Relationships Between Erythrocyte Osmotic Fragility and Vitamin C Nutriture in Adults with or without Type 2 Diabetes

by

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ABSTRACT

Diabetes is the 7th leading cause of death globally. In 2018, 34.2 million Americans had type 2 diabetes. Many symptoms of diabetes are similar to those of scurvy or vitamin C deficiency. Vitamin C marginality and inadequacy are more prevalent in Type 2 Diabetes/prediabetes than with normal glucose tolerance. Intracellular vitamin C inadequacy is suspected due to competition between dehydroascorbic acid and glucose at GLUT 1 and 3 cellular receptors. Erythrocyte osmotic fragility is noted in Gulo -/- knockout mice unable to synthesize endogenous vitamin C. The ascorbate deficient red blood cells presented with low cytoskeletal B-spectrin, spherocyte appearance, and impaired deformability. This cross-sectional study investigated the relationships between diabetes status, erythrocyte osmotic fragility, and serum vitamin C status.

Participants were aged 18-65, non-smoking, reported no unresolved health complications, and denied prior vitamin C supplementation. Those with T2D indicated diagnosis of >1 year. All participants provided written informed consent and the study was approved by the local Institutional Review Board in January 2021. Participants provided one fasted blood sample. Erythrocyte osmotic fragility was measured via UV/Vis spectrophotometry with various concentrations of sodium chloride (0.85% - 0.10%) to induce osmotic stress. In addition, plasma was extracted and mixed 1:1 with 10% (w/v) metaphosphoric acid in 2 mmol/L disodium EDTA and centrifuged. The supernatant was stored at -80°C until analysis with isocratic reverse-phase UV-HPLC separation.

Participant characteristics did not differ significantly between groups apart from age (p< 0.01) and HbA1c (p=0.002). Data are presented for adults with T2D (n=14; 36% female; 55.5±8.2 y; 31.5±9.0 kg/m2; HbA1c: 7.4±1.9%; plasma vitamin C: 36.0±12.2 uM) and without T2D (n=16; 69% female; 38.7±13.5 y; 26.8±6.6 kg/m2; A1c: 5.4±0.3%; plasma vitamin C: 34.8±10.9uM). Erythrocyte osmotic fragility was significantly elevated (+4.4% hemolysis) in adults without T2D at 0.35% saline (p=0.039). Greater VC status (>30 uM) was associated with lower hemolysis at 0.35% NaCl (p=0.031). Erythrocyte osmotic stability was linked to greater vitamin C intake at 0.20% saline in those without T2D (p =0.019).

In this pilot study, vitamin C status did not differ significantly by diabetes status. Vitamin C status was directly linked to erythrocyte osmotic stability in adults without T2D.

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TABLE OF CONTENTS

Page

CHAPTER

1 INTRODUCTION 1

Overview 1

Purpose of the Study 2

Research Aim and Hypothesis 2

Definitions of Terms 3

Delimitations and Limitations 4

2 REVIEW OF LITERATURE 5

Overview 5

Diabetes 5

Diabetes and Vitamin C 8

Vitamin C in the Body 11

Vitamin C Transport and Recycling in the Human Body 13

Vitamin C DRIs, Ascorbate Deficiency, and Scurvy 15

Similarities Between Scurvy and Diabetes Symptoms 17

Vitamin C and Erythrocyute Fragility 18

Testing for Erythrocyte Osmotic Fragility 20

Conclusion 21

3 METHODS 22

Participants 22

Study Design 23

Procedures 24

Independent and Dependent Variable Descriptions 25

Measurements 25

Statistical Analysis 26

Page

CHAPTER

4 DATA AND RESULTS 27

Recruitment 27

Descriptive 27

Outcomes 28

5 DISCUSSION 35

6 CONCLUSION 38

Funding Sources 39

REFERENCES 40

APPENDIX

A IRB APPROVAL 46

B SAMPLE SIZE JUSTIFICATION 48

C ELIGIBILITY SURVEY 50

D SERUM ASCORBATE ANALYSIS- BLOOD PROCESSING 52

E SERUM ASCORBATE ANALYSIS- HPLC PROTOCOL 54

F ERYTHROCYTE OSMOTIC FRAGILITY TESTING 56

CHAPTER 1

INTRODUCTION

Overview

Many symptoms of diabetes are similar to those of scurvy, a disease thought to be left in the past. Bleeding gums, poor wound healing, fatigue, and joint pain are common symptoms of both conditions. Vitamin C deficiency and mild scurvy are much more prevalent than often thought and the relationship to diabetes is of recent interest. Individuals with diabetes may be able to reduce their symptoms and health complications by supplementation with vitamin C, which could improve their overall quality of life. However, prior to a clinical trial intervention, the relationships between type 2 diabetes, blood vitamin C concentration, and erythrocyte osmotic fragility need to be further investigated.

Evidence shows that hyperglycemia, as seen in type 2 diabetes, reduces the ability of vitamin C to enter the erythrocyte due to competitive inhibition with glucose. The oxidized form of vitamin C, dehydroascorbic acid (DHA) and glucose both enter the erythrocyte via GLUT 1 & 3 receptors because of structural similarities.[1] Under conditions of chronic hyperglycemia, erythrocytes become ascorbate deficient due to poor DHA uptake and the inability to recycle vitamin C. These pathways will be discussed in chapter 2. Additional evidence shows that ascorbate deficient erythrocytes are more fragile and susceptible to lysis under conditions of osmotic stress.[2]

Previous studies have investigated the relationships between chronic hyperglycemia (type 2 diabetes) and ascorbate deficiency as well as between ascorbate deficiency and erythrocyte osmotic fragility. An animal study by Tu et al. measured erythrocyte hemolysis in Gulo-/- knockout mice. The mice in the intervention group were fed a diet supplemented with vitamin C as they are unable to synthesize ascorbic acid. After 14 weeks, blood was collected from both the intervention and control groups and erythrocytes were isolated for analysis. The results revealed that mouse erythrocytes were more osmotically fragile, susceptible to lysis, had spherocyte appearances, and had impaired deformability.[2] A study by Lino et al. quantified vitamin C levels in type 2 diabetes. It was found that the average serum vitamin C level in

subjects with type 2 diabetes was 0.49 mg/dL while the healthy controls had a plasma concentration of 0.68 mg/dL.[3] For comparison, the reference range for serum concentrations is 0.50-0.70 mg/dL.[4] These difference in vitamin C concentrations evoke the idea that human diabetic erythrocytes may be more susceptible to lysis under osmotic stress than healthy ones.

Individuals with type 2 diabetes may be experiencing symptoms of ascorbate deficiency but assume they are complications of diabetes. Completion of this research benefits the diabetes population by potentially identifying ascorbate deficiency as a root cause of symptoms. This research idea was derived from a commentary by James M. May, “Famine to Feast: Low Red Cell Vitamin C Levels in Diabetes.”[5]

Purpose of the Study

The purpose of this study is to determine how the presence of type 2 diabetes influences erythrocyte osmotic fragility and serum vitamin C concentration. There is a gap in the literature; no past studies have investigated both vitamin C concentration and erythrocyte fragility in individuals with and without type 2 diabetes.

Research Aim and Hypothesis

The aim of this research will be to further diabetes research and determine if there are relationships between type 2 diabetes, vitamin C status in the body, and erythrocyte health. Because many symptoms of diabetes reflect those of mild scurvy, completing this research will give rise to future research that may allow healthcare professionals to make evidence-based recommendations on vitamin C intake and supplementation.

The available literature allows us to make evidence-based predictions about how erythrocyte fragility and vitamin C concentrations will differ between the those with diabetes and healthy individuals. The null hypothesis states that erythrocyte fragility and blood vitamin C concentration will not be significantly different between study groups. The alternate hypothesis for this study is as follows: individuals with type 2 diabetes mellitus will have greater erythrocyte

osmotic fragility and lower blood vitamin C concentrations than healthy controls. It is predicted that the results of the trial will reject the null hypothesis and accept the alternative hypothesis.

Definitions of Terms

Antioxidant- a compound that protects against free radicals by donating an electron to an electron recipient

Ascorbic acid- another name for vitamin C (reduced/active form)

Cofactor- a non-protein compound that is responsible for catalyzing enzyme activity (e.g. vitamins)

Deformability- the ability for cells to change shape without rupturing in response to external stress

Erythrocyte- another name for red blood cells (RBCs)

Hemoglobin- protein component of erythrocytes that carry oxygen to organs and tissues and transport carbon dioxide back to the lungs

Hemolysis- rupturing (or lysis) of red blood cell membranes and the release of cytoplasmic contents

Hypotonic- solutions that have a lower solute concentration relative to intracellular levels

Osmotic fragility- a measurement that reflects the propensity of red blood cells to rupture when exposed to various concentrations of sodium chloride in solution[6]

Polymorphism- a variation at a single base pair within a DNA sequence that may result in an alternative phenotype[7]

Redox reaction- chemical reaction in which one species is **reduced** (gains electrons) and the other is **oxidized** (loses electrons)

Serum- liquid component of the blood that remains after the **formed elements** (cells and cellular components) and clotting factors have been removed

Delimitations and Limitations

Various delimitations have been set to narrow the scope of the study. For study inclusion, participants must be between the ages of 18 and 65, have not taken vitamin C supplements within 3 months of study participation, be non-smoking, not pregnant, and not be diagnosed with other active health conditions that may impact erythrocyte fragility status. Additionally, those who indicate a diagnosis of type 2 diabetes must have had the disease for at least one year. The study will take place at Arizona State University's Downtown Phoenix campus and participants should live near the Phoenix, AZ metropolitan area.

The study will have a few known limitations. One major limitation will be the current pandemic, COVID-19. Participants may be difficult to recruit due to fear of the pandemic. Another limitation will be that it is near impossible to recruit perfect participant matches for each study group. There will be differences in factors such as physical activity level, diet, age, gender, and ethnicity. It may also be challenging to determine whether hyperglycemia directly caused ascorbate deficiency or if deficiencies are independent of diabetes. However, if this study were longitudinal, it may be possible to determine. Despite these limitations, it is expected that this study will still further vitamin C, diabetes, and erythrocyte research and help bridge the literature gap that currently exists.

CHAPTER 2

REVIEW OF LITERATURE

Overview

The relevant and available literature studying the relationships between low vitamin C concentration and erythrocyte (red blood cell) osmotic fragility reveals a promising correlation between the two. The relationship between intracellular and plasma vitamin C levels and diabetes is also well supported by experimental evidence. Research on the relationship between vitamin C status and erythrocyte fragility does exist, however, almost all the studies are animal studies and/or in vitro studies. There is a literature gap between the two study topics, and though it can be hypothesized that the vitamin C status of individuals with diabetes contributes to RBC fragility, further research in humans is needed. At the conclusion of the commentary by James M. May, “Famine to Feast: Low Red Cell Vitamin C Levels in Diabetes,” he suggests a future study on whether ascorbate deficiency accounts for increased rates of deformability and lysis in diabetes.[5] This thesis will address that research question in a cross-sectional study.

