A model to study the effects of whole life chronic exposure to arsenic or cadmium on the development of adult metabolic syndrome: initial characterization of hepatic changes.

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A MODEL TO STUDY THE EFFECTS OF WHOLE LIFE CHRONIC EXPOSURE TO ARSENIC OR CADMIUM ON THE DEVELOPMENT OF ADULT METABOLIC SYNDROME: INITIAL CHARACTERIZATION OF HEPATIC CHANGES

By

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A Thesis
Submitted to the Faculty of the School of Medicine of the University of Louisville In Partial Fulfillment of the Requirements for the Degree of

Master of Science in Pharmacology and Toxicology

Department of Pharmacology and Toxicology
University of Louisville
Louisville, KY

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A MODEL TO STUDY THE EFFECTS OF WHOLE LIFE CHRONIC EXPOSURE TO ARSENIC OR CADMIUM ON THE DEVELOPMENT OF ADULT METABOLIC SYNDROME: INITIAL CHARACTERIZATION OF HEPATIC CHANGES

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DEDICATION

This thesis is dedicated to

John P. Wise Sr.

for his constant support and guidance

and his unquestionable belief in me

as a person and a student.
ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Gavin Arteel, first for his guidance and support in my thesis research and also for his confidence in my abilities as a scientific researcher. I would also like to thank my other committee members, Dr. Lu Cai, Dr. Chris States, Dr. Michael Merchant and Dr. Rachel Neal, for their comments and assistance.
Metabolic syndrome (MetS) is a group of diseases affecting < 30% of adults. Although obesity is a major risk for the development of MetS, it does not account for all cases, suggesting contribution of other risk factors. We hypothesized that early life exposure to arsenic (As) or cadmium (Cd) may represent such a risk. The purpose of this study was to characterize a model to discern the effects of early life exposures to Cd and As on high fat diet (HFD)-induced MetS. Adult C57BL/6J mice were exposed to control or metals containing drinking water. Pregnant dams and offspring were continuously exposed to the same toxicants as their parents. At weaning, offspring were fed LFD or HFD and sacrificed 10 or 24 weeks later. Metal exposure caused time- and sex-dependent alterations in HFD-induced variables of liver damage. The initial results suggest that these toxicants enhanced obesity-induced liver injury.
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INTRODUCTION

Obesity is a growing epidemic, with more than one third of adults in the United States considered to be overweight (1). By the year 2050 the number of obese individuals in the United States is expected to double (2). Obesity is a risk factor for a spectrum of health conditions including type 2 diabetes, hypertension, non-alcoholic fatty liver disease (NAFLD), and cancer (1). A combination of these obesity-induced conditions can lead to yet another growing epidemic known as metabolic syndrome.

Metabolic syndrome is a multifactorial condition that affects over 47 million people in the United States (3). Adults diagnosed with metabolic syndrome are at a five times higher risk of mortality (3). As defined by the World Health Organization, metabolic syndrome diagnosis requires the manifestation of any of the three following clinical conditions: hyperglycemia resulting from insulin resistance, hypertension, central obesity, and dyslipoproteinemia as a result of high triglyceride levels or low levels of high-density lipoprotein (HDL) (4). Although obesity is a primary risk factor for the development of these conditions, not all obese individuals develop metabolic syndrome directly linked to obesity, indicating that the risk for developing metabolic syndrome is influenced by other genetic and/or environmental factors.

Recently, environmental research has shifted from investigating the impact of a single chemical on human health to an ‘exposure biology’ approach. By investigating the impact of low to moderate chronic exposures in contrast to high acute exposures, this approach takes into account the multiple factors that may play a role in the development of a final phenotype. The impact of one exposure on the responses to other exposures
and/or diseases is critical. In this context, environmental exposure to toxicants, such as arsenic and cadmium, have been shown to influence various aspects of metabolism (5). However, to date, knowledge on the health effects of cadmium and arsenic has resulted mostly from adult and/or postnatal exposures.

While very important, these studies do not take into account that exposure to contaminated drinking water is a life-long, multi-generational event. In accordance with the 'Barker hypothesis', which evolved from the observations that famine-induced low birthweight correlated with increased risk of heart disease later in life (6), a number of studies have reported that an increased risk of developing adulthood diseases is correlated with adverse stimuli in utero that result in permanent physiological and metabolic changes (7). In this context, the impact of early life exposures to cadmium or arsenic on metabolic syndrome, and the consequences of these exposures in conjunction with high fat diets has not been investigated.

**Arsenic**

Arsenic is a naturally occurring, ubiquitous metalloid that continues to be number one on the Agency for Toxic Substances and Disease Registry (ATSDR) list of hazardous environmental chemicals (8). Chronic exposure to high concentrations of arsenic have been associated with a variety of health problems including skin lesions, neurotoxicity, diabetes, hepatotoxicity, and skin, lung, and bladder cancers (9). Inorganic arsenic is a natural drinking water contaminant that enters groundwater through the erosion of rocks and soils (10). In 2003 it was estimated that nearly 4000 public drinking water suppliers in the United States had wells with arsenic levels greater than the maximum contaminant level (MCL) of 10 μg/L. Based on census data from 1950-1999, this means that over 50 million person-years of exposure to arsenic contaminated drinking water above the current MCL occurred by 2003 (11). Many rural communities
have artesian water supplies that are unregulated by the Clean Water Act. These unregulated water supplies often have high levels of arsenic and are in communities where the prevalence of obesity is high; therefore posing potential overlap between arsenic exposure and obesity (12).

