Arsenic and Zinc Deficiency Induced Changes in Pancreatic Beta Cells

by
Annie Lihui Cao

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented April 19, 2019
Commencement June 2019

AN ABSTRACT OF THE THESIS OF

Annie Lihui Cao for the degree of Honors Baccalaureate of Science in Microbiology presented on April 19, 2019. Title: Arsenic and Zinc Deficiency Induced Changes in Pancreatic Beta Cells.

Abstract approved: _____

Emily Ho

Pancreatic beta cells produce and release insulin, a vital hormone that regulates blood glucose levels, and their dysfunction contributes to the development of *diabetes mellitus*. Dietary zinc deficiency and exposure to the toxicant inorganic arsenic have both independently been associated with diabetes, although the effects of their combination on pancreatic beta cell health and function remain unknown. We hypothesized zinc deficiency increases the toxicity associated with arsenic exposure, causing a greater susceptibility to DNA damage and disruption of insulin production. In a cell culture model, zinc deficiency increased *Ins1* gene expression and insulin production, but decreased cell proliferation. Arsenic exposure also decreased cell proliferation, and increased mRNA levels of genes involved in stress response and DNA damage. Zinc deficiency attenuated this response to arsenic and increased DNA double-strand breaks. Co-exposure did not decrease insulin levels beyond what was found with arsenic alone but did result in a further decline in cell proliferation, and increased beta cell apoptosis. These results suggest zinc deficiency and arsenic, both independently and in combination, adversely affect pancreatic beta cell health and both factors should be considered in the evaluation of health outcomes for susceptible populations.

Key Words: arsenic, insulin, pancreatic beta cell, zinc deficiency

Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; *Hmox1, heme oxygenase 1*; *Ins, insulin*; *Mt1, metallothionein 1*; *Mt2, metallothionein 2*; *Neurod1, neuronal differentiation 1*; *Ogg1, 8-hydroxyguanine DNA glycosylase*; *Pdx1, pancreatic and duodenal homeobox 1*; qPCR, quantitative real-time PCR; *p53, Tumor Protein 53*; T1D, type I diabetes; T2D, type II diabetes; ZA, zinc adequate; ZD, zinc deficient; *Znt8, zinc transporter 8*

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Annie Lihui Cao, Author

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Chapter 1 | Introduction of Thesis

Diabetes and Pancreatic Beta Cells

Diabetes mellitus, commonly known as diabetes, is a group of disorders characterized by high blood glucose (hyperglycemia) [1]. Diabetes has a worldwide prevalence of about 400 million people [2]. In the United States, diabetes affects approximately 29.1 million people, and about 86 million people are at risk for developing diabetes [3]. There are two types of diabetes: Type I diabetes (T1D) occurs when the body cannot produce sufficient levels of insulin, and Type II diabetes (T2D) occurs when the body becomes resistant to insulin [4]. Although T1D is commonly associated with children, more adults than children live with T1D, and it can occur with every age and race [5]. T2D accounts for 95 percent of all diabetes cases, and there are an estimated 350 million cases of T2D worldwide [3]. Since diabetes is a chronic disease, it can affect many organs over time leading to other negative health outcomes such as cardiovascular disease, vision loss, amputations, and end-stage kidney disease [3]. Diabetes is diagnosed by measuring high glucose levels in the blood, serum, or plasma of the patient, and risk factors that contribute to the onset of this disease can include lifestyle, diet, and/or genetics. Treatment for T1D requires insulin therapy, usually in the form of injections, while T2D can often be managed by healthy eating, exercise, and medications that increase insulin sensitivity [6].

Beta cells are found in the pancreas and comprise about 50-70 percent of cells in pancreatic islets in humans [7]. Pancreatic beta cells are important in the pathogenesis of both types of diabetes because beta cells produce insulin, the hormone responsible for decreasing blood glucose levels, and beta cell dysfunction and loss of beta cell mass are characteristics of both T1D and T2D [4]. In T1D, beta cell destruction occurs because of autoimmune destruction, and in T2D, tissues such as muscle and adipose cannot respond to insulin effectively to increase glucose uptake

(reviewed in [8]). In T2D, environmental and genetic stresses on the pancreatic beta cell can lead to increases in insulin output to offset the decreases in insulin action, but over time, this can begin to cause beta cell failure [9].

Zinc and Zinc Deficiency

Zinc is a micronutrient essential for development, cellular metabolism, enzyme function, protein and DNA synthesis, and cell division (reviewed in [10]). Foods that are high in zinc include oysters, beef, crab, pork, turkey, beans, chicken and yogurt [11]. Zinc is involved in immune system function as well as supporting physical and mental development and growth during pregnancy, childhood, and adolescence [12-14]. Because the human body does not contain zinc stores, all zinc required for the body must be obtained through the diet [15]. The average adult requires about 11 mg of zinc per day for men and 8 mg per day for women (11 mg per day if pregnant), but limited access to zinc-rich foods such as animal meats and shellfish can lead to inadequate intake of zinc, and eventually zinc deficiency [15, 16]. Severe zinc deficiency in human populations is uncommon because most diets contain some amount of zinc, but it can be caused by conditions that lead to zinc loss or impaired uptake, such as severe burns, prolonged diarrhea, and acrodermatitis enteropathica, a genetic disorder of an intestinal zinc transporter. Effects of severe zinc deficiency include slowed growth and development, skin rashes, anemia, and immune system deficiencies [11, 17]. Marginal zinc deficiency is much more common, and affects around two billion people worldwide due to individuals eating a diet low in zinc [18]. Marginal zinc deficiency can also occur as a result of GI, liver, and renal disorders, and can lead to increased susceptibility to infection, growth and developmental problems in children, and reproductive issues in adults (reviewed in [19-22]). In several cell types, zinc deficiency has been associated

with increased levels of oxidative stress and increased susceptibility to DNA damage because zinc is an essential cofactor and structural component of antioxidant defense proteins and DNA repair enzymes [23]. Zinc also plays an important role in beta cell function and insulin secretion, and zinc deficiency has been associated with diabetes [24, 25]. In a clinical study, zinc supplementation helped relieve symptoms in both T2D patients and mice (reviewed in [24]). In pancreatic beta cells, zinc deficiency has been associated with a malfunctioning superoxide dismutase, which may contribute to the oxidative stress associated with zinc deficiency [26]. Zinc deficiency affects about 17.3 percent of the world population, but rates range anywhere from 7.5 to 30 percent across different countries [27]. Zinc deficiency is an important public health problem because it is attributed to significant morbidity and mortality for children under the age of five, causing about 453,000 deaths per year [28]. Zinc supplementation has been proven an effective intervention for marginal zinc deficiency in developing countries, but its use is not widespread.

Inorganic Arsenic Exposure

The element arsenic exists in two forms: organic, which is found in foods, and inorganic, which is naturally found in air, soil, and water [29]. Inorganic arsenic is about one hundred times as toxic as organic arsenic [30]. The most common form of exposure to inorganic arsenic (henceforth referred to as arsenic) for humans is through water, which is used for cooking, cleaning, drinking, bathing, and other activities of daily living [30]. Once ingested, arsenic is absorbed by the body and enters the bloodstream where it can travel and accumulate in various tissues [30]. Chronic exposure to arsenic has been associated with many adverse health effects, such as skin lesions, vascular diseases, reproductive and neurological effects, and increased risk of skin, lung, bladder, and prostate cancers [31].

Arsenic exposure has been associated with both T1D and T2D. In an animal study, T1D mice were found to be more susceptible to arsenic uptake, and in human populations, arsenic in drinking water or its metabolized form in the body was associated with T1D [32, 33]. A study that sampled US adults found that, after adjusting for diabetes risk factors and seafood intake, higher concentrations of total urine arsenic were positively associated with T2D [34, 35]. In cell culture studies, arsenic exposure was associated with increased reactive oxygen species, which lead to increased DNA damage and decreased expression of genes that respond to mitochondrial oxidative stress [36]. Arsenic exposure in contaminated groundwater affects more than 200 million people worldwide, and most people affected reside in low-resource countries and the rural United States [31, 37, 38]. Most of the water in public water systems is purified for arsenic, but people can still be exposed to arsenic contamination through private wells.

Significance and Aim

Zinc deficiency and arsenic exposure have been reported to co-exist in populations all over the world, including communities in Bangladesh, India, Taiwan, the Navajo Nation, and the Pacific Islands [39-45]. Despite their significant overlap in populations, the interaction with zinc deficiency and arsenic co-exposure has not been studied extensively. In addition, although zinc deficiency and arsenic exposure have both been independently associated with T1D and T2D, little information is known about the interaction between zinc deficiency and arsenic exposure, and how their interaction affects the pancreas and insulin production. Recently, our group demonstrated that, with zinc deficiency and arsenic co-exposure, expression of the insulin gene significantly decreased in a developing zebrafish embryo, as well as enhanced the oxidative stress response in monocytes [46, 47].