Diabetes

Diabetes mellitus is the 7th leading cause of death globally.[8] In 2018, 34.2 million Americans, which accounts for 10.5% of the US population, had diabetes.[8] Every year, there are approximately 1.5 million new cases of diabetes.[8] Of the total reported cases in 2018, it was estimated that 7.3 million went undiagnosed.[8] Undiagnosed diabetes may lead to uncontrolled symptoms and overall worsening of the condition. Only 1.6 million of the reported 34.2 million cases (4.7%) were deemed type 1 diabetes, the autoimmune form.[8]

Type 1 diabetes mellitus is often considered “childhood diabetes.” 80-90% of childhood and adolescent diabetes cases are type 1, however, the prevalence of type 2 diabetes is growing in this population.[9] Type 1 diabetes is an autoimmune condition in which the pancreatic beta cells are destroyed by the immune system.[10] Beta cells are responsible for synthesizing and secreting insulin in response to glucose presence in the blood stream. Insulin is an anabolic hormone that signals various effector organs including liver, muscle, and fat cells to uptake

extracellular glucose.[11] Doing so prevents severe hyperglycemia and allows the cells to utilize the glucose for their specific functions. Due to the destruction of pancreatic beta cells, individuals with type 1 diabetes do not produce any insulin.[11] Instead, they must provide their body with supplemental, external insulin via needle injection or an insulin pump. In this literature review and study, type 1 diabetes will not be the focus and individuals with this form of diabetes will be excluded from the study. The reason for this is because those with autoimmune conditions are more likely to have other conditions that may impact vitamin C status or erythrocyte health.

Type 2 diabetes mellitus is a microvascular angiopathy characterized by chronic hyperglycemia resulting from impaired insulin secretion, insulin action, or both.[11,2] In this form of diabetes, insulin does bind to cellular receptors, however, the receptor is not activated due to insulin resistance. Due to poor insulin response, glucose transporting into cells is downregulated. GLUT 4 is known as the insulin-responsive glucose transporter isoform. The intracellular signaling pathway responsible for the translocation of GLUT 4 transport proteins is not activated and the tissues cannot take up glucose.[12] Exercise can help temporarily activate GLUT 4 and assist in glucose uptake. This is why exercise is recommended for blood sugar management in type 2 diabetes. As a result of insulin resistance, there is a gradual exhaustion of the pancreas, which leads to reduced insulin production and secretion. Insulin availability in the body decreases as the pancreas can no longer keep up with insulin demand.[11]

Metformin, along with diet and lifestyle changes, is the first line of medication treatment for type 2 diabetes. Metformin is a known antioxidant that works by reducing hepatic glucose production, reducing intestinal glucose absorption, and improving insulin sensitivity.[13] If lifestyle changes are not made and the disease progresses, supplemental insulin may be required and larger doses may be necessary due to insulin resistance. Studies have found that weight loss, specifically reductions in visceral adipose tissue, results in improved insulin sensitivity.[14] Insulin doses can often be reduced following weight loss. People with type 2 diabetes will experience uncontrolled hyperglycemia and further complications, such as diabetic ketoacidosis, if no intervention takes place.

Common symptoms of type 2 diabetes include the 3 P's: polyuria, polyphagia, and polydipsia which are frequent urination, frequent hunger, and frequent thirst, respectively.[11] Additionally, those with type 2 diabetes may experience various microvascular complications as well as endothelial dysfunction. These include complications include neuropathy, nephropathy, and retinopathy.[11] Neuropathy is defined as nerve damage that may lead to sensory loss and limb impairment.[11] Reduced sensation in the lower extremities may result in poor awareness of foot sores and injuries. Amputation of affected and wounded areas may be required if not treated promptly. Individuals with diabetes are encouraged to "check their feet" regularly to prevent this from occurring. Nephropathy, also known as diabetic kidney disease, is the result of damaged blood vessels within the kidneys.[11] Consequences of nephropathy include fatigue, anemia, poor cognition, and life-threatening electrolyte imbalances.[11] Diabetic kidney disease may progress into complete renal failure if not diagnosed early and controlled. Retinopathy results from poor vascular profusion to the retina, which is the back layer of the eye. Ocular blood vessel damage may progress and worsen into complete blindness.[11] Early diagnosis via regular dilated eye examinations as well as proper intervention will help prevent advanced conditions and preserve vision.

Diabetes is also strongly associated with the development of cardiovascular disease and increased risk of experiencing a myocardial infarction or stroke.[15,16] Increased glucose levels lead to a cascade of vascular complications: endothelial cell damage, impaired oxygen delivery and perfusion, and tissue hypoxia.[2]

Fortunately, not everyone with type 2 diabetes is dependent on supplemental insulin. Though the development of this disease is heavily dependent on genetics, the onset may be prolonged with lifestyle modifications. Following onset, the disease can be managed with a healthy diet and lifestyle and the need for insulin injections may be avoided. However, successful disease management depends on the individual's compliance with diet changes, exercise routines, smoking cessation (if applicable), and anti-diabetic medications.

Risk factors for type 2 diabetes can be divided into modifiable and non-modifiable categories. Weight status is the primary modifiable risk factor.[15] According to Mayo Clinic,

successive modifiable factors include diet, physical activity, hypertension, and abnormal cholesterol and triglyceride levels.[15] Non-modifiable risk factors, those that cannot be controlled by the individual, include family history of the disease, race or ethnicity, age, and history of gestational diabetes or polycystic ovarian syndrome.[15] Additionally, there is a single nucleotide polymorphism (SNP) in the *TCF7L2* gene that is strongly associated with the development of type 2 diabetes.[17] Evidence shows that among individuals with the *TCF7L2* gene variant, rs7903146, there is a greater prevalence of type 2 diabetes and metabolic syndrome.[17] A study by Cropano et al. revealed those carrying the *TCF7L2* rs7903146 allele had significantly decreased β-cell responsivity to glucose (Caucasians P=0.003; African Americans P=0.002, Hispanics P=0.035).[18] Although a genetic polymorphism is a non-modifiable factor, maintaining a healthy diet and lifestyle will help prevent against the development and onset of type 2 diabetes.

Diabetes and Vitamin C

As mentioned previously, diabetes is characterized by chronic hyperglycemia. It is known that the oxidized form of vitamin C, dehydroascorbic acid (DHA), enters erythrocytes via membrane GLUT transporters.[2,1,19(p1)] GLUT transporters are a family of 14 facilitative glucose transporters that allow glucose and some other molecules and metabolites to enter cells across the plasma membrane.[20] DHA is able to enter erythrocytes via GLUT 1 & 3 transporters due to structural similarities with glucose.[1] However, in cases of hyperglycemia, glucose competes with DHA for entrance into the cell. Under chronic conditions, as with diabetes, erythrocytes become ascorbate deficient which may result in increased fragility and impaired deformability.[5,2] Impaired deformability has been linked to reduced levels of beta spectrin, which is a cytoskeletal protein in the plasma membrane.[2] Under conditions of ascorbate deficiency, reduced membrane beta spectrin has been observed.[2] This is the basis for James M. May's proposition for a future study. In areas of higher oxidative stress and therefore greater DHA concentrations, DHA will have a better chance of competing with glucose at the GLUT transporter for entrance into the cell. Past research has shown a direct relationship between serum ascorbate concentrations and

erythrocyte ascorbate concentrations.[2] This indicates that if there is a high level of ascorbate in plasma, it can be assumed that intracellular erythrocyte concentrations will be higher as well.

Those with type 2 diabetes have a greater baseline requirement for vitamin C. This is primarily due to oxidative stress due to low grade inflammation. The antioxidant properties of vitamin C will be discussed in the following section. Because those with type 2 diabetes have a greater need for vitamin C because the redox uptake is greater, these individuals have lower average plasma concentrations. Normal body pool concentrations for humans are near 20 mg/kg (ppm) while normal serum concentrations are between 0.50-0.70 mg/dL.[4] A study by Lino et al. found the average level of plasma vitamin C in individuals with diabetes to be 0.49 mg/dL, which on the low end of the normal range.[3] Consistent with normal reference ranges, researchers found that the healthy controls had a vitamin C concentration of 0.68 mg/dL.[3] Another study by Wilson et al. showed that individuals with prediabetes and type 2 diabetes had a significantly greater prevalence of plasma vitamin C deficiency and inadequacy compared to those with normal glucose tolerance.[21] Below is a graph from that study depicting the differences in vitamin C plasma levels among those with normal glucose tolerance (NGT), prediabetes, and T2DM (figure 1).

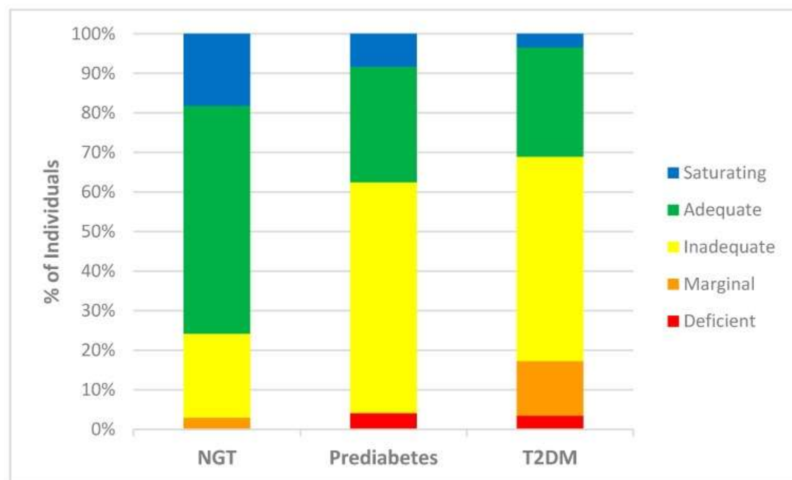

Figure 1. Plasma vitamin C status of individuals within study groups[21]

There are three proposed mechanisms for causing low plasma vitamin C levels in diabetes.[21] Microalbuminuria, a common consequence of diabetes, is when protein albumin leaks through the glomerular filtration barrier and into urine.[22] Those with this condition excrete increased levels of ascorbate along with albumin due to a leaky kidney filtration barrier caused by hyperglycemia and inflammation. The second mechanism is the competition between glucose and DHA at the GLUT transporter that was mentioned earlier in this section. The transport of DHA into the cell allows for intracellular recycling back to ascorbic acid. Third, the increased level of oxidative stress depletes vitamin C stores. These three mechanisms are well supported by previous research.

In addition to diabetes, there are other variables that may affect ascorbate levels. Obesity, pregnancy, smoking, and polymorphisms are known to cause increased vitamin C requirements. During pregnancy or if lactating, a mother can require up to 85 mg/d and 120 mg/d, respectively.[23] This is because vitamin C passes through the placenta during pregnancy and human milk during lactation. Smoking increases needs by 35 mg/day due to elevated levels of oxidative stress in the body.[23] Individuals who are affected by any of these factors will be excluded from the study to prevent skews in the data.

A polymorphism is a variation at a single base pair within a DNA sequence that may result in an alternative phenotype.[7] Such variations are common, affecting at least 1% of the population, and can be transmitted across generations. There are polymorphisms at the sodium-dependent vitamin C transporters 1 and 2 (SVCT1 and SVCT 2) that influence serum vitamin C concentrations.[24] SVCT1 is responsible for bulk transporting of vitamin C throughout the human body and SVCT2 is responsible for transport into various tissue cells.[25] The polymorphisms affecting the *SLC23A1* and *SLC23A2* genes are believed to reduce the renal threshold for ascorbic acid, thereby increasing urinary excretion, and impair vitamin C transport into cells, respectively.[24,25] An individual with either of these genetic variations will likely have lower serum vitamin C levels and may need to supplement to prevent deficiency.

Vitamin C in the Body

Vitamin C is an essential water-soluble vitamin that is readily found in many fruits and vegetables.[23] Because it is a water-soluble vitamin, the body does not store it and serum levels must be replenished daily. Excess amounts are expelled via urine and the risk of toxicity is significantly lower than with fat-soluble vitamins. Humans, bats, guinea pigs, pigs, and nonhuman primates must provide their bodies with exogenous vitamin C due to historical inactivation of the enzyme L-gulono-lactone oxidase (GLO).[19,26] Because of this change, these species have adapted to have greater concentrations of uric acid, copper, and zinc superoxide dismutases, which enhance oxidative protection.[27] Other species have the ability to biosynthesize endogenous vitamin C due to active GLO and are able to maintain a strong protection against oxygen toxicity.[17] Fortunately, humans have developed the ability to recycle vitamin C within the body via redox reactions within cells.[28] This adaptation greatly reduces the amount of vitamin C required from the diet to meet biological needs.

