The Effects of Arsenic and Selenium on Pancreatic β-cell Function

BY

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THESIS

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I dedicate this work to the One who walked with me every step of the way.

CONTRIBUTION OF AUTHORS

This thesis represents unpublished work conceptualized, designed and performed by the author, Christie J. Kang in collaboration with individuals Christopher Carmean designed and performed the Seahorse experiments, Margaret Shulz prepared samples and performed the qPCR experiments.

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LIST OF ABBREVIATIONS

AS	Arsenic
As_2O_3	Arsenic trioxide
β-cell	Pancreatic beta-cell
CDNA	Complementary DNA
DNA	Deoxyribonucleic acid
EF	Elongation factor
EPA	Environmental protection agency
ER	Endoplasmic reticulum
FRET	Fluorescence resonance energy transfer
GLP1	Glucagon-like-peptide 1
GLUT	Glucose transporter
GPx1	Glutathione peroxidase 1
GSIS	Glucose-stimulated insulin secretion
iAs	Inorganic arsenic
IP-GTT	Intraperitoneal glucose tolerance test
iSe	Inorganic selenium
KRB	Krebs-ringer buffer
MCL	Maximum contaminant level
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species

SBP2	SECIS-binding protein 2
Se	Selenium
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence element
T2DM	Type 2 diabetes mellitus

SUMMARY

This study aimed to investigate how selenium and selenoprotein status affect arsenic-induced β-cell dysfunction and potentiate the development of Type 2 diabetes mellitus (T2DM). T2DM is a complex metabolic disorder characterized by high blood glucose levels due to inadequate insulin secretion from pancreatic β-cell dysfunction. Conventionally accepted risk factors, such as genetics and lifestyle choices, do not account for the rapidly growing rate at which T2DM affects individuals on a global scale. Environmental exposures and their association with disease is a growing area that is elucidating associations such as arsenic and diabetes. This is a generally positive association, but the varying epidemiological data begs to question what additional factors cause such variation. Selenium is another element found in the environment just as readily at arsenic, but unlike arsenic it is essential to create a set of unique proteins for biological functions. The profile of this set of proteins, also known as selenoproteins, may affect arsenic-induced diabetes, particularly regarding β-cell dysfunction.

The following experiments explored selenoproteins' role in arsenic-induced β-cell dysfunction *in vitro*. Our studies exhibited upregulation of selenoproteins in β-cells with arsenic treatment, however upregulating selenoproteins with selenium did not cause β-cell dysfunction. Furthermore, co-treatment of arsenic and selenium showed varying results. Our results suggest that the relationship between arsenic, selenium and selenoproteins in the context of β-cells is highly nuanced and complex, requiring further research to better characterize these interactions.

1. **INTRODUCTION**

1.1 **Background**

Diabetes mellitus is a chronic metabolic disease that globally affects an estimated 451 million adults and almost half of people living with diabetes are undiagnosed (Cho et al., 2018). This statistic is inclusive of all forms of diabetes, however, type 2 diabetes mellitus (T2DM) is significantly more prevalent, accounting for 90-95% of diabetes cases (CDC, 2017). T2DM is known as a complex multifactorial disease largely characterized by chronic hyperglycemia, insulin resistance and β-cell dysfunction (Unnikrishnan, Pradeepa, Joshi, & Mohan, 2017). In consequence to the elevated circulating glucose, there is vascular dysfunction and small blood vessel damage that can lead to increased risk of cardiovascular disease, blindness, kidney failure and limb amputation (Fonseca, 2009). The most commonly-known indicators of T2DM risk include genetics in conjunction with certain dietary and lifestyle factors usually resulting in obesity and impaired cardiovascular health. However, these known contributing factors do not account for the alarming increase in T2DM prevalence. Therefore, it is important to investigate other forms of risk that predispose individuals or contribute to the progression of T2DM.

Epidemiological studies have indicated an association between arsenic exposure and diabetes. Arsenic is naturally found in the ground and water and is a carcinogen known to cause disease through increased reactive oxygen species (ROS) production and DNA damage. The most common form of arsenic exposure is through drinking water, and in populations that have had arsenic exposure through drinking water have seen a positive correlation with diabetes (WHO, 2011). Further studies have shown that arsenic lowers insulin secretion in β-cells as well as induces β-cell apoptosis. However, the mechanisms responsible for arsenic-induced β-cell dysfunction are still unknown.

Another environmental exposure with a longstanding known interaction with arsenic is selenium (H. J. Sun et al., 2014). Selenium is a naturally occurring metalloid in the earth's crust, but unlike arsenic, selenium is an essential element for metabolic regulation and thyroid function (Campbell et al., 2008; Jablonska et al., 2016). It is taken up from the environment as sodium selenite and incorporated into a family of selenoproteins exhibiting a variety of functions including antioxidant activity, protein folding, and selenium transport. The process of producing selenoproteins involves the conversion of a serine amino acid to selenocysteine by replacing the hydroxyl group with a phosphate, then selenium, from hydrogen selenide. This amino acid has a specific tRNA (Sec-tRNA) that recognizes UGA, conventionally a stop codon. To bypass translational termination, the mRNA coding for the selenoprotein has a stem loop structure called a selenocysteine insertion sequence element (SECIS) that recruits SECIS-binding protein 2 (SBP2) which recruits an elongation factor (EF) that binds to Sec-tRNA and incorporates the selenocysteine at the stop codon. The loss of SBP2 results in lower levels of all selenoproteins and is thus a reasonable target to investigate the effects of selenoprotein loss on diabetes progression (Cox et al., 2013; Hellwege et al., 2014; Yu et al., 2016).

