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The effects of nicotinamide riboside on postprandial oxidative stress and vascular function

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The effects of nicotinamide riboside on postprandial oxidative stress and vascular function

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

This thesis is dedicated my parents, who have always been there to pick me up when I am down. They have supported me tirelessly throughout my journey at Iowa State and I could never have made it this far without them. Thank you, mom and dad.

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NOMENCLATURE

ADPR - Adenosine Diphosphate Ribose
ANOVA – Analysis of Variance
ATP – Adenosine Triposphate
BIA – Bio-Electrical Impedance Analysis
BMI – Body Mass Index
cm – Centimeter
CVC – Cutaneous Vascular Conductance
CVD – Cardiovascular Disease
dL – Deciliter
DNA - Deoxyribonucleic acid
H_2O_2 - Hydrogen Peroxide
HDL – High-Density Lipoprotein
HFM – High Fat Meal
HIF1α – Hypoxia Inducing Factor 1-Alpha
Hz – Hertz
IDL – Intermediate-Density Lipoprotein
Kg – Kilogram
LDL – Low-Density Lipoprotein
LSCI – Laser Speckle Contrast Imaging
LXR – Liver Receptor X
MDA – Malondialdehyde
Mg – milligrams
ml – Milliliter
mmHg – Millimeter of Mercury
MRI - Magnetic Resonance Imaging
NAAD - Nicotinic Acid Adenine Dinucleotide
NAD+ - Nicotinamide Adenine Dinucleotide
NADH - Nicotinamide Adenine Dinucleotide Hydride
NAM – Nicotinamide
NAMN – Nicotinic Acid Mononucleotide
NAMPT - Nicotinamide Phosphoribosyltransferase
NHANES – Nation Health and Nutrition Examination Survey
nm – Nanometer
NMN- Nicotinamide Mononucleotide
NR - Nicotinamide Riboside
O_2^- - Superoxide Radical
OH^- - Hydroxyl Radical
PARP - Poly(ADP-ribose) Polymerases
PBMC - Peripheral Blood Mononuclear cells
PGC1α - Pparg Coactivator 1-Alpha
PORH – Post Occlusive Reactive Hyperemia
PPAR - Peroxisome Proliferator-Activated Receptor

REDOX – Reduction/Oxidation
ROS- Reactive Oxygen Species
SIRT – Silent Information Regulator
SREB - sterol regulatory element binding proteins
T2D – Type 2 Diabetes
TBARS - Thiobarituric Acid Reactive Substance Assay
TCA Cycle – Tricarboxylic Acid Cycle
TG – Triglycerides
Trp- Tryptophan
VLDL Very Low-Density Lipoprotein
VO_2 - Maximal Oxygen Consumption Rate

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ABSTRACT

High-fat meal (HFM) consumption increases oxidative stress in humans. The metabolic cofactor nicotinamide adenine dinucleotide (NAD+), has been identified as a key regulator of oxidative stress. Older individuals suffer from low NAD+ levels and experience age-related increases in oxidative stress. Nicotinamide Riboside (NR), a newly discovered NAD+ precursor has demonstrated the ability to raise NAD+ in older adults. Nicotinic Acid (NA), another NAD+ precursor is established as one of the oldest anti-atherogenic supplements that improves vascular function. Similarities between NR and NA indicate NR may have potential vascular benefits yet to be discovered. We hypothesized that one week of NR supplementation would reduce postprandial oxidative stress and improve vascular function following consumption of a HFM (1050 kcal, 72g fat) in old and young participants. We performed a double-blind, placebo-controlled crossover study with 16 participants, divided into young (n=13) and old (n=3) groups, assessing one week of 250mg 2x/day NR on a lipid peroxidation indicator of oxidative stress. Microvascular function was determined with blood flow measurements of post occlusive reactive hyperemia (PORH), analyzed using laser speckle contrast imaging (LSCI). NR supplementation did not significantly affect levels of postprandial lipid peroxidation indicators of oxidative stress (MDA). No differences in postprandial PORH measures of microvascular function were identified for treatment, time and interaction. Plasma MDA, glucose, and triglycerides all increased postprandially with significantly higher levels reported in the old group compared to young ($p<0.001$, $p=0.04$, $p<0.001$, respectively). One week of NR supplementation was well tolerated in all participants but had no effect on postprandial oxidative stress and microvascular function.

Elevated levels of oxidative stress in older adults are consistent with previous findings. Further examination of NR and its effects on postprandial oxidative stress and vascular function must be completed before conclusive results are established.

CHAPTER 1. GENERAL INTRODUCTION

In the past two decades, obesity and diabetes have become immensely prevalent in the United States as well as globally. These diseases are associated with a number of medical complications including insulin resistance, dyslipidemia, development of cardiovascular disease, and inflammation (Ali & Crowther, 2005). Westernized diets, larger portion sizes, and increased processed food accessibility are all major contributors to the current food environment that perpetuates excess consumption of saturated fats by large portions of the population. According to NHANES, over 60% of Americans consume more than the Dietary Guidelines for Americans recommended daily amount of saturated fat of less 10% of total calories coming from saturated fats (CDC, 2019). It has been established that diets rich in saturated fats contribute to higher levels of inflammation as well as hyperlipidemia (Enos et al., 2013). Elevated levels of LDL cholesterol and triglycerides (TGs) are common problems in developed countries, with over 95 million Americans having reported total cholesterol values over the desired range of <200 mg/dL (CDC, 2020). Because of the prevalence of this issue, a number of strategies have been developed to combat elevated LDL and triglyceride levels. Lifestyle changes such as altering dietary intake and increasing physical activity levels have been recognized as effective methods of ameliorating hyperlipidemia (Kelly, 2010). Pharmaceutical interventions including statins, bile acid sequestrants, and fibrates are commonly prescribed medications that function as effective lipid lowering medications (Pahan, 2006). Improving blood lipid levels via supplementation is also a common strategy. One supplement commonly used for this purpose is nicotinic acid (NA).

Nicotinic acid, also described as niacin or vitamin B3, has long been recognized as an effective cholesterol and lipid lowering supplement (Carlson, 2005; Julius & Fischer, 2013).

Although remarkably effective, nicotinic acid is associated with a severe flushing response that be extremely uncomfortable(Stern et al., 1991). Once in the body, NA is converted into nicotinamide adenine dinucleotide (NAD+). Nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP+) are essential for cellular function due to their key role as cofactors in a number of metabolic reactions. They function in multiple reduction and oxidation reactions throughout metabolism due to their unique ability to transfer electrons by accepting and donating hydrogen molecules.

Along with its role as a central cofactor in metabolism, NAD+ also functions as a regulator of oxidative stress. Oxidative stress is defined an imbalance between free radicals and antioxidant defenses in the body. Free radicals are molecules with unpaired electrons that can cause cellular and DNA damage when produced in high concentrations (Pizzino et al., 2017). Free radicals, also known as reactive oxygen species (ROS), can be produced as a result of a number of different circumstances. Free radical generation can be a product of inflammation, oxidative phosphorylation, radiation, tobacco smoke, pollution and more (Phaniendra et al., 2015). The damage caused by oxidative stress can eventually lead to the development of many chronic diseases such as cancer, diabetes, arthritis, cardiovascular diseases, and chronic inflammation (Uttara et al., 2009). The production of free radicals which lead to oxidative stress can be significantly accelerated or attenuated via dietary intake (Fang et al., 2002). Multiple studies have reported that consumption of meals high in saturated fats, can result in an increased postprandial oxidative stress response (Chan, 2016; Kesh et al., 2016). Because the typical western diet is rich in saturated fats, developing strategies that can reduce oxidative stress following the consumption of a high fat meal could be beneficial for the health of the general population.

While oxidative stress can develop from external carcinogens as well as consumption of high fat meals, aging may also be associated with changes is oxidative stress levels (Cui et al., 2012). As individuals age, they see a decline in overall NAD+ levels which may be a mechanism related to increasing oxidative stress with aging (Massudi et al., 2012). Because of the function of NAD+ as a reduction/oxidation reaction (redox) regulator in the cell, as well as its role as an activator of systems that potentially reduce oxidative stress (Brunet et al., 2004; Imai & Guarente, 2016), it can be postulated that increasing NAD+ levels in older adults may have an impact on their overall levels of oxidative stress.

Recently discovered NAD+ precursor nicotinamide riboside (NR) has been identified as an effective supplement in raising cellular NAD+ concentrations in older adults without producing adverse side effects (Martens et al., 2018). As described earlier, studies have indicated the consumption of a meal high in saturated fat leads to an immediate increase in postprandial oxidative stress (Chan, 2016; Kesh et al., 2016). The elevated levels of oxidative stress following the consumption of a HFM likely reflects a common position large portions of the American population are in multiple times throughout each day. The purported ability of NR to raise NAD+ concentrations in older adults, potentially impacting oxidative stress levels, justifies the assessment of NR supplementation on postprandial oxidative stress following the consumption of a high-fat meal.

Because testing of NR in humans only began in 2016, the capability of NR to function in a similar role to corresponding NAD+ precursor nicotinic acid has not yet been investigated. The long-established ability of NA to reduce blood lipid levels and lower the risk of cardiovascular disease (Ruparelia et al., 2011), raises questions about the capability of NR to function in a

similar manner. The investigation of NR and its ability to reduce blood lipid levels and improve vascular function is justified by the current lack of literature on this topic.

In this study, our goals were to 1) determine the impact of one week of 500 mg daily nicotinamide riboside supplementation on oxidative stress following the consumption of a high fat meal. The second aim was to 2) determine whether one week of 500 mg daily nicotinamide riboside supplementation was able to impact vascular function measured with laser speckle contrast imaging, assessing forearm post occlusive reactive hyperemia (PORH). We hypothesized that one week of NR supplementation would result in lower oxidative stress values compared when compared to placebo. We also hypothesized that NR supplementation would improve postprandial microvascular function assessed using PORH.