Vitamin C functions in the body as both a cofactor and an antioxidant.[28,29] It is important to maintain vitamin C concentrations within a specific biological range to ensure necessary bodily functions can be carried out.

Vitamin C functions as an intracellular cofactor, catalyzing many enzyme activities. One of its major cofactor roles is in the synthesis of collagen, especially during wound healing.[30] In states of vitamin C deficiency, collagenous structures are weakened and wound healing can be prolonged and unsuccessful. This can present itself as bleeding gums, poor healing wounds, and epiphyseal and metaphyseal plate lesions in bone.[26] Vitamin C is also required for carnitine synthesis.[26] Carnitine is an amino acid derivative that plays a role in energy production by transporting long chain fatty acids to the mitochondria where they are broken down.[31] When fatty acid bonds are broken, energy (ATP) is produced and becomes available for use. Under conditions of low vitamin C, an individual will likely feel fatigued due to inability to synthesize sufficient carnitine.[26] Vitamin C is also a cofactor in the synthesis of norepinephrine.[26] Norepinephrine, also known as noradrenaline, is a catecholamine hormone and neurotransmitter that elevates heart rate, blood flow, blood pressure, and encourages lipolysis and glycogenolysis

to release energy.[32] This neurotransmitter helps the body respond to stress and exercise by ensuring there is energy available to carry out necessary actions.

Vitamin C also functions as an antioxidant in the human body. It is a reversible reducing agent that plays a major role in protecting cells from oxidative stress.[29] It serves as a scavenger that partakes in redox reactions to reduce the amount of reactive oxygen species (ROS) in the body. As an antioxidant, ascorbic acid has the ability to donate an electron to an electron recipient. As a result, the ROS is reduced while ascorbic acid is oxidized to dehydroascorbic acid (DHA).[26,29] Redox reactions and the specifics of the ascorbic acid recycling pathways will be discussed further in a later section. Oxidative stress is known to have many negative effects on human health. Some of the detrimental effects include DNA damage, which may give rise to cancer, increased risk of cardiovascular disease and atherosclerosis, links to neurological diseases (Alzheimer's disease and Parkinson's disease), and increased inflammation that may contribute to lung diseases, rheumatoid arthritis, and renal failure.[33] In relation to heart health, ascorbic acid protects against low-density lipoprotein (LDL) cholesterol oxidation.[16] Oxidized LDL can progress to plaque, which can build up and develop into atherosclerosis. LDL is known as the "bad" cholesterol, whereas high-density lipoprotein (HDL) is considered "good." Protection from LDL oxidation reduces an individual's risk for myocardial infarction and stroke, decreases arterial stiffness, as well as improves blood lipid profiles and endothelial health. Additionally, vitamin C promotes enzyme activity by maintaining metal ions in their reduced forms as well as aids in non-heme iron absorption.[26]

Inadequate vitamin C status has been found to correlate with the prevalence of cancer, heart disease, and lung disease. A dose response meta-analysis by Luo et al. found that an increase of 100 mg/day of vitamin C was significantly associated with a 7% reduction in lung cancer risk.[34] This is due to the antioxidant capabilities of vitamin C. As an antioxidant, ascorbic acid can reduce oxidative species that contribute to cancer development and progression. As just mentioned, in cardiovascular disease (CVD), vitamin C's antioxidant potential prevents oxidative changes to low-density lipoproteins (LDL).[16,35] Further, vitamin C has been found to improve endothelial nitric oxide production.[36] Nitric oxide is a potent vasodilator that promotes

improved blood perfusion and reduces blood pressure. A study by Park et al. investigated the relationship between vitamin C intake and chronic obstructive pulmonary disease (COPD) prevalence in Korean patients. It was found that in those who were heavy smokers, COPD risk was significantly reduced in the highest vitamin C quartile (Q3) compared to the lowest quartile (Q1).[37] An article by Hemila et al. discusses the protective effects of vitamin C against lung infections such as pneumonia.[38] The article explains how vitamin C promotes the functions of phagocytes and lymphocytes in immunity and protects against infection.[38] Vitamin C has many crucial roles in maintaining human health, therefore, deficiency and inadequacy should be screened for and addressed promptly.

Vitamin C Transport and Recycling in the Human Body

Cell membrane transporters SVCT1 and SVCT2 are responsible for transporting vitamin C throughout the body. However, erythrocytes do not contain these transporters. In order for vitamin C to enter RBCs, the oxidized form, DHA, must instead be transported by a GLUT (1 or 3) receptor. DHA is the oxidized product of a redox reaction involving ascorbic acid and an oxidized species. Once DHA is inside the erythrocyte, it is reduced back to ascorbic acid. This is referred to as the bystander effect; the surrounding erythrocytes take in DHA and immediately convert it back to ascorbic acid, "trapping it" inside the cell.[39] This conversion of oxidized vitamin C back to available ascorbic acid is considered as vitamin C recycling. Experimental evidence suggests that vitamin C recycling occurs approximately every 3 minutes.[40,41] It is estimated that actual plasma DHA levels are very low, between 0-2 μM and approximately only 1-2% of total ascorbate levels.[34] Nualart et al. performed an analysis of this process. In addition to this quantification, glucose was observed to partially block DHA uptake by the bystander cells (not specifically focused on erythrocytes).[39] This observation is consistent with the proposed mechanism mentioned earlier. Other analyses have found DHA levels to be several times greater in the plasma of those with diabetes than in those without diabetes.[42] This information suggests that in the case of diabetes, high glucose levels will hinder DHA uptake more severely, causing greater erythrocyte ascorbate deficiency and thus worsen erythrocyte fragility.

There are various enzyme systems that permit vitamin C recycling to occur. The reduction of vitamin C can occur in different tissues such as the liver, kidney, brain, white blood cells, and erythrocytes. These different tissues house the bystander cells that participate in redox reactions with ascorbate free radicals (AFRs). These reactions each require different vitamin or mineral cofactors and electron carriers to occur.

There are four ways that ascorbate can be recycled in the human body: thioredoxin reductase, mitochondrial or microsomal AFR reductases, glutathione oxidation, and/or 3a-hydroxysteroid dehydrogensase.[43] Thioredoxin reductase is a selenium dependent enzyme. The AFR is reduced to ascorbate with the help of NADPH and a selenium cofactor.[43] Mitochondrial or microsomal AFR reductases convert AFR back to ascorbate via an NADH and riboflavin (vitamin B2) dependent reaction.[43] The third enzyme system is glutathione oxidation, which can occur three different ways: direct, via glutaredoxin, or protein disulfide isomerase (PDI). This system allows for a two-electron reduction back to ascorbate.[43] This reduction is dependent on glutathione (GSH) or PDI to occur. Lastly, DHA can be recycled back to ascorbate via thioredoxin reductase or by 3a-hydroxysteroid dehydrogenase. This system is niacin (vitamin B3) and NADPH dependent.[43] Below is a diagram of the different enzyme systems that facilitate vitamin C recycling in various human tissues (figure 2).

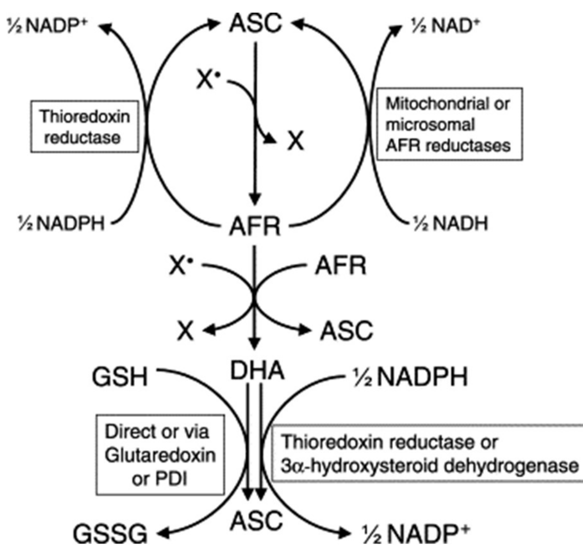

Figure 2. Schematic of intracellular ascorbate recycling.[43]

Vitamin C DRIs, Ascorbate Deficiency, and Scurvy

The current daily recommended intakes (DRIs) for vitamin C are 90 mg/d for men and 75 mg/d for women.[23] The current DRIs are believed to only be sufficient to support the role of ascorbic acid as cofactor. In order for the full antioxidant capabilities to be carried out, it is hypothesized that intakes greater than the DRIs are needed. Due to being unable to synthesize endogenous vitamin C, humans must consume 90% of their daily intakes from fruits and vegetables.[44] Supplementation of the vitamin is an additional way to ensure adequate exogenous intake. Research suggests that 75 mg/d is sufficient to prevent scurvy[45], 90 mg/d will support antioxidant functions[46], and 100 mg/d will saturate the body pool.[47] Other sources recommend supplementing with 200-500 mg/d to completely saturate the serum with vitamin C.[26] This would fully promote antioxidant function and allow total protection against scurvy. The tolerable upper limit (TUL) for vitamin C is 2000 mg/d and it is unlikely for someone to consume this amount unless “megadosing” via supplementation.[23] Further, excess water soluble vitamins are excreted in urine rather than stored in the body. However, if someone were to encounter toxicity, they would likely experience gastrointestinal upset characterized by diarrhea, nausea, vomiting, heartburn, and abdominal cramps, as well as headache and/or insomnia.[23,26] These symptoms will usually subside after one to two weeks of discontinuation.

Without supplementation, the richest sources of vitamin C are fresh fruits and vegetables. Excellent sources of vitamin C are those that provide >90mg/100g. In this category are guava (228 mg/100g), yellow bell pepper (184 mg/100g), kiwifruit (93 mg/100g), kale (93 mg/100g), and broccoli (89 mg/100g).[26] Good sources of vitamin C are those that provide 50-60mg/100g and include papaya, strawberries, oranges and fresh orange juice, and cauliflower.[26] Unfortunately, the most commonly consumed fruits and vegetables are apples, bananas, potatoes, grapes, and tomatoes which are considered poor sources that only provide 5-16 mg/100g.[26]

Additionally, one must consider the availability of the vitamin at the time of consumption. Processed and non-fresh versions of foods contain less available vitamin C than their fresh counterparts. Light, air, heat, and other food processing methods can degrade the vitamin.[26] An experiment by Nursal et al. quantified the amount of available vitamin C in spinach prior to and

after cooking. The results revealed that cooking resulted in vitamin C losses up to 60%.[48] Another analysis by Johnston, et al. investigated the differences in oxidation rates of vitamin C in frozen/reconstituted as well as ready-to-drink orange juices. The decomposition rates of both juices were similar at 2% per day after opening.[49] It was also found that frozen/reconstituted orange juices contained 86 mg/cup and the vitamin C content decreased by approximately 50% after 4 weeks of storage.[49] Ready-to-drink orange juices only contained 27-65mg initially, and after 4 weeks of storage, vitamin levels ranged between 0-25 mg/cup.[49] The study revealed that ready-to-drink orange juices contain significantly lower vitamin C than expected. Researchers suggest that such juices should be purchased at least 3-4 weeks prior to the marked expiration date and consumed within one week of opening to receive adequate vitamin amounts.

