June 2024

Cornus officinalis extract activates the Keap1/Nrf2 pathway and delays T1D onset in the NOD mouse

Justin D. Fletcher
University of South Florida

Cornus Officinalis Extract Activates the Keap1/Nrf2 Pathway and Delays T1D Onset in the NOD Mouse

by

Justin D Fletcher

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Molecular Biosciences
College of Arts and Sciences
University of South Florida

Major Professor: Brant Burkhardt, Ph.D.
Mark Atkinson, Ph.D.
Stanley Stevens , Ph.D.
Meera Nanjundan , Ph.D.

Date of Approval:
June 7 , 2024

Keywords: ethnopharmacology , autophagy , Type 1 diabetes,

DEDICATION

I would like to dedicate this work to my loving family who have supported me throughout my academic career and have been instrumental in getting me to the place I am today. I would also like to dedicate this work to my loving wife Riya, I cannot thank you enough for you love, compassion and understanding through my highest of highs and lowest of lows throughout my graduate career. You have been my rock and I don’t know if I could have gotten through this entire experience without you.

ACKNOWLEDGMENTS

I would like to thank my many peers and mentors that have supported and guided me through my graduate career at USF. First, I would like to thank Dr.Burkhardt for taking me into his lab. I was forced to change labs due to my previous PI leaving the university and I cannot thank you enough for giving me a chance. I know it can always be a risk taking someone into your lab that you did not directly accept into the program, so it does mean a lot that you believed in me enough to take me in. In addition, graduate school can be an absolutely horrific experience. However, I had you as a mentor which I can genuinely say made my graduate experience challenging but ultimately rewarding educational experience.

I would also like to thank my committee members, Dr.Mark Atkinson, Dr.Meera Nanjundan, and Dr.Stanley Stevens. Thank you for your guidance and expertise as it was instrumental in getting me here today.

I would like to thank my past and current lab peers: Grace, Sama and Anna. All your hard work was genuinely appreciated. I will always appreciate our in-depth conversations on any number of vastly different topics and I am happy to consider all of you friends.

Finally, I would like to thank the USF Chemical Purification Analysis and screening (CPAS) core facility for letting me use their Agilent 1100 HPLC with Diode Array Detector and Agilent 6120 Single Quadrupole Spectrometer.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disorder that results in the destruction of the insulin producing pancreatic β cells. The only therapeutic option following T1D clinical onset is exogenous insulin supplementation. At present, there is no cure for T1D and attempted interventional therapy has been focused on modulating the immune system. Few attempts have been pursued at preserving β cell function and survival. Here we provide a novel approach using extract from the fruit of the *Cornus officinalis* (*CO)* tree to preserve β cell function through the prevention of β cell stress. CO has been used in traditional Chinese medicine to treat several aliments with contemporary research demonstrating CO has a therapeutic effect in type 2 diabetes. My in vitro findings using a human pancreatic cell line demonstrate that CO induces a robust activation of the critical Keap1/Nrf2 antioxidant pathway and inhibit hydrogen peroxide induced cell death. To further extrapolate these findings, we then performed an in vivo analysis in which we treated the non-obese diabetic mouse (NOD) with an oral gavage of CO. We found that CO significantly delayed T1D onset, hyperglycemia, pancreatic insulitis and preserved c-peptide secretion indicating a preservation of β cell mass and function. Individual composition analysis was performed using HPLC and mass spectrometry to reveal specific fractions within CO are inducing the biological effects. Taken together, our study has demonstrated the therapeutic potential and mechanism of action for CO as an interventional therapy of T1D.

CHAPTER ONE:

INTRODUCTION

1.1 Review of the Etiology and Pathophysiology of Diabetes Mellitus

1.1.1 Anatomy of the pancreas and glucose stimulated insulin secretion

The pancreas is located in the upper abdomen sandwiched between the duodenum and the spleen. The pancreas is divided into 5 distinct parts: head, uncinate process, neck, body and the tail [1]. The pancreas has several different functions with much of the pancreatic tissue being devoted to its exocrine function. The cells responsible for the exocrine function are known as acinar cells and make up 85% of the pancreas by mass [1]. These acinar cells are arranged into acinus ("grape like" bundles of acinar cells) which produces, stores, and secretes digestive enzymes involved in protein, lipid and carbohydrate digestion [1]. These digestive enzymes are then secreted into the duodenum using a complex ductal system eventually resulting in the deposition of the digestive enzymes [2].

Although much of the tissue of the pancreas is devoted to its exocrine function by mass, the remaining endocrine tissue of the pancreas plays a critical role in maintaining the healthy homeostasis of the human body. The endocrine tissues of the pancreas are organized into micro-organs known as the islet of Langerhans [3]. The islet of Langerhans is composed of a considerable number of different endocrine cells including: epsilon (ε) cells (<1% of islet), pancreatic polypeptide (PP) cells (<2% in the

islet[4]) , delta (δ) cells (5-10% of islet), alpha (α)-cells (15–20% of islet) and beta (β-cells) (50-80% of islet) [3]. These islets are connected to the ductal network of the pancreas which allow for the exchange and detection of nutrients and the release of endocrine hormones.

Of the many cell types of the pancreatic islets the ε cells are the most recently to be discovered with its discovery only occurring in 2002 [5]. ε cells are characterized by their ability to secrete ghrelin [5], an important hormone involved in appetite stimulation[6]. Interestingly more recent findings have also shown that ghrelin plays a role in glucose homeostasis by inhibiting insulin secretion [7].

PP cells are simply named by their ability to secrete pancreatic polypeptide(PP). PP seems to play a complex multi-faceted role in the human body[8]. However in the islet, the secretion of PP has been established to play a regulatory role by inhibiting the secretion of glucagon[9] and somatostatin [10] as well as being implicated in insulin static actions as well [11]. Recent data has also suggested that PP may also play a protective role by preventing apoptosis in the β-cell [12]. δ cells are another regulatory cell of the islet identified by their ability to secrete somatostatin [13]. Somatostatin is necessary for proper glucagon regulation as somatostatin secretion is needed to inhibit glucagon secretion in the presence of glucose [14].

α-cells play a critical role in blood glucose regulation and were one of the original cell types to be identified in the islet of Langerhans [15]. When the circulating blood glucose levels are low (Below 70mg/dl) the α-cells secrete glucagon resulting in gluconeogenesis and glycogenolysis in the liver [16]. This results in the release of

glucose into the blood and restoring glucose homeostasis to typical levels of 80-100 mg/dl.

Conversely when the blood glucose levels are high (>100mg/dl) the β-cells come into play to restore glucose homeostasis. β-cells are characterized by their ability to produce the endocrine hormone insulin in response to hyperglycemic blood glucose levels (>100mg/dl). β-cells have this response due to the expression of the glucose transporter 2 (GLUT2) receptor which facilitates the diffusion of glucose from the circulating blood into the cytoplasm of the β-cells [17]. The glucose is then phosphorylated by glucokinase producing glucose 6 phosphate which in turn enters glycolysis[18]. Through glycolysis pyruvate is produced which then subsequently enters the citric acid cycle which in turn increases the intracellular ATP/ADP ratio [17]. Due to the increase in ATP to ADP, an ATP sensitive K channel is closed resulting in the plasma membrane becoming depolarized which then allows an influx of Ca^{2+} due to the opening of the voltage dependent Ca^{2+} channel. The increase in the Ca^{2+} causes the fusion of the insulin granules to the plasma membrane and the subsequent secretion of insulin into the pancreatic ductal network [17].The secretion of insulin into the blood stream causes skeletal muscle cells and adipocytes to uptake glucose and promote hepatic glucose uptake and synthesize glycogen [19]. By inducing the uptake of glucose, insulin decreases the blood glucose levels which is why any disruptions in the function of the β-cell is associated with debilitating disease.

1.1.2 Type 1 vs Type 2 Diabetes

Type 1 (T1D) diabetes and type 2 diabetes (T2D) are chronic illnesses that affect millions of people with worldwide prevalence only expected to increase over the

following decades [20]. Diabetes mellitus is characterized by the dysregulation of glucose metabolism and insulin secretion resulting in hyperglycemia [21] and has been associated with a decrease in quality of life and a substantial increase in healthcare costs. Insulin is a critical endocrine hormone involved in glucose metabolism and is directly responsible for the glucose uptake of the myofibers and adipocytes [22]. For this reason dysregulation of insulin secretion or insulin sensitivity is a critical aspect of the pathogenesis of T1D and T2D.

Even though both T1D and T2D result in hyperglycemia, the underlying causes are what differentiate T1D from T2D. T2D is the most prevalent with over 30 million people living with T2D in the US alone according to the CDC. T2D is unique in the fact that even though genetic factors play a role in T2D, environmental/lifestyle factors present the biggest risk for T2D pathogenesis [23]. T2D pathogenesis is characterized by two distinct factors: Insulin resistance and β-cell dysfunction. During T2D pathogenesis insulin resistance is characterized by the body's reduced responsiveness to insulin secretion [24]. Although the exact mechanism for the cause of insulin resistance is not entirely defined, there are several factors that are thought to play a role including: defects in insulin signaling [25], defects in the glucose transporter [26] and lipotoxicity [27]. Insulin resistance is usually followed by β-cell dysfunction brought upon by: oxidative stress [28], protein aggregate formation [29] and increased concentrations of circulating free fatty acids [30]. These factors result in β-cell death and the need for the patient to take exogenous insulin similar to individuals with T1D.

Even though T1D and T2D can result in the destruction of the β-cell and the need for exogenous insulin, T1D is unique in that T1D pathogenesis has a distinct immunological

element. Due to this, T1D is characterized as an autoimmune disease resulting in the destruction of the insulin secreting β-cells [31].

1.1.3 T1D Diagnosis, progression and long-term consequences of the disease

T1D is a long-term chronic disease with distinct stages of disease progression. Before the clinical onset of T1D symptoms the presence of autoreactive antibodies can be detected years before the clinical onset of T1D [32]. These autoreactive antibodies target a number of β-cell antigens including: glutamate decarboxylase, islet antigen 2, zinc transporter 8 [32], and tetraspanin [33]. The presence of 2 of autoantibodies targeting β-cell antigens in children is correlated with an 84% risk of T1D by 18 years of age [32] and in adults, 96% of adult onset diabetes cases have been shown to have at least 1 autoantibody present [34] . For this reason, the first stage of T1D is the presence of 2 autoantibodies with normal blood sugar levels. This is followed by Stage 2 which is the presentation of abnormal blood sugar readings. Stage 3 is the clinical diagnosis of T1D as determined by two consecutive blood glucose readings above 126 mg/dl after fasting. Stage 4 is long term T1D and any complications associated with chronic T1D.

Traditionally T1D has been considered as a childhood disease with a T1D diagnoses occurring during the adolescent years but, recent data has shown that up to 42% of T1D onsets occur after the age of 30 [35]. This indicates that T1D onset can happen at almost any age and unfortunately many adult individuals diagnosed with T2D may have T1D [36]. Regardless of age there are some common symptoms of T1D (Especially in children) including: polyuria (Frequent urination), polydipsia(excessive thirst) and unintended weight loss. Also, a life-threatening symptom can develop called ketoacidosis (KDA). KDA develops after the pancreas is no longer able to produce

insulin resulting in the body no longer being able to uptake glucose from the blood stream. Due to the body no longer being able to use glucose as an energy source, fats are broken down by the liver at a very fast rate resulting in a buildup of ketones in the blood and subsequent toxicity due to the blood becoming acidic.

Once a patient is displaying these symptoms there are several factors that must be considered before a diagnosis is given. First the fasting blood glucose must be above 126mg/dl or a random blood glucose concentration (with symptoms present) must be above 200mg/dl for a diagnosis to be given [37]. A glucose tolerance test may also be given in which a patient is given a solution with 75g of glucose dissolved and the plasma glucose concentration is then measured after two hours. A reading of over 200mg/dl 2 hours after the glucose solution is given is considered to be diabetic [37]. Another commonly used method to determine a diabetes diagnosis is measuring the concentration of glycated hemoglobin (HbA1c). Hemoglobin is normally found in the erythrocytes and is used to transport oxygen but in the presence of glucose hemoglobin becomes glycated [38]. Since the blood glucose levels is directly proportional to the amount of HbA1c, the percentage of HbA1c is useful indicator of blood glucose levels with an Hb1Ac level of 6.5% considered to be diabetic. Since red blood cells live an average of around 4 months [39], the percentage of HbA1c gives an average of the blood sugar levels over the previous 90 days making an Hb1Ac test a great way to get a blood glucose measurement that is not affected by short term changes in blood glucose [38].

Unfortunately, once T1D is diagnosed it becomes a lifelong chronic disease as there is no currently available cure for T1D. Due to the lifelong nature of T1D, chronic

hyperglycemia has been associated with several microvascular complications. These complications manifest themselves as retinopathy, neuropathy, and nephropathy [40] as well as cardiovascular disease [41].

Chronic hyperglycemia contributes to microvascular complications through several mechanisms. One mechanism is through the increased formation of diacylglycerol (DAG) which is a known activator of protein kinase C (PKC) [42]. Activation of the PKC pathway causes a number of detrimental effects including: increased production of transforming growth factor (TGF)-β (resulting in basement membrane thickening), activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase resulting in an increased production of reactive oxygen species (ROS) (promotes oxidative stress in the cardiovascular tissues) [42]. Chronic hyperglycemia also leads to the synthesis of advanced glycation products (AGEs) [43]. The AGEs than act as a ligand for the receptor for advanced glycation end products (RAGE) resulting in a signaling cascade that activates pro inflammatory pathways and causes oxidative stress [44]. This then exacerbates cardiac fibrosis in diabetic individuals resulting in cardiovascular disease [45]

1.1.4 T1D potential causes: a complex mix of environment and genetics

Although T1D is considered an autoimmune disease, T1D does not follow a simple autoimmune model instead T1D is an incredibly complex interplay between genetic, metabolic and immune factors resulting in the destruction of pancreatic β-cells [31] that even to this day is still not perfectly understood. Genetics seems to play a significant role in the onset of type 1 diabetes [46] but interestingly, studies in identical twins have shown that only 65% of twins at risk for T1D both develop T1D [47] demonstrating that

although genetics play a major role, environmental factors seem to play a critical role as well. In terms of the genetic factor, the genetic aspect of T1D is mostly due to the presence of unique haplotypes of the human leukocyte antigen (HLA) class 2. Since the HLA is directly responsible for antigen presentation between the antigen presenting cells and the adaptive immune system, mutations in the HLA have been associated with numerous autoimmune diseases [48]. In T1D two distinct HLA class 2 haplotypes (HLA DR3 and HLA DR4-DQ8) have been associated with 50% of cases in which disease heritability was involved [49].

These are not the only genes that have been associated with T1D though, as genome wide studies have also shown that over 60 non HLA loci are associated with T1D onset. Most of these loci are involved in the immune system and play a role in insulin gene expression in the thymus, T cell activation and viral response[46]. Of the non HLA loci associated with T1D there is a particular interest in PTPN22 and insulin VNTR. PTPN22 is a negative regulator of the T cell and B cell receptor and single nucleotide polymorphism (SNP) in PTPN22 has been found across multiple studies looking at non HLA loci involved in T1D [50]. Another non HLA loci of interest in T1D susceptibility is the insulin variable number of tandem repeats (INS-VNTR) gene. INS-VNTR is a genetic element associated with the proinsulin gene promoter region and is directly responsible for proinsulin expression based on the number of 14-bp consensus element repeats [51]. Interestingly, haplotypes with a short number of repeats (26-63 repeats) have been found to be associated with a higher risk of T1D while haplotypes with a large number of repeats (141-209 repeats) are associated with a decreased risk [51]. This is due to the larger number of repeats being associated with high expression of

proinsulin in the thymus resulting in better selection in thymus for autoreactive proinsulin-reactive T cells. This better selection in the thymus results in the deletion of autoreactive proinsulin-reactive T cells while haplotypes with a short number of repeats in INS-VTNR allow for more proinsulin reactive T cells to enter the peripheral blood due to low expression in the thymus [51].

Sadly, T1D incidence has been increasing in both prevalence and incidence [52] and genetic factors alone cannot explain this trend, implicating that other factors may also be involved. Many environmental exposures are associated with type 1 diabetes including: β-cells stress brought upon by childhood obesity[53, 54], exposure to certain viruses such as enteroviruses [55] and even decreased gut microbiome diversity has been implicated [53].

One of the most widely studied environmental factors related to T1D is infection by enteroviruses. Studies have shown that individuals with recent onset of T1D have been found to have Enteroviral major capsid protein VP1 and RNA present in their islets [56]. The mechanism by which the enteroviruses exactly cause T1D is still controversial and heavily debated but one mechanism with considerable evidence is that infection by enterovirus creates interferonopathy conditions within the islet microenvironment [57]. Overexpression of the interferons (IFNs) brought upon by the enterovirus infection has several detrimental effects in the β-cells with IFN-α causing: ER stress, reduced insulin content and an increase in the pro insulin to pre insulin ratio [58]. Furthermore, IFN-α is associated with one of the hallmarks of T1D, hyperexpression of HLA 1 in the β-cells[59].IFNs also increase expression of a number of proteins involved in transporting and loading the HLA-1 through the ER including: the chaperone proteins (TAP1,

TAP2, TPBP) as well as the editing enzyme ERAP 1 [60]. This suggests that not only do the IFNs cause the hyperexpression of HLA-1 they are also involved increasing the transport and loading of antigens on the HLAs. In addition, TAP and ERAP1 have been shown to play a role in the generation of preproinsulin (PPI) epitopes which act as autoreactive antigens [61]. This production of autoreactive antigens, hyperexpression of HLA-1 and increased loading and processing of HLA-1 brought upon by enterovirus infection in the β-cells makes them the perfect target for autoreactive cytotoxic CD8 T cells and is why enterovirus infection is associated with T1D onset.

As mentioned earlier the causes of T1D are a complex mix of environment and genetics and although enteroviruses have been shown to have a well-established role in T1D onset, β-cell stress also plays a major role in T1D onset and will be discussed in detail in section 1.2.

1.2. T1D and Oxidative Stress

1.2.1 β-cells are extremely susceptible to oxidative stress

Over the past decade there has been a substantial amount of evidence elucidating the link between T1D and oxidative stress, with many of the studies examining β-cells dysfunction having shown that an excess of reactive oxygen species (ROS) and reactive nitrogen species (RNS) result in β-cell dysfunction [62]. Interestingly the harmful effect of the ROS or RNS is entirely dependent on concentration, as low concentrations of ROS are the products of normal cellular metabolism and are involved in a number of signaling pathway [63]. Unfortunately, at high concentrations caused by disruptions in the antioxidant response or excess production by physiological process,

reactive molecules will cause cellular damage through lipid peroxidation, protein oxidation and DNA damage [64].

Sadly, β-cells are extremely susceptible to the damage brought upon by oxidative stress [65]. This is due to many of the inherent characteristics of the β-cell. β-cells express the GLUT2 receptor which is necessary for the efficient uptake of glucose to allow the β-cell to detect glucose and release insulin. In the presence of a high concentration of glucose, β-cells uptake large amounts of glucose resulting in the accumulation of ROS [66]. This causes stress to the β-cell and combined with the fact that β-cells express low levels of free radical detoxifying enzymes such as catalase, super oxide dismutase (SOD) and glutathione peroxidase when compared to other tissues [67] oxidative stress in the β-cell is further exasperated. Furthermore, not only does the β-cell express low levels of antioxidant enzymes it has also been shown that the β-cell is very poor at repairing the damage cause by ROS with β-cells being significantly less capable of doing so when compared to liver cells [68]. The β-cells poor capability to repair DNA damage combined with the low expression of antioxidant enzymes make β-cells extremely susceptible to the oxidative stress brought upon by hyperglycemia or inflammation.

1.2.2 β-cell stress precedes insulitis

Although T1D is considered an autoimmune disease and historically the immune system has taken most of the blame for causing T1D, recent research has suggested that the β-cells themselves also play a role in the development of islet autoimmunity[69]. One such avenue as to which β-cells contribute to their own demise is through oxidative stress. Oxidative stress in the β-cell comes from several different sources, as discussed

before hyperglycemia can lead to ROS generation, but even the mitochondria itself is considered to be a major source of ROS generation as ROS production in the mitochondria is part of normal metabolic processes. Due to ROS generation being a part of normal metabolic function in the mitochondria, dysregulation of the mitochondria results in the accumulation of ROS and has been implicated in T1D pathogenesis [70]. Intriguingly, more recent evidence is suggesting that endoplasmic reticulum (ER) stress can also contribute to the production of ROS and oxidative stress [71]. This is due to the ER in the β-cell having a high rate of insulin synthesis which leads to inherent ER stress which in turn means any kind of additional oxidative stress will lead to ER dysfunction and distressed β-cells. Due to the dysregulation of either the ER or mitochondrial function causing oxidative stress which β-cells are extremely susceptible to, preventing oxidative stress is considered a promising target for diabetes treatment [62].

Part of the reason that preventing stress in the β-cells is critical to T1D treatments is that current research seems to be indicating that stressed β-cells produce danger signals that precede insulitis. One of the hallmarks of T1D is hyper expression of HLA class 1 and evidence suggest that HLA class 1 hyperexpression in the insulin secreting islets of the pancreas is brought upon by β-cell stress [72]. This hyperexpression causes the β-cell to become much more “visible” to autoreactive $CD8^{+}$T cells resulting in increased β-cell death. Furthermore, stressed β-cells have also been shown to have increased expression of the chemokine CXCL10 [73]. CXCL10 is directly responsible for the recruitment of leukocytes [74] and when combined with the hyper expression of HLA class 1 by stressed β-cells, the critical role of β-cell stress in insulitis is demonstrated. Interestingly, in a humanized mouse model β-cell stress was shown to be critical in the

onset of insulitis as high levels of insulin specific autoreactive T cells only appeared after the mice had been primed for an autoimmune response and had been given streptozotocin to induce beta cell stress [75].

1.2.3 Oxidative stress in the β-cell increases proteome diversity

As discussed before, β-cells are very susceptible to oxidative stress and stressed β-cells promote inflammation in the islets. Interestingly, oxidative stress can cause epigenetic changes that lead to autoimmunity [76] and oxidative stress has been implicated in a number of autoimmune diseases [77]. T1D is no different as β-cell stress is known to cause the formation of neo-antigens which vastly increases the proteome diversity of the β-cells [78]. Neo-antigens are proteins that are not normally produced by the β-cells meaning that there is no immune tolerance against these proteins and for this reason neo-antigens have been found to be strongly immunogenic [79]. The creation of the neo-antigens arise from a number of aberrant cellular processes in stressed β-cells including: alternative splicing [80], translational mistakes [81], peptide fusion [82] and post translational modifications [83]. In the case of neoantigens created by aberrant post translational modifications (PTMs) , oxidative stress seems to be playing a direct role [84].

Oxidative stress in the β-cells directly causes the creation of neo-antigens through a number of aberrant PTMs including: deamidation, oligomerization, citrullination, carbonylation and general spontaneous oxidation [84].Of the many PTMs caused by oxidative stress much of the current evidence is pointing to deamidation playing a major role in the creation of neoantigens. Deamidation converts neutral amino acids such as asparagine and glutamine into their acidic counterparts aspartic acid and glutamic acid.

Although this process can happen spontaneously, enzymes in the transglutaminase family are directly involved in deamidation with the most common enzyme in this family being tissue transglutaminase 2 (TG2) [85]. Interestingly, TG2 has been found to have increased expression under conditions of oxidative stress [86] and since TG2s activity is governed by intracellular calcium [87] (which oxidative stress has also been found to increase [88]), The activity and expression of TG2 are both directly increased by oxidative stress. Furthermore, in the context of T1D aberrant TG2 expression is much higher in the pancreas of the T1D mouse model (non-obese diabetic mouse (NOD)) when compared to other tissues of the mouse which demonstrates the importance of deamidation in T1D. [89]. This is further reinforced by the fact deamidated neoantigens derived from glutamic acid decarboxylase 65-kDa isoform (GAD65) and islet antigen 2 (IA-2) have been found to be immunogenic in T1D patients [79, 90].

In addition to deamidation, transglutaminases can also catalyze the creation of protein crosslinks between lysine and glutamine residues [91]. Interestingly, Chromogranin A (A protein involved in vesicle storage and insulin secretion in the β-cells) forms oligomers in the presence of TG2 which have been found to be recognized by T cell clones from the NOD mouse [92]. T cells isolated from patients with T1D were also found to recognize the TG2 modified Chromogranin A [93] indicating that oligomerization plays a role in T1D onset. When considering the effects of aberrant TG2 expression caused by oxidative stress in the β-cells, it becomes clear that oxidative stress is directly contributing to creation of neoantigens and the resulting autoimmunity seen in T1D highlighting the importance of preventing oxidative stress in the β-cell.

1.2.4 Pathways that protect the β-cells from oxidative stress are crucial in preventing T1D onset

As discussed earlier oxidative stress plays a critical role in T1D onset and for this reason improving β-cell resistance to oxidative stress has been a promising avenue for drug development projects for T1D [94]. Of the many pathways that the cell has for dealing with the accumulation of reactive oxygen species, the evolutionarily conserved mechanism of the nuclear factor-erythroid 2 p45-related factor 2 (NRF2) and its cytoplasmic repressor, Kelch-like ECH-associated protein 1 (KEAP1) pathway is absolutely critical [95]. The Keap1/Nrf2 pathway functions by using a unique mechanism which is dependent on Keap1 binding to Nrf2[95]. Under unstressed conditions Keap1 binds to Nrf2 resulting in the ubiquitination of Nrf2 and subsequent degradation by the proteosome. This is a quick process as under unstressed conditions the half-life of Nrf2 is around 20-40 minutes [96]. Under conditions of oxidative stress Keap1 binding to Nrf2 is inhibited through a complex mechanism of cysteine modifications caused by ROS [97] as well as direct inhibition by phosphorylated p62 [98]. With Keap1 inhibited, Nrf2 is released which in turn allows Nrf2 to translocate to the nucleus and bind to the antioxidant response element (ARE) within the promoter region of antioxidant genes. Upon Nrf2 binding to the ARE the transcription of a number of antioxidant genes is promoted including superoxide dismutase (SOD) [99] and heme oxidase (HO) [95] both of which are critical antioxidant enzymes with SOD being directly involved in the breakdown of ROS [100].

Due to the Keap1/Nrf2 pathway playing such an important role in protection from oxidative stress, there is a growing amount of research showing that Nrf2 signaling

plays an important role in diabetes [101]. In T1D the Keap1/Nrf2 pathway plays a crucial role in preventing T1D onset, Research has shown that if the Nrf2/Keap1 pathway is inhibited there is a significant promotion of the progression of T1D in mice [102]. Furthermore, a knockout of Keap1 resulting increased activation of Nrf2 significantly suppressed the onset of diabetes in a T2D model [103].

Although the Keap/Nrf2 pathway plays a critical role in the antioxidant response and the breakdown of ROS, a pathway is needed to repair or recycle dysfunctional organelles that come because of oxidative stress. This is where autophagy, also known as cellular recycling comes into play. In the cell there are a number of different forms of autophagy including microautophagy, macroautophagy, and chaperone-mediated autophagy [104]. Of the many forms of autophagy, proper regulation of macroautophagy seems to be playing a critical role in preventing diabetes onset [105]. macroautophagy is characterized by the formation of a cytosolic double membrane vessel known as an autophagosome which is needed to sequester and transport cargo to the lysosome for degradation [104]. In the β-cell a specific form of macroautophagy known as selective macroatuophagy is critical for maintaining β-cell homeostasis [105]. Selective autophagy differs from non-selective autophagy in the fact that during selective autophagy, ubiquitinated organelles are recognized and bound by a scaffolding protein like p62 which then targets the organelle for degradation by autophagy [106]. In the β-cell, selective autophagy allows for the degradation of dysfunctional organelles and is critical for maintaining β cell function as recent evidence has shown that autophagic function is significantly reduced in the NOD mouse [107] implying proper autophagic function is needed to prevent T1D. When combined with the antioxidant effects of the

Keap1/Nrf2, it becomes apparent that pathways that help the β-cell deal with oxidative stress are critical in preventing T1D. Experiments presented in this dissertation further this point by demonstrating compounds which activated the Keap1/Nrf2 pathway protect β-cells from oxidative stress and delay T1D onset in a T1D mouse NOD model.

1.3. T1D Treatment and Ethnopharmacology

1.3.1 The T1D Interventional window

Standard care for T1D has mostly revolved around treating the affected individual with insulin following clinical presentation of hyperglycemia after the destruction of the pancreatic β-cells by autoimmunity. Therapies which promote β-cell survival may remove the need for exogenous insulin by preventing the mass loss of pancreatic β-cells during T1D onset.

As discussed before, the exact cause of T1D is complex and varied involving a mix of environmental factors causing β-cell stress and genetic factors promoting autoimmunity. Regardless of the exact cause, all T1D patients have an autoimmune response which promotes infiltration of the pancreatic islets with leukocytes. This invasion of the islets results in a substantial increase in cytokine release which creates a toxic inflammatory microenvironment that attenuates β-cell function and induces β-cell apoptosis [108]. Furthermore, it has been well established that inflammation causes oxidative stress in inflamed tissues [109] further exacerbating the damage to the β-cells under these conditions.

This destructive autoimmune process precedes clinical identification of measurable metabolic disorders (hyperglycemia or elevated HbA1c) providing a potential treatment

window. Clinical T1D follows the development of autoantibodies in 90% of individuals against pancreatic β-cell antigens [110] with research showing that individuals who express two or more of these antibodies have an 84% chance of developing T1D by the age of 18 [32]. This research has resulted in individuals displaying advanced β-cell autoimmunity (multiple autoantibody types) but without T1D symptoms being classified as having an early stage of T1D progression. As discussed earlier there are three stages to T1D progression: Stage 1: presence of two autoantibodies with no symptoms, Stage 2: autoantibodies with abnormal blood glucose levels, Stage 3: clinical onset of T1D. As autoantibodies in stage 1 can be detected years before clinical onset in stage 3 [111], a generous interventional window is created as the autoantibodies are easily detectable and a large amount of Pancreatic β-cells still remain functional**.** As discussed before, oxidative stress plays a critical role in T1D progression and compounds that may prevent or help the β-cells deal with the oxidative inflammatory environment may provide a new avenue of interventional therapies when given during this interventional window.

1.3.2 Current preventative interventional therapies

Numerous attempts have been explored to prevent or inhibit the onset of T1D but largely have been unsuccessful. These include several immunomodulatory strategies evaluated for the intervention of T1D. For example, an extensive trial evaluating cyclosporin A was shown to promote T1D remission, but only for a limited time and eventually insulin dosing was increased [112]. Numerous other clinical interventional studies have utilized anti-CD3 , anti-CD2034 monoclonal antibodies; antithymocyte globulin (ATG), and inhibitors of interleukin-1 (IL-1). All of which failed to preserve

pancreatic B-cell mass and C-pepide indicating that immunotherapies alone may not be sufficient as a therapeutic approach for interventional T1D therapy [113].