The potential overlap between arsenic and obesity was investigated by Tan and colleagues (13) in the liver, a major site of arsenic metabolism and a known target of arsenic toxicity. Chronic exposure to low concentrations of arsenic was shown to be a critical factor that promotes the progression of NAFLD, a feature of metabolic syndrome, induced by a high fat diet (13;14). Another example of the association of arsenic with metabolic syndrome can be seen with work done by Srivastava et al (15) in which prenatal arsenic exposure accelerated the development of atherosclerosis in ApoE -/- mice. There is, however, very little information about the interactions between early-life, in utero exposures to arsenic and obesity in adults that results in metabolic syndrome.

**Cadmium**

Cadmium, ranked number 7 on ATSDRs list of environmental chemical hazards (8) is another metal of concern. Cadmium is a ubiquitous, naturally occurring metal that is widely used in the production of batteries, pigments, plastics, and galvanized products. Anthropogenic sources of cadmium include mining, production of phosphate fertilizers, burning of fossil fuels, and incineration of household wastes (16). For non-smokers, the main source of cadmium exposure is through diet, with more than 80% of food-derived cadmium coming from cereals, vegetables, and potatoes (16;17). Cigarette smokers are exposed to high concentrations of cadmium via inhalation because cadmium bioaccumulates in the leaves of tobacco plants. Cadmium exposure is associated with a number of health effects including renal toxicity, cardiovascular disease, osteoporosis, and cancer in adults (17).
Cadmium accumulates in the renal cortex and the liver, with a whole body half-life between 15 and 30 years (18). Although acute and chronic exposures to cadmium are well known models of liver failure in rodent studies, liver-related outcomes as a result of cadmium exposure are not well characterized in humans; however there is evidence that environmental exposures to cadmium are associated with NAFLD in men, but not women (19). Also unclear is the association between cadmium exposure and obesity, a risk factor for NAFLD, as the existing data are contradictory in human studies (20). Hence, there is very little information about the interactions between early-life, in utero exposures to cadmium and obesity in adults that results in metabolic syndrome.

The purpose of the current study was to design and characterize a two-hit model of multigenerational (F₀ and F₁) exposures to environmental toxicants, specifically cadmium and arsenic, followed by consumption of a high fat diet (F₁) in order to begin to define the mechanistic interaction of these exposures with obesity, and subsequent metabolic syndrome. The results presented here are the initial characterization of the hepatic changes observed with the model, as the liver is a major target for arsenic metabolism and toxicity as well as cadmium accumulation and toxicity (21; 22).
MATERIALS AND METHODS

Animals and Exposures

Six week old male (n=20) and female (n=40) C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the University of Louisville's Institutional Animal Care and Use Committee. Diets were switched from standard laboratory chow to AIN-76A purified diet (Envigo TD 160377, Madison, WI) after one week of acclimation in the barrier facility. Food and deionized water provided ad libitum. At 10 weeks of age mice were started on a drinking water regimen of either deionized water alone (control) or toxicant-containing water (100 ppb Arsenic, 500 ppb cadmium, or 5 ppm cadmium).

Sodium meta(arsenite) (Fluka, Mexico City, Mexico) was used to make a stock solution of 1000 ppm arsenic. Stock solution was prepared in deionized water and 100 μl aliquots were stored at -80°C. Frozen, aliquoted stock solution was thawed and added to deionized water to a final concentration of 100 ppb arsenic and given to mice ad libitum. Fresh 100 ppb arsenic containing water was given twice a week to minimize oxidation of As(III) to As(V). Water consumption was recorded weekly. The concentration of 100 ppb arsenic is within the range of arsenic concentrations seen in both drinking water supplies and unregulated artesian wells.

Cadmium chloride (Alfa Aesar, Tewksbury, MA) was used to make a stock solution of 1000 ppm cadmium. Stock solution was prepared in deionized water and
100 μl aliquots were stored at -80°C. Once a week frozen, aliquoted stock solution was thawed and added to deionized water to a final concentration of either 500 ppb or 5 ppm cadmium and given to mice ad libitum. Water consumption was recorded weekly. Based on the literature, 5 ppm cadmium is one of the lowest concentrations of cadmium tested with results supportive of a metabolic syndrome phenotype; therefore we used 5 ppm cadmium as a proof of concept as well as a ten times lower concentration of 500 ppb cadmium.

At 12 weeks of age mice were placed into breeding triples (1 male to 2 females) within each drinking water exposure (Figure 1). Pregnant dams and offspring were continuously exposed to the same toxicants as their parents after weaning. At weaning, offspring were also fed either a low-fat diet (Envigo TD 160377 - 13% fat, Madison, WI) or high-fat diet (Envigo TD 09682 – 42% fat, Madison, MI) (Figure 1). Offspring were sacrificed 10 or 24 weeks after weaning.

Mice were anesthetized with ketamine/xylazine (100/15 mg/kg i.p.) and blood was collected from the vena cava prior to sacrifice via exsanguination. Blood samples were centrifuged for 1 min at 13,400 rpm and citrated plasma was stored at -80°C for further analysis. Organs were harvested from each mouse in the same sequence (Figure 2). Portions of the pancreas, heart, lung, liver, spleen, kidney, epididymal fat pads, and testis/ovary were snap-frozen in liquid nitrogen, fixed in 10% neutral buffered formalin for subsequent sectioning and mounting on microscope slides, and used for metals analysis.

**Histology and Clinical Chemistry**

Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using standard kits (Thermo Fisher Scientific, Waltham, MA). Formalin fixed, paraffin-embedded sections were cut at 5 μm and
mounted on glass slides. Sections were deparaffinized with Citrisolv (Thermo Fisher Scientific, Waltham, MA) and rehydrated through graded ethanol washes. Sections were then stained with hematoxylin and eosin (H&E) and slides were dehydrated through graded ethanol rinses, washed in Citrisolv and mounted with Permount (Thermo Fisher Scientific, Waltham, MA).

