Protective Effects of Zinc Supplementation for Diabetes-Induced Vascular Damage

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ABSTRACT

Protective Effects of Zinc Supplementation for Diabetes-Induced Vascular Damage Diabetes

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The leading cause of mortality in diabetic patients is cardiovascular disease developed through the accumulation of damage done by oxidative stress. Hyperglycemic conditions activate pathogenic pathways, including mitochondrial dysfunction, which generate reactive oxygen species. The endogenous antioxidant defense system is unable to adequately mitigate this damage as diabetes is linked with zinc deficiency, and zinc plays a crucial role in the functionality of the antioxidant system. The effects of zinc supplementation in the prevention of diabetic cardiomyopathy have been documented in previous research. However, the protective capacity of zinc supplementation in the vasculature has yet to be established. We hypothesized that a four-week zinc supplementation intervention would enhance mitochondrial respiration, reduce protein oxidation, and increase the expression of several important antioxidants (superoxide dismutase (SOD), zinc-induced metallothionein (MT), catalase, and peroxiredoxin-2 (Prx2)) in the vasculature of streptozotocin-induced diabetic mice when compared to diabetic controls. Results revealed that mitochondrial respiration was significantly more efficient in the zinc-supplemented group. Additionally, it was found that the intervention group experienced significantly reduced levels of protein oxidation as well as a significant upregulation in both SOD and MT expression. No differences were observed among levels of catalase and Prx2. These findings suggest that zinc supplementation may have the potential to prevent deleterious changes in mitochondrial health and act in a protective capacity against oxidative damage in the vasculature.

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Introduction

Introduction

Pathophysiology of Diabetes Mellitus

Chronic hyperglycemia is the main feature used to characterize and diagnose the metabolic disorder diabetes mellitus (DM) [Inzucchi SE, 2012]. The prevalence of DM has risen at a dramatic rate over the past three decades [World Health Organization, 2016]. Previous projections estimated that 430 million people will be affected by the year 2030, when in fact, this is the current number of individuals with DM [World Health Organization, 2016; Rochette et al., 2014]. Currently, the International Diabetes Federation predicts that there will be a staggering 642 million affected by 2040 [Ranasinghe et al., 2018]. For the United States alone, this represents a total health care cost of $327 billion dollars for diagnosed DM in 2017 [American Diabetes Association (ADA), 2018]. Consequently, the ADA found that a diabetic pays more than double the average medical expenditure compared to healthy. Considering the current predictions for future DM prevalence, these costs will likely continue to increase and thus place a huge economic burden on society.

The health and socioeconomic impact of DM continues to skyrocket despite the progress science has made in identifying risk factors, tailoring treatment plans and diabetes management; the complications linked to this disorder are largely responsible for this paradox. [ADA, 2014; Domingueti et al, 2016; Zaccardi et al., 2015]. Risk of myocardial infarction, peripheral vascular disease, and stroke are among the many cardiovascular diseases that are doubled for an individual with DM [Domingueti et al., 2016; Zaccardi et al., 2015]. As a result, a more comprehensive understanding of the pathways involved in the process from diabetic onset to a fatal cardiac event is needed. Understanding the importance of prophylactic agents, such as zinc, become paramount in contributing towards the reduced risk of cardiovascular complications.

As previously mentioned, DM is characterized by chronically elevated blood glucose levels as a result of either insulin resistance or insulin deficiency [Rochette et al., 2014; Zaccardi et al., 2015]. Consequently, this hyperglycemia promotes the activation of numerous pathways leading to an overproduction of damaging waste products including reactive oxygen species (ROS). This generation of ROS, together with the lack of endogenous antioxidant defense that diabetics experience, culminates in massive oxidative stress throughout the body, including the

vasculature via lipid peroxidation and oxidative damage done to cells, proteins, enzymes, and DNA [Cruz et al., 2015; Rochette et al., 2014]. The result is chronic inflammation leading to the deterioration of the endothelial function which is tightly linked to the development of cardiovascular diseases (CVD) [Rochette et al., 2014]. Consequently, cardiovascular complications are the leading causes of death and morbidity in diabetics as there are currently no specific treatments tailored to diabetes-induced CVD [Lu et al., 2015].

There are two predominant classifications of DM: Type 1 (T1) and Type 2 (T2), with T2DM accounting for ~90-95%% of all cases in the United States [ADA, 2014; Zaccardi et al., 2015]. T1DM is an autoimmune disease which targets the pancreas by the progressive destruction of the beta cells in the islets of Langerhans [Saberzadeh-Ardestani et al., 2018; Zaccardi et al., 2015]. As a result, the individual develops an insulin deficiency which ultimately leads to hyperglycemia [DiMeglio et al., 2018]. The onset of T1DM has been identified by both genetic and environmental factors. Beta-cell destruction is closely linked to the genetic region of the human leukocyte antigen, albeit there are other immune system genes that have also been found to play a guiding role [Steck & Rewers, 2011]. Current treatment includes management via insulin injections and meal planning [Diabetes Canada, 2019]. The development of T2DM is due to lifestyle and genetic factors whereby insulin resistance forces the body to overproduce insulin in an attempt to overcome insufficient glucose uptake through faulty receptors. Concurrent to this, beta-cell apoptosis occurs in the pancreas and the body is thus unable to compensate for the insulin resistance [Diabetes Canada, 2019; Rochette et al., 2014].

Despite the etiological differences between T1DM and T2DM, the vascular complications of the resulting chronic hyperglycemia remain the same: onset of oxidative stress through production of ROS along with decreased activation of the antioxidant defense system [Cruz et al., 2015; Rochette et al., 2014]. Diabetic retinopathy, neuropathy, and nephropathy are common consequences of diabetes-induced endothelial dysfunction; however cardiovascular complications are the leading causes of death among both T1DM and T2DM [Domingueti et al., 2016; Rochette et al, 2014].

Diabetes and the Vasculature

The homeostatic functioning of the blood vessels in the periphery is managed through vascular tone, which in turn is regulated by the endothelial cells [Sena et al., 2013]. Endothelial cells make-up the lining of the vascular system while smooth muscle cells support the vessel structure [Sturtzel, 2017]. To maintain regulation of vascular homeostasis, the endothelium produces and secretes vasoactive substances as well as growth factors that influence the various layers of the vessels, particularly the vascular smooth muscle cells [Hu et al., 2018]. Endothelial cells also contribute to cardiovascular homeostasis by actively mediating immune responses. As a result, due to the integral nature of endothelial cells within the cardiovascular system, they are intrinsically disease-causing when dysfunctional [Sturtzel, 2017].

In response to hyperglycemic conditions, endothelial cells themselves are at risk of developing intracellular hyperglycemia as glucose can passively diffuse into the cell without the need for insulin action [Domingueti et al., 2016]. The accumulation of intracellular glucose activates a secondary metabolic pathway that alters the redox potential and ultimately manifests as oxidative stress [Domingueti et al., 2016; Sena et al., 2018]. Homeostatic ROS concentrations act in the immune response systems as secondary messengers in various signaling pathways [Schieber & Chandel, 2014]. However, under hyperglycemic conditions, vascular oxidative stress causes the overproduction of ROS (superoxide anion $O_2^{\bullet-}$) which combines with nitric oxide (NO), decreasing NO bioavailability - the end result is endothelial dysfunction [Sena et al., 2018]. Consequently, endothelial cells activate an inflammatory response which increases the expression of chemokines, cytokines, adhesion molecules, and proteases; this creates prolonged vascular inflammation [Domingueti et al., 2016; Sena et al., 2018]. These circumstances then lead to an increase of apoptotic cells, extracellular matrix remodeling, and further endothelial dysfunction [Sena et al, 2018]. This dysfunction at the cellular level then initiates vascular dysfunction, which ultimately results in both micro- and macro-vascular diseases [Domingueti et al., 2016; Rochette et al., 2014; Sena et al., 2018].

The progression of endothelial dysfunction is tightly bound to diabetes-induced hyperglycemia resulting in retinopathy, neuropathy, nephropathy, peripheral artery disease, and coronary heart disease [Hu et al., 2018]. The culmination accelerates atherosclerotic-complications and

increases the risk of myocardial infarction, stroke, and ischemic heart disease [Rochette et al., 2014; Sena et al., 2018]. The lack of cellular protection against oxidative stress is thus the reason why these cardiovascular complications are the leading cause of morbidity and mortality among diabetics [Domingueti et al., 2016; Rochette et al., 2014].

Oxidative Stress and Diabetes

Generation of Reactive Oxygen Species

A disruption in the balance between ROS generation and endogenous antioxidant mechanisms is the underlying process in the manifestation of oxidative stress. When the bioavailability of ROS exceeds the buffering capacity of ROS scavengers or the antioxidant system, oxidative stress becomes an issue as it leads to the activation of stress-sensitive intracellular signaling pathways [Fiorentino et al., 2013; Incalza et al., 2018; Sena et al, 2018]. ROS represents chemically reactive molecules containing oxygen, it includes various free radicals and non-free radicals that all have oxidizing effects ($O_2^{\bullet-}$, NO, hydrogen peroxide (H_2O_2)) [Moris et al., 2017; Panth et al., 2016]. The direct damage done by ROS affects lipids, proteins, or DNA and can result in irreversible oxidative alterations [Fiorentino et al., 2013]. The predominant sources of ROS include: dysregulation of mitochondrial complexes, NADPH oxidases (Nox), and "uncoupled" endothelial NO synthases (eNOS) in vascular cells [Incalza et al., 2018; Moris et al., 2017; Sena et al., 2018].

Mitochondria are double membrane organelles that mediate the mechanism of oxidative phosphorylation (OXPHOS) by transferring electrons through complexes in order to produce energy in the form of adenosine triphosphate (ATP) [Yu et al., 2012]. The mitochondria are also the main source of ROS which are generated from this electron transport chain (ETC) as a by-product [Yu et al., 2012]. As depicted in Figure 1, this occurs via the leakage of electrons in Complex I and III which results in the partial reduction of molecular oxygen to $O_2^{\bullet-}$ instead of water [Incalza et al., 2018]. Mitochondrial dysfunction and subsequent ROS over-production occurs under hyperglycemic conditions [Fiorentino et al., 2013.]. Ultimately, the ETC becomes blocked at Complex III and electrons are then donated to molecular oxygen which generates $O_2^{\bullet-}$ [Rolo & Palmeira, 2006].

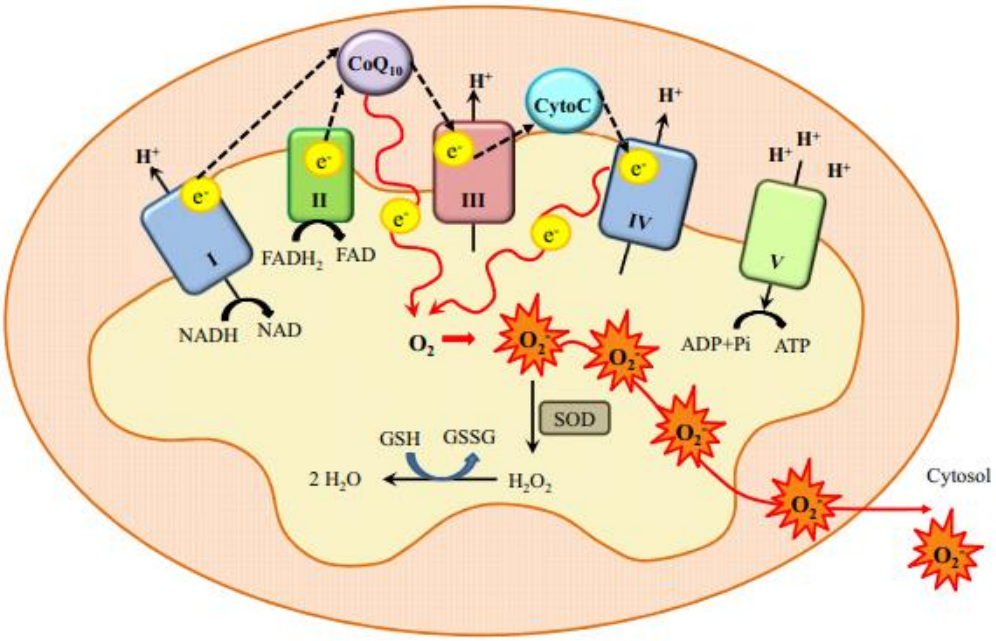

Figure 1: Increased levels of NADH and $FADH_2$ enter the ETC under hyperglycemic conditions. The voltage gradient across the mitochondrial membrane is altered and electron transfer is blocked in Complex III. Consequently, electrons are passed to coenzyme Q_{10} and thus to molecular oxygen (O_2) producing superoxide ($O_2^{\bullet-}$) [Fiorentino et al., 2013].

In addition to ROS production in mitochondria, the single function of the NOX family is to produce ROS by catalyzing the oxidation of molecular oxygen to $O_2^{\bullet-}$ [Bedard et al., 2007]. The main isoform found in endothelial cells is NOX4. Normally, the process of $O_2^{\bullet-}$ and subsequent H_2O_2 generation aids in the proliferation, survival, and migration of endothelial cells [Kim & Byzova, 2014]. However, when the system undergoes pathological levels of oxidative stress NOX4-produced ROS increases the output of pro-inflammatory cytokines, chemokines, and adhesion molecules [Incalza et al., 2018]. This further contributes to the clinical risk factors for atherosclerosis and vascular damage [Sena et al., 2018].

Another mechanism that leads to the generation of ROS is via the eNOS pathway. Vascular homeostasis is regulated, in part, by eNOS which produces NO; this process requires an abundance of the cofactor tetrahydrobiopterin (BH_4) [Alp & Channon; 2004]. Under conditions where ROS concentrations are abnormally high, BH_4 is oxidized to BH_2 and eNOS produce $O_2^{\bullet-}$ instead of NO – a condition known as "uncoupling" [Incalza et al., 2018; Santilli et al., 2015]. Additionally, $O_2^{\bullet-}$ reacts with NO to produce another strong oxidant called peroxynitrite; this

contributes to both mitochondrial dysfunction and endothelial dysfunction alike [Diers et al., 2013; Li & Fostermann, 2014].

Abnormal mitochondrial respiration, NOX4 overexpression, and the "uncoupling" done by eNOS all contribute to the development of diabetes-induced cardiovascular damage. The consequences and effects of these factors produce a vicious cycle which only exacerbates the destruction done to all tissues involved [Incalza et al., 2018; Panth et al., 2016].

Pathways Leading to Tissue Damage

Conditions developed through DM involve free radical accumulation in the vasculature which induces prolonged vessel inflammation and ROS generation [Moris et al., 2017]. As a result, $O_2^{\bullet-}$ overproduction, caused by hyperglycemia, leads to the activation of four pathways (Figure 2) through which tissue damage occurs: the polyol pathway; the hexosamine pathway; activation of protein kinase C (PKC); and increased formation of advanced glycation end-products (AGEs) [Brownlee, 2005].

Nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH) are two antioxidant neutralizing species important to the maintenance of physiological ROS levels [Rochette et al., 2014]. However, under hyperglycemic conditions the polyol pathway experiences an increased shift in glucose flux by four to five times that of homeostatic levels [Katakami, 2018]. Subsequently, glucose is converted to polyalcohol sorbitol which decreases NADPH and GSH levels, leading to the overproduction of $O_2^{\bullet-}$ and the exacerbation of oxidative stress [Giacco & Brownlee, 2010]. Next, sorbitol is metabolized to fructose and other metabolites that trigger a cascade of intracellular imbalances creating precursors of AGEs, and the activation of PKC [Fiorentino et al., 2013]. The increased supply of glucose into the hexosamine pathways generates the capacity to deregulate cellular functions. For instance, it inhibits normal eNOS activity in the endothelial cells and favors the expression of pro-inflammatory and pro-coagulant genes [Fiorentino et al., 2013; Katakami, 2018]. Chronic hyperglycemia causes ROS-induced activation of PKC. In turn, this promotes the activity of pro-oxidant NADPH oxidase, impaired NO production, and inhibits eNOS expression [Moris et al., 2017]. An increase in the activation of the PKC pathway plays a large role in ROS production

contributing towards diabetic atherosclerosis and cardiomyopathy [Giacco & Brownlee, 2010]. Finally, higher intracellular glucose concentrations promote the binding of AGEs to their receptors (RAGE) [Katakami, 2018]. This pathway triggers ROS production and the activation of nuclear factor kB (NF-kB) which causes pathological changes in gene expression [Chilelli et al., 2013; Giacco & Brownlee, 2010].