1.2 **Hypothesis**

To explore the paradigm of how selenoproteins modulate arsenic-induced β-cell dysfunction**, the central hypothesis of this application is that optimal selenium and selenoprotein profile hamper arsenic-induced β-cell dysfunction and hinder the development of diabetes**. This work will test the hypothesis that selenoproteins are direct responders of arsenic-induced injuries, such as ROS and ER stress, and negate the negative effects of arsenic upon upregulation.

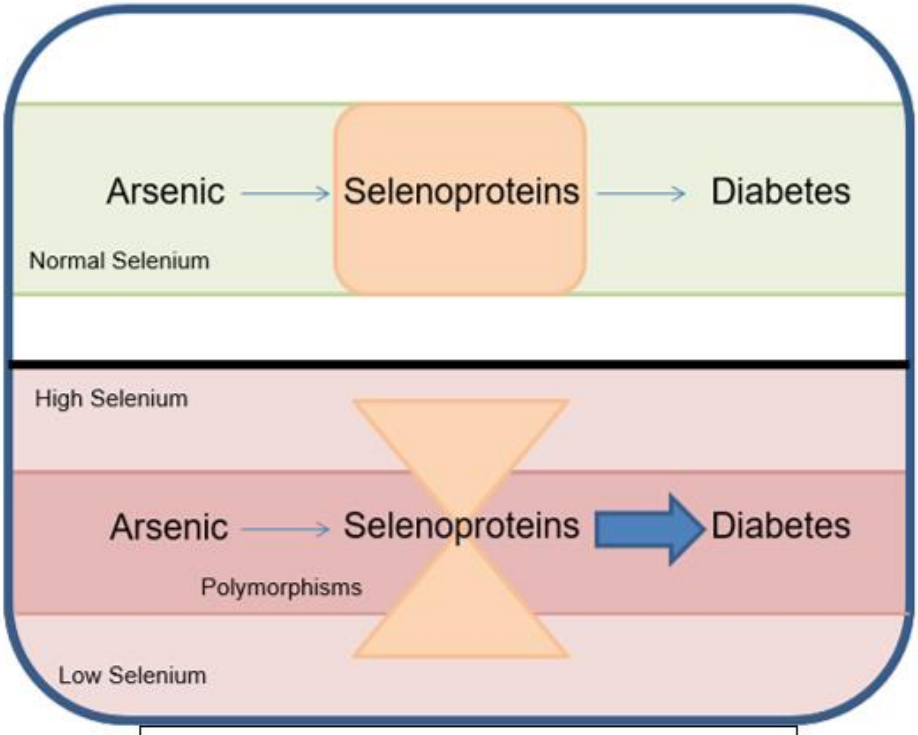

Fig. 1: Proposed model of selenoprotein-modulated arsenic-induced diabetes

Figure 1 provides the conceptual scheme of normal and aberrant selenoprotein expression and levels, induced by a range of selenium availability or polymorphisms of specific selenoproteins or translation factors, that modulate the association of arsenic-induced diabetes. Selenoprotein levels are affected by selenium availability, therefore an acceptable level of selenium must be present to express “normal” levels of selenoproteins. The top portion of the

figure represents a range of selenium that produces selenoproteins at a normal level, and the presence of these selenoproteins mitigate arsenic's drive towards diabetes. However, the lower portion of the figure represents selenium levels that are either too high or too low or polymorphisms present in selenoprotein translational factors, such as SBP2, that cause irregular levels of selenoproteins that result in arsenic more efficiently driving β-cell dysfunction and diabetes.

1.3 **Significance of Study**

Though the impact of diabetes on human health has long been understood, the mechanisms leading to disease are not fully known. This project will help further understand the contribution of a common environmental toxicant in the etiology of diabetes, as well as factors that modulate these effects. Understanding this will help develop more innovative therapies. Clinically, this research could pinpoint genetic predispositions to diabetes (such as polymorphisms of selenoproteins or proteins involved in selenoprotein transcription/translation) and raise consciousness to particular diets to prevent and lower blood glucose (such as eating the right amount of selenium). Additionally, this research can affect awareness for the individual of what is safe living environments as well as make policy change to prevent further water pollution from human activities like fracking and have more active water quality surveillance.

2. REVIEW OF LITERATURE

2.1 **Diabetes and Environmental Risk Factors**

Type 2 diabetes mellitus is a widespread cause of morbidity and mortality, characterized by insulin resistance and hyperglycemia (Fonseca, 2009). The underlying hallmark of diabetes mellitus lies in the progressive loss of pancreatic β-cell function and mass. In the progression of T2DM, β-cells initially produce compensatory amounts of insulin in response to persistently elevated peripheral insulin resistance. Sustaining this level of production without mitigating insulin resistance results in the deterioration of the β-cells' secretory capacity and ultimately to hyperglycemia (Kahn, 2001; Y. Wu, Ding, Tanaka, & Zhang, 2014). This process is augmented by chronic exposure to free fatty acids and high glucose that lead to β-cell apoptosis (Rojas et al., 2018). However, the increasing prevalence of diabetes is not accounted for solely by the presence of these traditional risk factors.

Though pancreatic β-cell death is a hallmark of diabetes mellitus, the mechanisms of which differ between type 1 and type 2 diabetes (Cnop et al., 2005). While type 1 is largely an autoimmune response, modes of type 2 diabetes β-cell dysfunction and death include glucotoxicity, lipotoxicity, apoptosis, autophagy and pyroptosis (Rojas et al., 2018). Arsenic causes injury by elevating reactive oxygen species which causes DNA damage, endoplasmic reticulum (ER) stress, protein misfolding and apoptosis (Lu et al., 2011; Zhu et al., 2014). However, arsenic-associated T2DM occurs after chronic exposure, suggesting that there are mechanisms that counter these injurious actions, which are not clearly identified.