The aim of this thesis was to determine the effects of zinc deficiency, arsenic exposure, and their combination on pancreatic beta cell growth and function. We hypothesized that zinc deficiency would exacerbate the toxicity associated with arsenic exposure in a pancreatic beta cell model, causing an increase in susceptibility to oxidative stress and disruption of insulin production. We also evaluated the effects of zinc deficiency and arsenic exposure on the expression of genes that regulate the response to oxidative stress and insulin production. We found that zinc deficiency or arsenic exposure both independently adversely affected pancreatic beta cell health, and the combination decreased cell proliferation and increased DNA double strand breaks and apoptosis.

Chapter 2

Zinc Deficiency and Arsenic Co-exposure in Cultured Pancreatic Beta Cells

To be submitted to the journal *Toxicology Letters*

1 Introduction

Pancreatic beta cells produce and release insulin, a vital hormone that regulates blood glucose levels, and their dysfunction contributes to *diabetes mellitus*, a chronic metabolic disorder characterized by hyperglycemia [1, 48, 49]. There are two types of diabetes; Type I diabetes (T1D) occurs when the pancreatic beta cell cannot produce sufficient levels of insulin, and Type II diabetes (T2D) occurs when the body becomes resistant to insulin. Given their important role in the body, it is important to maintain beta cell health and function. The essential micronutrient zinc plays an important role in beta cell function [24, 25]. Zinc is required for normal insulin production and is involved in glucose metabolism [50]. Zinc concentrations in the pancreatic beta cell are among the highest in the body, making it an important element to the organ, and zinc deficiency in model organisms decreases zinc levels in the pancreas [51-53]. Zinc is packaged with crystallized insulin in the secretory vesicles of the pancreatic beta cell, and the two are released together into the intracellular space [51]. Zinc deficiency has been correlated with diabetes. In a clinical review, diabetic patients had lower serum zinc levels compared to normal patients, and low zinc levels in drinking water has been associated with T1D in children [1, 54]. Zinc deficiency in other cell types has been associated with increased levels of oxidative stress and increased susceptibility to DNA damage because zinc is an essential cofactor and structural component of antioxidant defense proteins and DNA repair enzymes [23, 26]. The effect of zinc deficiency on health is an important avenue of research because it is estimated that 17.3 percent of the world's total population is zinc deficient, but regional zinc deficiency can range anywhere from 7.5 percent

in affluent countries like the United States and those in Europe, to 30 percent in low-resource countries in Africa, South America, and Southeast Asia [27]. While zinc plays an important role in insulin secretion, there is an incomplete understanding of how zinc deficiency affects pancreatic beta cell health and how it may increase susceptibility to damage caused by exposure to environmental toxicants.

Long-term ingestion of inorganic arsenic (henceforth referred to as arsenic) may be associated with development of T1D and T2D (reviewed in [29, 55, 56]). Arsenic is found in drinking water at concentrations higher than the 10 μg/L (10 ppb) World Health Organization recommendation in many countries around the world, including Argentina, Chile, Mexico, China, India, Bangladesh, Vietnam, and the United States [38, 57]. Contaminated drinking water is the major source of arsenic exposure for millions of people worldwide, and in the United States, concentrations of arsenic greater than 3,000 ppb have been found in private wells [58]. At the molecular level, arsenite, the soluble arsenic ion, has a high affinity for sulfhydryl groups, and can form covalent bonds with the disulfide bridges of insulin, insulin receptors, glucose transporters, and enzymes that regulate glucose metabolism, thus decreasing their function [59]. In cells, arsenic increases levels of reactive oxygen species and other free radicals, causing oxidative stress and DNA damage (reviewed in [59]). Furthermore, arsenic has been shown to accumulate in the pancreas, and is associated with pancreatic beta cell damage and dysfunction [36, 56].

Arsenic contamination in the groundwater often co-exists with regions in the world where people are prone to zinc deficiency [39-42]. For example, more than 50 percent of women evaluated in studies in Bangladesh and the Navajo Nation had low serum zinc levels and came from regions where wells contained elevated arsenic levels [43-45]. While arsenic exposure and zinc deficiency share hallmarks, like associations with oxidative stress and DNA damage, little

information is known about the interaction between zinc deficiency and arsenic exposure, and how their interaction affects pancreatic beta cell health and insulin production. This is of interest because zinc deficiency and arsenic co-exposure decreased the expression of the insulin gene in zebrafish embryos, suggesting a possible combination effect of these exposures in a developmental model [46].

The aim of this study was to determine the effects of zinc deficiency, arsenic exposure, and their combination on pancreatic beta cell viability and function. We hypothesized that zinc deficiency would exacerbate the toxicity associated with arsenic exposure in a pancreatic beta cell model, causing an increase in susceptibility to oxidative stress and disruption of insulin production. We evaluated the effects of zinc deficiency and arsenic exposure on cell health and examined the expression of genes that regulate insulin production and the response to toxicant exposure. We found that both zinc deficiency or arsenic exposure adversely affected pancreatic beta cells independently, and that their combination decreased cell proliferation, and increased DNA double strand breaks and apoptosis.

2 Methods

2.1 Cell Culture and Treatment

INS-1 rat insulinoma pancreatic beta cells were obtained from AddexBio (San Diego, CA). INS-1 cells were maintained in 5% CO_2 at 37°C with RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin-streptomycin, and 0.05 mM 2-mercaptoethanol. Zinc adequate (ZA) and zinc deficient (ZD) media were prepared by using a chelator to remove zinc from the FBS as previously published [60-62]. FBS was incubated with Chelex® 100 Resin (10% w/v) (Bio-Rad Laboratories,

Hercules, CA) at 4°C overnight with continuous stirring. To make ZA media, zinc (4 μM $ZnSO_4$) was added back to the media containing Chelex-treated FBS to obtain similar levels of zinc found in media with non-Chelex-treated FBS. No zinc was added back to the ZD media. Chelex treatment can also remove other divalent metals like calcium. As in previous studies using this depletion strategy, calcium (200 μM $CaCl_2$) was added back to all Chelex-treated media to similar levels found in non-Chelex treated media [61].

For all assays, INS-1 cells were cultured in either ZA media (4 μM $ZnSO_4$) or ZD media (0 μM $ZnSO_4$) for five days before exposure to arsenic. INS-1 cells (2.5 x 10^6 cells per dish) were plated in triplicate for each treatment. Sodium arsenite ($NaAsO_2$, Sigma-Aldrich) was diluted with the appropriate media, and cells were exposed for 24 h at concentrations of either 0, 50, or 500 parts per billion (ppb). These concentrations were chosen because 50 ppb arsenic was the limit set by the Environmental Protection Agency for drinking water up until 2001. Furthermore, arsenic can still be found at the 50-500 ppb concentrations in various groundwater sources around the world [37]. Cell proliferation and death were determined by using a trypan-blue exclusion assay (Media Tech Inc., Herndon, VA) and a hemocytometer.

2.2 Metal Analysis

Zinc, calcium, copper, iron, magnesium, and selenium, essential metals in human health, were evaluated in media and treated cells as previously described with minor modifications [63]. Cell pellets (containing 1.5×10^7 INS-1 cells) were digested in 0.5 mL of ultrapure 70% nitric acid, incubated, and shaken overnight. Samples were then diluted with Chelex-treated nanopure water to 7% nitric acid, centrifuged, and analyzed with Prodigy High Dispersion inductively coupled

plasma-optical emission spectrometry (ICP-OES) instrument (Teledyne Leeman Labs, Hudson, NH, USA) against known metal standards (Ultra Scientific, Kingstown, RI).

2.3 Gene Expression

Expression of genes related to insulin regulation, oxidative stress response, and DNA damage were analyzed using quantitative real-time PCR. Total RNA was collected from INS-1 cells using a standard Trizol extraction method (Life Technologies) as previously published [46, 60, 61]. cDNA was synthesized using 1 µg of total RNA and SuperScript III First-Strand Synthesis SuperMix (Life Technologies). Real time PCR was accomplished using primers that amplify all known transcript isoforms of each gene as a single product of expected size. Rat-specific primer sequences were as indicated (Supplemental Table 1). Reactions were performed using Fast SYBR Green Mastermix (Life Technologies) on 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 95°C for 20 s, followed by 40 cycles of denaturing at 95°C for 1 s, annealing and extension at 58°C for 20 s, followed by a standard dissociation curve. A dilution series of 10^3, 10^4, 10^5, 10^6, and 10^7 copies of template DNA served as internal standard for quantification. Data represent the copy number of the gene of interest normalized to the copy number of the housekeeping gene, *18s*, and then expressed relative to the mean levels found in ZA cells with no arsenic exposure.