Hyperglycemia induces a continuous production of ROS that creates massive oxidative stress whereby local antioxidant systems are overtaken by ROS bioavailability [Sena et al., 2018]. All the major pathways linked to the development and progression of diabetic complications are emphasized by oxidative stress [Rochette et al., 2014]. With the beneficial role of antioxidants suppressed by the production of ROS, therapeutic options are needed to boost the capacity of the endogenous defense systems such that the balance between the two is restored.

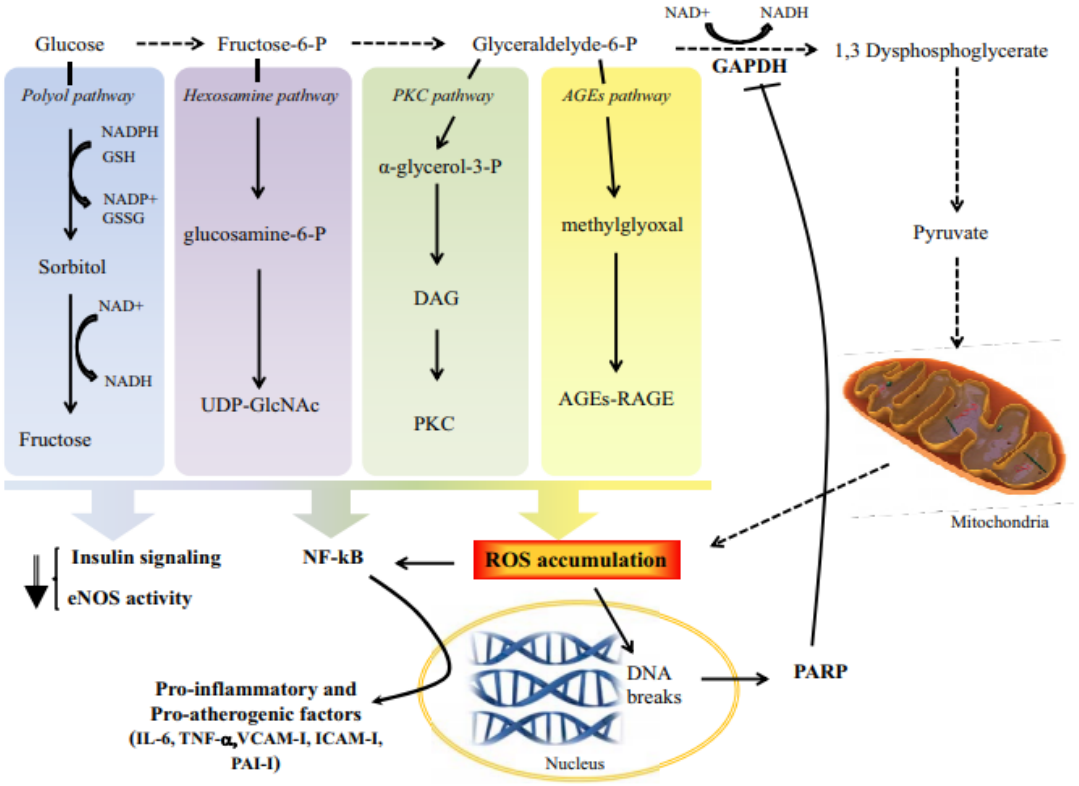

Figure 2: Four main pathways are activated under hyperglycemic conditions: polyol, hexosamine, PKC, and AGEs. The consequences include impaired eNOS activity, the accumulation of ROS, and the expression of pro-inflammatory and pro-coagulant factors. Hyperglycemia-induced mitochondrial dysfunction also promotes ROS production. The resulting oxidative stress triggers NF-kB pathways and induces damage to DNA [Fiorentino et al., 2013].

Antioxidants and Diabetes

Endogenous Antioxidant Defense Systems

Cellular antioxidant compounds and enzymes work as a part of an innate system to remove ROS via metabolic conversion [Park et al., 2018]. Antioxidant enzymes refer to mitochondrial magnesium-superoxide dismutase (SOD), cytoplasmic copper/zinc-SOD, glutathione peroxidase (GPX), thioredoxin peroxidase peroxiredoxin-2 (Prx2), and catalase while non-enzymatic defenses include vitamins A, C, and E, thioredoxin and metallothionein (MT) [Park et al., 2018]. All versions of SOD convert $O_2^{\bullet-}$ into H_2O_2 which is subsequently converted into water and molecular oxygen by catalase, GPX, or Prx2 [Devasagayam et al., 2004; Johansen et al., 2005; Sailaja et al.,2003; Wood et al., 2003]. The endogenous antioxidant defense systems are not meant to dismantle ROS altogether, but to simply maintain the homeostatic balance between the two, such that pathological pathways are not activated [Sena et al., 2018].

Previous treatment of diabetes-induced cardiovascular complications focused on vitamins A, E, and B antioxidants with little success as it is difficult to maintain proper antioxidant levels and correct tissue distribution [Faria & Persaud, 2017; Park et al. 2018]. However, there is evidence suggesting that other antioxidants, such as MT, could play a pivotal role in protecting against the destruction done by oxidative stress [Chiaveri & De Ley, 2010].

Human MTs are metal-binding small proteins rich in cysteine that participate in a multitude of functions. MTs have been recognized to participate in detoxification of heavy metals, trace element homeostasis, cell expression, redox signaling, and more notably, DNA protection against ROS and oxidative stress [Chabosseau & Rutter, 2016; Chiaveri & De Lay, 2010; Park et al. 2018]. Previous studies, using a murine model, have demonstrated that upregulation of cardiac MT plays an important antioxidant role in protecting and preventing diabetic cardiomyopathy [Miao et al., 2013; Wang et al., 2006]. The underlying mechanism that promotes MT expression is mediated through zinc supplementation, which on its own, also induces several other beneficial pathways that protect against cardiovascular damage [Chabosseau & Rutter, 2016; Miao et al., 2013; Wang et al., 2006]. This indicates a shift in the way to approach antioxidant therapeutic treatments, from targeting upregulation of the antioxidant itself, towards

an understanding of all the mechanistic pathways that are involved in the process [Cruz et al., 2015].

Zinc and Diabetes

Zinc homeostasis

As alterations in zinc status contribute to pathogenic changes, homeostatic mechanisms exist to closely regulate zinc absorption, distribution, availability, and excretion [Hu et al.,2018; Woodruff et al.,2018]. The ZnT and ZIP families of zinc transporters, along with zinc-sensitive MTs facilitate the influx, outflux, and compartmentalization of Zn^{2+} in response to physiological changes, for example, during a state of increased oxidative stress or inflammation [Chabosseau & Rutter, 2016; Myers et al.,2012; Zalewski et al.,2019]. ZnT8, ZIP 7, and MT have been specifically highlighted as potential therapeutic targets due to their participation in cell signaling processes involved in DM disease pathways [Adulcikas et al.,2019; Huang et al.,2019; Wang et al.,2006].

Zinc as an antioxidant

The mineral zinc participates in the antioxidant defense system through various protective mechanisms by acting as an essential cofactor for more than 300 enzymes [Cruz et al., 2015]. Notably, zinc is central for the SOD enzyme, an important ROS scavenger [Saharia & Goswami, 2013]. Studies in diabetic rodents have shown that zinc supplementation increases the activity of SOD, demonstrating that low zinc concentrations can lead to impaired antioxidant defense [Cruz et al., 2015]. Another action of zinc includes the modulation of signaling cascades that are beneficial to antioxidant defenses [Oteiza et al., 2012]. Zinc also impacts glutathione metabolism through the regulation of glutamate ligase enzyme expression; this enzyme neutralizes free-radicals on its own and also acts as a cofactor for another important scavenger: GPX [Cruz et al.,2015; Oteiza, 2012].

Diabetes and Zinc Deficiency

In healthy individuals, normal zinc serum levels are generally considered >70 mcg/dL while concentrations below that level are deemed too low and potentially detrimental [Farooq, 2019].

Previous studies have indicated that zinc serum and diabetes are associated, whereby TIDM and T2DM patients exhibit significantly lower values [Basaki et al., 2012; Jansen et al., 2012]. A suggested mechanism for this deficiency is that hyperglycemia interferes with the active transport of zinc in the renal system and causes increased loss of zinc in urine [Jawawardena et al., 2012]. In a recent descriptive cross-sectional study, zinc serum levels were evaluated in 200 diabetic patients and 192 controls. Mean serum zinc was significantly lower in diabetic patients versus controls: 66.54 +11.328 mcg/dL and 82.63 +12.194 mcg/dL, respectively ($p<0.001$) [Farooq, 2019]. It was thus concluded that serum zinc level is negatively associated with poor glycemic control [Chabosseau & Rutter, 2016; Farooq, 2019]. Furthermore, a prospective cohort study in the United States examined 82000 women and found that there was a 17% increased risk of developing diabetes associated with low zinc intake compared to women ingesting sufficient amounts of zinc [Fukunaka & Fujitani, 2018; Sun et al., 2009].

Recent research has underscored the dynamic role of zinc in cellular secondary messaging which controls insulin signaling and mechanisms that are responsive to changes in glycemic conditions [Myers et al., 2012; Norouzi et al., 2017]. As physiological levels of zinc are tightly regulated, this would suggest that abnormal levels of the mineral increase susceptibility to the induction of significant pathological effects [Norouzi et al., 2017]. Consequently, zinc deficiency can now be classified as a risk factor for diabetes, and as such, it is necessary that research be directed at better understanding zinc supplementation and zinc homeostasis [Fukunaka & Fujitani, 2018].

Zinc Supplementation in the Prevention of Cardiovascular Complications

Previous research has focused on the use of zinc supplementation and its effects on cardiac tissue. Lu et al. [2015] used diabetic rats to assess zinc supplementation; they found that there was partial protection against diabetic cardiomyopathy through the decrease in oxidative stress and autophagy in cardiac tissue. Wang et al. [2006] used diabetic mice to also investigate the effects of zinc supplementation against diabetic cardiomyopathy but through an MT mechanism. There was an increase in cardiac MT due to zinc binding and they also found that zinc supplementation protected cardiac cells against free fatty acid cytotoxicity. Wang et al. [2017] looked at the role of various zinc levels in diabetic cardiomyopathy development and progression in diabetic mice. Mice that were fed a zinc deficient diet had: increased non-fasting blood

glucose levels and intraperitoneal glucose tolerance; decreased levels of hepatic zinc and ejection fraction percentage; and increased cardiac fibrosis and left ventricle mass. The group fed a diet with adequate zinc levels did not experience these effects and the diet with additional zinc supplement levels had no significant changes when compared to the adequate zinc diet. Zinc deficient mice also had increased expression of inflammatory markers while the zinc supplemented diet alleviated this when compared to the adequate zinc diet. Ranasinghe et al. [2018] conducted a double-blind randomized control trial with 200 pre-diabetic human subjects to assess effects of zinc supplementation over a year. The zinc group received a capsule containing 20mg elemental zinc daily, while the control group was given a placebo. Significant decreases were found in fasting plasma glucose, 2-hour oral glucose tolerance test, and plasma glucose in the zinc group compared to their controls. 25% of the control group versus 11% in the zinc group went on to develop diabetes; however, significance levels for disease progression were not clear.

Evidence from previous research demonstrate that zinc supplementation could be a viable preventative and protective therapy against diabetes-induced cardiomyopathy and cardiac damage. However, studies have yet to examine the effects that this mineral has on preventing vascular damage. Additionally, research has not used immunoblotting and mitochondrial respiration techniques for verification that proteins themselves are upregulated and less oxidized due to zinc supplementation. Without healthy vessels, the success of zinc supplementation against cardiac damage is limited and unresolved. The destruction done to the vasculature through diabetes-induced oxidative stress would still ultimately lead to fatal cardiac events. Therefore, the interaction between zinc supplementation and vascular damage requires further investigation.

Project Overview

The prevalence of diabetes mellitus is rising at a rapid rate and the associated costs of the disease itself along with resulting complications are already currently a burden on society and the health care system. Diabetes-induced cardiovascular diseases remain the predominant cause of morbidity and mortality [Lu et al.,2015]. Emerging evidence has demonstrated the consequences of zinc dysregulation and its subsequent effect on the mechanisms that promote many pathogenic changes including oxidative stress, inflammation, endothelial dysfunction, and vascular damage.

Although there is evidence supporting the cardioprotective impact that zinc supplementation offers in diabetic subjects, there remains insufficient research targeting the vasculature. The primary goal of this thesis is to provide novel insight on the role of zinc supplementation in the prevention of diabetes-induced injury to the blood vessels and to explore the mechanisms of zinc status which favor the manifestation of oxidative stress. Diabetes demonstrates an imbalance between reactive oxygen species and the antioxidant defense system. Due to the critical link between zinc and the antioxidant system, the hypothesis of our experiments was that zinc supplementation will promote the upregulation of antioxidants, increase mitochondrial respiration, and reduce protein oxidation in the vasculature of zinc supplemented mice. After four weeks of zinc supplementation through the drinking water, mitochondrial respiratory capacity was examined in the aortic vessels. Immunoblotting will determine mitochondrial and antioxidant content, and the degree of protein oxidation in the tissue extracted from both the zinc supplemented and control group. This comparison allowed us to quantify the extent to which zinc supplementation plays a role in the prevention and protection of diabetic vascular tissue.

II

Zinc Homeostasis in Diabetes Mellitus and Vascular Complications*

Stephanie MacKenzie and Andreas Bergdahl

*This project has been submitted to the Journal of Cardiovascular Pharmacology and Therapeutics

Abstract

Oxidative stress represents an impaired metabolic system that promotes damage to cells and tissues. This is the predominant factor that leads to the development and progression of diabetes and diabetic complications. Research has indicated that zinc plays a consequential mechanistic role in the protection against oxidative stress as zinc is required for the proper functioning of the antioxidant system, the suppression of inflammatory mediators, and the modulation of zinc transporters. Recently, the mechanisms surrounding ZnT8, ZIP7, and metallothionein have shown to be of particular pathogenic importance and are considered as potential therapeutic targets in disease management. The literature has shown that zinc dysregulation is associated with diabetes and is considered as a leading contributor to the deleterious vascular alterations exhibited by the disease. Although further investigation is required, studies have indicated the favorable use of zinc supplementation in the protection and prevention of oxidative stress and its consequences over the course of the condition. This review aims to provide a comprehensive account of zinc homeostasis, the oxidative mechanisms governed by zinc status, current therapeutic targets, and the impact of zinc supplementation in the prevention of disease-onset and in mitigating vascular complications.

Introduction

Diabetes mellitus (DM) is a metabolic disease defined as chronic hyperglycemia caused by insulin resistance (IR) or compromised insulin production [Rochette et al.,2014]. These hyperglycemic conditions activate several pathways that generate reactive oxygen species (ROS), with the main source stemming from mitochondrial dysfunction and ER stress along with the polyol, hexosamine, protein kinase C (PKC), and advanced glycation end products (AGEs) pathways [Fiorentino et al.,2013]. Consequently, the over-production of ROS (superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and NADPH-Oxidase (NOX)) coupled with reduced antioxidant capacity promotes a pathological imbalance that leads to oxidative stress and inflammation [Cruz et al.,2015; Fiorentino et al.,2013; Marreiro et al.,2017]. Stemming from these alterations, a wide array of diabetic complications are provoked including diabetic nephropathy, neuropathy and retinopathy, as well as the development of cardiovascular diseases (CVD) [Hu et al.,2018; Incalza et al.,2018]. In addition, individuals with diabetes exhibit a significantly increased risk of heart disease and stroke, and cardiovascular complications are the leading cause of morbidity and mortality within this population [Domingueti et al.,2016].