2.2 **Growing Arsenic Exposure**

Arsenic is a known carcinogen that has been widely studied for its association with disease. This element is a naturally occurring metalloid in the earth's crust that predominantly exists in inorganic forms [As(III) and As(V)] in the environment, but it is biologically processed in animals by reduction and methylation. Modes of exposure include pesticide use, foods grown in arsenic-contaminated water, most notably, rice grown in high-arsenic regions of southeast Asia, seafood raised in high-arsenic waters and human activities such as mining and fracking which increase arsenic seepage into water sources, particularly well water. It is now estimated that 140 million individuals worldwide are exposed to arsenic-contaminated water with still more water sources, particularly well water, being discovered to be contaminated with arsenic (WHO, 2018). The association between environmental arsenic and cancer and other diseases has prompted the Environmental Protection Agency (EPA) to lower maximum contaminant levels (MCL) in the water. The evidence indicating association between arsenic and diabetes has grown significantly in population studies as well as animal and *in vitro* studies showing diabetic phenotype with arsenic exposure (Boquist, Boquist, & Ericsson, 1988; Kirkley et al., 2018; Liu et al., 2014).

2.3 **Arsenic and Diabetes**

The most common source of arsenic exposure to humans is through drinking water, seeped into well water or other unregulated drinking water sources, found near fracking/mining/electricity plants. Since the mid-1990s, nearly 20 countries have studied the association of arsenic exposure from drinking water with the prevalence of diabetes (Coronado-Gonzalez, Del Razo, Garcia-Vargas, Sanmiguel-Salazar, & Escobedo-de la Pena, 2007; J. W.

Huang, Cheng, Sung, Guo, & Sthiannopkao, 2014; Idrees & Batool, 2018; Jovanovic et al., 2013; Kim et al., 2013; Lai et al., 1994; Lee & Kim, 2013; Rahman, Tondel, Ahmad, & Axelson, 1998; Sripaoraya, Siriwong, Pavittranon, & Chapman, 2017) and the data generally indicate a positive association. The history of arsenic's effects on human health dates back before its association with diabetes. From the late 1700s, arsenic was first tested as a cure for a variety of diseases and symptoms (Fowler, 1786; Willan, 1787) however in the early 1800s, it was soon discovered to be a poison and a carcinogen – a fact that still holds true today, but ironically arsenic, as arsenic trioxide, is also used as a chemotherapeutic for acute myeloid leukemia. The use of arsenic as a treatment is not too far from arsenic's original association with diabetes. In 1873, La France Medicale published that several doctors treated their diabetic patients with arsenic resulting in diminished sugar in the urine ("Treatment of Diabetes by Arsenic," 1873). They explained the success by describing the underlying physiology of diabetes being a "permanent relaxation of the capillaries" and that arsenic was "suitable, from the good effects of that drug in intermittent fever…" ("Treatment of Diabetes by Arsenic," 1873). Several other doctors who did not have success with the treatment method were described of having patients with different types of diabetes. However, arsenic's reputation as a treatment for diabetes would decline as it did for most diseases and was quickly identified as a harmful substance that promiscuously injured biological functions once exposed. The study of how arsenic affected biological functions picked up in the late-1900s with the predominant use of rats, mice, and broiler chickens, with the occasional study in rhesus monkeys, crayfish, cow, pig, and geese. Arsenic metabolism was studied using these models (Marafante, Vahter, & Dencker, 1984) which led to increasing findings of impaired insulin secretion and signaling in rodent pancreata (Diaz-Villasenor, Sanchez-Soto, Cebrian, Ostrosky-Wegman, & Hiriart, 2006; Paul et al., 2008).

Further study into the molecular events of arsenic exposure and altered insulin secretion in established cell lines from mouse (MIN6), rat (RIN-5F, INS-1), hamster (HIT-T15) observed physiology *in vivo* (Lu et al., 2011; Q. Sun et al., 2018; W. Wu et al., 2018; Yen et al., 2007). Of the many pathways arsenic could be altering, the growing consensus is that arsenic's main modes of injury are through oxidative stress and endoplasmic reticulum (ER) stress, which induce apoptosis and possibly autophagy. These areas where arsenic cause injury are crucial for proper insulin secretion in the β-cell since insulin undergoes a series of protein cleaves before becoming mature insulin packaged in vesicles ready to be released triggered by the metabolism of glucose. Whether or not arsenic directly affects proteins involved in metabolism is still being understood, but it is suggested that though the arsenate form of arsenic might directly affect GLUT transporters, the damage largely comes from the oxidative stress. In the event of injury, the cell is equipped with protein that can counteract such insults and a unique group of them, called selenoproteins, happen to have specific proteins that counter the oxidative and ER stresses caused by arsenic.

2.4 **Selenium's Role in Health**

Selenium is an important element for maintaining health. Selenium deficiency can lead to diseases, such as Keshan disease, while excess selenium can lead to toxicity. Selenium in its free form is able to bind to other elements to form a complex and be excreted, which is protective of any toxic elements such as arsenic. Selenium is also an essential element for the proper production of proteins such as selenoproteins.

Selenoproteins are proteins in which selenocysteine is incorporated into their amino acid sequence where it plays a critical role in protein function. One of the first identifications of

selenoproteins in mice used radioactive selenium to locate the site of selenium incorporation in mammary epithelial cells. (Danielson & Medina, 1986) This effort led to the identification of 25 selenoproteins with molecular weights ranging from 12-78 kDa. Subsequent studies elucidated the biosynthetic process required for selenoprotein synthesis, including the fundamental requirement for adequate selenium levels (Arthur, Nicol, Grant, & Beckett, 1991).