**Metals Analysis**

Each liver sample (20-100mg wet-weight) was digested in 1 mL of 70% concentrated trace metal grade nitric acid in an 85°C water bath for 4 h. After digestion, 34 mL of deionized water was added to each sample. The digested samples were filtered using a 100 μm filter. Metal levels were assessed by ICP-MS (Thermofisher ICPMS X series II), calculated and presented as ng/g wet tissue.

**Statistical Analysis**

Results are reported as means ± standard error mean (SEM; n = 3-10). ANOVA with Bonferroni’s post-hoc test (parametric data) or the Holm-Sidak or Dunn’s Tests (for non-parametric data) were used for the determination of statistical significance among treatment groups, as appropriate. In vivo: a, p < 0.05 compared to diet; b, p < 0.05 compared to toxicant.
Figure 1. Multigenerational exposure to arsenic and cadmium in conjuncture with diet. Adult male and female C57BL/6J mice on defined, low-fat diets were exposed to control drinking water, or water containing metals (100 ppb As, 500 ppb Cd, or 5 ppm Cd) for >2 weeks before being established into breeding pairs. Pregnant dams and offspring were continuously exposed to the same toxicants as their parents after weaning. At weaning, offspring were also fed either a low- or high-fat diet (LFD or HFD, respectively) for 10 or 24 weeks.
Figure 2. Scheme of organ harvest. F₁ offspring were sacrificed at 10 or 24 weeks after being on low fat or high fat diet. On day of sacrifice mice were weighed and anesthetized with ketamine/xylazine (100/15 mg/kg i.p.). Blood collection was followed by exsanguination and decapitation at which point the brain and eyes were collected and the rest of the body was processed in the order seen in the figure starting with the pancreas and ending with the reproductive organ(s).
RESULTS

**Effect of HFD and toxicant exposure on body weight.**

All animals gained weight as the study progressed and there was no mortality or morbidity associated with any group throughout the course of the study. As expected, male mice fed HFD gained more weight compared to those fed LFD (Figure 3A and B). For example, male mice exposed to control water on HFD gained on average 0.42 grams more body weight per week compared to LFD fed male mice. This trend was also seen in male mice exposed to 100 ppb arsenic, 500 ppb cadmium, and 5 ppm cadmium on HFD with an average body weight gain of 0.40, 0.44 and 0.45 grams per week, respectively, compared to LFD fed male mice.

Compared to control, body weight gain was not altered by arsenic exposure in male mice fed LFD or HFD. Cadmium exposure did not altered body weight in male mice fed LFD. However, HFD in combination with cadmium exposure caused male mice to gain less weight compared to control mice at multiple weigh-ins points throughout then study. For example, HFD fed male mice exposed to 500 ppb cadmium gained significantly less weight compared to controls after 5, 8, 9, and 13 weeks of diet \( (p = 0.006, 0.007, 0.031, \text{ and } 0.017, \text{ respectively}) \). In addition, body weights of HFD fed male mice at 5, 7, 8, 19, and 20 weeks of diet were significantly lower than controls \( (p < 0.001, 0.011, 0.007, 0.036, \text{ and } 0.036, \text{ respectively}) \).

Similar to the male cohort, female mice fed HFD gained more weight compared to those fed LFD (Figure 4A and B); however, to a lesser degree. For example, female mice exposed to control water on HFD gained an average of 0.13 grams more body
weight per week compared to LFD female mice, which is approximately 70% less weight gain per week compared to males in the same exposure group. Female mice exposed to 100 ppb arsenic, 500 ppb cadmium, and 5 ppm cadmium on HFD gained 0.29, 0.38, and 0.27 grams more body weight on average compared to LFD fed female mice, which is approximately 27, 14, and 30 percent less weight gain per week compared to males in the same exposure group, respectively.

In contrast to males, arsenic did impact bodyweight in females. Like males, arsenic exposure did not alter bodyweight in female mice fed LFD. However, arsenic increased weight gain in HFD fed mice compared to control throughout the study, with a final average weight gain of 5.8 grams more in arsenic-exposed female mice (Figure 4B). Also, in contrast to males, cadmium impacted bodyweight in females. Independent of diet, 500 ppb cadmium exposure caused female mice to gain less weight during first half of the study. After 5, 7, 8, 9, and 10 weeks of LFD, female mice exposed to 500 ppb cadmium gained significantly less weight compared to controls (p = 0.020, 0.017, <0.001, 0.043, and 0.048, respectively). LFD fed female mice exposed to 5 ppm cadmium also gained less weight compared to controls. However, this lack of weight gain was observed throughout the study and not only in the first half as seen in the 500 ppb cohort. Compared to control, cadmium exposure did not affect body weight gain in HFD fed female mice.