Vitamin C deficiency is among the leading deficiencies in the United States. The CDC's Second National Report on Biochemical Indicators of Diet and Nutrition estimated that 6% of Americans are deficient.[50] In severe and chronic cases, deficiency may progress to scurvy. Though scurvy is much less common in modern times, milder cases are believed to be more prevalent than expected. Leading up to the end of the 18th century, undernourished sailors were greatly affected by scurvy and it caused millions of deaths. Sailors were more likely to die to scurvy than to storms, shipwreck, combat, and other diseases combined.[51] It was estimated that up to 50% of sailors would die from scurvy on a single voyage.[52] The high prevalence of scurvy was a consequence of poor access to vitamin C rich foods, such as fresh fruits and vegetables. Symptoms of scurvy include bleeding gums, fatigue, scaly skin, dry hair/skin/nails, compromised dental health, weakened immunity, and joint pain.[26,44,53] As for the cure, a case study of a patient with scurvy determined that a dose of 300 mg/d of vitamin C for 1 month cures a majority of the complications associated with the deficiency. Complete recovery from the condition takes approximately 3 months and vitamin C intake must be sufficient throughout that period and after.[54]

The reason that complete recovery may take 3 months is likely because erythrocytes have a lifespan of up to 120 days.[55] A study by Huang et al. measured average red blood cell lifespan in participants with diabetes and healthy controls. It was found that the average

erythrocyte lifespan was significantly shorter in the diabetic group than the control group (86.08 ± 18.13 days versus 103.6 ± 22.02 days, $p = 0.00$).[56] Although diabetic erythrocytes have a shorter lifespan by approximately 17.5 days, an intervention duration of 120 days should be used to ensure complete erythrocyte turnover and recovery. It is important to ensure complete turnover because RBCs do not contain a nucleus and cannot synthesize proteins in response to oxidative stress.[55] Erythrocytes depend on antioxidants, such as vitamin C, to counter such stress. This information may be useful for treating scurvy patients who also have diabetes

Similarities Between Scurvy and Diabetes Symptoms

As mentioned previously, risk of ascorbate deficiency is significantly elevated with diabetes. Interestingly, many symptoms of diabetes are similar to those of vitamin C deficiency. This makes it difficult to distinguish the cause of the symptoms one may be experiencing. For that reason, vitamin C supplementation is recommended to reduce symptoms and/or rule out deficiency as the cause.

Vitamin C plays a key role in collagen synthesis. Collagen is an essential structural component of blood vessels. When vitamin C availability is lacking, collagen production is inadequate and vessels become weak and leaky which can result in abnormal bleeding.[57] Diabetes may result in damaged blood vessels and therefore poor vascular perfusion and impaired oxygen delivery. Individuals with diabetes may bruise easily and experience long lasting bruises due to weak and leaky blood vessels. Similarly, a common clinical manifestation of scurvy is presence of hematomas, or internal bleeding, on upper thighs and legs.[57]

Spongy and bleeding gums are another symptom of scurvy caused by poor collagen synthesis. Individuals with diabetes are at a greater risk for gum disease and often experience bleeding gums, a result of weak blood vessels. Greater oral and dental problems may arise and worsen if proper care and intervention are not initiated promptly.

Healing of open wounds may be prolonged in patients with scurvy and/or diabetes.[58,59] Collagen synthesis is necessary for proper wound healing. Those with diabetes may be experiencing neuropathy and because of this, foot injuries may go unnoticed for a long time.

These poor healing wounds may become infected and amputation may be required to prevent sepsis. In addition, poor immune function is a complication of both scurvy and diabetes, making infection likeliness greater and more severe.

Fatigue is another common symptom of scurvy and diabetes.[57,60] Vitamin C is needed for the synthesis of carnitine, a necessary component in energy production. In scurvy, fatigue is a direct result of poor vitamin C status. However, in diabetes, fatigue may be a result of ascorbate deficiency or due to poor glucose uptake and cellular utilization. Prolonged fatigue may hinder an individual's self-management of other symptoms and lead to worsening of the condition.

Vitamin C and Erythrocyte Fragility

Erythrocyte osmotic fragility is an evaluation of the stability of the erythrocyte membrane when exposed to osmotic stress.[61,6] It is a measurement of red blood cell (RBC) hemolysis when exposed to various concentrations of sodium chloride.[6] Measurements are often taken as mean osmotic fragility, which is defined as the NaCl concentration that causes 50% of cells to hemolyze.[62] Percent hemolysis is calculated using values collected via UV/Vis spectrophotometry. Methods for measuring hemolysis will require whole blood exposure to hypotonic solutions. Solutions that are more hypotonic have a lower solute concentration relative to intracellular levels. Such solutions will increase erythrocyte hemolysis due to osmosis of water into the cell to establish solute equilibrium.

Prior research has provided substantial evidence that diabetes mellitus diminishes erythrocyte deformability, which is the ability for the cell to change shape without rupturing in response to stress. Erythrocyte deformability plays a key role in microvascular blood flow as well as capillary perfusion.[5,63] In a diabetic environment, erythrocytes have been observed to have greater rates of fragility and cell death.[5,2,64] Studies by Prakash et al. and Rownak et al. show increased and early RBC hemolysis in diabetic groups compared to controls.[65,66] As mentioned in a prior section, diabetes is a microvascular disease. Tissue hypoxia is a direct result of compromised erythrocyte function due to increased fragility and impaired deformability.[2] Another

result of abnormal deformability is anemia, which also causes hypoxia via decreased oxygen delivery to tissues.[5]

An animal study by Tu et al. measured erythrocyte hemolysis in ascorbate deficient, Gulo-/- knockout mice.[2] These mice require dietary ascorbic acid to survive as they are unable to synthesize their own. Researchers found that erythrocyte ascorbate levels have an inverse correlation with deformability and a positive association with fragility. Membranes of ascorbate deficient RBCs were osmotically sensitive, had spherocyte-like appearances, and contained lesser amounts of B-spectrin.[2] A spherocyte is an erythrocyte with a shape that resembles a sphere rather than a biconcave disk and has a decreased surface area to volume ratio. B-spectrin is a protein found in the erythrocyte membrane that is needed to maintain normal cytoskeletal structure and function.[5] With ascorbate replenishment, the mouse erythrocytes were less osmotically fragile, restored normal shape, and B-spectrin levels were restored. Approximately 30 years ago, an analysis of erythrocyte membranes of humans with type 1 and 2 diabetes showed lower levels of oxidized B-spectrin. These findings were consistent with decreased deformability. In his review, James May concludes that the reduced levels of B-spectrin is a cause for the morphed shape and increased membrane sensitivity.[5]

An analysis of RBCs from CPD-preserved human blood was done by Czubak et al. The focus of the study was to determine if sodium ascorbate and Trolox, a water-soluble analog of vitamin E, prevented storage-induced hemolysis and altered membrane permeability. The donor blood samples were supplemented with varying concentrations of sodium ascorbate/Trolox and stored in the refrigerator for 20 days. The results showed that supplementation of the RBC storage solution with a combination of sodium ascorbate (25 μM) and Trolox (125 μM) significantly inhibited RBC hemolysis.[67] Although this was an in vitro study, the results suggest that vitamin C supplementation may prevent erythrocyte hemolysis.

Another animal study by Kraus et al. looked at the effects of vitamin C, vitamin E, and beta carotene supplementation on rat erythrocyte fragility. The rats used for the study were zinc deficient. Zinc is an metal antioxidant known to play a role in maintenance of biological membranes.[68] It was found that there was a significant improvement in fragility with vitamin C

supplementation.[69] This study is similar to this thesis because individuals with diabetes are known to be deficient in vitamin C, an antioxidant. The positive response of zinc deficient rat erythrocytes to vitamin C supplementation increases the confidence that humans with diabetes will experience improvements in erythrocyte fragility.

An intervention study by Duranti et al. investigated the effects that chronic supplementation of the polyphenolic flavonoid, quercetin, has on oxidative damage after exercise. In past research, quercetin has shown to be a powerful antioxidant in both in vitro and in murine models.[70] Blood samples were collected immediately following exercise at baseline and after 2 weeks of intervention. The results revealed a significantly higher time to 50% erythrocyte hemolysis in the supplemented group compared to the control group.[70] This study strengthens the belief that vitamin C (antioxidant) supplementation will improve RBC fragility in humans with diabetes.

Another clinical trial by Sultana et al. focused on the effects of oral vitamin E supplementation on RBC fragility of hemolytic anemic patients with G6PD deficiency and healthy controls. The study included both adults and children. Half of the participants who had hemolytic anemia received supplementation while the other half received a placebo. Mean baseline fragility was significantly higher in patients suffering from hemolytic anemia than the healthy controls.[71] After vitamin E supplementation of 800 IU/day (adults) or 400 IU/day (children) for 60 consecutive days, blood samples were taken again. The results revealed that RBC fragility was significantly improved in the supplemented hemolytic anemia group compared to the non-supplemented hemolytic anemia group.[71] Vitamin E and C have very similar roles in maintaining membrane stability and integrity. Because of that, the results of this trial strengthens the proposal of this thesis.

Testing for Erythrocyte Osmotic Fragility

In this study, one blood sample will be collected. Erythrocyte osmotic fragility will be measured based on lysis under varying NaCl concentrations. It is known that erythrocyte lysis is greater under hypotonic conditions, causing the erythrocytes to swell due to greater solute

concentrations within the cell. In 1947, Parpart et al. developed the original methods to quantify RBC osmotic fragility.[72] The methods for this study will be based on modified, successful, and repeatable methods from the available literature. Fragility will be measured at the NaCl concentration associated with the 50% erythrocyte hemolysis point. Hemolysis will be determined using a UV/Vis spectrophotometer. The intensity of light transmitted through a hypotonic media containing erythrocytes will be measured at $\lambda = 540$ nm as this is the wavelength at which hemoglobin, which is released into the media upon hemolysis, absorbs light.[62]

Conclusion

After a comprehensive review of the available literature addressing this topic, it can be concluded that further research is needed in humans with type 2 diabetes. There is a substantial amount of research completed on the relationships between diabetes and ascorbate levels, ascorbate levels and RBC fragility, and supplementation of various antioxidant compounds on RBC fragility. In studies that closely resemble this thesis, animal or in vitro models were utilized. This thesis will fill the gap in the literature and provide additional information on the relationships that exist between serum vitamin C levels, erythrocyte osmotic fragility, and presence of type 2 diabetes mellitus in humans

CHAPTER 3

METHODS

Participants

The inclusion criteria for this study were males and females diagnosed with type 2 diabetes for at least one year and healthy individuals without diabetes who are aged 18-65. Participants with diabetes were asked to report their most recent HgbA1c values. Recruitment was focused in the Phoenix Metropolitan Area as participants needed to be willing to commute to the test site. The study was based out of Wexford Innovation Center located within Arizona State University's Downtown Phoenix campus.

Exclusion criteria included smoking, pregnancy, vegetarianism, age <18 or >65, and active or unresolved health complications such as cardiovascular disease (CVD), chronic kidney disease (CKD), cancer, respiratory diseases, and/or recent injury or surgery. Individuals with type 1 diabetes were not eligible for participation due to increased risk of additional autoimmune disorders. These factors all increase one's baseline requirement for vitamin C and may influence results. Research volunteers were also excluded if they had been regularly taking vitamin C supplements greater than ~60 mg/d, which is a standard dose in a multivitamin, within 3 months prior to the study. Once it was determined that a research volunteer was qualified for the study, they were emailed for scheduling. Informed consent was sent to each participant prior to the study appointment. This study was approved by the Arizona State University institutional review board (IRB) in January 2021 and the approval can be found in Appendix A.