The selenocysteine (Sec) amino acid is encoded by UGA, traditionally a stop codon. To incorporate this unique amino acid, specialized machinery is required to overcome the stop codon. A special tRNA, Sec-tRNA, sits on the UGA codon to receive the selenocysteine. (Jung et al., 1994) In conjunction with the Sec-tRNA, a secondary structure in the 3" untranslated region of the mRNA, known as Sec insertion sequence (SECIS), recruits a transcription factor, SECIS binding protein 2 (SBP2), which then recruits an elongation factor (EF) that binds to the selenocysteine and recognizes the Sec-tRNA to incorporate the selenoamino acid into the polypeptide chain (Copeland, Fletcher, Carlson, Hatfield, & Driscoll, 2000; Donovan, Caban, Ranaweera, Gonzalez-Flores, & Copeland, 2008; Low, Grundner-Culemann, Harney, & Berry, 2000).

The selenoprotein family is comprised of 25 different proteins in humans, all containing a selenocysteine but with varying functions including reactive oxygen scavenging, metabolism, protein folding, and element transport. They localize to different parts of the cell, but there is evidence that some are mobile. Their full characteristics and functions are still being understood, but an alteration of one or a cluster of selenoproteins could result in the dysregulation of normal processes such as insulin secretion and glucose homeostasis.

Studies investigating that effects of specific selenoproteins in diabetes has largely focused around selenoprotein P (SelP), the selenium transport protein. The general consensus is

that there is an observed upregulation of SelP in T2DM but not gestational diabetes (Altinova et al., 2015; Misu et al., 2012; Yang et al., 2011), which has led to the idea of SelP being a reasonable target of the disease. However, GPx1, an antioxidant selenoprotein, is elevated in times of stress, logically to counter the present stress. It is possible that selenoprotein P could be acting in the same way.

Specifically, high levels of SelP and SelS, known for ER stress response and inflammation, are associated with insulin resistance (Hellwege et al., 2014; Ishikura et al., 2014; Yu et al., 2016; Zeng et al., 2012). However, modest selenium supplementation does not seem to increase the risk for T2DM in pregnant women and those with low selenium levels. (Mao et al., 2016; Rayman et al., 2012) Overall, the studies indicate a "Goldilocks" effect, in that, selenium and selenoprotein levels have an optimal range for maintaining metabolic homeostasis. If either selenium intake or selenoprotein levels increase, which usually is one resulting from the other or the presence of a polymorphism, there is risk of insulin resistance and development of T2DM, but an optimal level of selenium must be maintained for normal health.

2.5 **Selenium, Selenoproteins and Arsenic-Induced Diabetes**

In the context of diabetes, selenium deficiency, genetic alterations that reduce the levels or selenoproteins and their activity and/or arsenic-induced loss of selenoproteins, could predispose to arsenic-induced β-cell dysfunction (Ganyc et al., 2007; Z. Huang, Li, Zhang, & Zhang, 2009; Meno, Nelson, Hintze, & Self, 2009; Talbot, Nelson, & Self, 2008).

Previous studies in the lab have shown that arsenic exposure to mice increase glucose intolerance *in vivo* and decreases β-cell function without altering system insulin sensitivity (Kirkley et al., 2018). This was shown using C57BL/6 mice that were given sodium arsenite

(NaAsO2), a species of inorganic As^{3+} [As(III)], at 50 mg/L in their drinking water for 8 weeks. Intraperitoneal glucose tolerance tests (IP-GTTs) showed arsenic reducing insulin secretion after 8-week exposure (Kirkley et al., 2018). To understand the impact of selenoproteins on the effects of arsenic exposure on glucose homeostasis, transgenic mice generated with global haploinsufficiency for selenocysteine insertion sequence binding protein 2 (SBP2) on the C57BL/6 background were given sodium arsenite (NaAsO2), a species of inorganic As^{3+} [As(III)], at 50 mg/L in their drinking water for 4 and 8 weeks. IP-GTT measurements showed that lower levels of SBP2 with arsenic exposure worsened glucose tolerance and was further worsened with longer exposure. Glucose stimulated insulin secretion (GSIS) revealed that arsenic treated $SBP2^{+/-}$ mice had decreased first-phase insulin, indicating more advanced β-cell dysfunction. Given the physiological results *in vivo*, we looked specifically in the β-cell to further elucidate the mechanisms underlying the complex relationship between selenoproteins and arsenic-induced β-cell dysfunction.

We have a growing understanding of selenium and arsenic in the context of diabetes, but what we know about arsenic's relationship with selenoproteins is very limited to *in vitro* models including mouse embryonic stem cells, human liver, human lung, human skin fibroblast. What we know about arsenic and selenoproteins in β-cells is almost non-existent. However, previous *in vivo* studies showed that selenoprotein levels affect arsenic-induced diabetes. This supports further study into this novel axis of arsenic and selenium/selenoproteins in β-cells and how each play a crucial role in the pathogenesis of diabetes.

3. MATERIALS AND METHODS

Materials

Reagents and chemicals

Sodium arsenite and sodium selenite were purchased from MilliporeSigma (Darmstadt, MA)]. Dextrose, trypsin, GSIS reagents (NaCl, KCl, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $NaHCO_3$, $CaCl_2 \cdot 2H_2O$, NaOH, HEPES, Triton-X100) from Fisher (Waltham, DE) and MilliporeSigma, high-glucose DMEM [MilliporeSigma] (Darmstadt, DE), sodium pyruvate [Invitrogen) (Carlsbad, CA), 1.25µL 2-mercaptoethanol [Fisher] (Waltham, DE).