As of the writing of this dissertation there are many T1D therapies being investigated [114], with the first cell based therapy using allogenic islet transplantation being approved in 2023 [115]. Currently there is only one major FDA approved interventional therapy designed to preserve beta cell function. This treatment is an anti-CD3 antibody known as teplizumab[116]. Although teplizumab is an anti CD3 antibody, treatment with teplizumab does not result in complete depletion of a patient's T cells instead evidence suggests that teplizumab works by a unique agonistic mechanism. Preclinical trials have suggested that teplizumab acts as a partial agonist to T cells resulting in changes in differentiation and circulation of activated T cells [117]. These changes result in the exhaustion of CD8+ T cells [118], which in turn causes a decrease in tumor necrosis factor-α and interferon-γ production, both of which are associated with T cell mediated β-cell death [118]. What is especially interesting about teplizumab is that evidence shows that this effect is mostly limited to activated CD8 T cells and spares regulatory T cells and memory T cells meaning that the preservation of β-cell function is not associated with a long term consequence on immune function[119].

Although teplizumab is a groundbreaking therapy, it still has several limitations. When evaluated during a clinical trial teplizumab had a significant effect on delaying the onset of T1D when compared to placebo, however 43% of the treatment group still progressed to clinical diabetes [120]. As discussed earlier the β-cell itself seems to be playing a significant role in T1D progression and just focusing on modulating the immune system will not be enough to completely prevent the onset of T1D thus

compounds that can preserve β-cell function by promoting β-cell viability should be investigated.

Taken together, despite the most recently approved interventional therapy and cell based therapeutic for T1D, there is very strong demand for safe and effective novel T1D interventional therapies. My research has demonstrated that the highly utilized *Cornus officinalis* may provide a therapeutic resource for the treatment of T1D and is thoroughly investigated in this proposal.

1.3.3 Source and preparation of *Cornus Officinalis*

Ethnopharmacological approaches have been shown to be instrumental in treating numerous diseases [121, 122] and has resulted in the discovery of new and novel therapies for debilitating diseases like cancer and diabetes [123]. One ethnopharmacological approach which seems to hold a lot of promise is the extract from the fruit of the *Cornus Officinalis* (CO) tree. CO originates from east Asia and has been used in traditional Chinese (TCM) medicine for millennia, with its traditional uses including nourishing the kidney and liver [124].

The CO extracts we used in our experiments were sourced from Evergreen pharmaceuticals located in Taiwan. As per Evergreen, to create the CO extract the fruits of the *Cornus Officinalis* tree are harvested in early winter when the pericarp of the berries is a bright red (**Figure 1**). Fruits are then baked over a soft fire and dried (**Figure 1**) and milled into a powder. Quality control analysis is then determined by TLC analysis to ensure Loganin content within the CO extract.

1.3.4 Individual constituents and biological effects of *Cornus Officinalis*

Interestingly, crude extract and individual compounds extracted from CO have been shown to have numerous pharmacological effects. In whole CO extract our lab had shown that the top ten most abundant compounds include: Cornuside II, Ellagic Acid, Malic Acid, Morroniside diglycoside, Morroniside, Loganin, Loganic Acid, Gallic Acid, Sweroside and Tartaric Acid [125]. Out of the most abundant compounds found in CO, Loganin and Morroniside are of particular interest as they have been shown to have distinct bioactive effects [126]. These bioactive effects were demonstrated when CO treatment in a Streptozotocin (STZ) induced diabetes mouse model significantly reduced fasting blood glucose and the symptoms of diabetes related complications like polyphagia and polydipsia were found to be alleviated [127]. Furthermore, CO has also been shown to have renal protective effects through the inhibition of AGE/RAGE formation in the STZ induced diabetes mouse model [126]. This data so far seems to suggest that CO can protect the cell from stress and was further reinforced when compounds isolated from CO where found to have a strong antioxidant effect as shown by the fact that Morronoside (Isolated from CO) can protect a human neuroblastoma cell line from hydrogen peroxide induced cell death [128].Due to the ability of CO to alleviate symptoms of diabetes in a STZ induces mouse model and compounds isolated from CO preventing Hydrogen peroxide induced cell death, CO seemed to be a promising source for new T1D interventional therapeutics.

1.3.5 *Cornus officinalis* promotes pancreatic β-Cell viability

Due to the aforementioned findings our lab decided to investigate and determine if CO could induce potentially beneficial effects in a human pancreatic β cell line (1.1B4). Upon investigation we found that CO induced a number of potentially beneficial effects in the 1.1B4 cells. Our lab demonstrated that CO induced increased metabolic activity and cell viability as measured by an MTT assay in a time and dose dependent manner [125]. This increase in viability was than further elaborated on when our lab showed that CO treatments as short as 2 and 4 hours substantially increased the 1.1B4 cells mitochondrial respiratory capacity [125]. With our lab demonstrating that CO could induce 1.1B4 cell viability the question remained if CO had any form of biologically relevant protective effects. To test this 1.1B4 cells were treated with a cytokine trio of interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β) followed by a treatment with CO [125]. Interestingly CO was shown to rescue the 1.1B4 cells in a time and dose dependent manner demonstrating that CO may have a lot of promise as a T1D interventional therapy.

After these experiments two critical questions remained: What was the mechanism of action for the biological effects of CO? Would CO extract have any effect in an *in vivo* T1D model? To address these questions is where my personal contributions to the field of endocrinology and diabetes begin.

A. 

B. 

Figure1: (A) Representative image of the fruit of the *Cornus Officinalis* tree in early winter (B) Representative image of the CO fruit after being baked and dried (images Provided by Evergreen pharmaceuticals)

CHAPTER 2:

PROTEOMIC EXAMINATION OF CORNUS OFFICINALIS STIMULATED 1.1B4 HUMAN PANCREATIC CELLS REVEALS ACTIVATION OF AUTOPHAGY AND KEAP1/NRF2 PATHWAY

Note to Reader: I was a co first author on this paper and share first authorship with Arielle Tawfik. This Chapter (except for sections 2.7 and 3.7) was published in molecular and cellular endocrinology: Sharp-Tawfik, A.[1], Fletcher, J. D[1]., Guergues, J.[2], Marelia-Bennett, C.[3], Wolf, T. J., Coiner, A. M., Zhang, Y. C., Stevens, S. M., Jr. & Burkhardt, B. R. (2022) Proteomic examination of Cornus officinalis stimulated 1.1B4 human pancreatic cells reveals activation of autophagy and Keap1/Nrf2 pathway, Mol Cell Endocrinol. 557, 111773. PMID: 36100124

2.1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease that results in the targeted apoptotic and necrotic destruction of the insulin-producing pancreatic β-cells [129-131]. The precise etiology and pathogenic mechanisms resulting in T1D are not fully elucidated but are being investigated by numerous T1D observational studies [130]. The primary therapy for T1D is the use of exogenous insulin, and although the technology for insulin delivery has improved significantly since inception in 1922, there are very few interventional therapies available. However, promising results have recently been observed with the investigational monoclonal anti-CD3 antibody teplizumab which

demonstrated improved pancreatic β-cell function and delayed clinical onset among high-risk relatives of individuals with T1D [118, 132]. Autoimmune mediated targeting and destruction of the pancreatic β-cells is typically a process that takes years and precedes eventual clinical identification and measurable metabolic disorder (hyperglycemia or elevated $HbA1_c$).

For development of T1D interventional therapy, an approach is required that will slow or prevent the autoimmune destruction of pancreatic β-cells while preserving and enhancing the function of existing β-cells. Therefore, identification and development of novel and safe T1D interventional strategies and modalities are urgently needed. Natural products, mainly phytochemicals represent an abundant resource for novel therapeutic candidates, especially with treatment for T1D [133]. Specifically, *Cornus officinalis* (CO) has shown antidiabetic properties *in vitro* and *in vivo* [134-136]. CO has been utilized for medicinal purposes for over 2,000 years with more than 20 traditional Chinese medicine (TCM) prescriptions containing CO to treat a range of renal and endocrine disorders with an extensive composition of biologically active compounds [137]. Our earlier findings indicated CO is capable of stimulating pancreatic β-cell viability, metabolic activity and expression of critical regulators of pancreatic β-cell differentiation and function, such as Nuclear Factor of Activated T-Cells (NFAT) [125]. The composition of CO is highly complex as our prior High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis identified almost 300 compounds with the most abundant being cornuside, ellagic acid, malic acid, morroniside, loganin, loganic acid, gallic acid, sweroside and tartaric acid., The abundance of potential etiological compounds makes elucidating a mechanism of action

extremely challenging. Therefore, a comprehensive proteomic approach is well suited to understand and elucidate the potential network of causative molecules involved in this CO-induced biological process.

To address this complex question, a global proteomic approach was incorporated to delineate biological effect via abundance and phosphorylation status of proteomic networks. Therefore, we analyzed the full proteome as well as the phosphorylation events upon CO induction in the human pancreatic β-cell line, 1.1B4, by quantitative label-free mass spectrometry (MS). 1.1B4 cells have been proven as a useful model of human beta cells. They display characteristics of normal pancreatic β-cells that express human insulin, β-cell enriched genes, glucose responsiveness, and stability in culture for biological study like human islets [138-140]. Label-free proteomics can measure significant changes within a complex cellular mixture encompassing an entire proteome or phophoproteome. Determining both are equally important in elucidating cellular networks and anticipated activation states. Following MS analysis, Ingenuity Pathway Analysis (IPA) was utilized to determine predicted molecules and pathways involved in CO's biological effect. The proteomic examination revealed significantly and differentially expressed cellular networks involved with autophagy and the Keap1/Nrf2 pathway. Significant increased phosphorylation of the selective autophagy receptor of p62 was identified upon CO stimulation. Bioinformatic analysis of the global proteomic investigation of differentially and expressed proteins predicted an increased activation of the Keap1/Nrf2 pathway along with identified increased expression of significantly and differentially expressed antioxidant response proteins such as heme oxygenase 1 (HO-1) and superoxide dismutase 1 (SOD-1). Further validation by immunoblotting and

immunofluorescence revealed increased markers of autophagy such as LC3-II and phospho-p62 upon CO stimulation along with nuclear localization of Nrf2. To our knowledge, this is the first report ever examining the biological impact of CO via a proteomic approach. Our manuscript provides a novel and compelling potential mechanism of action induced by CO that may be promoting both pancreatic β-cell viability and protection from cytotoxic physiological conditions as observed with early onset of T1D.

2.2 Materials and Methods

2.2.1. Preparation of *Cornus Officinalis*

CO was sourced from Evergreen Herbs International, LLC (kindly provided by Dr. Clare Zhang). Individual composition of this commercial source as determined by HPLC/MS has been previously published by our laboratory [141]. The CO extract utilized in the biological applications was prepared as previously described [125].

2.2.2. Cell Line culture and treatment

Cell culturing was performed as previously stated detailed [125]. Experiments were performed at passage number 12-15. Cells were seeded at 2 x 10^6 cells in a T75 flask and incubated overnight at 37°C with 5% CO_2 to allow the cell adherence. Cells were then treated with H_2O (vehicle control) or CO at 500 and 1000µg/mL for 2, 6, 12, and 24 hr. Cells were trypsinized and washed with 1X Phosphate Buffered Saline (PBS) prior to being stored in -80°C until processing for MS analysis.

2.2.3. Proteomic and phosphoproteomic analysis

Samples were processed using the suspension trap (S-trap) midi-kits as the protocol instructed with certain buffer modifications as previously described to prepare for MS analysis [142, 143]. An average of 661 µg of protein were loaded and processed, with 50 µg of tryptic peptides reserved for a global analysis, while the remaining peptides were further enriched for phosphopeptides using the High-Select TiO_2 Phosphopeptide Enrichment Kit (Thermo). A 2 cm x 75 µm ID PepMap C18 trap column (Thermo) was used to pre-concentrate digested samples before running through a 55°C-heated 75 cm x 75 µm ID C18 PepMap column (Thermo) on an Ultimate 3000 UPLC system (Thermo) with a 120-min gradient from 2-28% B (B was 0.1% formic acid in 80% acetonitrile:20% water). Mass spectrometric analysis was performed on a hybrid quadrupole-Orbitrap instrument (Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer, Thermo) operated in data-dependent acquisition (DDA) mode using a top-10 method. Full MS spectra in the range of 375-1200 m/z were acquired at 70,000 resolution followed by MS/MS scans at 17,500 resolution.

MaxQuant (version 1.6.6.0) was used to search raw files against the Uniprot protein database for Homo Sapiens, UP000005640 with 71607 entries. Variable modifications of N-terminal protein acetylation and methionine oxidation as well as the constant modification of cysteine by carbamidomethylation were applied as search parameters. The additional database of known contaminants provided with MaxQuant was also applied with a first search tolerance of 20 ppm followed by a main search tolerance of 4.5 ppm. A decoy search strategy using reversed sequences was utilized to achieve protein and peptide FDR values of less than 1%. LFQ-based quantitation was enabled,

on the global proteomic analysis samples, with a minimum ratio count of 1, and the "match-between-runs" feature using default settings was employed to increase protein identification. For phosphopeptide-enriched samples that were analyzed, the additional Phospho (STY) modification was added as a variable modification to the database search.

Reverse sequences, contaminant sequences, and proteins only identified by modification were then filtered out of the resulting proteinGroups text file, yielding the initial identification of 28,774 phosphorylation sites (including redundant hits before localization probability filtering) and 6,161 global proteins for phosphorylation site quantitation and global protein expression analysis, respectively. Perseus (version 1.6.15.1) was then used twice to separately analyze and further filter the global proteome and the enriched phosphopeptides data sets. For global analysis, values were log2 transformed and any proteins that did not have at least 70% valid values across all treatments and replicates were removed. This filtering yielded 5,063 quantifiable global proteins. For the phosphosite analysis, an initial filtration was performed where any sites with a location probability less than 75% were removed before expanding the site table. Values were then also log2 transformed and any phosphosites that did not have at least 70% valid values across all treatments and replicates were removed yielding 7,121 phosphosites. The remaining missing values for both analyses were then replaced with the imputation function using width and downshift parameters of 0.3 and 1.8, respectively, for the global set and 0.6 and 1.6, respectively, for the phosphosite analysis to best fit the low abundance values of the Gaussian curve.

The average intensity ratio of treatment over control by matched time point was then calculated in Excel along with a Welch's t-test (p-value < 0.05) and z-score (z-value > 1). To limit the false discovery rate while maintaining sensitivity, statistically significant differentially expressed proteins were filtered using both Welch's t-test and z-score cutoffs [144, 145]. Finally, less stringent lists, filtered by Welch's t-test only, containing protein identification and average ratio of each comparison, were then uploaded to Ingenuity Pathway Analysis (IPA). This provided a larger pool to increase the bioinformatic analysis prediction of upstream regulators, over-represented canonical pathways, and other biological and disease functions ($p < 0.05$, Fisher's exact test).

2.2.4. Western analysis of LC3, phospho-p62, SOD-1, HO-1 and GAPDH

Cells were seeded in a 6 well plate and treated with CO. Cells were pelleted and resuspended in lysis buffer (50 mM ammonium bicarbonate solution with 50 mg of SDS). Lysates were heated at 95°C for 4 min and sonicated for 6 rounds at 200 amp for 3 seconds each round. Protein concentrations were quantified using the Pierce 660nm protein assay (Thermo Fisher Scientific). Protein samples were electrophoresed on a 4-20% TGX SDS PAGE gel (Bio-Rad) using the Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad) and transferred to a nitrocellulose membrane using the iBlot dry transfer system (Invitrogen). The nitrocellulose membrane was blocked using 5% milk-Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST) for 2h. The membranes were incubated overnight with primary antibodies for the detection of LC3 (Cell Signaling Technology #4108, 1:500), phosphorylated p62 (Cell Signaling Technology #13121, 1:500), total p62 (Cell Signaling Technology #5114, 1:500), SOD-2 (Cell Signaling Technology #13141, 1:500), HO-1 (Cell Signaling Technology #5061S,

1:500) and GAPDH (Cell Signaling Technology #5174S,1:500). Based on information provided by Cell Signaling Technology, the LC3 antibody detects both LC3A/B and cross-reactivity may exist with LC3C. Membranes were incubated in secondary antibody followed by chemiluminescent detection (SuperSignal West Femto, Thermo Fisher Scientific). Membranes were then imaged using the Amersham™ Imager 600 (GE Healthcare Life Sciences). Densitometric analysis for measurement of pixel density from band detection upon western analysis was performed using ImageJ version 1.53r [146].

2.2.5. Immunofluorescence of cytosolic LC3 and nuclear localization of Nrf2

To perform the immunofluorescent assay, cells were seeded in a 12 well plate at 1 x 10^6 cells per well. Each well also contained a 15mm diameter circular coverslip of 1.5mm thickness. The cells were then treated with CO at previously described concentrations. Media was removed and cells were washed with 1X PBS. After washing with PBS, the wells were incubated with 500 µl of a 4X formaldehyde solution for 10 min followed by a 1X PBS wash. Cells were then incubated with 500 µl of a 0.25% Triton X 100 solution. After the incubation, wells were rinsed with 1X PBS and incubated for 1 hour with 30 µl of a 1:500 dilution of the LC3 antibody (Cell Signaling Technologies, Cat. No. #4108) in 1X PBS. For Nrf2 detection, incubated for 1 hr with 30 µl of a 1:500 dilution of the primary Nrf2 antibody (Cell Signaling Technologies, Cat. No. 12721s) in 1X PBS. Cells were then incubated for 1 hr in 40 µl of a 1:1000 dilution of the Alex Fluor 488 secondary antibody (Thermo Fisher, Cat. No. #A27034) in 1X PBS. Cells were then washed for a final time with PBS. Coverslips were then removed and fixed onto a microscope slide using a DAPI stain and allowed to set for 20 m. Coverslips were then sealed onto the microscope slide using commercially available nail polish and incubated

overnight. Slides were imaged using an immunofluorescent microscope (Nikon Eclipse Ti2).

2.2.6 Cell viability determination by MTT assay

Cells were seeded in a 96 well plate with 100ul of a 5 X10^4 cell suspension in each well. Cells were then left untreated (NT group), treated with water (Vehicle control (VC) and a CO concentration of 32 ug/ml for 4 hours. After the initial treatments the CO treated cells and a control group were treated with 500um H2O2 for 4 hours. After the H2O2 treatments, we then used a commercially available MTT kit to perform the MTT assay (Promega, Cat No.G4000). In brief, the dye was added in a 15:100 ratio for each well and then was incubated at 30C for 4 hours. The solubilization solution was then added in a 1:1 ratio and incubated overnight. Absorbance of each well of the 96 well plate was then measured at 570nm and the data was inputted onto graphpad prism and statistical analysis was performed by T test.

2.3 Results

2.3.1 Phosphoproteomic analysis of CO-treated 1.1B4 cells

to elucidate a biological mechanism of action for therapeutic application of CO, we utilized quantitative mass spectrometry to evaluate the proteome of CO-induced 1.1B4 cells at varying time points and concentrations. 1.1B4 cells were treated with vehicle control (H_2O) or 500 µg/mL and 1000 µg/mL of CO for 2, 6, 12, and 24 hr followed by protein purification and run on a hybrid quadrupole-Orbitrap instrument (Q Exactive Plus, Thermo). The overall experimental design and methodology for proteomic processing and analysis are shown in **Figure 2** and discussed in Materials and

Methods. As also mentioned previously, our proteomic filtering method resulted in the identification of 5,063 quantifiable proteins, with the phosphopeptide analysis identifying 7,121 quantifiable sites. Differentially expressed proteins upon CO treatment compared to vehicle control treated 1.1B4 cells were analyzed by performing a Welch's t-test and z-score for each timepoint. All significantly differentiated proteins had a p-value of at least <0.05 and a z-score >1. In addition to the examination of CO induced changes in protein abundance, we also evaluated phosphorylation status of the proteome to further characterize the CO-induced mechanism of action. A complete listing of the experimental data obtained from the proteomic analysis listing significantly and differentially expressed global proteins and phosphoproteins collected from all time points post-treatment are shown in Supplementary **Tables 1 and 2**.

Statistical analysis of the phosphoproteomic data revealed that one of the most significantly induced phosphoproteomic changes observed was with autophagy adaptor, p62 (also known as sequestosome-1) (Fig. 2). P62 was significantly hyperphosphorylated at 6, (**Figs. 3A and 3B**), 12 (**Figs. 3C and 3D**), and 24 hr (Figs 2E and 2F) following CO treatment at both concentrations as shown in the volcano plots. Numerous phosphosites within p62 were shown to be increased upon CO treatment at positions of 152, 226, 269, 272, 275, 276, 277, 288 and 328. This result is summarized and detailed in **Figure 4A** showing the temporal relationship of increased phosphorylation of p62 upon CO treatment. For example, a 6 hr exposure of 1.1B4 cells to CO resulted in 3 identified phosphorylated sites whereas a 24 hr exposure found 5 phosphorylated sites within p62 (**Fig. 4A**). To further validate this proteomic finding, immunoblotting was performed on cell.

lysate for detection of both total and phospho-p62 following CO stimulation (**Fig. 4B**). Western analysis revealed that total p62 levels decreased upon CO stimulation (**Fig. 4B**) as compared to both no treatment (NC) and vehicle control (VC) conditions. A statistically signficant decrease of total p62 was observed at the highest dose and time exposure based on densitometric analysis (**Fig. 4C**). In contrast, the ratio of phospho-p62 to total p62 increased signficantly (**Fig. 4D**). In summary, our proteomic findings in combination with the western validation have suggested that CO stimulation of 1.1B4 cells increases p62 phosphorylation with a decrease in total p62 indicating induced autophagy later supported in these results by further validation of autophagic markers.

2.3.2. Proteomic prediction of CO-induced Nrf2 activation

Ingenuity Pathway Analysis (IPA) was performed on the proteomic dataset of differentially expressed proteins across all conditions and times in relation to control treatment. A significantly predicted pathway across the CO treatments was Nrf2 activation **(Fig. 5).** IPA analysis on global significantly and differentially expressed proteins identified and predicted Nrf2 activation upon CO stimulation for 24 hr at 500 (20 proteins within network) (Fig. 4A) and 1000 μg/ml (31 proteins within network) **(Fig 5B).** Nrf2 activation was significantly shown at other times post-treatment as well (data not shown). Significance of activity prediction was determined by Welch's t-test with by an activation z-score of 2.616 and 2.854 for 500 and 1000 ug/ml treatment, respectively. The significantly and differentially expressed proteins associated with Nrf2 activation are listed in both Table 1 and 2. Significant proteomic findings across dosages regarding Nrf2 associated proteins were very consistent. Eighty-five percent (17 out of 20) of the differentially expressed proteins at 500 μg/ml were also identified at the 1000 μg/ml

dosage **(Tables 1 and 2 Respectively)**. Numerous significantly and differentially expressed proteins governed by Nrf2 and associated with several cellular functions such as increased antioxidant stress response, (SOD2, GCLC, GCLM) and autophagy (SQSTM1 (p62), MAP1LC3B, MafF) were identified across treatment conditions.

2.3.3. CO-induced autophagosome formation in 1.1B4 cells

To further validate the CO-induced biological pathways identified by the proteomic analysis, we examined cellular distribution of LC3 within CO treated cells by immunofluorescent assay to determine autophagy by the presence of autophagosomes **(Fig. 6).** 1.1B4 cells were treated with CO at previously described concentrations in addition to hydroxychloroquine (HCQ) to serve as positive control for visualization of LC3 cytosolic puncta. HCQ inhibits autophagy by blocking the fusion of autophagosomes with lysosomes, therefore inducing an accumulation of autophagosomes. Upon treatment of CO, an increase in cytoplasmic LC3 puncta was markedly observed (**Fig. 6**, Panels E and F) as compared to water treated controls (**Fig. 6,** Panels A and B) as determined by immunofluorescence shown by representative images. LC3 puncta distribution was consistent with distribution observed with HCQ treatment (positive control) (Fig. 5, Panels C and D). Overall, upon CO stimulation, increased LC3 positive puncta were observed suggesting an increased formation of autophagosomes.

2.3.4. CO-induced formation of LC3-II in 1.1B4 cells

LC3 is conjugated to phosphatidylethanolamine (PE) to form LC3-II, which is localized to isolation membranes and autophagosomes [21]. LC3-II is a frequently utilized autophagosome marker because the amount of LC3-II proportionally reflects the number of autophagosomes. Our MS data analysis revealed that LC3 was significantly increased at 24 h post-treatment **(Fig. 5).** However, our proteomic approach is unable to distinguish between LC3-I and LC3-II, therefore we further discerned LC3 specific expression by size distribution. Despite the increased molecular weight, LC3-II has a faster mobility than LC3-I by SDS-PAGE and will appear at approximately 14 kDa instead of 16 kDa as observed with LC3-I [147]. Therefore, we measured the expression for both LC3-I and LC3-II by western analysis in CO stimulated 1.1B4 cells **(Fig. 6G).** Increased LC3-II conversion was observed upon CO induction in 1.1B4 cells at both at both the 500 and 1000 μg/ml concentrations following 3 and 6 hr treatment as compared to mock treated controls as shown by western analysis (**Fig. 6G.** Lanes 3-6). Densitometric analysis revealed statistically significant differences (ANOVA, $p<0.05$) for LC3-II conversion upon CO stimulation at the highest dose and time post-exposure (**Fig. 6H**).

2.3.5. Validation of Nrf2 activation in CO stimulated 1.1B4 cells

Our prior experiments indicated that p62 would be phosphorylated and IPA predicted Nrf2 would be activated upon CO stimulation. Phosphorylation of p62 promotes the Keap1/Nrf2 pathway during selective autophagy [98]. Under normal cellular conditions, the transcription factor Nrf2 is degraded via the ubiquitin-proteasome pathway. Nrf2 has a binding partner, Keap1 that serves as an adaptor of the ubiquitin ligase complex that

targets Nrf2. Upon phosphorylation, p62 interacts with the Nrf2-binding site of Keap1 and competitively inhibits this interaction. As a result, Nrf2 translocates to the nucleus to enhance the transcription of numerous cytoprotective genes such as HO-1 (also demonstrated to be increased by CO within our study, **Fig. 4**), among many others [148]. In addition, Nrf2 is primarily governed through the regulation of its subcellular distribution instead of increased production or induction [149]. Our IPA analysis suggested activation of Nrf2 but to further explore this pathway this needed to be investigated by evaluation of cellular distribution upon CO induction. Therefore, we performed immunofluorescent analysis on the subcellular distribution of Nrf2 within CO-stimulated 1.1B4 cells (**Fig. 7**). A lower concentration of CO stimulation was utilized in these experiments as before CO stimulation was utilized in these experiments as before because initial optimization detected higher backgrounds of immunofluorescence upon visualization. In the absence of CO stimulation (no treatment) (**Fig. 7, Panel A**) or vehicle treatment (**Fig. 7, Panel B**), Nrf2 was not found to be localized within the nucleus as shown by lack of overlap with DAPI staining. However, upon CO stimulation at either concentration or time post-treatment, Nrf2 staining was identified within the nucleus in much greater abundance than observed in no treatment or vehicle treated conditions (**Fig. 7,** Panels C-F). In summary, CO stimulation of 1.1B4 cells may promote the redistribution of Nrf2 to the nucleus and supports the predicted activation and significant antioxidant response as earlier indicated by the proteomic analysis.

2.3.6. Measurement of downstream targets of Nrf2 in CO-stimulated 1.1B4 cells

Nrf2 is a critical transcriptional activator that serves a central role in the expression of many cytoprotective genes in response to oxidative stress such as SOD-2 and HO-1

among numerous others [150]. Our proteomic analysis revealed that indeed SOD-2 and HO-1 was increased upon CO stimulation (**Fig. 5**. Therefore, we performed western analysis following independent experiments measuring CO stimulation of 1.1B4 cells and determined SOD-2 and HO-1 protein expression (**Fig. 8**). CO increased levels of both SOD-2 and HO-1 particularly at the highest concentration and time after exposure as compared to no or vehicle treatment controls (**Fig. 8A**). Statistically significant differences (ANOVA, $P<0.05$) for both SOD-2 and HO-1 expression were measured upon CO stimulation at the highest dose and time post-exposure (**Figs. 8B and 8C**).

2.3.7 CO stimulated 1.1B4 cells are protected from H_2O_2 induced oxidative stress

Since we had demonstrated that CO stimulates the critical Keap1/Nrf2 pathway antioxidant pathway resulting in the upregulation of antioxidant enzymes such as SOD-2 and HO-1 (**Fig 8**), we wanted to determine if CO treatment could protect the 1.1B4 cells from oxidative stress. To test this, we measured cell viability by MTT assay before and after treatment with H_2O_2. After treatment with H_2O_2 CO demonstrated a substantial protective effect as absorbance at 570nm was significantly different (Unpaired T test, P value <.0001) in the CO and H_2O_2 treated group when compared to the group treated with just H_2O_2 alone(**Fig 9**)**.** Due to absorbance of the formazan dye in the MTT assay being directly proportional to metabolic activity and thus viability, the retention of absorbance at 570nm in the CO treated group after treatment with H2O2 exhibits CO's ability to protect the 1.1B4 cells from oxidative stress.

2.4.Discussion

CO is one of the most popular components that has been used in ethnopharmacological approaches or TCM for thousands of years with various pleiotropic effects [137, 151]. However, the biological mechanisms of action for CO are still being fully elucidated and very complex since CO in of itself is a highly complex mixture [137, 141]. Our quantitative proteomic investigation followed up with further *in-vitro* validation has suggested the utility of this approach in unraveling complex pathways for drug discovery. Despite the wide use of CO in diabetic ethnopharmacological approaches, no other studies have been reported that have examined the impact of whole CO extract on a global proteome scale particularly in endocrine pancreatic cells. However, one investigation has reported the evaluation of loganin, an iridoid glycoside extracted from CO, in relation to improving outcomes such as memory loss and anxiety in a transgenic murine model of Alzheimer's disease [152]. By performing 2D-DIGE combined with MALDI-TOF-MS/MS, they were able to identify 28 differentially expressed proteins in loganin- treated mice as compared to wild-type mice. Ten of which were found to be associated with inflammatory response, energy metabolism, and synaptic proteins. The proteomic identified network was then confirmed by western analysis. Taken together, the overall utility of this approach certainly has merit in not only identifying molecular mechanisms but also regarding drug discovery as was employed with our approach. Finally, our generated proteomic dataset can provide a useful resource for further pursuit of novel CO-induced cellular mechanisms.

One of the most striking and significant findings of our proteomic investigation was the CO-induced increased phosphorylation and abundance of p62 identified by the

proteomics and validated by immunoblotting and immunofluorescence. p62 has numerous functions but was initially identified as a part of sequestosome-1 which serves as a storage vesicle for ubiquitinated proteins [153] and is considered as an adaptor protein involved in selective autophagy [154]. After complexing to ubiquitinated proteins, p62 will oligomerize and interact with the autophagosomal membrane protein LC3 (also identified and validated by our analysis) and thereby delivering cargo destined to be removed via the autophagosome [155]. LC3 exerts several roles in autophagy such as selection of cargo, membrane fusion and autophagosome formation. The interaction between p62 and LC3 is necessary for the regulated autophagic degradation of polyubiquitinated proteins. However, p62 has numerous roles besides autophagy including regulation of the antioxidant response, inflammation and cell survival [156-158]. Examining the structure of the p62 protein, numerous functional domains have been identified that serve well within the nature of p62 not only serving as an autophagy adaptor but also providing a critical signaling cornerstone in several biological pathways [159, 160]. Autophagy is an essential mechanism in pancreatic β-cells to overcome oxidative stress caused by proinflammatory cytokines highly expressed within the pancreas of early onset T1D such as TNF-α, IFN-γ and IL-1β. The depletion of autophagy in a mouse model caused reduced pancreatic β-cell mass and insulin function due to induction of apoptosis [161].