The sample size for this study was justified by reviewing the available literature and utilizing the Harvard.edu sample size calculator for a parallel trial (Appendix B). The sample size calculation for significant results determined an ideal sample size of N=40 with n=20 per group. There are not many studies that are similar and comparable to this thesis, and because of that, the sample size used was an estimate. Unfortunately, due to limited time and COVID-19, only N=30, $n_{healthy}$=16, $n_{diabetes}$=14 were recruited.

Study Design

This study utilizes a cross-sectional design. Dr. Carol Johnston and MS student Ciara Lundy maintained the master list containing participant names with respective coded identifiers. Participants were recruited via listservs, flyers in doctor's offices and around ASU campuses, advertisements on MyASU, social media, and word of mouth. Survey responses were screened manually, and if eligible, individuals were reached out to for appointment scheduling. COVID-19 precautions were taken; temperature checks and COVID questionnaires were completed upon arrival to the study site and participants were asked to reschedule if they felt sick. During the appointment, participants were asked to sign an informed consent, participate in height and weight assessment, complete a food frequency questionnaire (FFQ) and health history/physical activity recall, and provide a fasted blood sample. Participants were informed of the expectations and responsibilities for study participation within the initial invitation. Participation in this study was voluntary and individuals were able to withdraw at any time. The study flow chart is included below (figure 3).

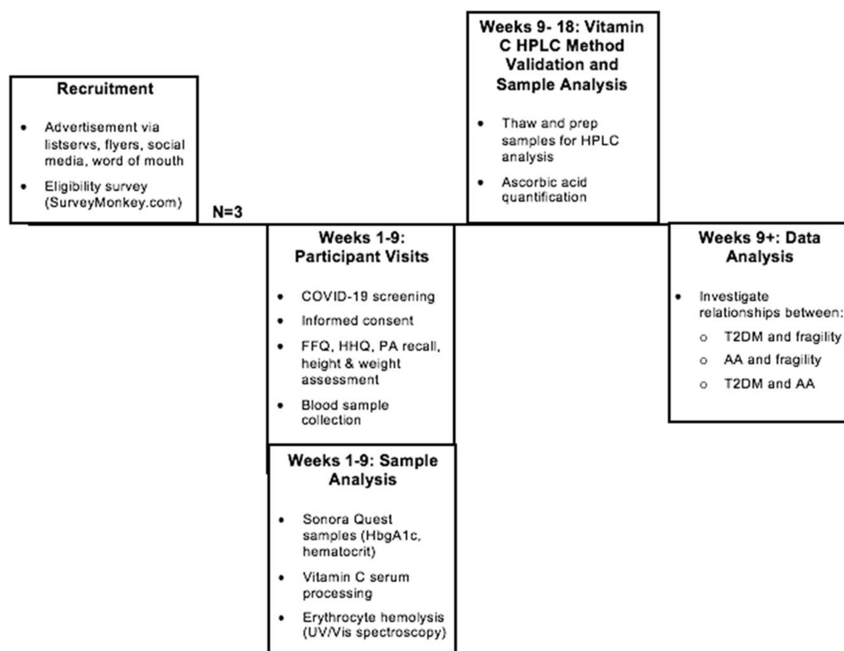

Figure 3. Study Flow Chart

Procedures

Fasted blood samples were collected at the end of study visits allowing for immediate processing and analyzing of samples. Whole blood was collected into 2 purple top vacutainers containing K2EDTA preservative; one for hematocrit and HgbA1c testing at Sonora Quest and the other for on-site analysis. Within an hour of the blood draw, erythrocyte fragility analysis was carried out and vitamin C samples were prepared for future analysis.

Preparation of serum vitamin C samples started with centrifuging the whole blood at 4 degrees C for 8 minutes at 2800 g. The serum supernatant was then removed and mixed with an equal volume of 10% (w/v) metaphosphoric acid (MPA) in 2 mmol/L disodium EDTA. This mixture was then vortexed and stored on ice for 15 minutes prior to centrifuging at 4 degrees C for 10 minutes at 4700 g. The supernatant was aliquoted into micro-centrifuge tubes for storage at -80 degrees C until reverse-phase HPLC-UV analysis could be performed. This protocol can be found in Appendix D.

Stored samples were thawed and 50 uL aliquots were transferred to 1.5 mL micro-centrifuge tubes. Equal volumes of 5 mmol/L Tris (2-carboxyethyl) phosphine (TCEP) and HPLC grade water were added to sample tubes and kept in the dark for 20 minutes at room temperature to react. TCEP was used as a reducing agent so that total vitamin C content could be measured. Samples were then centrifuged at 16,000 g for 5 minutes. Centrifuge tubes were kept on ice and the supernatant was transferred into HPLC vials for analysis. Samples were analyzed immediately or kept in the refrigerated auto-sampler for up to 4 hrs. Analysis was carried out using a Waters Alliance e2695 HPLC equipped with a photodiode array detector and an Agilent Zorbax Eclipse XDB-C18 column and guard. The column was held at 25 degrees C and sample volume was set to 20 uL. Separation was achieved using an isocratic 1.8 mmol/L sulfuric acid mobile phase with a flow rate of 0.8 mL/min. This protocol was adapted from Robitaille and Hopper et al. and can be found in Appendix E.

Erythrocyte hemolysis was measured via a Thermo Fisher Genesys UV/VIS spectrophotometer. Thirteen aqueous NaCl solutions ranging from 0.10%-0.85% (w/v) were prepared using ultrapure water. For each participant, 20 uL aliquots of whole blood were added

to 2 mL of each of the 13 NaCl solutions in centrifuge tubes. These tubes were gently inverted several times to mix without causing mechanical hemolysis. They were then incubated at room temperature (20 degrees C) for 30 minutes to allow for osmotic stress-induced hemolysis to occur. Samples were centrifuged at 4 degrees C for 10 minutes at 2000 g. The supernatant of each tube was then transferred to plastic micro-cuvettes and absorbance was measured at 540 nm. A 0.9% NaCl solution was used as the blank. The absorbance measurements were recorded for data analysis. This protocol was adapted from the methods of Parpart et al. and can be found in Appendix F.

Independent and Dependent Variable Descriptions

The independent variable for this study was the presence of type 2 diabetes. The dependent variables were erythrocyte osmotic fragility, which is expressed as a percentage of lysed cells in various concentrations of NaCl solution, and blood serum vitamin C concentration. The expected relationship between the variables was that erythrocyte fragility would be greater and vitamin C concentration would be lower in the type 2 diabetes group than in the control group. If these relationships are true, then it can be inferred that erythrocyte osmotic fragility has an inverse relationship with serum vitamin C concentration.

Measurements

Prospective research volunteers completed an eligibility survey though SurveyMonkey.com. Survey questions can be found in Appendix C. Heights were measured using a stadiometer and body masses were measured with a bio-electrical impedance analysis scale (Tanita). To quantify lysed cells (% hemolysis), absorbance was measured via UV-Vis spectrophotometry and percentage of lysed cells was calculated. The formula used to calculate % hemolysis is below:

Hemolysis (%) = {Abs [Supernatant of each buffer] - Abs [Supernatant of 0.85% buffer]} x 100/Abs [Supernatant of 0.1% buffer] - Abs [Supernatant of 0.85% buffer]

To quantify serum vitamin C content, blood serum was processed and measured via HPLC-UV. Serum concentrations were calculated from AUC (area under the curve) to mg/dL.

Statistical Analysis

Statistical analysis was performed using IBM SPSS (version 26 for Macintosh; SPSS, Chicago, IL) and Microsoft Excel. Data is reported as mean ± standard deviation for descriptive statistics, hemolysis, and blood vitamin C concentrations for each study group. Data was tested for normality and found to be not normally distributed. Because of this, Mann-Whitney U tests were performed to determine differences amongst the groups. The significance level was set at $\alpha=0.05$ (95% confidence).

CHAPTER 4

DATA AND RESULTS

The study hypothesis, stating that erythrocyte osmotic fragility would be greater and serum ascorbic acid would be lower in individuals with type 2 diabetes compared to controls, was rejected. However, when comparing all the variables, other relationships were discovered that will contribute to understanding the role of vitamin C in cellular integrity.

Recruitment

Recruitment for this study utilized an eligibility survey through SurveyMonkey.com. The link to the survey was shared through social media (LinkedIn, Nextdoor, Instagram, Reddit, and Facebook), flyers on local mailboxes and around the ASU downtown Phoenix campus, a MyASU staff portal banner, and word of mouth. The recruitment phase was continuous and lasted from one week before study visits started through the end of the data collection phase (July 2021 to September 2021).

Descriptive

Of the 56 total survey respondents, 22 indicated that they had type 2 diabetes for over a year. More women (n=36) than men (n=20) responded to the survey. The average age of respondents was 42.6 years, with a median age of 44 years. Six respondents did not meet inclusion criteria, primarily due to reported vitamin C supplementation leaving 50 respondents eligible for the study. One qualifying respondent decided not to participate due to the location of the study site and another declined participation due to COVID-19 concerns. Eighteen eligible respondents did not reply to the study invitation email. A final count of 30 qualifying respondents agreed to move forward with the study. Scheduled participants were sent a copy of the informed consent, which was signed prior to data collection, prior to their appointed study visit. No participants withdrew from the study after being scheduled. A consort diagram summarizing this process is included below (figure 4).

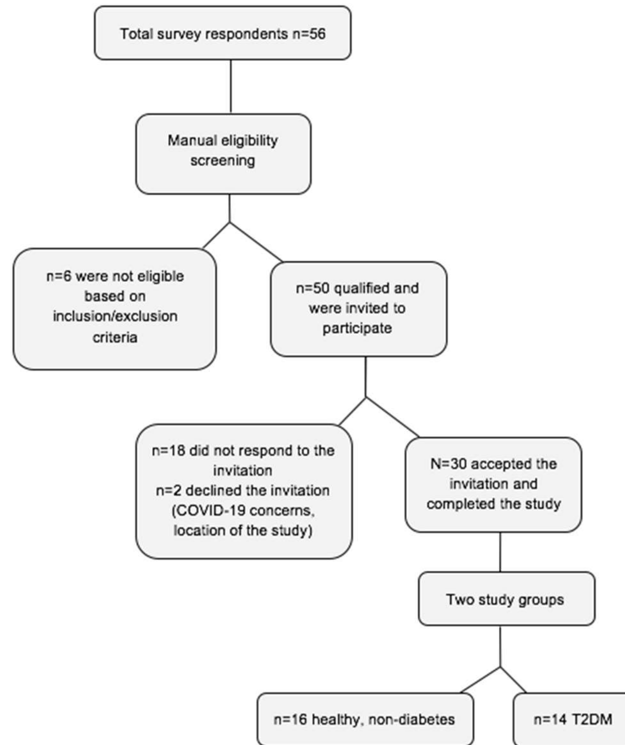

Figure 4**.** Consort Diagram

Of the 30 participants that completed the study, 73% (n=22) identified as Caucasian, 10% (n=3) identified as Asian, 10% (n=3) identified as Hispanic, 3% (n=1) identified as Pacific Islander, and 3% (n=1) identified as African American. 46.6% of the participants were male, and the average age of study group was 46.5 years (SD=14 years), with a median age of 51.5 years (range: 21 to 64 years). Participant characteristics did not differ significantly between groups apart from age ($p< 0.01$) and HbA1c ($p=0.002$).

Outcomes

The original hypothesis expected erythrocyte osmotic fragility to be greater and serum ascorbic acid levels to be lower in individuals with type-2 diabetes. When dividing the population based on the presence or absence of type 2 diabetes, a relationship contrary to the hypothesis was found. Significance was determined at the 95% confidence level with $p<0.05$. Mann-

Whitney U test was used due the data not being normally distributed and the sample size was small (n<30). Hemolysis at 0.35% NaCl was 4.4% lower for the type 2 diabetes group (*p*=0.039).

