

THE EFFECTS OF ARSENIC AND HEAVY METAL MIXTURES ON PANCREATIC β -
CELL INSULIN SECRETION

by

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ABSTRACT

Naishal Patel: The Effects of Arsenic and Heavy Metal Mixtures on Pancreatic β -cell Insulin Secretion

(Under the direction of Dr. Miroslav Styblo)

Diabetes mellitus is a known metabolic disorder characterized by altered insulin secretion or responsiveness. Recently, a potential association between diabetes and environmental exposures has been described, in which environmental compounds have been identified as potential diabetogens. With diabetes prevalence and industrial growth rising in many middle- and low-income countries, it is imperative we look further into the association between heavy metals and diabetes mellitus. Interactions between iAs^{III} and heavy metals may exhibit differing effects on insulin secretion machinery than just individual heavy metals. In previous studies, trivalent inorganic arsenic (iAs^{III}) has been identified as an inhibitor of insulin secretion in isolated mouse islets. In addition to iAs^{III} , heavy metals, notably zinc (Zn), manganese (Mn) and cadmium (Cd), have also been found to be associated with alteration of insulin secretion. INS-1 (832/13) rat insulinoma cells were treated for 24 hours with varying concentrations of each of the aforementioned heavy metals. When co-exposed with Zn and Cd, iAs^{III} acted as the primary inhibitor of insulin secretion in the β -cells. Co-exposure of Mn and 1 μM iAs^{III} , exhibited a significant decrease in insulin secretion at the 25 and 50 μM Mn concentrations compared to 1 μM iAs^{III} exposure alone. There was no significant difference in insulin secretion between the Mn and iAs^{III} co-exposure and Mn alone treatment groups at the 25 and 50 μM Mn concentrations, suggesting Mn as a primary driver of insulin secretion inhibition at higher concentrations. Additionally, 24-hour 1 μM iAs^{III} exposure to INS-1 (832/13) cells caused a significant decrease in mitochondrial oxygen consumption rate (OCR) under energy-demanding conditions, identifying mitochondria as targets for iAs^{III} in β -cells. This study examines the impact of heavy metals and their mixtures with iAs^{III} on insulin secretion in INS1 (832/13) rat pancreatic β -cells. From these results, we conclude that water mixtures containing certain heavy metals in mixture with iAs^{III} may pose significant danger to pancreatic β -cell function.

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“Never give up on what you really want to do. The person with big dreams is more powerful than the one with all the facts.”

–Albert Einstein

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LIST OF ABBREVIATIONS

DM	diabetes mellitus
GLUT2	glucose transporter 2
TCA	tricarboxylic acid
NADH	nicotinamide adenine dinucleotide
FADH ₂	flavin adenine dinucleotide
ETC	electron transport chain
OCR	oxygen consumption rate
ATP	adenosine triphosphate
ADP	adenosine diphosphate
iAs ^{III}	trivalent inorganic arsenic
iAs ^V	pentavalent inorganic arsenic
MAs ^{III}	trivalent methylarsonite
DMAs ^{III}	trivalent dimethylarsinite
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
SAB	secretion assay buffer
GSIS	glucose stimulated insulin secretion
ELISA	enzyme-linked immunosorbent assay
DMSO	dimethyl sulfoxide
ROS	reactive oxygen species

CHAPTER 1: INTRODUCTION

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic disorder that is characterized by abnormal glucose homeostasis and is associated with many secondary health outcomes. Individuals living with DM have increased risks of many unfavorable conditions including: blindness, kidney failure, heart disease, stroke and loss of body extremities (CDC, 2014). DM prevalence has doubled from 1980 to 2014 and has accounted for about 422 million deaths worldwide in 2014 (IDF, 2015). In 2014, DM constituted 12% of global health expenditures, summing up to a grand total of \$673 billion (IDF, 2015). Currently, DM is known to be associated with various risk factors including obesity, lack of physical activity, unhealthy diet, high blood pressure and pro-diabetic genetic traits. These risk factors have been widely recognized as the major targets of diabetes prevention. However, some DM cases may develop as the result of environmental toxins. In order to further investigate this claim, research on the potential causes of DM, similar to this study, needs to continue in order to prevent the exacerbation of DM-related complications.

DM is typically characterized as either Type I or Type II. Type I diabetes is an autoimmune disorder primarily involving pancreatic β -cell destruction and can result in chronic hyperglycemia due to defects in insulin secretion. Type II diabetes also results in chronic hyperglycemia, but is normally caused by either insulin resistance of target tissues or diminished insulin release (Sivitz and Yorek, 2010).

1.2 Pancreatic β -cell Insulin Secretion

The pancreatic β -cell is the primary cell type involved in insulin secretion, and its dysfunction plays a central role in the development of DM. The main function of the β -cell is to

secrete insulin in response to increased glucose loads via glucose uptake by the GLUT2 transporter. Following uptake, glucose is metabolized via glycolysis and the tricarboxylic acid (TCA) cycle. The oxidative coenzymes produced, NADH and FADH₂, then travel to the electron transport chain (ETC), where ATP synthase increases ATP production, resulting in an increased ATP:ADP ratio. A rise in the ATP:ADP ratio closes an ATP-sensitive K⁺ channel, depolarizing the β-cell plasma membrane. Depolarization of the plasma membrane opens voltage-gated Ca²⁺ channels, allowing extracellular Ca²⁺ to rush into the β-cell. Ultimately, the process ends in the Ca²⁺ ions leading to exocytosis of secretory vesicles and the release of insulin into the bloodstream (Antonioli et al., 2015).

1.3 Mitochondria and Insulin Secretion

Recently, mitochondria have been suggested to play an important role in regulating K_{ATP}-dependent and K_{ATP}-independent insulin secretion (Wollheim, 2000). Dysfunction in mitochondria, for this reason, could potentially be associated with inhibition of insulin secretion. A recent review has identified many environmental compounds as having a role in mitochondrial dysfunction (Meyer et al., 2013). Studies within the review have recognized some of the compounds being examined in this thesis as having some toxic effect on mitochondria in various cell types and include: arsenic, manganese and cadmium (Bowman et al., 2011; Dopp et al., 2008; Garceau et al., 2010; Sokolova et al., 2005; Zheng et al., 1998).

1.4 Arsenic

Arsenic (As) is a naturally-occurring toxic metalloid with no known physiological purpose and is one of the less abundant minerals of the Earth's crust (Nordstrom, 2002). Arsenic can be found in either the organic or inorganic form, with inorganic arsenic (iAs) primarily being found in groundwater reservoirs and certain food sources like rice and apple juice (FDA, 2013;

Nriagu and Lin, 1995). Many countries including the United States of America have iAs present at high levels in the groundwater (WHO, 2016). Both the EPA and WHO regulations have agreed upon a drinking water standard for As at 10 ppb (DEP, 2012; US EPA, 2009). However, well water is not regulated and has been identified to have high levels of iAs in certain regions (González-Horta et al., 2015; Gronberg, 2011; Sanders et al., 2014). There are two oxidation states at which iAs is found: trivalent (iAs^{III}) or pentavalent (iAs^V). Humans have been shown to reduce iAs^V to iAs^{III} as an intermediate step during iAs metabolism, with iAs^{III} being more toxic than iAs^V (Styblo et al., 2000). Many epidemiological studies have highlighted the toxic effects iAs may have on pancreatic β -cells ultimately resulting in their dysfunction (Liu et al., 2014) and have found an association between iAs exposure and DM (Mendez et al., 2016). One study has established a dose-dependent inhibition of insulin secretion by INS-1 (832/13) β -cells in response to chronic low-level iAs^{III} exposure (Fu et al., 2010). Additional research has indicated that methylated metabolites of iAs, methylarsonite (MA^{III}) and dimethylarsinite (DMA^{III}), are even more potent inhibitors of β -cell function (Douillet et al., 2013). Studies in other cell types have shown that many essential components of the insulin secretory pathway are susceptible to the effects of iAs^{III} . These targets include: GLUT2, hexokinase, dihydrolipoamide, Calpain-10 and insulin granule exocytosis (Liu et al., 2006; Pysher et al., 2007; Tseng, 2004; Díaz-Villaseñor et al., 2008; Turner, 2007). Research on the mechanisms of iAs^{III} effects on β -cells is limited, however, and will be further investigated in this thesis.

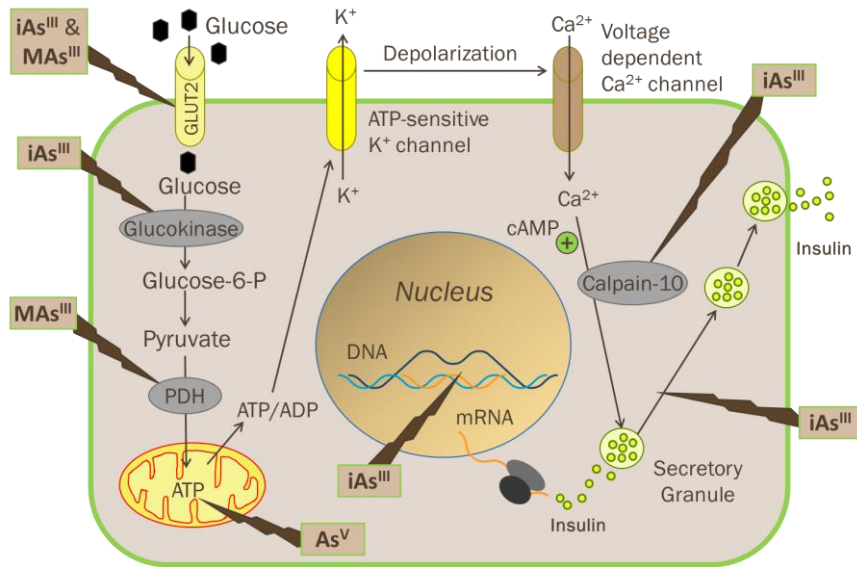


Figure 1.1: Potential Mechanisms for Arsenic Inhibition of Insulin Secretion

1.5 Manganese

Manganese (Mn), a naturally occurring brittle heavy metal, is found in the Earth's crust in over 100 minerals (WHO, 2004). Common environmental sources of Mn include industrial emissions, soil erosion and volcanic emissions, while dietary sources include mostly nuts and leafy vegetables (Higdon, 2001). Mn can also occur naturally in surface and groundwater due to Mn-rich soils (WHO, 2011a). The EPA has established a secondary drinking water standard for Mn at 0.05 mg/L, which many countries besides the USA have also adhered to (US EPA, 2009; WHO, 2011a). Studies testing groundwater levels, especially in South Asian countries, have found well water exceeding US-EPA limits (Akter et al., 2016; Kumar et al., 2016).