**Effects of HFD and toxicant exposure on hepatic arsenic concentrations**

In male mice, hepatic arsenic concentrations were not altered by diet or toxicant exposure (Figure 5A and B). However, hepatic arsenic concentrations were lower in all groups at 24 weeks post weening compared to 10 weeks with decreases in arsenic
Figure 3. Effects of diet and toxicant exposure on body weight of F1 male mice. F1 generation male mice were treated as described in Material and Methods and body weight was monitored weekly. Body weight gain of male mice on a low fat (Panel A) or a high fat diet (Panel B), exposed to either control drinking water or toxicant containing drinking water is shown. Body weights are shown as means ± SEM for each group (n = 3-17). # indicates a significant difference (p<0.05) between control and arsenic. * indicates a significant difference (p<0.05) between control and 500 ppb cadmium. ‡ indicates a significant difference (p<0.05) between control and 5 ppb cadmium.
Figure 4. Effects of diet and toxicant exposure on body weight of F₁ female mice. 
F₁ generation female mice were treated as described in Material and Methods and body weight was monitored weekly. Body weight gain of female mice on a low fat (Panel A) or a high fat diet (Panel B), exposed to either control drinking water or toxicant containing drinking water is shown. Body weights are shown as means ± SEM for each group (n = 3-20). # indicates a significant difference (p<0.05) between control and arsenic. * indicates a significant difference (p<0.05) between control and 500 ppb cadmium. † indicates a significant difference (p<0.05) between control and 5 ppb cadmium.
concentrations ranging from 50% in the LFD fed, 5 ppm cadmium exposed males, to 98% in the HFD, 5 ppm cadmium exposed males. This same trend was seen in female mice with decreases in hepatic arsenic concentrations ranging from 68% in the LFD fed, control water exposed females to 100% in the LFD fed, 500 ppb exposed females. Also, as seen in the male cohort, female hepatic arsenic concentrations were not altered by diet or arsenic exposure (Figure 6A and B). However, at 10 weeks post-weaning it was observed that exposure to 500 ppb cadmium, independent of the diet type (i.e. LFD or HFD), significantly increased arsenic concentrations in the liver compared to control (p = 0.023) (Figure 6A). Interestingly at 24 weeks post-weaning this phenotype was no longer seen.

**Effects of HFD and toxicant exposure on hepatic cadmium concentrations**

Hepatic cadmium concentrations in male mice, 10 weeks post-weaning, were significantly greater in all three toxicant exposed groups, compared to control and independent of diet type (i.e. LFD or HFD) (As: p = 0.005; 500 ppb Cd: p < 0.001; 5 ppm Cd: p < 0.001) (Figure 7A). As expected there was a concentration-dependent increase in hepatic cadmium load with increased cadmium exposure concentration. These trends were also seen 24 weeks post-weaning, with one exception. Although hepatic cadmium concentrations are greater after exposure to 500 ppb cadmium, independent of diet, a HFD significantly blunted the effect (p = 0.002) (Figure 7B).

As seen in the male cohort 10 weeks post-weaning, hepatic cadmium concentrations in female mice were significantly greater in all three toxicant exposure groups, compared to control and independent of diet type (As: p = 0.025; 500 ppb Cd: p < 0.001; 5 ppm Cd: p < 0.001) (i.e. LFD or HFD); however toxicant exposed female mice had between 18 and 33% more cadmium in their liver compared to males (Figure 8A).
Figure 5. Arsenic concentrations in the liver of male F1 mice. F1 generation male mice were treated as described in Material and Methods. After 10 weeks (Panel A) or 24 weeks (Panel B) of being on diet mice were sacrificed and a portion of the liver was processed for metals analysis via ICP-MS. Arsenic concentrations (ng/g) are shown as means ± SEM for each group (n = 3-8). There were no statistical differences among groups.
Figure 6. Arsenic concentrations in the liver of female F1 mice. F1 generation female mice were treated as described in Material and Methods. After 10 weeks (Panel A) or 24 weeks (Panel B) of being on diet mice were sacrificed and a portion of the liver was processed for metals analysis via ICP-MS. Arsenic concentrations (ng/g) are shown as means ± SEM for each group (n = 2-10). b, p < 0.05 compared to deionized water.
**Figure 7. Cadmium concentrations in the liver of male F₁ mice.** F₁ generation male mice were treated as described in Material and Methods. After 10 weeks (Panel A) or 24 weeks (Panel B) of being on diet mice were sacrificed and a portion of the liver was processed for metals analysis via ICP-MS. Cadmium concentrations (ng/g) are shown as means ± SEM for each group (n = 3-8). a, p < 0.05 compared to LFD; b, p < 0.05 compared to deionized water.
This dimorphic margin increased over time with female hepatic cadmium concentrations increasing to between 20 and 85% more cadmium 24 weeks post-weaning, compared to the corresponding male groups (Figure 8B). Twenty-four weeks post-weaning, female hepatic cadmium concentrations remained significantly greater in all toxicant exposure groups, compared to control, as seen in male mice (As: p = 0.013; 500 ppb Cd: p < 0.001; 5 ppm Cd: p < 0.001). However, not seen in males, HFD fed female mice exposed to 5 ppm cadmium had significantly greater hepatic cadmium concentrations compared to LFD females in the same exposure group (p < 0.001).

**Effect of HFD and toxicant exposure on liver weight**

In male mice, at 10 weeks post-weaning, there was no effect of HFD, arsenic or 500 ppb cadmium exposure on liver-to-body weight ratios (Figure 9A). Independent of diet, exposure to 5 ppm cadmium significantly increased liver size in male mice (p = 0.003). By 24 weeks post weening, this phenotype was gone; however HFD significantly increased liver size compared to LFD in all exposure groups (As: p = <0.001; 500 ppb Cd: p < 0.001; 5 ppm Cd: p < 0.001) (Figure 9B). For example, there was a 2-fold increase in the liver size of HFD fed mice exposed to control and arsenic containing water compared to LFD. In female mice, at both 10 and 24 weeks post weening, neither HFD nor toxicant exposure altered liver-to-body weight ratios (Figure 10A and B).