2.4 Insulin and Oxidative DNA Damage Quantification by ELISA

A Rat/Mouse Insulin ELISA kit (EMD Millipore, Billerica, MA) was used to measure the amount of insulin released by cells into the culture media. The assay was performed following manufacturer's recommendations with the exception that control media was substituted for matrix

Primer Sequences for qRT-PCR

Gene	Forward	Reverse
18s	GGACCAGAGCGAAAGCATTTGC	CGCCAGTCGGCATCGTTTATG
Glut2	GGTGTTCCTCTGGATGACCG	GTCAACGAGAGGCTCTTTGC
Ins1	CCAAGTCCCGTCGTGAAGT	CTCCAGTTGGTAGAGGGAGC
Mt1	CAAGAAGAGCTGCTGCTC	CACAGCACGTGCACTTGTCC
Mt2	ACCCCAACTGCTCCTGTG	CACTTGTCCGAAGCCTCTTT
Neurod1	AAGACGCATGAAGGCCAATG	GAGACGAGGTCTGGGCTTTT
Ogg1	CAACATTGCTCGCATCACTGG	ATGGCTTTAGCACTGGCACATACA
p53	GCGTTGCTCTGATGGTGA	CAGCGTGATGATGGTAAGGA
Parp	TGTGAACTCCTCTGCACCAG	AGCTGAGGCAGACACATCCT
Pdx1	CCTTTCCCGAATGGAACCGA	AGGCTGTACGGGTCCTCTTA
Znt8	TCGAGCAGAGATCCTCGGTG	TGCTCTGAAACACATCCCCC

Supplemental Table 1: Forward and reverse primer sequences used to complete quantitative real-time PCR in rat INS-1 cell samples.

solution. A standard curve was generated with insulin concentrations of 0.2, 0.5, 1, 2, 5, and 10 ng/mL (EMD Millipore) and samples were diluted 1:500 to be in range of the standard curve. Insulin content was determined with Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA) at 450 nm and values were normalized to the number of viable cells per dish.

For oxidative DNA damage, genomic DNA was collected from INS-1 cells using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). Presence of 8-hydroxy-2'-deoxyguanosine (8-OHdG) on DNA was analyzed using EpiQuik 8-OHdG DNA Damage Quantification Direct Kit (Colormetric) (EpiGentek, Farmingdale, NY) and was reported per 300 ng genomic DNA and quantified relative to mean levels found in ZA cells without arsenic. Assays were performed according to manufacturer's instructions.

2.5 Protein Immunoblot Analysis

Protein was harvested from treated cells with radioimmunoprecipitation assay (RIPA) protein lysis buffer (Thermo Fisher) supplemented with protease inhibitor cocktail (Thermo Fisher) and processed as previously described [64]. Equal amounts of protein were separated on NuPage 4-12% Bis-Tris SDS-PAGE gels (Thermo Fisher) and blotted to a nitrocellulose membrane (Bio-Rad, Hercules, CA) in accordance with the manufacturer's protocol (Thermo Fisher). Membranes were blocked overnight with 2-5% BSA in TBST at 4°C and then probed for the indicated proteins following standard protocols using ɣ-H2AX (1:2,000, Santa Cruz Biotechnology, Dallas, TX), BAX (1:500, Santa Cruz Biotechnology), BCL2 (1:2,000, Santa Cruz Biotechnology), cleaved PARP (1:5,000, Millipore Sigma, Burlington, MA), and β-actin (1:50,000 Sigma-Aldrich, St. Louis, MO) antibodies. Goat anti-rabbit (1:10,000 dilution), goat anti-mouse (1:10,000), or donkey anti-goat (1:10,000) secondary antibodies (Santa Cruz Biotechnology) were also used using

standard conditions. Membranes were incubated in SuperSignal West Femto Reagent (Thermo Fisher) and developed on the ChemiDoc MP imaging system for visualization (Bio-Rad). Densitometric analyses were performed on the native membrane image using Image Lab 4.0 software (Bio-Rad). The densitometric value for each sample and protein of interest was normalized to the corresponding level of β-actin and expressed relative to the mean amount found in control ZA cells exposed to no arsenic on the same blot.

2.6 Statistical Analysis

Graphs were created and statistical significance was determined with GraphPad Prism software (La Jolla, CA). Data represent the mean fold-change from three independent experiments ± the standard error of the mean. Significant differences between control and treatment groups were measured by two-way ANOVA which tested for main effects of zinc status, arsenic exposure, or an interaction. Bonferroni post-tests were used for pairwise comparisons.

3 Results

3.1 Zinc levels in pancreatic beta cells cultured with zinc deficient media

We confirmed that zinc levels in the ZD media were significantly lower than that of ZA media (Figure 1A). No significant differences were found between ZA and ZD media in calcium, copper, iron, magnesium, and selenium levels (Supplemental Table 2). A six-day zinc deficiency treatment alone (i.e. with no arsenic) resulted in a 46.2 percent reduction in zinc levels in cells (Figure 1B). While not statistically significant, arsenic alone reduced zinc levels in ZA cells and increased zinc levels in ZD cells (trend for an interaction, $p=0.0605$) (Figure 1B). Importantly, ZD cells always had significantly less zinc than the ZA cells when compared to the same concentrations of arsenic.

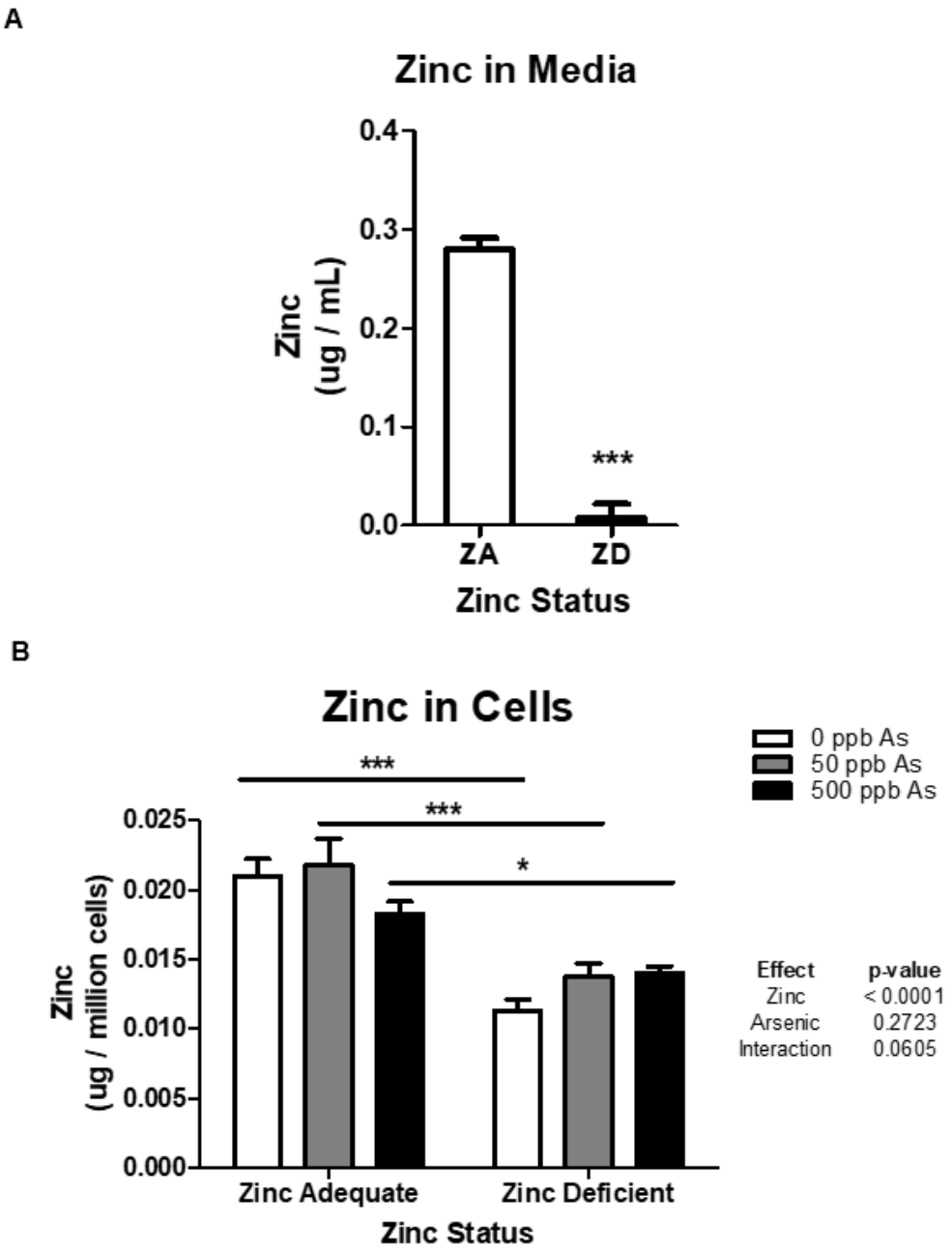


Figure 1. Zinc levels in zinc deficient media and INS-1 cells after 6-day zinc deficiency treatment. Cells were grown in zinc adequate (ZA) or zinc deficient (ZD) media for five days, followed by 24 h arsenic (As) exposure in ZA or ZD media. Zinc levels were measured by ICP-OES in (A) media, and (B) beta cells. Data represent mean zinc level (± SEM) where n = 9 per group from three independent experiments. A) Unpaired t-test was used to test for a significant

difference between ZA and ZD media. B) Two-way ANOVA was used to test for main effects of zinc status and arsenic exposure. Lines indicate significant differences between treatments, as determined by Bonferroni post-test, *P<0.05, ***P<0.001.