Due to its extensive role in the body, Mn is not treated with the same urgency as potent environmental toxins (i.e. As and Cd) for good reason. Within the human body, Mn serves roles as an activator and cofactor for many enzymes. Mn exists in either the 2+ (more common) or 3+ oxidation state in the body and has been shown to accumulate in specific tissues, including pancreatic β -cells (Rorsman et al., 1982). Not much is known regarding the specific role of Mn

in β -cell insulin secretion, but Mn supplementation has been shown to increase insulin secretion in isolated islets from C57BL/6J mice fed a high fat diet (Lee et al., 2013).

1.6 Cadmium

Cadmium (Cd) is a naturally occurring heavy metal with no known physiological purpose. In the environment, Cd occurs as a part of Zn ores and is only found in the 2+ oxidation state (WHO, 2011b). Naturally, Cd is found in surface water and groundwater, but normally is not found in particularly high concentrations (WHO, 2011b). Nevertheless, higher Cd levels can be found in areas containing a large amount of Zn-galvanized pipes, Cd-containing solders, low pH soft water or high industrial activity (WHO, 2011b). US EPA regulations mandate Cd in drinking water to be limited to 5 ppb, while the WHO has 3 ppb as the guideline value for Cd in drinking water (US EPA, 2009; WQA, 2013). In developed countries, dietary Cd is the highest source of Cd exposure in the United States (WHO, 2011b). Cd in the diet is mainly obtained from leafy vegetables, potatoes, soybeans and sunflower seeds (CDC, 2012). Although Cd may not be found in particularly high concentration in many areas, it is important to consider chronic accumulation of the heavy metal in bodily tissues, especially the pancreas.

Cd over the average human's lifespan, has been shown to accumulate in organs for unknown reasons. Although Cd has been shown to preferentially accumulate in the liver and kidneys, it has also been documented to accumulate in pancreatic β -cells (ATSDR, 2008; El Muayed et al., 2012). Chronic Cd accumulation in the β -cells results in inhibition of insulin secretion at low concentrations without significant decreases in cell viability (El Muayed et al., 2012). This would suggest Cd has some inhibitory effects on either insulin production or secretion machinery after chronic exposure and is worth examining in this study.

1.7 Zinc

Zinc (Zn) is an abundant element in the Earth's crust, which can be found either in surface water, groundwater or even tap water due to Zn leakage from pipes and fittings (WHO, 2003). Zn has been limited to 5 ppm by a secondary drinking water standard by the US EPA, whereas the WHO does not report a specified guideline value for Zn in drinking water (US EPA, 2009; WHO, 2011c).

Of Zn's many physiological roles, some are specifically related to insulin storage and secretion. One of the most well-known functions of Zn in regards to insulin, is the Zn-mediated crystallization of insulin. Insulin crystallization allows stable storage of insulin, protecting it from degradation (Li, 2014). Insulin secretion has been shown to release Zn as well for paracrine signaling within islets (Nygaard et al., 2014). Zn is also a key functional component of Zn finger proteins, which assist in gene regulation of various enzymes in the majority of cells including β -cells (Henry et al., 2014).

One study using the INS-1E rat pancreatic β -cell line identified 24-hour Zn supplementation to increase insulin secretion using physiologically relevant Zn concentrations (Nygaard et al., 2014). The same study then showed higher Zn concentrations inhibiting insulin secretion via cytotoxic effects.

1.8 Arsenic and Heavy Metal Interaction

Trivalent iAs and some heavy metals, namely Zn, Mn and Cd, have been found to be present naturally in groundwater across the world. Although there are other methods of exposure, populations drinking unregulated private well water in the US and living in specific regions within industrializing countries are at risk for high levels of exposure to these compounds (Sanders et al., 2014). In the case of Cd and Mn, it is crucial to keep in mind that they have the

potential to accumulate in certain bodily tissues, including those of the pancreas. More specifically, in the insulin-secreting pancreatic β -cells.

In addition to understanding the effects of these compounds alone on β -cells, it is also imperative to recognize that these metals and metalloids may coexist as complex mixtures in nature. Coexistence in mixture allows for the possibility for interactions among the heavy metals and iAs^{III} , which have yet to be studied. Further research into this topic will help gather knowledge on whether an interaction between compounds in complex mixtures exists and if so, the mechanism by which it acts. This thesis will attempt to determine if Zn^{2+} , Mn^{2+} or Cd^{2+} interact with iAs^{III} to affect INS-1 (832/13) rat pancreatic β -cell insulin secretion differently from that of iAs^{III} alone. The results from this study will aim to explain the effects of naturally occurring compound mixtures on pancreatic β -cells, and how they could potentially contribute to DM.

CHAPTER 2: MATERIALS AND METHODS

2.1 *INS-1 (832/13) β -cell Culture*

A rat pancreatic β -cell line, INS-1 (832/13), passage number 45-60 was used in this study. Cells were cultivated in RPMI 1640 medium with 10% fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate and 0.05 mM β -mercaptoethanol (all from Sigma, St. Louis, MO) at 37°C with 5% CO₂. Cells were grown in T75 flasks and refed every two days with complete RPMI 1640 medium until splitting.

2.2 *Treatment*

Cells were plated at a concentration of 1,000,000 cells per well in a 12-well tissue culture-treated plate and were allowed to incubate for 24 hours at 37°C with 5% CO₂ prior to treatment. For Cd exposures, cells were treated with 1 mL of 1, 2 or 5 μ M CdCl₂ with or without 1 μ M iAs^{III}. Mn exposed cells were treated with 1 mL of 12.5, 25 or 50 μ M MnCl₂ with or without 1 μ M iAs^{III}. Cells exposed to Zn were treated with 1 mL 6.25, 25, 50 or 200 μ M ZnCl₂ with or without 1 μ M iAs^{III}. After administration of the respective treatment concentrations, cells were incubated at 37°C with 5% CO₂ for 24 hours before analysis. All compounds used were from Sigma, St. Louis, MO.

2.3 *MTT Cell Viability Assay*

INS-1 cells were plated at a concentration of 1,000,000 cells per well in a 12-well plate and treated for 24 hours with metals or arsenic as indicated. Following treatment, cells were washed with 1 mL phenol red-free RPMI 1640 media. Each well was then incubated in 1 mL MTT media containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phenol red-free RPMI 1640 media containing the respective heavy metal and

iAs concentrations for each treatment group. Cells were placed in an incubator at 37°C and 5% CO₂ for 1 hour. Following incubation, the MTT media was aspirated and 500 µL dimethyl sulfoxide (DMSO) was added to each well. The plate was allowed to sit for 20 minutes to allow complete dissolution of the formazan product by DMSO. Absorbance was read at 570 nm and corrected using a measurement at 630 nm.

2.4 Glucose Stimulated Insulin Secretion (GSIS) Assay

Following 24-hour exposure to metals, a GSIS assay was performed and metal treatment was carried throughout the entire experiment. RPMI 1640 medium was replaced with 1 mL of 0 mM glucose Secretion Assay Buffer (SAB) solution containing 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES buffer, 2.5 mM CaCl₂, 0.2% bovine serum albumin, 25.5 mM NaHCO₃ and sterile dH₂O. After washing with 1 mL 0 mM glucose SAB, cells were incubated in 0 mM glucose SAB for 1 hour at 37°C with 5% CO₂. After incubation, 0 mM glucose SAB medium was replaced with 1 mL 2.5 mM glucose SAB medium and incubated for 1 hour. Following incubation, a 500 µL sample of medium was collected after the incubation period and immediately stored at -20°C. Medium was then replaced with 16.7 mM glucose SAB medium and incubated for 2 hours. A sample of 500 µL was collected after the incubation period and immediately stored at -20°C. Each well was then rinsed with 1 mL DPBS, followed by the addition of 100 µL RIPA lysis buffer, containing protease inhibitor, to each well. Cells were scraped off and the lysate was then collected in Eppendorf tubes and stored at -20°C. Insulin concentrations of the frozen samples were determined using a Rat/Mouse Insulin ELISA kit (EZRMI 13-K) from Millipore Sigma (Billerica, MA).

2.5 Seahorse XF Mitochondrial Stress Test

The Seahorse XFe96 Bioanalyzer (Agilent, Santa Clara, CA) was used to measure β -cell mitochondrial respiration. 50,000 cells per well were plated on a 96-well Seahorse plate and treated as indicated above. Seahorse medium was prepared using 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.16 mM MgSO_4 , 20 mM HEPES buffer, 2.5 mM CaCl_2 , 0.2% bovine serum albumin, 25.5 mM NaHCO_3 and sterile dH_2O . All media were buffered to pH 7.40 using 1 M NaOH. Treated cells were placed in 0 mM glucose SAB for 40 minutes and incubated at 37°C with 5% CO_2 . The 0 mM glucose SAB was then replaced with 2.5 mM glucose SAB (without NaHCO_3) and incubated at 37°C without CO_2 . The Seahorse XFe96 Bioanalyzer was then used to analyze the mitochondrial oxygen consumption rate (OCR) at baseline and then in response to sequential addition of 16.7 mM glucose SAB (without NaHCO_3 and BSA), 2 μM oligomycin, 4 μM FCCP (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) and 1 μM antimycin A/rotenone mix (all from Sigma, St. Louis, MO).