Primers

GPx1, SelP, SelF [IDT] (San Jose, CA).

Cell line

MIN6-K8 was a generous gift provided by Dr. Seino from Osaka University at P19.

Methods

Cell culture

MIN6-K8 were cultured in high-glucose DMEM [MilliporeSigma] (Darmstadt, DE) supplemented with 10% FBS, 1% sodium pyruvate [Invitrogen) (Carlsbad, CA), and 1.25 µL 2-mercaptoethanol [Fisher] (Waltham, DE). Cells were maintained at 37˚C in 5% CO_2 incubator. Media was changed every 48 hours.

Glucose-stimulated insulin secretion assay

MIN6-K8 cells were plated on clear treated 24-well plates at 200,000 cells/well. Cells were then treated with arsenic, selenium or both for a specified time. Arsenic treatment was fresh every 24 hours to account for oxidation. Selenium treatment was fresh every 48 hours.

Cell media was discarded and replaced with KRB supplemented with 2.8 mM glucose. Cells were incubated in agitating 37˚C water bath for 1 hour. KRB was discarded and replaced with KRB with different glucose concentrations. Cells were incubated in an agitating water bath at 37˚C for 1 hour. KRB was manually swirled, then 100 µL of KRB was collected for insulin quantification. KRB was discarded out, and 300 µL of 1x triton added to each well for cell lysis and total insulin content quantification. Cells with triton were frozen in a -80˚C freezer for further freeze fracture. Cells lysed with triton were collected for total insulin content quantification.

Fluorescence resonance energy transfer

High-range insulin assay from CisBio (Darmstadt, DE) and Synergy H1 microplate reader [BioTek] (Winooski, VT) were used to quantify insulin measured via FRET analysis. Samples were loaded on high-profile 384-well white plate with adhesive plate covers to avoid overnight evaporation. Plates were analyzed after 24 hours of samples incubating with CisBio antibody mixture.

Seahorse metabolic assay

Cells were plated on 24-well seahorse plates and treated with arsenic for 48 hours before performing the mito-stress test from Agilent (Santa Clara, CA). An hour before analysis, cells were incubated in a non-CO_2 37˚C incubator, then the seahorse plate was transferred to the Seahorse machine where the cells were challenged with metabolic enzyme inhibitors oligomycin, FCCP, and rotenone/antimycin A (reagents provided by Agilent). Post stress test, media was discarded and cells were lysed using RIPA. Protein was measured to normalize individual well readings.

Quantitative polymerase chain reaction

Cells were treated with arsenic and selenium for up to 72 hours then collected using Omega E.Z.N.A. Total RNA Kit II (Norcross, GA). RNA was isolated and used to make cDNA using Quantabio qScript cDNA Synthesis Kit (Beverly, MA) and PCR machine. Resulting cDNA was used for qPCR on BioRad plates and qPCR reagents, and primers from IDT.

Immunoblotting

Cells were treated then collected with RIPA supplemented with protease inhibitors and phosphatase inhibitors. Cells in RIPA were scraped and moved to microcentrifuge tubes. Tubes were spun down to separate debris from supernatant and supernatant were moved to fresh tubes. From the supernatant, 5 µL was used for BCA to measure protein concentration. Samples were then prepared with RIPA and LDS in separate tubes, boiled at 95˚C for 5 minutes on heat block, then placed in a gel and separated by electrophoresis. After separating the protein by gel electrophoresis at 180V for about an hour, protein was transferred onto PVDF membranes using BioRad transfer kit at 110V for 80 minutes. Blots were incubated and agitated with 10% BSA [MilliporeSigma] and 1x TBST for one hour in room temperature. Blots were then incubated and agitated with 1˚ and 2˚ antibodies in 10% BSA and 1x TBST and analyzed using chemilluminesence.

Analysis

Two-way ANOVA analysis was used on GSIS and qPCR results.

4. RESULTS

4.1 **Arsenic induces β-cell dysfunction**

To strengthen the association between arsenic and diabetes, previous studies have shown that arsenic directly induces β-cell dysfunction. In order to reinforce these findings as well as validate our *in vitro* model as an appropriate cell line for our investigation, MIN6-K8 cells were treated with 0.1 µM, 0.5 µM, and 1.0 µM sodium arsenite in growth media for 48 hours. Insulin production and release were measured using GSIS and FRET analysis to determine any changes in β-cell function (Figure 2). The lowest concentration of inorganic arsenic (iAs) did not affect insulin release, however the two higher concentrations resulted in statistically significant decreases in insulin levels. These results agree with data produced by other groups using MIN6 cells as well as indicate that our model is appropriate for our investigation.