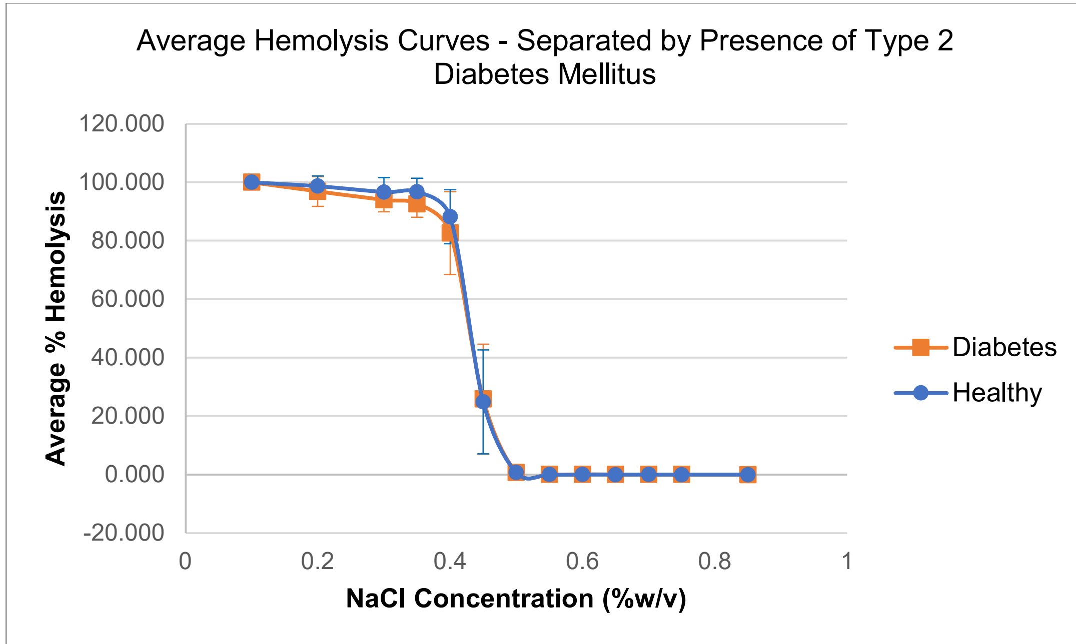

Figure 5. Overlay graph of average hemolysis curves- diabetes vs healthy.

Table 1. Table containing hemolysis means and standard deviations for diabetes vs healthy. P-values were calculated using Mann-Whitney U test and significance was determined at *p*<0.05.

[NaCl] (%w/v)	Diabetic		Healthy		*p*-value*
	Mean % Hemolysis	SD	Mean % Hemolysis	SD	
0.85	0.000	0.000	0.000	0.000	N/A
0.75	0.035	0.206	-0.002	0.171	0.604
0.7	0.068	0.196	0.061	0.243	0.952
0.65	0.076	0.313	0.026	0.161	0.984
0.6	0.089	0.214	0.066	0.254	0.631
0.55	0.028	0.204	0.058	0.349	0.952
0.5	0.766	1.068	0.690	1.118	0.697
0.45	25.875	18.815	24.871	17.763	0.952
0.4	82.663	14.160	88.201	9.261	0.327
0.35	**92.628**	**4.580**	**96.732**	**4.621**	**0.039**
0.3	93.971	4.023	96.698	4.922	0.093
0.2	96.878	5.065	98.714	3.419	0.238
0.1	100.000	0.000	100.000	0.000	N/A

*Significant p-value at P<0.05 (2-tailed).

As shown in table 2, there was a significant difference in HbA1c between the groups (*p*=0.0004). There were also significant differences in hemolysis at 0.35% NaCl (p=0.038) and complete hemolysis (*p*=0.033).

Table 2. Table showing means and standard deviations for HbA1c, hematocrit, and hemolysis. *P*-values were calculated using Mann-Whitney U test and significance was determined at *p*<0.05. Hemolysis data are percentages expressed as decimals.

Test items	Reference values	Mean ± SD		*p*- value*
		Diabetes *n* = 14	Non-diabetes *n* = 16	
HbA1c (%)	3.0-5.9	7.379 ± 1.874	5.413 ± 0.326	**<0.001**
Hematocrit (%)	M: 38.3-48.6 W: 35.5-44.9	45.750 ± 5.667	44.400 ± 3.237	0.724
Hemolysis 0.35 NaCl		92.628 ± 4.580	96.732 ± 4.621	**0.038**
Initiate Hemolysis		0.450 ± 0.000	0.450 ± 0.000	1.000
Hemolysis 50 %		0.427 ± 0.012	0.428 ± 0.008	0.789
Complete Hemolysis		0.200 ± 0.127	0.303 ± 0.107	**0.033**
Plasma Vitamin C (uM)	Deficiency: <11 Marginal: 11-28 EAR Average: 38**	36.002 ± 12.246	34.824 ± 10.949	0.841

*Significant p-value at *P*<0.05 (2-tailed).
**If one is consuming the estimated average requirement (75 mg/d) plasma VC averages 38 uM.

Further, a significant difference in erythrocyte osmotic fragility at 0.35% NaCl was found via Mann Whitney when dividing the population at the median serum ascorbic acid concentration, 33 uM. Participants with lower vitamin C concentrations (n=16) displayed greater erythrocyte osmotic fragility in comparison to the participants with higher vitamin C status (n=14) (*p*=0.031). Note that diabetes was nearly equally represented in each group. Those with better vitamin C status (>33 uM) had 4% lower hemolysis at 0.35% NaCl. However, the results from this study do not show vitamin C status to differ significantly by diabetes status.

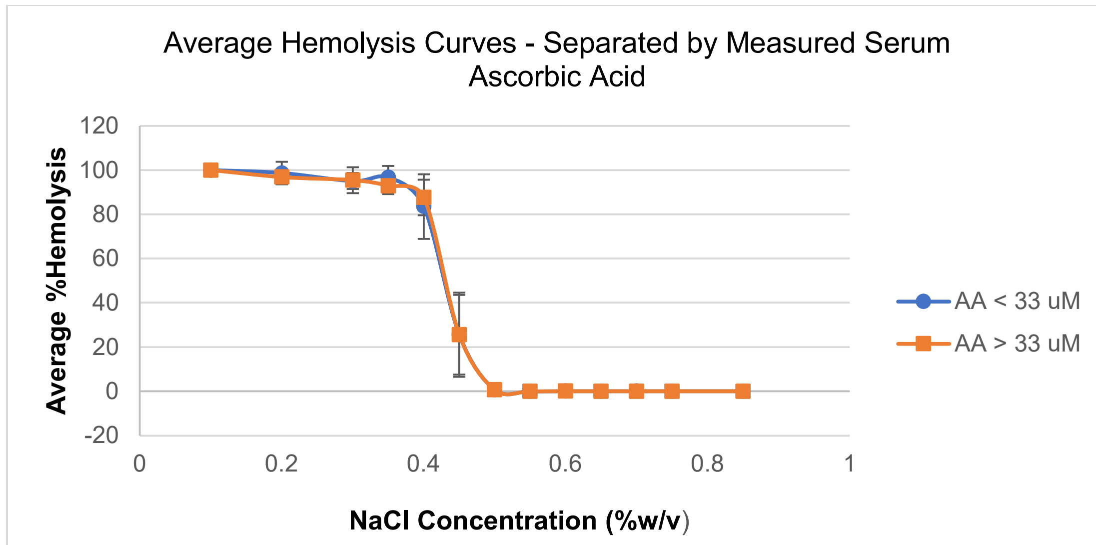

Figure 6. Overlay graph of average hemolysis curves- serum AA > 33 uM vs serum AA < 33 uM.

Table 3. Table containing hemolysis means and standard deviations for serum AA > 33 uM and serum AA < 33 uM. *P*-values were calculated using Mann-Whitney U test and significance was determined at $p<0.05$

	Serum AA <33 uM		Serum AA >33 uM		
[NaCl] (%w/v)	**Mean % Hemolysis**	**SD**	**Mean % Hemolysis**	**SD**	***p*-value***
0.85	0.000	0.000	0.000	0.000	N/A
0.75	0.006	0.195	0.021	0.188	0.711
0.7	0.073	0.276	0.056	0.143	0.741
0.65	0.038	0.306	0.051	0.143	0.285
0.6	0.076	0.299	0.083	0.136	0.555
0.55	0.047	0.370	0.038	0.163	0.555
0.5	0.752	1.343	0.703	0.738	0.582
0.45	25.601	18.991	25.600	17.966	0.984
0.4	83.602	14.616	87.708	8.014	0.646
0.35	**96.772**	**5.153**	**92.581**	**3.815**	**0.031**
0.3	95.060	3.622	95.488	5.810	0.881
0.2	98.705	5.070	96.917	3.210	0.303
0.1	100.000	0.000	100.000	0.000	N/A

*Significant p-value at $P<0.05$ (2-tailed).

When separating the healthy group into subgroups based on daily dietary vitamin C intake, a significant difference in % hemolysis was found at 0.20% (p=0.0198). This difference

can be seen in Figure 7. Significance was not found when doing the same for the diabetes group or the entire study group.

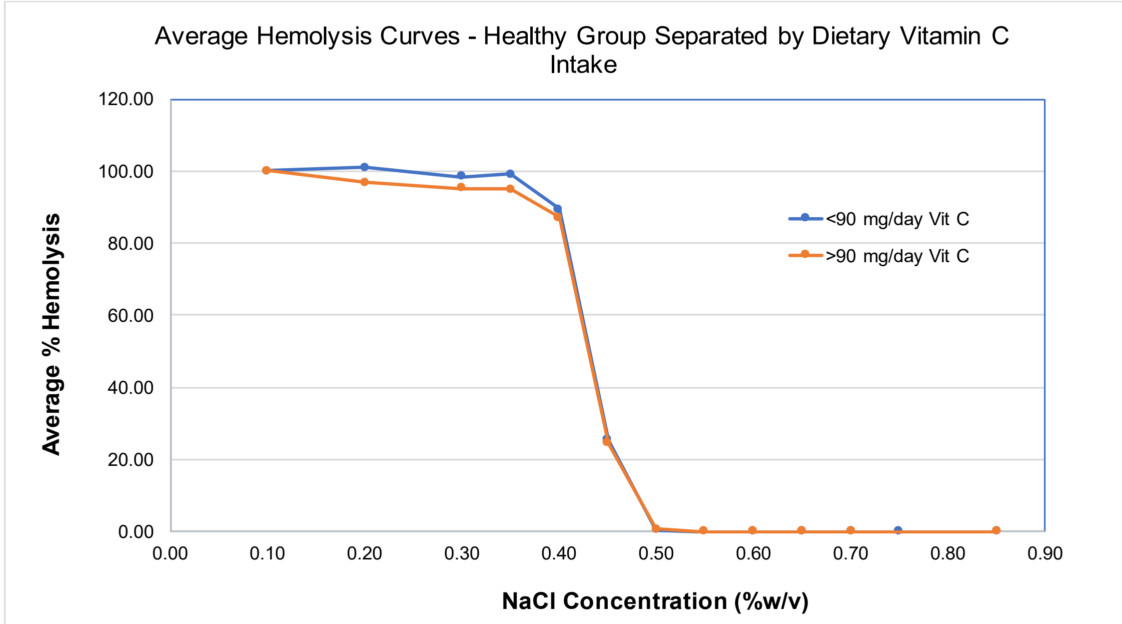

Figure 7. Overlay graph of average hemolysis curves- healthy individuals separated based on daily dietary vitamin C intake.