The divalent metal cation, zinc (Zn^{2+}) exhibits multiple physiological actions as a cofactor for over 300 enzymes and 2000 transporters [Marreiro et al.,2017; Zhao et al.,2019]. As Zn^{2+} can be found in approximately 10% of human proteins, many types of physiological activity depend on Zn^{2+} homeostasis [Zhao et al.,2019]. Zn^{2+} plays an important role in the antioxidant defense system that helps maintain a homeostatic balance by neutralizing ROS. Consequently, a Zn^{2+}-deficient state reduces the buffering capability of the endogenous defense system in response the over-production of ROS exhibited in DM [Chabosseau & Rutter, 2016].

Zn^{2+} acts as an anti-inflammatory agent, provides structural stability to cell membranes, is an important regulator of gene expression, is needed for the correct functioning of glucose and lipid metabolism, mediates cell signaling pathways for cell proliferation and homeostasis, functions as an insulin mimetic and participates in the synthesis, storage, and secretion of insulin, inhibits pro-oxidant enzymes (nicotinamide adenine dinucleotide phosphate oxidase (NADPH-Oxidase)), and is critical for the expression of superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase, and metallothionein (MT), potent antioxidants that act as ROS scavengers [Adulcikas et

al.,2019; Chabosseau & Rutter,2016; Marreiro et al.,2017; Norouzi et al.,2017; Olechnowicz et al.,2018]. Moreover, recent literature has demonstrated an association between Zn^{2+} deficiency and diabetes - a distorted cycle where decreased Zn^{2+} bioavailability subsequently reduces the body's ability to combat rising levels of pro-inflammatory, pro-apoptotic and pro-oxidant agents [Farooq,2019; Fukunaka & Fujitani,2018; Myers et al.,2012]. Fundamentally, the association between DM and Zn^{2+} status is regulated by oxidative stress, inflammation, modulation of Zn^{2+} transporters, and impaired glucose and lipid metabolism [Olechnowicz et al.,2018].

Studies using diabetic mice or rats have observed that Zn^{2+} supplementation is cardioprotective and decreases oxidative stress as well as inflammatory markers [Liu et al.,2015; Wang et al., 2006; Wang et al.,2017]. Zn^{2+} supplementation in gestational diabetes was shown to reduce fasting plasma glucose and insulin levels [Li & Zhao,2019]. And several studies in humans indicated that inadequate intake of Zn^{2+} may increase the risk of diabetes onset [Ranasinghe et al.,2015; Shan et al.,2014; Sun et al.,2009]. This suggests that Zn^{2+} supplementation, particularly in Zn^{2+}-deficient populations, may prevent disruptions of glucose homeostasis [Fukunaka & Fujitani,2018]. However, further investigation is required in order to ascertain the precise efficacy of Zn^{2+} supplementation.

Zn^{2+} homeostasis impacts both the pathophysiology of DM as well as the consequences of this metabolic disease. As such, the development of therapeutic solutions depends upon access to clarity regarding zinc's properties and the extent to which Zn^{2+} can mediate diabetes-induced pathogenic changes and protect against cardiovascular complications. This review will examine the functional role of Zn^{2+} and its transporters, the association of Zn^{2+} status with inflammation, oxidative stress, DM, and the possible mechanisms surrounding zinc's protective role following supplementation.

Zinc Homeostasis

As Zn^{2+} plays a diverse role in cellular processes including cell signaling, enzymatic activity, and gene transcription, homeostatic mechanisms are required to tightly control Zn^{2+} absorption, distribution, intracellular availability, and excretion [Myers et al.,2012; Woodruff et al.,2018]. The cation Zn^{2+} cannot cross lipid bilayers and consequently physiological levels are maintained

by three groups of proteins which regulate inflow, outflow, and compartmentalization of Zn^{2+}: the ZnT and ZIP families of Zn^{2+} transporters and the Zn^{2+}-sensitive metallothioneins (MTs) [Chabosseau & Rutter,2016; Myers et al.,2012; Woodruff et al.,2018; Zalewski et al.,2019]. The ZnT family (SLC30A) are a group of 10 (ZnT1-ZnT10) cation diffusion facilitators that transport Zn^{2+} ions towards the extracellular space or from the cytosol into organelles [Adulcikas et al.,2019; Fukunaka & Fujitani,2018]. The ZIP family (SLC39A), ZIP1-ZIP14, pass Zn^{2+} into the cytoplasm from the extracellular space or from intracellular organelles; [Adulcikas et al.,2019; Fukunaka & Fujitani,2018; Norouzi et al.,2017]. Zn^{2+} binds with MTs until homeostatic conditions change such that Zn^{2+} is required to be released and redistributed in the cells (e.g. in a state of oxidative stress, Zn^{2+} is released from its complex with MT for antioxidant purposes) [Marreiro et al.,2017; Myers et al.,2012].

Movement of the cation is facilitated within a bimodal framework of Zn^{2+} signaling. Early zinc signaling (EZS) is independent of gene transcription and results in a rapid fluctuation of intracellular Zn^{2+} levels via efflux from the organelles into the cytosol [Norouzi et al.,2017] Late zinc signaling (LZS) is slower than the response of EZS because it consists of transcriptional changes in genes and includes the use of storage proteins or transporters. Together, both systems regulate processes involved in metabolism, cell differentiation, proliferation, and growth [Farooq,2019; Fukunaka & Fujitani,2018; Myers et al.,2012; Norouzi et al.,2017; Olechnowicz et al.,2018]. In the liver or muscle, evidence of Zn^{2+} on cellular signaling is exemplified through the inhibition of protein tyrosine phosphatase 1B (PTP1B). This protein negatively regulates insulin signaling pathways

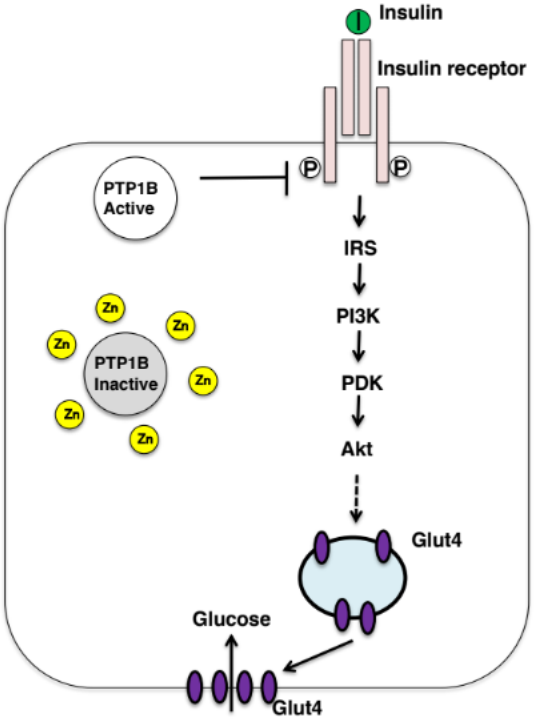

Figure 3. Zinc acting as an insulin-mimetic through its direct effect on the insulin-signaling pathway. IRS, insulin receptor substrate; PI3K, phosphatidylinosol-3-kinase; PKD, protein kinase D; Akt, protein kinase B [Fukunaka & Fujitani, 2018]

whereas Zn^{2+} can extend the insulin signal through the insulin receptor via the inhibition of PTP1B [Adulcikas et al.,2019; Norouzi et al.,2017]. The human MT family consists of 12 operational MTs, with MT1 and MT2 as the major isoforms in the pancreas; these molecules bind Zn^{2+} with high affinity incorporating up to 7 Zn^{2+} ions per molecule [Chabosseau & Rutter,2016; Choi et al.,2018; Mondragon & Bergdahl,2018; Zhao et al.,2019]. As MTs are equipped with reversible dissociation, they can act as Zn^{2+} donors or acceptors [Chabosseau & Rutter,2016; Choi et al.,2018; Mondragon & Bergdhal,2018; Zhao et al.,2019]. Downstream cell signaling is induced by releasing Zn^{2+} either via the oxidation of the sulfur donors in the MT molecule or through the interaction of MT with nitric oxide (NO) [Choi et al.,2018].

Zinc Distribution in the β-cell

To understand the molecular pathways involved in DM and disease processes like IR it is necessary to uncover the contribution that Zn^{2+} and Zn^{2+} transporter mechanisms make towards cell signaling. This will provide the comprehension necessary to delineate the important molecules or pathways that have the greatest therapeutic potential [Norouzi et al.,2017].

Generally, mammalian cells cannot withstand high concentrations of Zn^{2+} as it can quickly cause toxicity, however a small amount is necessary to maintain homeostatic functioning – hence the classification of Zn^{2+} as an essential micronutrient. Unlike elsewhere in the body, a healthy pancreas contains relatively high levels of the mineral [Chabosseau & Rutter,2016]. The predominant source of pancreatic Zn^{2+} is found within the β-cells; specifically contained in the

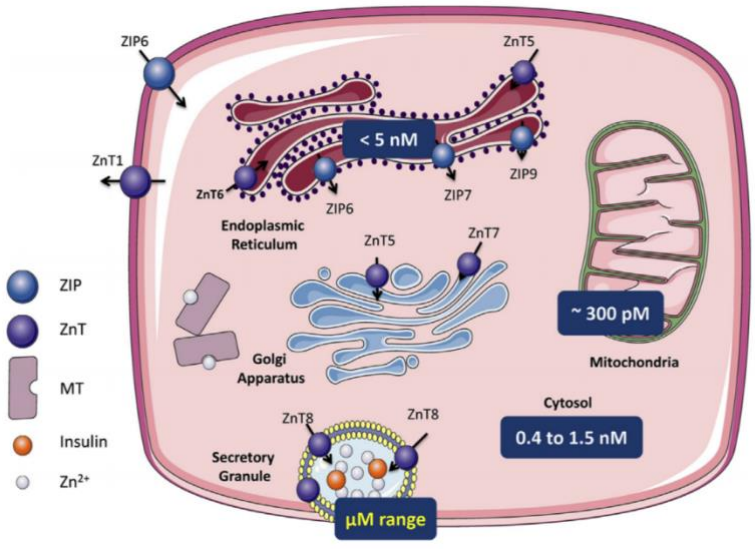

Figure 4: Zinc homeostasis in pancreatic beta cells includes multiple ZnT and ZIP transporters with ZnT8 transporters specific to the ISGs of pancreatic cells [Chabosseau and Rutter, 2016].

dense core of insulin secretory granules (ISG). This presence highlights the critical nature of Zn^{2+} for insulin processing and storage [Chabosseau & Rutter,2016; Fukunaka & Fujitani,2018].

As shown in Figure 4, ZIP6 transports Zn^{2+} from the extracellular area into the β-cell, while ZnT1 moves Zn^{2+} from the cytosol towards the extracellular space. ZnT5 and ZnT6 move Zn^{2+} from the cytosol into the endoplasmic reticulum (ER), while ZIP6, ZIP7, and ZIP9 perform the reverse. ZnT5 and ZnT7 transport Zn^{2+} from cytosol to the Golgi apparatus and ZIP7, ZIP9, ZIP11, and ZIP13 work moving Zn^{2+} out of the Golgi into the cytosol (not pictured) [Norouzi et al.,2017].

ZnT8 is expressed with high specificity to pancreatic cells; it is a transmembrane protein of the ISG in islet beta and alpha cells and moves Zn^{2+} from the cytosol into these cells [Williams & Long,2019]. The selective site and function of ZnT8 establishes its immediate role in glucose homeostasis and insulin biology.

Within the β-cell, an inactive preproinsulin single-chain molecule is produced in the rough endoplasmic reticulum (ER) and is subsequently cleaved to form proinsulin [Huang et al.,2019]. Transporter ZnT8 imports this into the ISG where maturation occurs followed by a second cleavage generating a c-peptide along with a native insulin molecule [Chabosseau & Rutter,2016]. With adequate levels of Zn^{2+} and insulin, a hexamer is formed and to obtain maximum storage capacity within the secretory vesicle, the hexamerization process decreases insulin solubility producing crystallized proinsulin [Chabosseau & Rutter,2016; Huang et al.,2019]. When blood glucose levels are high, the hexamers are converted into active monomers and expelled into the extracellular medium while concurrently freeing-up a considerable concentration of Zn^{2+}. Implications of whether these ions act downstream to further manage the actions of insulin or proceed to other tasks independent of insulin are unclear [Chabosseau & Rutter,2016].

Zinc Transporters: Glucose Homeostasis, Insulin Resistance, and Immunity

ZnT8

Due to the specificity and purpose of ZnT8 within the pancreatic β-cells, recent literature has

examined the potential therapeutic and diagnostic roles of this transporter. Huang et al. [2019] reviewed the functions of ZnT8 in diabetes, highlighting common gene polymorphisms and mutations that may increase the risk of Type 2 diabetes mellitus (T2DM) or have protective effects respectively. Carriers of the SLC30A8 risk allele have lower insulin secretion, less conversion of proinsulin to insulin, decreased insulin sensitivity, and attenuated β-cell function. Meanwhile, other SLC30A8 mutations were linked with the therapeutic efficacy of antidiabetic drugs. The review by Nourazi et al. [2017] shared similar evidence where gene polymorphisms of ZnT8 and carriers of certain risk alleles played a part in the pathogenesis and likelihood of developing T2DM. Fukunaka and Fujitani [2018] suggested that ZnT8 levels determine the risk of T2DM development in mouse models. Electron microscopy analyzed the presence of dense ISGs in β-cells and found that ZnT8-KO mice present irregular granules with distorted or empty cores. These mice also display mild glucose intolerance compared with the control group. The authors performed a pancreas perfusion experiment which indicated that insulin secretion is enhanced in ZnT8-KO mice, but the majority of the insulin produced was degraded in the liver. This indicates that the ZnT8 transporter may play a role regulating insulin clearance in the liver. The authors concluded that ZnT8 is a critical actor in insulin delivery to peripheral organs and subsequently on overall glucose metabolism.

Meanwhile, other literature investigated the relationship between the ZnT8 transporter and the incidence of Type 1 diabetes mellitus (T1DM). Of note, Williams & Long [2019] examined the capacity of ZnT8 autoantibodies (ZnT8A) to predict T1DM through its use as a biomarker to reflect insulin secretory capacity in diabetic patients. They found that accurate measurement of ZnT8A is an efficient method to identify those who are at risk of this disease. However, the review found contrasting evidence as to whether ZnT8A could effectively act as biomarker for therapeutic effect.

In addition to these contributions, further literature focused on the mechanism of ZnT8 in the pathogenesis of DM. Specifically, in a review by Myers et al. [2012], ZnT8 was found to play a role in the pathogenesis of both T1 and T2 diabetes. It was found that ZnT8A targeted ZnT8 in 60%-80% of new onset instances in T1 patients. The β-cell dysfunction and cytotoxicity associated with T1DM has been linked to these autoantibodies [Choi et al.,2018]. In T2DM,

ZnT8 overexpression was linked with increased glucose-stimulated insulin secretion versus downregulation which was consistent with reduced insulin secretion under hyperglycemic conditions. Furthermore, ZnT8 knock-out (KO) mice experienced problems with glucose intolerance and ZnT8 null mice had diet-dependent issues of glucose tolerance, insulin secretion, and body weight. Adulcikas et al. [2019] demonstrated comparable results in ZnT8 null mice: decreased insulin secretion, compromised glucose tolerance, and impaired β-cell function.

Based on previous research, it is clear that ZnT8 is a key player in insulin biology and by extension, glucose homeostasis, making it a prime target for diabetic therapies. Yet, the literature above underscores the complexity of this task. Perhaps a more coherent picture could be obtained by pairing ZnT8 information with the knowledge emerging on other Zn^{2+} transporters?