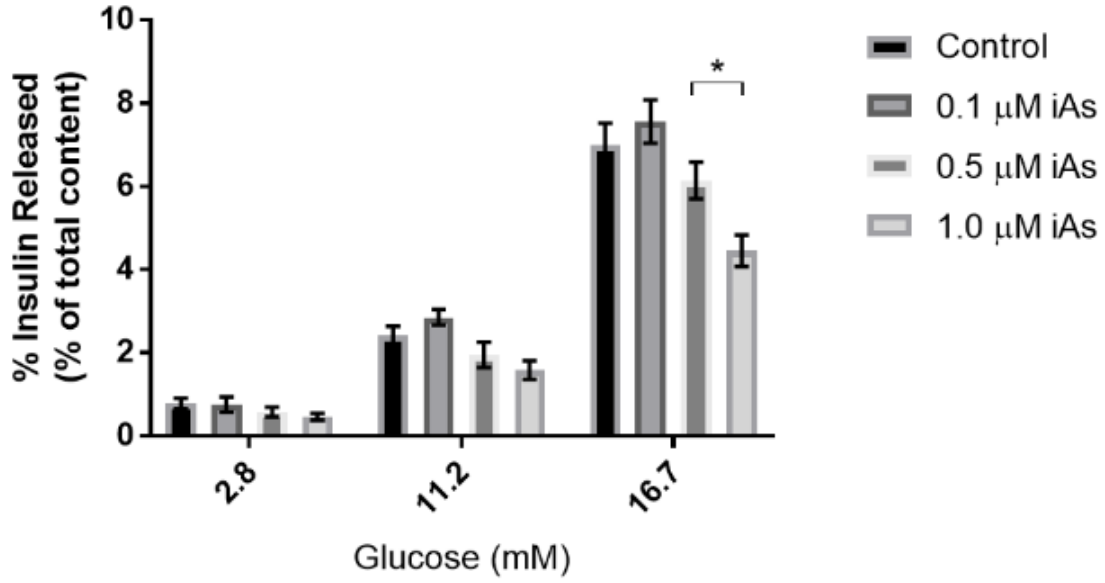

Figure 2. Arsenic induces β-cell dysfunction. Increasing arsenic concentrations lowered insulin production in MIN6-K8 cells after 48 hours of exposure. ANOVA results were significant ($p<0.05$*, n=6)

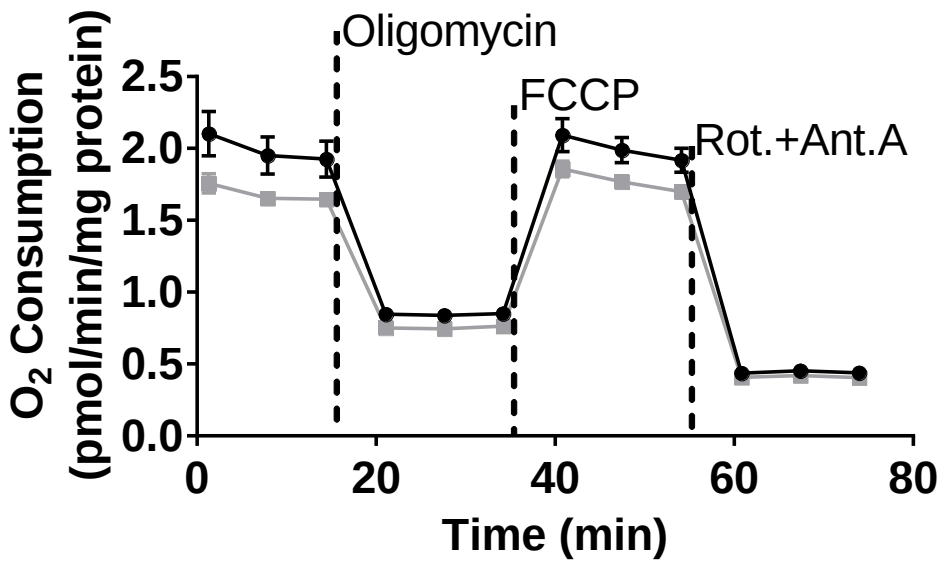

Figure 3. Arsenic did not alter aerobic metabolism. MIN6-K8 did not exhibit significantly altered oxidative metabolism with arsenic treatment. (n=3)

We then investigated whether or not arsenic was targeting the mitochondria of the β-cell to cause dysfunction. We tested oxidative metabolism using the oxidative stress test, using chemicals oligomycin, FCCP, and rotenone/antimycin A, that challenge different components of the electron transport chain to determine any alterations. Resulting effects were captured by the Seahorse analyzer (Figure 3).

This test did not show statistically significant alterations in mitochondrial metabolism with arsenic treatment in MIN6-K8 cells. The glycolytic stress test was also attempted on these cells to observe any changes in the anaerobic metabolism enzymes, but the cells apoptosed during the glucose starvation period that was necessary for the assay. These results showed that arsenic does not immediately cause injury to the mitochondria, but other events are occurring to cause lower insulin production and secretion. In the event of injury, the cell responds by upregulating components to counter the event, whether it is increased reactive oxygen species or protein misfolding.

4.2 **Arsenic induces upregulation of selenoproteins**

To investigate other modes of cell injury, select selenoprotein transcripts were measured using qPCR to observe which modes of injury arsenic was causing in the β-cell. Antioxidant GPx1 as well as SelP and SelF were selected to be measured. MIN6-K8 cells were treated with 0.1 μM, 0.5 μM, and 1.0 μM sodium arsenic. In the case of increased reactive oxygen species, GPx1 expression was expected to be elevated. In the case of ER stress, SelF was expected to be elevated. In the case of observed elevated SelP, that would indicate that β-cell has selenium transporters and that arsenic is causing the cell to increase selenium uptake (Hill et al., 2012), whether for the production of selenoproteins or to be utilized in a different pathway (Figure 4). The resulting data showed increased levels of GPx1 and SelF with all three concentrations of arsenic. SelP also elevated with the higher two concentrations of arsenic. These results indicate that arsenic may be causing both oxidative stress as well as ER stress. The increased SelP also indicates that β-cells have the ability to transport selenium into the cell for utilization. Immunoblotting was performed to measure protein levels of these selenoproteins, but there were negligible differences with arsenic treatment.

4.3 **Selenoproteins do not alter β-cell function**

Given the observation that GPx1, SelP, and SelF gene expression elevated with arsenic treatment, it is possible to hypothesize that the upregulation of these selenoproteins plays a part in β-cell dysfunction. To test this, we first mimicked the upregulation of general selenoproteins by treating the MIN6-K8 cells with 10 nM, 50 nM and 100 nM sodium selenite for 48 hours. Increased inorganic selenium (iSe) availability is expected to increase selenoprotein gene expression.