Table 4. Table containing hemolysis means and standard deviations for healthy group separated by dietary intake <90 mg/d and >90 mg/d. *P*-values were calculated using Mann-Whitney U test and significance was determined at $p<0.05$.

[NaCl] (%w/v)	<90 mg/day vitamin C		>90 mg/day vitamin C		*p*-value*
	Mean % Hemolysis	SD	Mean % Hemolysis	SD	
0.85	0.00	0.00	0.00	0.00	N/A
0.75	0.03	0.18	-0.02	0.17	0.7496
0.70	0.08	0.27	0.04	0.24	0.9124
0.65	0.05	0.20	0.00	0.13	0.3421
0.60	0.07	0.28	0.06	0.25	0.6745
0.55	-0.02	0.29	0.12	0.40	0.6745
0.50	0.50	0.50	0.84	1.45	0.6745
0.45	25.39	14.21	24.47	20.97	0.4593
0.40	89.44	12.04	87.24	7.06	0.3421
0.35	99.09	4.65	94.90	3.89	0.0910
0.30	98.57	4.18	95.24	5.19	0.1676
0.20	**100.99**	**2.52**	**96.95**	**3.02**	**0.0198**
0.10	100.00	0.00	100.00	0.00	N/A

*Significant p-value at $P<0.05$ (2-tailed).

Interestingly, as shown in figure 8, there is a weak positive correlation between BMI status and serum vitamin C ($r^2=0.269$). It also was also found via Mann-Whitney that serum vitamin C status was significantly different based on BMI status ($p=0.010$).

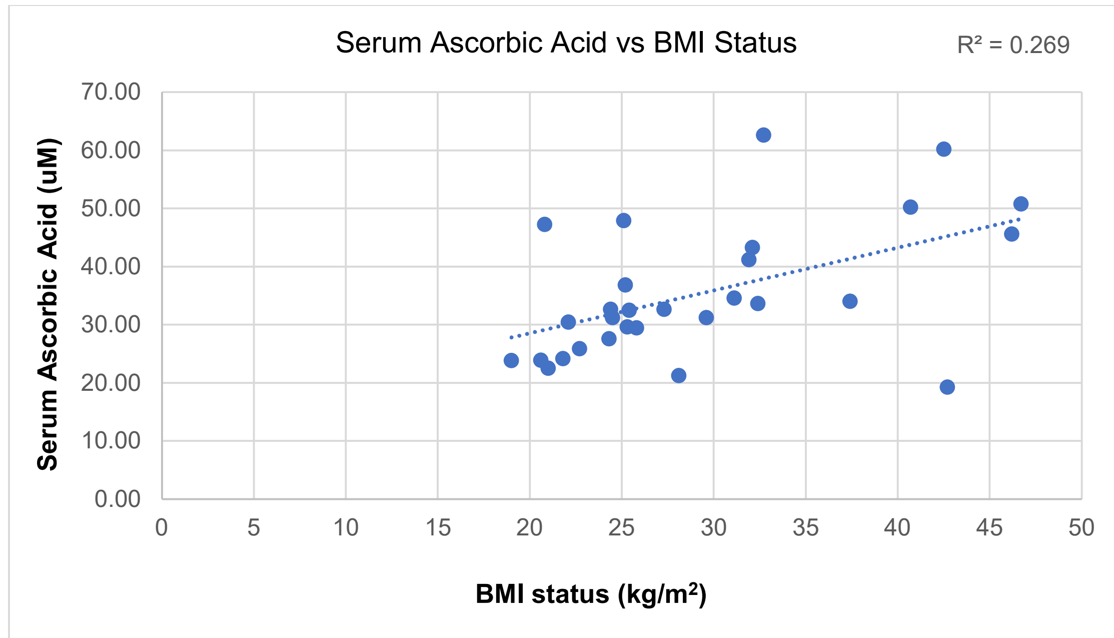

Figure 8. Scatter plot showing a weak positive relationship between BMI status and serum ascorbic acid levels.

Interestingly, as shown in figure 9, serum vitamin C levels do not correlate with dietary vitamin C intake. It should be mentioned that no outliers were found in the data.

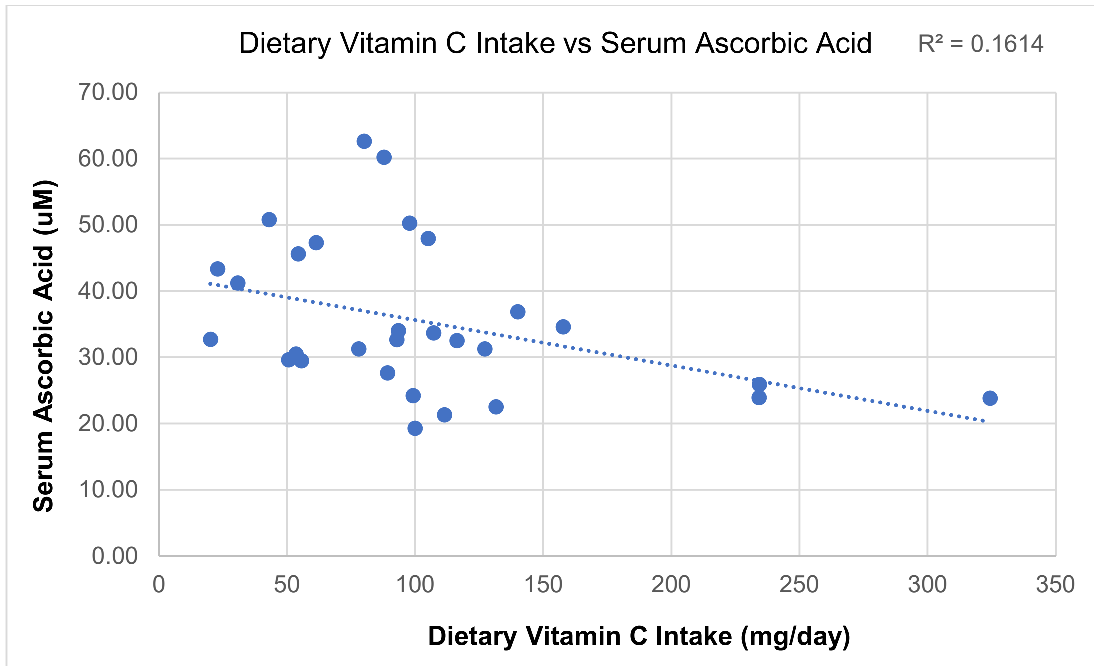

Figure 9. Scatter plot showing no relationship between dietary vitamin C intake and serum ascorbic acid levels.

Further, as shown in figure 10, there was no relationship found between HbA1c percent and serum ascorbic acid levels.

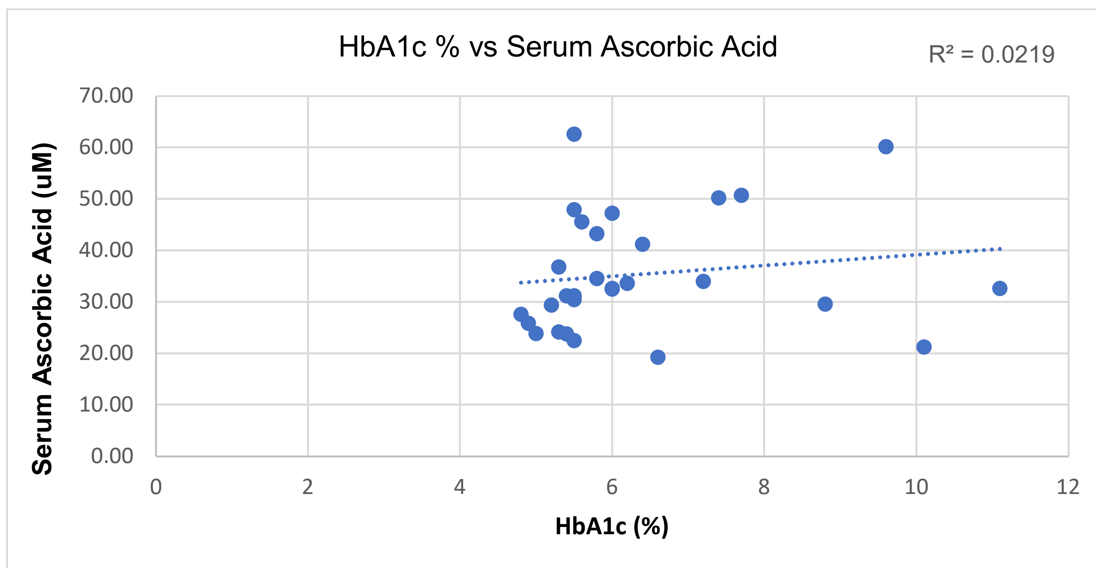

Figure 10. Scatter plot showing no relationship between HbA1c percent and serum ascorbic acid.

CHAPTER 5

DISCUSSION

The original hypothesis of this cross-sectional pilot study anticipated erythrocyte osmotic fragility to be higher and serum vitamin C to be lower in those with type 2 diabetes compared to non-diabetic individuals. This hypothesis was derived from a conjecture of James May and was not based on experimental evidence.

The results of this study revealed a different relationship between erythrocyte fragility and diabetes status. It was found that the erythrocytes of those with diabetes had lower fragility than those without diabetes. Upon researching this relationship, it was discovered that these findings were consistent with some of the available literature. A paper by McMillan et al. explains that when erythrocytes contain greater amounts of glycosylated hemoglobin, the intracellular fluid becomes more viscous.[73] The increased viscosity causes the erythrocytes to be less deformable and more rigid, reducing the ability to change shape and requiring a greater pressure gradient when moving through capillaries. Impaired cell structure and function will impact oxygen delivery to peripheral tissues. Having erythrocytes with impaired deformability contributes to the development of microvascular complications such as neuropathy, nephropathy, and retinopathy. These complications are primarily caused by impaired blood flow and vascular abnormalities.

A study by Knychala et al. found that there is an increased blood sodium chloride concentration in women with poor glycemic control.[74] Osmotic stress is induced in hypotonic environments. If hyperglycemia is associated with elevated sodium concentration, then it would explain why erythrocyte osmotic stability is better in diabetes. It is important to note that these results were found in women and may not be consistent across both sexes.

Though diabetic erythrocytes are less likely to lyse under osmotic stress, this does not mean they are healthier cells. As mentioned in chapter 2, diabetic erythrocytes have a shorter lifespan and lower survival rate than healthy ones. Erythrocytes do not have a nucleus and cannot repair themselves when damaged. Further, the consequential development of microvascular complications can compromise one's health. We originally believed hemolysis to be an indicator of poor cell health, however, this concept is much more complicated than

originally thought. Based on our study results and the other available literature, it seems that diabetic red blood cells are able to withstand greater osmotic stress but are otherwise not optimal.

Serum ascorbic acid levels were measured via isocratic reverse-phase UV-HPLC. The results from this study did not show a relationship between daily dietary intake and serum levels. This does not make sense as the only way for humans to maintain serum ascorbic acid levels is by consuming dietary vitamin C via food or supplementation. A positive relationship was expected between intake and serum levels. The food frequency questionnaire used for data collection was not validated, which may explain why no statistically significant relationship was found between intake and serum levels. It is also possible that even with instruction, participants may have completed the FFQ inaccurately. The questionnaire asked about fruit, vegetable, and fortified food intake in the past week. It is possible that participants filled out the form reflecting their usual intake rather than their recent intake. Because vitamin C is a water-soluble vitamin, it is not stored in the body. If recent intake is lower than usual intake, but usual intake was recalled, then the relationship between intake and serum levels will altered and not be genuine.