ZIP7

While T2DM indicates major metabolic dysfunction and aberrant blood glucose levels, IR is the precursor to this disease marked through the body's inability to respond properly or in a timely fashion [Adulcikas et al.,2019]. IR is induced by the distortion of accurate cell signaling and effective glucose uptake into peripheral tissues [Norouzi et al.,2017]. ZIP7 is responsible for transporting Zn^{2+} out of the ER and Golgi into the cytosol; it also controls cell signaling pathways (of note, the insulin receptor substrate-phosphoinositide-3-kinase-protein kinase B

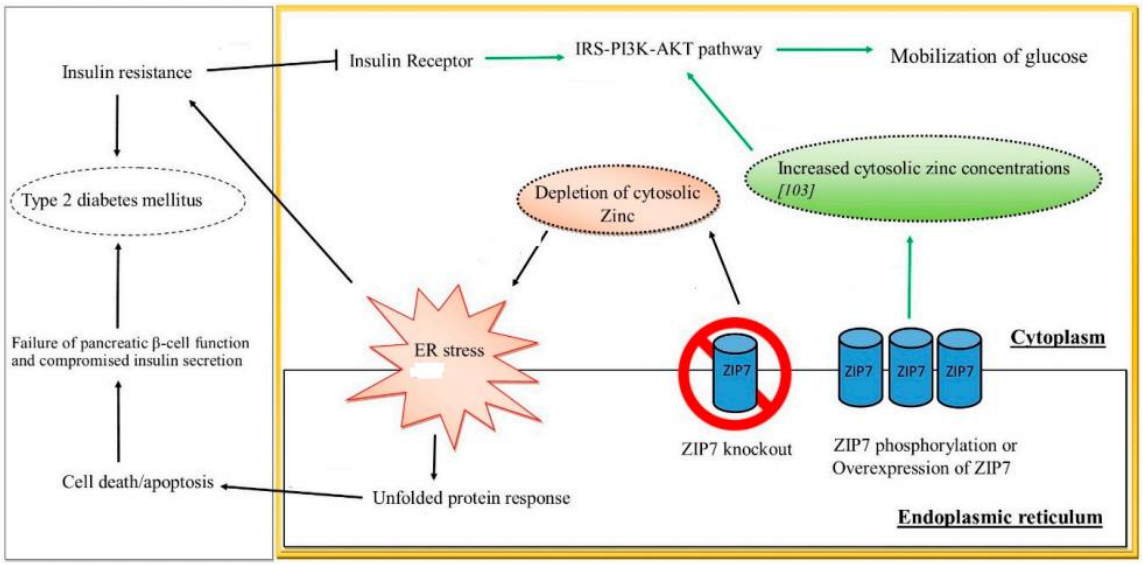

Figure 5: The effects of ZIP7 on glucose mobilization, ER stress, IR, and T2DM as depicted through various regulatory pathways [Adapted from Adulcikas et al.,2019].

(IRS-P13K-AKT) pathway) akin to insulin that initiates glucose uptake in skeletal muscle [Adulcikas et al.,2019; Norouzi et al.,2017]. Literature suggests that ZIP7, as linked with ER stress and cell signaling pathways, is at the root of IR-associated distortions.

As seen in Figure 5, ZIP7 action increases cytosolic Zn^{2+} concentrations which participate in a signaling pathway that elicits glucose mobilization and metabolism [Adulcikas et al.,2019; Anzilotti et al.,2019]. In this regard, Zn^{2+} acts as an insulin mimetic through the phosphorylation of AKT and resultant mobilization of Glut4 transporters to facilitate the influx of glucose in skeletal muscle [Norouzi et al.,2017] In a Zn^{2+}-depleted cytosolic environment, ER protein folding becomes compromised activating the unfolded protein response (UPR); the rate of folding is thus reduced, but if ER stress does not resolve, apoptosis is triggered [Adulcikas et al.,2019].

When tested, the ablation of the transporter led to a significant reduction in cytosolic Zn^{2+} levels, increased ER zinc content, cell proliferation abnormalities, and ER stress [Woodruff et al.,2018]. These findings were congruent with the research done by Anzilotti et al. [2019], where mass spectrometry performed in cells with the loss of ZIP7 displayed low concentrations of cytoplasmic Zn^{2+} and increased Zn^{2+} concentrations within the ER. As a result of Zn^{2+} depletion, ER stress can enhance ROS production and subsequent oxidative stress while byproducts of ER stress are also known to activate inflammatory pathways leading to endothelial dysfunction [Fiorentino et al.,2013]. Therefore, normal Zn^{2+} status and ensuing ZIP7 expression is crucial to curtail ROS generation, impaired glucose uptake, β-cell failure, and IR [Adulcikas et al.,2019; Norouzi et al.,2017].

Prior research demonstrated that low ZIP7 levels in skeletal muscle cause a significant reduction in Glut4 expression, in the phosphorylation of AKT, and defective glycogen synthesis [Myers et al.,2013]. Other studies indicated that hyperglycemic conditions caused the upregulation of ZIP7 in order to alleviate ER stress in pancreatic islet β-cells of mice and in rat cardiomyocytes [Bellomo et al.,2011; Tuncay et al.,2019]. The association between ZIP7 and the pathogenesis of DM was further examined by Norouzi et al. [2019] in insulin-resistant skeletal muscle and mice fed a high-fat diet. IR was created by the treatment of skeletal muscle with an insulin receptor inhibitor or palmitate and mice were fed a high-fat diet or normal chow for 10 weeks.

Immunoblotting conducted on increasing concentrations of glucose in normal skeletal muscle cells confirmed that glucose upregulates ZIP7 expression. However, the expression of ZIP7 and Glut4 were suppressed in the insulin-resistant cells. Furthermore, the high-fat diet mice exhibited a significant reduction of ZIP7 and Glut4 expression compared to the control mice when skeletal muscle tissue was analyzed from both cohorts.

While current literature suggests that ZIP7 may be a contributing factor in the pathogenesis of T2DM. Zn^{2+}-mediated mechanisms involved in skeletal muscle glycemic control require further investigation, particularly in vivo studies of mice and humans.

Metallothionein

MT is a potent ROS scavenger that offers significant protection against DM and DM-induced cardiovascular injury [Miao et al.,2013]. Studies on Zn^{2+} supplementation in diabetic mice show that the expression of MT is significantly induced by cellular Zn^{2+} levels [Miao et al.,2013; Wang et al.,2006]. This mechanism has been further confirmed as Zn^{2+} supplementation up-regulates the expression of MT and consequently decreases diabetes-induced vascular complications [Wang et al.,2006]. Research has shown that MT single nucleotide polymorphisms are related to various pathological processes; 3 of which are linked with a significant increase in T2DM prevalence due to reduced MT antioxidant capabilities [Zhao et al.,2019]. Zn^{2+} effectively determines MT levels through the stimulation of responsive metal transcription factor 1 (MTF-1); this transcription factor directly regulates the expression of MT [Fiorentino et al.,2013].

A review by Choi et al. [2018], describes the importance of MT's antioxidant capabilities whereby reduced MT activity contributes to the pathology of DM. These decreased concentrations were linked with an increased susceptibility to hyperglycemia and oxidative stress in T2DM. Concurrently, overexpression of MT in streptozotocin-induced diabetic mice presented with lower levels of β-cell DNA damage and oxidative stress. While this classical view of MT as an antioxidant has been confirmed, the review by Park et al. [2018] suggests new mechanisms of action for MT.

First, the authors proposed that MT may counteract oxidative stress by reversing mitochondrial dysfunction - the main source of ROS. Mitochondrial dysfunction is a primary contributor to many diabetic issues including β-cell impairment, IR, endothelial dysfunction, and vascular damage [Incalza et al.,2018; Park et al.,2018]. The precise mechanism surrounding MTs' effects on the respiratory chain are unclear, but studies have demonstrated that MT protects against mitochondrial superoxide ($O_2^{\bullet-}$) over-production and exerts myocardial anti-apoptotic effects induced by mitochondrial dysfunction [Cai et al.,2006; Kang et al.,2003; Park et al.,2018]. Other promising diabetic MT protective mechanisms suggested by Park et al. [2018] include: the mitigation of oxidative-induced ER stress via suppression of signaling pathways, the maintenance of cellular autophagy to prevent apoptosis, and the possible anti-inflammatory action of extracellular MTs in the immune system and neuronal health.

Interestingly, Choi et al. [2018], suggests the use of MT in the treatment of DM and DM-induced CVDs. As Zn^{2+} homeostasis plays a prominent role in disease pathology and diabetic complications, an accurate method to analyze Zn^{2+} levels is required. Recent literature has proposed the use of Zn^{2+} transporters or Zn^{2+}-binding proteins (MTs) in circulating blood cells as biomarkers of Zn^{2+} status. As Zn^{2+} itself is not a reliable indicator the expression of MT in leukocytes appears to be the favorable option due to the rapid and dose-dependent manner in which MT responds to changing Zn^{2+} levels. In addition, Zn^{2+} transporters and MTs may also hold diagnostic and prognostic abilities for CVDs. The authors describe how emerging research on extracellular vesicles (EVs) from human liquid biopsies could act as disease-markers, in this case, specifically EVs from endothelial or cardiac tissue. Further investigation to link EVs with a specific Zn^{2+} transporter or MT, and CVDs will be required in order to uncover the true diagnostic and prognostic value of Zn^{2+} status. However, studies have shown that stress-induced levels of MT have been detected in endothelial exosomes, and Zn^{2+} transporters were present among other samples of EVs.

Other ZnT/SLC30A Transporters

The efflux of Zn^{2+} in smooth muscle cells is regulated by the ZnT1, ZnT5, and ZnT9 transporters [Choi et al.,2018]. Studies show that levels of ZnT1 and ZnT2 are impacted by dietary intake of

Zn^{2+} while ZnT4 expression is not diet-dependent [Myers et al., 2012]. ZnT3 transporters are predominantly located within neurons, transporting Zn^{2+} to the synaptic vesicles of glutaminergic hippocampal neurons [Fukunaka & Fujitani,2018; Zhao et al.,2019]. ZnT3 null mice have been shown to demonstrate reduced insulin gene expression and secretion [Myers et al.,2012]. Overexpression of ZnT5 and ZnT7 can prevent cellular death in hyperglycemic conditions, whereas the inhibition of these transporters are linked to an increase in apoptosis [Zhao et al.,2019]. ZnT5 was also found to be abundantly expressed in human endothelial cells while ZnT9 was highly expressed in human and rat hearts [Choi et al.,2018]. Some studies suggested that ZnT7 plays a redundant role of ZnT8 in the pancreas but this remains unclear as ZnT7-KO and ZnT8-KO studies illustrate inconclusive results [Fukunaka & Fujitani,2018; Zhao et al.,2019].

Other ZIP/SLC39A Transporters

The ZIP1 transporter, regulated by testosterone and prolactin, is linked with rapid cellular accumulation and uptake of Zn^{2+} in cells [Myers et al.,2012]. ZIP1 and ZIP13 are also the dominant SLC39A transporters expressed in human endothelial tissue [Choi et al.,2018]. ZIP6 impacts insulin secretion in pancreatic β-cells, whereby down-regulation of the transporter results in dysfunctional insulin secretion in response to glucose [Liu et al.,2015]. ZIP8 action increases intracellular Zn^{2+} levels and may also play a role in lung epithelial cells [Myers et al.,2012]. Zn^{2+} passed to macrophages and monocytes by ZIP8 under inflammatory conditions highlights its key role in protecting against inflammation [Olechnowicz et al.,2018]. ZIP13 is associated with beige adipocyte synthesis and energy metabolism through the use of Zn^{2+} to inhibit adipocyte browning [Chabosseau et al.,2018]. It was found that ZIP13-KO mice produce higher levels of beige adipocytes and consequently improves glucose metabolism and insulin tolerance [Fukunaka & Fujitani,2018]. ZIP14 can transport Zn^{2+}, iron, and manganese, with upregulation occurring under proinflammatory conditions (stress, acute infection, inflammation) when there are elevated concentrations of interleukin-6 (IL-6) and NO [Aydemir & Cousins,2018; Myers et al.,2012]. The acute phase response during inflammation will up-regulate ZIP14 for rapid intake of plasma Zn^{2+} into the organs, primarily the liver, to limit Zn^{2+} availability for invading pathogens [Jarosz et al.,2017]. Additionally, it has been noted that ZIP1,

ZIP7, ZIP13, and ZIP14 are highly expressed in human heart tissue [Choi et al.,2018]. A summary of zinc transporters, regulators, and effects can be found in Table 1.

Table 1: Zinc transporters, regulators, and effect

Zinc Transporter	Regulators	Effect
ZnT1	Metal-responsive mode of regulation; dietary intake of zinc	Efflux of zinc in smooth muscle cells
ZnT2	Metal-responsive mode of regulation; dietary intake of zinc	Zinc transport in vesicles and lysosomes of pancreas, kidney, testis, epithelial cells, small intestine
ZnT3	Glucose status	Transport of zinc to synaptic vesicles
ZnT4	Unaffected by changes in dietary zinc uptake; regulated by extracellular zinc concentrations	Transport of zinc in the trans-Golgi network and in the cytoplasmic vesicular compartment
ZnT5	Glucose status; zinc-responsive elements	Transport of zinc into Golgi lumen for storage
ZnT7	Glucose status	Transport of zinc to Golgi apparatus in retina, liver, epithelial cells, small intestine; may play a redundant role of ZnT8
ZnT8	Glucose status	Regulation of zinc in the secretory vesicles of pancreatic β-cells
ZnT9	Expressed in low levels in response to dietary intake of zinc	Export of zinc out of myocytes; efflux of zinc in smooth muscle cells
ZIP1	Testosterone and prolactin	Uptake of zinc into cells
ZIP6	Estrogen stimulation, glucose status	Downregulation leads to poor insulin secretion
ZIP7	Glucose status	Increases cytosolic zinc concentrations that participate in glucose mobilization and metabolism
ZIP8	Glucose status, TNF-∝ in lung epithelial cells	Increases intracellular zinc levels
ZIP13	Gene mutation leads to loss of function	Inhibition of adipocyte browning
ZIP14	Acute phase response during inflammation; IL-6	Rapid intake of plasma zinc into the organs

Zinc, Inflammation, Oxidative Stress, and Vascular Complications

Zn^{2+} deficiency, a risk factor for DM, is closely associated with increased levels of oxidative stress and the generation of inflammation [Olechnowicz et al.,2018]. A vicious cycle between inflammation and oxidative stress is crafted through pro-inflammatory transcription factors (e.g. nuclear factor kappa-light-chain-enhancer of activated B cells: NF-kB) inducing ROS production and inflammatory cytokine release (IL-6, etc.), which in turn, intensifies oxidative stress [Marreiro et al.,2017]. The NF-kB signaling pathway regulates expression of proinflammatory cytokines, acute phase proteins (C-reactive protein: CRP), matrix metalloproteinases (MMPs), and is responsible for genes that dictate apoptosis, proliferation, cell adhesion, tissue remodeling, cellular-stress responses, inflammatory processes, and immune responses [Jarosz et al.,2017; Olechnowicz et al.,2018]. Within this framework, the accumulation of vascular oxidative stress results in endothelial dysfunction whereby cells activate an inflammatory response that further perpetuates the cycle [Domingueti et al.,2016; Sena et al.,2018]. These circumstances lead to increases of apoptotic cells, extracellular matrix remodeling, and further endothelial dysfunction [Sena et al, 2018]. Dysfunction at the cellular level triggers vascular dysfunction which ultimately results in both micro- and macro-vascular diseases [Domingueti et al.,2016; Rochette et al,2013; Sena et al.,2018].