CHAPTER 2. REVIEW OF LITERATURE

2.1 Cholesterol and Atherosclerosis

In the United States, over 95 million adults over the age of 20 have total cholesterol levels higher than the desired value of <200 mg/dL, with 29 million Americans having total cholesterol level above 240 mg/dL (Carroll et al., 2017). Generally, cholesterol is comprised of multiple different lipoproteins which function as a main lipid transport mechanism within the body. These lipoproteins, produced in the liver, are essential in the absorption and transport of dietary lipids in the small intestine, lipid transport from the liver to surrounding tissues, and transport of lipids from the peripheral tissues back to the liver (Feingold & Grunfeld, 2000). Low-density lipoprotein (LDL) contain a majority of the body's cholesterol in the blood, transporting lipids away from the liver to peripheral tissues. Chronic high levels of LDL cholesterol can lead to the development of plaque buildup on arterial walls, a process is known as atherosclerosis (Libby et al., 2002). High levels of LDL are commonly associated with metabolic disease, obesity, and type two diabetes. Conversely, high-density lipoprotein (HDL) functions as a carrier of cholesterol from peripheral tissues back to the liver, an anti-atherogenic mechanism which actively prevents plaque buildup (Rye et al., 2009). Very low-density lipoprotein (VLDL) and intermediate density lipoprotein (IDL) are also lesser pro-atherogenic particles, though targeting decreases in LDL or increases in HDL are often the primary mechanisms of pharmaceutical strategies for reducing cholesterol(Feingold & Grunfeld, 2000).

A number of risk factors can contribute to elevated cholesterol levels including age, family history, smoking, elevated blood pressure, poor diet, lack of physical activity, the development of heart disease, obesity, and diabetes (CDC, 2020.) High cholesterol is often treated using a variety of strategies. Increasing physical activity, limiting foods high in saturated

fats, and eating a varied diet including fresh fruits and vegetables, are common non-pharmacological approaches to the management of cholesterol (Mannu et al., 2013). While these strategies can be effective, high cholesterol is also regularly managed using medications. A variety of cholesterol lowering medications utilizing different mechanisms are frequently used in conjunction with lifestyle changes to manage elevated cholesterol levels. Perhaps the most commonly prescribed medications are statins, which lower LDL cholesterol by reducing cholesterol biosynthesis in the liver (Stancu & Sima, 2001). Bile acid sequestrants, another class of medications, function to reduce cholesterol by binding to bile acids in the intestine forcing the liver to increase production of bile. As a result of cholesterol utilization in the synthesis of bile in the liver, circulating LDL cholesterol levels are significantly decreased (Einarsson et al., 1991). Apart from pharmaceutical approaches, various forms of supplementation are commonly used to manage cholesterol. One such supplement is Omega-3 fatty acid. Omega-3 fatty acids occur naturally in fish are often taken in either pill or liquid form. They function to reduce VLDL cholesterol synthesis, ultimately impacting overall blood lipid levels (Chris Bradberry & Hilleman, 2013).

2.2 Niacin and NAD+

Another supplement that has proven to reduce cholesterol and is nicotinic acid (NA), also known as niacin or vitamin B3. Nicotinic acid has been utilized as an effective cholesterol reducing agent since 1955 (Djadjo & Bajaj, 2019). NA functions to improve the cholesterol profile by reducing hepatic synthesis of VLDL, increasing HDL synthesis, and inhibiting lipolysis in adipose tissue (Aboulsoud, 2014). While nicotinic acid was the first established cholesterol lowering drug, its initial discovery was tied to the treatment of pellagra.

In the 20th century pellagra was a widespread cause of death. Symptoms of severe cases of pellagra are categorized by the classic '4 D's', Dermatitis, Diarrhea, Dementia, and death. In 1926, chronic vitamin B3 deficiency was identified as the cause of pellagra, and by 1938 the U.S. government began fortifying flour with niacin, eventually leading to is essential eradication from the developed world (Savvidou, 2014). One of the prominent issues associated with vitamin B3 deficiency is depleted levels of NAD+. Upon consumption of vitamin B3 or nicotinic acid, it is converted, through a number of steps, into nicotinamide adenine dinucleotide (NAD+), an essential metabolic regulator and signaling molecule (Carles Cantó et al., 2015). The substantial complications related to the advanced stages of vitamin B3 deficiency demonstrate the importance of NAD+ as it relates to human health. One of the primary functions of NAD+ is as a co-enzyme for metabolic pathways. Its role of electron transfer by way of accepting and donating hydrogen molecules is essential in a number of reactions required for metabolic function. NAD+ and nicotinamide adenine dinucleotide phosphate (NADP+), the same molecule with an added phosphate group, either accept or donate a hydrogen in multiple essential metabolic processes including glycolysis, the TCA cycle, lactate fermentation and oxidative phosphorylation. In these reactions, NAD+ gains a hydrogen, and is reduced to become NADH, NAD's reduced form, NADH, can then be utilized by the electron transport chain, thus participating as a key substrate in mitochondrial ATP production (Yang & Sauve, 2016). As a result of processes such as glycolysis, NADH equivalents are generated in the cytoplasm.

In order to maintain an adequate NAD+/NADH balance, two immediate NADH oxidation routes are utilized in metabolism. The first path is the transfer of NADH into the mitochondria via shuttling mechanisms such as the malate-aspartate shuttle where it is then oxidized by the electron transport chain contributing to the generation of ATP (L. R. Stein &

Imai, 2012). The second pathway involves NADH oxidation to NAD+ via lactate dehydrogenase, acting as a cofactor in the reaction, assisting in the production lactate from pyruvate (Yang & Sauve, 2016). NAD+ and NADH can also influence metabolic rate via a feedback system determined by the ratio of NAD+ to NADH. The electron transport chain can inhibit the oxidation of NADH to NAD, thus lowering the ratio of NAD+ to NADH. This altered ratio impacts the α-ketoglutarate/citrate ratio, which can eventually limit acetyl-coA from entering the TCA cycle, consequently, NADH feedback playing a significant role in determining rate of catabolism and energy production (McLain et al., 2011).

2.3 NAD+, Sirtuins, and PPAR

NAD+ also functions as an essential molecule for the activation of other substrates. One such substrate is the NAD+ dependent Sirtuin or silent information regulator (SIRT) family of deacetylases which rely on the availability of NAD+ for activation (Imai & Guarente, 2016).

SIRT 1,6, and 7 function as regulators of gene expression and are located in the nuclei of cells. SIRT 3,4, and 5 can be found in the mitochondria, while SIRT 2 is localized in the cytoplasm (C. Cantó & Auwerx, 2011). Sirtuin activation is accelerated when the body is experiencing a nutritional deficit, prompting metabolic adaptations that improve metabolic efficiency and increase utilization of non-CHO energy sources (Zhu et al., 2013). Upon activation, SIRT1 drives enhanced lipid oxidation as well as increases mitochondrial biogenesis in cells(Chang & Guarente, 2014). SIRT3, located in the mitochondria targets proteins involved in fatty acid oxidation, deacetylating the proteins during periods of fasting resulting in an increased breakdown of fatty acids (Ansari et al., 2017). SIRT1, the most studied sirtuin is a potent activator of PGC1α via deacetylation. PGC1α is a central regulator in mitochondrial biogenesis, consequently the activation of PGC1α via SIRT1 improves mitochondrial biogenesis and

enhances overall mitochondrial function(Cheng et al., 2018). The sirtuins can also greatly influence glucose metabolism via multiple mechanisms. The first is via PGC1α deacetylation by SIRT1. The deacetylation and consequent activation of PGC1α can influence glycolysis via the mitigation of the expression of several glycolytic genes (Houtkooper et al., 2012). Additionally, SIRT1, SIRT3 and SIRT6 all possess the ability to hinder glucose oxidation through the TCA cycle via deacetylation of hypoxia inducing factor 1 α (HIF1α) (Houtkooper et al., 2012). SIRT1 is able to influence lipid metabolism via a number of deacetylation mechanisms. One described mechanism is again via the activation of PGC1α, a co-activator of the peroxisome proliferator-activated receptor (PPAR) transcription factors. Eventually, the activation of the PPAR transcription factors regulates lipid metabolism via the control of gene expression (Duncan, 2011). SIRT1 can also represses lipid synthesis and promote lipolysis via direct deacetylation pathways that alter gene expression. One such pathway is the deacetylation and subsequent down regulation of sterol regulatory element binding proteins (SREBP), resulting in subsequent reductions of activity in pathways that promote fat storage and lipid synthesis (Ye et al., 2017). In mice, SIRT1 has been shown to interact with liver receptor x (LXR), promoting deacetylation, eventually significantly contributing to the overall regulation of blood cholesterol and triglyceride levels (Li et al., 2007).

Mounting evidence indicates up-regulation of SIRT1 in humans may be anti-atherogenic (Chi et al., 2014; Stein & Matter, 2011), in part due to sirtuin involvement in metabolic regulation. The immense role the sirtuins have in metabolism, combined with the potential ability to increase sirtuin activity via supplementation of NAD+ precursors, implies the possibility of NAD+ supplementation to provide a variety of benefits for overall human health and metabolism.

Besides its redox role in metabolism and sirtuin activation, NAD+ is also an essential substrate for the poly(ADP-ribose) polymerases (PARPs). PARPs function as important DNA damage sensors that play a critical role in repair of damaged DNA sites (Fouquerel et al., 2014). PARPs are one of the major consumers of NAD+ in the body, with studies indicating that prolonged activation of PARP1 can lead to NAD+ depletion, eventually, resulting in long-term detrimental metabolic effects (Murata et al., 2019). The requirement of NAD+ in PARP activation paired with the notion that increased PARP activity can deplete NAD+, only further demonstrates the crucial role of NAD+ in cellular function.

Maintenance of adequate NAD+ can protect against PARP1 mediated cell death. Consequently, the elevation of NAD+ levels has the potential to ameliorate PARP-1 mediated cell death resulting from NAD+ depletion (Alano et al., 2004). The functions of NAD+ as both a key metabolic cofactor, essential in energy production, as well as an important signaling and activating substrate for both sirtuins and PARPs, indicate that the consumption of NAD+ increasing supplements may have a variety of potential health benefits for humans.

Nicotinic acid (NA), discussed earlier as a common supplement taken to reduce blood cholesterol and triglyceride levels, has demonstrated the ability to increase expression of SIRT1 in animals (Y. Li et al., 2015). This study found daily nicotinic acid supplementation in rabbits led to significantly increased expression of SIRT1 compared to the control. This group carried out an additional experiment that investigated the effects NA supplementation on rabbits with induced acute vascular inflammation. The NA supplemented group had significantly higher levels of SIRT1 expression, while also achieving reductions in vascular inflammation levels, indicating increased SIRT1 expression via NA was one mechanism responsible for changes in vascular function in rabbits.