In this regard, 9 total sites within p62 were identified by the proteomics to be significantly phosphorylated upon CO induction in a dose and time-dependent manner. Phosphorylation of p62 increases the binding affinity of Keap1 subsequently allowing the release and stabilization of Nrf2 [98]. Nrf2 will then translocate to the nucleus to

promote the transcription of numerous cytoprotective genes through heterodimerization with small Maf proteins (Maf was also shown to be significantly increased by CO in our findings, Tables 1 and 2). In addition, both Keap1/Nrf2 bind to LC3 to create the autophagosome for degradation and subsequent recycling of toxic material induced by oxidative stress. The ability of CO to promote phosphorylation of a pleiotropic protein such as p62 may explain in part why CO can also have such broad ranging effects in numerous tissues.

Of the phosphorylated sites identified within p62 by our study, only T269 and S272 have been previously identified experimentally and reported in UniProt database. To our knowledge, S226 and S275 have been reported in the literature but not listed within UniProt. Therefore, 5 other potential phosphosites within p62 have been identified. Interestingly, Linares *et al.* revealed that p62 phosphorylation at T269/S272 is necessary to coordinate the amino acid response to mTORC1 activation, most likely due to binding of p62 with components of the mTORC1-signaling complex [162]. The authors also speculate that this initial activation of mTORC1 may promote phosphorylation of S351 of p62 and ultimately promote Nrf2 translocation to the nucleus and stimulation of antioxidant response. Phosphorylation of murine p62 at S351 corresponds to S349 in human p62. Phosphorylation of human S349 p62 has been demonstrated to stimulate Nrf2 activation and several cytoprotective genes [98]. Our findings certainly support this potential mechanism of site specific p62 phosphorylation in relation to Nrf2 activation. However, we did not identify this crucial site of S349 as being phosphorylated in our proteomic approach which dampens our conclusions. The closest site we identified as being significantly phosphorylated was S328 and this has

yet to be described in the literature. Another discrepancy we observed in our data is that we found increased total p62 by our proteomic approach at 24 hrs., yet our western validation revealed a decreased p62. This may be explained my longitudinal discrepancy between points of measurement. Total p62 can serve as an excellent marker of autophagic flux and based on our western analysis it does appear that CO is indeed stimulating this effect especially when combined with our other findings (increased LC3-II and cytosolic puncta). The increase in p62 observed by the proteomics may be the result of the positive feedback loop that has demonstrated that Nrf2 can stimulate p62 expression [156]. Since we have demonstrated that Nrf2 is stimulated by CO, it is possible that this Nrf2 may ultimately increase the levels of p62 following 24 hr treatment and explain the discrepancy.

The use of CO as an interventional treatment for T1D holds much promise in that the very mechanisms that are potentially deficient in the early onset of this disease such as either autophagy or Nrf2 activation in the pancreatic islets may be alleviated by chronic use of CO application. Several recent studies have recently shown that autophagy is impaired in both human and mouse models of T1D. Muralidharan *et al.* demonstrated that pancreatic islet autophagy is impaired in human T1D [107]. Furthermore, based on the increased quantity of telolysosomes (lysosomes containing undigestible cellular components) in the pancreatic β-cells of autoantibody-positive individuals they indicated that impaired autophagy may precede T1D clinical onset. Therefore, application of autophagy promoting, or restorative interventional agents may thwart further pancreatic β-cell destruction and autoimmune progression in T1D. A recent review by Muralidharan *et al.* has further detailed and summarized the studies demonstrating a potential role for

β-cell autophagy in the onset and progression of T1D [163]. The authors also conclude that therapeutic approaches aimed at promoting autophagy may inhibit pancreatic β-cell death and inhibit T1D progression.

Another key facet from our data is the predicted and validated increased activation of Nrf2 upon CO induction. The Keap1/Nrf2 pathway is considered the most critical antioxidant signaling pathway that counteracts oxidative stress and reactive oxygen species [95]. The precise etiological mechanisms for T1D onset are not completely understood but the widespread belief is the combination of genetic predisposition and environmental factors eventually contribute to uncontrolled pancreatic β-cell autoimmunity [131]. Increasing evidence is indicating that increased oxidative stress within pancreatic β-cells serves a precipitating role in the development of T1D [164]. A recent investigation examined the impact of the Keap1/Nrf2 signaling pathway in the progression of T1DM [102]. Investigators examined an alloxan- (ALX-) stimulated T1D, wild-type, and Nrf2 knockout ($Nrf2^{-/-}$) C57BL/6J murine models. $Nrf2^{-/-}$ mice displayed increased incidence of T1D and exhibited more severe pancreatic β-cell damage. In conclusion, the authors suggested that Keap1/Nrf2 signaling in pancreatic β-cells may serve as a therapeutic target for T1D.

Taken together, our findings within this report suggest that CO may promote highly protective mechanisms within pancreatic β-cells via both autophagy and the Keap1/Nrf2 pathways via the adaptor molecule of p62. Use of phytochemicals as potential activators of the Nrf2 pathway is an active area of investigation and warrants further exploration to identify novel therapeutic agents and strategies to promote cell survival and limit oxidative stress [165], even in relation to treatment of type 2 diabetes (T2D)

[166]. Further *in-vivo* studies are necessary to evaluate efficacy for potential use in the interventional treatment of T1D as a pancreatic β-cell preservation therapy.

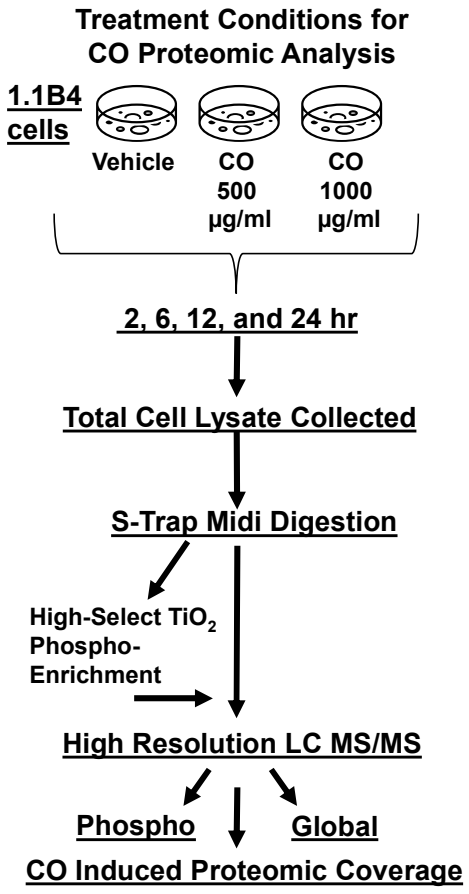


Figure 2: Experimental outline of proteomic approach. 1.1B4 cells were treated with CO at the concentrations of 500 and 1000 μg/ml for 2, 6, 12 and 24 hr. Following treatment, cell lysates were collected and S-Trap™ midi digestion was performed along with High-Select™ TiO_2 Phosphopeptide Enrichment for phosphorylation identification. Following respective purification, samples were analyzed on the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. Experiment was performed in biological triplicates. Further purification details are provided in Materials and Methods.

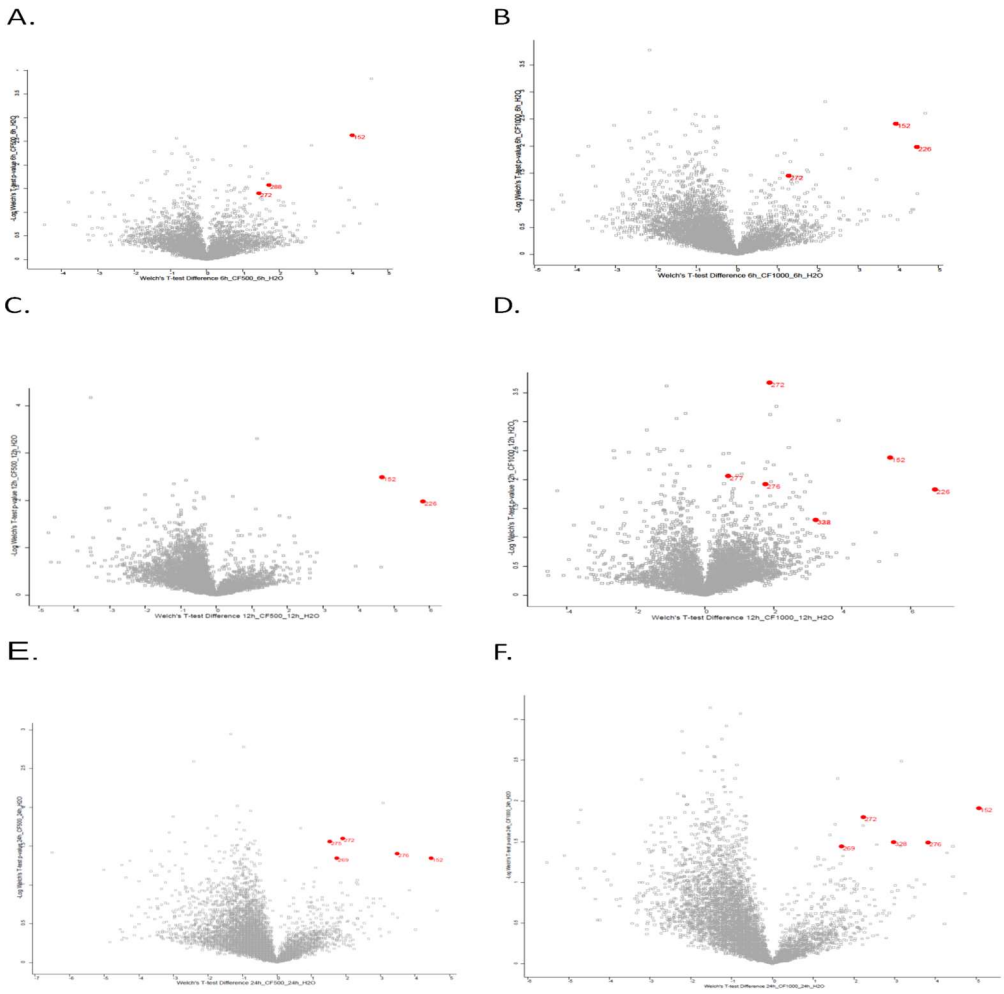


Figure 3: Increased phosphorylation of p62 following CO treatment. Volcano plots showing the phosphoproteomic analysis of human 1.1B4 pancreatic β-cells after 500 and 1000 ug/ml of CO treatment, respectively, following (A and B) 6, (C and D) 12 (E and F) 24 hr. Phosphosite and the q value as calculated by [-log(p) vs. t-test difference]. The averages of the phosphoproteomic expression data of CO-treated cells were compared with the averages of the no treatment group (N=3). Red circles show phosphosites that are significantly increased with the specific phosphorylated p62 position numerically identified.

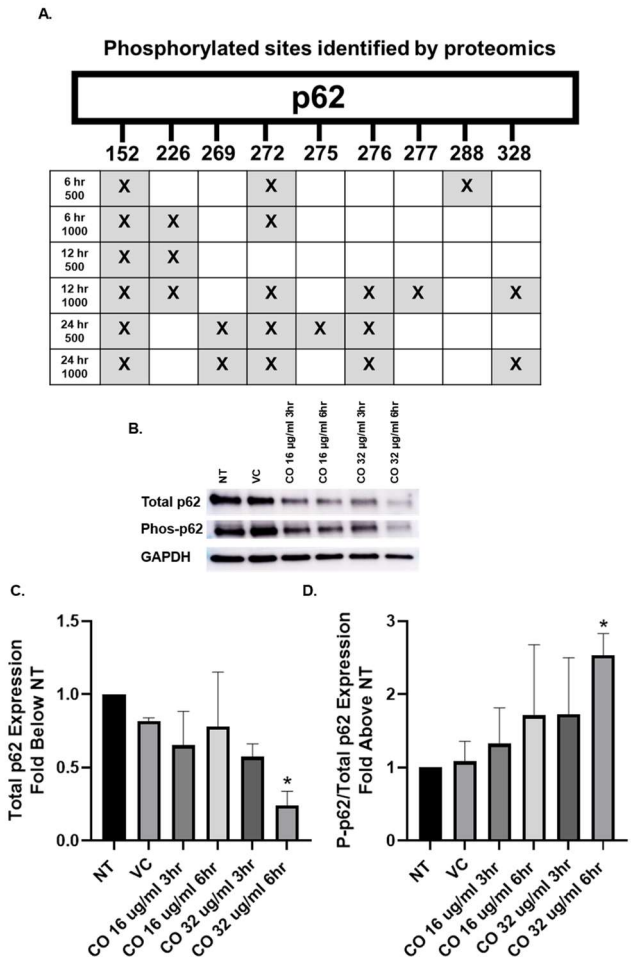


Figure 4: CO treatment of 1.1B4 cells increases p62 phosphorylation. (A) Summary of p62 phosphorylation as determined by proteomic analysis. The identified amino acid positions of p62 are designated numerically. An X with gray shading denotes identified phosphorylation with the treatment condition designated on left. (B) Representative western analysis of both total and phosphorylated p62 as determined by antibody against total p62 and phospho-p62 at positions 269 and 272 (Cell Signaling), respectively. Treatment conditions are denoted above lanes. Lanes: 1- No Treatment (NT), 2- Vehicle Control (VC), 3 and 4- CO treatment at 16 µg/ml following 3 and 6 hr incubation, respectively; 5 and 6- CO treatment at 32 µg/ml following 3 and 6 hr incubation, respectively. Densitometric analysis of immunoblots following 3 independent experiments as determined by ImageJ. (C) Fold expression of total p62 above NT normalized to GAPDH. (D) Fold expression of phospho-p62 normalized to total p-62 and GAPDH. Data are shown as mean ± SE. * denotes $P < 0.05$ from NT and VC treatments based on one-way ANOVA.

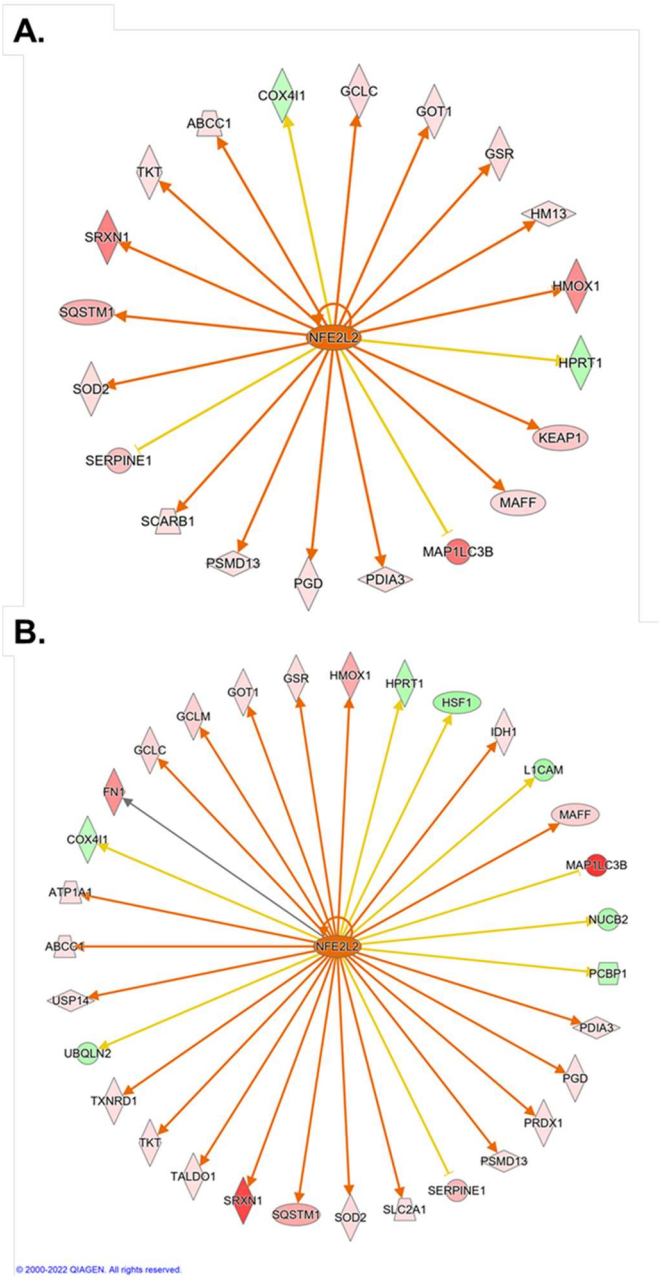


Figure 5: CO induced Nrf2 predicted activation in 1.1B4 cells. IPA analysis on significantly and differentially expressed proteins predicts Nrf2 (shown as NFE2L2, shaded in orange) activation upon treatment of 1.1B4 cells with (A) CO 500 μg/ml (20 proteins within network) and (B) CO 1000 μg/ml (31 proteins within network) for 24 hr. Significance was determined by Welch's t-test with a z-score of 2.616 and 2.854 for 500 and 1000 ug/ml treatment, respectively. Gene symbols are denoted in surrounding nodes. Red and green shading denotes increased and decreased protein expression, respectively as compared to time point matched no treatment control. An orange line connecting predicted Nrf2

Table 1: List of differentially expressed proteins associated with Nrf2 activation as determined by proteomic analysis of CO induced 1.1B4 cells at 500 µg/ml treatment for 24 hr.

	Gene symbol	Protein	Up/Down†
1.	ABCC1	Multidrug resistance-associated protein 1	Up*
2.	COX4I1	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	Down*
3.	GCLC	Glutamate--cysteine ligase catalytic subunit	Up*
4.	GOT1	Aspartate aminotransferase, cytoplasmic	Up*
5.	GSR	Glutathione reductase, mitochondrial	Up*
6.	HM13	Minor histocompatibility antigen H13	Up*
7.	HMOX1	Heme oxygenase 1	Up*
8.	HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	Down*
9.	KEAP1	Kelch-like ECH-associated protein 1	Up
10.	MAFF	Transcription factor MafF	Up*
11.	MAP1LC3B	Microtubule-associated proteins 1A/1B light chain 3B;Microtubule-associated proteins 1A/1B light chain 3 beta 2	Up*
12.	PDIA3	Protein Disulfide Isomerase Family A Member 3	Up*
13.	PGD	6-phosphogluconate dehydrogenase, decarboxylating	Up*
14.	PSMD13	26S proteasome non-ATPase regulatory subunit 13	Up*
15.	SCARB1	Scavenger receptor class B member 1	Up
16.	SERPINE1	Plasminogen activator inhibitor 1	Up*
17.	SOD2	Superoxide dismutase [Mn], mitochondrial	Up*
18.	SQSTM1	Sequestosome-1 (p62)	Up*
19.	SRXN1	Sulfiredoxin-1	Up*
20.	TKT	Transketolase	Up

† Indicates whether protein was either significantly up- or downregulated as compared to vehicle treatment.

* Indicates proteins was also significantly and differentially expressed at the 1000 ug/ml concentration in association with increased Nrf2 activation

Table 2: List of differentially expressed proteins associated with Nrf2 activation as determined by proteomic analysis of CO induced 1.1B4 cells at 1000 µg/ml treatment for 24 hr.

	Gene symbol	Protein	Up/Down†
1.	ABCC1	Multidrug resistance-associated protein 1	Up
2.	ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	Up
3.	COX4I1	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	Down
4.	FN1	Fibronectin 1	Up
5.	GCLC	Glutamate--cysteine ligase catalytic subunit	Up
6.	GCLM	Glutamate--cysteine ligase regulatory subunit	Up
7.	GOT1	Aspartate aminotransferase, cytoplasmic	Up
8.	GSR	Glutathione reductase, mitochondrial	Up
9.	HMOX1	Heme oxygenase 1	Up
10.	HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	Down
11.	HSF1	Heat shock factor protein 1	Down
12.	IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic	Up
13.	L1CAM	Neural cell adhesion molecule L1	Down
14.	MAFF	Transcription factor MafF	Up
15.	MAP1LC3B	Microtubule-associated proteins 1A/1B light chain 3B	Up
16.	NUCB2	Nucleobindin-2;Nesfatin-1	Down
17.	PCBP1	Poly(rC)-binding protein 1	Down
18.	PDIA3	Protein Disulfide Isomerase Family A Member 3	Up
19.	PGD	6-phosphogluconate dehydrogenase, decarboxylating	Up
20.	PRDX1	Peroxiredoxin-1	Up
21.	PSMD13	26S proteasome non-ATPase regulatory subunit 13	Up
22.	SERPINE1	Plasminogen activator inhibitor 1	Up
23.	SLC2A1	Solute carrier family 2	Up
24.	SOD2	Superoxide dismutase [Mn], mitochondrial	Up
25.	SQSTM1	Sequestosome-1 (p62)	Up
26.	SRXN1	Sulfiredoxin-1	Up
27.	TALDO1	Transaldolase	Up
28.	TKT	Transketolase	Up
29.	TXNRD1	Thioredoxin reductase 1, cytoplasmic	Up
30.	UBQLN2	Ubiquilin-2	Down
31.	USP14	Ubiquitin carboxyl-terminal hydrolase 14	Up

† Indicates whether protein was either up- or downregulated as compared to vehicle treatment.

* Indicates proteins was also significantly and differentially expressed at the 1000 ug/ml concentration in association with increased Nrf2 activation

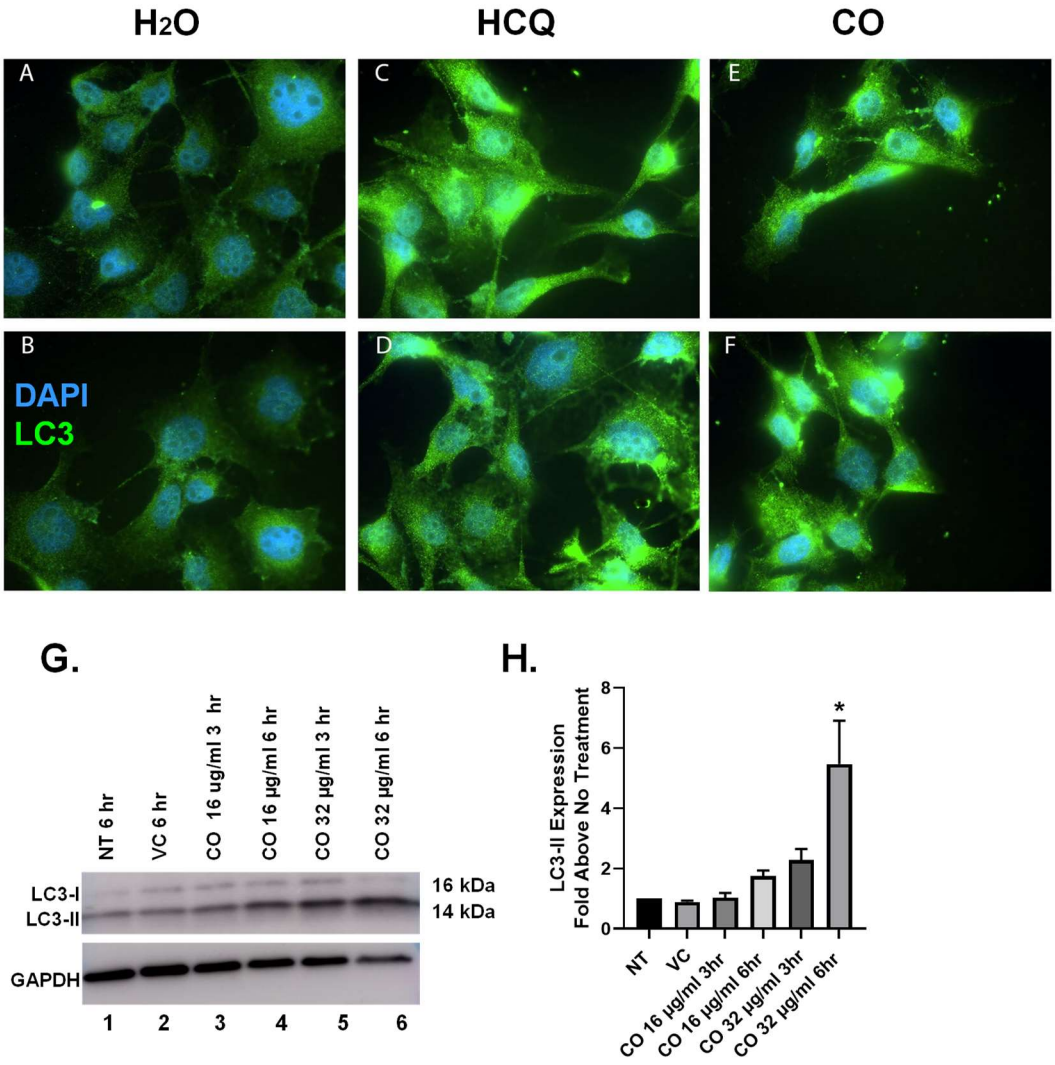


Figure 6: Increased LC3 expression upon CO treatment in 1.1B4 cells. (Panels A-F) Indirect immunofluorescence of LC3 in 1.1B4 cells. Treatment conditions are denoted above box inlays. Each treatment application was applied for 18 hr. (A and B) Water treatment; (C and D) hydroxychloroquine (HCQ) as a positive control at 25 µM and (E and F) CO treatment at 500 µg/ml. Representative images are shown with immunofluorescent staining for LC3 protein expression (green) and DAPI staining of nuclei (blue). (G) Western analysis of LC3. Western analysis was performed on protein lysates following various treatment conditions. LC3-I and LC3-II at 16 and 14 kDa respectively. Treatment conditions are shown above lanes. (H) Densitometric analysis of immunoblots following 3 independent experiments as determined by ImageJ. Protein expression of LC3-II was normalized to GAPDH and fold expression is shown as fold above normalized LC3-II expression of no treatment (NT) at 6 hr. Data are shown as mean ± SE. * denotes $P < 0.05$ from NT and VC treatments based on one-way ANOVA.

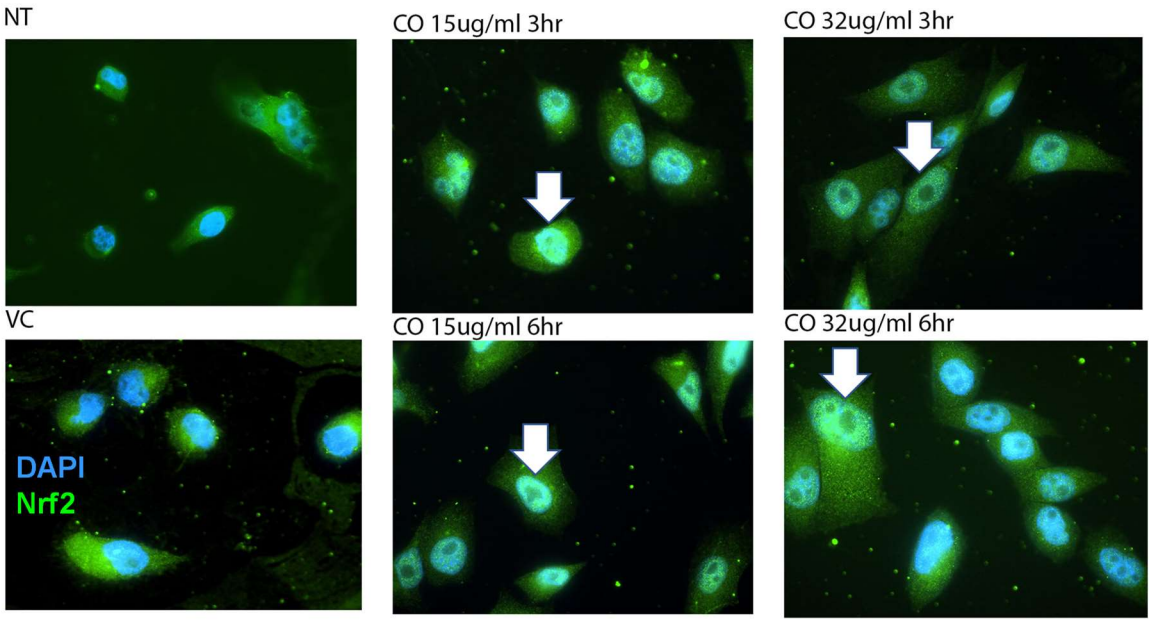


Figure 7: Localization of Nrf2 upon CO treatment in 1.1B4 cells. Indirect immunofluorescence of Nrf2 nuclear localization in 1.1B4 cells. Representative images are shown. Treatment conditions are denoted above box inlays. A: No Treatment, B: Water treatment as Vehicle Control, C and D: CO treatment at 15 µg of CO for 3 and 6 h, respectively and E and F: CO treatment at 32 µg/ml for 3 and 6 hr, respectively. Representative images are shown with immunofluorescent staining for Nrf2 protein expression (green) and DAPI staining of nuclei (blue). Arrows denotes example of Nrf2 localization to the nucleus.

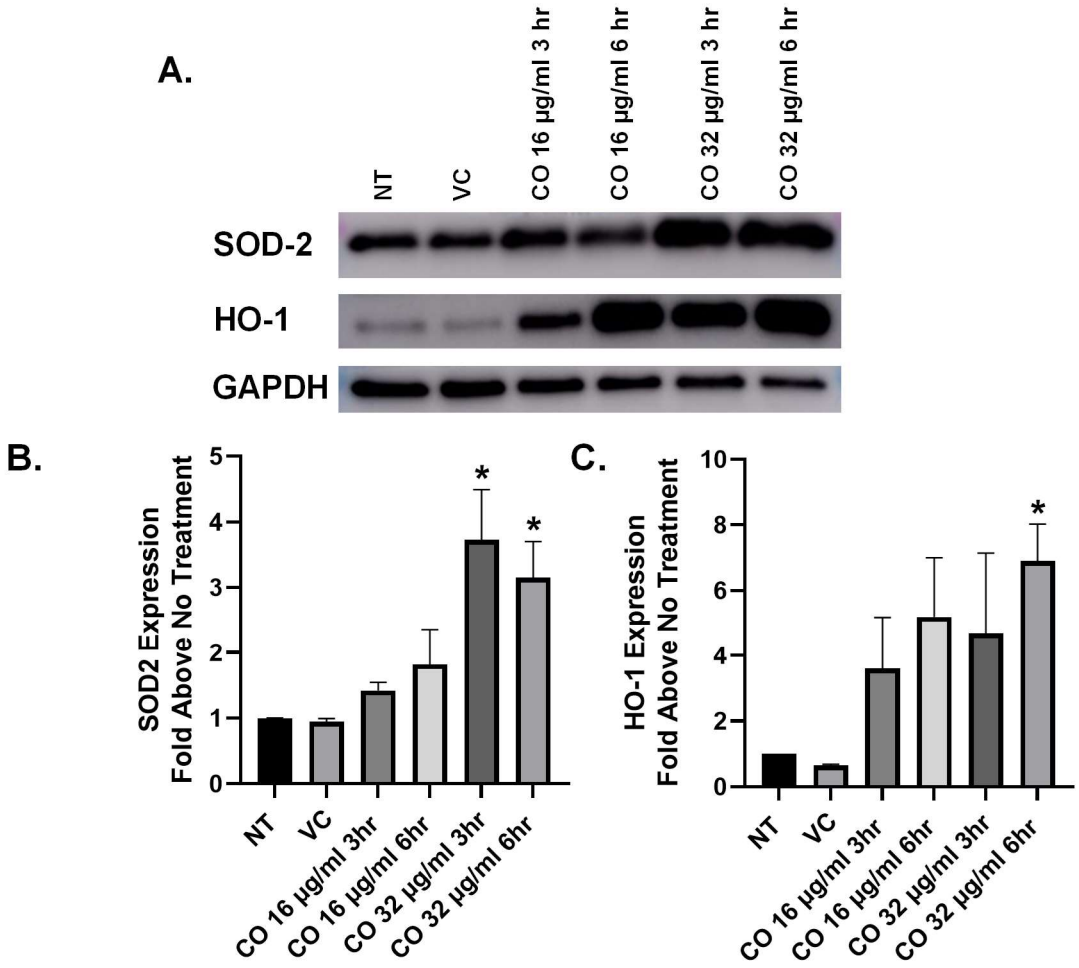


Figure 8: Increased CO-induced expression of SOD-2 and HO-1. Western analysis was performed on cell lysate obtained from CO treated cells as described earlier and immunoblotted using antibodies for either SOD-2 or HO-1. (A) Representative immunoblot with treatment conditions denoted above lanes and antibody utilized for detection labelled on left. (B and C) Densitometric analysis of immunoblot results using ImageJ software showing results as fold above normalized expression to GAPDH above no treatment (NT) condition for SOD-2 and HO-1. * denotes statistical significance ($p<0.05$) as determined by one-way ANOVA between treatment group and NT and VC conditions (n=3).