Amount of zinc, calcium, copper, iron, magnesium, and selenium in culture media and INS-1 cells

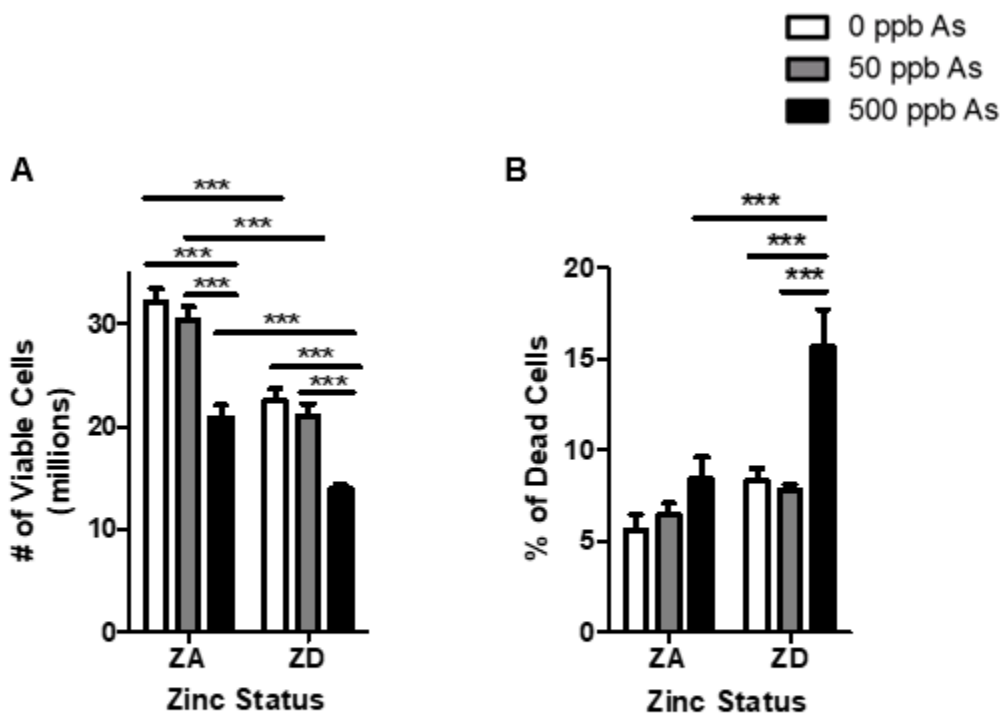
	Media			Cells 0 ppb As			Cells 50 ppb As			Cells 500 ppb As			Two-way ANOVA		
Element	ZA	ZD	Significance	ZA	ZD	Significance	ZA	ZD	Significance	ZA	ZD	Significance	Arsenic	Zinc	Interaction
Zinc	0.263	0.000	***	0.021	0.011	***	0.022	0.014	***	0.018	0.014	*	0.2723	< 0.0001	0.0605
Calcium	16.605	17.271	No	0.030	0.027	No	0.023	0.024	No	0.018	0.023	No	0.2934	0.8251	0.2318
Copper	0.009	0.005	No	ND	ND	No	0.0002	0.0004	No	0.0001	0.0002	No	0.2349	0.6441	0.6927
Iron	0.251	0.225	No	0.004	0.003	No	0.005	0.004	No	0.005	0.004	No	0.0202*	0.003**	0.9127
Magnesium	6.123	6.386	No	0.151	0.123	No	0.168	0.139	No	0.157	0.133	No	0.1799	0.0007***	0.9515
Selenium	0.119	0.115	No	0.005	0.003	No	0.004	0.004	No	0.005	0.004	No	0.5733	0.2081	0.554

Supplemental table 2: Data are mean values obtained by ICP-OES measurement in zinc adequate (ZA) or zinc deficient (ZD) cell culture media and INS-1 cells cultured in ZA or ZD media for five days followed by 24 h arsenic (As) exposure at 0, 50, or 500 ppb As. Media samples are μg of the element / mL of media, and INS-1 cell samples are μg of the element / 10^6 cells. Data is representative of three independent experiments and n = 9. Data were analyzed for significant differences by t-test (media) or two-way ANOVA with Bonferroni post-test (cells).

Zinc deficiency, arsenic exposure, or their combination, did not significantly affect calcium, copper, or selenium levels in the cells (Supplemental Table 2).

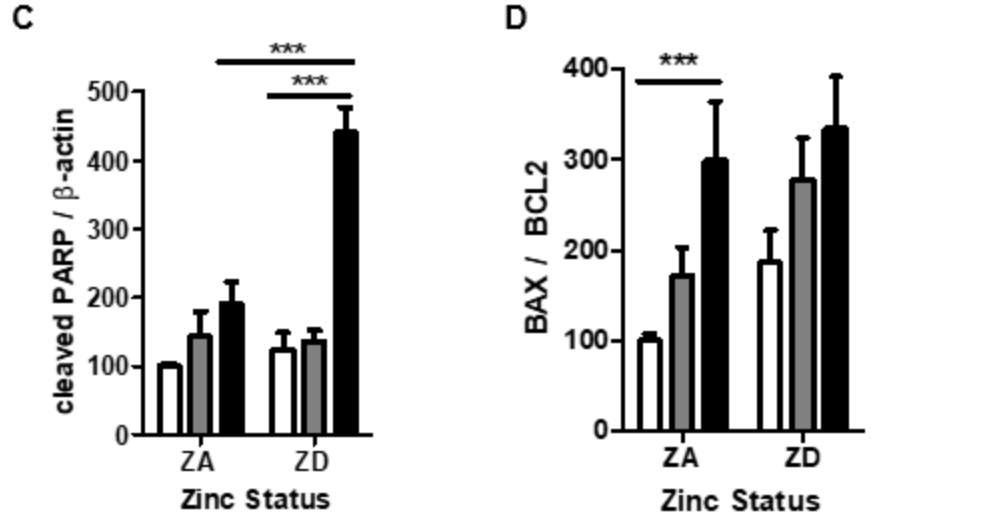
3.2 Zinc deficiency and arsenic co-exposure decreased pancreatic beta cell proliferation and viability

We next investigated if zinc deficiency and/or arsenic exposure affected pancreatic beta cell growth and survival. Zinc deficiency or arsenic exposure both significantly reduced the number of viable pancreatic beta cells by 30% and 35% respectively. The combination of zinc deficiency and 500 ppb arsenic resulted in a significant 33% further decrease in viable cells as compared to ZA cells with 500 ppb arsenic exposure (Figure 2A). In addition, zinc deficiency or arsenic both increased the percentage of non-viable cells, and the combination of zinc deficiency and 500 ppb arsenic produced a significant interaction and the greatest percentage of non-viable cells (Figure 2B). To determine if cell death was from apoptosis, we examined if cleaved PARP, a marker of apoptosis, changed with exposure. Cleaved PARP levels increased with zinc deficiency or arsenic exposure alone, but the fold change was modest (Figure 2C, Supplemental 3A). A significant interaction with zinc deficiency and arsenic exposure was found, and ZD cells with 500 ppb arsenic had a 2.3-fold increase in cleaved PARP as compared to ZA cells also exposed to 500 ppb arsenic (Figure 2C). We also examined the abundance of pro-apoptotic BAX protein and anti-apoptotic BCL2 protein and found no significant effect of zinc deficiency, arsenic exposure, or their combination on their levels (see Supplemental 3B). However, zinc deficiency or arsenic exposure alone significantly increased the ratio of BAX/BCL2 ratio by two- and three-fold respectively, but no interaction was observed, suggesting other mechanisms may contribute to the increase in apoptosis observed with zinc deficiency and arsenic co-exposure (Figure 2D).