While the potential for supplementation of NAD+ precursors influencing sirtuin activity is promising, nicotinic acid and other NAD+ precursors have demonstrated measurable benefits regarding improvements in blood cholesterol. A placebo controlled study with 71 participants investigating the long term efficacy of 2g of daily NA supplementation found that after 12 months, NA increased HDL cholesterol by 23%, while decreasing LDL cholesterol by 19% (Lee et al., 2009). The same study used magnetic resonance imaging (MRI) to analyze differences in carotid wall area and found that after 12 months, the NA group had significantly reduced (-1.1± 2.6 mm^2) carotid wall area compared to placebo (+1.2 ± 3.0 mm^2).

A review paper examining previous studies that investigated NA reinforced the efficacy of NA in treatment of cholesterol. In six studies which analyzed effectiveness of chronic NA supplementation, participants demonstrated a 25-40% increase in HDL cholesterol, a 30-40% reduction in LDL cholesterol, and a 15-30% reduction in plasma triglycerides (Carlson, 2005). Nicotinic Acid is one of the oldest lipid lowering medications, and while the literature supports its efficacy, a prominent disadvantage associated with NA is the severe flushing response associated with high dose supplementation (Gille et al., 2008).

2.4 NAD+ Precursor Pathways

NAD+ synthesis can result from several key precursor pathways. One of which, is previously discussed nicotinic acid, the most studied precursor. NA is converted to NAD+ via a three-step pathway known as the Preiss-Handler pathway where it is converted to nicotinic acid mononucleotide (NAMN), then nicotinic acid adenine dinucleotide (NAAD), before it is converted to NAD+ (Houtkooper et al., 2010). NAD+ can also be produced via a salvage pathway that utilizes nicotinamide (NAM), which is a byproduct of many NAD-consuming enzymes including sirtuins. Nicotinamide is converted into Nicotinamide mononucleotide

(NMN) by Nicotinamide phosphoribosyl transferase (NAMPT), which is then converted to NAD+. This salvage pathway plays a crucial role in maintaining NAD+ concentrations, especially when dietary sources of NAD+ precursors are limited (Sporty et al., 2009). NAD+ can also be synthesized by dietary tryptophan (Trp) via an eight-step de novo pathway, providing another source of NAD+ synthesis from the diet (Castro-Portuguez & Sutphin, 2020). The final pathway for NAD+ synthesis is via Nicotinamide Riboside (NR). NR utilizes a two-step pathway, being converted into NMN before then being synthesized into NAD+. The production of NAD+ via NR is similar to the salvage pathway. Instead of the conversion of NAM from NAD+ consuming enzymes, into NMN, NR from the diet is converted directly into NMN which is then converted into NAD+, making it an efficient two step pathway. The ability for NA to significantly increase NAD+ has been well documented (Bogan & Brenner, 2008). The recently discovered NAD+ precursor NR has only begun human testing within the last 5 years. Further investigation into the impact of NR on metabolic function and its potential as a lipid lowering agent are required.

2.5 Nicotinamide Riboside, a Recently Discovered NAD+ Precursor

Until recently, nicotinic acid was identified as the primary NAD+ precursor viable for supplementation. In 2016, the first human trials of nicotinamide riboside (NR), an NAD+ precursor vitamin were completed. Nicotinamide riboside is a naturally occurring compound found in milk and in minor amounts in unprocessed animal sources (S. A. Trammell et al., 2016). While Nicotinamide riboside had been identified for decades, it did not gain traction in the literature until recently. The first human study reported had multiple of significant discoveries regarding NR supplementation in humans (S. A. J. Trammell, Schmidt, et al., 2016). In the initial experiment, Trammell was the only participant, as side effects of NR in humans were unknown

at this point. Trammel found that an acute dose of 1000mg of NR, orally administered, had increased his NAD+ concentration by roughly 2.7-fold, eight hours post ingestion. He followed this experiment with a comparison of NAD+ precursors, NR, NA, and NAM, on hepatic NAD+ metabolism in mice. They found that was NR superior to NA and NAM in at increasing hepatic concentration of NAD+, NADP+ and number of other NAD+ intermediates. This finding indicated that NR supplementation resulted in greater NAD+ metabolism in the liver compared to other NAD+ precursors NA and NAM. Trammel went on to expand testing of NR in humans and found that compared to NA, oral consumption of NR did not result in a severe flushing response. Another finding of importance from this publication was the significant increase in adenosine diphosphate ribose (ADPR), a compound used to measure activity of NAD+ consuming enzymes. The increase in ADPR implied that in addition to the increasing NAD+ from NR supplementation, NR may provide additional up-regulation of NAD+ consuming activities such as sirtuin activation. The identification of NR as a functionally effective NAD+ precursor that is safe for human consumption, with limited side effects was an important first step for the progression of NR related studies in humans.

In recent years more studies investigating NR have been published. One of the earlier studies investigating NR from 2012, analyzed cultured mammalian cells and compared the different NAD+ precursors. They found NR dose-dependently increased NAD+ levels in mice and human cell lines (Carles Cantó et al., 2012). Importantly, they found NR and NA both increased NAD+ levels to similar concentrations, though NR did so without activating the GPR109A, the receptor associated with the flushing reaction caused by NA. This may have been the first indication that NR can be an effective NAD+ raising supplement without providing the side effects commonly associated with NA. This study also investigated the effect of NR on

Sirtuin activation, finding that SIRT1 and SIRT3 were both positively regulated by NR. This implies that the increase in NAD+ via NR, influences both nuclear and mitochondrial components, as SIRT1 and SIRT3 both saw positive regulation within their respective compartments.

2.6 Nicotinamide Riboside Studies in Mammals

In 2016, Trammell published another study examining how nicotinamide riboside supplementation influenced the development of type 2 diabetes (T2D) in mice (S. A. J. Trammell, Weidemann, et al., 2016). They gave mice raised with a high fat diet a dose of streptozotocin creating a model of T2D. The streptozotocin treated mice developed insulin resistance, hyperglycemia, and diabetic peripheral neuropathy. The treated prediabetic and T2D mice were divided into high fat diet (HFD) + NR, and HFD with no supplement groups. They found the NR HFD group had attenuated weight gain, improved glucose tolerance and reduced liver damage, delaying the progression of hepatic steatosis. Using corneal confocal microscopy, a technique used to assess development of diabetic peripheral neuropathy, they found NR had a neuroprotective effect, limiting the development of neuropathy in diabetic and prediabetic mice.

Another study involving NR and mice aimed to investigate the impact of NAD+ repletion of stem cells (Zhang et al., 2016). This study analyzed the impact altering NAD+ pools had on stem cell and mitochondrial function. They concluded that mitochondrial oxidative respiration was essential for the maintenance of multiple kinds of stem cells during aging. Importantly, they found that a depletion of NAD+ pools led to an eventual loss of mitochondrial function. Mice supplemented with NR to replenish the NAD+ pools, exhibited improvements in mitochondrial homeostasis and enhanced life spans compared to the control. These findings provide valuable

insight regarding the benefits of maintaining NAD+ pools on aging, and the potential for NR combat age associated reductions of NAD+ in humans.

Since Trammell's human pilot study in 2016, a number of human studies centered around NR supplementation have been published. A study examining the viability and efficacy of NR with middle aged and older adults (between ages of 55 and 79 years old) established that chronic NR supplementation, (500mg, 2x/day) for six weeks, had little to no adverse side effects in humans compared to NA (Martens et al., 2018). This study also demonstrated that NR significantly elevated levels of NAD+ in peripheral blood mononuclear cells (PBMCs) by roughly 60% compared to the placebo. This study further illustrated the capability of NR as a viable NAD+ raising supplement in humans with limited side effects compared to NA.

While Trammell and Martens established NR supplementation as an effective method of raising levels NAD+ in middle aged to older adults, the literature surrounding the testing of NR in human studies is relatively undeveloped. Only a handful of studies have further investigated the benefits of NR beyond its capacity to raise NAD+ levels.

One recent publication to do so analyzed the effect of NR supplementation on exercise performance and redox homeostasis (Dolopikou et al., 2020). An investigation of NR and its capacity to influence redox reactions had not yet been completed in humans. The likely depletion of NAD+ and NADP+ as humans age has been previously discussed (Verdin, 2015). Despite this, the investigation of NR supplementation in order to raise cellular levels of NAD+ and combat age-associated changes in redox homeostasis pools had not yet been investigated. The study by Dolopikou attempted to address the impact of NR on redox homeostasis and exercise performance in old and young adults, finding mixed outcomes. They postulated that, due to older adults having lower levels of NAD+ and NADP+, NR supplementation would increase these

levels, restoring reduction/oxidation homeostasis to optimal levels, ultimately increasing their exercise performance. They investigated the effects of an acute dose of 500 mg of NR on oxidative stress and exercise performance in old and young individuals. The results indicated the old group had higher overall levels of F_2-isoprostane levels, a measure of oxidative stress, compared to the young group. NR supplementation significantly reduced this measure of oxidative stress in the older group while no effect was seen in the young group. After NR supplementation, the old group saw increases in isometric peak torque compared to baseline and increased resistance to fatigue. The young group saw no changes in exercise performance. The findings from this study indicate that the ability for NR to increase cellular NAD+ levels had on reduced oxidative stress in older individuals by repairing their cellular redox capacity. These results are supported by previous findings that have demonstrated similar antioxidant properties of NAD+ precursor nicotinic acid (Ilkhani, 2016).

Nicotinamide riboside has potential to improve vascular function in mammals. A study investigating the potential vascular effects of nicotinamide mononucleotide (NMN), a similar NAD+ precursor, found that supplementation of NMN in mice, improved their overall vascular function (de Picciotto et al., 2016). The study demonstrated that supplementation of mice with NMN, reduced aortic stiffness in older mice and NMN activated arterial SIRT1 expression. As discussed previously NMN is an intermediate in the NAD+ synthesis pathway via NR. With the findings that NMN improved vascular function in mice, and the establishment of NA as a potent anti-atherogenic agent (Carlson, 2005), it can be hypothesized that supplementation of NR in humans may also potentially provide similar benefits. On account of this, examination of NR and its effects on vascular function require further investigation in human subjects.

One method that has gained tractionc as a valid indicator of microvascular function is post occlusive reactive hyperemia (PORH). PORH involves the occlusion of blood flow to a limb or area using a cuff, followed by the release of the cuff, increasing in skin blood flow to tissues that had been occluded. PORH is a method of assessing skin microvasculature which has been used to identify irregularities in vascular function associated with cardiovascular disease pathogenesis (Tee et al., 2004). PORH measured with laser speckle contrasting and laser doppler probing has been used to assess the effects of a number of supplements on microvascular function (Ashor et al., 2015; De Moraes et al., 2014), validating its ability to detect differences in blood flow as a result of supplementation.