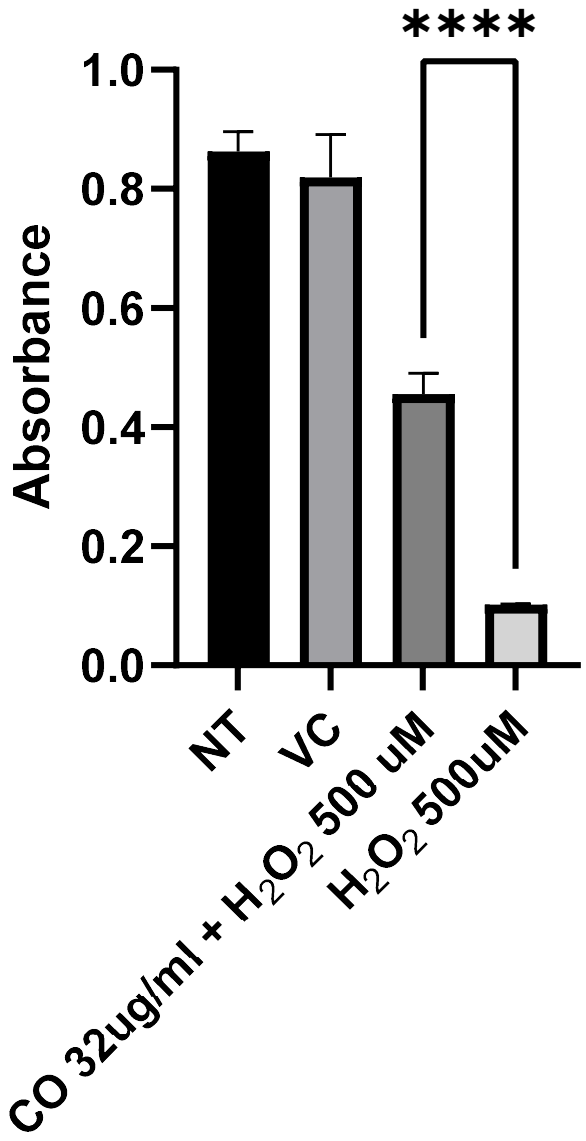


Figure 9: CO preserves 1.1B4 cell viability in the presence of H_2O_2. Bar graph representing the absorbance at 570nm after the designated treatments on the X axis. Data are shown as mean ± SE. **** denotes statistical significance ($P < 0.0001$) as determined by unpaired T test (N=4)

CHAPTER 3:

ORAL GAVAGE DELIVERY OF *CORNUS OFFICINALIS* EXTRACT DELAYS TYPE 1 DIABETES ONSET AND HYPERGLYCEMIA IN THE NON-OBESE DIABETIC (NOD) MOUSE

Note to Reader: I was the first author on this paper and this chapter was published in FEBS open bio in December 2023: Fletcher JD, Olsson GE, Zhang YC, Burkhardt BR. Oral gavage delivery of Cornus officinalis extract delays type 1 diabetes onset and hyperglycemia in non-obese diabetic (NOD) mice. FEBS Open Bio. 2023 Dec 21. doi: 10.1002/2211-5463.13758. Epub ahead of print. PMID: 38129973.

3.1.Introduction

Type 1 Diabetes (T1D) is an autoimmune disease initiated by genetic predisposition and environmental influences resulting in the specific destruction of the insulin producing pancreatic β-cells. Currently, there are over 1.6 million cases of T1D in the United States with a worldwide incidence rate that has been increasing since 1990 [167]. Unfortunately, the standard of care for T1D has not changed since the discovery of insulin 100 years ago and mostly revolves around treating T1D with exogenous insulin following clinical presentation of hyperglycemia which is considered a fasting blood glucose concentration above 126 mg/dL or random blood glucose concentration above 200 mg/dl [168]. The pathogenesis of T1D typically precedes clinical onset of symptoms due to the progressive destruction of the pancreatic β-cells by autoimmunity.

Approximately 90% of the pancreatic β-cell mass has been lost at the time of eventual clinical presentation which can take months to years thereby providing a potential interventional opportunity to halt the autoimmune process or salvage remaining functional pancreatic β-cells.

Ninety percent of T1D patients develop autoantibodies against pancreatic β-cell associated antigens, such as insulin autoantibodies (IAA), GAD autoantibodies (GADA), insulinoma-associated antigen-2 autoantibodies (IA-2A), and zinc transporter 8 autoantibodies (ZnT8A)[1]. Research has shown that individuals who express two or more of autoreactive antibodies have an 84% chance of developing T1D by the age of 18 however, it may take up to 15 years between the detection of the autoreactive antibodies and the onset of T1D symptoms [32]. Even in adult onset T1D which modern research has shown has a lower number of autoreactive antibodies, GAD autoantibodies still remain prevalent [169]. For this reason, between the detection of autoreactive antibodies and the clinical onset of T1D there is a generous interventional window for treatment. Currently, there is only one approved T1D interventional therapy known as Teplizumab. Unfortunately Teplizumab only had a median delay in the diagnosis of T1D of about 2 years[170] and with an expected high cost of $194,000 for treatment the search for cheaper, effective and safe T1D interventional therapies remains warranted.

Ethnopharmacological approaches provide a tremendous resource for the discovery of new therapeutics and have resulted in the discovery of important diabetes medications such as the type 2 diabetes medication, metformin [171]. One promising source of new therapeutics is from the frequently utilized herbal application of *Cornus officinalis* (CO).

CO extract contains a wide array of biologically active constituents that provide protective cellular properties [172-174]. Some of the biological actions of CO include protection from oxidative stress [175] and our current research has also shown that CO can protect a pancreatic cell line from cytokine induced cell death as well as increase viability and proliferation of a β-cell pancreatic cell line [125]. Furthermore, CO can induce the activation of the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway which controls the upregulation of crucial antioxidant enzymes such as Heme-oxidase 1(HO-1) and Superoxide dismutase-2 (SOD2) [176].

In T1D, damage mediated by oxidative stress is a major contributor to the pathogenesis of T1D as well as the diabetic complications that come as a result [177]. This is because oxidative stress plays a major role in T1D induced β-cell death. The autoimmune response in T1D results in the infiltration of macrophages, dendritic and T cells into the pancreatic islets. These immune cells release a number of proinflammatory cytokines including but not limited to interferon-gamma (IFN)-γ, tumor necrosis factor-alpha (TNF-α) and interleukin 1-beta (IL-1β). Furthermore, additional harmful reactive molecules such as nitric oxide (NO) and various other reactive oxygen species (ROS) are secreted. This detrimental combination of cytokines and ROS has been shown to play a major role in the apoptosis of the β-cells in both human and animal models with T1D [178]. This increase in ROS results in β-cell dysfunction and decreased insulin secretion [179] and when combined with the fact that β-cells normally have lower expression of antioxidant enzymes when compared to other tissues,[67] β-cells are extremely susceptible to oxidative stress. For this reason the activation of critical

antioxidant pathways such as the Keap1/Nrf2 pathway have been show to repress the onset of T1D by protecting the β-cells from the oxidative stress caused by inflammation [180].

Since we have shown that CO extracts can induce the Keap1/Nrf2 pathway and promote the stimulation of important antioxidant response genes such as HO-1 and SOD 1/2 in a pancreatic β-cell line, [176] we wanted to extrapolate these prior findings into in-vivo models of T1D to initially determine potential clinical efficacy. We hypothesize that CO may be able to delay onset of T1D in the non-obese diabetic (NOD) mouse and may prove useful in discovering new interventional therapeutics for T1D. To determine the therapeutic effect of CO in T1D, we utilized the well-established NOD mouse model. NOD mice are prone to developing spontaneous T1D with 90% of female and 52% of male NOD mice developing T1D by 30 weeks of age.[181] A measured dose of CO extract was delivered into NOD mice via oral gavage and efficacy was determined based on measurement of glucose levels, diabetic onset, pancreatic insulitis and C-peptide.

3.2.Materials and Methods

3.2.1 Animal husbandry

NOD/ShiLtJ (Strain #:001976) 8 wk. old female mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed at 25°C and 60% humidity and maintained on a 12-h light/dark cycle in the USF College of Medicine vivarium. Prior to experimentation, female NOD mice were allowed to acclimate to the housing facility for 2 weeks. Mice had unlimited access to laboratory chow (Research diets, 24% protein,

41% carbohydrates, and 24% fat) except for a 4 h. fast prior to glucose measurement whereby food but not water was removed. Husbandry and experimental procedures were reviewed and approved by the USF Institutional Animal Care and Use Committee under Protocols #IS00009521 and #IS00008962.

3.2.2 Preparation of *Cornus Officinalis*

Cornus Officinalis extract (CO) (Evergreen Herbs, Shan Zhu Yu, City of Industry, CA, USA) was kindly provided by Dr. Clare Zhang (Oriental Medicine, Tuscon, AZ, USA) and prepared by water extraction as previously described [141, 176]. A certificate of analysis provided by the supplier has indicated that the extract is pure and free of microbiological or heavy metal contamination. The solution was then centrifuged to pellet any insoluble material then filter sterilized with a 0.2-micron syringe filter. The extracts were then stored in 50 mL aliquots at 4°C until use at a concentration of 250 mg/ml that were subsequently delivered by oral gavage.

3.2.3 Oral gavage delivery of CO into NOD mice

27 female NOD mice were randomly split into 3 treatment groups, 10 mice receiving a CO treatment at 250 mg/ml at a volume of 200 µl once per day 5X per week during weekdays with no treatments over weekend, 10 mice receiving 200 µl of deionized water once per day 5 times per week, and the third group of 7 mice receiving no treatment or handling. Oral gavage treatment was started when mice were at 10 wks. of age. We chose this timepoint to initiate CO treatment because this is when the NOD mouse model typically begins development of inflammation (8–11 weeks) within the pancreas and precedes onset of cytotoxicity and eventual overt clinical diabetes [182].

It is at this stage of T1D in the NOD that we would parallel with that in human T1D for use of an interventional agent. Oral gavage administration of the NOD mice continued for 15 consecutive wks. Treatment was stopped when the mice were 25 wks. old. with final specimen collection and termination of experiment with mice aged 26 wks.

3.2.4 Glucose and C-peptide measurements

Fasting blood glucose (FBG) readings were taken every 4 days via tail vein method using the Contour Next glucometer (Ascensia Diabetes Care Inc. East Mississauga, Ontario, Canada). Body weight was measured every 7 days. One FBG measurement above 130 mg/dL was considered a hyperglycemic event and two consecutive FBG separated by at least 72 hours above 250 mg/dL was classified as diabetic onset upon which mice were subsequently humanely euthanized. 50 µl of blood was collected from each mouse via tail vein every 2 weeks using a heparin coated Microvette CB 300 LH (Fischer, ref. 16.443.100). The Microvette CB 300 LH tubes were then centrifuged for 10 minutes at 1000 x g for plasma collection. Plasma was then collected and aliquoted into Eppendorf tubes. 5 µl of plasma was then used to test for C-peptide concentrations were measured from collected plasma every 2 weeks during NOD animal study using the Crystal Chem C-peptide ELISA kit (Elk Grove Village, IL, USA, cat. 90050) following the manufacturer's instructions.

3.2.5 Immunohistochemistry/insulitis scoring

After the final blood glucose and weight readings at 26 weeks of age, the remaining mice were humanely euthanized by CO_2 asphyxiation and then exsanguinated via cardiac puncture with subsequent collection of pancreata. The isolated pancreata were

fixed in formalin for 24 hours, then formalin was removed and pancreata were immersed in PBS and shipped to the University of Florida Molecular Pathology Core. Fractions were sectioned and stained with hematoxylin and eosin (HE) to determine degree of insulitis per islet in mice treated with CO compared to control with light microscopy using Leica Aperio eSlide manager as previously described. Insulitis was scored from stage 0-4. Stage 0 was considered when there was no presence of insulitis with typical islet architecture. Stage 1 was considered when there was perinsulitis but no evidence of immune infiltrate into the islet itself. Stage 2 was considered with immune infiltrate in <50% of the total islet. Stage 3 was considered when immune infiltrate was >50% of the total islet. Stage 4 was considered when there was pronounced islet atrophy or 100% immune infiltration.

3.2.6 Insulitis immunophenotyping

IHC analysis to determine the immunological composition of the observed insulitis was performed as previously described [183]. In brief, paraffin processing was performed on blocks examined during the insulitis scoring for the various treatment groups. The staining series was designed to immunophenotype the insulitis for the cellular populations of total leukocytes [CD45], T-cells [CD3] and B-cells [CD20],

3.2.7 Multiplexing analysis measuring TNF-α and CXCL10

Cytokine levels were determined using a multiplexed bead immunoassay and measured with a Luminex MAGPIX instrument (Luminex, Austin, TX, USA) as previously detailed [184, 185]. In brief, TNF-α and CXCL10 were measured using magnetic bead assay (Mouse XL Cytokine Luminex Performance Premixed Kit, Bio-Techne, Minneapolis, MN,

USA) following manufacturer's instructions. Each assay was analyzed on the Luminex MAGPIX instrument to measure inflammatory concentrations followed by a 5-parameter logistic curve-fitting method from a standard curve of each respective analyte. Plasma samples were normalized by equivalent volume of commercially supplied dilution buffer and examined in duplicate per each assay run. Concentration was calculated by the StatLIA Immunoassay Analysis software (Brendan Technologies).

3.2.8 Statistical significance

Statistical significance was determined by log-rank test for trends and mixed effect analysis (Graphpad Prism version 5.01). P value less than 0.05 was considered significant.

3.3 Results

3.3.1 Experimental Design

To determine the biological impact of CO on T1D onset and hyperglycemia in the NOD model within a measured dosage, we established an experimental design that consisted of 3 treatment groups of: CO treated (CO, n=10), water treated (WT, n=10), and a no handling or treatment control group (NHT, n=7). CO and water were administered once per day for five consecutive days per week for 15 wks. into female NOD mice beginning at 10 wks. of age. The NHT group was included because our earlier experiments (unpublished observation) indicated that the procedure of oral gavaging delayed the onset of T1D in the NOD model. Therefore, we included the NHT group that was not

handled or gavaged during course of the study to account for the observed handling/procedure induced T1D delay in the NOD model.

3.3.2 Chronic oral gavage delivery of CO did not impair weight gain in NOD mice

Chronic oral gavage delivery of CO into the NOD model has not been documented before and therefore we wanted to ensure that neither the constant gavaging nor the CO extract in of itself, had any detrimental effects on the mice by way of food consumption or weight gain. Therefore, we measured the body weights of the NOD mice weekly starting at 15 weeks up until 25 weeks. NOD body weights among treatment groups were not statistically significant ($P > 0.05$, ANOVA) (Figure 10). No harmful effects were observed in the consistently gavaged groups (CO and WT) and body weights did not differ across experimental groups including the NHT group, suggesting that oral gavage delivery of CO did not impair weight gain and potentially food consumption.

3.3.3 Oral delivery of CO decreases incidence of T1D, glucose concentration and hyperglycemia

To determine if CO inhibited the onset of T1D or hyperglycemia in the NOD mouse, we measured the blood glucose via tail vein collection approximately every 3 to 4 days following a 4 hour fast. Diabetic onset was determined by two separate daily independent glucose readings ≥ 250 mg/ml. T1D incidence per group at 26 wks. was as follows: CO (30%), WT (60%) and NTH (86%) (**Figure 11A**). The NHT control group had the highest incidence of T1D with 6 out of the 7 mice having diabetic onset by 26 wks. of age. In addition, we observed T1D onset of approximately 50% by 19 wks. of

age in the NHT group as has been previously reported for the NOD model. Regarding the treatment groups, the diabetic onset between the WT and CO treated groups was significantly different, with the CO treated group showing a significantly higher proportion of non-diabetic mice (Log rank test, $P = 0.018$) (Figure 11A). The average glucose concentration ± SD are also shown from surviving NOD mice displayed by age (**Figure 11B**). The glucose concentration slopes were shown to be significantly different with P<0.0001. In addition, appearance of hyperglycemia (single glucose reading >130 mg/dl considered hyperglycemic event) was significantly delayed in the CO treated NOD mice as compared to WT and NTH groups ($P < 0.001$) (**Figure 12**). By 20 wks. of age, all mice in the WT group exhibited at least one reading of hyperglycemia, whereas the observation was not matched in the CO treated group until 26 wks. of age.

3.3.4 CO reduces insulitis in the NOD mouse

Insulitis is characterized as the influx of immune cells surrounding and within the pancreatic islets and is considered a distinguishing clinical characteristic of T1D [186]. Therefore, we examined insulitis in the endocrine pancreas of the surviving non-T1D NOD mice at 26 wks. of age from all treatment groups (CO; n=4, WT; n = 4, and NHT; n=1) by performing H&E staining of pancreatic sections obtained from surviving mice at the conclusion of the study at 26 wks. This was performed in collaboration with the University of Florida Molecular Pathology Core. Degree of insulitis was scored by staging criterion ranging from: Stage 1: Only perinsulitis observed, Stage 2: <50% insulitis, Stage 3: >50% insulitis, Stage 4: 100% insulitis for the pancreatic sections from the CO and WT group but not from NHT since only 1 was assessed. Representative images observed from our experiments are shown in **Figure 13A**. In the WT group,

most of the examined islets exhibited severe insulitis (73%), while CO treated group had mild to no insulitis (56% stages 0 or 1) (**Fig. 13B**). The insulitis score in the CO treated group (n=58 islets counted) was significantly lower when compared to the control WT group (n=26 islets) (1.83 ± 0.22 vs. 3.12 ± 0.32, respectively, $P < 0.001$, unpaired t-test) (**Fig. 13C**). In addition, we also performed immunophenotyping of the observed pancreatic insulitis via IHC analysis staining for CD45, CD3 and B220 serving as markers of lymphocytes, T-cells and B-cells respectively. No difference was observed among the treatment groups in terms of appearance and distribution with regard to the observed immunophenotyping (**Fig 14**). Differences in circulating levels of cytokines were also measured across surviving mice of completed regimen across treatments groups. Plasma was collected during the terminal collection and the concentration of TNF-α and CXCL10 was measured via multiplexing analysis for CO and WT treated groups. No significant differences were observed in circulating levels of both TNF-α and CXCL10 between both groups (**Fig 15**).

3.3.5 Increased circulating C-peptide in non-diabetic CO treated mice:

C-peptide is secreted along with insulin by pancreatic β-cells following enzymatic cleavage of the prohormone proinsulin to produce insulin and C-peptide in equimolar amounts [187]. C-peptide also more accurately represents portal insulin secretion rather than measurement of peripheral insulin[188] due to relatively fast peripheral clearance and first pass metabolism of insulin by the liver [189, 190]. As a result, C-peptide is commonly used as a measurement for insulin secretion and pancreatic β-cell function which is a direct measure of β-cell viability. Therefore, we wanted to examine if CO promotes β-cell preservation. For the reasons stated above we measured C-

peptide circulating levels every two weeks on the surviving mice from the ages of 14 to 26 wks (**Figure 16**). CO had a small but not significant increase in C-peptide secretion (as compared to WT group) that diminished around 22 weeks. However, at the conclusion of the study we observed a statistically significant increase in C-peptide concentration in the CO as compared to the WT group in the surviving mice at the conclusion of the study. (1.1±.15 vs .7±.15 ng/ml., $P < 0.05$ as determined by unpaired t-test. respectively)

3.4 Discussion

CO has been shown to have several beneficial effects in pancreatic β-cells including promotion of viability and metabolic activity in addition to cellular rescue from cytokine induced cell death [125]. Furthermore, our earlier findings demonstrated that CO could induce the Keap1/Nrf2 pathway resulting in increased expression of the critical antioxidant genes SOD2 and HO-1 in a human pancreatic β-cell line [176]. Since Nrf2 signaling has been shown to also repress the onset of T1D in the NOD mouse[180], we hypothesized that CO may promote these beneficial effects in the NOD model with potential translational value as an additive interventional therapy for T1D by inhibiting the progression of pancreatic β-cell destruction.

Through this study, we showed that CO extract delivered by oral gavage delays the onset of T1D in a greater percentage of NOD mice as compared to control groups WT and NHT. Our study utilized a novel approach employing oral gavage delivery of CO. Our recent literature evaluation examining oral gavage therapy of CO has identified only 4 manuscripts reporting this approach of CO delivery and to our knowledge, none have ever examined the impact of oral gavage therapy of CO in the NOD model

demonstrating the novelty of our approach in a T1D application [191-194]. Our results showed that chronic gavaging of CO had no effect on the weight gain of the mice which provides some evidence that this approach is not detrimental to the overall health of the mice **(Figure 10).** We assume that there were no differences in food consumption, but this was not exactly measured, and the limited conclusion is based overall similar linearity of weight again among all experimental groups especially that of the NHT control. Moreover, we showed that CO not only significantly reduced the T1D incidence **(Figure 11)** at 26 wks. but we also observed a delayed appearance of hyperglycemia **(Figure 12).** In addition, we also examined the degree of insulitis in the excised pancreata from the NOD mice at the conclusion of the study from surviving mice which revealed reduced severity of immune infiltration in the NOD mice associated with CO treatment **(Figure 13).** Taken together, these results do suggest that there was a preservation of β-cell function that is further supported by the significantly higher concentration of C-peptide secreted by CO treated NOD mice at the terminal time point of surviving mice **(Figure 16).** Our combined findings provide initial support of CO as a potential therapeutic agent that is safe and effective in the interventional treatment for stages 1 and 2 T1D. Stages 1 and 2 of T1D are characterized by the presence of autoimmunity against the pancreatic β-cell without any of the typical symptoms of T1D (Ketoacidosis, weight loss, fatigue etc) while stage 2 is differentiated from stage 1 by the presence of dysglycemia.[195] Despite the appearance of autoimmunity and dysglycemia during Stages 1 and 2 T1D, there is still sufficient and functional pancreatic β-cell mass to prevent the necessity for insulin treatment. Therefore,

therapies employed during these stages such as CO may be potentially implemented as a preventative and safe measure that can inhibit and delay T1D onset.

Currently the only FDA approved interventional therapy for T1D is an immunotherapy called teplizumab. Teplizumab is an anti CD3 antibody which targets the autoimmune response [170] but does not do much to restore beta cell function itself. Considering that β-cell stress has been shown to precede insulitis[196], trying to maintain β-cell homeostasis is crucial. β-cell homeostasis is so critical in fact that current research has shown that β-cell stress can result in the creation of “Neo-antigens” and the onset of autoimmunity [197]. For the reasons stated above, it is important to look at interventional treatments that not only prevent autoimmunity but also preserve beta cell function. CO may be beneficial in identifying further compounds which may help maintain β-cell homeostasis and could provide an avenue for new interventional therapies.

Even though we showed the potential therapeutic utility of CO for T1D preventative therapy there are still limitations that need to be addressed. The oral gavage delivery method had not been attempted before and even though it did not seem to have a detrimental effect there was a substantial delay in T1D onset between the no handling control and the water treated control. Going forward it will be necessary to devise a method for CO delivery that is not as invasive as oral gavaging and importantly not inducing an overall T1D delay in the NOD model.

Another critical limitation of our findings is that this reported study has not addressed the precise mechanism of how CO may be inhibiting T1D onset or hyperglycemia. CO has been reported to have numerous pleiotropic effects on both metabolism and

inflammation [137]. CO has been shown to promote glucose sensitivity through mechanisms such as promoting hepatic glucose uptake as well as anti-inflammatory effects via suppression of phosphorylation of STAT3 and NF-κB. Whether the metabolic or anti-inflammatory effects alone or both in combination explains in part our results have yet to be fully evaluated and are beyond the scope of this current study. However, this report was the critical first step to determine if there is biological efficacy of CO in a widely supported and tested model of T1D. Future studies examining the impact of CO on T1D should incorporate additional immunological markers such as examination of T-cell subsets, generation of Regulatory T cells (Tregs), and the impact on lymphocyte trafficking. Other studies have demonstrated that administration of CO in animal models can alter the intestinal microbiome and promote various metabolic effects [198, 199]. Therefore, further examination should be performed elucidating any changes in the gut microbiome during CO administration.

Our earlier analysis has revealed that CO is able to promote a robust antioxidant response by increasing expression of SOD-2 and HO-1 along with stimulating autophagy. We will need to confirm that this pathway is responsible for the T1D delay observed in the NOD mouse. Moreover, CO is a complex mixture of many different compounds and trying to identify individual compounds which are responsible for delaying T1D will be crucial for elucidating direct agonist actions of CO involve and efficacy in humans.

In summary, the extract from CO significantly delayed the onset of T1D in the NOD mouse. Although further research is needed to confirm the mechanism of action, CO

may be preserving beta cell function by promoting beta cell viability thus making CO a promising source for new potential interventional therapies.

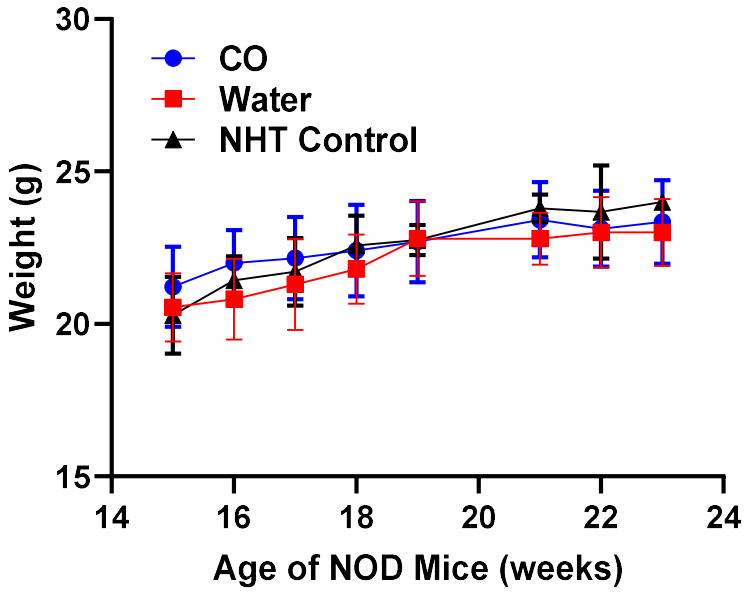


Figure 10: Chronic gavage delivery of CO does not impair weight gain in NOD mice. Body weights were measured weekly or biweekly starting at 15 wks. of age. Experimental groups consisted of CO treated (CO, n=10), water treated (WT, n=10), and no handling or treatment control group (NHT, n=7). All data are expressed as mean ±SD.

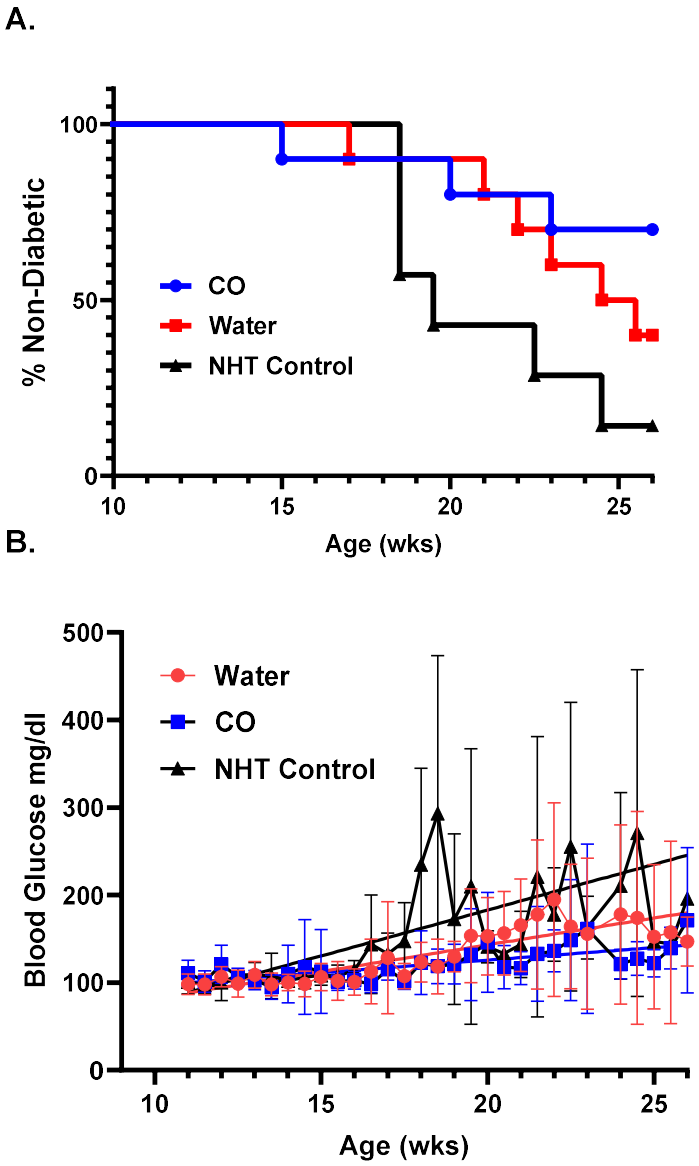


Figure 11: Oral gavage delivery of CO delays T1D onset and lowers average glucose concentration in NOD mice. Glucose concentration was determined from collected tail vein plasma by commercial glucometer (Contour Next). (A) Diabetic onset was defined as two consecutive readings above 250 mg/dl. Vertical axis shows total percentage of non-diabetic mice. Log-rank test for trend was significant with $P < 0.05$. (B) Average glucose concentration ± SD is shown from surviving NOD mice displayed by age. Line of best fit as determined by linear regression is shown. The glucose concentration slopes were shown to be significantly different with $P<0.0001$.