Serum vitamin C levels did not differ significantly by diabetes status. Past research shows individuals with type 2 diabetes to commonly display marginal vitamin C nutriture, a consequence of the reduced cellular uptake and recycling of dehydroascorbic acid. This reduced uptake is due to the competition that occurs between glucose and dehydroascorbic acid at the GLUT receptor under hyperglycemic conditions. However, a diagnosis of diabetes does not always indicate a higher A1C value. Though the average A1C of the diabetes group was significantly higher than that of the healthy group, some diabetics had A1C values that were within or very close to the healthy reference range. Further, diet could not be controlled. Because the FFQ was not validated, it is difficult to determine whether vitamin C intake was significantly different between the two groups. As mentioned previously, vitamin C intake should have a positive relationship with serum vitamin C levels. In the real world, diabetes status is not necessarily an indication of diet quality or vitamin C intake.

Though diabetes status was not found to be related to low vitamin C status, vitamin C nutriture was directly linked to erythrocyte osmotic stability in adults without type 2 diabetes. It is

possible that the same relationship was not found in the diabetes group because A1C, weight status/BMI, and possibly lifestyle were more variable than in the healthy group. This greater variability in the diabetes group decreases the validity of separating groups by diabetes status.

CHAPTER 6

CONCLUSION

In this small pilot trial, type 2 diabetes was not related to increased erythrocyte osmotic fragility or marginal vitamin C status. However, vitamin C nutriture was directly linked to erythrocyte osmotic stability in adults without type 2 diabetes only.

Though greater erythrocyte osmotic stability was not found to be correlated with higher vitamin C status across both groups, vitamin C still plays various critical roles in the body. Individuals with and without diabetes should continue to focus on consuming a balanced diet containing rich sources of vitamin C. In particular, people with diabetes would benefit from antioxidant intake as research shows diabetes to be associated with higher levels of oxidative stress throughout the body. Though it should be noted that levels of oxidative stress are highly dependent on diet, lifestyle, and other factors. For those who have limited access to fresh fruits and vegetables, supplementation with a multivitamin is another way to ensure adequate intake of this micronutrient.

One of the greatest limitations of this study was the COVID-19 pandemic. It is likely that fewer individuals applied to the study due to fear of contracting the virus during an in-person study visit. Another limitation is that there was variance in participant lifestyle factors and these factors were not controlled for. This was not an intervention study, and it is known that diet, physical activity levels, age, gender, and health status all impact vitamin C and erythrocyte health. A matched group design was not utilized as the amount of available and eligible participants was already low and selection bias may have occurred. Also, the FFQ used to estimate dietary vitamin C intake was not validated. It is consistent with literature for dietary intake and serum levels to be related, however no relationship was found. Despite these limitations, the data collected from this study was valid and is supported by the other available literature on this topic.

It would be interesting to do a future cross-sectional study investigating the effects of low vitamin C status on erythrocyte osmotic fragility in the healthy population. It is expected that greater vitamin C status will be related to greater erythrocyte stability because vitamin C improves

cytoskeletal beta-spectrin levels and cell deformability. It would also be interesting to measure serum NaCl levels as it is known that greater NaCl reduces osmotic stress. The results of this study suggest that greater vitamin C levels, which may be achieved through daily supplementation, will increase erythrocyte osmotic stability.

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APPENDIX A

IRB APPROVAL

ASU Knowledge Enterprise Development

APPROVAL: EXPEDITED REVIEW

Carol Johnston
CHS: Health Solutions, College of
602/496-2539
CAROL.JOHNSTON@asu.edu

Dear Carol Johnston:

On 1/14/2021 the ASU IRB reviewed the following protocol:

Type of Review:	Initial Study
Title:	Effects of Vitamin C Supplementation on Erythrocyte Osmotic Fragility in Individuals with Type 2 Diabetes Mellitus: A Randomized Controlled Trial
Investigator:	Carol Johnston
IRB ID:	STUDY00013197
Category of review:	
Funding:	Name: Graduate College (GRAD)
Grant Title:	
Grant ID:	
Documents Reviewed:	• ad and verbal script, Category: Recruitment Materials; • calendar, Category: Participant materials (specific directions for them); • consent, Category: Consent Form; • emails to respondents, Category: Participant materials (specific directions for them); • exit survey, Category: Participant materials (specific directions for them); • food questionnaire, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • health history questionnaire, Category: Screening forms; • KE approval, Category: Technical materials/diagrams; • online screener, Category: Screening forms; • physical activity form, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • protocol, Category: IRB Protocol; • test release, Category: Participant materials (specific directions for them);

The IRB approved the protocol from 1/14/2021 to 1/13/2022 inclusive. Three weeks before 1/13/2022 you are to submit a completed Continuing Review application and required attachments to request continuing approval or closure.

If continuing review approval is not granted before the expiration date of 1/13/2022 approval of this protocol expires on that date. When consent is appropriate, you must use final, watermarked versions available under the "Documents" tab in ERA-IRB.

In conducting this protocol you are required to follow the requirements listed in the INVESTIGATOR MANUAL (HRP-103).

Sincerely,

IRB Administrator

cc:
Ciara Lundy

APPENDIX B

SAMPLE SIZE JUSTIFICATION- HARVARD.EDU SAMPLE SIZE CALCULATOR FOR A PARALLEL TRIAL

	Author	Year	mean difference ± SD	n per group	Calculated n per group	Age range	Subject state	Test
1	Duranti et al.	2018	14.73 min. ± 18.4 min.	7 (14 total)	26 (52 total)	21 - 33	Men (exercising)	qercetin supplementation, time to 50% hemolysis
2	Czubak et al.	2017	15% hemolysis ± 15%	8	17 (34 total)	-	Donor blood samples	Blood sample supplementation of vitamin C and Trolox
3	Kraus et al.	1997	17 % hemolysis ± 21 % hemolysis	12	25 (50 total)	-	Zinc deficient rats	Vitamin C supplementation
4	Sultana et al.	2009	0.09% ± 0.04%	34 (102 total)	5 (10 total)	5 - 40	Hemolytic anemic patients with G6PD deficiency	Vitamin E supplementation
5	Tu et al.	2015	30% hemolysis ± 10% hemolysis	5	4 (8 total)	-	Ascorbate deficient mice	Vitamin C supplementation
	Average			13	15 per group	-		

APPENDIX C

ELIGIBILITY SURVEY- SURVEYMONKEY.COM

1. Have you been diagnosed with Type 2 Diabetes for over one year?

2. Please select your gender

3. What is your age?

4. What was your last A1c value?

5. Please enter your height and weight

6. Are you a vegetarian?

7. Do you take any dietary supplements? (Please list them)

8. Do you have any unresolved medical conditions such as heart disease, cancer, high blood pressure, kidney disease, infectious disease, etc.?

9. Do you take prescription medications? If yes, please indicate how long you have been taking the medication.

10. If female, are you pregnant, lactating, or do you anticipate becoming pregnant?

11. If you smoke, please select how many cigarettes you smoke per day.

12. Are you willing and able to travel to the ASU Downtown Campus to meet with research investigators on one occasion? (Roosevelt and 2nd Streets)

13. Are you willing to fast overnight and provide a morning blood sample on 1 occasion during this study?

APPENDIX D

SERUM ASCORBATE ANALYSIS- BLOOD PROCESSING

1. Precool centrifuge to 4 degrees Celsius.
2. Label microfuge tubes with Participant ID and collection date on side and cap of the tubes
3. Fill a cup/small cooler with ice.
4. Draw ~7 mL bood into a K2EDTA (purple top) vacutainer for hemolysis/vitamin C and 1 K2EDTA (purple top) for SQ.
5. Remove 1 mL whole blood for hemolysis assay – Conduct hemolysis assay immediately (protocol below).
6. Centrifuge sealed vacutainer at 4 degrees for 8 minutes at 2800 g – make sure to balance centrifuge with water tube and add bucket caps before starting centrifuge.
7. Remove plasma supernatant from vacutainer and add to 5 mL conical tube.
8. Add an equal volume (1-1.5 mL) plasma supernatant and MPA/NA2EDTA solution to 15 mL conical tube, vortex, and push into ice for 5 minutes.
9. Centrifuge again (make sure to balance centrifuge with water tube) at 4 degrees Celsius for 10 minutes at 4700 g.
10. Remove supernatant in 100 uL aliquots into labeled microfuge tubes.
11. Push microfuge tubes into ice then transfer samples to deep freezers.
12. Store in -80-degree Celsius freezer until analysis by UV-HPLC.

APPENDIX E

SERUM ASCORBATE ANALYSIS- HPLC PROTOCOL

MPA/EDTA (make fresh every 3 weeks)

- Dissolve 5.0 g *meta*-phosphoric acid (MPA) in 40 ml water (10% w/v final) and add 10 ml 10 mM Na2EDTA (372 mg/100 ml; 2.0 mM final). Stir or shake at room temperature until the solution is homogenous (~5 to 10 min) and store at 4°C.

TCEP solution:

- 5 mmol/L TCEP at pH 2. Mix 1.43325 g/L TCEP in HPLC grade water

Sulfuric Acid Mobile Phase:

- 0.176544g/L of sulfuric acid in HPLC grade water

1. Thaw samples (previous stored at -80 degrees C) and separate into aliquots of 50 uL in 1.5 mL Eppendorf tubes.
2. Add equal volumes of 5mmol/L TCEP in HPLC grade water to sample tubes and keep in dark for 20 minutes at room temperature to allow for reaction. This allows for measure of total vitamin C in samples.
3. Centrifuge samples at 16,000 g for 5 minutes, balancing with water tubes as needed. Keep centrifuge tubes on ice, remove 90 uL of supernatant, and transfer into auto injection vials. Inject sample vials into HPLC immediately or keep in refrigerated autosampler for up to 4 hrs. After completed sample run, rinse system using wash of 40% acetonitrile in water to flush mobile phase.
4. Prior to sample run, create a standard curve using a peak area linear regression from ascorbic acid standards (concentrations of 0 to 100 umol/L) made in 10% MPA in 2mmol NA2EDTA.

APPENDIX F

ERYTHROCYTE OSMOTIC FRAGILITY PROTOCOL

1. Bring 40 ml stock to 400 mL (.9% NaCl) with dH2O use to prepare dilutions
2. Pre-prepare NaCl dilutions in 50 mL conical tubes, enough for ~10 participants run in duplicate (dilution table below). Prepare dilutions as needed.

%NaCl	**Transfer of Stock**	**Volume of Water**
.90	50 mL	**0**
0.85	47.2	2.8
0.75	41.6	8.4
0.70	39	11
0.65	36.2	13.8
0.60	33.4	16.6
0.55	30.6	19.4
0.50	27.8	22.2
0.45	25	25
0.40	22.2	27.8
0.35	19.4	30.6
0.30	16.6	33.4
0.20	11	39
0.10	5.6	44.4

3. Add 2.0 mL of each (n=14) NaCl dilution to 15 mL conical tubes
4. Collect venous blood in 7 mL K2EDTA (Purple top vacutainer), Slowly invert tubes 10 times. Remove 1 mL blood transfer to microfuge tube (as mentioned in protocol for vitamin C processing).
5. Add 20 uL blood to each NaCl solution and gently mix by inverting several times.
6. Incubate at 20 C (room temperature) for 30 minutes.
7. Gently mix again by inverting and centrifuge at 2000 g for 10 min. at 4 degrees Celsius.
8. Remove supernatant from 15 mL conical tubes and transfer into cuvettes.

9. Use 0.9% NaCl solution as the blank.
10. Read and record absorption value at 540 nm.
11. Calculate Hemolysis:

    Hemolysis (%) = {Abs [Supernatant of each buffer] - Abs [Supernatant of 0.85% buffer]} x 100/Abs [Supernatant of 0.1% buffer] - Abs [Supernatant of 0.85% buffer]