A prominent method of quantifying data from PORH is utilizing laser speckle contrast imaging (LSCI). LSCI uses the reflection of a laser on red blood cell movement to provide indices of blood flow (Humeau-Heurtier et al., 2013). The ability to translate PORH data using laser speckle images, into quantifiable data, allows for analysis of blood flow and microvascular function of the imaged area, and potentially an indication overall vascular function.

2.7 NAD+ and Oxidative Stress

Oxidative stress is a situation in the body caused by a disparity between the production and accumulation of reactive oxygen species (ROS). Common reactive oxygen species are superoxide radicals (O_2^-), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2) (Pizzino et al., 2017). Most reactive oxygen species generated as byproducts from mitochondrial electron transport (Ray et al., 2012). ROS can also be generated as a result of activation of transcription factors, phosphorylation, apoptosis and differentiation. The accumulation of ROS is known to have a harmful impact on cellular structures such as proteins, lipids, and nucleic acids (Wu et al., 2013). Significant evidence exists that implicates oxidative stress has some degree of

responsibility for the onset and progression of a number of diseases including diabetes, cancer, atherosclerosis, and cardiovascular disease (Taniyama & Griendling, 2003). When ROS are kept at low or moderate concentration, these free radicals have valuable roles in the organism. An example of this is the need for free radicals to be utilized as a defense system against pathogens in some cells (Dröge, 2002). The delicate need to balance the concentration of ROS is why electron transfer via hydrogen movement from one molecule to another extremely important. The innate ability for NAD+ to perform this action is why it is regarded as one of the central regulators of redox homeostasis and essential regulator of the production of free radicals (Massudi et al., 2012). The capability of NR to increase cellular NAD+ pools suggests that NR supplementation has the potential influence oxidative stress, as evidenced by the previously mentioned study completed by Dolopikou (Dolopikou et al., 2020).

Oxidative stress can be influenced by a variety of external factors. Dietary intake plays a significant role in the development of oxidative stress. Evidence indicates that alterations in meal composition can influence postprandial oxidative stress (Chan, 2016; Roberts et al., 2002). Oxidative stress fluctuates significantly depending an individual's diet, though consumption of foods rich in saturated fats have been known to cause an acute spike in biomarkers that measure oxidative stress (Chan, 2016). The acute increase in oxidative stress caused by the consumption of a high fat meal provides an opportunity to investigate methods that have potential for decreasing postprandial oxidative stress.

NAD+ is a powerful regulator of oxidative stress in the body and increasing the overall amount of NAD+ via NR is a potential pathway to reducing postprandial oxidative stress. Oxidative stress can be quantified in humans using a number of different biomarkers.

One validated and frequently used biomarker is malondialdehyde (MDA), a natural byproduct of lipid peroxidation (Marnett, 1999). Lipid peroxidation is the oxidative degeneration of lipids on cell membranes by free radicals such as hydroxide (OH^-) or hydrogen peroxide (H_2O_2) causing damage to cells (Mylonas & Kouretas, 1999). The compounding effects of cellular damage occur after an accumulation of free radicals, this phenomenon is known as oxidative stress. MDA is an assessment of the rate at which lipid peroxidation is occurring, which provides an indication of oxidative stress status. The lack of literature surrounding NR as it relates to both oxidative stress and postprandial oxidative stress supports further investigation of the capacity of NR to function as a modulator of oxidative stress.

2.8 Conclusion

The first documented human trial of nicotinamide riboside was published in 2016. The early successes of NR documented by a number of animal and human studies (Brown et al., 2014; S. A. J. Trammell, Weidemann, et al., 2016), led to a growing amount of literature beginning to focus on NR. Despite this, the general body of literature focused on NR is limited, with even fewer studies published on NR in humans. This need for research on NR presents a number of different opportunities in terms of potential experiments. While its NAD+ precursor counterpart nicotinic acid is established in the literature as an effective blood cholesterol and lipid modifier (Julius & Fischer, 2013), NR has not been studied in that capacity. No long-term studies analyzing chronic NR supplementation and blood cholesterol and TGs exist, despite its potential to function in that capacity. The effect of NR on vascular function also requires investigation. Nicotinamide mononucleotide, an intermediate in the NR-NAD+ synthesis pathway has displayed the ability to improve vascular function in mice (de Picciotto et al., 2016).

This warrants further investigation of NR supplementation in the context of improving vascular function and health.

The body of literature on NR will certainly expand in the future as NR has only been approved for use in humans for less than five years. One area that requires analysis is the assessment of NR supplementation and its impact on oxidative stress. As mentioned earlier NAD+ is a key component in regulating oxidative stress, potentiating the idea that NR can improve oxidative stress regulation. Oxidative stress can be a result of a number of different circumstances. One common modality in which oxidative stress is produced is via the consumption of a high fat meal. Meals high in saturated fats are known to acutely spike oxidative stress biomarkers (Chan, 2016; Kesh et al., 2016). In assessing the impact of NR supplementation on postprandial oxidative stress, the goal is to provide an indication of the capacity of NR to limit increases in oxidative stress that results from common dietary situations in developed countries.

With this project we aimed to address the interaction between NR supplementation and postprandial oxidative stress as well as investigate the impact of NR supplementation on microvascular function using post occlusive reactive hyperemia measured with LSCI technology. We also determined the effects of NR on postprandial plasma glucose and triglycerides.

This study investigated the hypothesis that postprandial oxidative stress can be reduced with one week of 500 mg daily nicotinamide riboside supplementation. Additionally, we addressed the hypothesis that one week of 500 mg daily NR supplementation can improve forearm vascular function measured with post occlusive reactive hyperemia.

CHAPTER 3. INTRODUCTION

In developed countries westernized diets rich in saturated fats have become a staple of significant portions of the population. Increased saturated fat consumption has been implicated as a contributing factor in the development of diseases such including diabetes, obesity, and cardiovascular disease (Marshall & Bessesen, 2002; Phillips et al., 2012; Siri-Tarino et al., 2010). One of the purported mechanisms in which high saturated fat diets contribute to the pathogenesis of these disease states is via oxidative stress. Oxidative stress is a phenomenon in the body that occurs as a result of accumulation of free radicals or reactive oxygen species (ROS). Free radicals are molecules with an unpaired electron that are formed as metabolic byproducts of normal processes in cell metabolism, as well as via external sources (tobacco, pollution, radiation, medications (Phaniendra et al., 2015). Small concentrations of ROS in the body are not harmful, with free radicals actually playing an important role in the body's pathogen defense system (Dröge, 2002). Conversely, the over-accumulation of ROS in the body can lead to significant damage to proteins, nucleic acid, lipid membranes, and other essential cellular structures (Wu et al., 2013), this buildup of harmful free radicals is defined as oxidative stress. Reported levels of oxidative stress are known to spike following the consumption of a meal rich in fats (Ursini & Sevanian, 2002), a occurrence known as postprandial oxidative stress. It has been reported that alterations in meal composition influences levels of postprandial oxidative stress, with meals rich in saturated fats contributing to the largest increase in postprandial oxidative stress (Chan, 2016).

One potential regulator of oxidative stress is nicotinamide adenine dinucleotide (NAD+). NAD+ is an essential cofactor in several metabolic processes. NAD+ performs reduction and oxidation reactions by accepting and donating hydrogen molecules, a process which eventually

contributes to the production energy in the form of adenosine triphosphate (ATP), by oxidation of NADH to NAD+ at the electron transport chain (Yang & Sauve, 2016). Aside from its essential redox functionality in metabolism, NAD+ is also required for the sirtuin (SIRT) family of deacetylases (Imai & Guarente, 2016). SIRT1, the most studied of the sirtuins is essential for the deacetylation and activation of PGC1α, a powerful transcriptional coactivator that is a regulator of mitochondrial biogenesis. The capacity NAD+ has to serve as a cofactor in metabolism via redox reactions and perform as an essential activator of deacetylases that can function to reduce oxidative stress (Massudi et al., 2012), implies the maintenance of cellular NAD+ concentrations is crucial for limiting potential damage caused by oxidative stress.

In 2016, the first human trial of nicotinamide riboside (NR), an NAD+ precursor supplement, was completed. NR has recently gained traction due to its potential to increase NAD+ levels with limited side effects. Nicotinic acid (NA), also called niacin or vitamin B3, is another NAD+ precursor that was discovered in the early 20th century and was used to treat pellagra. Nicotinic acid has been established as one of the oldest lipid-lowering treatments and is still used today despite causing a strong flushing side effect in most users (Carlson, 2005). Due to its recent discovery, human trials of NR are still scarce, though, multiple studies that have been published have established NR supplementation as effective method for raising NAD+ levels in older adults with no significant side effects (Dolopikou et al., 2020; Martens et al., 2018). Because NAD+ levels decline with aging (Verdin, 2015), NR supplementation in older adults may be an effective way to combat this decline. Depleted NAD+ levels with aging is a potential contributing factor to the documented increase in age-associated oxidative damage to cells (Cui et al., 2012). Based on previously reported findings that chronic NR supplementation increases NAD+ in older and middle aged adults (Martens et al., 2018), it can be postulated that

NR supplementation could decrease oxidative stress levels in older adults by raising their NAD+ concentrations, specifically this improvement could be seen in postprandial situations that create significant increases in oxidative stress in a small period of time.

The effectiveness of nicotinic acid on lowering blood lipid levels is well documented (Julius & Fischer, 2013). Another NAD+ precursor nicotinamide (NMN) has displayed potential as a supplement that improves vascular function (de Picciotto et al., 2016). With earlier NAD+ precursors demonstrating long standing proven anti-atherogenic functions (Ruparelia et al., 2011), it can be hypothesized that supplementation of NR in humans may also provide similar benefits to its associated NAD+ precursors.