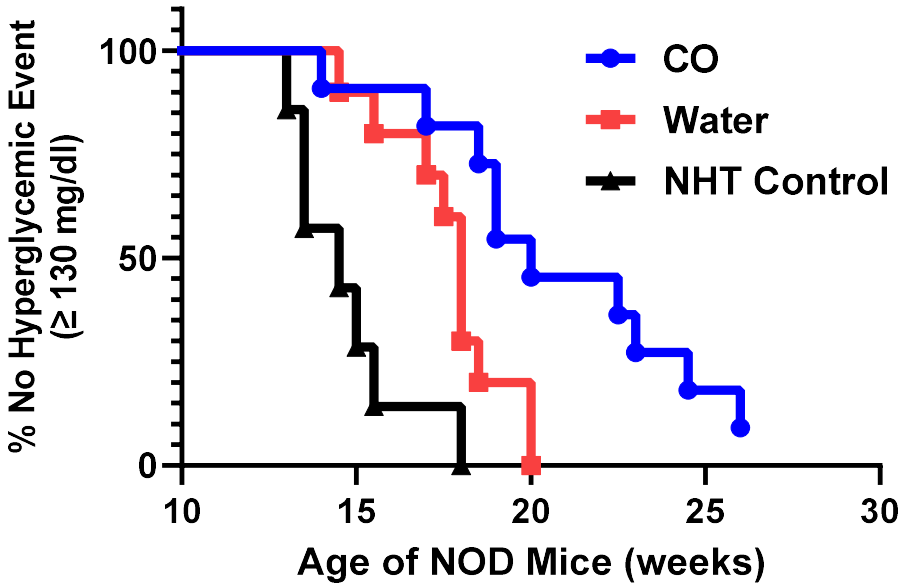


Figure 12: Oral gavage delivery of CO inhibits hyperglycemia in NOD mice. Hyperglycemia was defined by a single glucose measurement > 130 mg/dl following 4 hour fast as determined from tail vein collected plasma. Vertical axis shows percentage of mice without at least one hyperglycemic reading. Log-rank test for trend was significant with $P < 0.001$.

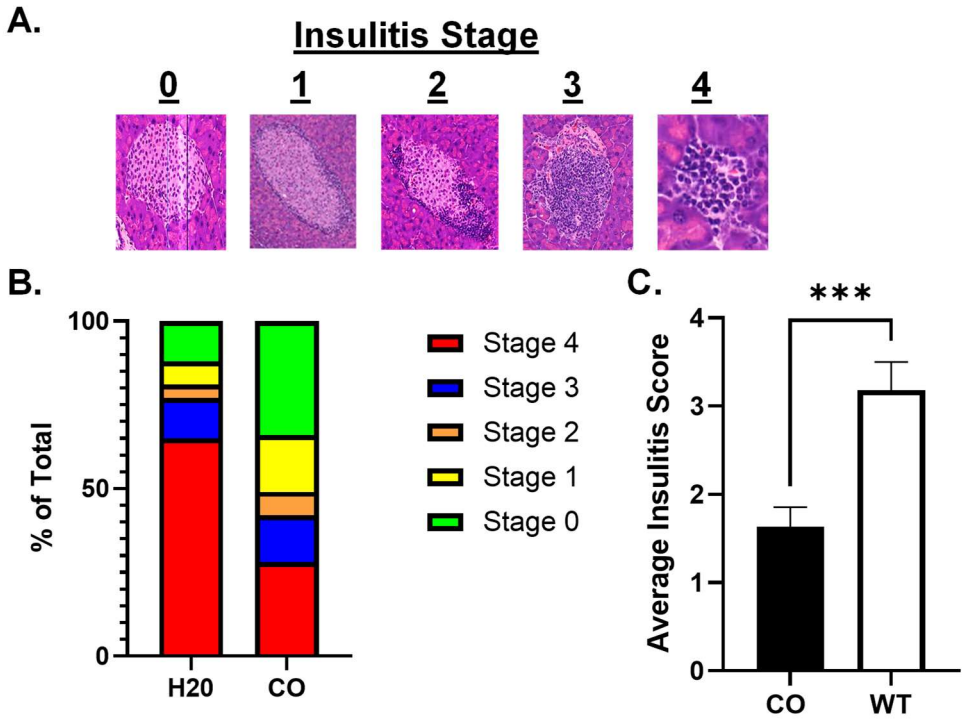


Figure 13: Decreased insulitis in CO treated NOD mice aged 26 wks. (A) Representative images of H&E-stained pancreatic sections obtained from CO treated NOD mice showing insulitis staging. (B) Insulitis stage percentage shown by insulitis category from all counted islets. (C) Average insulitis score of islets from both the CO and WT groups. All data expressed as mean ± SEM. $P < 0.001$ for average insulitis score between the CO and WT groups as determined by unpaired t-test.

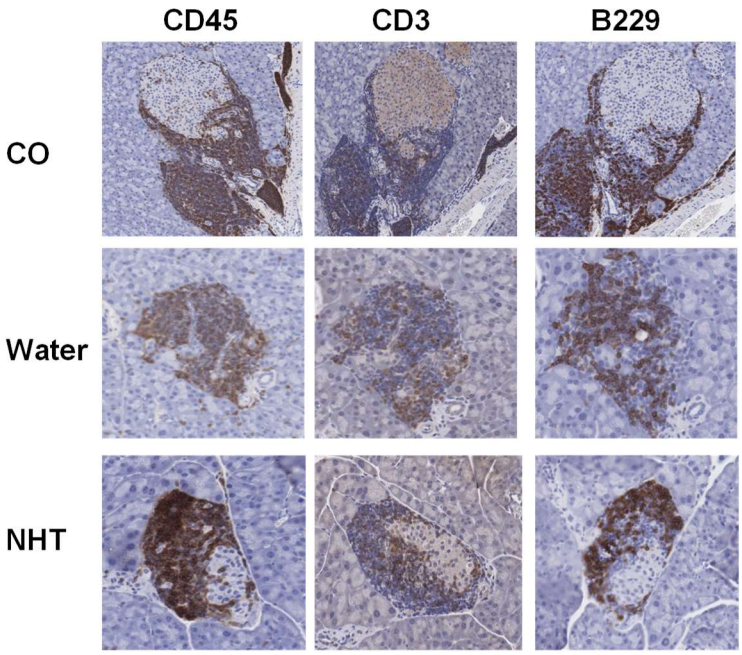


Figure 14: Immunophenotyping of pancreatic endocrine insulitis IHC staining was performed on pancreatic sections obtained from remaining mice following 15 treatment weeks. IHC analysis staining for CD45, CD3 and B220 serving as markers of lymphocytes, T-cells and B-cells respectively were utilized for evaluation of the endocrine pancreas from the CO (n=6), WT (n=4) and NHT (n=1) groups. Representative images are shown.

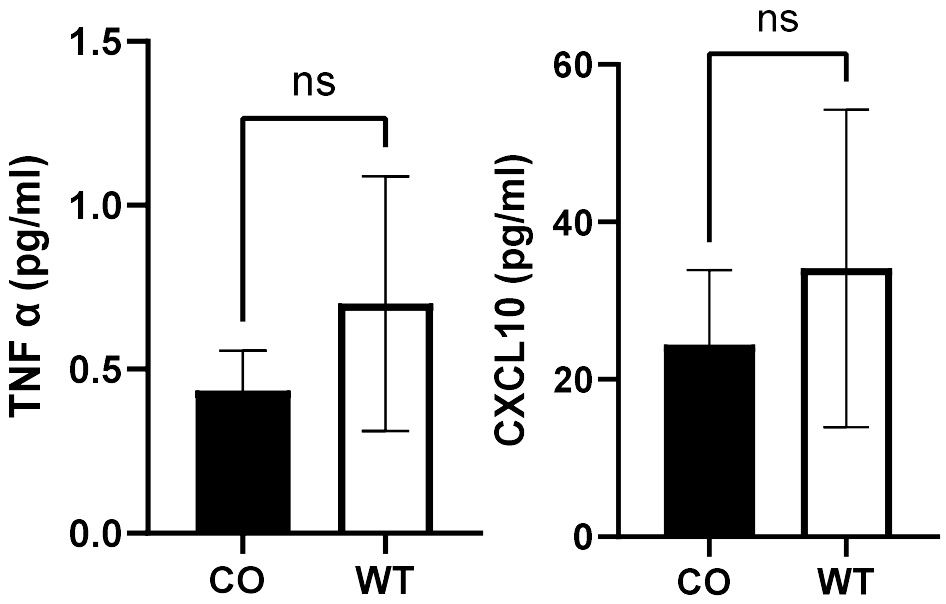


Figure 15: Measurement of circulating TNF-α and CLXL10 via multiplexing analysis. Plasma was collected from the surviving mice from the CO (n=7), WT (n=4) and NHT (n=1) treatment groups following 15 treatment weeks and examined for the concentration of TNF-α and CLX10. Measurements are shown as mean ± SD. $P > 0.05$.

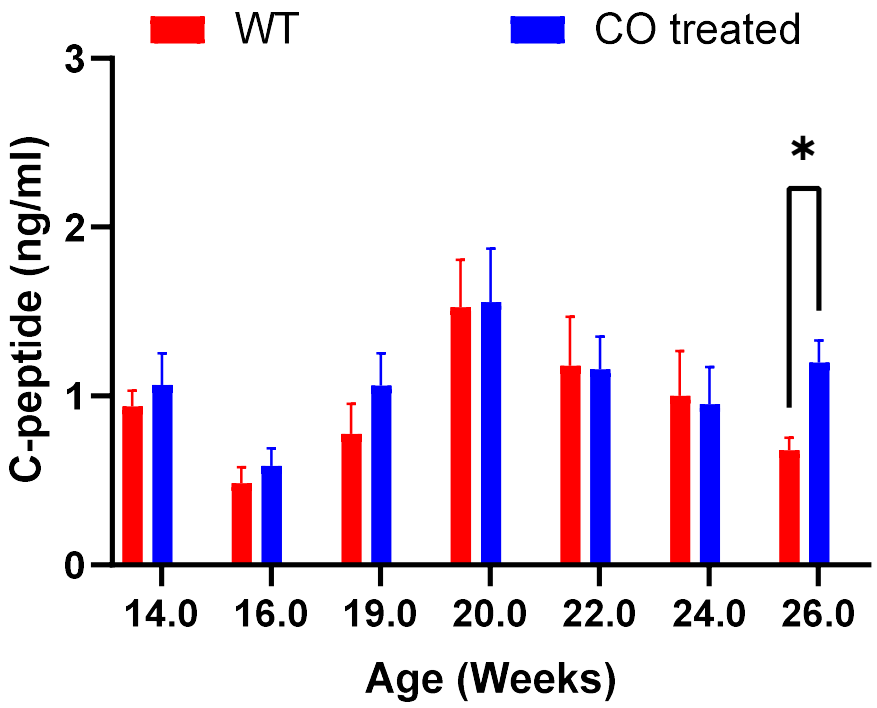


Figure 16: C-peptide levels measured from CO treated NOD mice. Tail vein plasma was collected from all surviving mice approximately every 3 weeks. C-peptide concentration was determined by commercial ELISA. Average C-peptide levels are shown as mean ± SEM. $P < 0.05$ as determined by unpaired t-test.

CHAPTER 4:

DETERMINING THE INDIVIDUAL COMPOUNDS RESPONSIBLE FOR THE BIOLOGICAL EFFECT OF CO

4.1 Introduction

Type 1 Diabetes (T1D) is an autoimmune disease initiated by a number of genetic and environmental factors resulting in recognition of the β cell by the immune system. This recognition is followed by obliteration of the β cell resulting in the subsequent loss of the body's ability to produce insulin. This loss of insulin production is recognized as the symptomatic stage of T1D and existing treatment mostly focuses on treating T1D after the loss of insulin. Currently there is only one FDA approved T1D interventional therapy designed to delay the clinical onset of T1D known as Teplizumab, an anti CD3 antibody that results in the exhaustion of $CD8^+$ T cells [116]. Teplizumab treatment was shown to have a significant preservation of β cell function two years after treatment but unfortunately the effect is greatly diminished after 7 years [119]. Thus, the search for new interventional therapies remains apparent to either use on their own or in conjunction with Teplizumab.

One reason that teplizumab may not be the end all interventional treatment for T1D may be due to the fact that current research is showing not just the immune system but the β cell it's self seems to be playing a critical role in T1D onset [69]. The leading theory is that β cell stress precedes insulitis due to β cell stress causes significant dysregulation

of normal cellular process resulting in the creation of “Neo antigens” [78]. These Neo antigens are created by a number of aberrant cellular process in stressed β cells and have been demonstrated to be immunogenic in T1D patients [79]. With oxidative stress in the β cell being directly linked to creation of neo antigens [84], compounds that can inhibit oxidative stress in the β cell may prove to be beneficial in delaying T1D onset.

One such compound is the extract from the fruit of the *Cornus officinalis* (CO) tree. Our lab has shown that CO has a number of beneficial effects both *in vitro* and *in vivo*. We have shown that treatment with CO extract increases viability in a beta pancreatic cell line [125] as well as activates the critical antioxidant pathway Keap1/Nrf2 [176]. Furthermore we demonstrated that treatment with CO extract was able to significantly delay T1D onset in the NOD mouse [200]. For this reason, we wanted to determine the individual compounds responsible for the biological effect of CO in the β cell.

Due to CO being a complex mixture of many different compounds, we aimed to try and elucidate what compounds were responsible for having this effect. To do this we used a high performance liquid chromatography (HPLC) approach to analyze all the compounds in the extract and make individual fractions of the extract. This was followed by cellular viability assay like MTT to determine if any individual fractions are responsible for our previously published effects. We then followed this up with an examination of downstream targets of the Keap1/Nrf2 pathway to demonstrate the individual fraction is indeed responsible for our previously published data.

4.2 Materials and Methods

4.2.1 Cell culture and treatments

1.1B4 were cultured in RPMI media supplemented with 10% Fetal Bovine serum and 1% Penicillin streptomycin. After the cells have been seeded for experiments, cells were incubated overnight at 37°C with 5% CO2 to allow for cell adherence. After 24 hours cells were then ready for treatment.

4.2.2 Preparation of chemicals and reagents

Cornus officinalis (C. officinalis) was acquired by Evergreen Herbs (City of Industry, CA, USA). 12 g of CO was dissolved in 12ml of H_2O and left on a rocker for 3 days to extract the compounds from CO into aqueous solution. The aqueous solution was then filtered using a 0.2um filter. The standards of Loganin (Cayman chemicals, Item No. 19997) and Morroniside (Cayman chemicals, Item No. 30711) were prepared by resuspending the solutions in water then filter sterilizing using a 0.2um filter. All other chemicals were of analytical grade and commercially available.

4.2.3 Analytical HPLC analysis

HPLC analyses were performed using a Hitachi LaChrom Ultra HPLC system (Hitachi ltd, Chiyoda City, Tokyo, Japan) with a diode array detector. The monitoring wavelength was set at 240 nm. A Hitachi LaChrom C18 column (150 mm × 4.6 mm, 5 µm) was used with a flow rate of 1.0 mL/min. The mobile phase consisted of water + 0.1% formic acid (Solvent A), and acetonitrile + 0.1% formic acid (Solvent B) starting with an EQ at 5% for one minute than using a gradient elution 5-20% solvent B from 1-15 minutes followed by an equilibration at 5% solvent B from 12-22 minutes. The column temperature was

maintained at 30°C, and 10 µL aliquots of the supernatants were injected into the LaChrom C18 column.

4.2.4 HPLC fractionation of *Cornus Officinalis* extract

HPLC Fractionation was performed were performed using an Agilent Technologies 1260 infinity HPLC system. (Aligent Technologies Inc., Santa Clara, California). The monitoring wavelength was set at 240 nm. A Gemini C18 110A column (250 mm × 21.2 mm) was used with a flow rate of 12 mL/min. The mobile phase consisted of water (Solvent A), and Methanol (Solvent B). The column temperature was maintained at 30°C, and 1000 µL aliquots of CO were injected into the Gemini C18 column followed by a gradient elution from 5-100% solvent B from 1-26 minutes followed by a wash with 100% solvent B from 26-30 minutes. An equilibration was then performed at 5% solvent B from 30.1-40 minutes. A total of 19 fractions were collected in scintillation vials and the solvent was evaporated away using compressed air. After all the solvent was evaporated away the samples were resuspended in nano pure water to use for cell treatments.

4.2.5 Mass spectrometric (LC/MS) analysis of *Cornus Officinalis* extracts

CO extracts and fractions were filtered with a .45 um filter to remove particulate. Then tandem high pressure liquid chromatography and mass spectrometry (LC-MS) was performed by Agilent 1100 HPLC with Diode Array Detector and Agilent 6120 Single Quadrupole Spectrometer using an ESI source. HPLC was performed using an Agilent Poroshell 120 C18 column (30mm X 4.6mm) at a flow rate of 1.3ml/min. We then ran the extract using a gradient starting at 90% H2O (+ 0.1% Formic acid) and 10%

Acetonitrile (+ 0.1% Formic acid) and going up to 100% Acetonitrile (+ 0.1% Formic acid) followed by a hold at 100% for 3.5 minutes.

4.2.6 MTT assay

Cells were seeded in a 96 well plate with 100ul of a 5 $X10^4$ cell/ml suspension in each well. Cells were then left untreated (NT group), treated with water (Vehicle control (VC) and the treatments. After the initial treatments, the MTT assay (Promega, Item No.G4000) was performed. In brief, the dye was added in a 15:100 ratio for each well and then was incubated at 30C for 4 hours. The solubilization solution was then added in a 1:1 ratio and incubated overnight. Absorbance of each well of the 96 well plate was then measured at 570nm, and the data was inputted onto graphpad prism and statistical analysis was performed by one-way analysis of variance (ANOVA) o comparing the treated groups to the NT group.

4.2.7 Western blotting

Cells were seeded in a 6 well plate and treated with CO. Cells were pelleted and resuspended in lysis buffer (50 mM ammonium bicarbonate solution with 50 mg of SDS). Lysates were heated at 95°C for 4 min and sonicated for 6 rounds at 200 amp for 3 seconds each round. Protein concentrations were quantified using the Pierce 660nm protein assay (Thermo Fisher Scientific). Protein samples were electrophoresed on a 4-20% TGX SDS PAGE gel (Bio-Rad) using the Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad) and transferred to a nitrocellulose membrane using the iBlot dry transfer system (Invitrogen). The nitrocellulose membrane was blocked using 5% milk-Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST) for 2h. The

membranes were incubated overnight with primary antibodies for the detection of HO-1 (Cell Signaling Technology #5061S, 1:500) and GAPDH (Cell Signaling Technology #5174S,1:500). Membranes were incubated in secondary antibody (Cell signaling Technologies #7074P2, 1:10000) followed by chemiluminescent detection (SuperSignal West Femto, Thermo Fisher Scientific). Membranes were then imaged using the Amersham™ Imager 600 (GE Healthcare Life Sciences). Densitometric analysis for measurement of pixel density from band detection upon western analysis was performed using ImageJ version 1.53r [141].

4.2.8 ELISA analysis of HO1

Cells were seeded in a 12 well plate using 1 ml of a 1 X 10^5 cells/ml solution. Cells were then allowed to attach overnight and treated with 250ul of the respective CO fractions (S1-S19), Whole CO extract (CO),water (VC) and 100 um of Loganin and Morroniside with remaining untreated cells (NT) used as a control. After 4 hours the media was removed from the wells and cells were washed with PBS and lysed using M-PER lysis buffer (Thermo Fischer Scientific, Item # 78501). An HO1 ELISA was then performed using a commercially available kit and procedures were followed according to the manufacturer's instructions (Thermo Fischer Scientific, Cat.# EEL060).

4.3 Results

4.3.1 Morroniside and Loganin have no effect on viability on the 1.1B4 cells

We have published before on the biological effects of CO both *in vivo* and *in vitro* but the exact compounds that cause this biological effect remain elusive. The literature seems to suggest that loganin and morroniside seem to be responsible for the biological effect of CO, with morroniside being shown to activate the Keap1/Nrf2 pathway in the mouse brain [201] and loganin being shown to protect Schwann cells from high glucose induced NLRP3 activation of the inflammasome and the resulting pyroptosis [202]. Due to the published literature demonstrating that these compounds extracted from CO seem to have a similar biological effect to what we have demonstrated, we decided to examine if loganin and morroniside are responsible for the biological effect we have published on in 1.1B4 β pancreatic cell line. To start we performed an MTT assay using loganin and morroniside and found that not only did it not have the same effect as CO, neither loganin nor morroniside had any significant measurable effect on viability **(Figure 17 A)**. Furthermore, as discussed earlier Morroniside can induce the Keap1/Nrf2 pathway in vitro so we measured if morroniside had any effect in inducing this pathway. This was assessed by measuring a predominant downstream target of the Keap1/Nrf2 pathway by immunoblot specifically, Heme oxygenase-1 (HO1) [203]. Morroniside had no significant effect on HO1 expression in the 1.1B4 cells. However, whole extract of CO **(Figure 17 B and C)** significantly increased HO-1 expression as determined by western analysis indicating the biological effect of CO on the 1.1B4 cells is not due to Morroniside on its own.

4.3.2 Analytical HPLC analysis of whole CO extract reveals many abundant compounds including loganin and morroniside

Due to Morroniside nor Loganin having much of an effect on the 1.1B4 cell line we decided to investigate to see what where the most abundant compounds in our CO extract. To do this we used an analytical HPLC system to determine which compounds in CO where the most abundant. As shown in the HPLC chromatograms made with Loganin and Morroniside standards, Morroniside elutes first with a retention time (RT) of 7.3 minutes while Loganin then elutes after with a RT of 9.8 minutes **(Figure 18 B).** In the whole CO extract, both Morroniside and Loganin are found to have prominent peaks with the Loganin peak being more prominent than Morroniside (**Figure 18 A**). Although this indicates that Loganin and Morroniside are among the most abundant compounds, we found that the most prominent peak was the peak with an RT of 3.3. Since this peak was quite prominent, we decided to see if these other abundant compounds could be responsible for our observed biological effect in CO.

4.3.3 Fractionation of CO reveals new compounds with a biological effect

To determine which individual compounds or combination of compounds was responsible for the biological effect we have demonstrated, we decided to use a time-based fractionation in which we injected 1000ul of whole CO extract (250mg/ml) onto a C18 column and collected a fraction every minute for 26 minutes **(Figure 19)**. 19 fractions were collected as the HPLC chromatogram displays no compounds being present in the fractions from the 20–26-minute time points. After the fractionation we decided to test the fractions we obtained (1-19) for biological effect. To do so we decided to use an MTT assay as CO had been demonstrated before to increase viability

when measured by MTT [125]. Interestingly we did see a significant increase in viability for fractions (12-14) (**Figure 20**) but this increase in viability was not as substantial as the CO fractions. Furthermore, these were the only fractions to have any significant effect on viability indicating that fractions 12-14 may contain the compound or compounds responsible for the biological effect.

To get a general idea of what the fractions contained, we decided to examine fractions 12-14 by HPLC. We found that when compared to the Morroniside and Loganin standards **(Figure 21 (A))** all three fractions had peaks with similar RTs to the standards. Fraction 12 had a prominent peak a RT 4.5 **(Figure 21 (C))** corresponding with the Morroniside peak in the standard while fractions 13 and 14 had a prominent peak at RT 7.5 **(Figures 21 E and G)** corresponding with the Loganin peak in the standard. Furthermore, we used mass spectrometry to determine the exact identity of the peaks with similar RT's to the standards. We confirmed that Morroniside (MS peaks: 450,451,440 (**Figure 22 B**)) was found in fraction 12 **(Figure 23 C)** and that Loganin (MS Peaks: 434,435,424 **(Figure 22 C))** was found in fraction 13 **(Figure 24 C)** and fraction 14 **(Figure 25 C)**, although the Loganin peak in fraction 13 was found to have quite a few CO eluents while in fraction 14 the Loganin was found to be quite pure.

Interestingly, when we examined the fractions using a diode array detector, we observed a prominent peak in the heatmap at an RT of 2.5 for fractions 12-14 **(Figure 21 D,F and H).** This peak did not absorb well at a wavelength of 240nm but did absorb well at the other wavelengths measured. We followed this up with a mass spectrometric analysis and found that for all the fractions examined there was a prominent peak at 0.4 minutes in the total ion chromatogram **(Figures 23,24,25)**. The mass spectra (MS) for

this peak displayed similar Ion fragmentation (MS Peaks 133,274,288) in fractions 12-14 **(Figures 23B,24B, 25B)** indicating this was the same compound in each fraction. This data seems to demonstrate that there may be a combinatorial effect as although neither Loganin nor Morroniside had much of an effect on their own but when combined with the other abundant compounds present in these fractions there was a similar effect on viability when measured by MTT.

4.3.4 Individual fractions of CO induce the Keap1/Nrf2 pathway

To identify the individual compound responsible for the biological effect we decided to examine if any individual fraction of CO could induce the Keap1/Nrf2 pathway as whole CO extract had been shown to do previously[176]. To determine the effect of the individual fractions on Keap1/Nrf2 activation we used an ELISA to measure HO1 expression as HO1 is a known downstream target of Nrf2 activation [203]. We found that HO1 expression was significantly higher in several individual fractions **(Figure 26)**. Fractions 12-14 had the highest expression and was comparable to HO1 expression in the whole CO extract. Interestingly, this mirrors the MTT data **(Figure 20)** as fractions 12-14 had the greatest effect on viability. Furthermore, fractions 12-16 seem to have an inverse relation between the concentration of the peak with an RT of 2.5 **(Figure 21: D,F,H)** and the expression of HO1 demonstrating a potential dose dependent response **(Figure 26)**. Also, Morroniside and Loganin were found to have no effect on their own **(Figure 26)** further reinforcing that the biological effect of CO on the 1.1B4 cells is either due to a combinatorial effect or an entirely different compound than what has been published.

4.4 Discussion

We had shown previously that CO had a substantial biological effect as demonstrated in our earlier paper which revealed that CO treatment had a robust activation of the Keap1/Nrf2 Pathway [176]. We then followed this up by providing evidence for clinical significance as we demonstrated that CO could delay the onset of T1D in the NOD mouse model [200]. One important question remaining was what compound or combination of compounds were responsible for CO's biological effect? We hypothesized that either Loganin and Morroniside may be responsible as both have been isolated from CO and have been shown to be of biological significance [204] [202] but interestingly our results seem to indicate this is not the case.

We examined 1.1B4 cells after treatment with Loganin and Morroniside and found that they had no effect on viability **(Figure 17),** nor did they activate the Keap1/Nrf2 pathway. We then used an analytical HPLC system to find the most abundant compounds and found that although Loganin and Morroniside were abundant in whole CO extract, there were many other high intensity peaks indicating there was a number of other abundant compounds as well **(Figure 18)**. Due to this we decided to fractionate the whole CO extract **(Figure 19)** and test the individual fractions for biological effect. Interestingly we found 3 fractions (12-14) that have a similar effect on viability as whole CO extract **(Figure 20),** and these three factions caused a very significant increase in expression of HO1 indicating an activation of the Keap1/Nrf2 pathway. We then examined these fractions by HPLC and mass spectrometry, we found that although they contained Loganin and Morroniside there was another compound that was highly abundant in all three fractions **(Figures 21-25).** Considering that Loganin and Morroniside had no effect

on their own this seems to indicate that there is a combinatorial effect or that a different compound is responsible for the biological effect on CO.

Future studies will need to address whether the effect of CO is combinatorial or caused by an individual compound as the fractions that were biologically relevant contained Loganin and Morroniside as well as another compound that was highly abundant. **(2.5 RT peak, Figure 21 B-H)** Furthermore, the 2.5 RT peak **(Figure 21 B-H)** will need to be elucidated as our data suggests that it has a substantial biological effect. HNMR and highly sensitive Mass Spectrometry we will need to be used to identify the structure as this compound has not been clearly defined in the literature. By identifying the structure, the compound can then be purified and tested for biological significance on its own and the individual compound responsible for CO's biological effect may be identified.

In summary, we clearly show that neither loganin nor morroniside on their own is responsible for the biological effect in the 1.1B4 cells. Instead, we show that a previously uncharacterized compound may be responsible either in concert with Loganin and Morroniside or on its own for the biological effect of CO.

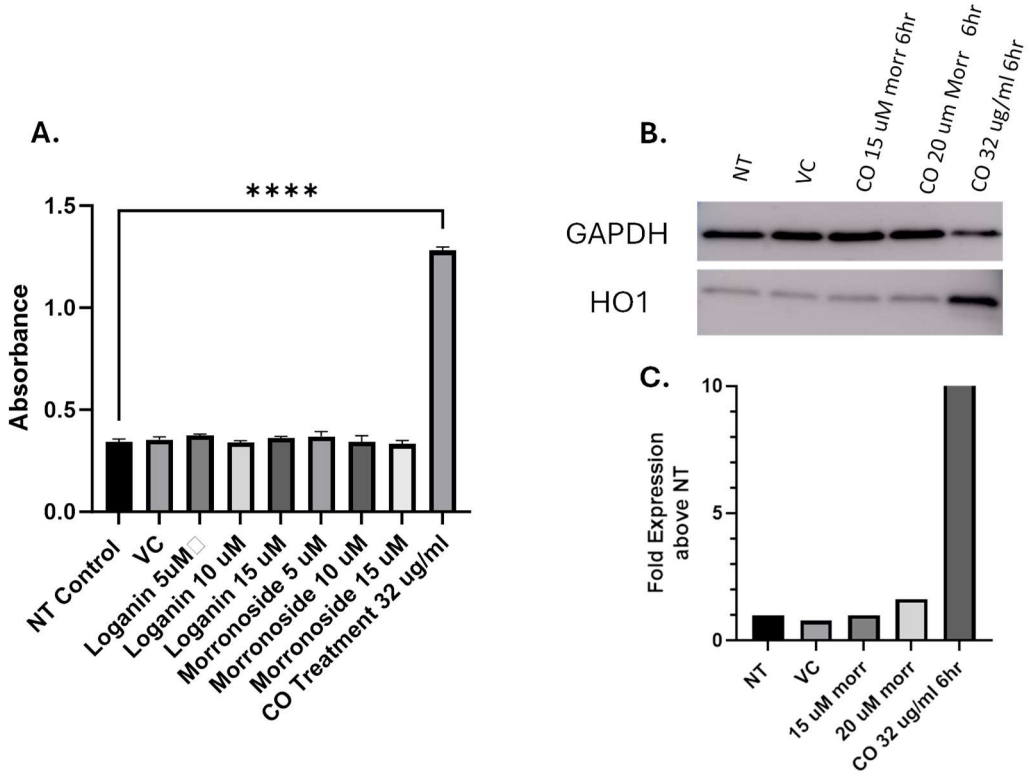
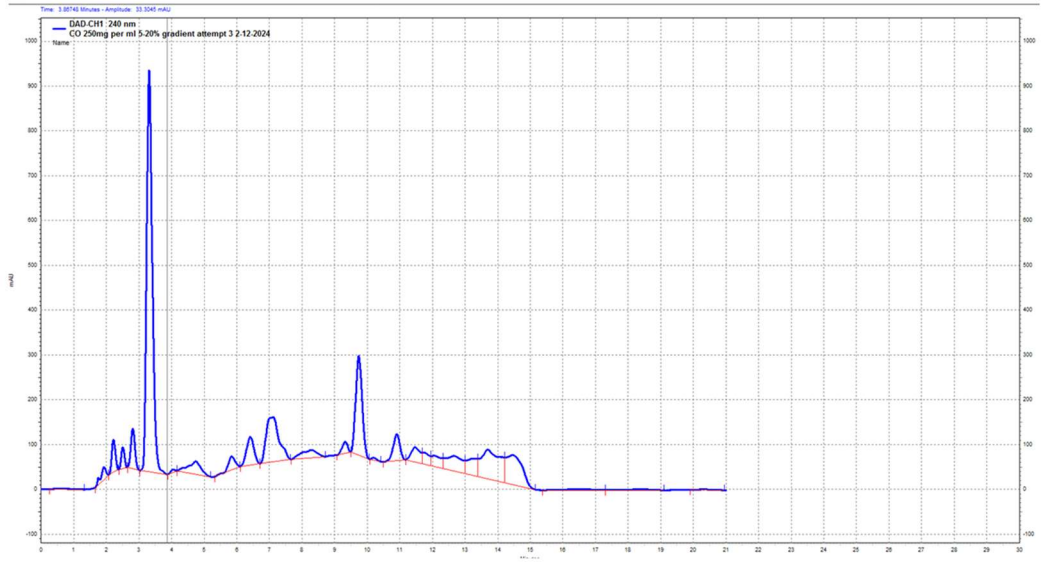


Figure 17: Loganin and Morroniside do not activate the keap1/Nrf2 pathway on the 1.1B4 cells. (A) MTT assay with the absorbance measured at 570nm after the designated treatments on the X axis. Data are shown as mean ± SEM. **** denotes statistical significance ($P < 0.0001$) as determined by unpaired T test (N=3). **(B)** Western blot analysis looking at HO1 expression. GAPDH was used as a loading control. **(C.)** densitometric analysis of the WB in **(B)**

A.