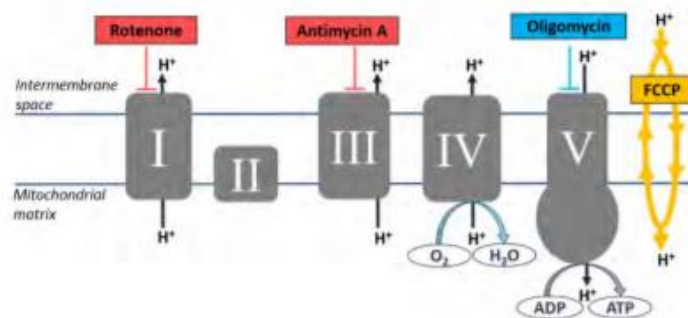


Figure 2.1: Action of Seahorse Mitochondrial Stress Test Reagents. (Seahorse Bioscience, 2009)

Oligomycin, antimycin A and rotenone are all inhibitors of the ETC, acting at complexes V, III and I, respectively. FCCP, on the other hand, is an uncoupler of the inner mitochondrial membrane. Action by these compounds mimics certain physiological conditions and allow us to measure key parameters of mitochondrial respiration based on how OCR responds.

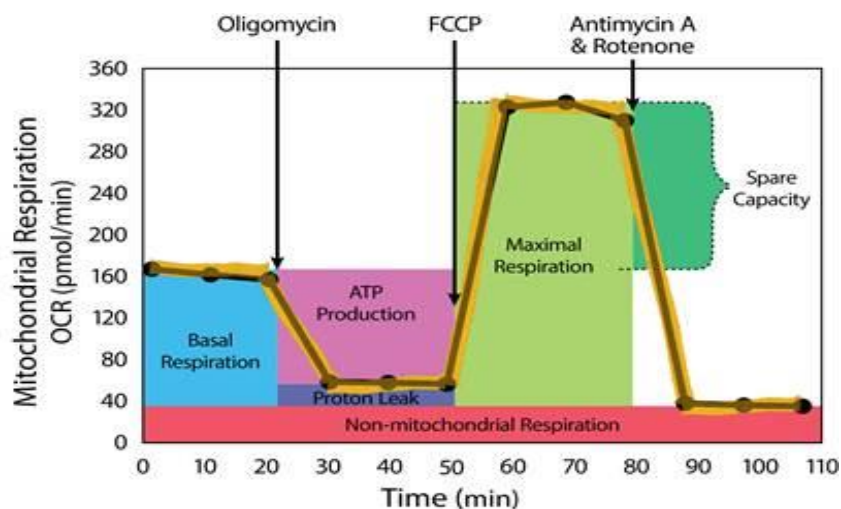


Figure 2.2: Seahorse XF Cell Mitochondrial Stress Test profile of mitochondrial respiration parameters.

(Seahorse Bioscience, 2009)

As seen in Figure 2.2, the inhibition of ATP synthase by oligomycin allows for the measurement of ATP production based on the decrease in OCR. This measurement allows for quantification of the amount of oxygen being used for ATP synthesis but does not measure ATP production itself. Additionally, there is some inevitable proton leak contributing to the measured OCR at this point, which is accounted for after subtracting the OCR due to non-mitochondrial respiration. Next, uncoupling by FCCP results in the breakdown of the proton gradient across the inner mitochondrial membrane. The resulting massive proton influx increases OCR to the maximum possible value capable by the cell's mitochondria. This condition would be analogous to physiological energy-demanding situations. Finally, antimycin A/rotenone administration results in the inhibition of complexes I and III, essentially abolishing mitochondrial OCR. The remaining OCR measured can be attributable to non-mitochondrial respiration.

2.6 Statistical Analysis

All MTT and GSIS assay data was analyzed using Microsoft Office Excel 2013. P-values were calculated using a 2-tailed t-test. For MTT results, means and standard deviations were calculated using either $n = 3$ or $n \geq 2$, which were obtained by testing each treatment group with

2 technical replicates in biological triplicate or in the case of Zn, 3 technical replicates in biological duplicate. Percent absorbance of the control group was checked for significant differences to the control group in all treatment groups (“*” or “****”) and to 1 μM iAs^{III} alone in mixture treatment groups (“a”) using a two-tailed t-test. For GSIS results, means and standard deviations were also calculated using $n=3$ from trials using the 2 technical replicates in biological triplicate setup. Insulin secretion of each treatment group was checked for significant differences in four ways: all groups to the control group (“*” or “****”), each mixture group to 1 μM iAs^{III} alone (“a”), each mixture to its identical heavy metal concentration alone (“\$” or “\$\$\$”) and each heavy metal concentration compared to a lower exposure level (“%”; i.e. 25 μM Mn to 12.5 μM Mn). For Seahorse data, Wave 2.3.0 software was used for analysis. Means and standard deviations were calculated in Microsoft Office Excel 2013 using $n=3$ by testing each treatment group with 3 technical replicates in biological triplicate. P-values for Seahorse data were calculated in GraphPad Prism 7.03 using a 2-way ANOVA test.

CHAPTER 3: RESULTS

3.1 MTT Assay and Cell Viability

MTT colorimetric assays measuring cell metabolic activity were used to determine any changes in INS-1 (832/13) cell viability in response to 24-hour heavy metal and iAs^{III} exposure.

3.1 MTT Assay and Cell Viability

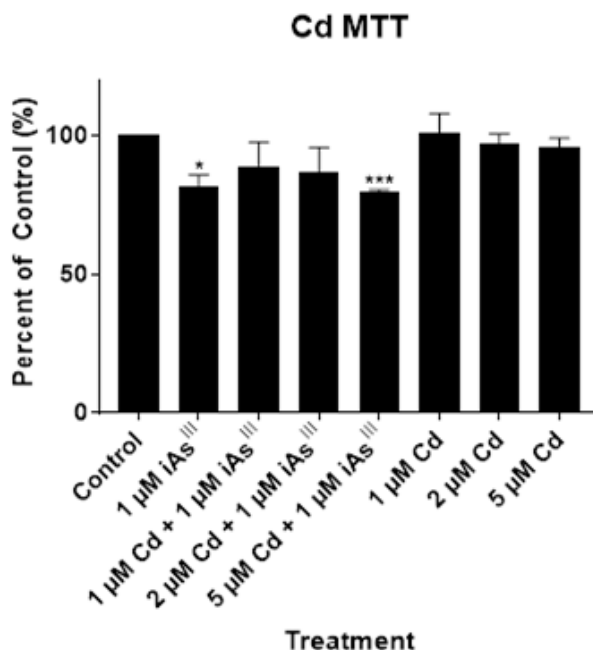


Figure 3.1: Effects of Cadmium on Cell Viability. 24-hour exposure to Cd in conjunction with iAs^{III} or alone does not significantly alter cell metabolism levels in INS-1 (832/13) β -cells. $n \geq 2$ * $p < 0.05$, *** $p < 0.001$ (Metal vs. Control).

After Cd exposure at 1, 2 and 5 μM Cd for 24 hours, INS-1 (832/13) cells did not exhibit any decrease in cell metabolic activity (Figure 3.1). A 24-hour exposure to only 1 μM iAs^{III} resulted in a 19% decrease in metabolic activity of treated cells compared to the untreated group (Figure 3.1). There was also a 20% decrease in metabolic activity of cells chronically exposed to 5 μM Cd + 1 μM iAs^{III} (Figure 3.1). There was no significant difference in cell viability levels

between those exposed to 1 μM iAs^{III} alone and those exposed to 1-5 μM Cd exposures in conjunction with 1 μM iAs^{III} .

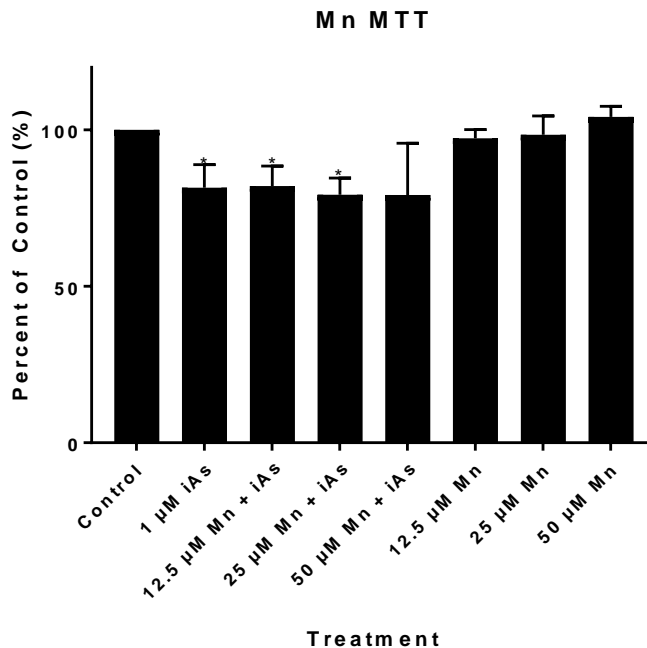


Figure 3.2: Effects of Manganese on Cell Viability. 24-hour exposure to Mn in conjunction with iAs^{III} or alone does not significantly alter cell metabolism levels in INS-1 (832/13) β -cells. $n = 3$; * $p < 0.05$ (Metal vs. Control).