Post occlusive reactive hyperemia (PORH) has gained traction as an effective way to measure vascular function via assessment of microvascular response to occlusion of blood flow. PORH consists of occlusion of blood flow to a limb or area using a cuff, the release of the cuff results in corresponding hyperemic response to the tissues that had been occluded. and can be measured using laser speckle contrast imaging. PORH methods have of microvascular measurement have demonstrated the ability to function as an important indicator of cardiovascular disease pathogenesis (Tee et al., 2004)., while also being utilized for assessment of effects of medications and supplements on microvascular function (De Moraes et al., 2014)

One method utilized for the assessment of PORH is laser speckle contrast imaging (LSCI). With an LSCI camera instrument, measurement of blood flow is assessed via reflection of red blood cell movement which generates a dynamic speckled image, allowing fluctuations in blood flow to be identified (Humeau-Heurtier et al., 2013). The ability to translate PORH data using laser speckle images into quantifiable data allows for analysis of blood flow and

microvascular function of the imaged area. This measurement can be assessed to provide a potential outlook of overall vascular function.

While the amount of literature on NR will certainly continue to grow in the coming years, to our knowledge no studies have been published examining the effects of NR supplementation on postprandial oxidative stress and microvascular function. In this study, our aim was to address the impact of NR supplementation on postprandial oxidative stress and microvascular function in both young and old participants. We assessed the effects of one week of 500 mg daily NR supplementation on postprandial oxidative stress by measuring lipid peroxidation assessed by measuring plasma MDA levels following the consumption of a high fat meal. Microvascular blood flow was assessed postprandially using post occlusive reactive hyperemia measured with laser speckle contrast imaging. We hypothesized that with NR supplementation, participants would see a postprandial reduction in plasma MDA levels indicating reduced oxidative stress. Additionally, we also hypothesized that NR would improve PORH measures of postprandial microvascular function, and reductions in plasma triglycerides would be identified.

CHAPTER 4. METHODS

4.1 Participants

Sixteen participants, ages 18-30 and 60-70 were recruited to participate in the study. Participants were non-smokers, that did not have high blood pressure (<140/90 mmHg). Participants were excluded if they were taking any medication that interfered with blood pressure, heart function, or immune function. Individuals with a known metabolic (e.g. Type II diabetes or CVD) or immunologic (e.g. Cancer, HIV, autoimmune) disease were excluded from the study. Individuals with food allergies to egg, milk, soy, or wheat were unable to participate in the study. Those who were pregnant or planning to become pregnant during the study as well as individuals with a pacemaker or other implanted device were excluded from the study. Individuals inclusion or exclusion from the study were based on responses to initial screening questions and a medical history questionnaire. All participants involved in the study were informed of any potential risks involved with the experiment and signed an informed consent document. Participants did not receive compensation for taking part in the study.

4.2 Design and ethics statement

The study design consisted of a randomized, double blind, placebo-controlled, crossover design to investigate the effects of one week of nicotinamide riboside supplementation on postprandial vascular function and oxidative stress. Participants reported to the laboratory on three separate occasions. Each of the three visits utilized an identical protocol across all visits. An overview of the study design is depicted in Figure 4.2. All procedures involving human subjects were reviewed and approved by the Iowa State University Institutional Review Board. Written Informed consent was received from all subjects prior to participation.

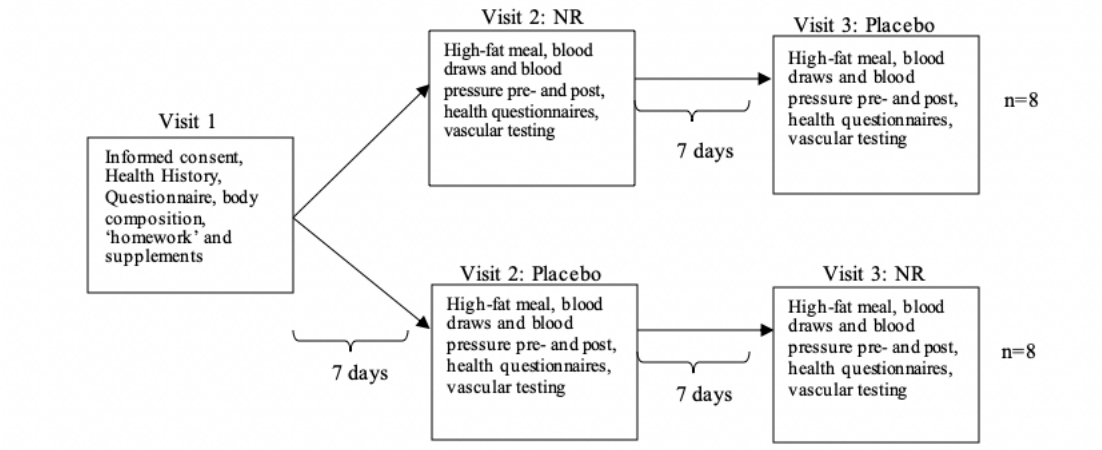

Figure 4.2 Study Design

4.3 Protocol

Visit 1

Investigator read and explained the informed consent. If signed, participants completed a health history questionnaire and using this the investigator determined if they should be included or excluded from the study. If they were included, the participant completed a body composition assessment. Body composition was determined using bioelectrical impedance analysis (BIA; InBody720). This instrument used a low level electrical current to determine lean body mass and body fat percentage. Height was determined using a stadiometer and weight using a beam platform scale. Waist and hip circumference were measured at the navel and widest portion of the hips respectively. At the conclusion of visit 1, participants were given a pedometer and pedometer log and were instructed to record their daily steps for the 7-days between visit 1 and visit 2. They were also given a food log and instructed to record, in detail the food consumed for the 72 hours leading up to the second visit. Finally, they were given a supplement container, in it,

were two pills for each day, that contained either nicotinamide riboside or placebo and a supplement log to document consumption.

Between Visits 1 and 2

Participants were asked to consume the supplements twice each day. It was recommended that they were consumed once in the morning, once in the evening for 7 days between visits 1 and 2. Documentation of supplement usage on the supplement log form was required. The provided pedometer was to be worn each day between the visits and step counts were recorded in the pedometer logs. Using the 72-hour food diary, participants documented, in detail, what foods they consumed and the quantity of each food that was consumed in the 3 days leading up to visit 2. Participants were asked to refrain from alcohol and caffeine consumption as well as heavy exercise for 24 hours prior to the appointment. Finally, participants were asked to report to the second visit fasted for a minimum of 12 hours.

Visit 2

Visit 2 was scheduled in the morning, 7 days following visit 1. Participants were asked to bring the pedometer and food logs as well as the empty supplement container to visit 2. Upon arrival to the lab, the participants entered a bed that had the individual reclining slightly. The participant rested for 15 minutes before an initial blood pressure was taken. A catheter was then placed in the arm for blood sampling. A baseline blood draw and occlusion vascular function test was conducted (as described below). Following the fasted baseline blood sampling, blood pressure, and forearm blood flow measure, participants were given 10 minutes to consume a 1050 kcal high fat meal described in the specific measures section. Over the course of the next 3 hours, blood samples were taken 30 min, 1h, 2h, and 3h post meal. Blood pressure was taken approximately every 15 minutes until 3 hours post meal. Forearm blood flow testing was

completed 30 min, 1.5h, and 3 hours post-meal. At the end of Visit 2 participants were given a second pedometer, supplement, and food log to fill out between visits 2 and 3. They were given a new supplement container of either supplement or placebo (crossing over from what they had before). Finally, they were given a copy of their initial food log in order to help them repeat their diet consumed prior to visit 2.

Between Visits 2 and 3

Participants were asked to repeat the same protocols described between visits 1 and 2 in an attempt to control for potential confounding variables. The goal for the participants was for the only meaningful difference from visit 2 to visit 3 being the change in supplementation (from NR to placebo or vice versa).

Visit 3

Participants were asked to take the provided supplement for 7 days as they did before visit 2. Visit 3 was conducted following the same procedure as visit 2. The participants returned the pedometer, and pedometer log, as well as the food diary, supplement log, and supplement container. A visualization of the data collection timeline in visits 2 and 3 is depicted in Figure 4.3.

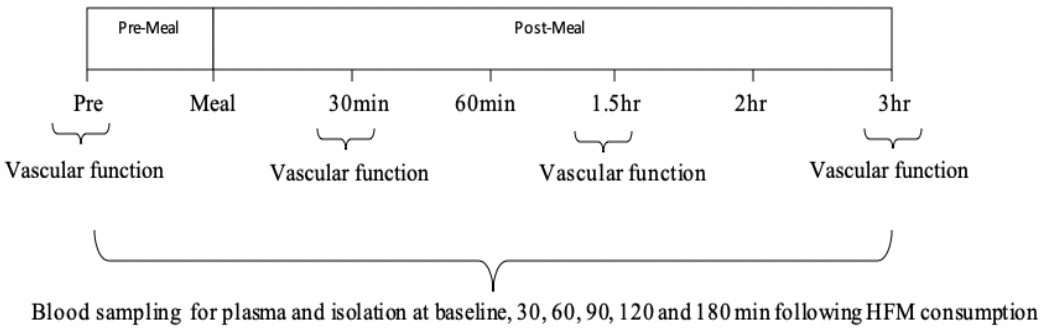

Figure 4.3 Data Collection Timeline

4.4 Specific Measures and Materials

Blood Sampling (Visit 2 and Visit 3)

For blood collection, a catheter was inserted into the participants arm at the beginning of the visits. Sampling for analysis of plasma glucose, TGs, and oxidative stress were taken at baseline, 30 minutes, 1 hour, 2 hours, and 3 hours post consumption of the high-fat meal. Blood samples taken were processed into both blood serum and plasma. Plasma and serum from each timepoint were frozen and stored at -80°C until utilized for analysis. The total amount of blood taken during each meal visit was 120 ml. Plasma glucose was measured using a Glucose hexokinase assay kit (Millipore Sigma, Shanghai, China). Plasma triglycerides were measured with triglyceride liquid regent assay (Pointe Scientific, Canton, MI, USA)

Blood Pressure and Heart Rate (Visit 2 and 3)

Once sitting in a semi-recumbent position, a blood pressure cuff was placed on the participant's upper arm, and an initial blood pressure was measured at baseline. Blood pressure was measured with automatic sphygmomanometer every 15 minutes postprandially. Heart rate was assessed with standard 3-lead electrocardiogram. Hear rate was measured continuously throughout the duration of the study.