The presence or absence of Zn^{2+} regulates NF-kB transcription. Zn^{2+} deficiency induces activation of NF-kB, promoting oxidative stress, whereas Zn^{2+} homeostasis alleviates inflammation and oxidative damage [Jarosz et al.,2017] This is accomplished via the anti-inflammatory, antiapoptotic, Zn^{2+}-protein complex A20 and the peroxisome proliferator-activated receptors (PPARs) [Jarosz et al.,2017; Marreiro et al.,2017; Olechnowicz et al.,2018]. A study with endothelial cells demonstrated that cells with high Zn^{2+} content had upregulated expression of A20 which decreased NF-kB activity and thus suppressed production of pro-inflammatory cytokines [Prasad,2014]. Moreover, Zn^{2+} supplementation was shown to increase the concentration of A20 and PPAR-α inhibiting NF-kB signaling, and thus reducing the levels of ROS, inflammatory cytokines, and adhesion molecules that would otherwise lead to the development of atherosclerosis [Bao et al.,2010]. Another mechanism of Zn^{2+}-induced NF-kB inhibition is accomplished via Zn^{2+} ion transport through the up-regulation of ZIP8 into macrophages and monocytes during inflammatory conditions [Olechnowicz et al.,2018]. These

Zn^{2+} ions block the downstream activity of IkB kinase complex (IKK) and ergo is unable to phosphorylate and degrade IkB - the inhibitor of NF-kB; ultimately this prevents the translocation of NF-kB and its ability to target gene expression [Jarosz et al.,2017]. Zn^{2+} is also a known inhibitor of N-methyl-D-aspartate (NMDA); where Zn^{2+} deficiency activates this receptor to produce elevated levels of intracellular calcium which, in turn, promotes neuronal cells to release substance P, causing increased concentrations of inflammatory cytokines, free radicals, and oxidative stress [Marreiro et al.,2017]. Additionally, Zn^{2+} status contributes to inflammation and atherosclerosis by its regulation of Zn^{2+}-dependent MMPs, where inadequate levels of Zn^{2+} initiate pathological signaling pathways [Olechnowicz et al.,2018].

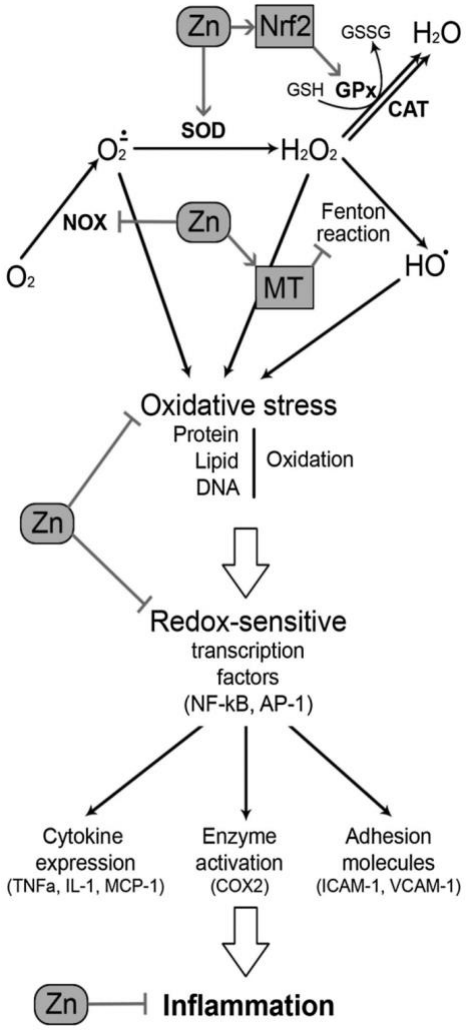

Figure 6: The various pathways in which zinc influences oxidative stress and inflammation [Adapted from Olechnowicz et al.,2017].

Furthermore, oxidative stress can impair protein folding in the ER, and conversely, improper protein folding brought on by ER stress (as a result of Zn^{2+} depletion) can enhance ROS production [Fiorentino et al.,2013]. This highlights the critical nature of Zn^{2+} homeostasis such that the ER Zn^{2+} transporter ZIP7 is properly expressed to mitigate ROS generation, impaired glucose uptake, β-cell failure, and IR [Adulcikas et al.,2019; Norouzi et al.,2017]. Induction of ER stress byproducts are also known to participate in the activation of an NF-kB pathway that facilitates inflammation, endothelial dysfunction, and the progression of diabetic complications [Fiorentino et al.,2013].

In the endogenous defense system, depicted in Figure 7, Zn^{2+} has the capacity to influence antioxidant functioning through direct and indirect methods. Zn^{2+} directly inhibits the pro-oxidant enzyme NADPH-Oxidase and also contributes to proper functioning of many antioxidant ROS scavengers; the metal has a strong influence on the expression of MT, it is a structural

component of SOD, and it promotes the expression of an enzyme (glutamate cysteine ligase) involved in GPX synthesis [Marreiro et al.,2017]. The transcription factor, nuclear factor erythroid 2-related-factor 2 (Nrf2) controls the expression of the genes that encode for these antioxidants and others; and it is Zn^{2+} that regulates Nrf2 activity [Jarosz et al.,2017]. Studies in Zn^{2+}-deficient mice have shown a significant reduction in Nrf2 expression in conjunction with elevated levels of oxidative damage [Zhao et al.,2011].

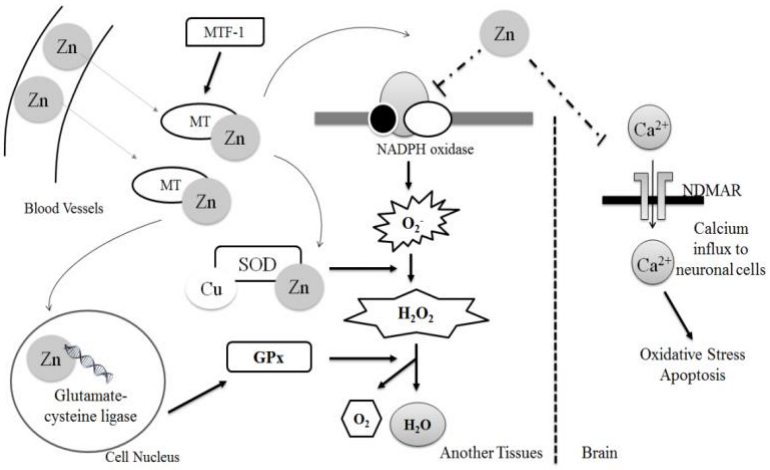

Figure 7: The mechanistic role of zinc in the antioxidant defense system [Marreiro et al.,2017].

As a result, Zn^{2+} deficiency provokes an environment where the body is unable to defend against an accumulation of oxidative damage. This oxidative stress induces endothelial dysfunction and atypical changes in vascular smooth muscle cells; promoting the propagation and migration of these cells in atherosclerotic lesions [Fiorentino et al.,2013]. However, healthy Zn^{2+} status and the regulation of Nrf2 mitigate the damage done to cells and vessels per the expression of antioxidants while buffering the ROS generated from hyperglycemia, inflammation, and ER stress [Marreiro et al.,2017].

It is clear that Zn^{2+} deficiency in DM promotes rampant expression of immune mediators while enhancing oxidative stress and an immune response that is unable to be constrained; the outcome leads to many pathological changes that aggravate disease progression and cardiovascular complications [Olechnowicz et al.,2018].

Zinc Supplementation

Emerging evidence on the complex yet critical nature of Zn^{2+} homeostasis in the pathophysiology of diabetes has shifted research focus towards the analysis of therapeutic Zn^{2+}

supplementation and its associated mechanistic pathways.

A large portion of previous research has focused on the use of Zn^{2+} supplementation relative to diabetic cardiomyopathy. Lu et al. [2015] used diabetic rats to assess the effects of Zn^{2+} supplementation on cardiac tissue. Their results demonstrated partial protection against diabetic cardiomyopathy through decreased oxidative stress and autophagy in cardiac tissue. Wang et al. [2006] also investigated the consequences of Zn^{2+} supplementation against diabetic cardiomyopathy in a murine model but through a mechanism driven by MT. They found an increase in cardiac MT due to Zn^{2+} binding and results indicated that Zn^{2+} supplementation protected cardiac cells against free fatty acid cytotoxicity. Another study in db/db mice by Wang et al. [2017] looked at the role of various Zn^{2+} levels (deficient, adequate, or supplemented) in diabetic cardiomyopathy development and progression. Mice fed a Zn^{2+}-deficient diet had: increased non-fasting blood glucose levels and intraperitoneal glucose intolerance; decreased levels of hepatic Zn^{2+} and ejection fraction percentage; and increased cardiac fibrosis and left ventricle mass. The group receiving adequate Zn^{2+} levels did not experience these effects and the cohort taking additional Zn^{2+} supplementation had no significant changes when compared to the adequate Zn^{2+} diet. Zn^{2+}-deficient mice also had increased expression of inflammatory markers while the Zn^{2+} supplemented diet alleviated this compared to the adequate Zn^{2+} diet. It was shown by Cooper-Capetini et al. [2017] that Zn^{2+} supplementation in mice fed a high-fat diet promoted pancreatic β-cell function as seen through revived glucose-stimulated insulin secretion, glucose tolerance, and improved HOMA-β. Additionally, Zn^{2+} supplementation was found to upregulate MTs in MDCK cells and subsequently alleviate oxidative stress and apoptosis [Li et al.,2019]. T1-diabetic mice treated with Zn^{2+} experienced a significant reduction in hepatic oxidative stress, ER stress, and cell death compared to the controls [Liang et al.,2015]. In addition, progression of diabetic nephropathy in rats was relieved by Zn^{2+} supplementation by reducing the overexpression of molecular markers associated with oxidative stress [Barman et al.,2018].

In humans, a double-blinded randomized control trial was conducted with 200 pre-diabetic subjects to assess effects of Zn^{2+} supplementation over a year [Ranasinghe et al.,2015]. The Zn^{2+} group received 20 mg of Zn^{2+} daily, while the control group was given a placebo. Significant

decreases were found in fasting plasma glucose, 2-hour oral glucose tolerance test, and plasma glucose in the Zn^{2+} group compared to their controls. 25% of the control group versus 11% in the Zn^{2+} group went on to develop diabetes; however, differences in significance levels for disease progression were unclear. Zn^{2+} supplementation was also studied in women with gestational diabetes; reducing subjects' serum insulin, insulin resistance, and fasting plasma glucose [Olechnowicz et al.,2018]. Furthermore, a meta-analysis of five randomized controlled trials, 263 subjects, examining Zn^{2+} supplementation in gestational diabetes concluded that although LDL and total cholesterol were unaffected, treatment decreased measurements of insulin, fasting plasma glucose, and HOMA-IR [Li & Zhao,2019]. In the United States, a prospective cohort study examined 82,000 women and found that insufficient intake of Zn^{2+} caused a 17% increased risk developing diabetes versus women with acceptable amounts of Zn^{2+} [Fukunaka & Fujitani,2018; Sun et al.,2009]. A study in China suggested that glucose tolerance and risk of diabetes is controlled by the interaction of ZnT8 dysfunction and reduced levels of plasma Zn^{2+} [Shan et al.,2014]. However, in other cases, the link between Zn^{2+} status, glucose metabolism, and IR lacked clarity [Olechnowicz et al.,2018]. While most of these results indicate that Zn^{2+} supplementation, particularly in Zn^{2+}-deficient populations, may prevent disruptions of glucose homeostasis, efficacy of such supplementation remains inconclusive and requires further investigation [Fukunaka & Fujitani,2018].

Conclusion

The full extent of zinc's role in the molecular mechanisms involved in the pathogenesis and pathophysiology of many chronic diseases, including diabetes, has not yet been fully uncovered [Marreiro et al.,2017]. Yet, current literature acknowledges the deleterious effects of dysregulated Zn^{2+} homeostasis in diabetes and its role in the development of both microvascular and macrovascular complications [Choi et al.,2018; Fiorentino et al.,2013; Jarosz et al.,2017; Marreiro et al.,2017]. Inadequate levels of Zn^{2+} result in impaired antioxidant functioning, cytokine over-expression, chronic inflammation, ROS accumulation and oxidative damage, distorted lipid and glucose metabolism, ER stress, β-cell defects and apoptosis, IR, endothelial dysfunction, and the evolution of cardiovascular complications [Olechnowicz et al.,2018]. Zn^{2+} depletion is now a hallmark characteristic of DM and is at the core of many pathogenic changes driven by hyperglycemia-induced oxidative stress and DNA damage. As such, clinical

management of Zn^{2+} nutrition over the course of the condition is paramount in maintaining Zn^{2+} homeostasis and subsequent regulation of MT and Zn^{2+} transporter expression [Barman et al.,2017].

Future research is required for the expression of Zn^{2+} transporters and MT as biomarkers for Zn^{2+} status, along with additional analyses of EVs from liquid biopsies [Choi et al.,2018]. More prospective cohort studies are needed to ascertain whether Zn^{2+} supplementation is indeed an effective agent in the prevention of diabetes onset [Fukunaka & Fujitani,2018]. And further investigation of Zn^{2+} supplementation in humans is necessary such that a clinical standard of care, relative to Zn^{2+} therapy, may be implemented jointly with other treatments.

Although the complete mechanistic relationship between Zn^{2+} status and occurrence of chronic disease remains obscured, studies have established that Zn^{2+} deficiency, by induction of inflammation and oxidative stress, promotes the onset/progression of many conditions (DM, metabolic syndrome, obesity, cancer, kidney disease, neurodegeneration, atherosclerosis, CVDs) [Fukunaka & Fujitani,2018; Jarosz et al.,2017; Marreiro et al.,2017]. The comprehensive effect of Zn^{2+} homeostasis on the immune system is undeniable; this suggests that restoring abnormal levels of the metal through supplementation may prove to be an effective therapeutic measure, not only for diabetes, but for overall human health.

III

Zinc Supplementation Enhances Vascular Mitochondrial Respiration in Diabetes Mellitus*

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*This project has been submitted to the journal Antioxidants

Contributions

Stephanie MacKenzie: preparation of manuscript, experimental techniques

Dana-Rae Reguis Yadao: experimental techniques

Andreas Bergdahl: editing of manuscript, experimental techniques

Abstract

Mitochondria are organelles predominantly associated with energy production via oxidative phosphorylation (OXPHOS). This process uses glucose-based substrates to generate adenosine triphosphate (ATP) while concurrently producing small amounts of harmful reactive oxygen species (ROS) that are neutralized through the endogenous antioxidant system. However, the hyperglycemic conditions exhibited in diabetes mellitus induce pathological changes that alter energy metabolism, disproportionately increasing levels of ROS with the main source stemming from mitochondrial dysfunction. Previous research has highlighted the critical role of zinc towards the effectiveness of the antioxidant system. Additionally, studies have demonstrated that zinc-supplementation acts in a protective capacity against diabetic cardiomyopathy. Due to the wide-ranging impact of mitochondrial health on cellular functions, the analysis of zinc supplementation on mitochondrial OXPHOS becomes paramount. This study investigated the effects of zinc supplementation on mitochondrial function in diabetic male mice following a four-week intervention. Mitochondrial oxidative capacity and density of mitochondria were analyzed. Results revealed that mitochondrial respiration is significantly more efficient in the zinc-supplemented group compared to the controls while no significant differences were observed in mitochondrial density. These findings suggest that zinc supplementation may have the potential to prevent deleterious alterations in mitochondrial health by augmenting mitochondrial capacity.