INS-1 (832/13) cells exposed for 24 hours to Mn at 12.5, 25 and 50 μM exhibited no significant differences in cell metabolic activity compared to the control (Figure 3.2). A 24-hour exposure to only 1 μM iAs^{III} resulted in an 18% decrease in metabolic activity of treated cells compared to the untreated group (Figure 3.2). The 12.5 and 25 μM Mn and iAs^{III} co-exposures both decreased metabolic activity by 21% (Figure 3.2). There was no significant difference in cell viability between cells exposed to 1 μM iAs^{III} alone and groups co-exposed to 12.5-50 μM Mn and 1 μM iAs^{III} .

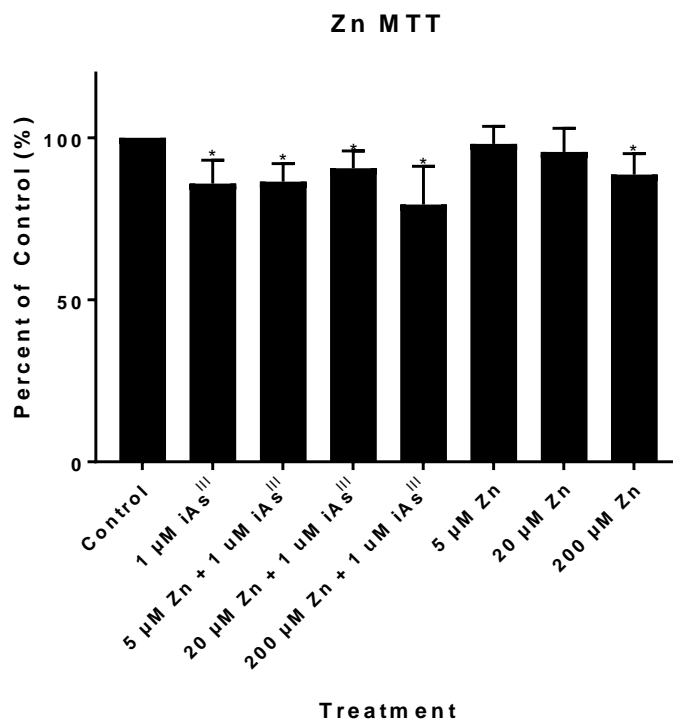


Figure 3.3: Effects of Zinc on Cell Viability. 24-hour exposure to Zn in conjunction with iAs^{III} or alone does not significantly alter cell metabolism levels in INS-1 (832/13) β -cells. $n \geq 2$; * $p < 0.05$ (Metal vs. Control).

INS-1 (832/13) cells exposed for 24 hours to 5 μM Zn alone exhibited a 5% decrease in metabolic activity compared to the control (Figure 3.3). A more pronounced decrease of 12% metabolic activity was observed after the chronic 200 μM Zn exposure (Figure 3.3). The 1 μM iAs^{III} exposure resulted in a 14% decrease in metabolic activity compared to the untreated group (Figure 3.3). Co-exposure of all tested Zn concentrations and 1 μM iAs^{III} did not elicit a significant decrease in cell viability compared to that of 1 μM iAs^{III} alone.

3.2 GSIS after Arsenic and Metal Exposure

Exposure to arsenic has been shown to elicit a dose-dependent inhibition of insulin secretion by pancreatic β -cells (Fu et al., 2010). In order to examine the impact of common co-contaminant heavy metals and arsenic, a 24-hour co-exposure model was employed followed by a GSIS. Similar to other studies, untreated INS-1 (832/13) cells exhibited approximately a 10-

fold response to high glucose incubation (16.7 mM glucose) from basal low glucose incubation (2.5 mM glucose) (Figures 3.4-3.6).

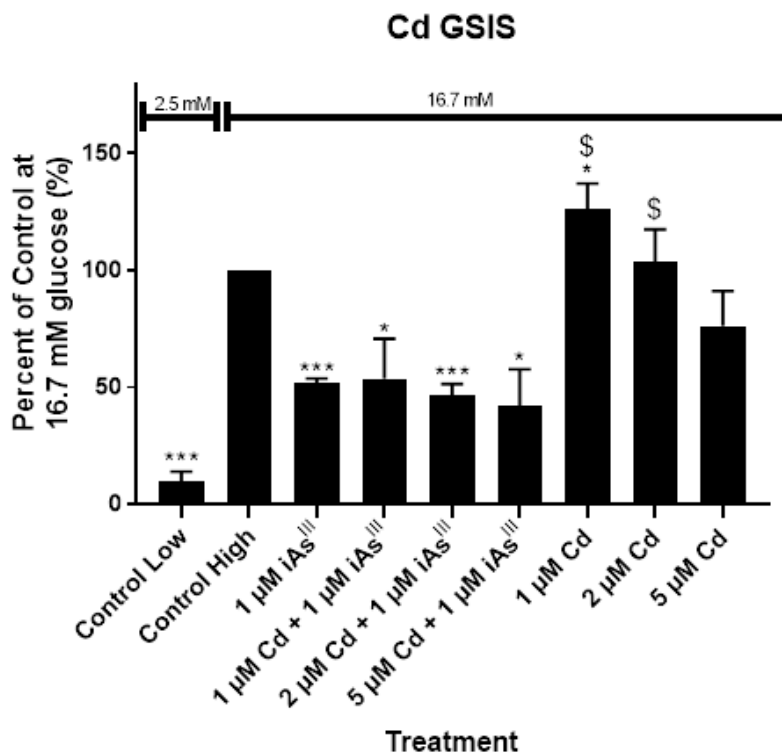


Figure 3.4: Glucose Stimulated Insulin Secretion following 24-hour exposure to iAs^{III} and Cd. 24-hour exposure to Cd in conjunction with iAs^{III} significantly inhibits GSIS in INS-1 (832/13) β-cells. n = 3; *p < 0.05, ***p < 0.001 (Metal vs. Control); “\$” p < 0.05 (Metal vs. Metal + iAs^{III}).

INS-1 (832/13) cells exposed for 24-hours to 1 μM Cd alone displayed a 26% increase in insulin secretion compared to untreated cells (Figure 3.4). Exposures to 2 μM and 5 μM Cd suggest a downward trend inhibition in percent insulin secretion relative to the 1 μM Cd exposure level, but didn't show significant differences (Figure 3.4). 24-hour exposure to 1 μM iAs^{III} alone resulted in a 48% decrease in insulin secretion. Interestingly, beginning with the 1 μM iAs^{III} exposure, co-exposure of increasing Cd concentrations suggests a downward trend in inhibition of insulin secretion; however, no differences were statistically significant (Figure 3.4).

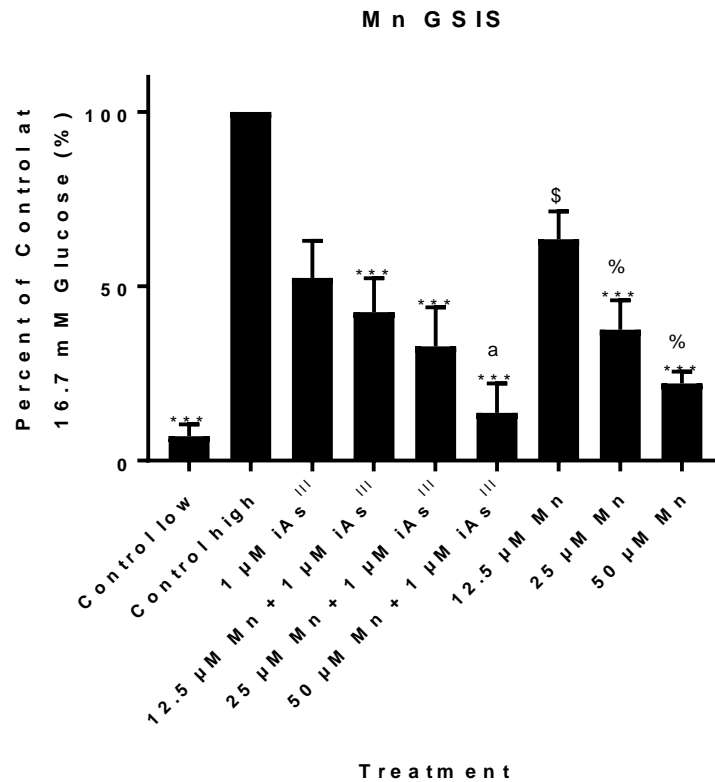


Figure 3.5: Glucose Stimulated Insulin Secretion following 24-hour exposure to iAs^{III} and Mn. 24-hour exposure to Mn in conjunction with iAs^{III} and alone significantly inhibits GSIS in INS-1 (832/13) β -cells. n = 3; *p < 0.05, ***p < 0.001 (Metal vs. Control); “a” p < 0.05 (Metal vs. iAs^{III}); “\$” p < 0.05 (Metal vs. Metal + iAs^{III}); “%” p < 0.05 (Metal vs. Previous Concentration Metal).

INS-1 (832/13) cells displayed a 14-fold increase in average percent insulin secretion at 16.7 mM glucose from that seen at 2.5 mM glucose (Figure 3.5). A 24-hour exposure of cells to 12.5, 25 and 50 μM Mn alone resulted in a dose-dependent inhibition of average insulin secretion by 37%, 62% and 78%, respectively, from that witnessed in the control group (Figure 3.5). 24-hour exposure to 1 μM iAs^{III} resulted in a 48% decrease in insulin secretion compared to the control group (Figure 3.5). Co-exposure to the aforementioned concentrations of Mn in conjunction with 1 μM iAs^{III} elicited an inhibition of average insulin secretion by 57%, 67% and 86%, respectively, from that secreted by control cells (Figure 3.5). The average insulin secretion for 25 and 50 μM Mn exposures alone did not show significant differences from the insulin

secretion from cells co-exposed at identical concentrations of Mn with 1 μM iAs^{III} ; however, exposure to 12.5 μM Mn elicited significantly higher amounts of insulin than co-exposure to 12.5 μM Mn and 1 μM iAs^{III} (Figure 3.5). Co-exposure of 50 μM Mn and 1 μM iAs^{III} for 24 hours resulted in a significant inhibition of average percent insulin secretion compared to that resulting from exposure to 1 μM iAs^{III} alone.