Effect	p-value
Zinc	< 0.0001
Arsenic	< 0.0001
Interaction	0.4059

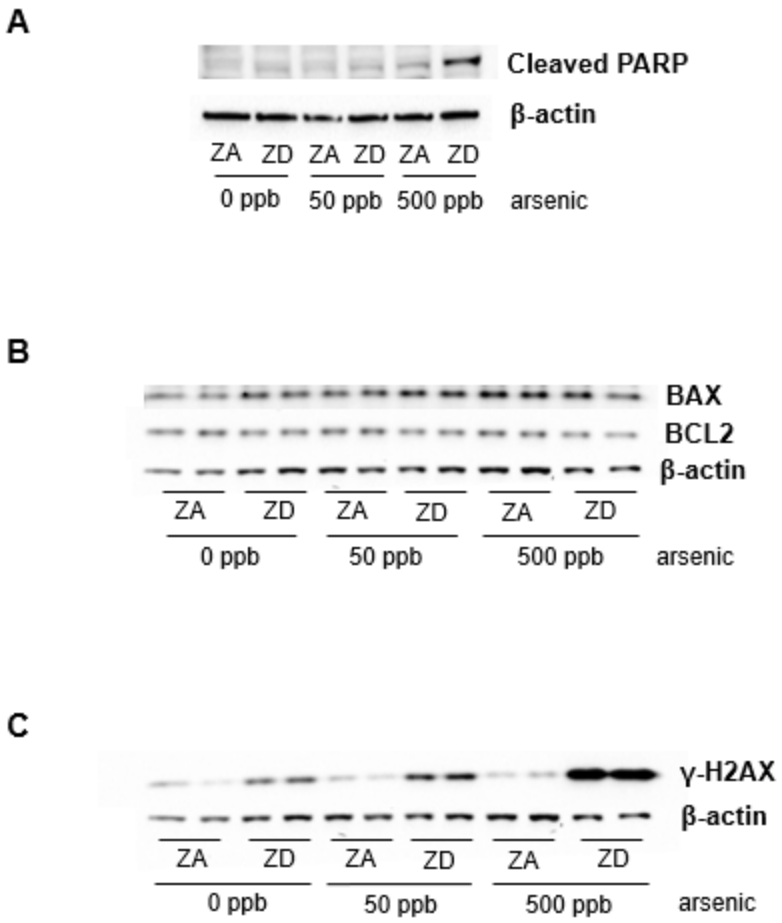
Effect	p-value
Zinc	0.0001
Arsenic	< 0.0001
Interaction	0.0282



Effect	p-value
Zinc	0.0004
Arsenic	< 0.0001
Interaction	< 0.0001

Effect	p-value
Zinc	0.0471
Arsenic	0.0023
Interaction	0.7178

Figure 2. Zinc deficiency and arsenic co-exposure decreased cell viability and increased apoptosis. Zinc adequate (ZA) or zinc deficient (ZD) INS-1 cells were treated with arsenic (As) for 24 h. A-B) Cell proliferation and cell death were determined by trypan-exclusion assay and hemocytometer. C-D) Expression of cleaved PARP, BAX, and BCL2 was measured at the protein level by Western blotting, quantified by densitometry and expressed relative to the housekeeping gene *β-actin*. Data represent mean fold-change of ZA control (0 μM As) ± SEM where n = 8-9 per group from three independent experiments. Two-way ANOVA tested for main effects of zinc status, arsenic exposure, or an interaction, followed by Bonferroni post-test for pairwise comparisons where lines indicate significant differences between treatment groups, ***$P<0.001$.



Supplemental Figure 3: Representative images of Western blots. Protein was harvested from zinc adequate (ZA) and zinc deficient (ZD) cells exposed to either 0, 50, or 500 ppb arsenic for 24 h. A) Apoptosis was determined by measuring cleaved PARP expression levels. B) BAX and BCL2 protein levels were measured to obtain the BAX/BCL2 ratio. C) DNA damage was quantified by measuring γ-H2AX abundance. All proteins of interest were normalized to expression of the housekeeping protein β-actin.

3.3 Zinc deficiency suppressed the oxidative stress response following arsenic exposure and increased DNA damage

To explore why pancreatic beta cells experienced slower growth and increased apoptosis, we next evaluated the expression of genes that respond to oxidative stress and DNA damage. *Metallothioneins 1* and *2* (*Mt1* and *Mt2*) regulate the intracellular concentrations of essential metals like zinc and can protect cells and tissues from reactive oxygen species and bind heavy metals with sulfhydryl groups [65, 66]. Increasing concentrations of arsenic induced the expression of *Mt1* and *Mt2* in a dose-dependent manner, resulting in a 100+-fold increase at 500 ppb arsenic (Figures 3A, 3B). An interaction was found for *Mt1* mRNA expression because zinc deficiency impaired *Mt1* expression under various treatments and a significant difference between ZA and ZD cells was found at 0 and 50 ppb arsenic concentrations. A similar, but less pronounced pattern was observed for *Mt2* where an increase in *Mt2* expression was significant at 500 ppb arsenic for both ZA and ZD cells, and a trend for interaction between zinc deficiency and arsenic was found. Arsenic also induced the expression of the oxidative stress responsive gene *heme oxygenase 1* (*Hmox1*) at the transcript level (Figure 3C) [67, 68]. ZA cells expressed significantly more *Hmox1* than did ZD cells at 500 ppb arsenic.

DNA repair genes *8-hydroxyguanine DNA glycosylase* (*Ogg1*) and *Tumor Protein P53* (*p53*) mRNA levels significantly increased in ZA cells at 500 ppb arsenic, but this effect was not present in ZD cells (Figure 4A, 4B). Because zinc deficiency can alter the efficiency of repair enzymes without necessarily changing the abundance of the enzyme, we moved on to examine indicators of DNA damage [62, 69]. Zinc deficiency significantly increased DNA damage from oxidative stress (8-OHdG), by 12.6%, but no changes were found related to arsenic exposure or the combination of zinc deficiency and arsenic exposure (Figure 4B). We also looked at the abundance

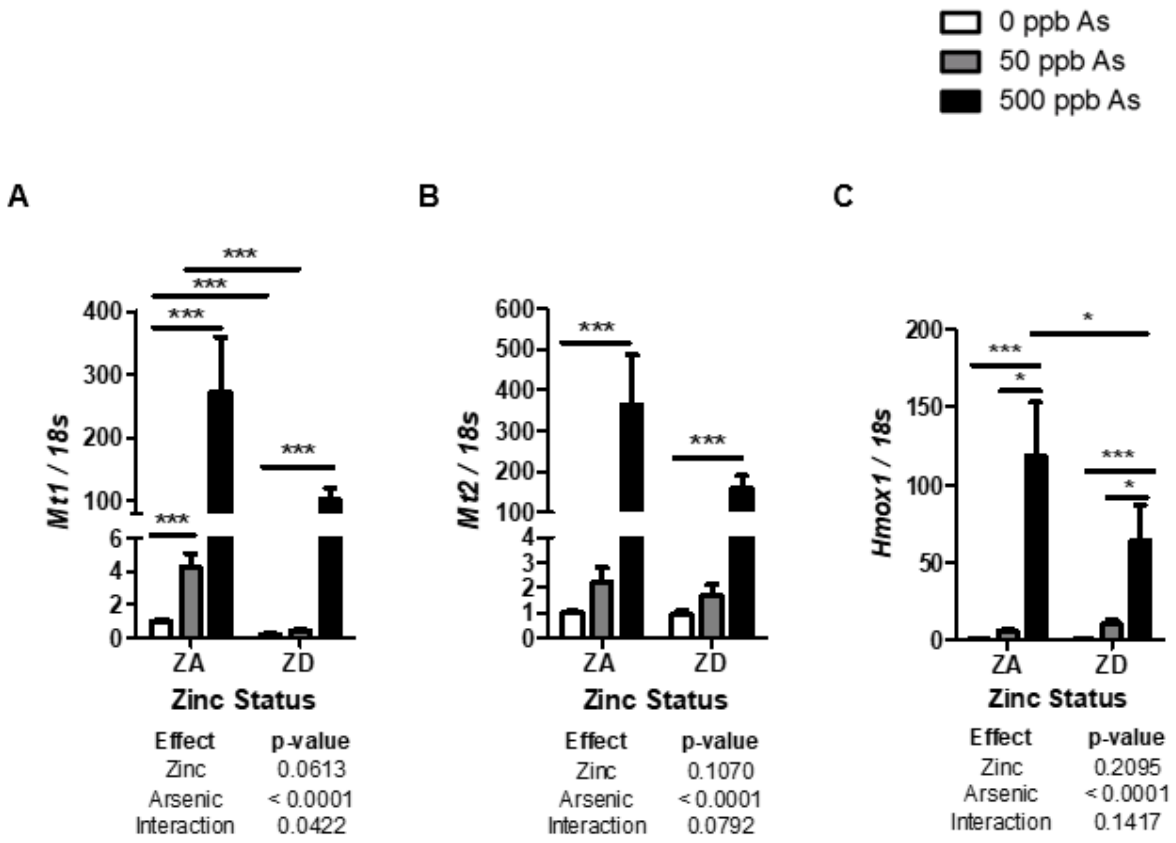


Figure 3. Zinc deficiency impaired the protective response to arsenic exposure. INS-1 cells were given a five-day zinc adequate (ZA) or zinc deficient (ZD) media treatment and then exposed to arsenic for 24 h in either ZA or ZD media. Expression of *Mt1*, *Mt2*, and *Hmox1* was measured (A-C) at the mRNA level by qRT-PCR and expressed relative to the housekeeping gene *18s*. Data represent mean fold-change (± SEM) compared to ZA control (0 µM As) where n = 6-9 per group from three independent experiments. Two-way ANOVA tested for main effects of zinc status, arsenic exposure, or an interaction, followed by Bonferroni post-test for pairwise comparisons where lines indicate significant differences between treatment groups, **P*<0.05, ****P*<0.001.