Post Occlusive Reactive Hyperemia and Laser Speckle Contrast Imaging (Visit 2 and 3)

For microvascular assessments, participants arrived to the laboratory and were positioned in a semi-recumbent manner, resting for a minimum of 10 minutes prior to baseline assessment. After a minimum of 2-minutes of steady baseline skin blood flow was established, a pneumatic blood pressure cuff was inflated on the non-dominant arm to 200 mmHg for 5 minutes. After 5

minutes the blood pressure cuff was released. Flux measurements of skin blood flow were continuously recorded throughout the hyperemic response using a laser speckle contrast imager (LSCI), Moor Instruments, Axminister, UK). The LSCI was placed 15-20 cm above the surface of the forearm. LSCI measures the reflection of a laser on red blood cell movement to provide indices of blood flow. LSCI recorded flux data at 25 Hz at a wavelength of 785 nm. Camera to forearm distance and area of analysis were replicated for both visits. The site for skin blood flow measurement was standardized to a 10 cm region defined 3-13 cm below the antecubital fossa for all participants.

Flux measurements were normalized with blood pressured by dividing flux by mean arterial pressure (diastolic pressure + pulse pressure) to determine cutaneous vascular conductance (CVC). LSCI data assessed were CVC baseline, CVC peak flux, 15 second flux, 3-minute area under curve. This data was collected at baseline and 0.5, 1.5, and 3 hours post consumption of the high fat meal. This data was used to determine the impact of nicotinamide riboside supplementation on postprandial forearm microvascular function.

High Fat Meal (Visit 2 and 3)

After baseline fasting blood sampling and vascular testing were completed, participants were instructed to consume a standardized high fat meal in fewer than 10 minutes. The meal consisted of two sausage, egg, and cheese sandwiches (Jimmy Dean, Tyson Foods, Springdale AR), one honey bun (Little Debbie, McKee Foods, Collegedale, TN), and one 16.9 oz bottle of water. The meal contained 1050 calories (62%) fat, 72g of total fat; 30 g of saturated fat, and 235 mg of cholesterol, 1.5g of polyunsaturated fat, and 4g of monounsaturated fat.

MDA TBARS Assay

Oxidative stress was assessed using blood plasma measurements of lipid peroxidation byproduct malondialdehyde (MDA). MDA levels were obtained using a thiobarituric acid reactive substance assay kit (TBARS) (Cayman Chemicals, Ann Arbor, MI). Plasma MDA measurements were assessed at baseline and 0.5, 1, 2, and 3 hours following consumption of the high fat meal.

Statistical Analysis

Data are presented as mean ± SEM. Statistical differences in fasting between placebo and nicotinamide riboside were determined using a Student's t-test. A two-way repeated measures analysis of variance was used to compare effects of placebo and NR at various timepoints following the meal. Data analysis was performed using SPSS (SPSS version 24, IBM, NY, USA)

CHAPTER 5. RESULTS

5.1 Anthropometric and Physiologic Characteristics

Anthropometric and physiologic characteristics of all but one member of the study population (n=15) are outlined in Table 5.1. Participants were categorized into two distinct age groups, younger adults (n=13, mean age= 22.3 years old), and older adults (n=3, mean age=64.0 years old). Participant race distribution was 11 white, 2 Asian, 2 Latino, and 1 Pacific Islander.

Table 5.1. Participant Anthropometric and Physiologic Characteristics

Variable	Mean	Std. Error
Mass (kg)	82.5	4.3
Height (m)	1.75	0.03
BMI (kg/m2)	26.5	0.8
Age (years)	30	4
Percent body fat	22.3	1.8
Systolic blood pressure*	119	3
Diastolic blood pressure*	69	3

*Systolic and diastolic blood pressure reported from fasting baseline values without supplementation. Blood pressure units are reported in mmHg.

5.2 Post Occlusive Reactive Hyperemia Assessment of Microvascular Function

No reported effects of NR supplementation on microvascular function following the consumption of a HFM were identified with each of the assessment variables (Baseline CVC, Peak CVC, 15s flux, 3 min AUC), p=0.874, p=0.219, p=0.244, p=0.403, respectively. Significant differences in PORH measurements of microvascular function at different timepoints were also not identified, in each of the four assessed variables, p=0.358, p=0.653, p=0.45, p=0.342, respectively. Finally, no interaction between NR supplementation and time of measurement on the effects of microvascular function were reported, p=0.224, p=0.478, p= 0.355, p=0.874 respectively. Reported means and significance values are displayed in Table 5.2.

Table 5.2. Effects of Nicotinamide Riboside on Forearm Post Occlusive Reactive Hyperemia Blood Flow Measured with LSCI

	PLACEBO				NICOTINAMIDE RIBOSIDE				ANOVA		
Variable	Fasting	30 min	90 min	180 min	Fasting	30 min	90 min	180 min	Treatment	Time	Interaction
Baseline CVC	0.83±0.5[1]	0.86±0.07	0.91±0.09	0.89±0.07	0.85±0.05	0.89±0.57	0.86±0.06	0.86±0.05	0.874	0.358	0.224
Peak CVC	2.68±0.17	2.59±0.16	2.66±0.15	2.63±0.14	2.65±0.14	2.67±0.14	2.71± 0.14	2.74±0.15	0.219	0.653	0.478
15s flux	214.3±13.2	304.1±101.9	214.2±16.9	211.0±15.6	213.1±12.4	200.5±12.7	203.9±12.8	209.1±13.2	0.244	0.450	0.355
3min AUC	23811±1186	23007±1444	23836±1706	24843±1632	22757±1785	21585±1864	23207±1408	23534±1272	0.403	0.342	0.874

[1]Statistics are shown as mean± SEM. Units expressed are PU/mmHg, PU are arbitrary units indicating velocity of red blood cells. All values analyzed were normalized with participants mean arterial pressure(mmHg) during the measured timepoints. Significance was determined with $P<0.05$. Differences in LSCI assessed blood flow reflect changes in forearm microvascular function. CVC (Cutaneous Vascular Conductance), LSCI (Laser Speckle Contrast Imaging), AUC (Area under the curve).

5.3 Plasma Triglycerides

Postprandial plasma triglycerides (TG) were analyzed in 11 subjects. No significant effects were identified between postprandial NR and placebo plasma TG measurements ($p=0.624$). Significant increases in plasma TG were seen over time ($p<0.001$) Significant differences between the young and old groups were identified in TG AUC values ($p<0.001$). Finally, an interaction between age and time in our plasma TG data nearly reached significance ($p=0.051$). Postprandial effects of NR on plasma TG values are depicted in Figure 5.3.

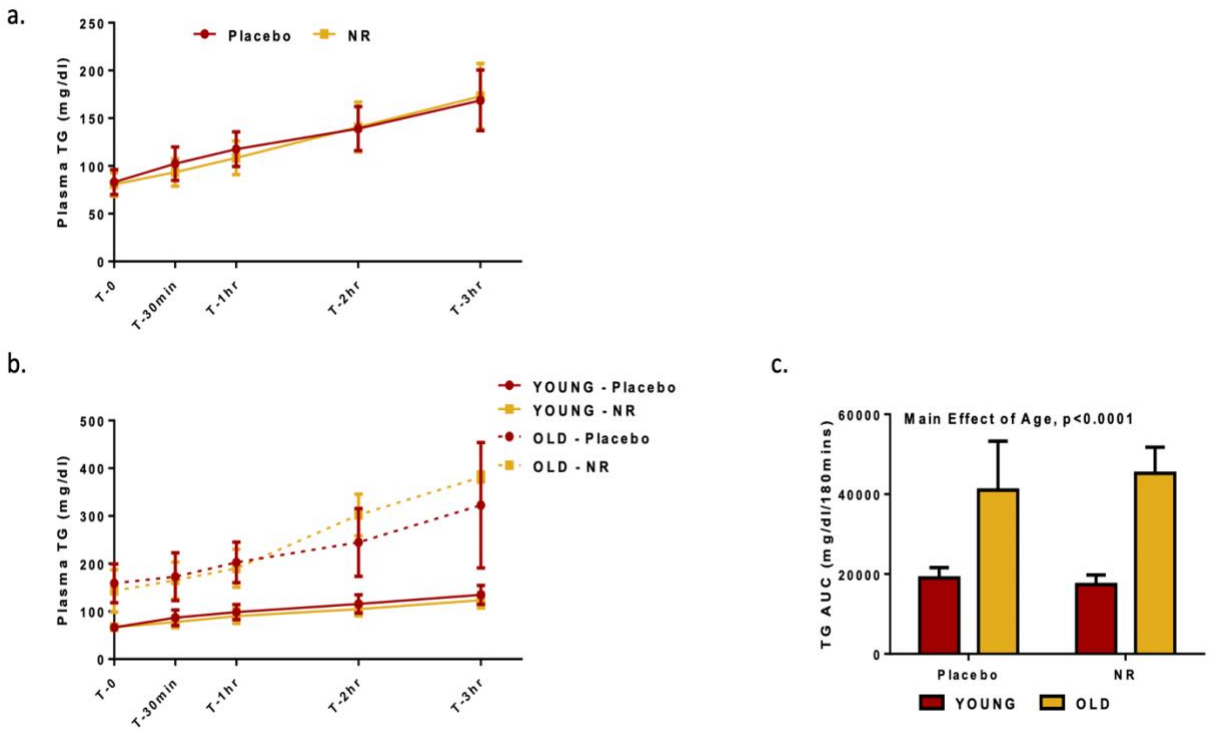

Figure 5.3 (a)The effect of NR on postprandial plasma TG levels in all participants (b) age related changes in postprandial plasma TG values (c) mean plasma TG area under curve values separated by age. Significance was determined with $p<0.05$ ($n=11$)

5.4 Plasma Glucose

Postprandial plasma glucose was also assessed in 11 subjects. No significant effects were identified between postprandial NR and placebo plasma glucose values (p=0.831). The old group had significantly elevated postprandial plasma glucose levels compared to the young group (p=0.04). Significant changes in plasma glucose over time following consumption of the HFM were identified (p<0.001), though no interaction between age time was identified for plasma glucose (p=0.756). Postprandial effects of NR supplementation on plasma glucose are shown in Figure 5.4.