Introduction

Mitochondria participate in a variety of essential cellular functions including regulation of energy, apoptosis signalling, and calcium homeostasis [Amuda &Hotamisligil, 2015; Wada & Nakatsuka, 2016]. This organelle is predominantly associated with oxidative phosphorylation (OXPHOS) - the transfer of electrons through complexes to produce energy in the form of adenosine triphosphate (ATP) [Yu et al., 2012]. During OXPHOS, mainly glucose-based substrates are required to generate ATP while concurrently producing small amounts of harmful reactive oxygen species (ROS) which are ultimately buffered through antioxidant defense [Panth et al., 2016]. However, under hyperglycemic conditions, increased levels of NADH and $FADH_2$ enter the mitochondrial electron transport chain (ETC) altering the voltage gradient across the mitochondrial membrane [Fiorentino et al., 2013]. Consequently, ROS is produced in the ETC as mitochondrial function is impaired, resulting in the partial reduction of molecular oxygen to superoxide ($O_2^{\bullet-}$) instead of water [Incalza et al., 2018; Yu et al., 2012]. The hyperglycemic conditions characterized in diabetes mellitus (DM) provoke the activation of several pathways that participate in the generation of ROS; with the main source stemming from mitochondrial dysfunction [Fiorentino et al., 2013]. As a result, a vicious cycle is established: the subsequent generation of ROS leads to further mitochondrial dysfunction which in turn produces more ROS [Panth et al., 2016]. The consequences and effects of these factors produce a self-perpetuating spiral that exacerbates the destruction done to all tissues involved [Incalza et al., 2018; Panth et al., 2016]. Due to the wide-ranging impact of mitochondrial health on cellular functions, the analysis of mitochondrial OXPHOS becomes paramount towards understanding the pathophysiology of many diseases including oxidative stress, aging, and diabetes [Kuznetsov et al. 2019].

Previous research has focused on the use of zinc supplementation in diabetic cardiomyopathy. Lu et al. [2015] demonstrated that zinc supplementation had partial cardioprotective effects in diabetic rats through decreased oxidative stress in cardiac tissue. Wang et al. [2006], using a murine model, found that zinc prevented diabetic cardiomyopathy via the up-regulation of the zinc-binding protein metallothionein (MT) - a potent antioxidant. Wang et al. [2017] investigated the role of various zinc levels (deficient, adequate, and supplemented) on diabetic cardiomyopathy in mice. Results confirmed that zinc deficiency contributes to many of the

pathogenic alterations seen in diabetic cardiomyopathy and that adequate intake of zinc could protect against these changes.

DM is associated with zinc deficiency, whereby diabetics exhibit markedly lower levels of the mineral compared to healthy individuals [Chabosseau & Rutter, 2016; Fukunaka & Fujitani, 2018]. Zinc plays an important role in maintaining the functionality of the endogenous antioxidant system which is key in mitigating the deleterious effects of mitochondrial ROS [Cruz et al.,2015]. The combination of a defective antioxidant system due to a zinc deficiency and the over-production of ROS caused by mitochondrial dysfunction results in oxidative stress displayed through lipid peroxidation and oxidative damage to cells, proteins, enzymes, and DNA [Cruz et al.,2015; Rochette et al.,2014]. This promotes the production of inflammatory mediators, which in turn, creates a persisting state of chronic inflammation that consequently elicits endothelial dysfunction - the main prerequisite in the development of cardiovascular diseases [Fiorentino et al.,2013]. The effects of oxidative stress on mitochondrial health and the vasculature coupled with a zinc deficiency that stunts the ability of the antioxidant defense system to properly neutralize ROS are the reasons why the leading cause of morbidity and mortality in DM is cardiovascular disease [Lu et al.,2015; Marreiro et al.,2017].

These conclusions indicate that zinc deficiency seen in DM greatly enhances the consequences of oxidative stress on cardiac tissues and would also suggest that zinc supplementation may have therapeutic value. However, little is known of zinc's effects on mitochondrial health and the prevention of mitochondrial dysfunction that is tightly associated with DM.

The objective of this study was to investigate the effects of zinc supplementation on mitochondrial function in streptozotocin (STZ) induced diabetic mice following a four-week intervention. Mitochondrial oxidative capacity and density of mitochondria was analyzed. Given that diabetics exhibit an imbalance between ROS and the buffering capacity of the antioxidant defense system, in addition to the crucial role of zinc in the antioxidant system, we hypothesized that zinc-supplemented diabetic mice belonging to the intervention group will exhibit greater

mitochondrial respiration and higher levels of mitochondrial density.

Materials and Methods

Animal Care

Mice were obtained from the Concordia University breeding colony. For the purpose of the study, age-matched (2-4 months), healthy male C57B1/6 mice were individually housed in a room with a 12-hour light/dark cycle; temperature was kept at a constant 22°C. Mice were randomly assigned into two groups: diabetic given regular tap water, which served as the control (CON), and the diabetic group with zinc supplementation; receiving an estimated daily dose of 2.5 mg Zn/kg in the drinking water (Zn). Blood-glucose concentration was measured 2 days post-injection from the mouse-tail-vein by the One Touch Verio Flex glucometer. Diabetes was induced and confirmed prior to the onset of the 4-week protocol via a peritoneal injection (150mg/kg) of streptozotocin (STZ). Outputs above 14mmol/L were taken as diabetic. Mice had continual access to standard rodent chow and water or zinc-water dependent upon group designation. The Concordia University Animal Research Ethics Committee approved all procedures (#30000259) and all measures were certified by the Canadian Council on Animal Care.

Experimental Protocol

Over a period of 4 weeks, the zinc group received an estimated daily dose of 2.5 mg Zn/kg in the form of $ZnSO_4$ administered to their drinking water. This specific dosage of zinc was provided on the basis of previously accepted experimental protocols [Miao et al., 2013; Wang et al., 2006]. Calculations to achieve this dose via water ingestion was based on previous research [Yadao, MacKenzie & Bergdahl, Manuscript in preparation] with respect to the average amount of water a diabetic mouse ingests over the course of one week.

Throughout the duration of the experiment, zinc mice had their remaining zinc-water emptied three times a week and were given fresh zinc-supplemented water. The respective tap water for the control group was also provided three times per week.

Tissue Permeabilization and Preparation

Upon completion of the 4-week protocol, the animals were euthanized using CO_2. The aortic vessels were harvested and promptly stored in an ice-cold buffer solution to avoid degradation of cell surface proteins. The solution (BIOPS) contained the following in mmol/L: CaK_2EGTA 2.77, K_2EGTA 7.23, Na_2ATP 5.77, $MgCl_2 \cdot 6H_2O$ 6.65, taurine 20, phosphocreatine 15, imidazole 20, dithiothreitol 0.5, MES 50; pH 7.1. Adipose and connective tissue were removed using a stereo microscope and the vessels denuded with a sponge in ice-cold BIOPS buffer. Once tissues were fragmented into smaller sections, the plasma membrane was permeabilized for 30 minutes in 2 ml BIOPS buffer that contained 50µg/ml of saponin and underwent two 10-minute washes in 2 ml of ice-cold Mir05 buffer [Larsen et al.,2015]. The Mir05 buffer contains (in mmol/L): EGTA 0.5, K-lactobionate 60, $MgCl \cdot 6H_2O$ 3.0, Taurine 20, KH_2PO_4 10, HEPES 20, sucrose 110, BSA 1g/1, pH 7.1.

Mitochondrial Respiration

Oxygen consumption was measured using a polarographic oxygen sensor (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) at 37°C in MiR05. Around 2.0-2.5 mg of tissue (wet weight) was put inside either chamber of the Oxygraph. In order to correct for any errors in either chamber from one day to the next, the tissue collected was tested alternatively between the two chambers. The DatLab On-line program [Gnaiger, 2012] resolved O_2 flux; and to avoid O_2 diffusion limitations all experiments were conducted in hyperoxygenated levels. In order to examine each mitochondrial complex independently, a protocol was used [Yadao, MacKenzie & Bergdahl., Manuscript in preparation] which directed that the following substrates were to be injected into each chamber sequentially: malate (2mmol/L); pyruvate (2 mmol/L); glutamate (10mmol/L); ADP (5 mmol/L); 20µl succinate (10mmol/L); oligomycin (2µg/ml), and antimycin A (2.5 µM). Complex I, state 2 respiration was studied via the addition of malate + pyruvate + glutamate; this highlights the ADP-restricted, non-phosphorylating basal resting state. Complex I, state 3 was studied through the presence of ADP. This provided an index of oxidative phosphorylation (OXPHOS). Maximal state 3 respiration was analyzed through the simultaneous activation of Complex I and Complex II by injection of succinate. Complexes V and III were blocked by the inhibitors oligomycin and antimycin A, respectively. The Oroboros DatLab

software noted and analyzed respiration rates and O_2 consumption in each condition for a period of 3-5 minutes while the actual data presented reflects the average respiration rate calculated over the last minute of this period.

Immunoblotting and Immunoflorescence

The aortic vessels underwent cell lysis by using a lysis buffer according to Rocha et al. (2015) containing (in mmol/L): NaCl 250, HEPES 50, EGTA 1, NaF 1, glycerol 10%, Triton X-100 1%, $MgCl_2$ 1.5, $Na_4P_2O_7$ 10, Na_3VO_4 800 µmol/L, and (pH=5). For 10 minutes the solution was centrifuged at 13000 g, after which the supernatant was obtained. Protein levels were assessed using the Pierce BCA Protein Assay Kit (Thermo Scientific, Mississauga, Ontario, Canada) with 10µg of protein samples initially separated on a 12.5% SDS-PAGE. This was followed by transfer with 10mmol/L sodium tetraborate buffer onto a nitrocellulose membrane (0.45 µm, 162-0115, Bio rad). An effective load and transfer were confirmed through the use of a Ponceau S staining. Over a 1-hour period, 5% BSA in TBS-T buffer (10 mmol/L Tris-HCL, pH 7.5, 150 mmol/L NaCl, 0.05% Tween 20) was utilized as a membrane block at room temperature. An overnight incubation period 4°C proceeded with the antibody for voltage dependent anion channel (VDAC; 1:1000, ab14734 Abcam) along with total OXPHOS rodent antibody cocktail (1:2000, MS604 MitoSciences). The blots derived from this process were then washed and incubated with horseradish-peroxidase-conjugated secondary antibodies (anti-mouse, ab6728 Abcam). A chemiluminescence system was used to visualize and examine relative protein expression (Immun-Star Chemiluminescent, 1705070; Bio-Rad, Mississauga, Ontario, Canada) while bands were analyzed by ImageJ software.

Statistical Analysis

A two-tailed Student's *t* test was used to analyze the data to compare between the CON and Zn groups. The data obtained from immunoblotting and other figures are presented as means $\pm$SE. Statistical significance was $P < 0.05$. A Gibbs test and a Dixon test were conducted to identify outliers.

Results

Mitochondrial density

In order to control for differences in mitochondrial content between the Zn and CON groups, antibody-specific immunoblotting was conducted. The experiments were performed using an antibody that was particular to the voltage-dependent anion channel (VDAC). There were no significant differences in mitochondrial density between the diabetic control group and the diabetic zinc-supplemented group (CON: 100% ± 8.5% versus Zn: 98% ± 2.3%). This indicates that the effects exhibited at the functional level relate to the activity of the mitochondria themselves rather than the number of mitochondria.

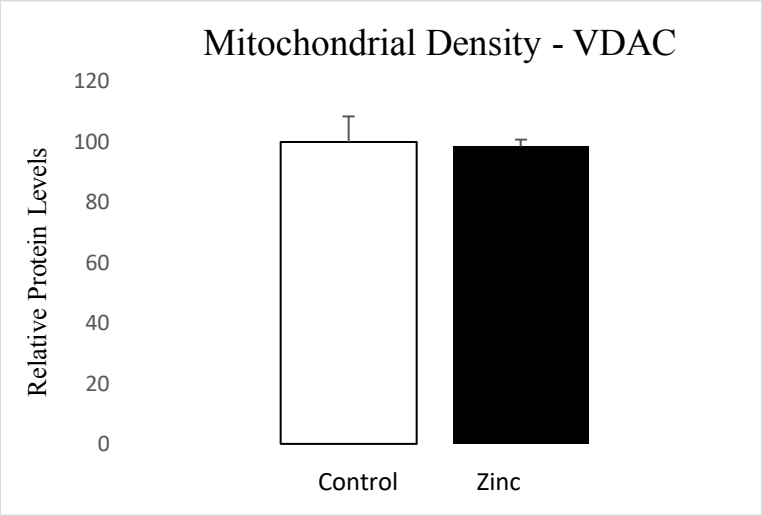


Figure 8: No significant differences were observed in relative protein levels between the diabetic control group and the diabetic zinc-supplemented group, 100% ± 8.5% and 98% ± 2.3%, respectively.

Mitochondrial Proteins

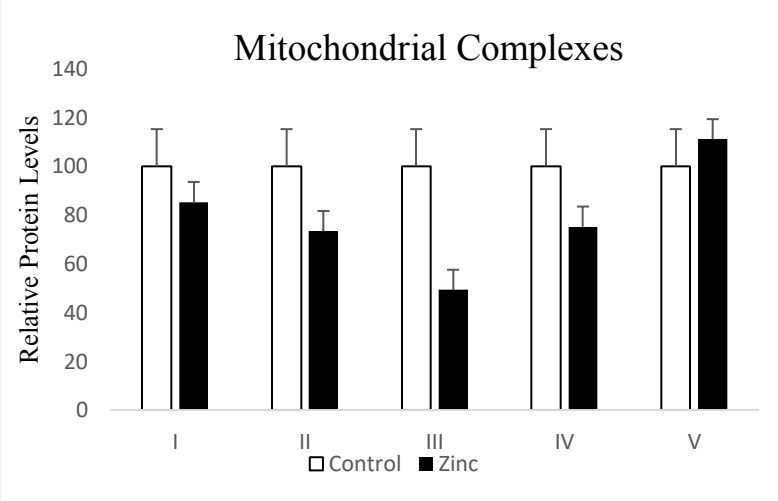
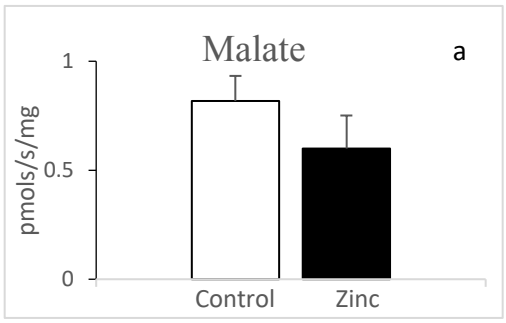


Figure 9: All complexes, in both the control and zinc cohorts, exhibited no significant differences in the expression of mitochondrial subunits. Immunoblots of mitochondrial complexes I-V were performed with total OXPHOS rodent antibody cocktail from MitoSciences (MS604).

Findings from immunoblots revealed no significant differences between the zinc and control cohorts among complexes I-V. Complex I (CON: 100% ± 20.5% versus Zn: 85% ± 6.8%) complex II (CON: 100% ± 15.7% versus Zn: 73% ± 5.6%), complex III (CON: 100% ± 18.0% versus Zn: 49% ± 14.7%), complex IV (CON: 100% ± 11.5% versus Zn: 75% ± 15.5%), and complex V (CON: 100% ± 15.2% versus Zn: 111% ± 8.3%). However, there was a trend towards downregulation in the zinc group for complex III, one of the ROS producing complexes.

Mitochondrial respiration

High resolution respirometry was used to measure rates of oxygen consumption. This data is indicative of mitochondrial function and respiratory capacity in permeabilized tissues. After the injection of malate, and subsequently pyruvate and glutamate, there were no significant differences observed in OXPHOS capacity for Complex I activation in an ADP restricted environment between the zinc and control groups. However, these results do exhibit a downwards trend, especially for pyruvate, which indicates a reduction in basal respiration (Zn: 0.60 ± 0.15 versus CON: 0.82 ± 0.12; Zn: 1.08 ± 0.24 versus CON: 1.67 ± 0.30; Zn: 1.33 ± 0.15 versus CON: 1.58 ± 0.26, respectively). After the addition of ADP, the zinc group demonstrated an OXPHOS capacity that was significantly increased, signifying more efficient use of substrates (Zn: 4.14 ± 0.52 versus CON: 2.56 ± 0.36, $p<0.05$). In addition, the acceptor control ratio (ACR), representing the degree of coupling between oxidation and phosphorylation in an activated Complex I, was found to be significantly elevated in the animals that received zinc (Zn: 3.18 ± 0.39 versus CON:1.49 ± 0.20, $p<0.01$). Meanwhile, the respiratory control ratio (RCR) that represents the degree of coupling between O_2 consumption and OXPHOS in Complex I+II was also significantly elevated in the animals that received zinc supplementation (Zn: 1.94 ± 0.11 versus CON: 1.49 ± 0.08, $p<0.01$). After the addition of succinate, an indicator of maximal oxygen utilization from the activation of Complex I + II, the zinc cohort exhibited significantly lower results (Zn: 7.03 ± 0.47 versus CON: 9.40 ± 0.72, $p<0.05$).