Figure 4

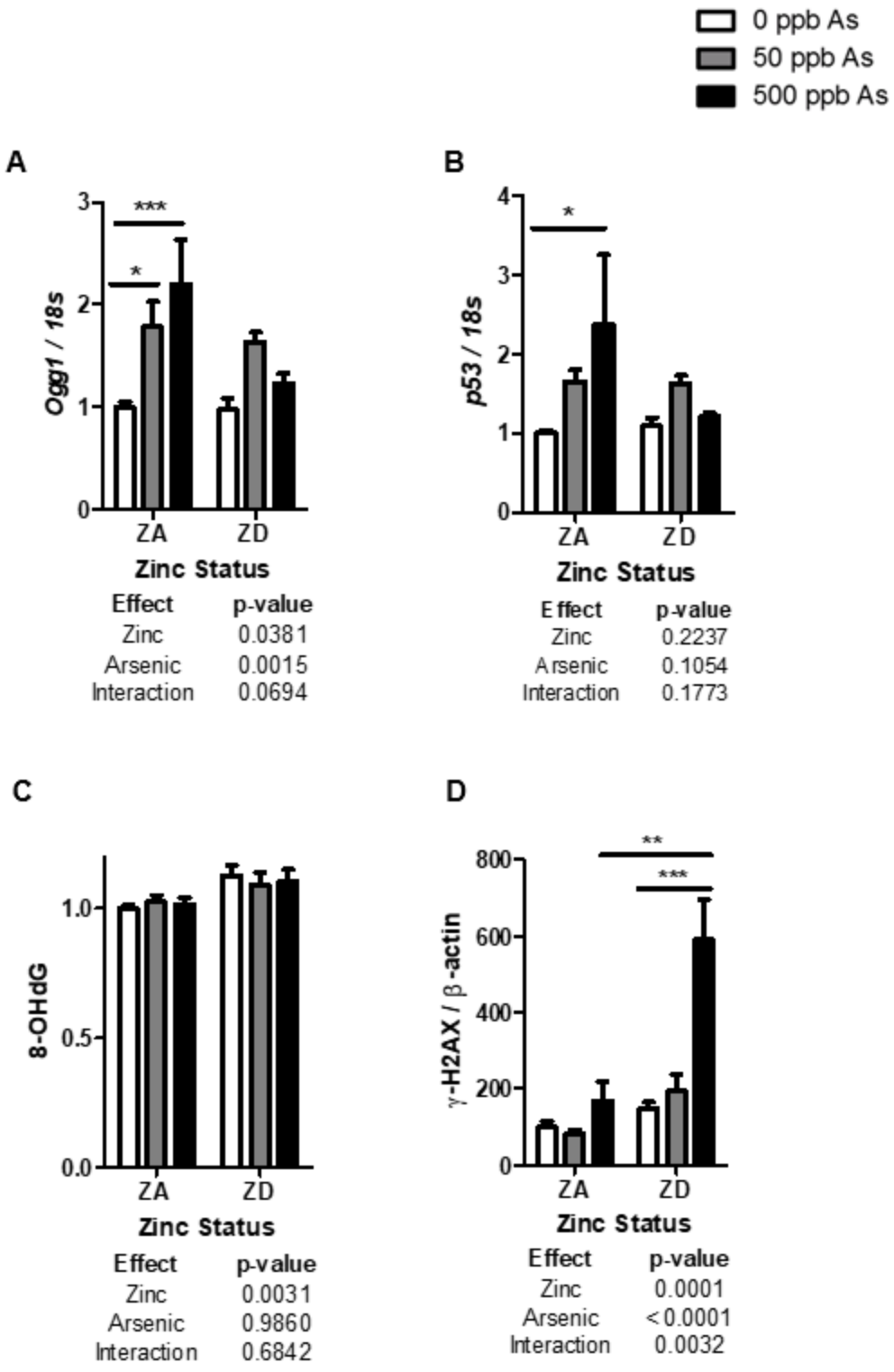

0 ppb As
50 ppb As
500 ppb As
A
Ogg1 / 18s
***
*
ZA
ZD
Zinc Status
Effect p-value
Zinc 0.0381
Arsenic 0.0015
Interaction 0.0694
B
p53 / 18s
*
ZA
ZD
Zinc Status
Effect p-value
Zinc 0.2237
Arsenic 0.1054
Interaction 0.1773
C
8-OHdG
ZA
ZD
Zinc Status
Effect p-value
Zinc 0.0031
Arsenic 0.9860
Interaction 0.6842
D
γ-H2AX / β-actin
**
***
ZA
ZD
Zinc Status
Effect p-value
Zinc 0.0001
Arsenic < 0.0001
Interaction 0.0032

Figure 4. Zinc deficiency increased the susceptibility of beta cells to DNA damage. INS-1 cells were grown in zinc adequate (ZA) or zinc deficient (ZD) media for five days, followed by 24 h arsenic (As) exposure in ZA or ZD media. A-B) Expression of *Ogg1* and *p53* was measured at the mRNA level by qRT-PCR. C) 8-hydroxy-2'-deoxyguanosine (8-OHdG) was measured with an ELISA. D) ɣ-H2AX abundance was measured by Western blotting, quantitated, and normalized to β-actin protein levels. Data represent mean fold-change (± SEM) compared to ZA control (0 μM As) where n = 8-9 per group from three independent experiments. Two-way ANOVA tested for main effects of zinc status, arsenic exposure, or an interaction, followed by Bonferroni post-test for pairwise comparisons where lines indicate significant differences between treatment groups, **P*<0.05, ***P*<0.01, ****P*<0.001.

of γ-H2AX, a marker of DNA double-stranded breaks, and found a significant effect of zinc deficiency, arsenic exposure, and an interaction for their combination [70, 71]. At 500 ppb arsenic, zinc deficiency caused a 2.5-fold increase in γ-H2AX levels as compared to ZA cells at the same arsenic exposure (Figure 4D, Supplemental Figure 3C).

3.4 Zinc deficiency increased insulin production while arsenic inhibited insulin release

Because zinc deficiency and arsenic co-exposure decreased beta cell health and DNA repair mechanisms in cells, we next explored how these effects would impact insulin production. *Ins* mRNA levels significantly increased with zinc deficiency for both 0 and 50 ppb arsenic concentrations (Figure 5A). Arsenic exposure at 500 ppb significantly decreased *Ins* mRNA levels in both ZA and ZD cells. We also examined the amount of insulin released into the media and expressed it relative to the number of viable cells. ZD cells released 51% more insulin on a per cell basis as compared to ZA cells when no arsenic was present (Figure 5B). Arsenic induced a significant and dose dependent decrease in insulin concentration in media, which resulted in a 70 or 72% decline with 500 ppb arsenic in ZA or ZD cells respectively. No combination effect of zinc deficiency and arsenic was observed on insulin concentrations in the media.

We also examined the expression of *Pdx1* and *Neurod1* which both encode transcription factors that regulate the insulin gene. In ZA cells, 50 ppb arsenic significantly increased the expression of *Pdx1* mRNA levels, but a significant decrease in expression was found at 500 ppb arsenic (Figure 6A). Zinc deficiency significantly decreased *Pdx1* mRNA levels at 50 ppb arsenic, as compared to ZA 50 ppb arsenic, and is primarily responsible for the significant interaction between zinc deficiency and arsenic exposure observed for this gene. *Neurod1* mRNA levels