**Effects of HFD and toxicant exposure on plasma AST: index of liver damage**

As expected by the observation of unchanged liver size, 10 weeks of HFD feeding did not affect AST levels in male or female mice (Figure 11A and 12A). In male mice, after 24 weeks of HFD feeding, although a trend towards greater AST levels was observed in the all groups, independent of exposure, AST levels were statistically higher.
Figure 8. Cadmium concentrations in the liver of female F₁ mice. F₁ generation female mice were treated as described in Material and Methods. After 10 weeks (Panel A) or 24 weeks (Panel B) of being on diet mice were sacrificed and a portion of the liver was processed for metals analysis via ICP-MS. Cadmium concentrations (ng/g) are shown as means ± SEM for each group (n = 1-10). a, p < 0.05 compared to LFD; b, p < 0.05 compared to deionized water.
Figure 9. High fat diet caused hepatomegaly in male mice independent of toxicant exposure. F1 generation male mice were treated as described in Material and Methods. After 10 weeks (Panel A) or 24 weeks (Panel B) of being on diet mice were sacrificed and body and liver weights were recorded. Liver-to-body weight ratios (LW/BW%) are shown as means ± SEM for each group (n = 3-8). a, p < 0.05 compared to LFD; b, p < 0.05 compared to deionized water.
Figure 10. Neither diet nor toxicant exposure significantly alters liver-to-body weight ratios in female mice. F1 generation female mice were treated as described in Material and Methods. After 10 weeks (Panel A) or 24 weeks (Panel B) of being on diet mice were sacrificed and body and liver weights were recorded. Liver-to-body weight ratios (LW/BW%) are shown as means ± SEM for each group (n = 4-10). There were no statistical differences among groups.
in only the cadmium exposed mice (500 ppb Cd: p = 0.007; 5 ppm Cd: p = 0.030) (Figure 11B). Neither HFD nor toxicant exposure altered AST levels in female mice after 24 weeks, which is reflective of the unchanged liver-to-body weight ratios (Figure 12B).

**Effect of HFD and toxicant exposure on liver health: histology**

Neither diet nor to toxicant exposure altered liver morphology or lipid accumulation in male or female mice at 10 weeks post weening, as expected by unaltered liver size and AST activity (Figure 13A and 14A). However, by 24 weeks post-weaning liver health was altered by both diet and metal exposure. In LFD fed male mice, arsenic exposure increased liver inflammation while exposure to both concentration of cadmium caused structural changes in the liver (Figure 13B, upper panels). HFD feeding increased lipid accumulation in the liver compared to LFD, causing both microvesicular and macrovesicular steatosis (Figure 13B, lower left panel). This pathology was enhanced by low dose Cd (500 ppb) and to a greater extent by high dose Cd (5 ppm), as indicated by larger, more numerous lipid droplets as well as necroinflammatory foci (Figure 13B, lower left panels). Arsenic exposure in combination with HFD increased microvesicular steatosis only.

In females, low dose Cd (500 ppb) exposure did not alter liver histology in mice fed LFD; however independent of diet, arsenic caused simple steatosis and high dose Cd (5 ppm) caused structural changes in the liver (Figure 14B, upper panels). HFD caused moderate alterations in liver histology in both control and Cd treated mice, as indicated by marginal increases in lipid accumulation and structural changes (Figure 14B, lower panels). HFD feeding enhanced the arsenic-induced steatosis seen with LFD feeding. Overall, all toxicant exposures tended to exacerbate the effect of HFD on liver histology and this effect was much more pronounced in males than females.
Figure 11. Plasma aspartate aminotransferase activity (AST) in male mice. F₁ generation male mice were treated as described in Material and Methods. After 10 weeks (Panel A) or 24 weeks (Panel B) of being on diet mice were sacrificed and plasma aspartate aminotransferase (AST) activity was measured as a marker of liver injury. AST activity (IU/L) is shown as means ± SEM for each group (n = 3-8). a, p < 0.05 compared to LFD.
Figure 12. Plasma aspartate aminotransferase activity (AST) in female mice.
F1 generation female mice were treated as described in Material and Methods. After 10 weeks (Panel A) or 24 weeks (Panel B) of being on diet mice were sacrificed and plasma aspartate aminotransferase (AST) activity was measured as a marker of liver injury. AST activity (IU/L) is shown as means ± SEM for each group (n = 3-10).
Figure 13. Effect of arsenic and cadmium on high fat diet induced liver injury in male mice. F1 generation male mice were treated as described in Material and Methods. Panel A shows representative photomicrographs of hematoxylin & eosin staining (H&E, 200x) of the liver tissue of male mice after 10 weeks of being on either LFD or HFD. Panel B shows representative photomicrographs of hematoxylin & eosin staining (H&E, 200x) of the liver tissue of male mice after 24 weeks of being on either LFD or HFD.
**Figure 14.** Effect of arsenic and cadmium in conjunction with high fat diet on liver injury in female mice. F₁ generation female mice were treated as described in Material and Methods. Panel A shows representative photomicrographs of hematoxylin & eosin staining (H&E, 200x) of the liver tissue of female mice after 10 weeks of being on either LFD or HFD. Panel B shows representative photomicrographs of hematoxylin & eosin staining (H&E, 200x) of the liver tissue of female mice after 24 weeks of being on either LFD or HFD.
DISCUSSION

Obesity, a major public health concern affecting over 312 million people worldwide, is a primary risk factor for the development of metabolic syndrome; however, only 65% of obese males and 56% of obese females meet metabolic syndrome criteria (23), indicating the influence of other risk factors such as genetics and/or environmental exposures. Previous studies have correlated environmental exposures to arsenic and cadmium with the development of several metabolic disease pathologies including type 2 diabetes, hypertension, and atherosclerosis (3;5;24). These studies, however, do not take into account that such environmental exposures can be life-long and multigenerational. Therefore the purpose of the current study was to establish an in vivo model to look at the effects of early life exposure to chronic, low dose arsenic or cadmium on the development of metabolic syndrome pathologies associated with high fat diets.