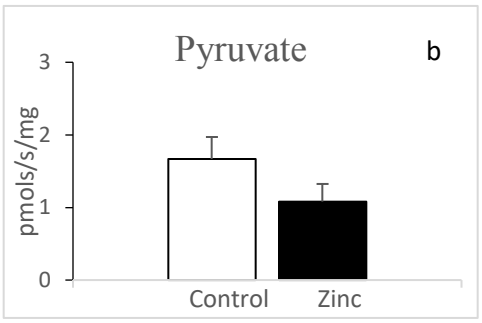


Figure 10: There is a downwards trend in OXPHOS capacity among the Zn groups and the CON groups as described in figure 4a, 4b, and 4c (Zn: 0.60 ± 0.15 versus CON: 0.82 ± 0.12; Zn: 1.08 ± 0.24 versus CON: 1.67 ± 0.30; Zn: 1.33 ± 0.15 versus CON: 1.58 ± 0.26, respectively).

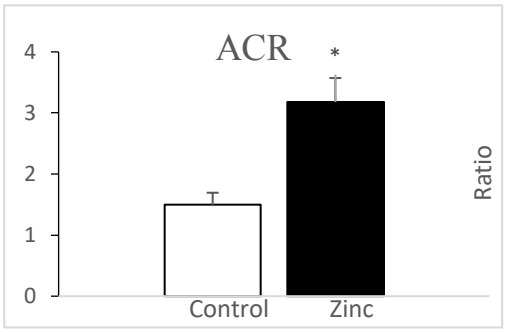


Figure 11: The ACR was significantly higher in the Zn group versus the CON group which is indicative of increased coupling between oxidation and phosphorylation in mice that received zinc supplementation.

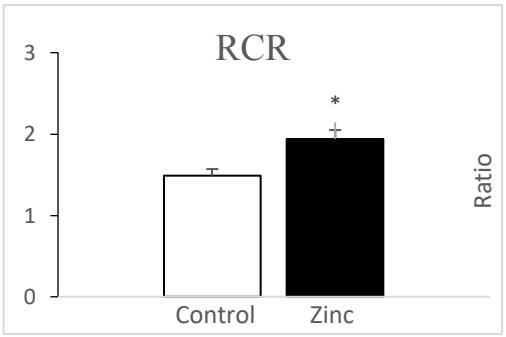


Figure 12: The RCR was significantly higher in the Zn group when compared to the CON group which is indicative of increased coupling between O_2 consumption and OXPHOS in mice that received zinc supplementation.

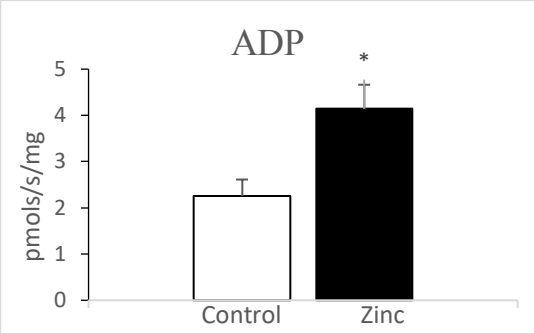


Figure 13: After the addition of ADP, the zinc group demonstrated an OXPHOS capacity that was significantly increased which represents a more efficient use of substrates

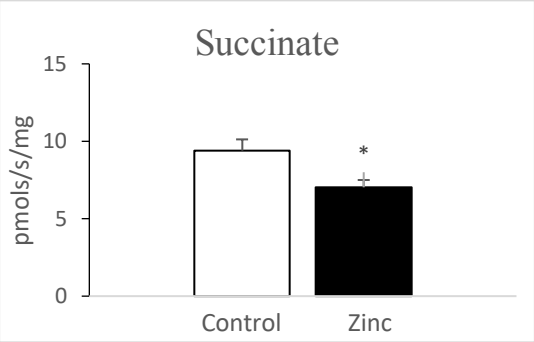


Figure 14: The zinc cohort exhibited a significantly lower maximal use of oxygen during activation of Complex I + II after the addition of succinate

Discussion

Recent literature demonstrates that zinc supplementation in diabetic mice play a protective role against the oxidative damage done to cardiomyocytes [Lu et al., 2015; Wang et al., 2006; Wang et al.,2017]. These studies focused on markers of oxidative stress including cardiac fibrosis, left ventricle mass, autophagy in cardiac tissue, and upregulation of cardiac metallothionein. However, to our knowledge, the preventative nature that zinc supplementation exhibits over the vasculature has not been previously confirmed. Studies have yet to implement immunoblotting and mitochondrial respiration techniques for verification of the role zinc supplementation has on mitochondrial density and capacity. The findings of this project show that following a 4-week zinc supplementation, mitochondrial respiration is significantly more efficient in the experimental group when compared to the controls. For a given amount of oxygen the zinc-supplemented subjects will produce more ATP than the control subjects. In this manner, zinc supplementation demonstrates a protective effect on the vasculature through the increased mitochondrial capacity of the zinc-supplemented mice.

In DM, hyperglycemia increases the influx of pyruvate into mitochondria subsequently augmenting the electrochemical potential difference generating ROS and mitochondrial hormesis which ultimately decreases mitochondrial capacity via reduction of oxidative phosphorylation and ATP generation [Wada & Nakatsuka, 2016]. Quality and capacity of mitochondrial function is a crucial focus in DM whereby overproduction of oxidants and proinflammatory and profibrotic cytokines stems from mitochondrial dysfunction [Wada & Nakatsuka, 2016]. Poor mitochondrial status can promote many pro-atherosclerotic changes in vascular smooth muscle cells via the impact of oxidative vascular injury; this causes cells to transition from a contractile to synthetic phenotype which shifts extracellular matrix production [Scheede-Bergdahl &Bergdahl, 2017]. The impact of mitochondrial function in vascular smooth muscle dedifferentiation is considered a critical factor in the early management of vascular disease associated with DM. Abhorrent mitochondrial metabolism increases the risk for the development of synthetic tissue and plaque, precursors to atherosclerosis and other cardiovascular issues. [Scheede-Bergdahl &Bergdahl, 2017]. However, this study has determined that zinc supplementation may prevent these alterations in mitochondrial health by augmenting mitochondrial capacity. Specifically, after the addition of ADP, the zinc group demonstrated an OXPHOS capacity that was significantly increased; this signifies a more efficient use of substrates accompanied with lower ROS production. These effects, seen on the functional level, represent the efficiency of the mitochondria themselves as there was no significant differences between the zinc and control groups in terms of mitochondrial density. Additionally, a decrease in maximal respiration from the activation of complex I+II is exhibited in Figure 8; the zinc cohort produces less waste products as this group has a lower use of maximal oxygen after the addition of succinate. Furthermore, after the injection of malate, pyruvate, and glutamate a downward trend of Complex I activation in an ADP-restricted environment is noted in the zinc group. This reduction in basal metabolic rate suggests that zinc supplementation affects the efficiency of mitochondria in a positive manner. These novel results further highlight the complex and dynamic function surrounding zinc supplementation, mitochondrial health, and the pathways in which they are involved. With diabetic vascular disease as the leading cause of renal failure, blindness, amputations, and cardiovascular disease, the importance of mitigating the deleterious effects of mitochondrial dysfunction become crucial [Widlansky & Hill, 2018]. Although this project did not demonstrate whether cells from the control cohort were synthetic,

future research could evaluate the effects of zinc supplementation and its ability to convert synthetic cells back into contractile cells, establishing a clear link between zinc and its protective nature in the vasculature.

IV

The Protective Effect of Zinc Supplementation on Vascular Damage and Antioxidant Expression

Stephanie MacKenzie, Dana-Rae Reguis Yadao, and Andreas Bergdahl

Contributions

Stephanie MacKenzie: preparation of manuscript, experimental techniques

Dana-Rae Reguis Yadao: experimental techniques

Andreas Bergdahl: editing of manuscript, experimental techniques

Abstract

Cardiovascular complications are the leading causes of morbidity and mortality in diabetic patients due to the oxidative stress that occurs when reactive oxygen species production overtakes the capacity of the endogenous antioxidant system. Previous research has identified the critical nature of zinc's contribution to key participants in this antioxidant defense system. Additionally, it has been shown that diabetics are zinc deficient as a result of excessive zinc loss in urine or inadequate dietary intake. Evidence also demonstrates that zinc supplementation is partially successful in the prevention of diabetic cardiomyopathy. As such, zinc supplementation may hold similar protective benefits for the diabetic damage done to the blood vessels. This study aimed to assess the efficacy of zinc supplementation as a protective therapy against the oxidative injury done to the aorta of diabetic male mice. This study investigated the effects of a four-week intervention of zinc supplementation on superoxide dismutase (SOD), zinc-induced metallothionein (MT), catalase, and peroxiredoxin-2 (Prx2) antioxidant expression, the preventative effects this has on the vasculature, and an analysis of smooth muscle cell dysfunction via oxidative protein markers. Immunoblotting techniques acted as quantitative analyses representing antioxidant levels and relative protein oxidation. Results revealed that the diabetic zinc-supplemented cohort exhibited a significantly lower level of protein oxidation compared to the diabetic controls. Furthermore, it was found that zinc-supplemented mice experienced a significant upregulation of both SOD and MT while no differences were observed among levels of catalase and Prx2. These findings highlight the protective role of zinc-supplementation with the potential for a more tailored treatment plan to protect and prevent diabetics from developing vascular complications.

Introduction

Diabetes mellitus (DM) is commonly characterized by hyperglycemia-induced overproduction of reactive oxygen species (ROS) paired with a defective antioxidant system resulting in oxidative stress [Park et al.,2018]. ROS are partially reduced forms of oxygen that serve essential physiologically functions, but which, when levels are not adequately buffered, can create structural damage and impair important biomolecules [van der Schaft et al., 2019]. As a result, DM patients are at an increased risk of cardiovascular disease (CVD) impelled by the repercussions of chronic hyperglycemia seen via activation of mitochondrial dysfunction and ER stress along with the polyol, hexosamine, protein kinase C (PKC), and advanced glycation end products (AGEs) pathways [Fiorentino et al.,2013; Thakur et al.,2018]. The accumulation of ROS (superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and NADPH-Oxidase (NOX)), promotes oxidative stress and inflammation which further impairs disease management and contributes to the development/progression of vascular complications [Fiorentino et al.,2013; Marreiro et al., 2017].

ROS is normally removed or neutralized, through metabolic conversion, by an innate system of antioxidant compounds and enzymes [Park et al.,2018; Sotler et al., 2019]. The enzymatic defense system includes mitochondrial magnesium-superoxide dismutase (SOD), cytoplasmic copper/zinc-SOD, glutathione peroxidase (GPX), thioredoxin peroxidase peroxiredoxin-2 (Prx2), and catalase. The non-enzymatic defense system includes vitamins A, C, and E, thioredoxin and metallothionein (MT) [Park et al., 2018]. SOD converts $O_2^{\bullet-}$ into H_2O_2 which is then efficiently changed into water and molecular oxygen by catalase, GPX, or Prx-2 [Devasagayam et al., 2004; Johansen et al., 2005; Sailaja et al.,2003; Wood et al., 2003]. The system does not eliminate ROS altogether, but works to maintain a balance such that pathological pathways are avoided [Sena et al., 2018].

The mineral zinc participates in the protective antioxidant defense system by acting as an essential cofactor for more than 300 enzymes [Cruz et al., 2015]. Notably, zinc is central to the effectiveness of the SOD enzyme; studies in diabetic mice and rats have shown that zinc supplementation increases the activity of SOD, demonstrating that low zinc concentrations can

lead to impaired antioxidant defense [Cruz et al., 2015; Saharia and Goswami, 2013]. Additionally, this mineral has been shown to modulate signaling cascades beneficial to antioxidant defenses, including the zinc-mediated release of the metal from MT for ROS scavenging purposes [Mondragon &Bergdahl, 2018; Oteiza et al., 2012]. Moreover, zinc impacts glutathione metabolism through the regulation of glutamate ligase enzyme expression; an enzyme that neutralizes free-radicals on its own and acts as a cofactor for GPX, another important scavenger [Cruz et al.,2015; Oteiza, 2012]. Recent literature has underscored the dynamic role of zinc in cellular secondary messaging, insulin signaling, and mechanisms that are responsive to changes in glycemic conditions [Myers et al., 2012; Norouzi et al., 2017]. As physiological levels of zinc are tightly regulated, this suggests that abnormally low levels increase susceptibility to the induction of significant pathological consequences [Norouzi et al., 2017]. Zinc deficiency is now classified as a risk factor for diabetes, and as such, it is necessary that research be directed at better understanding zinc supplementation and zinc homeostasis [Fukunaka and Fujitani, 2018].

The objective of this study was to investigate the effects of zinc supplementation on vascular damage in streptozotocin (STZ) induced diabetic mice following a four-week intervention. Immunoblotting was used to measure the amount of antioxidant protein expression (zinc-induced MT, SOD, catalase, and Prx2) in the aortic vessel while the level of smooth muscle cell dysfunction was analyzed via degree of protein oxidation. The results will reflect the extent of this minerals' protective capacity on the vasculature. Given that diabetics exhibit an imbalance between ROS and the buffering capacity of the antioxidant defense system, in addition to the crucial role of zinc in the latter, we hypothesized that zinc-supplemented diabetic mice belonging to the intervention group will exhibit greater increased levels of antioxidants (MT, SOD, catalase, and Prx2), and less smooth muscle cell dysfunction exhibited by reduced protein degradation.

Materials and Methods

Animal Care

Mice were obtained from the Concordia University breeding colony. For the purpose of the study, age-matched (2-4 months), healthy male C57B1/6 mice were individually housed in a room with a 12-hour light/dark cycle; temperature was kept at a constant 22°C. Mice were randomly assigned into two groups: diabetic given regular tap water, which served as the control, and the diabetic group with zinc supplementation; receiving an estimated daily dose of 2.5 mg Zn/kg in the drinking water. Diabetes was induced and confirmed prior to the onset of the 4-week protocol via a peritoneal injection (150mg/kg) of streptozotocin (STZ). Blood-glucose concentration was measured 2 days post-injection from the mouse-tail-vein by the One Touch Verio Flex glucometer. Outputs above 14mmol/L were taken as diabetic. Mice had continual access to standard rodent chow and water or zinc-water dependent upon group designation. The Concordia University Animal Research Ethics Committee approved all procedures (#30000259) and all measures were certified by the Canadian Council on Animal Care.

Experimental Protocol

Over a period of 4 weeks, the zinc group received an estimated daily dose of 2.5 mg Zn/kg in the form of $ZnSO_4$ administered to their drinking water. This specific dosage of zinc was provided on the basis of previously accepted experimental protocols [Miao et al., 2013; Wang et al., 2006]. Calculations to achieve this dose via water ingestion was based on previous research [Yadao, MacKenzie & Bergdahl, Manuscript in preparation] with respect to the average amount of water a diabetic mouse ingests over the course of one week.

Throughout the duration of the experiment, zinc mice had their remaining zinc-water emptied three times a week and were given fresh zinc-supplemented water. The respective tap water for the control group was also provided three times per week.