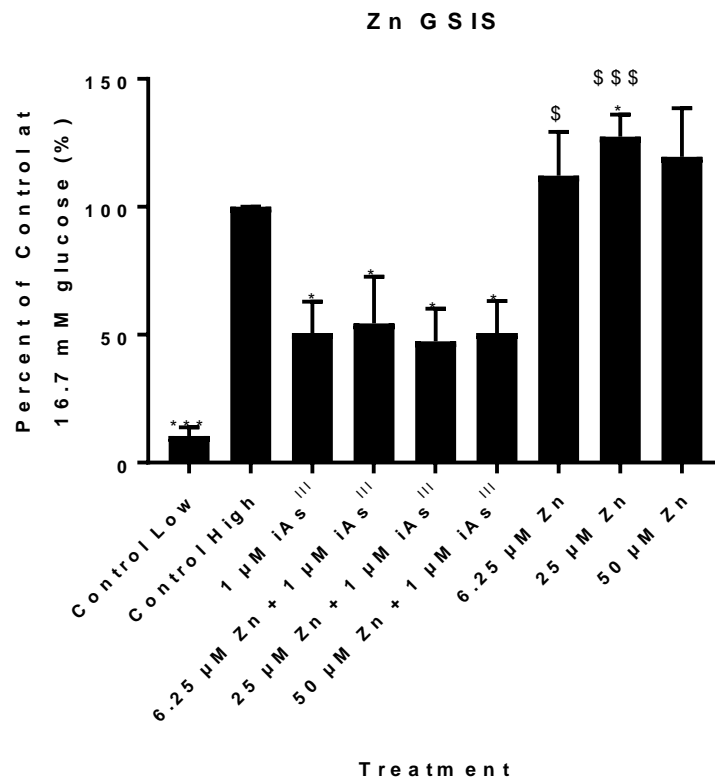


Figure 3.6: Glucose Stimulated Insulin Secretion following 24-hour exposure to iAs^{III} and Zn. 24-hour exposure to Zn alone increases GSIS, while co-exposure with iAs^{III} inhibits GSIS in INS-1 (832/13) β -cells. $n \geq 2$; * $p < 0.05$, *** $p < 0.001$; "\$" $p < 0.05$, "\$\$\$" $p < 0.001$.

INS-1 (832/13) cells exhibited a 16-fold increase in average percent insulin secretion at 16.7 mM glucose from that seen at 2.5 mM glucose (Figure 3.6). 24-hour exposure to 25 μM Zn elicited a 27% increase in GSIS compared to that of untreated cells (Figure 3.6). Cells exposed to the 6.25 μM Zn and 25 μM Zn concentrations displayed significantly higher insulin secretion

than those co-exposed to Zn and iAs^{III} at identical Zn concentrations (Figure 3.6). 24-hour exposure of cells to 1 μ M iAs^{III} alone resulted in a 49% decrease in percent insulin secretion compared to untreated cells (Figure 3.6). Co-exposure of Zn and 1 μ M iAs^{III} did not result in any significant change in percent insulin secretion compared to that seen in 1 μ M iAs^{III} exposure alone (Figure 3.6).

3.3 Mitochondrial Stress Test after Arsenic and Metal Exposure

In order to investigate potential mechanisms of insulin secretion inhibition by Mn, Cd and iAs^{III}, Seahorse XF technology was used to measure mitochondrial OCR in INS-1 (832/13) cells. The Seahorse mitochondrial stress test was performed to determine the OCR of β -cells in acute high glucose conditions (16.7 mM), and other various modulators of mitochondrial function. Response of the β -cells to the mitochondrial stress test trials can be seen in Figures 3.7, 3.8, 3.10 and 3.11.

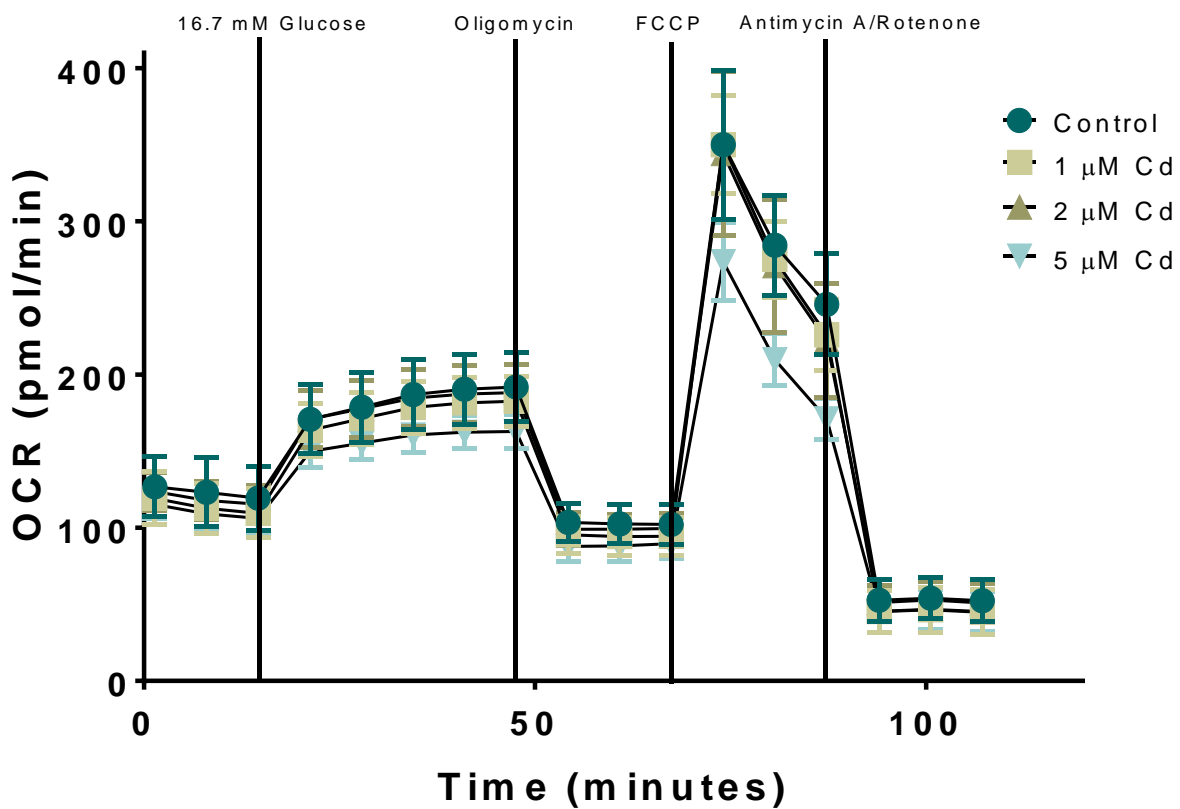


Figure 3.7: Cd Exposure Response to Mitochondrial Stress Test. Mitochondrial Stress Test response of INS-1 (832/13) cells exposed for 24-hours to Cd alone. n = 3.

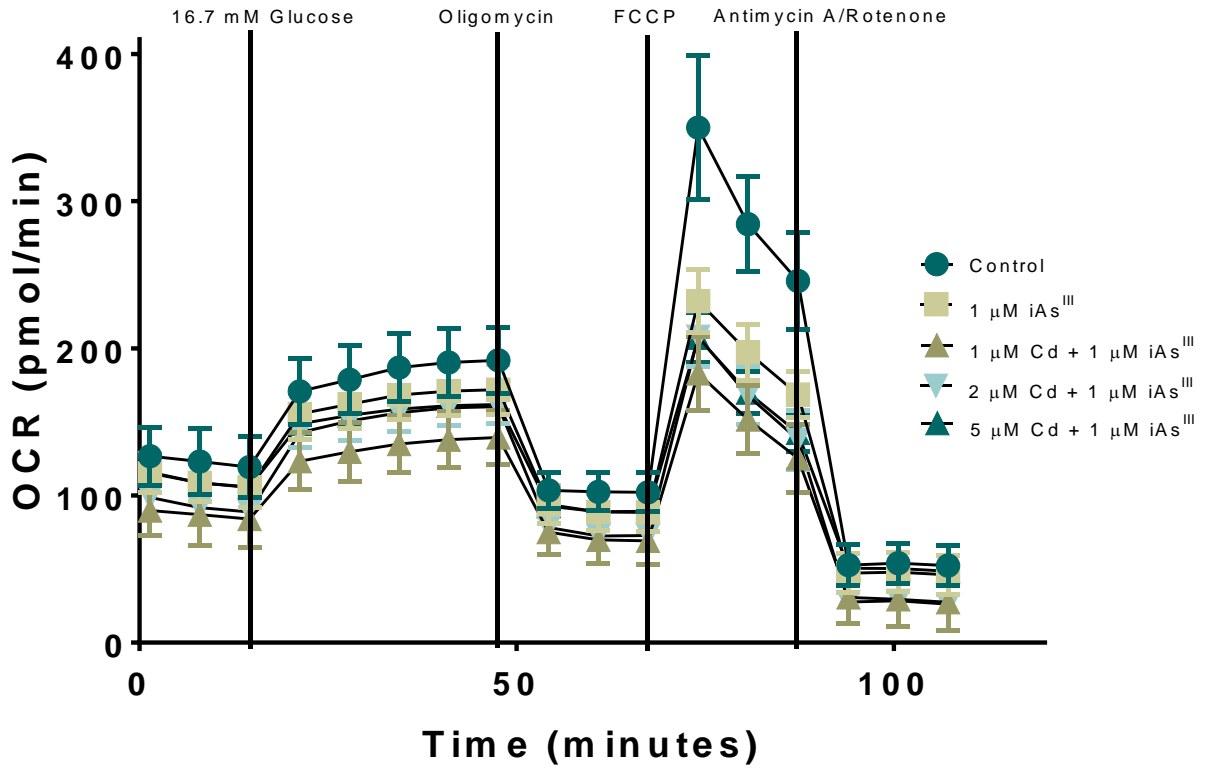


Figure 3.8: Cd and iAs^{III} Co-exposure Response to Mitochondrial Stress Test. Mitochondrial Stress Test response of INS-1 (832/13) cells co-exposed for 24-hours to Cd and iAs^{III} alone. n = 3.