The results presented here are the initial hepatic changes associated with the model. We choose the liver as the initial target organ, because as stated in the introduction, the liver is major site for arsenic metabolism and toxicity (21) and a major target organ of cadmium toxicity and accumulation (22). Importantly, endpoints were looked at in both male and female mice exposed in-utero and postnatally to arsenic or cadmium as there are significant difference in obesity and lipid metabolism between the genders (25). Initially these endpoints were to be examined after 10 weeks of diet, post-weaning; however the hepatic changes expected based on the literature were not observed (13;26-27) at this time point; therefore the study was carried out to 24 weeks.
Previously it has been shown that male mice fed a high fat, “Western Diet” (42% calories) for 10 weeks gained more weight and had enlarged livers with significantly more lipid accumulation compared to LFD fed mice (13). These results are consistent with other studies using this diet (26;27); however with our model, although we did see increases in average weight gain, we did not see hepatomegaly or lipid accumulation in the liver after 10 weeks of HFD feeding (Figures 3, 9, and 13).

It is important to point out that in the above contradictory studies mice were started on HFD between 6-8 weeks of age, whereas in our model, mice were started on HFD at weaning, or 3 weeks of age. Although a difference of 3-5 weeks may not seem like a long time, in “mouse days” it can be the difference between puberty and adulthood. A 3, 6, and 8-10 week old mouse is comparable to a 6 month old, 11.5 year old, and 20 year old human, respectively (28). Age-induced metabolic changes may account for these discrepancies (29).

By 24 weeks of being fed HFD we did indeed observe hepatic changes in male mice consistent with the previously reported HFD-induced fatty liver disease models (30) including hepatomegaly and micro-and macrovesicular steatosis (Figures 9 and 13). Although female mice did gain weight with HFD feeding (Figure 4), it was at a slower rate than male mice, and interestingly, HFD did not alter liver-to-body weight ratios (Figure 10) and only induced moderate lipid accumulation (Figure 14). This is not surprising as it is well established that there are sex differences in the susceptibility to various disease (25), and more specifically it has been shown that female mice are actually protected against HFD-induced metabolic syndrome (31).

The effects of two possible environmental obesogens, arsenic and cadmium, on HFD-induced liver damage was examined in both males and females. The influence of these toxicants on HFD-induced pathologies is poorly understood; however both arsenic and cadmium are known to impact liver physiology on their own (19;21).
Arsenic

The “environmental obesogen” hypothesis, suggests that the risk for obesity may increase as a result of environmental exposures (32). Arsenic is one such environmental pollutant that has been recognized as a risk factor for the development of health conditions associated with obesity such as type 2 diabetes, hypertension, and non-alcoholic fatty liver disease (1). Furthermore, obese individuals have been shown to metabolize arsenicals less efficiently than normal-weighted individuals; therefore, suggesting a link between obesity and susceptibility to arsenic toxicity (33). However in the literature there are substantial differences in the duration of treatment and the doses of arsenic used (34); therefore there is uncertainty underlining the mechanisms of arsenic toxicity and its role as an obesogen.

The contradictory results in the literature due to arsenic dose is best exemplified by hepatic arsenic concentrations. In our study we saw that neither HFD nor exposure to low level, 100 ppb arsenic resulted in arsenic accumulation in the liver (Figure 5 and 6). To the contrary, it has been shown that arsenic does accumulate in the liver in a concentration-dependent manner; however, the arsenic exposures were 250 to 500 times greater in the contradictory study compared the current study (35). Interestingly, Markowski et al (36) investigated the effects of multiple concentrations of arsenic and found that the offspring of female mice exposed to increasing concentrations of arsenic in drinking water, up to 20 ppm, had similar concentrations of arsenic in their liver compared to controls; however exposure to concentrations < 40 ppm resulted in hepatic accumulation of arsenic (36). In human studies, hepatic arsenic levels did not correlate with either the degree of fibrosis in the liver or the amount of arsenic in the drinking water (21). Although arsenic may not accumulate in the liver after exposure to environmentally relevant low doses, it is a known hepatotoxin.
One study showed that the offspring of female mice exposed to 100 ppb arsenic during pregnancy developed NAFLD and were at higher risk for developing metabolic syndrome (37). This study specifically looked at 36 week old male and female offspring of dams exposed to 100 ppb arsenic from embryonic day (ED) 6 until birth, and although a trend towards larger body weights was observed, there was no statistical difference compared to controls. Interestingly, although arsenic exposure did not lead to weight gain in either male or female mice, both prominent micro- and macrovesicular steatosis was observed (37). In the current study, arsenic exposure did not affect weight gain in male or female mice (Figures 3 and 4), which is also supported by other studies (13;15). Also, although prominent micro- and macrovesicular steatosis was not observed hepatic inflammation in male mice and simple steatosis in female mice was observed (Figures 1 and 14). The differences in histology may be an artifact of the shorter length of our study.

Another study in CD-1 mice showed that in utero exposure to low level arsenic (10 ppb) during the second half of gestation led to obesity in female offspring (38). Although this study does not consider the impact of a high fat diet, it does suggest that there are mechanism by which arsenic alone can increase the risk of obesity in female mice. In the model used in the current study, arsenic alone did not alter weight gain, but may have caused pathologically inert biochemical and/or physiological changes in utero that altered the ability of the offspring to metabolize a high fat diet.

