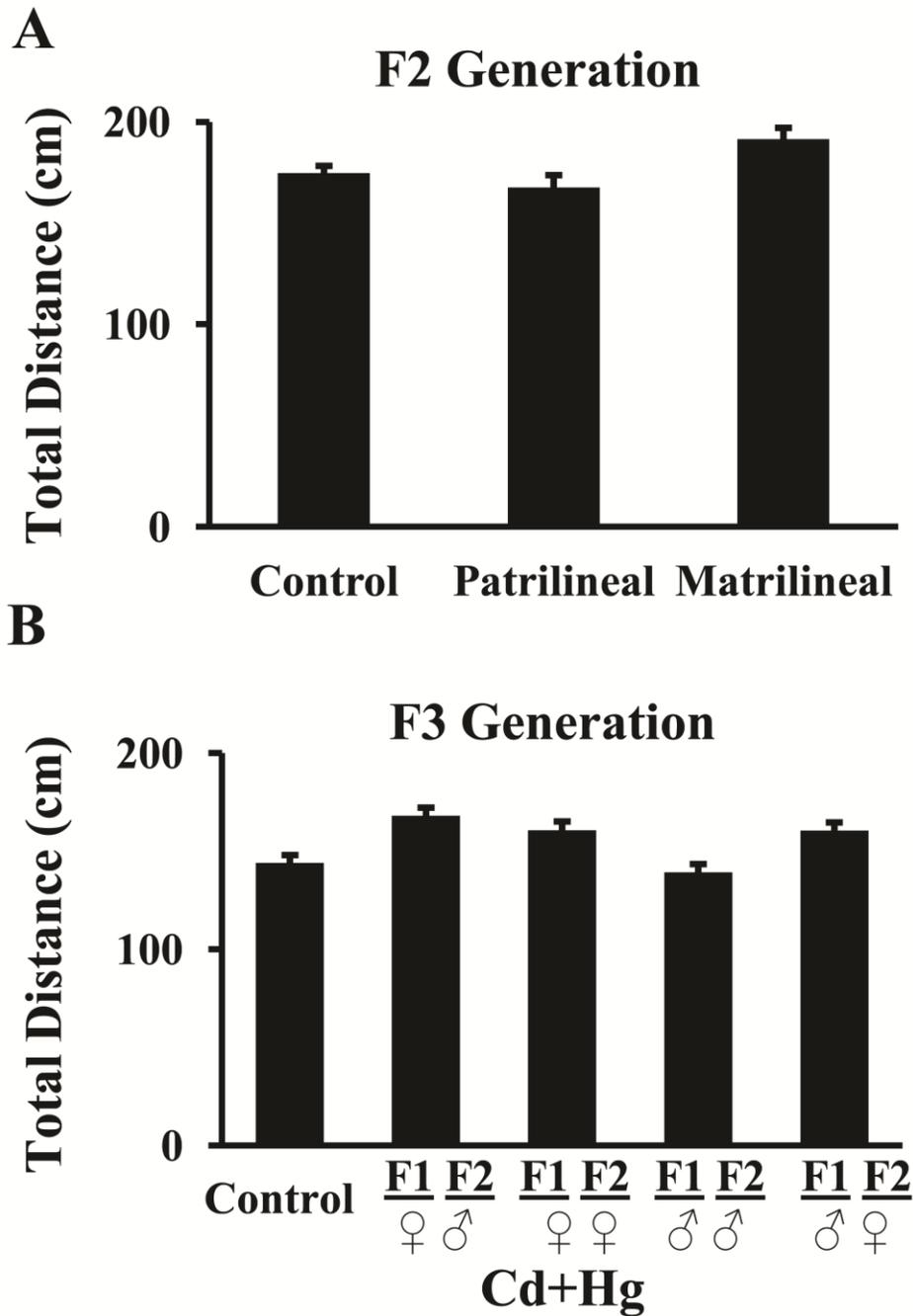


Figure A.10: Locomotor activity of F2 (A) and F3 (B) generation male offspring at 8 weeks of age. Total movement distance in the open field test system was tested (* $P < 0.05$ compared to controls; $n = 8-12$ per treatment). X-axis represents ancestral lineage in F2 and F3 generation. Data are presented as means \pm SEMs.

