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THE EFFECTS WATERMELON JUICE ON POST PRANDIAL VASCULAR ENDOTHELIAL FUNCTION AND BLOOD FLOW DURING HYPERGLYCEMIA

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THE EFFECTS WATERMELON JUICE ON POST PRANDIAL VASCULAR ENDOTHELIAL FUNCTION AND BLOOD FLOW DURING HYPERGLYCEMIA

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and Agricultural and
Mechanical college in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Kinesiology

by

Cullen Michael Vincellette

B.S. Louisiana State University, 2017

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Abstract

Impaired endothelial function and microvascular dysfunction are frequently observed in individuals with cardiovascular risk factors such as obesity, insulin resistance, and type 2 diabetes. Reduced nitric oxide (NO) bioavailability is considered to be central to the development of these risk factors. NO can be generated via enzymatic reaction via NO synthase which requires the amino acid arginine. Evidence suggests that the amino acid citrulline is more effective at increasing circulating arginine levels than arginine supplementation alone. The purpose of this pilot study is to determine the potential for citrulline-rich watermelon juice (WMJ) to attenuate the reduction in postprandial endothelial function associated with reduced skeletal muscle microvascular blood flow (MVBF) during hyperglycemia. Seventeen (12 female) non-obese (%Fat Mass= $27.72 \pm 7.89\%$) young adults (Age= 23 ± 3 y), with normal or overweight BMI (BMI= $23.48 \pm 3.22 \text{ kg/m}^2$) were enrolled in this study. In a randomized, crossover, double-blinded design, participants supplemented with WMJ or placebo (PLA) (average 12.8, 10-16 days) then underwent an oral glucose tolerance test (OGTT) after an overnight fast. Flow-mediated dilation (FMD) was greater after WMJ compared to PLA following the oral glucose challenge (treatment $p=0.038$). Baseline oxygen saturation (StO₂) was significantly higher in WMJ vs PLA ($p=0.031$) and the baseline StO₂ area under the curve (AUC) was significantly greater in WMJ vs PLA ($p=0.03$). Our findings suggest that WMJ supplementation is a potentially effective method for improving vascular responsiveness during hyperglycemia via improved skeletal muscle tissue oxygen saturation and improved resting endothelial function.

Chapter 1. Literature Review

1.1 Introduction

17.9 million people die each year from cardiovascular disease (CVD) which accounts for 31% of deaths worldwide with the majority of these coming from a heart attack and stroke [20]. Cardiovascular disorders are related to the heart and vessels and include coronary heart disease and rheumatic heart disease. Individuals with CVD have a higher likelihood of other risk factors such as endothelial dysfunction, hypertension, atherosclerosis, insulin resistance, obesity, and type 2 diabetes (T2D). These diseases and risk factors have strong vascular and endothelial components if improved could improve quality of life and reduce the frequency of premature death. CVD and many of the related diseases are known or suspected to be related to diminished arginine and nitric oxide (NO) bioavailability which contribute to vascular and endothelial dysfunction [33].

The vascular and endothelial systems work in conjunction as a network of vessels and muscles throughout the entire body. The vascular system is responsible for distributing blood and nutrients throughout the body to all of the tissues. The endothelium acts as a regulator as well as a barrier between the blood and other tissues of the body. Blood flow is largely affected by the vascular tone which can be vasodilated or vasoconstricted to change the amount of blood flow to an area. Vascular tone is altered by endothelium-derived or non-endothelium derived vasodilation or vasoconstriction.

Non-endothelium dependent changes in vascular tone are regulated by the autonomic nervous system. Individuals' sympathetic response to extrinsic stimuli such as stress, danger, or pain directly elevating vascular resistance [39]. The endothelium is innervated with neural

transmitters from the autonomic nervous system signals the change in vascular tone.

Neurotransmitters including norepinephrine and acetylcholine can act on the endothelium and counteract endothelial-dependent changes in the vasculature. Norepinephrine when bound to the α_2 -adrenergic receptors on the endothelium counteract the endothelium-dependent vasodilation and induce vasoconstriction of the endothelium smooth muscle cells [39]. Acetylcholine can mediate vasorelaxation by regulating NO release, but it can also inhibit NO production if activated on the M2 and M3 receptors [39]. Conversely, endothelium-dependent changes are mediated by the bioavailability of NO.

Diminished NO bioavailability is considered to play a role in nearly all cardiometabolic disease states. Patients with T2DM are at increased risk for developing micro- and macrovascular dysfunction which contributes to the increased risk of CVD in this group. This review will focus on the effects of hyperglycemia on vascular and endothelial function as well as a possible solution by increasing the bioavailability of NO. Citrulline has shown promising effects on NO bioavailability that could dampen the effects of cardiometabolic disease on the vascular and endothelial systems.