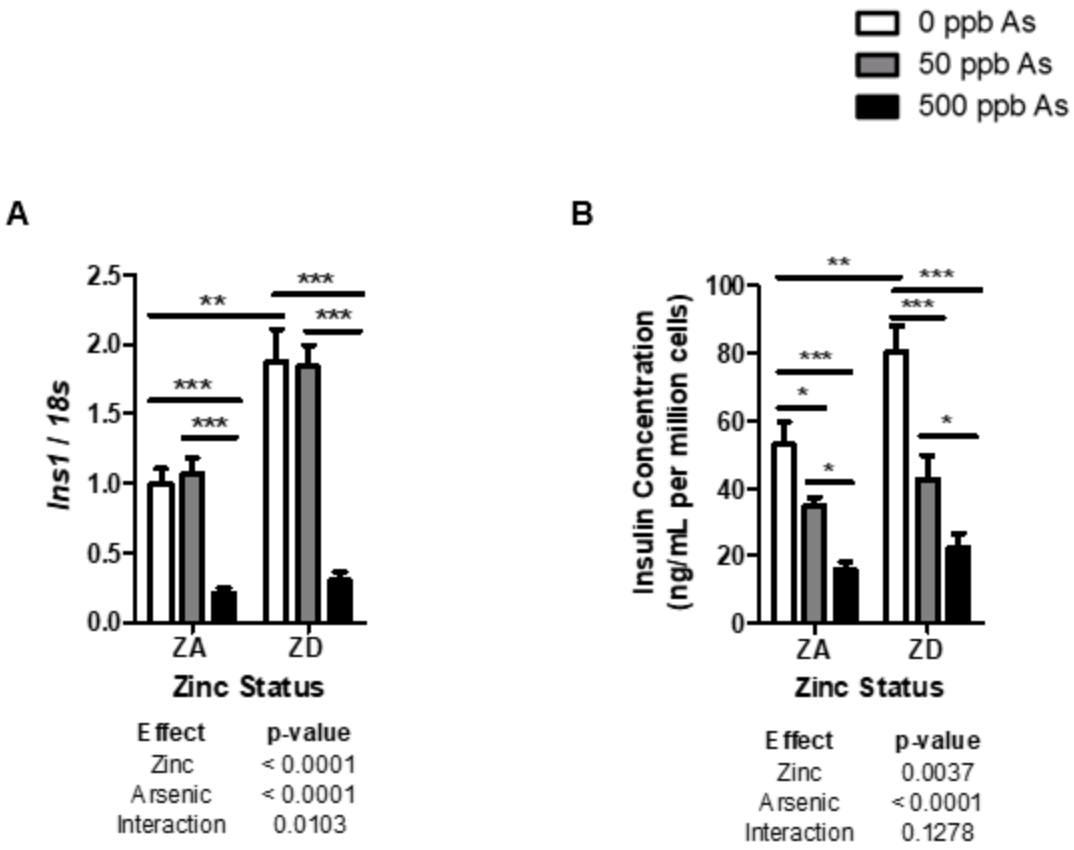


Figure 5. Zinc deficiency increased insulin levels while arsenic exposure decreased insulin production. Zinc adequate (ZA) or zinc deficient (ZD) INS-1 cells were cultured in media for five days, and then exposed to arsenic for 24 h in ZA or ZD media. A) Expression of *Ins1* was measured at the mRNA level by qRT-PCR and expressed relative to the housekeeping gene *18s*. B) Insulin was measured with an ELISA. Data represent mean fold-change (± SEM) compared to ZA control (0 µM As) where n = 8-9 per group from three independent experiments. Two-way ANOVA tested for main effects of zinc status, arsenic exposure, or an interaction, followed by Bonferroni post-test for pairwise comparisons where lines indicate significant differences between treatment groups, **P*<0.05, ***P*<0.01, ****P*<0.001.

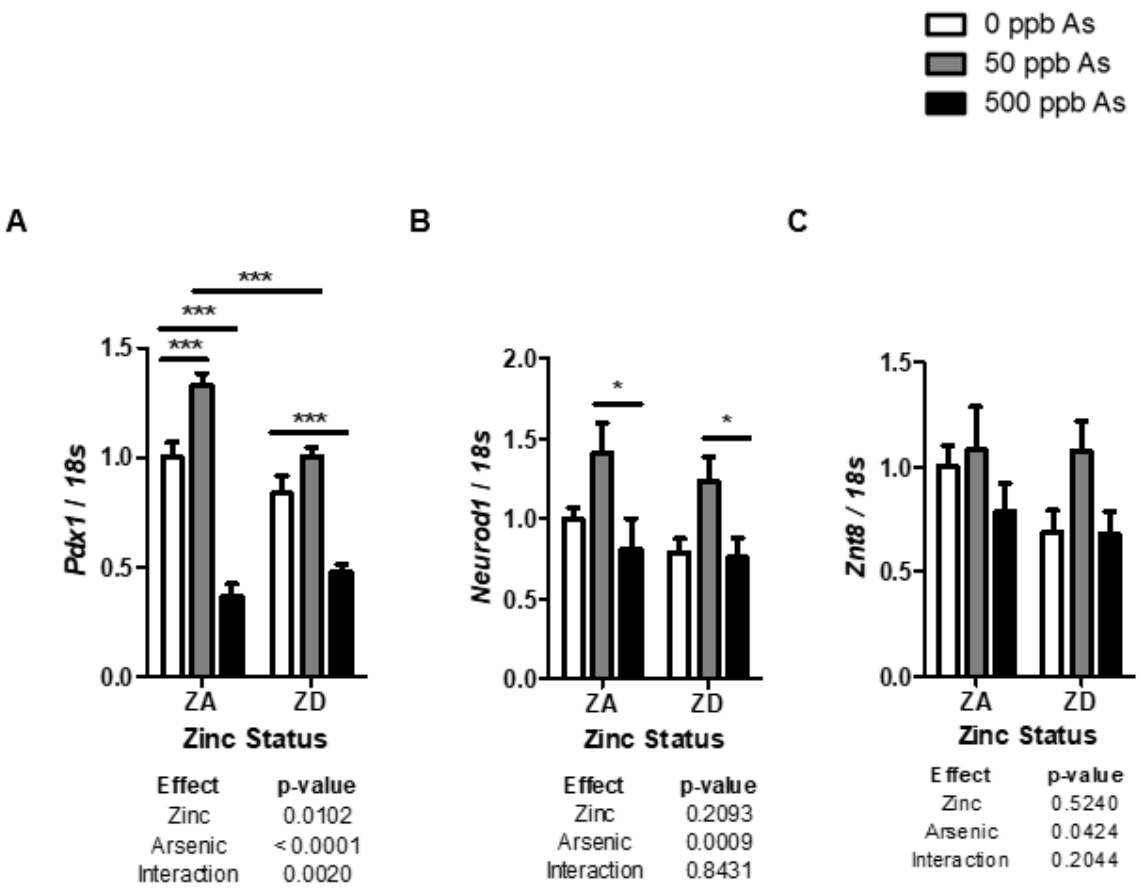


Figure 6. Zinc deficiency, arsenic, and their co-exposure altered *Pdx1* transcript levels. INS-1 cells were grown in zinc adequate (ZA) or zinc deficient (ZD) media for five days, followed by 24 h arsenic (As) exposure in ZA or ZD media. Expression of *Pdx1*, *Neurod1*, and *Znt8* was measured (A-C) at the mRNA level by qRT-PCR and expressed relative to the housekeeping gene *18s*. Data represent mean fold-change (± SEM) compared to ZA control (0 µM As) where n = 8-9 per group from three independent experiments. Two-way ANOVA tested for main effects of zinc status, arsenic exposure, or an interaction, followed by Bonferroni post-test for pairwise comparisons where lines indicate significant differences between treatment groups, **P*<0.05, ****P*<0.001.

increased at 50 ppb arsenic in both ZA and ZD cells, and significantly decreased between 50 and 500 ppb in both ZA and ZD cells (Figure 6B). We also evaluated the expression of *Zinc Transporter 8* (*Znt8*), a transporter specifically expressed in the secretory granules of beta cells that secrete insulin [72]. Zinc deficiency alone caused a modest and non-significant decrease in *Znt8* mRNA levels. There was a significant overall effect with arsenic but no significant changes in *Znt8* mRNA levels were found between ZA and ZD groups exposed to the same arsenic dose (Figure 6C).

4 Discussion

Here, we used a cell culture model to illustrate for the first time that zinc deficiency sensitizes pancreatic beta cells to the toxicity associated with arsenic exposure. Although a tissue culture model has inherent limitations, we showed that the zinc-deficient pancreatic beta cell's ability to produce a protective response to arsenic decreased at the mRNA level (*Mt1*, *Hmox1*, *p53*). While our results did not show a significant interaction for DNA damage caused by oxidative stress, we found significant interaction effects for an increase in DNA double strand breaks, a decline in cell proliferation, and an increase in apoptosis. We also showed that zinc deficiency alone decreased pancreatic beta cell proliferation, and increased *Ins* gene expression and insulin release in a cell culture model. Zinc deficiency and arsenic exposure can co-exist within human populations and understanding their interaction in the pancreas has important implications for understanding health outcomes for susceptible populations. More broadly, our work taken together with previous studies in various cell and animal models showed zinc deficiency and arsenic exposure can function independently or cooperatively to affect disease related parameters, like susceptibility to DNA

damage, declines in physical activity in developing embryos, and increases in inflammatory responses [46, 47, 73-75].