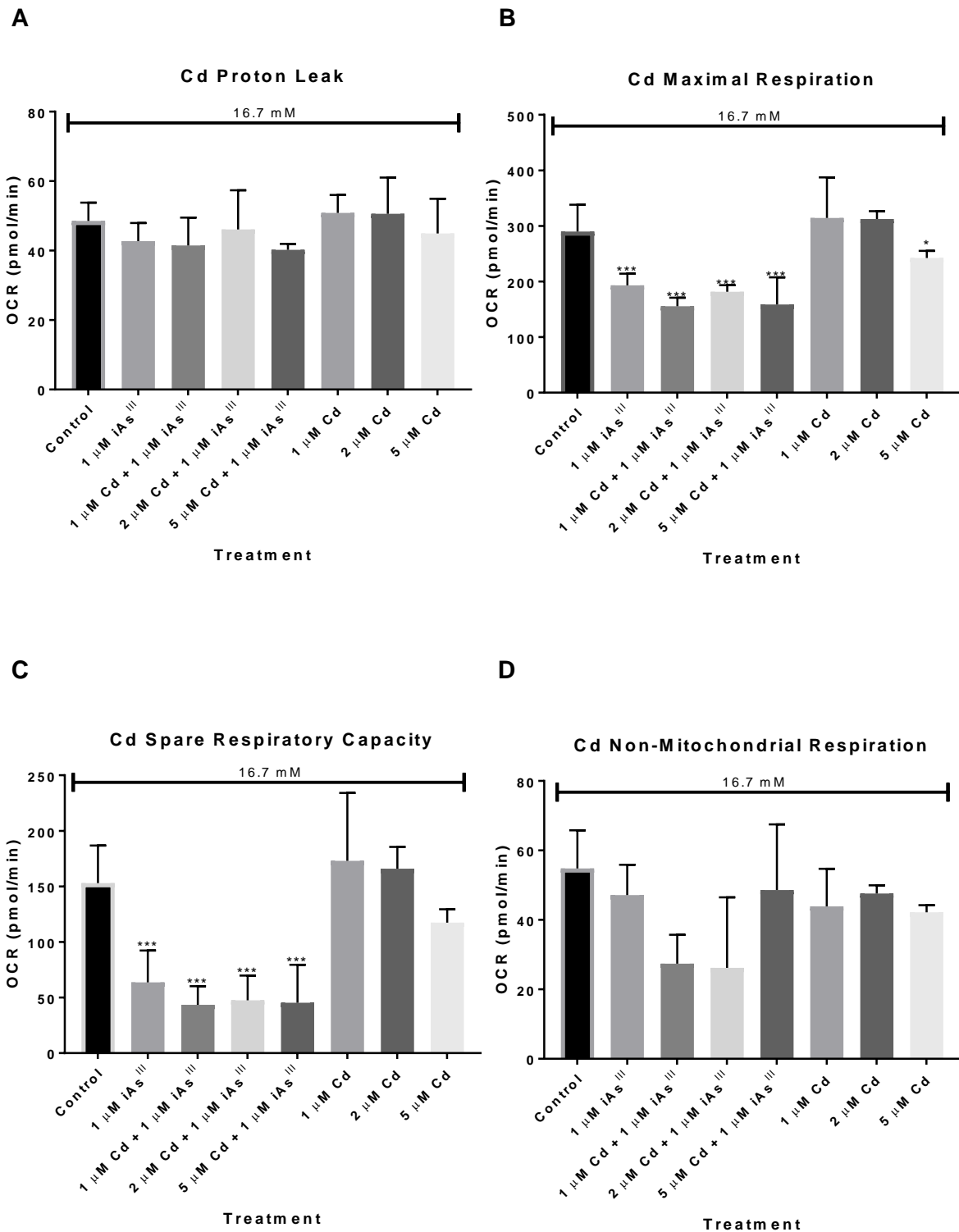


Figure 3.9: Effects of 24-hour iAs^{III} and Cd co-exposure on mitochondrial respiration. Inhibition of spare respiratory capacity and maximal respiration OCRs by 24-hour Cd and iAs^{III} co-exposure; n=3; *p \leq 0.05, ***p \leq 0.001 (Metal vs. Control).

The OCR in cells exposed to 1 μM iAs^{III} during periods of maximal respiration, induced by FCCP injection, was significantly decreased to 193 pmol/min compared to the control maximal respiration OCR of 290 pmol/min (Figure 3.8 B). 24-hour exposure to 5 μM Cd alone significantly decreased the OCR during maximal respiration conditions by 48 pmol/min (Figure 3.8 B). However, no Cd exposure alone showed a significant difference in OCR allotted as spare respiratory capacity in comparison with the control groups (Figure 3.8 C). 24-hour co-exposure to 1 μM iAs^{III} and 1, 2 and 5 μM Cd significantly inhibited maximal respiration (Figure 3.8 B). Cells exposed to 1, 2 and 5 μM Cd in conjunction with 1 μM iAs^{III} displayed maximal respiration OCRs of 156, 182 and 159 pmol/min, respectively (Figure 3.8 B). The decreases in OCR during maximal respiration translated into significant decreases in spare respiratory capacity as well. Compared to the 153 pmol/min OCR observed in untreated cells, a significant decrease to 64 pmol/min spare respiratory capacity OCR was seen in cells exposed to 1 μM iAs^{III} alone (Figure 3.8 C). Additionally, cells co-exposed with 1, 2 and 5 μM Cd and 1 μM iAs^{III} exhibited a significant decrease in spare respiratory capacity OCRs to 44, 47 and 45 pmol/min, respectively (Figure 3.8 C). Neither non-mitochondrial respiration nor proton leak were significantly altered by Cd or iAs^{III} exposure, indicating the changes in OCR are within the mitochondria.

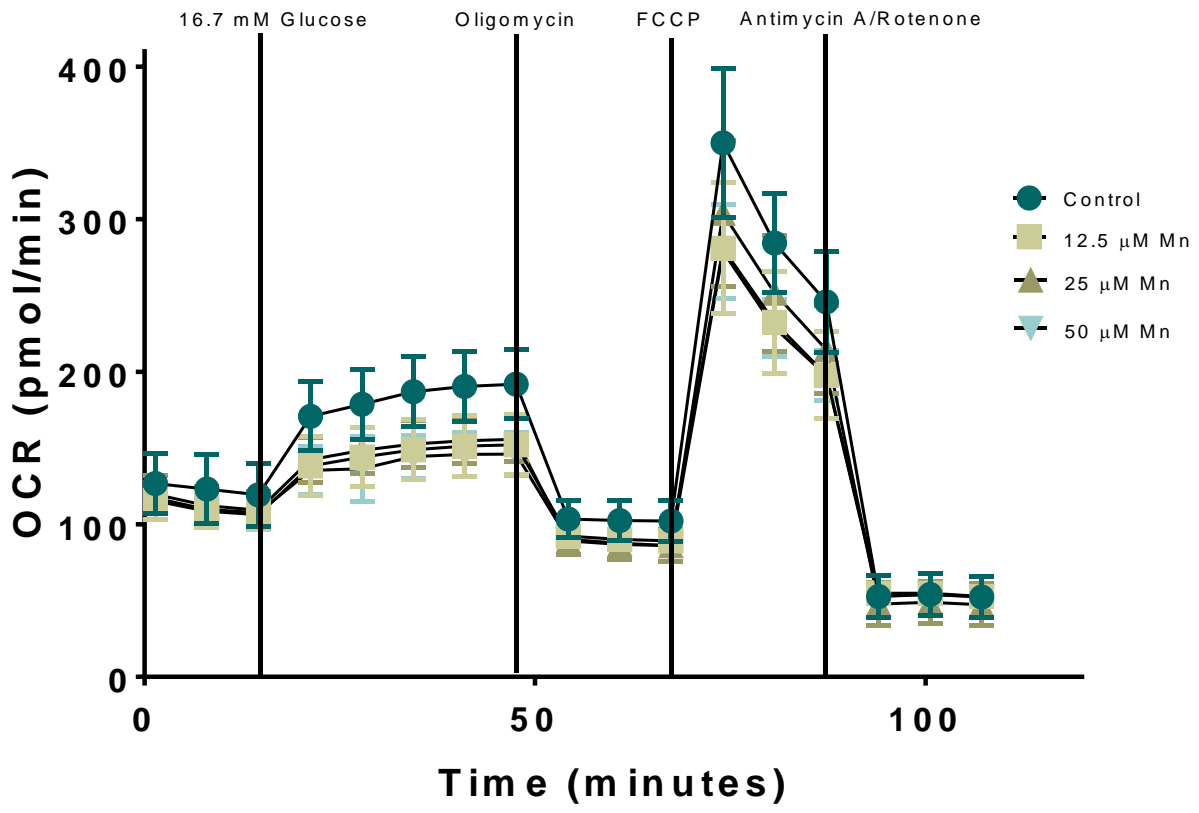


Figure 3.10: Mn Exposure Response to Mitochondrial Stress Test. Mitochondrial Stress Test response of INS-1 (832/13) cells exposed for 24-hours to Mn alone. n = 3.