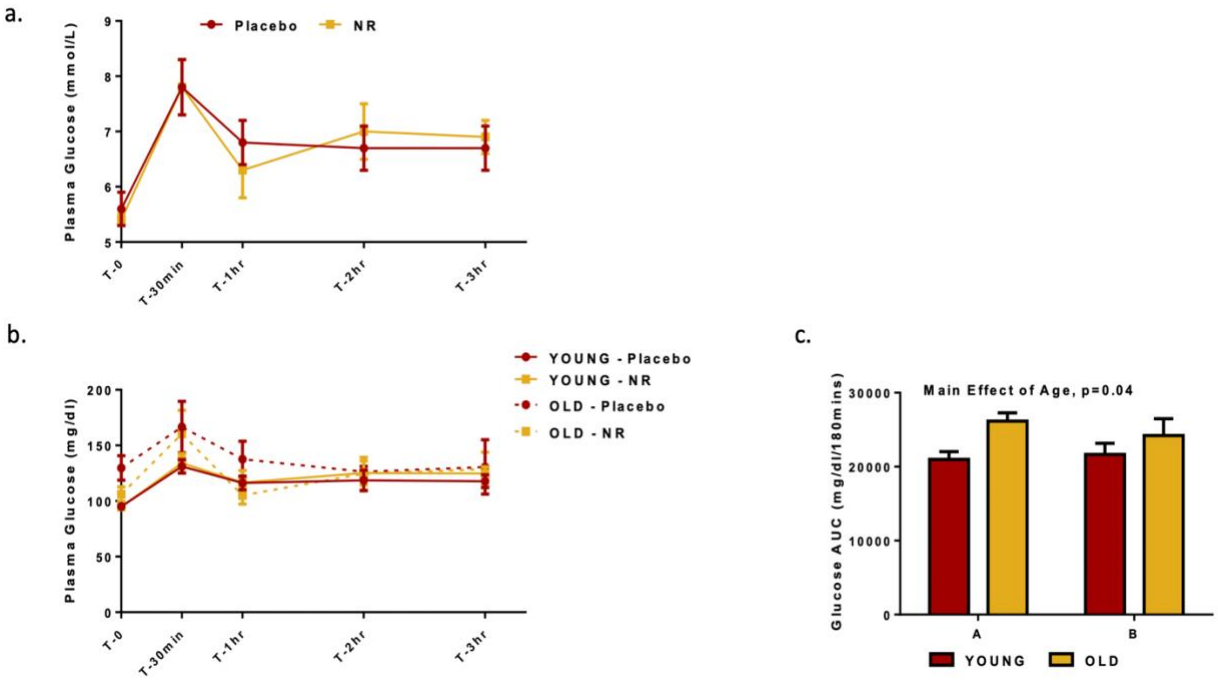

Figure 5.4 (a) The effect of NR on postprandial plasma glucose levels in all participants (b) age related changes in postprandial plasma glucose levels (c) mean plasma glucose area under curve values distinguished by age. Significance was determined with p<0.05 (n=11).

5.5 Postprandial Oxidative Stress

Determination of oxidative stress was measured with Malondialdehyde (MDA) to indicate lipid peroxidation. Plasma MDA was measured in 14 participants. No significant impact was identified between postprandial NR and placebo indicators of lipid peroxidation ($p=0.370$). Significant differences in MDA values between the old and young groups were identified in MDA area under the curve measurements ($p<0.001$). The effect of time on postprandial MDA was significant ($p<0.001$), though no interaction between time and age was identified ($p=0.423$). A comparison of placebo and NR effects on postprandial lipid peroxidation measured with MDA can be found in Figure 5.5.

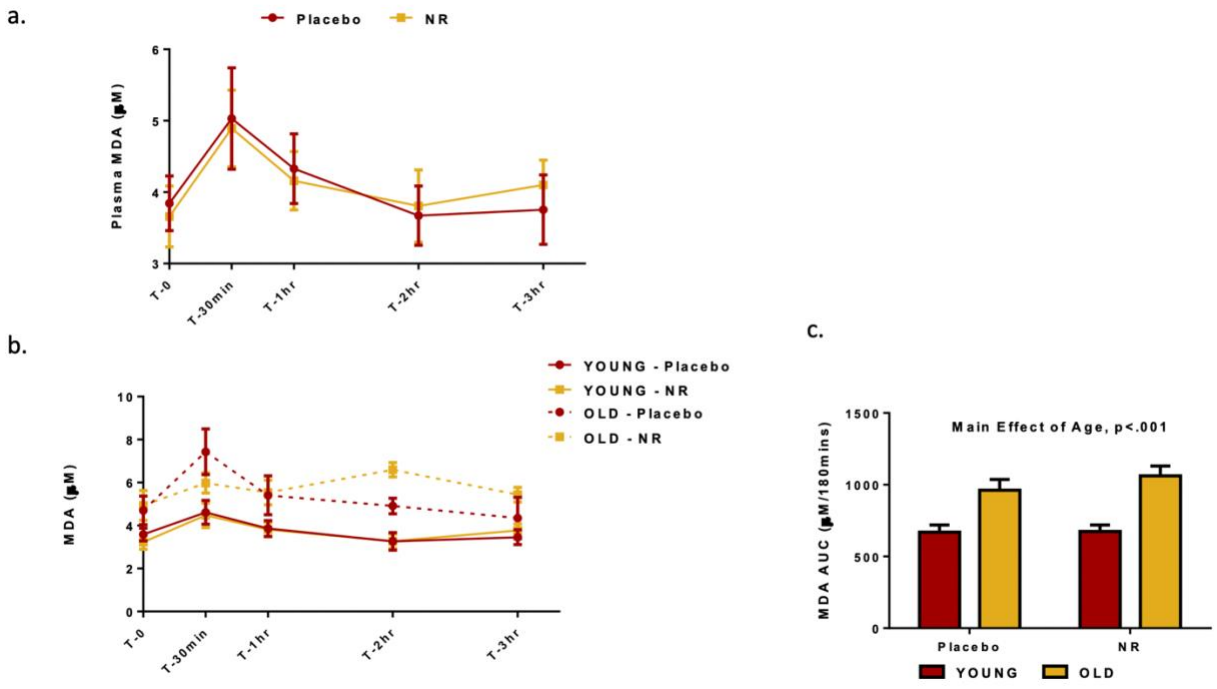

Figure 5.5 (a)The effect of NR on postprandial plasma malondialdehyde levels in all participants (b) age related changes in postprandial plasma malondialdehyde levels (c) mean plasma malondialdehyde AUC values separated by age. Significance was determined with $p<0.05$ (n=14).

CHAPTER 6. DISCUSSION AND LIMITATIONS

6.1 Discussion

One week of nicotinamide riboside supplementation did not have a significant effect on postprandial oxidative stress compared to placebo. Additionally, NR supplementation did not impact forearm microvascular function measured using laser speckle contrast imaging following the consumption of a high-fat meal. Postprandial plasma glucose and triglyceride values were also not affected by NR supplementation compared to placebo, Significant differences were established between young and old groups in postprandial plasma triglyceride ($p<0.001$), plasma glucose ($p=0.04$), and lipid peroxidation measured with MDA ($p<0.001$).

The inability of one week of 500 mg daily NR supplementation to produce meaningful differences in lipid peroxidation measurement of oxidative stress (MDA) and forearm microvascular function in young individuals was the most substantial finding in this study. The minimal effects seen by NR in young participants indicated that NR may not have impacted NAD+ concentrations in these participants, or that NAD+ was already sufficient in young individuals. One of the mechanisms that has been implicated with a rise in of oxidative stress with aging is the depletion of NAD+ levels (Imai & Guarente, 2016; Verdin, 2015). Consistent with previous findings, (Dolopikou et al., 2020), our results demonstrated that lipid peroxidation, an indicator of oxidative stress was significantly elevated in the older group of participants. NAD+ functions as a regulator of oxidative stress due to its dual functionality as a metabolic cofactor, regulating redox homeostasis in metabolism with electron transfer in various metabolic reactions, as well as its action as a signaling molecule and activator of other oxidative stress limiting compounds. One class of oxidative stress inhibiting molecules that are dependent on NAD+ for activation is the sirtuin family of deacetylases. Literature regarding the sirtuins

(SIRT1-7), has established the involvement of SIRT1 and SIRT3 in various important biological processes including regulation of energy utilization, mitochondrial biogenesis, inflammation, and apoptosis (Ansari et al., 2017; Chang & Guarente, 2014). Due to the requirement of NAD+ for sirtuin activity, it has been implicated that the decline in sirtuin activity in older adults is at least in part, due to depleted NAD+ levels older individuals (Imai & Guarente, 2016; Yuan et al., 2016). In this study we did not find NR supplementation to reduce lipid peroxidation indicators of oxidative stress in older adults. One previously published study did find that NR reduced levels of oxidative stress in older adults (Dolopikou et al., 2020), and because only three older adults were tested in our study, it is possible that with a larger sample, significant differences would have been established. While there is current lack of literature on human studies that have investigated the effects of NR on oxidative stress, the ability for NR to increase NAD+ levels in older adults, implies that with further testing, NR may demonstrate the ability to reduce postprandial oxidative stress. The results of this study reporting NR supplementation led to no significant differences in lipid peroxidation measurements of postprandial oxidative stress in all groups. Additionally, with our limited number of older adults tested(n=3), we were unable to establish definitive effects of NR on older adults. Further testing of NR and its effects on oxidative stress in older adults is required.

While we were unable to detect an impact of NR on oxidative stress for all participants, significant increases in lipid peroxidation following the consumption of a high fat meal were identified ($p<0.001$, Figure 5.5). The increase in postprandial oxidative stress aligns with reports from other publications that have documented similar changes (Chan, 2016; Sies et al., 2005). In our data, the rise in concentration of MDA values reflected similar increases in plasma glucose levels, each appearing to peak 30 minutes postprandially. The corresponding increases between

reported MDA and plasma glucose further confirms the previously described notion that oxidative stress can be dictated by an individual's diet (Chan, 2016; Kesh et al., 2016). The similarity between our reported postprandial MDA and plasma glucose curves (Figures 5.4 and 5.5), as well as established reports of increased postprandial oxidative stress (Chan, 2016; Perez-Martinez et al., 2010; Sies et al., 2005) indicate that our TBARS MDA assay results were a valid measure of determining oxidative stress.

One of the potential mechanisms in which NAD+ may regulate oxidative stress is the activation of sirtuins. Sirtuin activation is dependent on the presence of NAD+. SIRT1, the most studied of the sirtuin family has a number of different functions. SIRT1is responsible for the activation of a family of transcription factors known as FOXO, which promote the expression of stress response genes that can combat oxidative stress (Carles Cantó et al., 2015). SIRT1 is also responsible for deacetylating and activating PGC1α. PGC1α is a transcriptional coactivator that plays a large role in regulating mitochondrial biogenesis (Brunet et al., 2004). Additionally, PGC1α is a key regulator of a number of mitochondrial antioxidant genes that prevent oxidative injury and preserve mitochondrial function, consequently, participating in the prevention of oxidative stress (Rius-Pérez et al., 2020). NAD+ has the unique duality of functioning as a redox mediator itself, while also activating other compounds that play a role in modulating oxidative stress such as SIRT1. Despite our data indicating that NR supplementation did not reduce postprandial oxidative stress in older adults, the establishment of NR as mechanisms the oxidative stress reducing mechanisms demonstrated by NAD+, make further investigation into the effects of NR on oxidative stress necessary, specifically in older adults.