Interestingly, in that the current study female mice fed HFD, exposed to arsenic gained more weight on average compared to control (Figure 4B), suggesting that arsenic exposure may enhance the effects of HFD-induced weight gain in female mice. Although arsenic exposure has not been directly linked to increases in fat mass per se, arsenic does cause physiological and chemical changes, such as impairment of adipocyte
metabolism (32;39), that when challenged with a “second-hit”, such as a high fat diet, may enhance the risk of developing obesity.

This ‘second hit’ hypothesis has been well demonstrated in models of fatty liver disease (40). For example, subhepatotoxic exposure to arsenic has been shown to enhance HFD-induced liver damage (13). In the current study, HFD-induced hepatomegaly was unaltered by arsenic exposure in male mice (Figure 9); however, in contradiction to Tan et al (13) arsenic exposure enhanced microvesicular steatosis. One possible explanation for the contradiction is the utilization of different exposure models. Although both studies exposed mice to 100 ppb arsenic in drinking water, mice in our study were exposed in utero and postnatally (Figure 1), whereas in the Tan et al (13) study mice were only exposed postnatally. This explanation is supported by a study that looked at the effects of in utero (IU), postnatal (PN), and continual exposures (in utero + postnatal) (IU+) to 100 ppb arsenic combined with a high fat diet (41). It was concluded that NAFLD was present in both groups exposed to arsenic in utero, and the severity of the pathology was greater when exposure to arsenic was continued postnatally.

Taken together these initial findings suggest that “whole life” arsenic exposure enhances liver injury caused by HFD in male mice and to lesser degree in female mice, although arsenic exposure exacerbated HFD-induced weight gain in females only, suggesting sex-related differences in the mechanism of arsenic-induced hepatotoxicity and possibly the development of metabolic syndrome.

**Cadmium**

Metabolic syndrome has been associated with a number of toxic metals and metalloids, including mercury (42), lead (43), and arsenic (24). However, the association and underlying mechanism of cadmium exposure with metabolic syndrome pathologies, and to a greater extent obesity, is unclear. The human data on the role of cadmium
exposure in obesity is contradictory with studies showing negative, positive, and no correlations between exposure and weight gain. In animal studies, such as ours, cadmium exposure did not cause weight gain in male or female mice (Figures 3 and 4) (44; 45), whereas another study showed that female rats exposed to cadmium had significantly lower body weights compared to control (46). As seen in the arsenic literature, these discrepancies may be due, in part, to differences in route of exposure and exposure levels (20).

It is well known that cadmium accumulates in the liver due to high metallothionein (MT) levels, a metal binding protein with a high affinity for cadmium (47), and has a whole body half-life between 15 and 30 years (18). Therefore, it is not surprising that cadmium exposure resulted in a concentration-dependent increase in hepatic cadmium in both male and female mice at 10 and 24 weeks post-weaning (Figures 7 and 8). Female mice accumulated more cadmium in their livers than male mice, consistent with the literature that cadmium retention is generally higher in women than men (48; 49).

For more than a half a century it has been thought that women are more affected by cadmium than men in part due to greater body burden and in part due to cadmium-induced Itai-itai disease, which affects the kidney and bones and is mainly seen in women (49-52). We found that although cadmium concentrations were greater in the livers of female mice, histology showed alterations in the arrangement of normal sinusoidal architecture at both low (500 ppb) and high (5 ppm) cadmium exposures in male mice (Figure 13), but to a lesser extent in cadmium exposed females (Figure 14). Loss of normal parenchymal tissue architecture in the liver has been associated with cadmium exposure in male rodents, whereas studies on liver architecture in cadmium-exposed females is lacking.

Interestingly, in humans, cadmium-induced liver disease is more prevalent and more extensive in men than women (19; 53) regardless of the fact that cadmium
accumulates to a greater extent in the livers of females (52), which is consistent with the findings of this study. Exposure to environmental cadmium has been associated with a higher risk of hepatic necroinflammation, NAFLD, and NASH (non-alcoholic steatohepatitis) in males whereas in females exposure was associated with hepatic necroinflammation only, and to a lesser degree than that seen in the males (19). Although it is clear that chronic cadmium exposure induces hepatotoxicity, the mechanism(s) are not fully understood.

Hepatic gene expression profiling of adult mice that were exposed to low dose cadmium in utero and continuously throughout life showed enhanced expression of genes involved in fatty acid and lipid metabolism in male, but not female mice (54). Cadmium has also been shown to alter lipid and carbohydrate metabolism in adipocytes resulting in impaired adipogenesis. This alteration in adipose tissue physiology may induce an obesity-associated metabolic profile that promotes pathologies associated with metabolic syndrome, such as insulin resistance and type 2 diabetes (55).