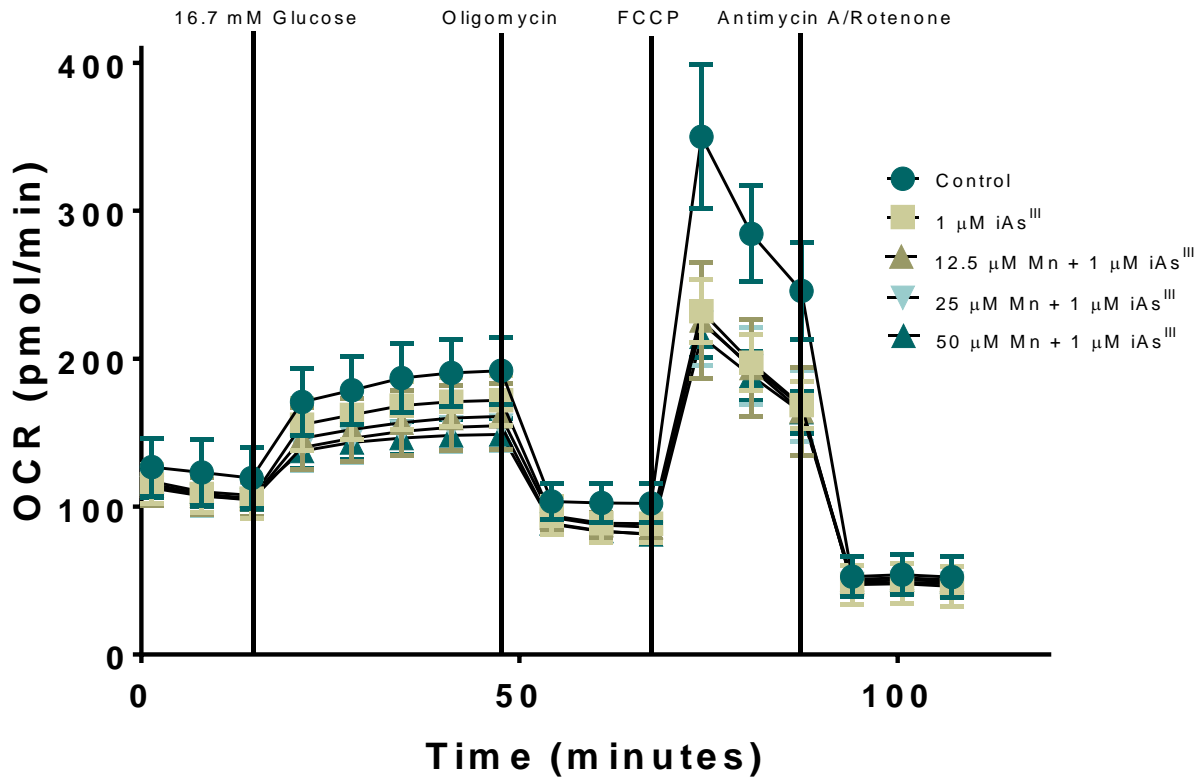


Figure 3.11: Mn and iAs^{III} Co-exposure Response to Mitochondrial Stress Test. Mitochondrial Stress Test response of INS-1 (832/13) cells co-exposed for 24-hours to Mn and iAs^{III} alone. n = 3.

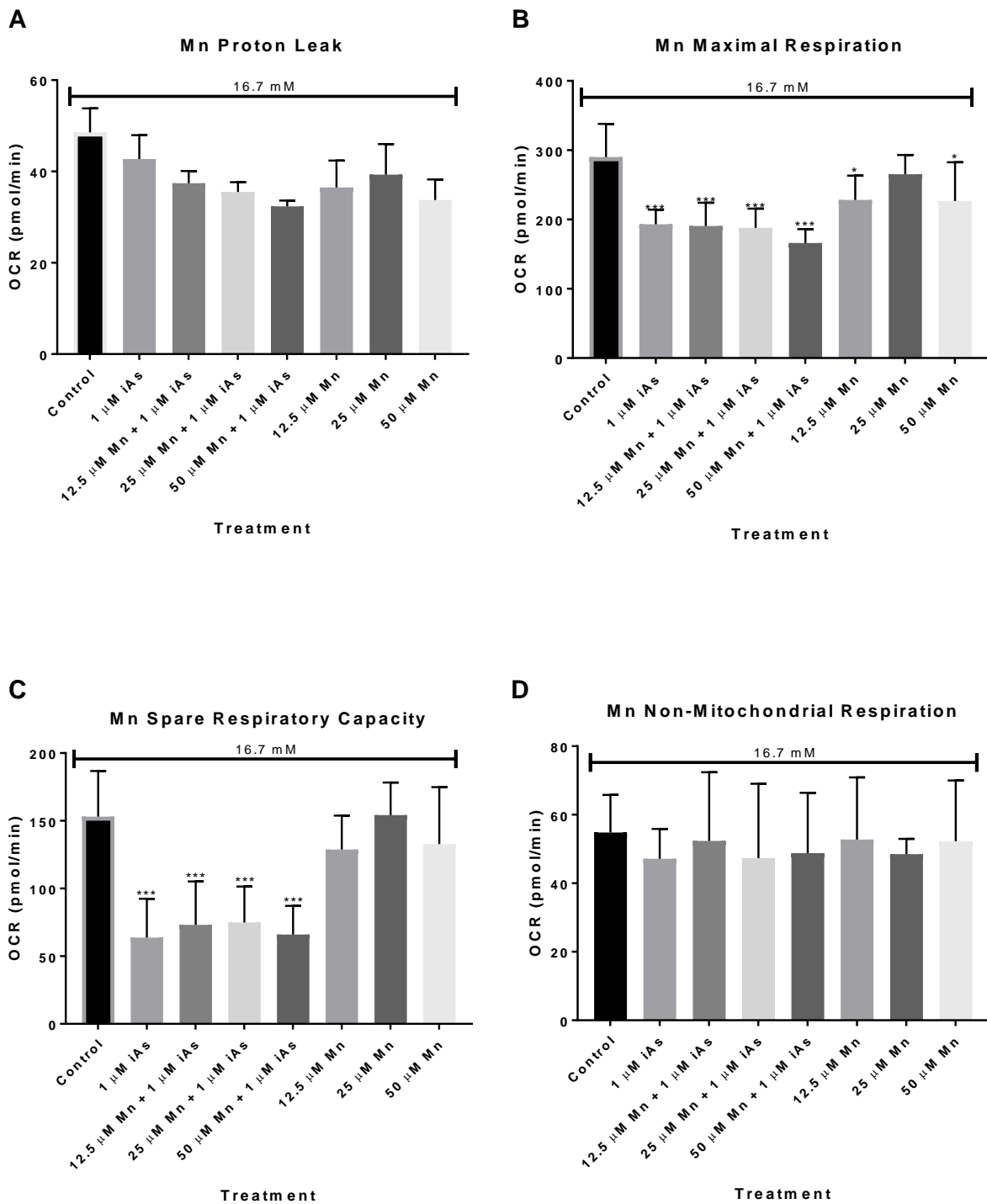


Figure 3.12: Effects of 24-hour iAs^{III} and Mn co-exposure on mitochondrial respiration. Inhibition of spare respiratory capacity and maximal respiration OCRs by 24-hour Mn and iAs^{III} co-exposure; $n=3$; * $p \leq 0.05$, *** $p \leq 0.001$ (Metal vs. Control).

24-hour exposure of Mn to INS-1 (832/13) β -cells did not result in a significant decrease in OCR for most of the parameters investigated. Exposure to 12.5 and 50 μ M Mn elicited a significant inhibition of OCR during maximal respiration by 62 and 63 pmol/min, respectively (Figure 3.9 B); however, these inhibitions did not translate to a significant decrease in spare respiratory capacity OCRs (Figure 3.9 C). 24-hour exposure of INS-1 (832/13) cells to 1 μ M iAs^{III} alone and in conjunction with 12.5, 25 and 50 μ M Mn all significantly decreased maximal respiration OCR from the control value by 97, 99, 102 and 124 pmol/min, respectively (Figure 3.9 B). Exposure to 1 μ M iAs^{III} alone and 12.5, 25 and 50 μ M Mn co-exposure with 1 μ M iAs^{III} exhibited a significant decrease in spare respiratory capacity OCRs at 89, 80, 78 and 87 pmol/min, respectively (Figure 3.9 C). Neither non-mitochondrial respiration nor proton leak were significantly altered by Mn or iAs^{III} exposure, indicating the changes in OCR are within the mitochondria.

CHAPTER 4: DISSCUSION

Pancreatic β -cell dysfunction and insulin resistance characterize Type II diabetes. Insulin resistance in peripheral tissues overworks β -cells which, over longer periods of time, can result in β -cell fatigue and death. Recently, diabetes has been linked to exposure to specific environmental toxins. These toxins, including some compounds investigated in this study, may disrupt critical points in the insulin secretion pathway and lead to inhibition of insulin secretion in β -cells.

Low or moderate exposure to iAs^{III} has been associated with Type II diabetes in human populations (Islam et al., 2012). Some studies have shown chronic low level iAs^{III} exposures cause isolated islet and β -cell dysfunction in mice (Douillet et al., 2013; Liu et al., 2014). In vitro research using INS-1 (832/13) β -cells has demonstrated 96-hour exposure to 0.5 μM iAs^{III} causing significant inhibition of insulin secretion (Fu et al., 2010). The hypothesized mechanism of action for inhibited insulin secretion in the INS-1 (832/13) cells was a change in gene transcription, causing altered ROS levels to impact GSIS (Fu et al., 2010). The current study attempts to observe the effect of 24-hour iAs^{III} exposure, as well as that of iAs^{III} and heavy metal co-exposure to the INS-1 (832/13) β -cell line.