B.

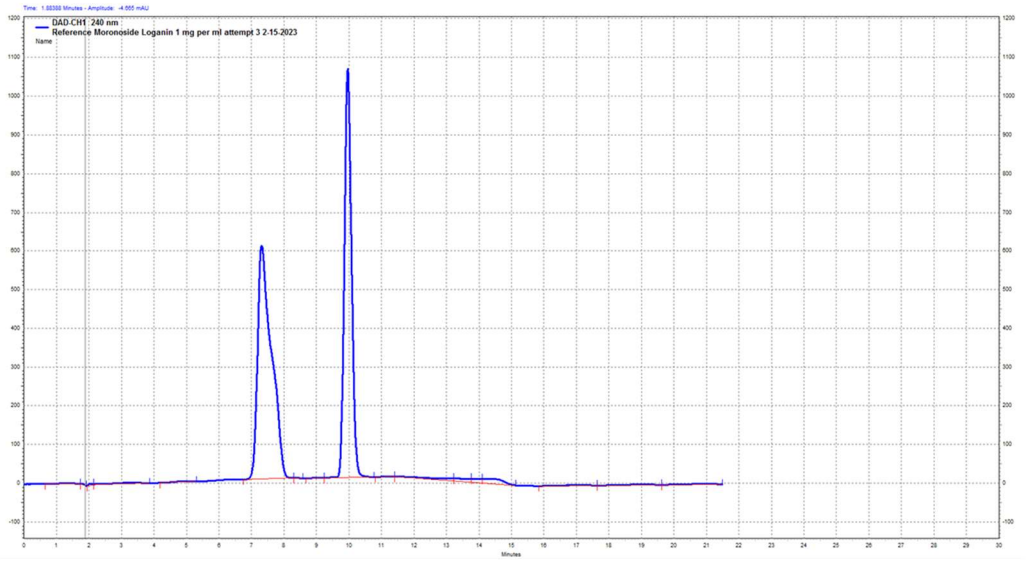


Figure 18: Analytical HPLC chromatograms of CO and Loganin and Morroniside standards **(A)** HPLC chromatogram of whole CO extract (250mg/ml)**.** Absorance measured at 240nm **(B)** HPLC chromatogram of the Morroniside and Loganin standards (1mg/ml). Absorbance measured at 240nm

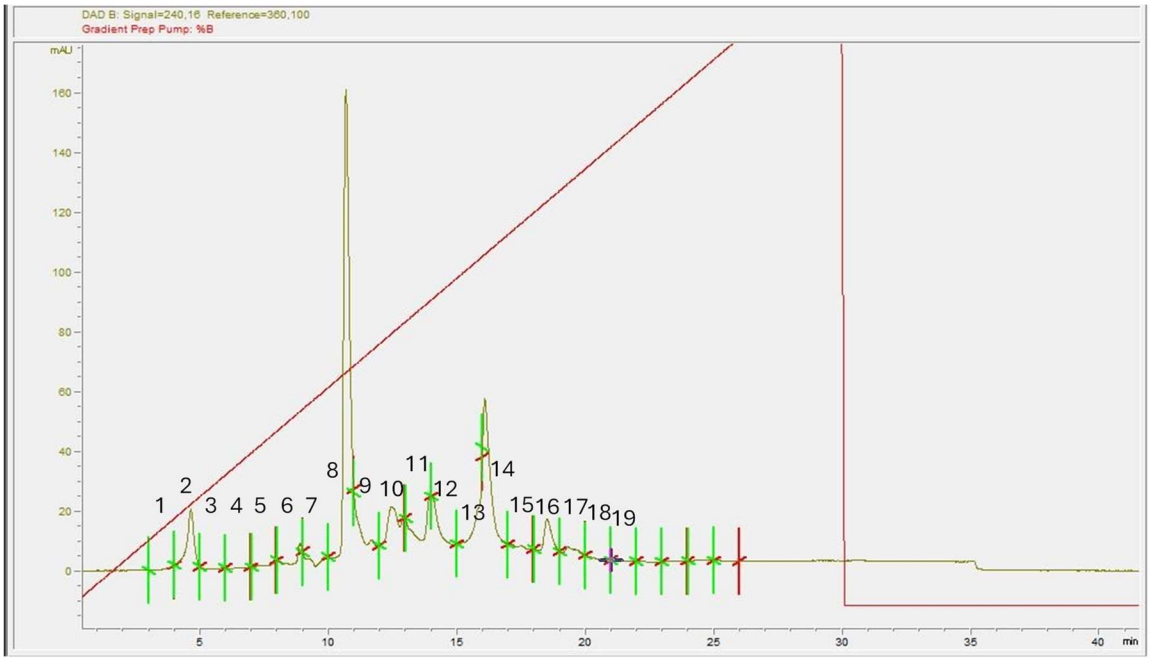


Figure 19: Preparative HPLC chromatogram of whole CO extract. Red line represents the concentration of the organic solvent. Labeled green columns represent where each time-based fraction was taken with a corresponding numerical designation.

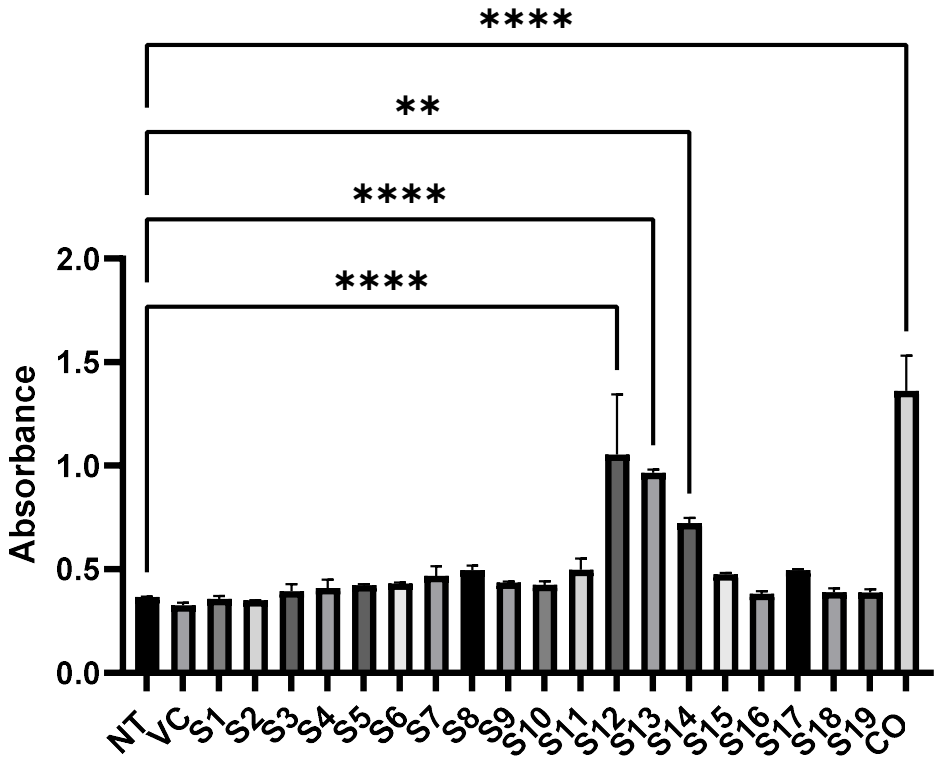


Figure 20: Individual Fractions (12-14) increase viability as measured by MTT in 1.1B4 cells. MTT assay with the absorbance measured at 570nm after the designated treatments on the X axis. Data are shown as mean ± SEM. **** denotes statistical significance ($P < 0.0001$) as determined by unpaired T test (N=3).

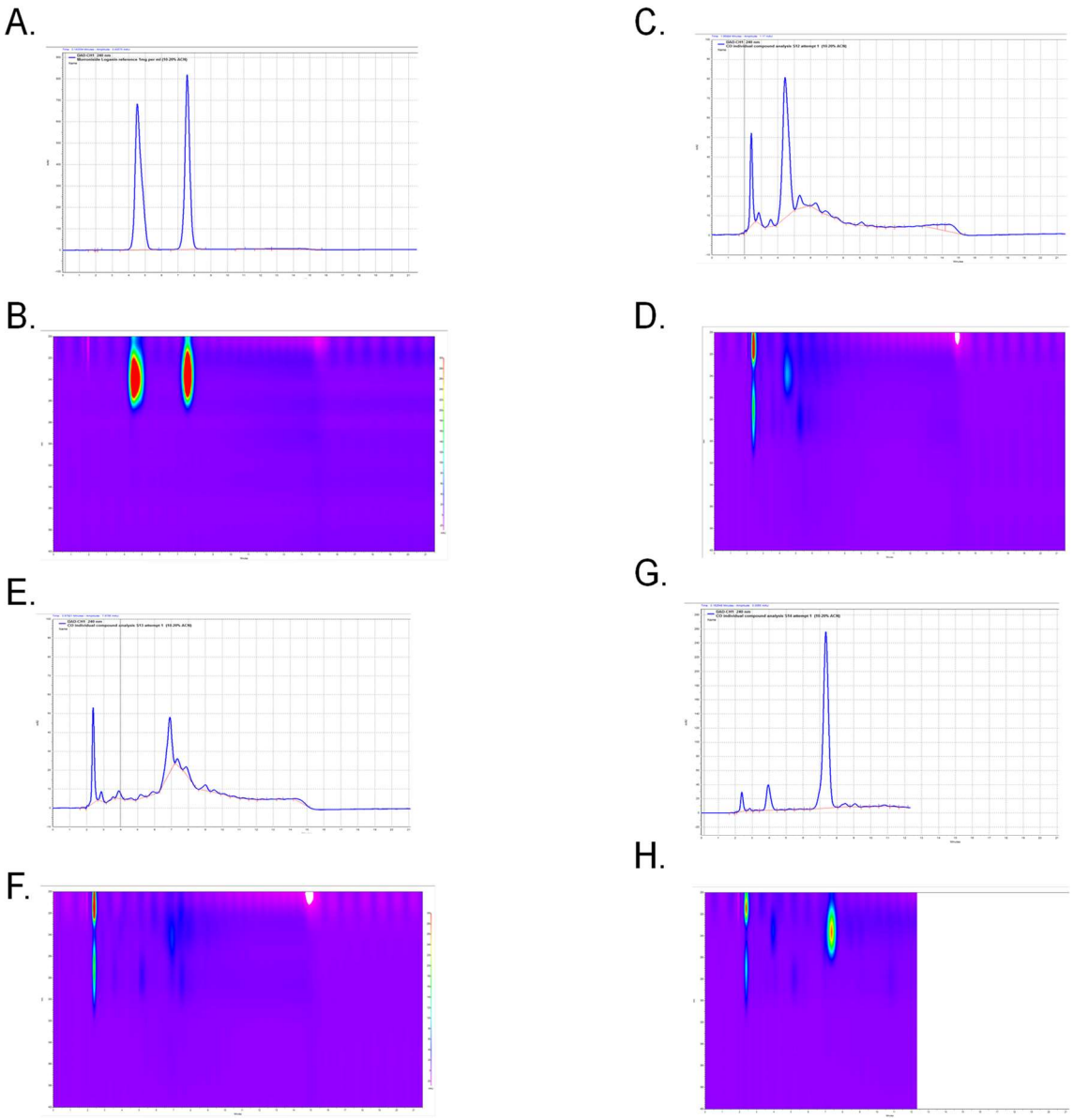


Figure 21: HPLC and Diode array Detector analysis of individual CO fractions: All HPLC absorbances measured at 240nm. All Diode array Detector (DAD) heatmaps monitored all wavelengths from 200-400nm. **(A)** HPLC chromatogram of Morroniside and Loganin standards. **(B)** Heat Map of DAD for Morroniside and Loganin standards **(C)** HPLC chromatogram of CO fraction 12 **(D)** Heat Map of the DAD for CO Fraction 12. **(E)** HPLC chromatogram of CO fraction 13 **(F)** Heat Map of the DAD for CO Fraction 13. **(G)** HPLC chromatogram of CO fraction 14 **(H)** Heat Map of the DAD for CO Fraction 14.

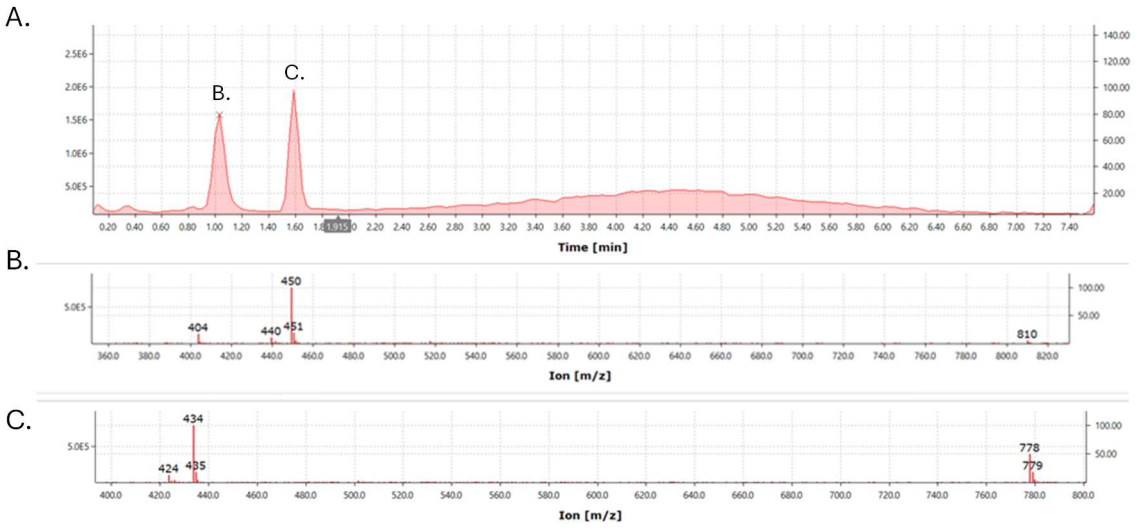


Figure 22: Mass spectrometric analysis of Loganin and Morronoside standards: (A) Total ion chromatogram of Morroniside and Loganin standards. **(B)** MS spectra of Morroniside peak from the total ion chromatogram labeled "B". **(C)** MS spectra of Loganin peak from the total ion chromatogram labeled "B".

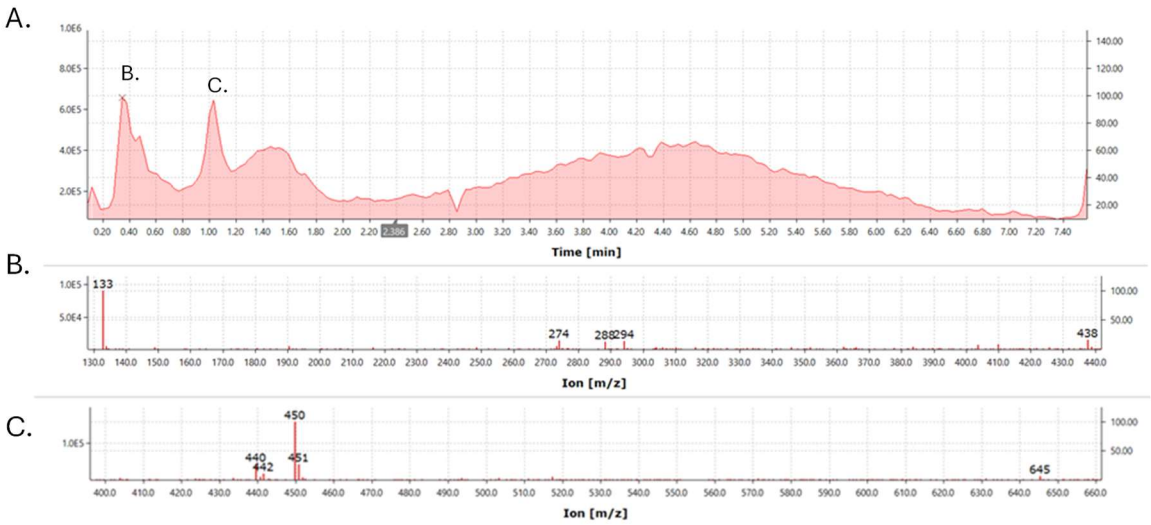


Figure 23: Mass spectrometric analysis of the 12th CO fraction : (A) Total ion chromatogram of fraction 12. **(B)** MS spectra of first peak from the total ion chromatogram labeled "B". **(C)** MS spectra of second peak from the total ion chromatogram labeled "B".

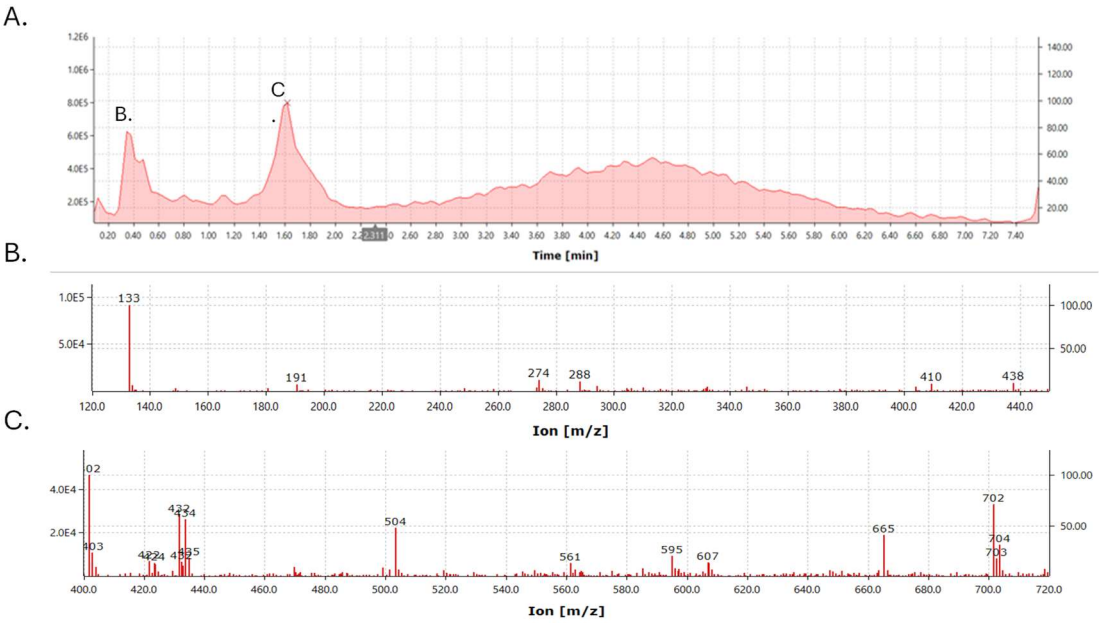


Figure 24: Mass spectrometric analysis of the 13th CO fraction : (A) Total ion chromatogram of fraction 13. **(B)** MS spectra of first peak from the total ion chromatogram labeled "B". **(C)** MS spectra of second peak from the total ion chromatogram labeled "B".

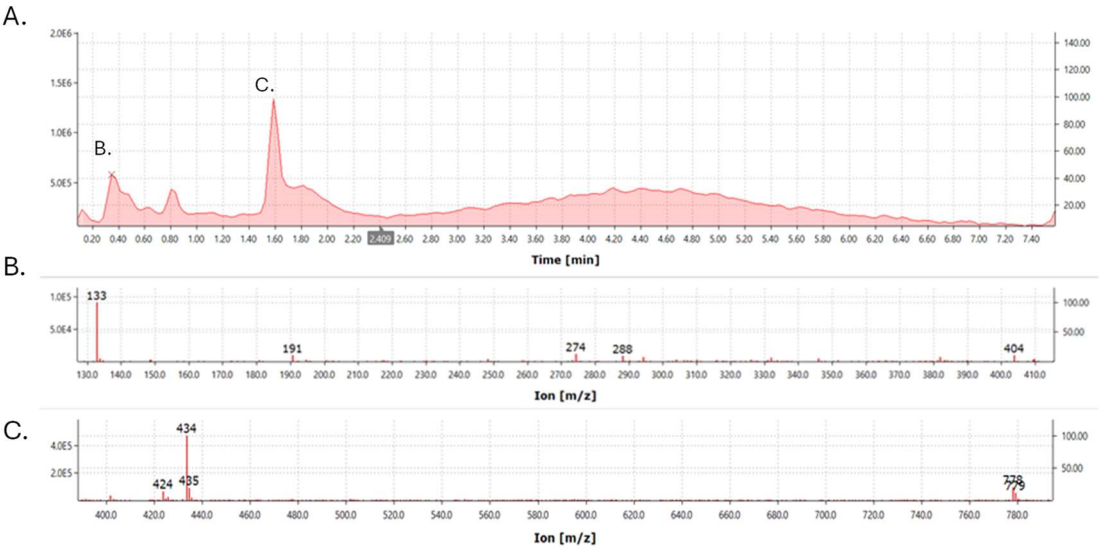


Figure 25: Mass spectrometric analysis of the 14th CO fraction : (A) Total ion chromatogram of fraction 14. **(B)** MS spectra of first peak from the total ion chromatogram labeled "B". **(C)** MS spectra of second peak from the total ion chromatogram labeled "B".

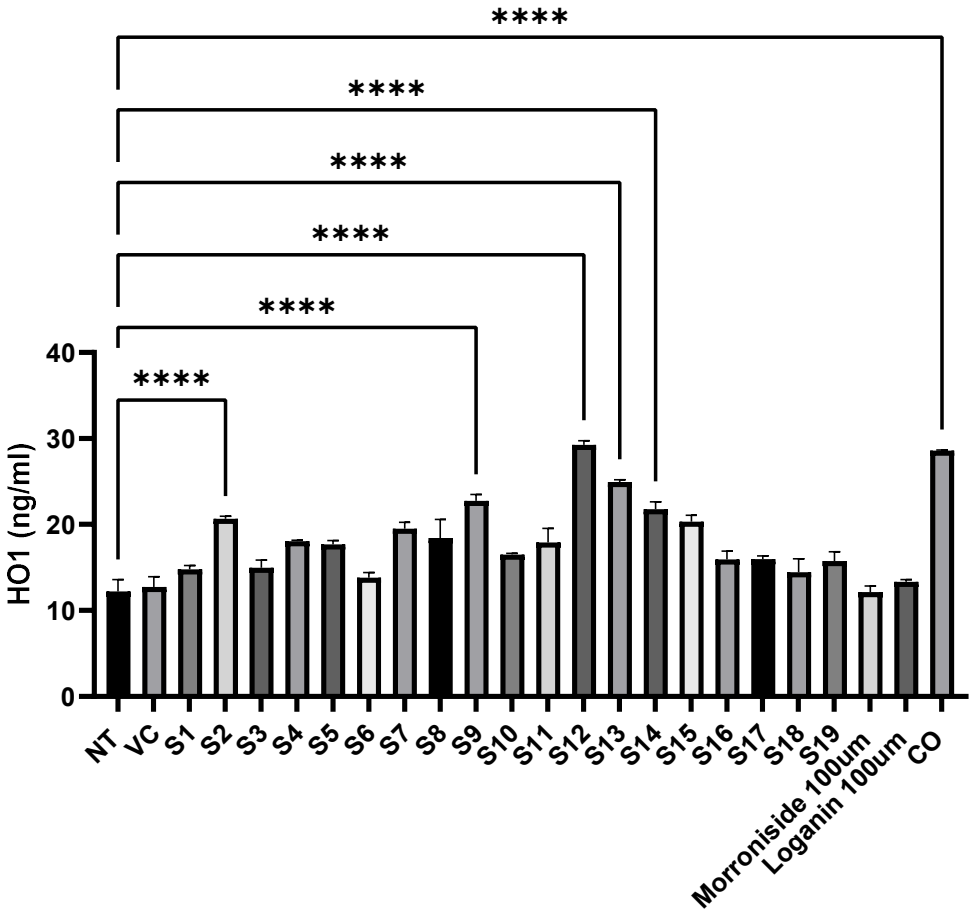


Figure 26: HO1 concentration (ng/ml) after CO treatment: Whole cell lysate was collected after corresponding treatments and HO1 concentration was determined by commercial ELISA. Average HO1 levels are shown as mean ± SEM. $P < 0.05$ as determined by one-way anova test.

CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Major Conclusions

The objective of this study was to determine the potential CO could have as a T1D interventional therapy. Much of the current literature focuses on the effect of CO in a T2D model with our lab being one of the few that is actively examining CO effect in a T1D model. The majority of our lab's previous research fixated on the beneficial effects CO had by promoting cellular viability and preventing cytokine induced cell death, however a mechanism of action and whether CO had any effect *in vivo* remained unclear. In chapter 2 we used a proteomic analysis to determine what pathways were being activated by CO treatment and found that there was a strong induction of autophagy and the Keap1/Nrf2 pathway. To further investigate the CO induced activation of autophagy we did an immunofluorescence and western blot analysis. We discovered a substantial increase in autophagosome formation and a significant increase in LC3II and P62 phosphorylation indicating a strong activation of autophagy. This was followed up by investigating the predicted activation of the Keap1/Nrf2 pathway. We found a substantial localization of Nrf2 to the nucleus upon CO treatment followed by a significant upregulation of the downstream antioxidant targets of the pathway: HO1 and SOD 2. Taken together this indicates a strong antioxidant response induced by CO and this was confirmed by showing that CO preserves cellular function in the presence of H2O2 induced oxidative stress. Thus, the combined activation of

autophagy and the Keap1/Nrf2 pathway by CO seem to protect the cell from oxidative stress and may be a potential mechanism of action.

As discussed in the introduction, oxidative stress seems to be playing a direct role in T1D progression. Since CO protects the cell from oxidative stress we decided to investigate if CO would have any effect in delaying T1D onset in vivo. In chapter 3 we used an oral gavage approach to ensure that each mouse received an equal dose of CO or water control. We discovered that CO significantly delayed T1D onset and had an overall delay in hyperglycemic events as well when compared to the water control. Furthermore, we saw that in the CO treated mice there was a significant reduction of insulitis suggesting that leukocyte recruitment was reduced. Interestingly, there were no significant changes in the different populations of leukocytes recruited to the islet indicating that the effect CO has is not modulating the immune system but instead is directly affecting the β-cell itself. This was further supported by a significant preservation of C peptide secretion in our CO treated mice indicating we were preserving beta cell function.

As a follow up to the in vivo study we wanted to try and identify individual compounds responsible for CO's biological effect. We anticipated that it could have been Loganin or Morroniside as both of these compounds were found in CO and had been published to have biological activity. Interestingly, in the 1.1B4 cell line these compounds had no effect on activating the Keap1/Nrf2 pathway indicating they had no biological effect on their own. We then used an analytical HPLC approach and found that although Loganin and Morroniside were abundant in the CO, there were many other compounds that where abundant as well. To test for biological activity, we then used a preparative

HPLC system to fractionate all the compounds in CO. After fractionation we tested the individual fractions for activation of the Keap1/Nrf2 pathway by looking at HO1 expression. We found that fractions 12-14 had a strong upregulation of the pathway. Interestingly, Upon LC/MS analysis we found that these fractions did contain Loganin and Morroniside as well as a third characterized compound. Considering the fact the neither Loganin nor Morroniside had any effect the activation of the Keap1/Nrf2 pathway on their own, this indicates that a combinatorial effect or an entirely new uncharacterized compound is responsible for CO's biological effect

Overall, our results suggest that CO's mechanism of action is through the activation of cytoprotective pathways like autophagy and the Keap1/Nrf2 pathway and activation of these pathways had a significant effect in delaying T1D an in vivo model displaying that CO may provide a promising avenue for new T1D interventional therapeutics.

5.2 Limitations of the Study

The studies throughout this dissertation have utilized the human pancreatic β cell line called 1.1B4. This cell line is a great cost-effective approach to relatively model human pancreatic β cells, however studies looking at the effect of CO treatment on primary β cells will be needed to ensure the physiological relevance of CO in humans. Furthermore, The NOD mouse model represents an acceptable model for modeling T1D that is autoimmune in nature, however studies have shown that promising reagents that reverse or delay T1D onset in the NOD mouse do not always translate directly into T1D delay in humans [205]. For this reason, CO extract and its constituents must be examined in a clinical trial or a model that better represents the human progression of T1D.

5.3 Future Directions

The studies within this dissertation have provided significant evidence that CO has a biologically relevant effect and may provide an avenue for developing new T1D interventional therapies. Future studies will need to address the remaining questions regarding CO's biological effect. A major question that needs to be addressed is whether the Keap1/Nrf2 pathway is responsible for delaying T1D in the NOD mouse. To test this, a Nrf2 knockout NOD mouse model will need to be developed using CRISPR/Cas9 or a Nrf2 inhibitor will be needed. After development of the Nrf2 knockout a similar experiment to our in vivo experiment will need to be done to determine if CO has any effect after Nrf2 has been knocked out or inhibited. If CO has no effect, it can be reasoned that Nrf2 signaling is critical to the biological effect of CO.

Furthermore, our LC/MS analysis demonstrated that there was an uncharacterized compound that seemed to have a biological effect but whether this effect was caused by a combinatorial effect remains unclear. This uncharacterized compound will need to be isolated and identified using an MS/MS and HNMR approach. Afterwards this compound will need to be tested on its own as well as in conjunction with Loganin and Morroniside to determine what exactly is responsible for CO's biological effect. This can be achieved by treating cells with either a combination of this compound + Loganin and Morroniside or just the compound its self, then using an ELISA to measure HO1 expression to determine activation of the keap1/ Nrf2 pathway.

Lastly, Identifying the effect compounds from CO have on primary human islets along with the effect CO has on specific cell types within the islets will be needed to distinguish CO's precise effects as well as determining clinical significance. For this,

pancreatic islets will need to be obtained from donors and the effects of CO on these islets will need to be compared to our current in vitro data. If the effect of CO is similar in primary human islets, CO may be a promising candidate alone or in combination with other treatments for clinical trials focused on interventional therapies for T1D.