Zinc is important for development, cellular metabolism, and enzyme function, and is an essential element in the pancreas [15]. Zinc homeostasis is tightly regulated in the beta cell, and while we focused on parameters related to insulin and did not find significant changes associated with *Znt8* transcript levels, *Znt8* is associated with pancreatic beta cell survival and is involved in the etiology of T1D (reviewed in [76]). In cultured human beta cells, ZNT8 and ZNT3 protein expression have been found to overlap the most with insulin protein expression compared to other zinc transporters, and ZNT3 may play a role in insulin maturation and secretion [77]. Because ZNT3 is uniquely expressed in human pancreatic beta cells and not those of other animals, we were unable to evaluate its expression, but exploring zinc transporters at the protein level in the context of pancreatic cells could be valuable future work. Zinc and its transport are linked to insulin, and previous animal studies have found that zinc deficiency had either no significant effects on serum insulin, or decrease serum insulin and insulin granules in pancreatic beta cells when compared to ZA mice, which is different from our findings [52, 78]. We found that ZD pancreatic beta cells released more insulin than did ZA cells, but our data is consistent with a study that found zinc deficient obese patients had increased insulin production [79]. Our data showing zinc deficiency increased 8-OHdG formation is consistent with studies in prostate epithelial cells and blood cells also reporting increased DNA damage with zinc deficiency[80, 81]. The beta cells' inability to repair DNA damage may contribute to the decreases in pancreatic beta cell survival and growth observed with zinc deficiency alone.

It is well known that arsenic exposure can cause impaired insulin secretion, induce oxidative stress, and is associated with an increased risk of diabetes (reviewed in [82, 83]. As expected, we

found a dose-dependent effect of arsenic exposure on gene expression, insulin release, and beta cell proliferation (reviewed in [58, 82, 84, 85]. While high concentrations of arsenic like 500 ppb are not encountered as often as 50 ppb in human populations, doses in excess of this concentration are regularly used in research, and arsenic can accumulate in the blood and pancreas making the physiological arsenic concentration exceed the exposure concentration (reviewed in [86, 87]). Consistent with the literature, we found that arsenic increased molecular indicators of oxidative stress and DNA damage (*Mt1*, *Hmox1, p53, Ogg1*) ([88, 89], reviewed in [85]). Beta cell destruction is the primary pathogenesis of T1D, and an animal study found that mice with T1D were more susceptible to arsenic uptake [34]. Interestingly, we found some parameters where zinc status did not affect pancreatic beta cell response to arsenic, showing that arsenic exposure can be the dominate environmental signal in the regulation of insulin release.

While zinc deficiency and arsenic exposure each have their own significant impact on pancreatic beta cells, many significant interactions occurred with their co-exposure. Notably, their interaction led to an increased impairment of the protective response to arsenic-induced toxicity. The inability of ZD cells to respond effectively to arsenic resulted in increased DNA double strand breaks, a decline in cell proliferation, and apoptosis, showing that co-exposure was detrimental to pancreatic beta cell health. In our study, *Hmox1* mRNA expression decreased with co-exposure, and a study that used a T1D animal model found that overexpression of the *Hmox1* gene provided protective effects against autoimmune diabetes by increasing beta cells' abilities to counteract apoptosis and inflammation [90]. Although we studied zinc depletion, other studies have shown that zinc supplementation reversed the arsenic-induced effects on repair enzymes and DNA damage [73, 91, 92]. Future supplementation studies could be helpful in determining if damage caused by arsenic can be reversed in pancreatic beta cells, and if this could restore insulin

production. Other significant interaction effects we found included the *Ins* gene and the transcriptional regulator *Pdx1* gene, but there was no interaction effect for insulin output. mRNA results for *Pdx1* and *Neurod1* also did not explain the lack of interaction, suggesting that there was disconnect from the messenger RNA and protein levels. Given this result, there are possibly other regulatory mechanisms of the *Ins* gene involved that we did not investigate, such as insulin-degrading enzyme (IDE), a zinc metalloprotease that controls insulin levels and whose reduced activity has been associated with T2D [93, 94]. The lack of interaction effect for insulin output may also be because the insulin was only measured for the 24 hours that arsenic was present. Because of the declines observed in cell proliferation and viability, it is possible an effect from the co-exposure on insulin release would be detected over a more extended time course (reviewed in [95]). Nevertheless, we did not pursue this work more due to confounding variables such as apoptotic cells releasing zinc into the media.

The increase in sensitivity of zinc-deficient pancreatic beta cells to arsenic exposure is consistent with our previous findings in animal and cell culture studies where zinc deficiency was also associated with a sensitization to arsenic exposure [46, 73, 74]. Furthermore, the combination of zinc deficiency and other toxic metal exposure has also been associated with chronic illnesses like hypertension [96, 97]. These studies support the idea that zinc deficient status and exposure to toxic metals may act synergistically to influence the onset of chronic diseases in susceptible populations. Importantly, development and progression of T1D is contributed to by both environmental and genetic factors. Our data and data showing that zinc deficiency has been associated with T1D support the idea that zinc deficiency alone, and when combined with other environmental toxicants like arsenic, could contribute to the environmental onset of T1D [98, 99]. Overall, our study found that the combination of zinc deficiency and arsenic exposure disrupted

cell health and proliferation in pancreatic beta cells and these conditions alone, and in combination, may promote insulin dysregulation and beta cell dysfunction.

Chapter 3 | Conclusion of Thesis

Summary of Findings

We found that pancreatic beta cells can be made zinc deficient within six days and that zinc deficiency significantly increased levels of a marker of oxidative stress and insulin. Arsenic decreased expression of the insulin gene, insulin release, and beta cell proliferation. Importantly, we found that with zinc deficiency and arsenic co-exposure, zinc's ability to provide a protective response to toxicity caused by arsenic was attenuated. Zinc deficient beta cells that were exposed to arsenic were less able to repair DNA damage which is one of many factors that can lead to cell death. We also observed increased apoptosis with co-exposure and interaction effects for the expression of insulin and a gene that regulates insulin at the transcript level.

Impact and Health Implications

These research findings are relevant for the millions of people around the world who are exposed to arsenic through contaminated groundwater and are potentially zinc deficient [39-45]. Understanding how these combinatorial factors impact pancreatic beta cell health and insulin is important for prevention of adverse health outcomes and effects on the pancreas. Zinc deficiency and arsenic co-exposure produce T1D-like symptoms consistent with beta cell dysfunction and decreasing beta cell mass. Due to an inability to repair DNA damage, beta cell proliferation significantly declined, and apoptosis significantly increased. Ultimately, this could result in less insulin released and increased blood glucose levels which can affect the organism as a whole. Beyond the pancreas, zinc deficiency and arsenic co-exposure can also cause health problems related to development, inflammation, and the gut microbiome [46, 47, 100]. In zebrafish, co-exposure of zinc deficiency and arsenic decreased swimming activity by 40 percent compared to

zinc adequate controls with no arsenic exposure [46]. The underlying conditions with co-exposure that lead to decreased activity are complex but could be related to altered eye development, nerve and muscle function, and energy metabolism. Arsenic can also decrease zinc levels in developing embryos, cells in culture, and in plasma of mice, and this decreased zinc status may contribute to an overall increased proinflammatory response [46, 47]. The inflammation study also found that zinc repletion did not restore zinc levels in cells, which implies that zinc deficiency can have a long-term impact and that more zinc may be needed to bring zinc levels back up to adequacy [47]. Finally, zinc deficiency and arsenic exposure can affect the physiology of the gut microbiome, and may affect an individual's susceptibility to pathogenic infections and gut inflammation [100]. These studies show that zinc deficiency and arsenic co-exposure can have impacts on multiple biological systems and health outcomes.

Future Directions

Because tissue culture has limitations in its applications, examining zinc deficiency and arsenic exposure in an animal model would provide a more accurate relationship of their co-exposure and its physiological effects. One of the differences between cell culture and a physiological model is that in cell culture, pancreatic cells are exposed to 500 ppb arsenic directly, which is likely higher than the concentration that may be found in pancreatic tissue of an animal that ingested the same concentration from contaminated water. However, arsenic can accumulate in tissues like the pancreas, and over time, the pancreas may exhibit changes that are similar to what we found in our study [36, 56]. The strength of the tissue culture model is that we were able to focus solely on the effects of zinc deficiency and arsenic exposure on pancreatic beta cell health. In an animal model, arsenic exposure and marginal zinc deficiency could be observed for longer periods of time which

would more accurately reflect co-exposure in human populations. Additionally, insulin release and glucose uptake could be examined in conjunction with other organs, such as the liver, to examine how the pancreas works with other organs to regulate glucose and insulin. Although zinc deficiency increased insulin release in cells, it is unclear whether this increase would compensate for increased glucose levels observed in a zinc deficient diabetic model [101, 102]. This is a question that an animal model could answer. Finally, it is important to test more zinc transporters because many exist, and we only tested one relevant to insulin release.

Final Conclusions

Overall, this study showed that zinc deficiency and arsenic exposure can both independently affect pancreatic beta cell health, but notably, co-exposure was associated with several significant interactions related to beta cell dysfunction. More specifically, zinc deficiency sensitized beta cells to the harmful effects of arsenic which resulted in a significant increase in DNA damage and apoptosis, and significant declines in proliferation with co-exposure. The results of this study can provide insight to the complex relationship between zinc deficiency and arsenic exposure and can be used when considering the health outcomes of susceptible populations.

Chapter 4 | References

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