24-hour exposure of INS-1 (832/13) cells to 1 μM iAs^{III} resulted in significant inhibition of insulin secretion without large changes in cell viability. This removes the possibility of cytotoxicity solely being responsible for the observed inhibition of insulin secretion. A previous study in isolated islets indicated 48-hour iAs^{III} exposure does not result in significant changes to islet insulin content, but instead severely inhibits the amount of insulin secreted by the islet

(Douillet et al., 2013). This suggests the reason for the observed inhibition of insulin secretion is due to disruption of insulin secretion machinery rather than decreased insulin biosynthesis.

There are currently many hypothesized targets of iAs^{III} within the insulin secretion pathway (Figure 1.1) based on studies in other cell types. As seen in Figure 1.1, iAs^{III} and its methylated metabolite (MAs^{III}) have been identified as possible modulators for many key steps in the insulin secretion pathway. Any of these identified steps could be a potential player in the inhibition of insulin secretion observed in the current study.

Along with iAs^{III} , Cd is another naturally-occurring environmental compound proposed to have toxic effects on the body (CDC, 2012). In certain regions, iAs^{III} and Cd have been found together in surface soils at high concentrations (Diawara et al., 2006). Other studies have also shown associations between increased urinary Cd and Type II diabetes incidence (Schwartz et al., 2003). One in vitro study went further and showed 72-hour Cd exposure to MIN6 β -cells resulting in Cd accumulation and inhibition of insulin secretion (El Muayed et al., 2012).

After a 24-hour exposure to 1 μM Cd alone, INS-1 (832/13) cells exhibited a significant increase in insulin secretion. There is no clear reason for the increase in insulin secretion. Both subsequent Cd exposure levels resulted in a downward trend in insulin secretion, but with no significant difference compared to untreated β -cells. This would suggest higher concentrations of Cd may cause significant inhibition of insulin secretion. 24-hour co-exposure of 1, 2 and 5 μM Cd and 1 μM iAs^{III} to the INS-1 (832/13) cells inhibited insulin secretion to similar levels observed at 1 μM iAs^{III} exposure alone. This suggests iAs^{III} acts as the primary driver of insulin secretion when co-exposed with the tested concentrations of Cd.

Chronic Mn exposure also inhibited GSIS of INS-1 cells in a dose-dependent manner without a significant decrease in cell viability. Interestingly, a downward trend in insulin

secretion is seen in the 24-hour Mn and iAs^{III} co-exposure groups with increasing Mn concentrations. It is important to note that co-exposure of 12.5 μ M Mn and 1 μ M iAs^{III} elicited a more potent inhibition of insulin secretion than that from 12.5 μ M Mn alone; however, 24-hour exposure of cells to 25 and 50 μ M Mn alone did not significantly differ from groups co-exposed to Mn and iAs^{III} at identical concentrations. This suggests that Mn-induced inhibition of insulin secretion overpowers that by iAs^{III} at higher concentrations, which identifies chronic Mn exposure as a potent inhibitor of GSIS.

Although Mn is identified as a heavy metal with many physiological uses in activating enzymes, not much is specifically known about its role in β -cell function. One study showed in vivo Mn supplementation could be protective against diet-induced diabetes by enhancing insulin secretion in islets (Lee et al. 2013). However, these results are contrary to those observed in the current study, exhibiting a severe dose-dependent inhibition of insulin secretion in INS-1 (832/13) β -cells following 24-hour Mn exposure. The differences seen between the two studies may be due to discrepancies in doses and differences of in vivo versus in vitro Mn exposure.

Similar to Cd, Mn and Ca fluxes in pancreatic β -cells have been shown to compete in the presence of D-glucose (Rorsman and Hellman, 1983). It is worth noting, however, that the study used 2.5 mM Mn²⁺ and 1.28 mM Ca²⁺ medium, which has a much greater concentration of Mn²⁺ than that used in the current study. For this reason, Mn-induced inhibition of insulin secretion observed in the INS-1 (832/13) cells is most likely not due to competition between Mn and Ca fluxes, due to the large ratio of Ca²⁺ to Mn²⁺ ions present in the SAB utilized in GSIS trials. Future studies will need to focus on evaluating the impact of Mn exposure on Ca changes within β -cells.

Unlike Cd or Mn, Zn has an identified role in β -cell function. Zn plays a role in insulin production, insulin crystallization for proper storage, Zn finger-dependent gene regulation and insulin granule translocation to the membrane for exocytosis (Li, 2014). One study using Zn supplementation on INS-1E β -cells found a significant increase in insulin secretion for concentrations up until 30 μ M Zn (Nygaard et al., 2014). Thus, it was hypothesized that Zn supplementation could combat the inhibitory effects of iAs^{III} exposure on insulin secretion by the β -cell. In agreement with Nygaard's study, a significant increase in insulin secretion compared to untreated β -cells was observed in the 25 μ M Zn exposure group. Nevertheless, there was no significant increase in insulin secretion when the same Zn exposures were co-exposed with 1 μ M iAs^{III} . These results identify iAs^{III} as the primary driver of reduced insulin secretion when found in mixture with Zn at the tested concentrations.

Recent studies have identified mitochondria as a target for environmental toxins, especially those with a cationic nature like Cd and Mn. Due to their divalent nature, it has been shown that both can accumulate within mitochondria in various tissues (Meyer et al., 2013; Rorsman et al., 1982). This is of special importance to studies exploring the effects of chronic exposures to these metals as lower exposure concentrations may induce significant effects over time. Cd, Mn and As have all been identified as possible mitochondrial toxicants (Meyer et al., 2013). Referring back to Figure 1.1, ATP production by mitochondria is a critical step in the insulin secretory pathway. Thus, it was hypothesized that iAs^{III} , Cd and Mn may be inhibiting insulin secretion by causing β -cell mitochondrial dysfunction.

24-hour exposure to 12.5 and 50 μ M Mn alone inhibited maximal respiration OCR. However, with the effects of Mn on mitochondrial function being minimal and in no identifiable trend, the explanation for why Mn drastically inhibits insulin secretion in a dose-dependent

fashion seems to lie elsewhere in the insulin secretion pathway. Additionally, Mn and iAs^{III} co-exposure inhibited mitochondrial function much more severely than Mn alone at the tested concentrations; however, the GSIS trials showed no difference in the amount of insulin secreted. This means that the primary mechanism of inhibition of insulin release by Mn is not in the ETC.

Contrary to Mn exposure, 5 μM Cd exposure alone did inhibit OCR during maximal respiration conditions. Inhibition of OCR in β -cells exposed to 5 μM Cd after FCCP injection mean the cells would not be able to respond as effectively during physiological condition of high energy demand. Since maximal respiration conditions were induced by FCCP, an inner mitochondrial membrane decoupler acting after Complex V in the ETC, it is difficult to determine which complex is being acted upon by Cd to elicit the inhibition of OCR.

When Cd and Mn treatments were administered in conjunction with 1 μM iAs^{III} , there was a drastic inhibition of maximal respiration and spare respiratory capacity OCR in the INS-1 (832/13) β -cells. As previously mentioned, due to the location of FCCP uncoupling, iAs^{III} can be acting on any of the ETC complexes. One recent study identified MAs and DMAs, methyl metabolites of As, as inhibitors of ETC complexes in rat hepatocytes, suggesting a possibility for iAs^{III} acting as an inhibitor of one or more ETC complexes in INS-1 (832/13) cells (Naranmandura et al., 2011). An inhibition of spare respiratory capacity again indicates that 24-hour 1 μM iAs^{III} renders β -cells unable to function efficiently under energy-demanding situations. However, even with a severe decrease in OCR available as spare respiratory capacity, no significant decrease in the amount of OCR going towards ATP production was observed.

This study found that 24-hour Cd exposure to INS-1 (832/13) β -cells results in a downward trend in insulin secretion without significant declines in cell viability. 24-hour Mn exposure, on the other hand, elicited a dose-dependent inhibition of insulin secretion, while Zn

exposure over the same period of time increased insulin release at a specific concentration. Co-exposure studies for the metals showed iAs^{III} as the primary driver of insulin secretion inhibition with the tested concentrations of Cd and Zn; however, at higher concentrations, 24-hour Mn and iAs^{III} co-exposure showed an overpowering inhibitory effect of Mn over iAs^{III} . Additionally, 24-hour iAs^{III} exposure, alone and in conjunction with the heavy metals, exhibited significant inhibition of maximal respiration OCR. Cd exposure alone also elicited an inhibition in maximal respiration OCR at the highest tested concentration. Mn exposure alone also showed inhibitory effects on maximal respiration however, not enough to explain the massive decreases in insulin secretion observed during the GSIS trials. This demonstrates that Mn and iAs^{III} primarily inhibit insulin secretion via actions on separate portions of the insulin secretory pathway.

In addition to the recognized effects of iAs^{III} exposure, chronic Cd and Mn exposures also show relevance in pancreatic β -cell dysfunction. Unfortunately, most research on the topic of Mn and Cd effects on β -cells uses high concentrations of the metals that could result in cytotoxicity. Further research using lower concentrations of these metals and chronic exposure times would better resemble real world exposures. Future studies on the effects of iAs^{III} , Mn and Cd on insulin secretion and their respective mechanisms should be carried out using isolated islets or an in vivo model. The use of either would allow a better representation of how interactions between compounds may be affecting β -cells and peripheral tissues.

Diabetes is a health concern worldwide and should be treated with the utmost importance. With increasing evidence of associations between environmental compound exposure and diabetes prevalence, especially in industrializing countries, it is crucial to further investigate this topic. Occurrence of these compounds is rarely ever alone, with complex mixtures many times found in surface soils (Diawara et al., 2006). This thesis sought to address the impact of heavy

metal co-exposures in the context of pancreatic β -cell function. Overall, it was determined that heavy metal co-exposures can increase the diabetogenic potential of iAs^{III} exposure, which could aid in understanding DM occurrence in human populations worldwide. Interactions between compounds exist and should be further studied to gain a better understanding of how they can affect the body.

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