REFERENCES

1. Atkinson, M. A., Campbell-Thompson, M., Kusmartseva, I. & Kaestner, K. H. (2020) Organisation of the human pancreas in health and in diabetes, *Diabetologia.* **63**, 1966-1973.
2. Pandol, S. J. (2010) in *The Exocrine Pancreas*, San Rafael (CA).
3. In't Veld, P. & Marichal, M. (2010) Microscopic anatomy of the human islet of Langerhans, *Adv Exp Med Biol.* **654**, 1-19.
4. Brereton, M. F., Vergari, E., Zhang, Q. & Clark, A. (2015) Alpha-, Delta- and PP-cells: Are They the Architectural Cornerstones of Islet Structure and Co-ordination?, *J Histochem Cytochem.* **63**, 575-91.
5. Wierup, N., Svensson, H., Mulder, H. & Sundler, F. (2002) The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas, *Regul Pept.* **107**, 63-9.
6. Sato, T., Nakamura, Y., Shiimura, Y., Ohgusu, H., Kangawa, K. & Kojima, M. (2012) Structure, regulation and function of ghrelin, *J Biochem.* **151**, 119-28.
7. Pradhan, G., Samson, S. L. & Sun, Y. (2013) Ghrelin: much more than a hunger hormone, *Curr Opin Clin Nutr Metab Care.* **16**, 619-24.
8. Zhu, W., Tanday, N., Flatt, P. R. & Irwin, N. (2023) Pancreatic polypeptide revisited: Potential therapeutic effects in obesity-diabetes, *Peptides.* **160**, 170923.
9. Aragon, F., Karaca, M., Novials, A., Maldonado, R., Maechler, P. & Rubi, B. (2015) Pancreatic polypeptide regulates glucagon release through PPYR1 receptors expressed in mouse and human alpha-cells, *Biochim Biophys Acta.* **1850**, 343-51.
10. Kim, W., Fiori, J. L., Shin, Y. K., Okun, E., Kim, J. S., Rapp, P. R. & Egan, J. M. (2014) Pancreatic polypeptide inhibits somatostatin secretion, *FEBS Lett.* **588**, 3233-9.
11. Bastidas, J. A., Couse, N. F., Yeo, C. J., Schmieg, R. E., Jr., Andersen, D. K., Gingerich, R. L. & Zinner, M. J. (1990) The effect of pancreatic polypeptide infusion on glucose tolerance and insulin response in longitudinally studied pancreatitis-induced diabetes, *Surgery.* **107**, 661-8.
12. Khan, D., Vasu, S., Moffett, R. C., Irwin, N. & Flatt, P. R. (2017) Influence of neuropeptide Y and pancreatic polypeptide on islet function and beta-cell survival, *Biochim Biophys Acta Gen Subj.* **1861**, 749-758.
13. Luft, R., Efendic, S., Hokfelt, T., Johansson, O. & Arimura, A. (1974) Immunohistochemical evidence for the localization of somatostatin--like immunoreactivity in a cell population of the pancreatic islets, *Med Biol.* **52**, 428-30.
14. Xu, S. F. S., Andersen, D. B., Izarzugaza, J. M. G., Kuhre, R. E. & Holst, J. J. (2020) In the rat pancreas, somatostatin tonically inhibits glucagon secretion and is required for glucose-induced inhibition of glucagon secretion, *Acta Physiol (Oxf).* **229**, e13464.
15. Habegger, K. M., Heppner, K. M., Geary, N., Bartness, T. J., DiMarchi, R. & Tschop, M. H. (2010) The metabolic actions of glucagon revisited, *Nat Rev Endocrinol.* **6**, 689-97.

16. Garcia, A., Williamson, J. R. & Cahill, G. F., Jr. (1966) Studies on the perfused rat liver. II. Effect of glucagon on gluconeogenesis, *Diabetes.* **15**, 188-93.
17. Fu, Z., Gilbert, E. R. & Liu, D. (2013) Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes, *Curr Diabetes Rev.* **9**, 25-53.
18. Suckale, J. & Solimena, M. (2008) Pancreas islets in metabolic signaling--focus on the beta-cell, *Front Biosci.* **13**, 7156-71.
19. Rahman, M. S., Hossain, K. S., Das, S., Kundu, S., Adegoke, E. O., Rahman, M. A., Hannan, M. A., Uddin, M. J. & Pang, M. G. (2021) Role of Insulin in Health and Disease: An Update, *Int J Mol Sci.* **22**.
20. Whiting, D. R., Guariguata, L., Weil, C. & Shaw, J. (2011) IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030, *Diabetes Res Clin Pract.* **94**, 311-21.
21. American Diabetes, A. (2013) Diagnosis and classification of diabetes mellitus, *Diabetes Care.* **36 Suppl 1**, S67-74.
22. Tokarz, V. L., MacDonald, P. E. & Klip, A. (2018) The cell biology of systemic insulin function, *J Cell Biol.* **217**, 2273-2289.
23. Kolb, H. & Martin, S. (2017) Environmental/lifestyle factors in the pathogenesis and prevention of type 2 diabetes, *BMC Med.* **15**, 131.
24. Lee, S. H., Park, S. Y. & Choi, C. S. (2022) Insulin Resistance: From Mechanisms to Therapeutic Strategies, *Diabetes Metab J.* **46**, 15-37.
25. Cusi, K., Maezono, K., Osman, A., Pendergrass, M., Patti, M. E., Pratipanawatr, T., DeFronzo, R. A., Kahn, C. R. & Mandarino, L. J. (2000) Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle, *J Clin Invest.* **105**, 311-20.
26. Herman, M. A. & Kahn, B. B. (2006) Glucose transport and sensing in the maintenance of glucose homeostasis and metabolic harmony, *J Clin Invest.* **116**, 1767-75.
27. Roden, M., Price, T. B., Perseghin, G., Petersen, K. F., Rothman, D. L., Cline, G. W. & Shulman, G. I. (1996) Mechanism of free fatty acid-induced insulin resistance in humans, *J Clin Invest.* **97**, 2859-65.
28. Roma, L. P., Pascal, S. M., Duprez, J. & Jonas, J. C. (2012) Mitochondrial oxidative stress contributes differently to rat pancreatic islet cell apoptosis and insulin secretory defects after prolonged culture in a low non-stimulating glucose concentration, *Diabetologia.* **55**, 2226-37.
29. Hull, R. L., Westermark, G. T., Westermark, P. & Kahn, S. E. (2004) Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes, *J Clin Endocrinol Metab.* **89**, 3629-43.
30. Cnop, M. (2008) Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes, *Biochem Soc Trans.* **36**, 348-52.
31. DiMeglio, L. A., Evans-Molina, C. & Oram, R. A. (2018) Type 1 diabetes, *Lancet.* **391**, 2449-2462.
32. Ziegler, A. G., Rewers, M., Simell, O., Simell, T., Lempainen, J., Steck, A., Winkler, C., Ilonen, J., Veijola, R., Knip, M., Bonifacio, E. & Eisenbarth, G. S. (2013) Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children, *JAMA.* **309**, 2473-9.

33. McLaughlin, K. A., Richardson, C. C., Ravishankar, A., Brigatti, C., Liberati, D., Lampasona, V., Piemonti, L., Morgan, D., Feltbower, R. G. & Christie, M. R. (2016) Identification of Tetraspanin-7 as a Target of Autoantibodies in Type 1 Diabetes, *Diabetes.* **65**, 1690-8.
34. Kawasaki, E. (2023) Anti-Islet Autoantibodies in Type 1 Diabetes, *Int J Mol Sci.* **24**.
35. Thomas, N. J., Jones, S. E., Weedon, M. N., Shields, B. M., Oram, R. A. & Hattersley, A. T. (2018) Frequency and phenotype of type 1 diabetes in the first six decades of life: a cross-sectional, genetically stratified survival analysis from UK Biobank, *Lancet Diabetes Endocrinol.* **6**, 122-129.
36. Hope, S. V., Wienand-Barnett, S., Shepherd, M., King, S. M., Fox, C., Khunti, K., Oram, R. A., Knight, B. A., Hattersley, A. T., Jones, A. G. & Shields, B. M. (2016) Practical Classification Guidelines for Diabetes in patients treated with insulin: a cross-sectional study of the accuracy of diabetes diagnosis, *Br J Gen Pract.* **66**, e315-22.
37. American Diabetes, A. (2018) 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018, *Diabetes Care.* **41**, S13-S27.
38. Eyth, E. & Naik, R. (2023) Hemoglobin A1C in *StatPearls*, Treasure Island (FL) ineligible companies. Disclosure: Roopa Naik declares no relevant financial relationships with ineligible companies.
39. Shemin, D. & Rittenberg, D. (1946) The life span of the human red blood cell, *J Biol Chem.* **166**, 627-36.
40. Virk, S. A., Donaghue, K. C., Cho, Y. H., Benitez-Aguirre, P., Hing, S., Pryke, A., Chan, A. & Craig, M. E. (2016) Association Between HbA1c Variability and Risk of Microvascular Complications in Adolescents With Type 1 Diabetes, *J Clin Endocrinol Metab.* **101**, 3257-63.
41. Verges, B. (2020) Cardiovascular disease in type 1 diabetes: A review of epidemiological data and underlying mechanisms, *Diabetes Metab.* **46**, 442-449.
42. Das Evcimen, N. & King, G. L. (2007) The role of protein kinase C activation and the vascular complications of diabetes, *Pharmacol Res.* **55**, 498-510.
43. Candido, R., Forbes, J. M., Thomas, M. C., Thallas, V., Dean, R. G., Burns, W. C., Tikellis, C., Ritchie, R. H., Twigg, S. M., Cooper, M. E. & Burrell, L. M. (2003) A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes, *Circ Res.* **92**, 785-92.
44. Bierhaus, A., Humpert, P. M., Morcos, M., Wendt, T., Chavakis, T., Arnold, B., Stern, D. M. & Nawroth, P. P. (2005) Understanding RAGE, the receptor for advanced glycation end products, *J Mol Med (Berl).* **83**, 876-86.
45. Burr, S. D. & Stewart, J. A., Jr. (2021) Rap1a Overlaps the AGE/RAGE Signaling Cascade to Alter Expression of alpha-SMA, p-NF-kappaB, and p-PKC-zeta in Cardiac Fibroblasts Isolated from Type 2 Diabetic Mice, *Cells.* **10**.
46. Pociot, F. & Lernmark, A. (2016) Genetic risk factors for type 1 diabetes, *Lancet.* **387**, 2331-2339.
47. Redondo, M. J., Jeffrey, J., Fain, P. R., Eisenbarth, G. S. & Orban, T. (2008) Concordance for islet autoimmunity among monozygotic twins, *N Engl J Med.* **359**, 2849-50.
48. Rajaei, E., Jalali, M. T., Shahrabi, S., Asnafi, A. A. & Pezeshki, S. M. S. (2019) HLAs in Autoimmune Diseases: Dependable Diagnostic Biomarkers?, *Curr Rheumatol Rev.* **15**, 269-276.

49. Noble, J. A. (2015) Immunogenetics of type 1 diabetes: A comprehensive review, *J Autoimmun.* **64**, 101-12.
50. Armitage, L. H., Wallet, M. A. & Mathews, C. E. (2021) Influence of PTPN22 Allotypes on Innate and Adaptive Immune Function in Health and Disease, *Front Immunol.* **12**, 636618.
51. Durinovic-Bello, I., Wu, R. P., Gersuk, V. H., Sanda, S., Shilling, H. G. & Nepom, G. T. (2010) Insulin gene VNTR genotype associates with frequency and phenotype of the autoimmune response to proinsulin, *Genes Immun.* **11**, 188-93.
52. Mayer-Davis, E. J., Lawrence, J. M., Dabelea, D., Divers, J., Isom, S., Dolan, L., Imperatore, G., Linder, B., Marcovina, S., Pettitt, D. J., Pihoker, C., Saydah, S., Wagenknecht, L. & Study, S. f. D. i. Y. (2017) Incidence Trends of Type 1 and Type 2 Diabetes among Youths, 2002-2012, *N Engl J Med.* **376**, 1419-1429.
53. Rewers, M. & Ludvigsson, J. (2016) Environmental risk factors for type 1 diabetes, *Lancet.* **387**, 2340-2348.
54. Ferrara, C. T., Geyer, S. M., Liu, Y. F., Evans-Molina, C., Libman, I. M., Besser, R., Becker, D. J., Rodriguez, H., Moran, A., Gitelman, S. E., Redondo, M. J. & Type 1 Diabetes TrialNet Study, G. (2017) Excess BMI in Childhood: A Modifiable Risk Factor for Type 1 Diabetes Development?, *Diabetes Care.* **40**, 698-701.
55. Geravandi, S., Richardson, S., Pugliese, A. & Maedler, K. (2021) Localization of enteroviral RNA within the pancreas in donors with T1D and T1D-associated autoantibodies, *Cell Rep Med.* **2**, 100371.
56. Krogvold, L., Edwin, B., Buanes, T., Frisk, G., Skog, O., Anagandula, M., Korsgren, O., Undlien, D., Eike, M. C., Richardson, S. J., Leete, P., Morgan, N. G., Oikarinen, S., Oikarinen, M., Laiho, J. E., Hyoty, H., Ludvigsson, J., Hanssen, K. F. & Dahl-Jorgensen, K. (2015) Detection of a low-grade enteroviral infection in the islets of langerhans of living patients newly diagnosed with type 1 diabetes, *Diabetes.* **64**, 1682-7.
57. Jean-Baptiste, V. S. E., Xia, C. Q., Clare-Salzler, M. J. & Horwitz, M. S. (2017) Type 1 Diabetes and Type 1 Interferonopathies: Localization of a Type 1 Common Thread of Virus Infection in the Pancreas, *EBioMedicine.* **22**, 10-17.
58. Lombardi, A. & Tomer, Y. (2017) Interferon alpha impairs insulin production in human beta cells via endoplasmic reticulum stress, *J Autoimmun.* **80**, 48-55.
59. Foulis, A. K., Farquharson, M. A. & Meager, A. (1987) Immunoreactive alpha-interferon in insulin-secreting beta cells in type 1 diabetes mellitus, *Lancet.* **2**, 1423-7.
60. Newby, B. N. & Mathews, C. E. (2017) Type I Interferon Is a Catastrophic Feature of the Diabetic Islet Microenvironment, *Front Endocrinol (Lausanne).* **8**, 232.
61. Kronenberg-Versteeg, D., Eichmann, M., Russell, M. A., de Ru, A., Hehn, B., Yusuf, N., van Veelen, P. A., Richardson, S. J., Morgan, N. G., Lemberg, M. K. & Peakman, M. (2018) Molecular Pathways for Immune Recognition of Preproinsulin Signal Peptide in Type 1 Diabetes, *Diabetes.* **67**, 687-696.
62. Dinic, S., Arambasic Jovanovic, J., Uskokovic, A., Mihailovic, M., Grdovic, N., Tolic, A., Rajic, J., Dordevic, M. & Vidakovic, M. (2022) Oxidative stress-mediated beta cell death and dysfunction as a target for diabetes management, *Front Endocrinol (Lausanne).* **13**, 1006376.
63. Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. & Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease, *Int J Biochem Cell Biol.* **39**, 44-84.

64. Acharya, J. D. & Ghaskadbi, S. S. (2010) Islets and their antioxidant defense, *Islets.* **2**, 225-35.
65. Lenzen, S. (2017) Chemistry and biology of reactive species with special reference to the antioxidative defence status in pancreatic beta-cells, *Biochim Biophys Acta Gen Subj.* **1861**, 1929-1942.
66. Fiorentino, T. V., Prioletta, A., Zuo, P. & Folli, F. (2013) Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases, *Curr Pharm Des.* **19**, 5695-703.
67. Lenzen, S., Drinkgern, J. & Tiedge, M. (1996) Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues, *Free Radic Biol Med.* **20**, 463-6.
68. Modak, M. A., Parab, P. B. & Ghaskadbi, S. S. (2009) Pancreatic islets are very poor in rectifying oxidative DNA damage, *Pancreas.* **38**, 23-9.
69. Roep, B. O., Thomaidou, S., van Tienhoven, R. & Zaldumbide, A. (2021) Type 1 diabetes mellitus as a disease of the beta-cell (do not blame the immune system?), *Nat Rev Endocrinol.* **17**, 150-161.
70. Chen, J., Stimpson, S. E., Fernandez-Bueno, G. A. & Mathews, C. E. (2018) Mitochondrial Reactive Oxygen Species and Type 1 Diabetes, *Antioxid Redox Signal.* **29**, 1361-1372.
71. Zeeshan, H. M., Lee, G. H., Kim, H. R. & Chae, H. J. (2016) Endoplasmic Reticulum Stress and Associated ROS, *Int J Mol Sci.* **17**, 327.
72. Wyatt, R. C., Lanzoni, G., Russell, M. A., Gerling, I. & Richardson, S. J. (2019) What the HLA-I!-Classical and Non-classical HLA Class I and Their Potential Roles in Type 1 Diabetes, *Curr Diab Rep.* **19**, 159.
73. Roep, B. O., Kleijwegt, F. S., van Halteren, A. G., Bonato, V., Boggi, U., Vendrame, F., Marchetti, P. & Dotta, F. (2010) Islet inflammation and CXCL10 in recent-onset type 1 diabetes, *Clin Exp Immunol.* **159**, 338-43.
74. Lee, E. Y., Lee, Z. H. & Song, Y. W. (2009) CXCL10 and autoimmune diseases, *Autoimmun Rev.* **8**, 379-83.
75. Tan, S., Li, Y., Xia, J., Jin, C. H., Hu, Z., Duinkerken, G., Li, Y., Khosravi Maharlooei, M., Chavez, E., Nauman, G., Danzl, N., Nakayama, M., Roep, B. O., Sykes, M. & Yang, Y. G. (2017) Type 1 diabetes induction in humanized mice, *Proc Natl Acad Sci U S A.* **114**, 10954-10959.
76. Zheng, X. & Sawalha, A. H. (2022) The Role of Oxidative Stress in Epigenetic Changes Underlying Autoimmunity, *Antioxid Redox Signal.* **36**, 423-440.
77. Smallwood, M. J., Nissim, A., Knight, A. R., Whiteman, M., Haigh, R. & Winyard, P. G. (2018) Oxidative stress in autoimmune rheumatic diseases, *Free Radic Biol Med.* **125**, 3-14.
78. Piganelli, J. D., Mamula, M. J. & James, E. A. (2020) The Role of beta Cell Stress and Neo-Epitopes in the Immunopathology of Type 1 Diabetes, *Front Endocrinol (Lausanne).* **11**, 624590.
79. Marre, M. L., McGinty, J. W., Chow, I. T., DeNicola, M. E., Beck, N. W., Kent, S. C., Powers, A. C., Bottino, R., Harlan, D. M., Greenbaum, C. J., Kwok, W. W., Piganelli, J. D. & James, E. A. (2018) Modifying Enzymes Are Elicited by ER Stress, Generating Epitopes That Are Selectively Recognized by CD4(+) T Cells in Patients With Type 1 Diabetes, *Diabetes.* **67**, 1356-1368.

80. Diez, J., Park, Y., Zeller, M., Brown, D., Garza, D., Ricordi, C., Hutton, J., Eisenbarth, G. S. & Pugliese, A. (2001) Differential splicing of the IA-2 mRNA in pancreas and lymphoid organs as a permissive genetic mechanism for autoimmunity against the IA-2 type 1 diabetes autoantigen, *Diabetes.* **50**, 895-900.
81. Kracht, M. J., van Lummel, M., Nikolic, T., Joosten, A. M., Laban, S., van der Slik, A. R., van Veelen, P. A., Carlotti, F., de Koning, E. J., Hoeben, R. C., Zaldumbide, A. & Roep, B. O. (2017) Autoimmunity against a defective ribosomal insulin gene product in type 1 diabetes, *Nat Med.* **23**, 501-507.
82. Delong, T., Wiles, T. A., Baker, R. L., Bradley, B., Barbour, G., Reisdorph, R., Armstrong, M., Powell, R. L., Reisdorph, N., Kumar, N., Elso, C. M., DeNicola, M., Bottino, R., Powers, A. C., Harlan, D. M., Kent, S. C., Mannering, S. I. & Haskins, K. (2016) Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion, *Science.* **351**, 711-4.
83. Storling, J., Overgaard, A. J., Brorsson, C. A., Piva, F., Bang-Berthelsen, C. H., Haase, C., Nerup, J. & Pociot, F. (2013) Do post-translational beta cell protein modifications trigger type 1 diabetes?, *Diabetologia.* **56**, 2347-54.
84. Lichti, C. F. & Wan, X. (2023) Using mass spectrometry to identify neoantigens in autoimmune diseases: The type 1 diabetes example, *Semin Immunol.* **66**, 101730.
85. Fesus, L. & Piacentini, M. (2002) Transglutaminase 2: an enigmatic enzyme with diverse functions, *Trends Biochem Sci.* **27**, 534-9.
86. Ientile, R., Caccamo, D. & Griffin, M. (2007) Tissue transglutaminase and the stress response, *Amino Acids.* **33**, 385-94.
87. Eckert, R. L., Kaartinen, M. T., Nurminskaya, M., Belkin, A. M., Colak, G., Johnson, G. V. & Mehta, K. (2014) Transglutaminase regulation of cell function, *Physiol Rev.* **94**, 383-417.
88. Ermak, G. & Davies, K. J. (2002) Calcium and oxidative stress: from cell signaling to cell death, *Mol Immunol.* **38**, 713-21.
89. Callebaut, A., Bruggeman, Y., Zamit, C., Sodre, F. M. C., Irla, M., Mathieu, C., Buitinga, M. & Overbergh, L. (2022) Aberrant expression of transglutaminase 2 in pancreas and thymus of NOD mice underscores the importance of deamidation in neoantigen generation, *Front Endocrinol (Lausanne).* **13**, 908248.
90. McGinty, J. W., Chow, I. T., Greenbaum, C., Odegard, J., Kwok, W. W. & James, E. A. (2014) Recognition of posttranslationally modified GAD65 epitopes in subjects with type 1 diabetes, *Diabetes.* **63**, 3033-40.
91. Greenberg, C. S., Birckbichler, P. J. & Rice, R. H. (1991) Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues, *FASEB J.* **5**, 3071-7.
92. Delong, T., Baker, R. L., He, J., Barbour, G., Bradley, B. & Haskins, K. (2012) Diabetogenic T-cell clones recognize an altered peptide of chromogranin A, *Diabetes.* **61**, 3239-46.
93. Gottlieb, P. A., Delong, T., Baker, R. L., Fitzgerald-Miller, L., Wagner, R., Cook, G., Rewers, M. R., Michels, A. & Haskins, K. (2014) Chromogranin A is a T cell antigen in human type 1 diabetes, *J Autoimmun.* **50**, 38-41.

94. Zhang, B., Zhai, M., Li, B., Liu, Z., Li, K., Jiang, L., Zhang, M., Yi, W., Yang, J., Yi, D., Liang, H., Jin, Z., Duan, W. & Yu, S. (2018) Honokiol Ameliorates Myocardial Ischemia/Reperfusion Injury in Type 1 Diabetic Rats by Reducing Oxidative Stress and Apoptosis through Activating the SIRT1-Nrf2 Signaling Pathway, *Oxid Med Cell Longev.* **2018**, 3159801.
95. Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A. & Dulak, J. (2016) Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism, *Cell Mol Life Sci.* **73**, 3221-47.
96. Kobayashi, M. & Yamamoto, M. (2006) Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species, *Adv Enzyme Regul.* **46**, 113-40.
97. Baird, L. & Yamamoto, M. (2020) The Molecular Mechanisms Regulating the KEAP1-NRF2 Pathway, *Mol Cell Biol.* **40**.
98. Ichimura, Y., Waguri, S., Sou, Y. S., Kageyama, S., Hasegawa, J., Ishimura, R., Saito, T., Yang, Y., Kouno, T., Fukutomi, T., Hoshii, T., Hirao, A., Takagi, K., Mizushima, T., Motohashi, H., Lee, M. S., Yoshimori, T., Tanaka, K., Yamamoto, M. & Komatsu, M. (2013) Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy, *Mol Cell.* **51**, 618-31.
99. Mohammadzadeh, M., Halabian, R., Gharehbaghian, A., Amirizadeh, N., Jahanian-Najafabadi, A., Roushandeh, A. M. & Roudkenar, M. H. (2012) Nrf-2 overexpression in mesenchymal stem cells reduces oxidative stress-induced apoptosis and cytotoxicity, *Cell Stress Chaperones.* **17**, 553-65.
100. Yasui, K. & Baba, A. (2006) Therapeutic potential of superoxide dismutase (SOD) for resolution of inflammation, *Inflamm Res.* **55**, 359-63.
101. Behl, T., Kaur, I., Sehgal, A., Sharma, E., Kumar, A., Grover, M. & Bungau, S. (2021) Unfolding Nrf2 in diabetes mellitus, *Mol Biol Rep.* **48**, 927-939.
102. Lou, Y., Kong, M., Li, L., Hu, Y., Zhai, W., Qi, X., Liu, Z. & Wu, J. (2021) Inhibition of the Keap1/Nrf2 Signaling Pathway Significantly Promotes the Progression of Type 1 Diabetes Mellitus, *Oxid Med Cell Longev.* **2021**, 7866720.
103. Uruno, A., Furusawa, Y., Yagishita, Y., Fukutomi, T., Muramatsu, H., Negishi, T., Sugawara, A., Kensler, T. W. & Yamamoto, M. (2013) The Keap1-Nrf2 system prevents onset of diabetes mellitus, *Mol Cell Biol.* **33**, 2996-3010.
104. Parzych, K. R. & Klionsky, D. J. (2014) An overview of autophagy: morphology, mechanism, and regulation, *Antioxid Redox Signal.* **20**, 460-73.
105. Pearson, G. L., Gingerich, M. A., Walker, E. M., Biden, T. J. & Soleimanpour, S. A. (2021) A Selective Look at Autophagy in Pancreatic beta-Cells, *Diabetes.* **70**, 1229-1241.
106. Tung, Y. T., Hsu, W. M., Lee, H., Huang, W. P. & Liao, Y. F. (2010) The evolutionarily conserved interaction between LC3 and p62 selectively mediates autophagy-dependent degradation of mutant huntingtin, *Cell Mol Neurobiol.* **30**, 795-806.
107. Muralidharan, C., Conteh, A. M., Marasco, M. R., Crowder, J. J., Kuipers, J., de Boer, P. & Linnemann, A. K. (2021) Pancreatic beta cell autophagy is impaired in type 1 diabetes, *Diabetologia.* **64**, 865-877.
108. Berchtold, L. A., Prause, M., Storling, J. & Mandrup-Poulsen, T. (2016) Cytokines and Pancreatic beta-Cell Apoptosis, *Adv Clin Chem.* **75**, 99-158.

109. Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P. & Malik, A. B. (2014) Reactive oxygen species in inflammation and tissue injury, *Antioxid Redox Signal.* **20**, 1126-67.
110. Simmons, K. M. & Michels, A. W. (2015) Type 1 diabetes: A predictable disease, *World J Diabetes.* **6**, 380-90.
111. Pietropaolo, M., Towns, R. & Eisenbarth, G. S. (2012) Humoral autoimmunity in type 1 diabetes: prediction, significance, and detection of distinct disease subtypes, *Cold Spring Harb Perspect Med.* **2**.
112. Martin, S., Pawlowski, B., Greulich, B., Ziegler, A. G., Mandrup-Poulsen, T. & Mahon, J. (1992) Natural course of remission in IDDM during 1st yr after diagnosis, *Diabetes Care.* **15**, 66-74.
113. Pathak, V., Pathak, N. M., O'Neill, C. L., Guduric-Fuchs, J. & Medina, R. J. (2019) Therapies for Type 1 Diabetes: Current Scenario and Future Perspectives, *Clin Med Insights Endocrinol Diabetes.* **12**, 1179551419844521.
114. Subramanian, S., Khan, F. & Hirsch, I. B. (2024) New advances in type 1 diabetes, *BMJ.* **384**, e075681.
115. (2023) First cell therapy for diabetes approved, *Nat Biotechnol.* **41**, 1036.
116. Herold, K. C., Gitelman, S. E., Gottlieb, P. A., Knecht, L. A., Raymond, R. & Ramos, E. L. (2023) Teplizumab: A Disease-Modifying Therapy for Type 1 Diabetes That Preserves beta-Cell Function, *Diabetes Care.* **46**, 1848-1856.
117. Long, S. A., Thorpe, J., Herold, K. C., Ehlers, M., Sanda, S., Lim, N., Linsley, P. S., Nepom, G. T. & Harris, K. M. (2017) Remodeling T cell compartments during anti-CD3 immunotherapy of type 1 diabetes, *Cell Immunol.* **319**, 3-9.
118. Sims, E. K., Bundy, B. N., Stier, K., Serti, E., Lim, N., Long, S. A., Geyer, S. M., Moran, A., Greenbaum, C. J., Evans-Molina, C., Herold, K. C. & Type 1 Diabetes TrialNet Study, G. (2021) Teplizumab improves and stabilizes beta cell function in antibody-positive high-risk individuals, *Sci Transl Med.* **13**.
119. Perdigoto, A. L., Preston-Hurlburt, P., Clark, P., Long, S. A., Linsley, P. S., Harris, K. M., Gitelman, S. E., Greenbaum, C. J., Gottlieb, P. A., Hagopian, W., Woodwyk, A., Dziura, J., Herold, K. C. & Immune Tolerance, N. (2019) Treatment of type 1 diabetes with teplizumab: clinical and immunological follow-up after 7 years from diagnosis, *Diabetologia.* **62**, 655-664.
120. Morris, A. (2019) Teplizumab delays onset of type 1 diabetes mellitus, *Nat Rev Endocrinol.* **15**, 437.
121. Tewari, D., Mocan, A., Parvanov, E. D., Sah, A. N., Nabavi, S. M., Huminiecki, L., Ma, Z. F., Lee, Y. Y., Horbanczuk, J. O. & Atanasov, A. G. (2017) Ethnopharmacological Approaches for Therapy of Jaundice: Part I, *Front Pharmacol.* **8**, 518.
122. Sharifi-Rad, J., Salehi, B., Stojanovic-Radic, Z. Z., Fokou, P. V. T., Sharifi-Rad, M., Mahady, G. B., Sharifi-Rad, M., Masjedi, M. R., Lawal, T. O., Ayatollahi, S. A., Masjedi, J., Sharifi-Rad, R., Setzer, W. N., Sharifi-Rad, M., Kobarfard, F., Rahman, A. U., Choudhary, M. I., Ata, A. & Iriti, M. (2020) Medicinal plants used in the treatment of tuberculosis - Ethnobotanical and ethnopharmacological approaches, *Biotechnol Adv.* **44**, 107629.
123. Hunt, J. T. (2009) Discovery of ixabepilone, *Mol Cancer Ther.* **8**, 275-81.
124. Dong, Y., Feng, Z. L., Chen, H. B., Wang, F. S. & Lu, J. H. (2018) Corni Fructus: a review of chemical constituents and pharmacological activities, *Chin Med.* **13**, 34.