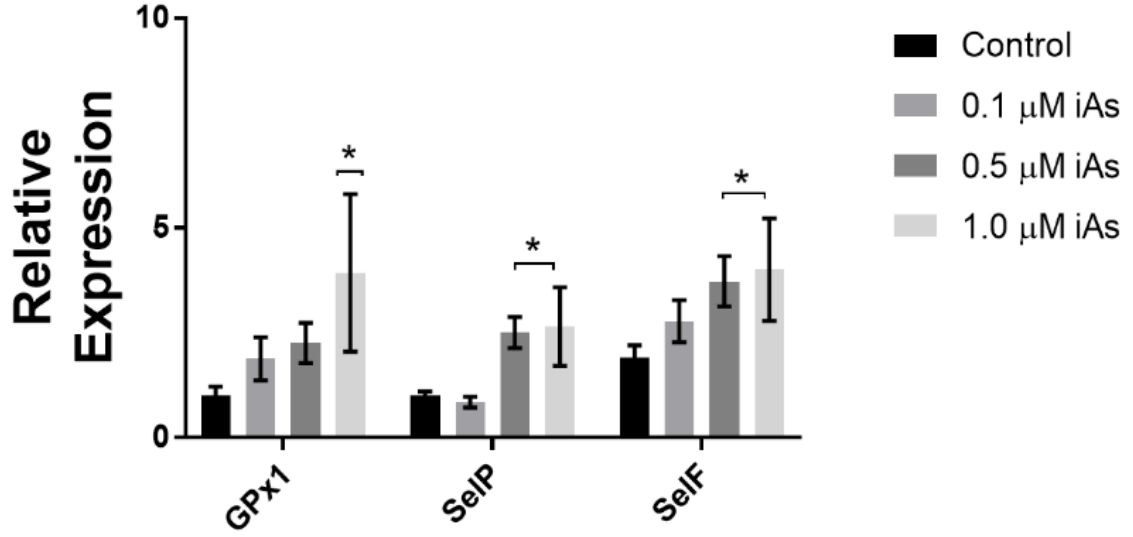

Figure 4. Arsenic treatment upregulates GPx1, SelP, and SelF transcription. MIN6-K8 cells increasingly transcribed more selenoproteins with increasing concentration of sodium arsenite. ANOVA results were significant ($p<0.05$*, n=3)

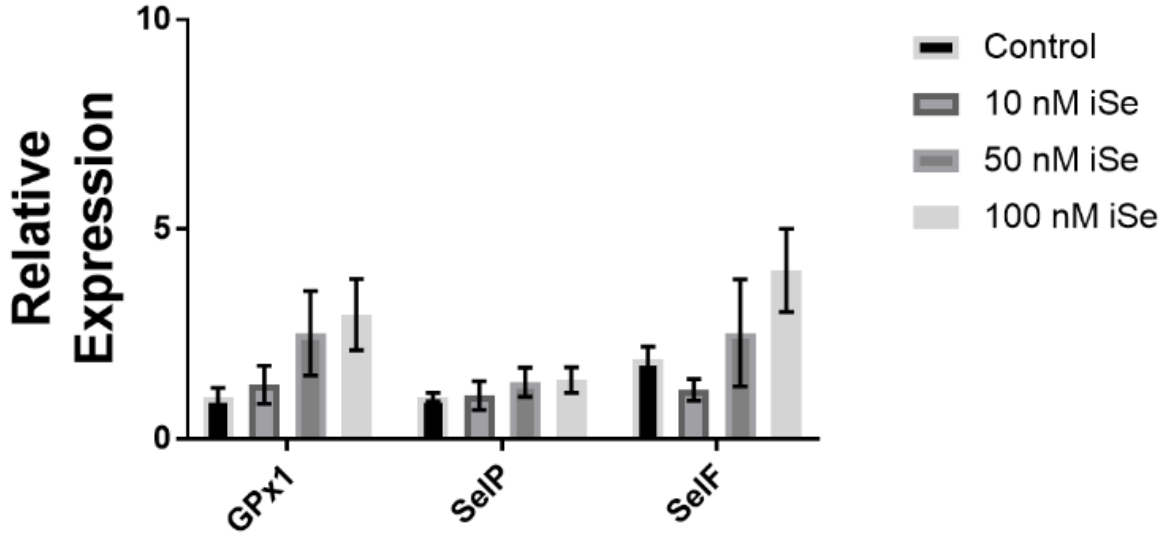

Figure 5. Selenium increases transcription of GPx1 and SelF. Increasing trends are not significant (ANOVA, $p\geq0.05$ n=3)

qPCR was used to establish that selenium exposure was increasing GPx1, SelP and SelF gene expression (Figure 5). The results did not show statistical significance in the expression change of the selenoproteins, however, there is an increasing trend of expression with increasing concentration of sodium selenite. To test if the upregulation of selenoproteins causes β-cell dysfunction, GSIS was measured on MIN6-K8 cells treated with 10 nM, 50 nM and 100 nM sodium selenite (Figure 6). The cells were treated with selenium for at least 72 hours before running the GSIS assay. Insulin was collected and measured to show that the increased expression of selenoproteins does not alter insulin production and release.