Due to the effects of cadmium on metabolic processes in male mice it is not surprising that in this study HFD-induced steatosis in male mice was exacerbated by cadmium exposure in a concentration-dependent manner (Figure 13). Observations that cadmium only caused moderate structural change in the liver of HFD-fed female mice (Figure 14) is supported by research that shows cadmium does not enhance expression of genes involved in fatty acid and lipid metabolism (54). Cadmium exposure potentiate other pathologies, such as LPS-induced inflammation linked to metabolic syndrome (56); therefore it reasonable to suggest, based on the findings from the current study, that cadmium exposure potentiates HFD-induced liver disease in the male mice, but not female mice.
SUMMARY AND CONCLUSIONS

STRENGTHS OF THIS WORK

The purpose of this work was to establish an *in vivo*, two-hit model to study the effects of whole life exposure (preconception to time of sacrifice) of arsenic or cadmium on the development and progression of high fat diet-induced metabolic syndrome pathologies. To our knowledge this is the first study with this exposure paradigm for either toxicant. As metabolic syndrome is a group of diseases affecting multiple organs in the body, it is important to point out that all of the work was performed in the whole animal. Tissue samples from over 10 organs were harvested from each animal, allowing for various endpoints to be examined both within and between (cross-talk) multiple organs as well as creating the possibility for collaboration. Additionally, the exposures were in both sexes which is important considering the National Institute of Health requirement for accounting for sex as a biological variable. Indeed, the initial characterization of the hepatic changes associated with this model revealed sexual dimorphism as well as established that exposure to arsenic and cadmium exacerbate HFD-induced liver disease, a pathology associated with metabolic syndrome.

CAVEATS AND WEAKNESSES

One of the weaknesses of this study is that the model does not account for *in utero* exposure alone or postnatal exposure alone, both of which are windows of susceptibility that may be differently impacted by toxicant exposure. Assessment of the contribution of arsenic and cadmium to the phenotypic changes *in utero*, postnatally, and
in combination may better elucidate the molecular processes involved in development of metabolic syndrome pathologies later in life.

After 10 weeks of diet the hepatic changes that were expected were not observed; therefore the study was carried out to 24 weeks. Due to this unexpected, but necessary extension of the study there were fewer animals in each exposure group at the 24 week time point, impacting statistical power. This extension also resulted in the need to order a new lot of HFD shortly after the 10 week sacrifice, adding another variable.

FUTURE WORK

This study has laid the foundation for studying the mechanism by which an early life exposure to cadmium or arsenic exacerbates disease phenotypes associated HFD-induced obesity, and subsequent metabolic syndrome. Numerous collaborations have resulted from this study and future work will build on our initial findings and examine other target organs of metabolic syndrome. For example, "omics" approaches have been initiated and cross-talk between organs is being considered.
REFERENCES


CURRICULUM VITAE

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Education
2003-2007 B.A. in Biology: cum laude, University of Maine at Farmington

Professional Experience
2015 - Current Doctoral Student, Department of Pharmacology and Toxicology, University of Louisville, Louisville, KY

2015 Research Associate, Wise Laboratory of Environmental and Genetic Toxicology, University of Southern Maine, Portland, ME

2010 – 2015 Environmental Scientist, Portland Water District 225 Douglass Street, Portland, ME

April –August 2010 Analytical Chemist, Katahdin Analytical Services 600 Technology Way, Scarborough, ME

2007-2010 Doctoral Student, Wise Laboratory of Environmental and Genetic Toxicology, University of Southern Maine, Portland, ME.

2005-2007 Research Assistant III, Dr. Ronald Butler – Spatial Ecology Laboratory, University of Maine at Farmington, Farmington, ME.

2005 Research Assistant III, The Jackson Laboratory-Genotyping Lab, Bar Harbor, ME.

Fall 2005 Tutor, Anatomy and Physiology, University of Maine at Farmington, Farmington, ME

Seminars/Platform sessions
2016 Platform Session. “One Environmental Health Perspective: Insight into the Cytotoxic and Genotoxic Effects of Hexavalent Chromium in Human, Aquatic

41
Reptile and Aquatic Mammal Skin Cells” 47th Annual Meeting of the International Association for Aquatic and Animal Medicine, Virginia Beach, VA. May 2016.


Seminar. "Zinc Chromate Induced Spindle Assembly Checkpoint Bypass and Chromosome Instability in Human Lung Cells.” Department of Applied Medical Science, University of Southern Maine, Portland, Maine, April 2009.

2008 Seminar. "Biomedical Science Research at the University of Southern Maine." 1st Annual Meeting of the Graduate School of Biomedical Sciences, University of Maine, Orono, May 2008.


**Honors and Awards**

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<tr>
<td>2017</td>
<td>Research!Louisville 2017, 2nd place poster in Masters Basic-Science</td>
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<td>Elected SOT Graduate Student Leadership Committee programming sub-committee vice-chair</td>
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<td>2016</td>
<td>Elected as SOT Metals Specialty Section Graduate Student Representative (2 year position)</td>
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<td>2015</td>
<td>Student Travel Award, Environmental Mutagenesis and Genomics Society</td>
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<td>Michael D. Wilson Scholar, University of Maine at Farmington</td>
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**Grants and Fellowships**

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<td>2008-2010</td>
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**Professional Memberships & Societies**

Ohio Valley Chapter, Society of Toxicology

Society of Toxicology

**Meetings Attended**

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<td>2008</td>
<td>Annual Meeting for the American Association for Cancer Research</td>
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Toxics and Tomorrow’s Children – University of Southern Maine
Thinking Matters - University of Southern Maine

2009
Annual Meeting for the Society of Toxicology
Thinking Matters - University of Southern Maine
36th Biological and Medical Science Symposium

Annual Meeting of the Northeast Regional Chapter of the Society of Toxicology

2015
54th Annual Meeting for the Society of Toxicology
5th Georgian Bay International Conference on Bioinorganic Chemistry
46th Annual Meeting of the Environmental Mutagenesis and Genomics Society
Research Louisville! – University of Louisville
21st Biennial Meeting of the Society of Marine Mammalogy

2016
55th Annual Meeting of the Society of Toxicology
47th Annual Meeting of the International Association for Aquatic and Animal Medicine
Research Louisville! – University of Louisville

2017
56th Annual Meeting of the Society of Toxicology
Research Louisville! – University of Louisville

Publications

Journals


Abstracts