125. Sharp-Tawfik, A. E., Coiner, A. M., MarElia, C. B., Kazantzis, M., Zhang, C. & Burkhardt, B. R. (2019) Compositional analysis and biological characterization of Cornus officinalis on human 1.1B4 pancreatic beta cells, *Mol Cell Endocrinol.* **494**, 110491.
126. Yokozawa, T., Kang, K. S., Park, C. H., Noh, J. S., Yamabe, N., Shibahara, N. & Tanaka, T. (2010) Bioactive constituents of Corni Fructus: The therapeutic use of morroniside, loganin, and 7-O-galloyl-D-sedoheptulose as renoprotective agents in type 2 diabetes, *Drug Discov Ther.* **4**, 223-34.
127. Han, Y., Jung, H. W. & Park, Y. K. (2014) Selective therapeutic effect of cornus officinalis fruits on the damage of different organs in STZ-induced diabetic rats, *Am J Chin Med.* **42**, 1169-82.
128. Wang, W., Sun, F., An, Y., Ai, H., Zhang, L., Huang, W. & Li, L. (2009) Morroniside protects human neuroblastoma SH-SY5Y cells against hydrogen peroxide-induced cytotoxicity, *Eur J Pharmacol.* **613**, 19-23.
129. Atkinson, M. A., Eisenbarth, G. S. & Michels, A. W. (2014) Type 1 diabetes, *Lancet.* **383**, 69-82.
130. Vehik, K., Fiske, S. W., Logan, C. A., Agardh, D., Cilio, C. M., Hagopian, W., Simell, O., Roivainen, M., She, J. X., Briese, T., Oikarinen, S., Hyoty, H., Ziegler, A. G., Rewers, M., Lernmark, A., Akolkar, B., Krischer, J. P., Burkhardt, B. R. & Group, T. S. (2013) Methods, quality control and specimen management in an international multicentre investigation of type 1 diabetes: TEDDY, *Diabetes Metab Res Rev.* **29**, 557-67.
131. Vehik, K., Ajami, N. J., Hadley, D., Petrosino, J. F. & Burkhardt, B. R. (2013) The changing landscape of type 1 diabetes: recent developments and future frontiers, *Curr Diab Rep.* **13**, 642-50.
132. Abbasi, J. (2021) Teplizumab Improves Beta Cell Function, Delays Type 1 Diabetes, *JAMA.* **325**, 1385.
133. Apaya, M. K., Kuo, T. F., Yang, M. T., Yang, G., Hsiao, C. L., Chang, S. B., Lin, Y. & Yang, W. C. (2020) Phytochemicals as modulators of beta-cells and immunity for the therapy of type 1 diabetes: Recent discoveries in pharmacological mechanisms and clinical potential, *Pharmacol Res.* **156**, 104754.
134. Chen, C. C., Hsu, C. Y., Chen, C. Y. & Liu, H. K. (2008) Fructus Corni suppresses hepatic gluconeogenesis related gene transcription, enhances glucose responsiveness of pancreatic beta-cells, and prevents toxin induced beta-cell death, *J Ethnopharmacol.* **117**, 483-90.
135. Park, C. H., Xu, F. H., Roh, S. S., Song, Y. O., Uebaba, K., Noh, J. S. & Yokozawa, T. (2015) Astaxanthin and Corni Fructus protect against diabetes-induced oxidative stress, inflammation, and advanced glycation end product in livers of streptozotocin-induced diabetic rats, *J Med Food.* **18**, 337-44.
136. Czerwinska, M. E., Bobinska, A., Cichocka, K., Buchholz, T., Wolinski, K. & Melzig, M. F. (2021) Cornus mas and Cornus officinalis-A Comparison of Antioxidant and Immunomodulatory Activities of Standardized Fruit Extracts in Human Neutrophils and Caco-2 Models, *Plants (Basel).* **10**.
137. Gao, X., Liu, Y., An, Z. & Ni, J. (2021) Active Components and Pharmacological Effects of Cornus officinalis: Literature Review, *Front Pharmacol.* **12**, 633447.

138. Vasu, S., McClenaghan, N. H. & Flatt, P. R. (2016) Molecular Mechanisms of Toxicity and Cell Damage by Chemicals in a Human Pancreatic Beta Cell Line, 1.1B4, *Pancreas.* **45**, 1320-9.
139. Green, A. D., Vasu, S., McClenaghan, N. H. & Flatt, P. R. (2015) Pseudoislet formation enhances gene expression, insulin secretion and cytoprotective mechanisms of clonal human insulin-secreting 1.1B4 cells, *Pflugers Arch.* **467**, 2219-28.
140. Vasu, S., McClenaghan, N. H., McCluskey, J. T. & Flatt, P. R. (2014) Mechanisms of toxicity by proinflammatory cytokines in a novel human pancreatic beta cell line, 1.1B4, *Biochim Biophys Acta.* **1840**, 136-45.
141. Sharp-Tawfik, A. E. & Burkhardt, B. R. (2019) Chemical profile dataset of Cornus officinalis from multiple sources using HPLC/MS, *Data Brief.* **25**, 104401.
142. Guergues, J., Zhang, P., Liu, B. & Stevens, S. M., Jr. (2019) Improved Methodology for Sensitive and Rapid Quantitative Proteomic Analysis of Adult-Derived Mouse Microglia: Application to a Novel In Vitro Mouse Microglial Cell Model, *Proteomics.* **19**, e1800469.
143. Zougman, A., Selby, P. J. & Banks, R. E. (2014) Suspension trapping (STrap) sample preparation method for bottom-up proteomics analysis, *Proteomics.* **14**, 1006-0.
144. Deeb, S. J., D'Souza, R. C., Cox, J., Schmidt-Supprian, M. & Mann, M. (2012) Super-SILAC allows classification of diffuse large B-cell lymphoma subtypes by their protein expression profiles, *Mol Cell Proteomics.* **11**, 77-89.
145. Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M. & Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data, *Nat Methods.* **13**, 731-40.
146. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis, *Nat Methods.* **9**, 671-5.
147. Barth, S., Glick, D. & Macleod, K. F. (2010) Autophagy: assays and artifacts, *J Pathol.* **221**, 117-24.
148. Taguchi, K., Motohashi, H. & Yamamoto, M. (2011) Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution, *Genes Cells.* **16**, 123-40.
149. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D. & Yamamoto, M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, *Genes Dev.* **13**, 76-86.
150. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M. & Nabeshima, Y. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements, *Biochem Biophys Res Commun.* **236**, 313-22.
151. Huang, J., Zhang, Y., Dong, L., Gao, Q., Yin, L., Quan, H., Chen, R., Fu, X. & Lin, D. (2018) Ethnopharmacology, phytochemistry, and pharmacology of Cornus officinalis Sieb. et Zucc, *J Ethnopharmacol.* **213**, 280-301.
152. Nie, L., He, K., Xie, F., Xiao, S., Li, S., Xu, J., Zhang, K., Yang, C., Zhou, L., Liu, J., Zou, L. & Yang, X. (2021) Loganin substantially ameliorates molecular deficits, pathologies and cognitive impairment in a mouse model of Alzheimer's disease, *Aging (Albany NY).* **13**, 23739-23756.

153. Shin, J. (1998) P62 and the sequestosome, a novel mechanism for protein metabolism, *Arch Pharm Res.* **21**, 629-33.
154. Lamark, T., Svenning, S. & Johansen, T. (2017) Regulation of selective autophagy: the p62/SQSTM1 paradigm, *Essays Biochem.* **61**, 609-624.
155. Ciuffa, R., Lamark, T., Tarafder, A. K., Guesdon, A., Rybina, S., Hagen, W. J., Johansen, T. & Sachse, C. (2015) The selective autophagy receptor p62 forms a flexible filamentous helical scaffold, *Cell Rep.* **11**, 748-58.
156. Jain, A., Lamark, T., Sjottem, E., Larsen, K. B., Awuh, J. A., Overvatn, A., McMahon, M., Hayes, J. D. & Johansen, T. (2010) p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription, *J Biol Chem.* **285**, 22576-91.
157. Moscat, J., Karin, M. & Diaz-Meco, M. T. (2016) p62 in Cancer: Signaling Adaptor Beyond Autophagy, *Cell.* **167**, 606-609.
158. Sanchez-Martin, P., Saito, T. & Komatsu, M. (2019) p62/SQSTM1: 'Jack of all trades' in health and cancer, *FEBS J.* **286**, 8-23.
159. Katsuragi, Y., Ichimura, Y. & Komatsu, M. (2015) p62/SQSTM1 functions as a signaling hub and an autophagy adaptor, *FEBS J.* **282**, 4672-8.
160. Emanuele, S., Lauricella, M., D'Anneo, A., Carlisi, D., De Blasio, A., Di Liberto, D. & Giuliano, M. (2020) p62: Friend or Foe? Evidences for OncoJanus and NeuroJanus Roles, *Int J Mol Sci.* **21**.
161. Jung, H. S., Chung, K. W., Won Kim, J., Kim, J., Komatsu, M., Tanaka, K., Nguyen, Y. H., Kang, T. M., Yoon, K. H., Kim, J. W., Jeong, Y. T., Han, M. S., Lee, M. K., Kim, K. W., Shin, J. & Lee, M. S. (2008) Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia, *Cell Metab.* **8**, 318-24.
162. Linares, J. F., Duran, A., Reina-Campos, M., Aza-Blanc, P., Campos, A., Moscat, J. & Diaz-Meco, M. T. (2015) Amino Acid Activation of mTORC1 by a PB1-Domain-Driven Kinase Complex Cascade, *Cell Rep.* **12**, 1339-52.
163. Muralidharan, C. & Linnemann, A. K. (2021) beta-Cell autophagy in the pathogenesis of type 1 diabetes, *Am J Physiol Endocrinol Metab.* **321**, E410-E416.
164. Delmastro, M. M. & Piganelli, J. D. (2011) Oxidative stress and redox modulation potential in type 1 diabetes, *Clin Dev Immunol.* **2011**, 593863.
165. Thiruvengadam, M., Venkidasamy, B., Subramanian, U., Samynathan, R., Ali Shariati, M., Rebezov, M., Girish, S., Thangavel, S., Dhanapal, A. R., Fedoseeva, N., Lee, J. & Chung, I. M. (2021) Bioactive Compounds in Oxidative Stress-Mediated Diseases: Targeting the NRF2/ARE Signaling Pathway and Epigenetic Regulation, *Antioxidants (Basel).* **10**.
166. Matzinger, M., Fischhuber, K. & Heiss, E. H. (2018) Activation of Nrf2 signaling by natural products-can it alleviate diabetes?, *Biotechnol Adv.* **36**, 1738-1767.
167. Group, D. P. (2006) Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999, *Diabet Med.* **23**, 857-66.
168. American Diabetes, A. (2021) 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2021, *Diabetes Care.* **44**, S15-S33.
169. Leslie, R. D., Evans-Molina, C., Freund-Brown, J., Buzzetti, R., Dabelea, D., Gillespie, K. M., Goland, R., Jones, A. G., Kacher, M., Phillips, L. S., Rolandsson, O., Wardian, J. L. & Dunne, J. L. (2021) Adult-Onset Type 1 Diabetes: Current Understanding and Challenges, *Diabetes Care.* **44**, 2449-2456.

170. Herold, K. C., Bundy, B. N., Long, S. A., Bluestone, J. A., DiMeglio, L. A., Dufort, M. J., Gitelman, S. E., Gottlieb, P. A., Krischer, J. P., Linsley, P. S., Marks, J. B., Moore, W., Moran, A., Rodriguez, H., Russell, W. E., Schatz, D., Skyler, J. S., Tsalikian, E., Wherrett, D. K., Ziegler, A. G., Greenbaum, C. J. & Type 1 Diabetes TrialNet Study, G. (2019) An Anti-CD3 Antibody, Teplizumab, in Relatives at Risk for Type 1 Diabetes, *N Engl J Med.* **381**, 603-613.
171. Sterne, J. (1958) [Blood sugar-lowering effect of 1,1-dimethylbiguanide], *Therapie.* **13**, 650-9.
172. Schreck, K. & Melzig, M. F. (2021) Traditionally Used Plants in the Treatment of Diabetes Mellitus: Screening for Uptake Inhibition of Glucose and Fructose in the Caco2-Cell Model, *Front Pharmacol.* **12**, 692566.
173. Ma, W., Wang, K. J., Cheng, C. S., Yan, G. Q., Lu, W. L., Ge, J. F., Cheng, Y. X. & Li, N. (2014) Bioactive compounds from Cornus officinalis fruits and their effects on diabetic nephropathy, *J Ethnopharmacol.* **153**, 840-5.
174. He, K., Song, S., Zou, Z., Feng, M., Wang, D., Wang, Y., Li, X. & Ye, X. (2016) The Hypoglycemic and Synergistic Effect of Loganin, Morroniside, and Ursolic Acid Isolated from the Fruits of Cornus officinalis, *Phytother Res.* **30**, 283-91.
175. Fernando, P., Piao, M. J., Zhen, A. X., Ahn, M. J., Yi, J. M., Choi, Y. H. & Hyun, J. W. (2020) Extract of Cornus officinalis Protects Keratinocytes from Particulate Matter-induced Oxidative Stress, *Int J Med Sci.* **17**, 63-70.
176. Sharp-Tawfik, A., Fletcher, J. D., Guergues, J., Marelia-Bennett, C., Wolf, T. J., Coiner, A. M., Zhang, Y. C., Stevens, S. M., Jr. & Burkhardt, B. R. (2022) Proteomic examination of Cornus officinalis stimulated 1.1B4 human pancreatic cells reveals activation of autophagy and Keap1/Nrf2 pathway, *Mol Cell Endocrinol.* **557**, 111773.
177. Haskins, K., Bradley, B., Powers, K., Fadok, V., Flores, S., Ling, X., Pugazhenthi, S., Reusch, J. & Kench, J. (2003) Oxidative stress in type 1 diabetes, *Ann N Y Acad Sci.* **1005**, 43-54.
178. Rojas, J., Bermudez, V., Palmar, J., Martinez, M. S., Olivar, L. C., Nava, M., Tomey, D., Rojas, M., Salazar, J., Garicano, C. & Velasco, M. (2018) Pancreatic Beta Cell Death: Novel Potential Mechanisms in Diabetes Therapy, *J Diabetes Res.* **2018**, 9601801.
179. Lightfoot, Y. L., Chen, J. & Mathews, C. E. (2012) Oxidative stress and beta cell dysfunction, *Methods Mol Biol.* **900**, 347-62.
180. Yagishita, Y., Uruno, A., Chartoumpekis, D. V., Kensler, T. W. & Yamamoto, M. (2019) Nrf2 represses the onset of type 1 diabetes in non-obese diabetic mice, *J Endocrinol*.
181. Makino, S., Kunimoto, K., Muraoka, Y., Mizushima, Y., Katagiri, K. & Tochino, Y. (1980) Breeding of a non-obese, diabetic strain of mice, *Jikken Dobutsu.* **29**, 1-13.
182. In't Veld, P. (2014) Insulitis in human type 1 diabetes: a comparison between patients and animal models, *Semin Immunopathol.* **36**, 569-79.
183. Campbell-Thompson, M., Fu, A., Kaddis, J. S., Wasserfall, C., Schatz, D. A., Pugliese, A. & Atkinson, M. A. (2016) Insulitis and beta-Cell Mass in the Natural History of Type 1 Diabetes, *Diabetes.* **65**, 719-31.

184. Kuehl, M. N., Rodriguez, H., Burkhardt, B. R. & Alman, A. C. (2015) Tumor Necrosis Factor-alpha, Matrix-Metalloproteinases 8 and 9 Levels in the Saliva Are Associated with Increased Hemoglobin A1c in Type 1 Diabetes Subjects, *PLoS One.* **10**, e0125320.
185. Sarkar, A., Kuehl, M. N., Alman, A. C. & Burkhardt, B. R. (2021) Linking the oral microbiome and salivary cytokine abundance to circadian oscillations, *Sci Rep.* **11**, 2658.
186. Gepts, W. (1965) Pathologic anatomy of the pancreas in juvenile diabetes mellitus, *Diabetes.* **14**, 619-33.
187. Jones, A. G. & Hattersley, A. T. (2013) The clinical utility of C-peptide measurement in the care of patients with diabetes, *Diabet Med.* **30**, 803-17.
188. Polonsky, K. S., Licinio-Paixao, J., Given, B. D., Pugh, W., Rue, P., Galloway, J., Karrison, T. & Frank, B. (1986) Use of biosynthetic human C-peptide in the measurement of insulin secretion rates in normal volunteers and type I diabetic patients, *J Clin Invest.* **77**, 98-105.
189. Brundin, T. (1999) Splanchnic and extrasplanchnic extraction of insulin following oral and intravenous glucose loads, *Clin Sci (Lond).* **97**, 429-36.
190. Field, J. B. (1973) Extraction of insulin by liver, *Annu Rev Med.* **24**, 309-14.
191. Xu, H. Q. & Hao, H. P. (2004) Effects of iridoid total glycoside from Cornus officinalis on prevention of glomerular overexpression of transforming growth factor beta 1 and matrixes in an experimental diabetes model, *Biol Pharm Bull.* **27**, 1014-8.
192. Zheng, Y., Vagal, S., Zhu, X. X., de Waele, C., Smith, P. F., Wang, G., Zhang, M. & Darlington, C. L. (2010) The effects of the Chinese herbal medicine EMF01 on salicylate-induced tinnitus in rats, *J Ethnopharmacol.* **128**, 545-8.
193. Jiang, Z., Jiang, X., Li, C., Xue, H. & Zhang, X. (2016) Development of an IgY Antibody-Based Immunoassay for the Screening of the CYP2E1 Inhibitor/Enhancer from Herbal Medicines, *Front Pharmacol.* **7**, 502.
194. Xu, M., Wu, H. Y., Liu, H., Gong, N., Wang, Y. R. & Wang, Y. X. (2017) Morroniside, a secoiridoid glycoside from Cornus officinalis, attenuates neuropathic pain by activation of spinal glucagon-like peptide-1 receptors, *Br J Pharmacol.* **174**, 580-590.
195. Insel, R. A., Dunne, J. L., Atkinson, M. A., Chiang, J. L., Dabelea, D., Gottlieb, P. A., Greenbaum, C. J., Herold, K. C., Krischer, J. P., Lernmark, A., Ratner, R. E., Rewers, M. J., Schatz, D. A., Skyler, J. S., Sosenko, J. M. & Ziegler, A. G. (2015) Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the Endocrine Society, and the American Diabetes Association, *Diabetes Care.* **38**, 1964-74.
196. Vig, S., Lambooij, J. M., Dekkers, M. C., Otto, F., Carlotti, F., Guigas, B. & Zaldumbide, A. (2022) ER stress promotes mitochondrial DNA mediated type-1 interferon response in beta-cells and interleukin-8 driven neutrophil chemotaxis, *Front Endocrinol (Lausanne).* **13**, 991632.
197. Kracht, M. J. L., Zaldumbide, A. & Roep, B. O. (2016) Neoantigens and Microenvironment in Type 1 Diabetes: Lessons from Antitumor Immunity, *Trends Endocrinol Metab.* **27**, 353-362.
198. Ju, C. G., Zhu, L., Wang, W., Gao, H., Xu, Y. B. & Jia, T. Z. (2022) Cornus officinalis prior and post-processing: Regulatory effects on intestinal flora of diabetic nephropathy rats, *Front Pharmacol.* **13**, 1039711.

199. Wang, L., Zhu, J. H., Jiang, X. D., Ma, Z. X. & Tao, J. H. (2022) Preventive effects of the Rehmannia glutinosa Libosch and Cornus officinalis Sieb herb couple on chronic kidney disease rats via modulating the intestinal microbiota and enhancing the intestinal barrier, *Front Pharmacol.* **13**, 942032.
200. Fletcher, J. D., Olsson, G. E., Zhang, Y. C. & Burkhardt, B. R. (2024) Oral gavage delivery of Cornus officinalis extract delays type 1 diabetes onset and hyperglycemia in non-obese diabetic (NOD) mice, *FEBS Open Bio.* **14**, 434-443.
201. Li, M., Zhang, J., Jiang, L., Wang, W., Feng, X., Liu, M. & Yang, D. (2023) Neuroprotective effects of morroniside from Cornus officinalis sieb. Et zucc against Parkinson's disease via inhibiting oxidative stress and ferroptosis, *BMC Complement Med Ther.* **23**, 218.
202. Cheng, Y. C., Chu, L. W., Chen, J. Y., Hsieh, S. L., Chang, Y. C., Dai, Z. K. & Wu, B. N. (2020) Loganin Attenuates High Glucose-Induced Schwann Cells Pyroptosis by Inhibiting ROS Generation and NLRP3 Inflammasome Activation, *Cells.* **9**.
203. Guerrero-Hue, M., Rayego-Mateos, S., Vazquez-Carballo, C., Palomino-Antolin, A., Garcia-Caballero, C., Opazo-Rios, L., Morgado-Pascual, J. L., Herencia, C., Mas, S., Ortiz, A., Rubio-Navarro, A., Egea, J., Villalba, J. M., Egido, J. & Moreno, J. A. (2020) Protective Role of Nrf2 in Renal Disease, *Antioxidants (Basel).* **10**.
204. Zhang, J. X., Wang, R., Xi, J., Shen, L., Zhu, A. Y., Qi, Q., Wang, Q. Y., Zhang, L. J., Wang, F. C., Lu, H. Z. & Hu, J. G. (2017) Morroniside protects SK-N-SH human neuroblastoma cells against H2O2-induced damage, *Int J Mol Med.* **39**, 603-612.
205. Reed, J. C. & Herold, K. C. (2015) Thinking bedside at the bench: the NOD mouse model of T1DM, *Nat Rev Endocrinol.* **11**, 308-14.

APPENDIX A: IACUC APPROVAL LETTERS

UNIVERSITY OF SOUTH FLORIDA
USF RESEARCH & INNOVATION

RESEARCH INTEGRITY & COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO:	Brant Burkhardt,
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	3/22/2021
PROJECT TITLE:	Cornus officinalis type 1 diabetes interventional therapy in non-obese diabetic mice Cornus officinalis T1D therapy
FUNDING SOURCE:	USF department, institute, center, etc.
IACUC PROTOCOL #:	M IS00008962
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC **APPROVED** your request to use the following animals in your **protocol for a one-year period beginning 3/22/2021:**

Non-obese diabetic mouse	NOD/ShiLtJ strain (commonly called NOD)	200
Non-obese diabetes-resistant (NOR)	Non-obese diabetes-resistant (NOR)	200

Please take note of the following:

• **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

• **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
PHS No. A4100-01, AAALAC No. 000434, USDA No. 58-R-0015

UNIVERSITY of SOUTH FLORIDA

MEMORANDUM

TO:	Brant Burkhardt,
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	1/12/2024
PROJECT TITLE:	Cornus officinalis type 1 diabetes interventional therapy in non-obese diabetic mice part 2 Cornus officinalis T1D therapy
FUNDING SOURCE:	USF department, institute, center, etc.
IACUC PROTOCOL #:	M IS00012671
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC **APPROVED** your request to use the following animals in your **protocol for a three-year period beginning 1/12/2024 and expiring on 1/12/2027:**

Non-obese diabetic mouse	NOD/ShiLtJ strain (commonly called NOD)	200
Non-obese diabetes-resistant (NOR)	Non-obese diabetes-resistant (NOR)	200

Please take note of the following:

• After three years, all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.

• All costs invoiced to a grant account must be allocable to the purpose of the grant.

UNIVERSITY of SOUTH FLORIDA

MEMORANDUM

TO:	Brant Burkhardt,
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	4/1/2024
PROJECT TITLE:	Cornus officinalis type 1 diabetes interventional therapy in non-obese diabetic mice part 2 Cornus officinalis T1D therapy
THREE YEAR EXPIRATION DATE:	1/12/2027
FUNDING SOURCE:	Federal government or major agency that awards grants based on peer-reviewed proposals (NIH, NSF, DOD, AHA, ACS, etc.) NIH National Institutes of Health
IACUC PROTOCOL #:	M IS00012671
PROTOCOL STATUS:	**Procedural Change (Verified)**

Your Modification concerning the above referenced IACUC protocol was reviewed by the Veterinarian Verification and Consultation process on **4/1/2024** for the following changes:

Modifications: Funding Information 2.1.1

Added: Federal Government

Removed: Internal

Modifications: Funding - Federal/Major Agency 2.2.1

NIH National Institutes of Health

Modifications: Funding - Federal/Major Agency 2.2.2

1R01AT011907-01A1

Modifications: Funding - Federal/Major Agency 2.2.3

Added: 1R01AT011907-01A1 grant narrative(0.01)

Adding a federal funding source to the protocol.

UNIVERSITY OF SOUTH FLORIDA
USF RESEARCH & INNOVATION

RESEARCH INTEGRITY & COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO:	Brant Burkhardt,
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	7/23/2021
PROJECT TITLE:	Cornus officinalis type 1 diabetes interventional therapy in non-obese diabetic mice Use of Cornus officinalis to prevent onset of type 1 diabetes
FUNDING SOURCE:	USF department, institute, center, etc.
IACUC PROTOCOL #:	R IS00009521
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC **APPROVED** your request to use the following animals in your **protocol for a one-year period beginning 7/23/2021:**

Mouse: NOD/ShiLtJ strain (commonly called NOD) (Ages will be from 5 to 30 weeks Weight will be from 15-30 grams Female only)	200

Please take note of the following:

• **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

• **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
PHS No. A4100-01, AAALAC No. 000434, USDA No. 58-R-0015
University of South Florida • 12901 Bruce B. Downs Blvd., MDC35 • Tampa, FL 33612-4799
(813) 974-7106 • FAX (813) 974-7091

APPENDIX B: PERMISSIONS

Previously published work has been cited within the text. Copyright permission has been given for all previously published work within this dissertation.

The following are the publishers and journals that have been cited and given permission:

Elsevier (Molecular and Cellular Endocrinology)

FEBS Open Bio

Copyright

Overview

In order for Elsevier to publish and disseminate research articles, we need certain publishing rights from authors, which are determined by a publishing agreement between the author and Elsevier.

For articles published open access, the authors license exclusive rights in their article to Elsevier where a CC BY-NC-ND end user license is selected, and license non-exclusive rights where a CC BY end user license is selected.

For articles published under the subscription model, the authors typically transfer copyright to Elsevier. In some circumstances, authors may instead grant us (or the learned society for whom we publish) an exclusive license to publish and disseminate their work.

Regardless of whether they choose to publish open access or subscription with Elsevier, authors have many of the same rights under our publishing agreement, which support their need to share, disseminate and maximize the impact of their research.

For open access articles, authors will also have additional rights, depending on the Creative

Commons end user license that they select. This Creative Commons license sets out the rights that readers (as well as the authors) have to re-use and share the article. Learn how articles can be reused and shared under these licenses.

This page aims to summarize authors' rights when publishing with Elsevier; these are explained in more detail in the publishing agreement between the author and Elsevier.

Irrespective of how an article is published, Elsevier is committed to protect and defend authors' works and their reputation. We take allegations of infringement, plagiarism, ethical disputes, and fraud very seriously.

Author rights

The below table explains the rights that authors have when they publish with Elsevier, for authors who choose to publish either open access or subscription. These apply to the corresponding author and all co-authors.

Author rights in Elsevier's proprietary journals	Published open access	Published subscription
Retain patent and trademark rights	√	√
Retain the rights to use their research data freely without any restriction	√	√
Receive proper attribution and credit for their published work	√	√
Re use their own material in new works without permission or payment (with full acknowledgement of the original article): Extend an article to book length	√	√

Author rights in Elsevier's proprietary journals	Published open access	Published subscription
Include an article in a subsequent compilation of their own work Re use portions excerpts and their own figures or tables in other works		

Use and share their works for scholarly purposes (with full acknowledgement of the original article): 1. In their own classroom teaching Electronic and physical distribution of copies is permitted 2. If an author is speaking at a conference they can present the article and distribute copies to the attendees 3. Distribute the article including by email to their students and to research colleagues who they know for their personal use 4. Share and publicize the article via Share Links, which offers 50 days free access for anyone, without signup or registration 5. Include in a thesis or dissertation (provided this is not published commercially) 6. Share copies of their article privately as part of an invitation only work group on commercial sites with which the publisher has a hosting agreement	√	√
Publicly share the preprint on any website or repository at any time	√	√

Author rights in Elsevier's proprietary journals	Published open access	Published subscription
Publicly share the accepted manuscript on non commercial sites	√	√ using a CC BY NC ND license and usually only after an embargo period (see Sharing Policy for more information)
Publicly share the final published article	√ in line with the author's choice of end user license	×
Retain copyright	√	×

Institution rights

Regardless of how the author chooses to publish with Elsevier, their institution has the right to use articles for classroom teaching and internal training. Articles can be used for these purposes throughout the author's institution, not just by the author:

Institution rights in Elsevier's proprietary journals (providing full acknowledgement of the original article is given	All articles
Copies can be distributed electronically as well as in physical form for classroom teaching and internal training purposes	√

Institution rights in Elsevier's proprietary journals (providing full acknowledgement of the original article is given	**All articles**
Material can be included in coursework and courseware programs for use within the institution (but not in Massive Open Online Courses)	√
Articles can be included in applications for grant funding	√
Theses and dissertations which contain embedded final published articles as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publication on ScienceDirect	√

Government rights

For US government employees, works created within the scope of their employment are considered to be public domain and Elsevier's publishing agreements do not require a transfer or license of rights for such works.

In the UK and certain commonwealth countries, a work created by a government employee is copyrightable, but the government may own the copyright (Crown copyright). Please find information about UK government employees publishing open access.

Find out more

- Download a sample publishing agreement for articles financed by journal subscriptions in English and French

- Download a sample publishing agreement for articles published open access with a commercial user license (CC BY) in English and French.
- Download a sample publishing agreement for articles published open access with a noncommercial user license (CC BY-NC-ND) in English and French.
- Download a sample publishing agreement for articles published open access with a noncommercial user license (CC BY-NC) in English and French.
- For authors who wish to self-archive see our sharing guidelines
- See our author pages for further details about how to promote your article
- See our hosting page for additional information on hosting research published by Elsevier
- For use of Elsevier material not defined here please see our permissions page or visit the Permissions Support Center
- If an author has become aware of a possible plagiarism, fraud or infringement we recommend contacting their Elsevier publishing contact who can then liaise with our in-house legal department
- If you are publishing in a society or third party owned journal, they may have different publishing agreements. Please see the journal's Guide for Authors for journal specific copyright information

Author Guidelines

GUIDE FOR AUTHORS

Information about *FEBS Open Bio*

Aims and Scope

Why publish in *FEBS Open Bio*?

Editorial Board

Contact Us

Reviewing process

Fast-track publication

Open access license and article processing charge

Education Section

Editorial Policies

Open access license and article processing charge

Open access license

This journal is Open Access; all articles will be immediately and permanently free for everyone to read and download.

If your paper is accepted, the author identified as the formal corresponding author for the paper will receive an email prompting them to login into Author Services; where via the Wiley Author Licensing Service (WALS) they will be able to complete the license agreement on behalf of all authors on the paper.

FEBS Open Bio o ers corresponding authors the following license agreement:
Creative Commons Attribution License CC BY

To preview the terms and conditions of this open access agreement, please visit http://creativecommons.org/licenses/by/4.0/.

Article processing charge

FEBS Open Bio is an Open Access journal with no subscribers. A fee is payable by the author or research funder to cover the costs associated with publication. This ensures each article will be immediately and permanently free for anyone to access. Authors transferring their papers from either *The FEBS Journal*, *FEBS Letters* or *Molecular Oncology* are entitled to a 10% discount. Authors will be asked to pay the article publication charge on acceptance of their article. For articles for the Education Section, the publication costs will be covered by FEBS and so there is no cost to the author.

Your institute or funder may have an agreement with Wiley to pay all or some of the article publication charge on your behalf. You can check here. Waivers and discounts are available to authors from countries eligible for the Research4Life programs. You can check the lists of countries here.

Questions related to Open Access publication fees and waivers should be sent to csopenaccess@wiley.com

Education Section

The Education section of *FEBS Open Bio* is devoted to research and review articles in molecular and cellular life sciences education. Research papers should include a hypothesis, methods, and validation of results. We welcome articles dealing with all areas of science education, such as best practice, innovative methods, assessment and feedback, use of technology, curriculum development, internationalization, skills development, and training or career planning. There is no cost to authors for this section as the publication costs will be covered by FEBS.