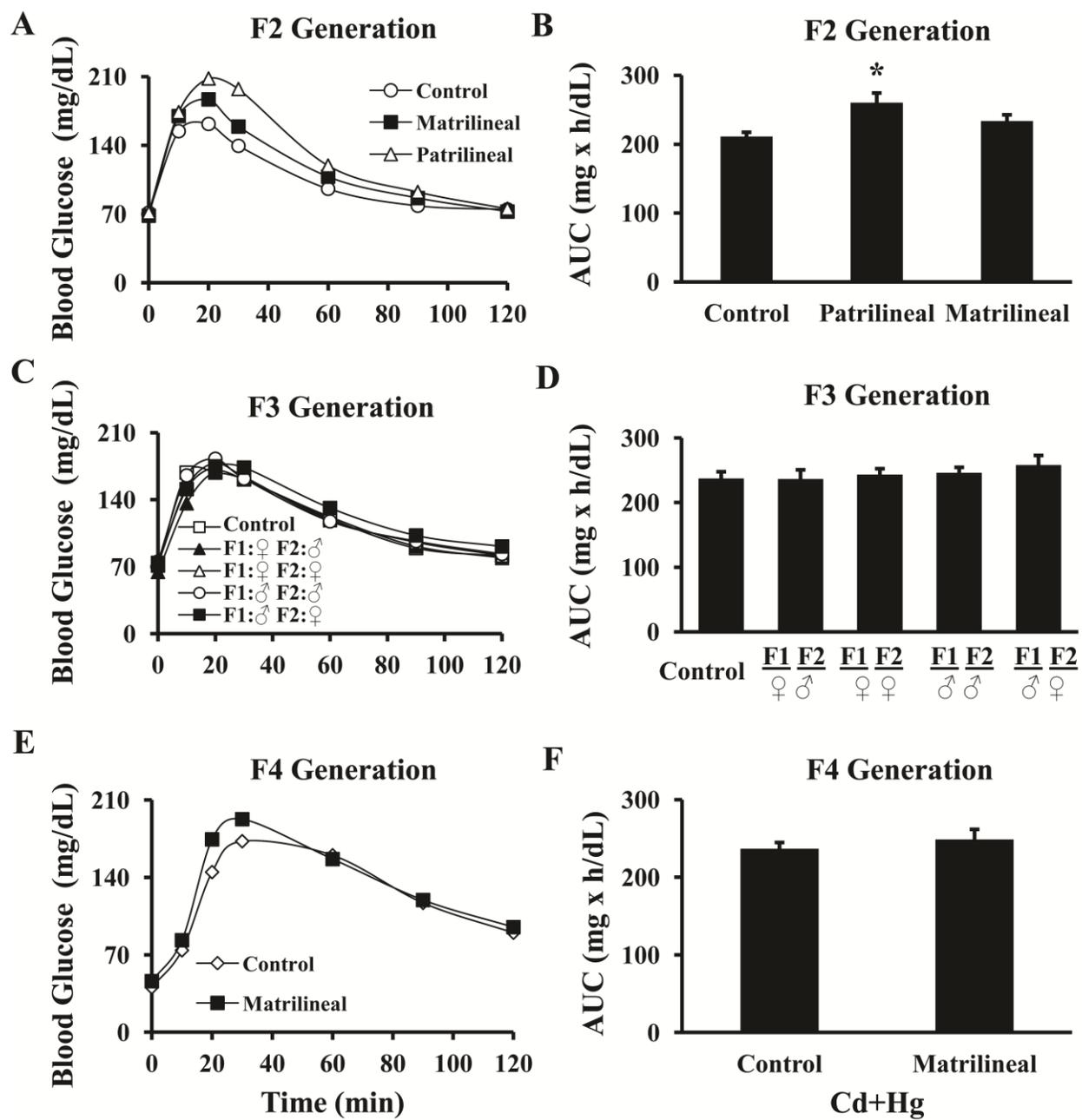


Figure A.11: Glucose tolerance and area under the curve values for female offspring (n=11-22, 17-20 and 13-21 per treatment in F2, F3 and F4 generations, respectively) Left Panel (A, C E):

Figure A.11 (cont'd). X-axis represents experimental duration in minutes and Y-axis represents blood glucose concentrations in mg/dL. Right Panel (B, D, F): Area under the curve (AUC) values for female offspring (* P<0.05 compared to controls). X-axis represents ancestral lineage of F2-F4 generations relative to F1 offspring of periconception Cd plus Hg treated females. Y-axis represents area under the curve values for plasma glucose in mg x h/dL of blood. Data are presented as mean \pm SEM.

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