Tissue Preparation

Upon completion of the 4-week protocol, the animals were euthanized using CO_2. The aortic arch was located and the vessel traced down along the spine, separating it from the connective tissue prior to removal and immediately placing it in iced sterile nominally Ca^{2+}-free Krebs solution containing 122 mmol/L NaCl, 15.5 mmol/L $NaHCO_3$, 4.7 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 1.2 mmol/L KH_2PO_4, 11.5 mmol/L glucose, 100 U/mL penicillin, and 100 Ig/mL streptomycin. Periaortic fibro-adipose tissue was carefully removed under a dissection microscope before the endothelial cells were removed and the vessels denuded with a sponge.

Immunoblotting and Immunofluorescence

The aortic vessels underwent cell lysis by using a lysis buffer according to Rocha et al. (2015) containing (in mmol/L): NaCl 250, HEPES 50, EGTA 1, NaF 1, glycerol 10%, Triton X-100 1%, $MgCl_2$ 1.5, $Na_4P_2O_7$ 10, Na_3VO_4 800 µmol/L, and (pH=5). For 10 minutes the solution was centrifuged at 13000 g, after which the supernatant was collected. Protein levels were assessed using the Pierce BCA Protein Assay Kit (Thermo Scientific, Mississauga, Ontario, Canada) with 10µg of protein samples initially separated on a 10% SDS-PAGE. This was followed by transfer with 10mmol/L sodium tetraborate buffer onto a nitrocellulose membrane (0.45 µm, 162-0115, Bio rad). An effective load and transfer were confirmed through the use of a Ponceau S staining. Over a 1-hour period, 3% BSA in TBS-T buffer (10 mmol/L Tris-HCL, pH 7.5, 150 mmol/L NaCl, 0.01% Tween 20) was utilized as a membrane block at room temperature. An overnight incubation period 4°C proceeded with the following primary antibodies: rabbit polyclonal anti–human CuZnSOD (#SOD-100) at 1:1000 dilution from Stressgen, USA. And from Abcam, United Kingdom, sheep polyclonal anti-human catalase (#ab8954, 1:1000 dilution), sheep polyclonal anti-Gpx1 (cat #ab8850, 1:1000 dilution) and rabbit polyclonal anti-human Prx2 (cat #ab15572, 1:10000 dilution). The blots derived from this process were then washed and incubated with HRP-conjugated goat polyclonal anti-rabbit (cat #1706615, 1:3000 dilution, Biorad) and HRP-conjugated donkey polyclonal anti-sheep (cat #ab6900, 1:3000 dilution, Abcam, United Kingdom). A chemiluminescence system was used to visualize and examine relative protein expression (Immun-Star Chemiluminescent, 1705070; Bio-Rad, Mississauga, Ontario, Canada) while bands were analyzed by ImageJ software.

Protein Oxidation as Detected through Protein Carbonyl Formation

The Protein Carbonyl Assay Kit (Western Blot; ab179020 Abcam) was used to determine the amount of oxidation that occurred to the proteins in the tissue samples. This tool utilizes an immunoblotting technique in which the individual oxidized proteins are separated and identified through SDS-PAGE. First, proteins were extracted from the tissue samples through an incubation period in 4 ml of extraction buffer (20-minute centrifugation at 18 000 g). Next, 12% SDS solution (2 ml) was used to denature the proteins, upon which they were incubated for 15 minutes with 2, 4-Dinitrophenylhydrazine (DNPH; 4 ml). A neutralization solution (4 ml) was added and then the samples were run on the gel. Proteins were then transferred to a membrane and incubated in a blocking solution (1 hour). After, the samples were incubated with 100 µl DNP antibody for 3 hours and washed. Subsequent incubation with HRP goat anti-rabbit secondary antibody (100 µl) for 1 hour and then washed. Finally, using the ECL, bands were visualized and the image was acquired.

Statistical Analysis

A two-tailed Student's *t* test was used to analyze the data to compare between the control and zinc groups. The data obtained from immunoblotting and other figures are presented as means ±SE. Statistical significance was $P < 0.05$. A Gibbs test and a Dixon test were conducted to identify outliers.

Results

Protein Oxidation

The amount of oxidation that occurred to the proteins in the collected tissue samples were assessed through an immunoblotting technique that separates and identifies oxidized proteins. Results revealed that when compared to the diabetic control group, the diabetic zinc-supplemented cohort exhibited a significantly lower level of protein oxidation (100 ± 2.80 and 90.29 ± 3.07 respectively).

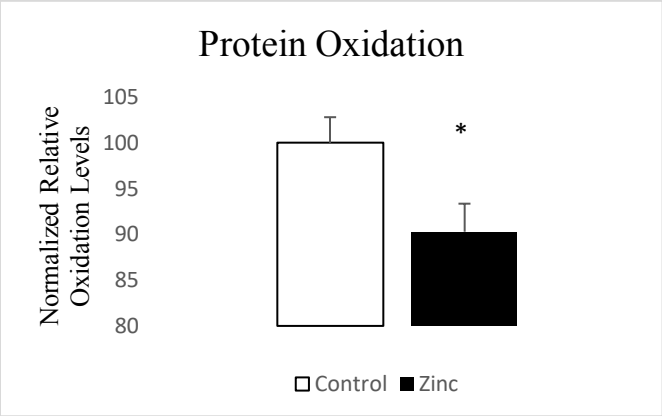


Figure 15: Data from Protein Carbonyl Assay Kit (Western Blot; ab179020 Abcam) significantly shows the zinc group has less protein oxidation versus the control group.

Oxidative Stress

Figure 16: Image developed from protein oxidation immunoblot. Diabetic control group (left) and the diabetic zinc-supplemented group (right). Data indicates that the vessels of the control group experienced significantly more oxidative stress when compared to the zinc group.

Immunoblotting and Immunofluorescence

Results from immunoblots demonstrate that the relative protein levels of SOD and MT in mouse aortic vessels are significantly increased in the zinc-supplemented cohorts; SOD control: 100 ± 10.67 versus SOD zinc: 150.39 ± 15.41, $p<0.05$; MT control: 100 ± 15.03 versus MT zinc: 145.02 ± 14.58, $p<0.05$. However, no significant differences were noted between the relative protein levels of catalase and Prx2 when the aortic vessels of the control and zinc groups were compared; catalase control: 100 ± 11.36 versus catalase zinc: 120.72 ± 21.61; Prx2 control: 100 ± 14.34 versus Prx2 zinc: 104.76 ± 14.88.

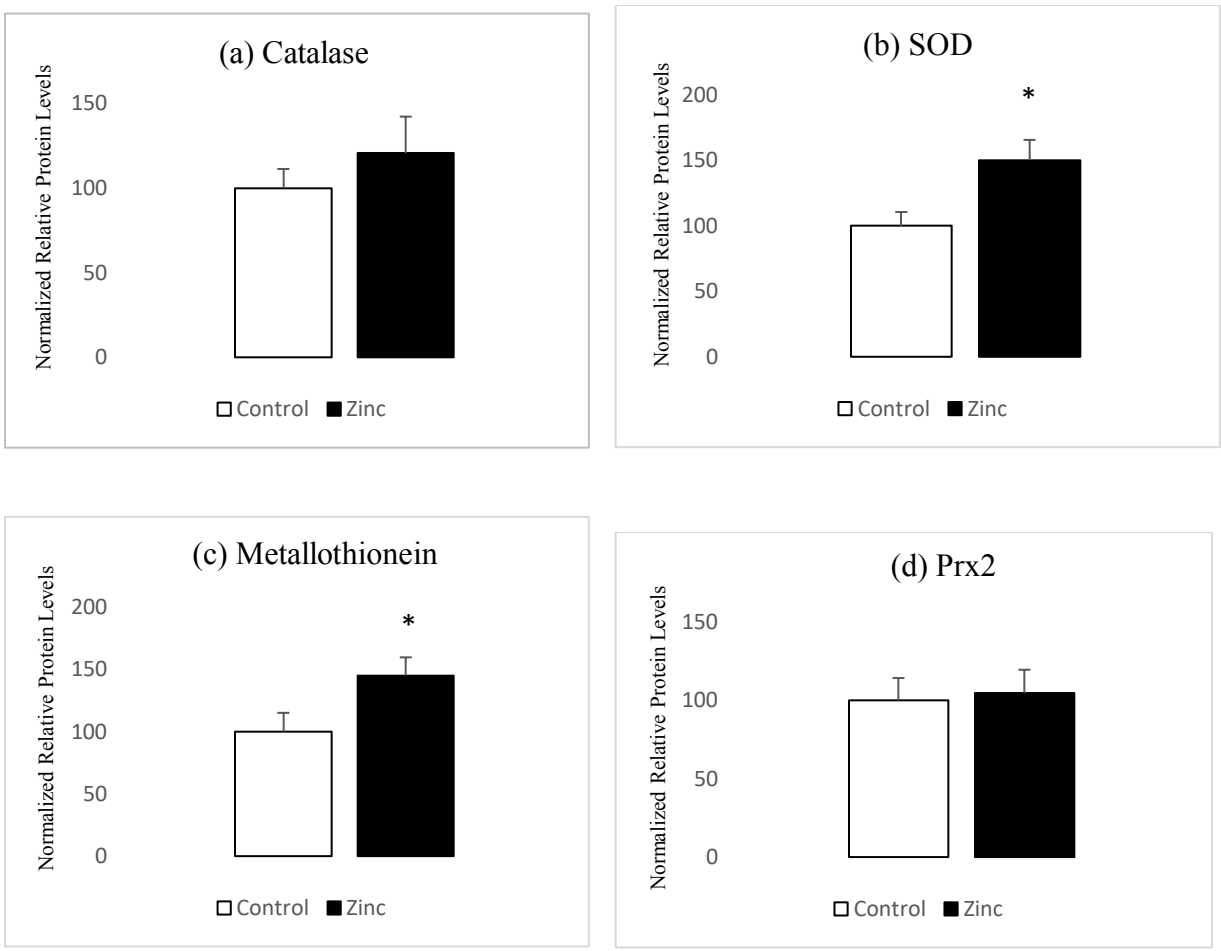


Figure 17: Relative amounts of (a) catalase, (b) SOD, (c) MT, and (d) Prx2 in aortic vessels isolated from STZ- induced diabetic mice zinc n=8, control n=9. The zinc group experienced significantly higher SOD and MT expression when compared to the control group. Meanwhile, no significant differences in catalase and Prx2 expression were established in the aortic vessels between the control and zinc groups

Discussion

The antioxidant enzymes SOD, catalase, MT, and Prx2 alter their levels of expression in response to the demands placed on the endogenous defense system. DM reduces the ability of this system to properly buffer the overproduction of pro-oxidant and pro-inflammatory species though aberrant modifications in the pathways that manage antioxidant production. Deficiencies linked with this metabolic disease highlight the shunted ability of the antioxidant system, specifically where the mineral zinc is involved.

Prior research has demonstrated zinc action on antioxidant expression in several areas of the body including the liver and kidneys. Miao et al. [2013] and Ozcelik et al. [2012] showed that zinc supplementation increases MT expression and activity in type 2 diabetic rats. Zhu et al. [2013] studied diabetic rats and demonstrated increases in both SOD and GPX activity, and liver function in response to zinc supplementation. However, to our knowledge, the value of zinc-supplementation on expression levels of SOD, MT, catalase, and Prx2 in aortic vessels of diabetic subjects have not been previously explored.

The findings of this study effectively demonstrate that zinc-supplementation may hold therapeutic value in the treatment of DM and has the potential to act as a preventative supplement with very few side effects. The vessels of the diabetic subjects who received zinc on a daily basis displayed a significant reduction in protein oxidation when compared to the diabetic control group. This protective capacity exerted by zinc highlights its crucial role in the prevention of cardiovascular issues that are routinely the cause of mortality in DM. Meanwhile, the mechanisms behind these novel results still remain somewhat unclear and future research should target these areas [MacKenzie et al., manuscript in preparation]. Zinc is well-known for playing a role in a multitude of processes and pathways in the body, and it is likely there are additional pathways that have yet to be identified as research on zinc transporters continues to develop. Our experimental regiment on relative antioxidant levels was consistent with previous research, and confirmed that both zinc-dependent SOD and MT expression can be up-regulated in response to zinc supplementation in the vessels of diabetic subjects. Yet, levels of catalase and Prx2 did not display similar dynamics as there were no significant differences seen among cohorts. This suggests that these antioxidant expression levels are not zinc-dependent. While plasma concentrations of thioredoxin are used as biomarkers for oxidative stress, Prx2 may not be considered as potent of a player in the antioxidant defense system compared with catalase and GPX [Ali et al.,2020; Wood et al.,2003].

Cardiovascular complications are the leading causes of morbidity and mortality in diabetic patients driven by the consequences of chronic oxidative stress and inflammation. Research has underscored the importance of zinc dynamics in DM and offers a therapeutic option through zinc

supplementation. The protective capacity of zinc now extends to the vasculature via reduction of protein degradation and subsequent reduction in smooth muscle cell dysfunction. The prevention of protein oxidation paired with increased expression of SOD and MT could help to reduce the vascular damage exhibited in DM and, as a result, reduce the risk of death and disease linked with CVD.

V

Concluding Remarks

Conclusion

The objective of my thesis was to describe the effects of zinc supplementation on vascular mitochondrial respiration, antioxidant expression, and protein oxidation. While further investigations focused on the contributing mechanisms driven by zinc deficiency in the development and progression of diabetes and vascular complications. These projects offer an understanding as to the protective efficacy of zinc supplementation and its value as a potential therapeutic treatment for diabetes that could ultimately decrease the associated risk of cardiovascular disease.

Diabetic markers of oxidative stress result from a combination of a defective antioxidant defense system, chronic levels of inflammation, endoplasmic reticulum stress, and mitochondrial dysfunction. Our results, derived from the comparison between the diabetic vessels of the experimental and control groups, support the concept that zinc plays a key role in reducing the mechanisms that produce oxidative disease markers. This was emphasized by the mitigation of vascular damage following zinc supplementation. However, this project was not without several limitations. The use of STZ to induce diabetes in mice reflects an untreated T1DM model which does not accurately reflect the T1 diabetic model among humans who do receive treatment and could also be considered as a poorly maintained T2DM model. Subsequently, the mortality rate among the diabetic mice was high, limiting the number of participating subjects. Additionally, within the constraints of time, we have made the assumption that the effects measured do have a positive impact on reducing vascular consequences seen in DM. However, we did not specifically test whether these vascular changes directly lower incidence of cardiovascular complications due to time limitations.

Zinc supplementation may offer a novel and affordable therapy in the approach to DM and its consequences. Yet, it is of relevance to remark upon the detrimental effects of excessive zinc supplementation which may result in the onset of unwanted and possibly harmful side-effects. However, as zinc deficiency is associated with diabetes, and the dysregulation of zinc metabolism provokes a myriad of deleterious pathways, it is clear that zinc nutrition needs to be a component of disease management. To further clarify the role of zinc and zinc supplementation over the course of the condition, further investigation is required. Future research should be

directed towards several aspects which encompass diabetes treatment. The accurate measurement of zinc status via reliable biomarkers and extracellular vesicles obtained from liquid biopsies. The administration of randomized controlled trials for zinc supplementation in humans and its effects on diabetic symptoms such that a standard of care may be established. Minimally invasive studies could be performed through the use of ultrasound to examine the effects of zinc supplementation on endothelial function. To perform more prospective cohort studies in pre-diabetics or individuals with insulin resistance to assess whether zinc supplementation is a legitimate strategy in the prevention of disease onset. And to continue to advance our knowledge with respect to the mechanisms that involve zinc, and how to utilize zinc transporters ZnT8 and ZIP7 in a therapeutic setting.

Overall, this thesis delivers unique observations about the protective effects of zinc supplementation in diabetic blood vessels. Moreover, it facilitates the identification of potential therapies that could be implemented in order to relieve the metabolic and vascular symptoms of diabetes. This includes the investigation into the advantageous effects of zinc supplementation on diabetic vascular tissue, the modulation of zinc transporters to ascertain an individuals' level of disease risk, and zinc's comprehensive role in the holistic management of diabetes and diabetic vascular complications.

VI

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