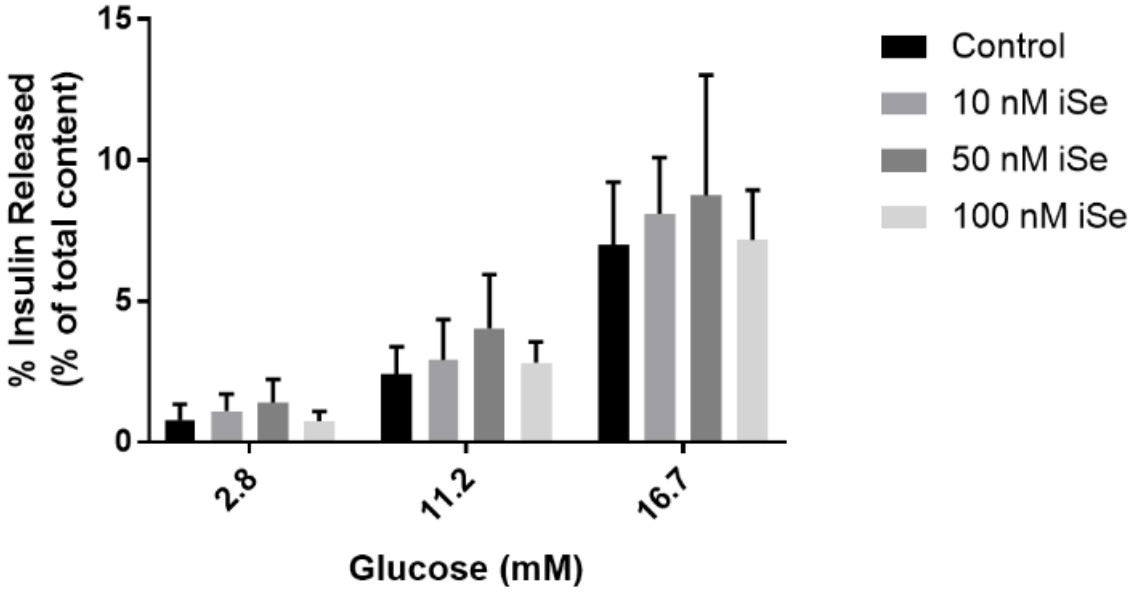

Figure 6. Selenium does not affect insulin secretion MIN6-K8 cells did not have altered β-cell function after 72hour selenium treatment (ANOVA, $p \geq 0.05$, n=6)

To test if selenium affects arsenic-induced β-cell dysfunction, MIN6-K8 cells were co-treated with sodium selenite and sodium arsenite, and insulin release was measured. The cells were pretreated with sodium selenite for 72 hours to allow time for the translation of selenoproteins, then co-treatment for 48hours. GSIS was performed, insulin measured, and results varied despite repeated experiments. There was indication that increasing selenium concentrations increasingly exacerbated arsenic's effects on β-cell function, but selenium also was suspected to negate arsenic's effects on β-cell function. Selenoprotein expression of GPx1, SelF and SelP were also variable with co-treatment. No conclusions can be made with the performed experiments without further trouble-shooting.

5. DISCUSSION

As seen in previous reports of arsenic inducing β-cell dysfunction, we observed in our study that arsenic induces β-cell dysfunction in MIN6-K8 cells, arsenic induces upregulation of selenoprotein gene expression not looked at and that the selenoproteins do not alter β-cell function. Co-treatment of selenium and arsenic showed varying results, which speaks to the complexity of how these elements are routed in the system. Selenium is known to bind to arsenic as a detoxifier, however, too much selenium is also known to exacerbate arsenic's injurious effects. Both outcomes were observed in the co-treatment experiments and need further investigation to comprehend the interactions.

The results of this study are important to note that though selenoprotein status may affect arsenic-induced β-cell dysfunction, free-floating inorganic selenium may have a preventative role on the effects of inorganic arsenic in the system. Selenoproteins' roles are more responsive to the damages already done by arsenic which could indicate that their presence is more impactful in hindering the continual development of diabetes. Both selenium and selenoproteins are important for countering arsenic on both preventative and responsive ends of β-cell damage, but the optimal dose of selenium for preventative measures and protective levels of certain selenoproteins need to be determined with further study.

Though MIN6 is a validated model for β-cell research, it presents several limitations, including the fact that 2-dimensional models cause cells to behave and communicate differently from their 3-dimensional origin. Specifically, for MIN6, the *in vitro* model excludes any endogenous cross talk between other cells in the islet and neighboring organs of the pancreas such as the liver. This type of cross talk might be crucial for how it reacts to arsenic and other toxicants *in vivo*. For these reasons, suggested future directions include using increasingly sophisticated

models to more-closely mimic β-cell physiology of humans. This would include using pseudo-islets that have a more 3-dimensional structure, using isolated mouse and human islets, and using *in vivo* models. After understanding the effects of iAs and iSe on β-cell function, further studies could look at the different forms of arsenic and selenium that are both organic and organic to see if there are major differences in the effects of the different forms. On a molecular level, future studies could explore how the effects of arsenic and selenium affect endocrine cell communication within the islet (between α, β, γ, δ-cells) as well as the communication with exocrine cells, and which pathways are most affected. Beyond arsenic, mercury, lead, and cadmium are also known to cause poisoning and disease so other toxins could be studied separately and together to see if multiple exposures have different effects on β-cell function.

6. CONCLUSION

Arsenic induces β-cell dysfunction in MIN6-K8 cells and as a response to the injurious effects of arsenic, GpPx1, SelP and SelF gene expression is upregulated. The upregulation of these selenoproteins do not contribute to the loss of β-cell function. Though selenium is known to detoxify arsenic, co-treatment of selenium and arsenic showed varying results which indicate a complex interaction in both intracellular and extracellular contexts. Varying results under the same condition could also suggest a methodological issue that needs to be troubleshooted. Future studies using 3-dimensional models will help verify these results and elucidate this complex relationship.

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