As expected, significant increases were reported in plasma glucose concentrations 30 minutes following the consumption of the meal ($p<0.001$). Plasma glucose concentrations were

unaffected by nicotinamide riboside supplementation in both age groups. The older group had significantly higher postprandial plasma glucose values than the younger group ($p=0.04$, Figure 5.4). The finding that older adults had elevated plasma glucose compared to the young group is consistent with previous reports of age-related changes in postprandial blood glucose concentrations (Fraze et al., 1987). One mechanism responsible for the reported age-related changes in plasma glucose is likely caused by alterations in insulin function associated with aging. Insulin, one of the primary regulators of blood glucose, has demonstrated reduced activity in older adults with age-related beta-cell dysfunction leading to impaired insulin secretion being a key mechanism responsible for increases in blood glucose with aging (De Tata, 2014).

Our data demonstrated NR had no effect on postprandial plasma triglyceride levels. We did, however, see a significant increase in plasma TG values in the old group compared to the young ($p<0.001$). It has been established nicotinic acid is an effective lipid lowering supplement (Carlson, 2005). NR, a related NAD+ precursor had not yet been studied in this capacity. Our results indicated one week of supplementation of NR did not affect plasma TG levels both fasting and postprandially. Long-term chronic consumption of NR and its effects on plasma TG and cholesterol requires investigate in order to better understand if NR is an effective lipid lowering alternative to NA.

Post occlusive reactive hyperemia measured using laser speckle contrast imaging has been previously utilized in the assessment of supplements on microvascular function (De Moraes et al., 2014). Previous literature has described measurement of microvascular function as a potentially effective method for determining vascular function as it related to the pathogenesis of cardiovascular disease (Roustit & Cracowski, 2012). In this study microvascular function was

assessed using PORH measured with LSCI to assess the impact of NR supplementation on microvascular function.

No reported differences in microvascular function were identified between NR and placebo in both young and old participants identified at any point throughout the study (Table 5.2). Furthermore, we also saw no differences in PORH LSCI data between time all time points. A difference in the values between fasting and postprandial PORH results was expected as previous studies have reported decreased postprandial blood flow (Baron et al., 1990). No differences were reported in PORH LSCI blood flow measurements between any of the timepoints. The inability PORH LSCI blood flow data to detect differences in microvascular function following the consumption of a high-fat meal led to the conclusion that measurement of PORH with LSCI was not an effective measurement tool for assessing the effects of NR supplementation on microvascular function. While LSCI PORH measurements have been establish as effective tools to measure vascular function, for the purposes of this study, the detection of microvascular function retrieved using LSCI was not sensitive enough to establish expected differences in fasting and postprandial blood flow and therefore no definitive conclusions regarding the NR as it relates to vascular function can be observed from this study.

Further postprandial assessment of nicotinamide riboside is required to establish definitive effects of NR on postprandial oxidative stress and microvascular function. Additionally, chronic supplementation of NR should be further investigated to determine its effects on blood triglycerides and cholesterol.

6.2 Limitations

There were several limitations in this study that must be noted. The first limitation of the study was the sample. The study had 16 participants, 13 young participants with an average age of 22.3 years old, and 3 older participants with an average age of 64 years old. The unequal group sizes and small group of older adults made identifying significant differences in response to NR between older and younger groups extremely unlikely. A more balanced sample size including an equal number of young and old participants would have been ideal. In future studies investigating NR, assessing the effects of supplementation on middle aged and older adults should be a priority. Previous publications have indicated NR raises NAD+ levels in older and middle aged adults (Dolopikou et al., 2020; Tee et al., 2004), with the knowledge of age related NAD+ decline, NR may provide significant anti-aging benefits including oxidative stress reduction and therefore, more experiments focusing on NR and older adults need to be completed.

The second major limitation of the study was in the study design. The study took place over three visits with one week of NR or placebo supplementation between visits 1-2 and 2-3, between weeks of supplementation, no washout period was included in the study. Incorporating a one-week washout period between beginning supplementation would eliminate any potential carryover effects of NR for those who received it during the first visit.

A third substantial limitation was the inability of laser speckle contrast imaging measurements of PORH to detect expected changes in blood flow. While LSCI and PORH have both been validated as effective methods to assess microvascular function (Bezemer et al., 2010; De Moraes et al., 2014; Roustit & Cracowski, 2012), In this study, the data retrieved from LSCI

measurements of PORH failed to detect significant differences in microvascular function between fasting flood flow and all postprandial timepoints. The inability to detect said differences indicated that our LSCI measurements of PORH were likely not sensitive enough to detect any potential microvascular changes that may have occurred as a result of NR supplementation. Future studies assessing the effect of NR on microvascular function may benefit from more sensitive techniques of measurement such as skin video microscopy to measure capillary density (Houben et al., 2017)

A third limitation of the study was our inability to complete glucose (n=11), triglyceride (n=11), and oxidative stress (n=14), measurements in all participants. The smaller sample size tested likely reduced our ability to determine any significant differences resulting from the supplementation of NR of plasma glucose, triglyceride, and MDA values. Additionally, because not all of the participants were assessed for these outcomes, the data that has been reported is not reflective of the entire population of the study.

Another potential limitation of the study was the dose of NR given and the length of supplementation. In this study participants consumed 250mg NR 2x/day for one week. Though the body of literature on NR testing in human subjects is still extremely scarce, a study published in 2018 that was successful in raising NAD+ levels with NR supplementation, had participants consume 1000mg/day for six weeks (Martens et al., 2018). While no current standard dose of NR has been established, it is possible that future studies assessing NR supplementation on oxidative stress would see a greater impact using higher doses of NR over longer periods of supplementation compared to our study.

The final limitation in this study was the inability have precise control over participants dietary intake and exercise during the study. We attempted to combat the potential issue of

dietary intake and exercise by providing participants with pedometers to measure fluctuations in activity levels. We also provided participants a food log to track dietary intake for the 72-hours prior to the study. After the second visit, a copy of that food log was provided, and participants were instructed to try to the best of their ability to recreate their diet from before visit 2. Despite this, differences in dietary intake in the period of supplementation before visits could still have an impact on oxidative stress. Previous studies have described the impact of dietary intake on oxidative stress (Chan, 2016; Perez-Martinez et al., 2010) While it is impractical to entirely dictate the diet of participants for the leading up to postprandial oxidative stress testing, one potential solution could involve providing participants a form of standardized meal for the two days prior to testing, ensuring that all participants dietary intake is the same for the two days before testing.

CHAPTER 7. GENERAL CONCLUSION

One week of 500 mg daily nicotinamide riboside supplementation did not have an effect on postprandial oxidative stress measured using lipid peroxide biomarker malondialdehyde, nor did it effect postprandial microvascular function assessed with post occlusive reactive hyperemia measured using laser speckle contrast imaging. No effects of NR were reported on plasma triglyceride and glucose levels. Significantly elevated levels of oxidative stress, plasma glucose and triglycerides were recognized in older adults compared to the younger group.

No significant side effects from supplementation were reported. Similar studies assessing the effects of NR on oxidative stress and vascular function are needed in order to determine the effectiveness of NR supplementation in these capacities. The findings in the current study warrant further research on nicotinamide riboside and its function as an NAD+ elevating supplement and its potential influence postprandial oxidative stress and vascular function.

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APPENDIX. INSTITUTIONAL REVIEW BOARD APPROVAL FORMS

IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY

Institutional Review Board
Office for Responsible Research
Vice President for Research
2420 Lincoln Way, Suite 202
Ames, Iowa 50014
515 294-4566

Date: 1/8/2018

To: Dr. Rudy Valentine
243 Forker Building

CC: Dr. James Lang
103R Forker

From: Office for Responsible Research

Title: Effects of nicotinamide riboside on vascular function and oxidative stress following a high-fat meal

IRB ID: 17-603

Approval Date:	1/8/2018	**Date for Continuing Review:**	12/18/2019
Submission Type:	New	**Review Type:**	Full Committee

The project referenced above has received approval from the Institutional Review Board (IRB) at Iowa State University according to the dates shown above. Please refer to the IRB ID number shown above in all correspondence regarding this study.

To ensure compliance with federal regulations (45 CFR 46 & 21 CFR 56), please be sure to:

- **Use only the approved study materials** in your research, **including the recruitment materials and informed consent documents that have the IRB approval stamp.**
- **Retain signed informed consent documents for 3 years after the close of the study,** when documented consent is required.
- **Obtain IRB approval prior to implementing** <u>any</u> **changes** to the study by submitting a Modification Form for Non-Exempt Research or Amendment for Personnel Changes form, as necessary.
- **Immediately inform the IRB of (1) all serious and/or unexpected adverse experiences** involving risks to subjects or others; and **(2) any other unanticipated problems involving risks** to subjects or others.
- **Stop all research activity if IRB approval lapses,** unless continuation is necessary to prevent harm to research participants. Research activity can resume once IRB approval is reestablished.
- **Complete a new continuing review form** at least three to four weeks prior to the **date for continuing review** as noted above to provide sufficient time for the IRB to review and approve continuation of the study. We will send a courtesy reminder as this date approaches.

Please be aware that IRB approval means that you have met the requirements of federal regulations and ISU policies governing human subjects research. **Approval from other entities may also be needed.** For example, access to data from private records (e.g. student, medical, or employment records, etc.) that are protected by FERPA, HIPAA, or other confidentiality policies requires permission from the holders of those records. Similarly, for research conducted in institutions other than ISU (e.g., schools, other colleges or universities, medical facilities, companies, etc.), investigators must obtain permission from the institution(s) as required by their policies. **IRB approval in no way implies or guarantees that permission from these other entities will be granted.**

Upon completion of the project, please submit a Project Closure Form to the Office for Responsible Research, 202 Kingland, to officially close the project.

Please don't hesitate to contact us if you have questions or concerns at 515-294-4566 or IRB@iastate.edu.