1.2 Nitric Oxide

Discovered in 1987, NO is a vasoprotective molecule that is synthesized by endothelial nitric oxide synthase (eNOS) which can be a major component to fighting cardiovascular disease [21]. NO is responsible for changing the tone of the vasculature regulating hemodynamics of the associated tissues. Endothelium-derived NO is synthesized from the amino acid L-arginine by eNOS, yielding L-citrulline as a byproduct [2]. After NO is synthesized, NO diffuses across the endothelial cell membrane, entering the vascular muscle cells where soluble guanylate cyclase (sGC) is activated leading to an increase in cyclic guanosine monophosphate (cGMP) synthesis

[4]. cGMP acts as a secondary messenger that controls the vascular tone and platelet function by mediating the effects of NO [4]. This process makes NO a potent vasodilator while also suppressing platelet aggregation, leukocyte migration and cellular adhesion to the endothelium, and attenuates vascular smooth muscle cell proliferation and migration [22].

Nitric oxide undergoes rapid oxidation to form nitrite, and further oxidation into nitrate. Conversely, NO can be generated the reduction of nitrate and nitrite by a series of biological reactions in the body (25, 26). This reaction along with oral ingestion are the two main sources of nitrate/nitrites in human metabolism [24]. Once ingested, foods naturally high in nitrates such as beetroot, spinach, and celery are broken down by the saliva and reduced from nitrate to nitrite and are absorbed through the gastrointestinal tract entering circulation [23]. Nitrite is then reduced to NO in the cells and tissues by several proteins and enzymes [24]. Carlstrom et al (2015) suggests that higher chronic doses of nitrates could be detrimental to endothelial function by increasing the formation of reactive nitrogen oxides as well as decreasing the amount of sGC while smaller and acute doses are beneficial [23].

1.3 Citrulline

Citrulline is a non-essential amino acid that has been shown to increase the bioavailability of NO production by increasing the bioavailability of arginine, a precursor to NO. Citrulline is also an important component in the urea cycle in the liver and kidneys [6]. While being mainly used in NO production, citrulline has been used as a biomarker for functional small bowel enterocyte mass due to being almost exclusively synthesized in the small intestine [31]. In addition, increased plasma citrulline has been noted as a potential marker for renal function due to the kidneys being the main site of citrulline conversion to arginine [32]. Citrulline is typically supplemented by watermelon juice, watermelon extract, pharmaceutical grade citrulline, or

citrulline conjugated with malate (1:1 ratio) [30]. The main food source of citrulline is watermelon, particularly the rind and seeds have the highest concentrations, with 1.6-3.5 g/kg of citrulline per whole watermelon [1]. Citrulline is effective at doses as low as 1g/day and as high as 10g/day with no known toxicity [1, 50]. With the possible endothelial benefits, watermelon products (juice, extract, etc.) should be considered as a natural, relatively cost-effective alternative to pharmaceutical-grade citrulline.

The oxidative production of NO and citrulline requires the three main nitric oxide synthase enzymes (eNOS, iNOS, and nNOS) [6]. Arginine is hydrolyzed yielding NO and citrulline. Citrulline is then sustained in the endothelial cells yielding more arginine to create NO [7]. Citrulline is coproduced with NO as an end product of NOS activity, which can be then be recycled into arginine for additional NO production in this citrulline- NO cycle [16]. When ingested, approximately 40% of arginine is catabolized by intestinal bacteria then passes through the liver where arginase breaks down arginine yielding urea and ornithine accounting for another 10-15% extraction [3, 15]. Unlike arginine, citrulline is not acted on by first-pass extraction in the liver by arginase enzyme allowing for citrulline to be converted to argininosuccinate and subsequently arginine in the kidneys by argininosuccinate lyase [55]. This process yields more circulating arginine allowing eNOS to produce NO.

NO is an integral part of endothelial physiology, leading to increased vasodilation, therefore improving blood pressure and serum cholesterol along with several other factors. Citrulline can be converted to arginine in the kidneys, bypassing the uptake of the liver allowing for an increase in NO downstream. Conversely, ingested arginine will be taken up by the liver, and converted into urea and ornithine, thereby diminishing the amount of arginine that can be used to create NO.

1.4 Vasoprotective effects

Individuals with diabetes, impaired glucose tolerance, and even normal individuals post glucose intake suffer from impaired endothelium-dependent NO mediated vasorelaxation [11]. Endothelial dysfunction has been shown to possibly be linked with low levels of arginine as well as a possible increased production of oxygen-derived free radicals that are likely associated with repeated or prolonged bouts of postprandial hyperglycemia [10]. Kwano et al. (1999) found a decreased association in brachial artery vasodilation with increased plasma levels of thiobarbituric acid reactive substances (TBARS) that parallel the plasma glucose levels in the respective groups, while serum nitrite/nitrate levels remained the same post glucose loading [10]. Diseases that cause impaired endothelial function and increase reactive oxygen species (ROS) such as atherosclerosis and diabetes, have been shown to cause eNOS uncoupling, which causes eNOS to produce the oxygen-derived free radical superoxide instead of NO which lead to a reduction in eNOS activity [27, 21]. During periods of high oxidative stress, superoxide is produced. Superoxide then reacts with NO to produce peroxynitrite. The production of peroxynitrite leads to the oxidation of tetrahydrobiopterin (BH_4) into BH_2 which cannot be recycled back into BH_4 , causing the uncoupling of eNOS and reducing oxygen to superoxide and diminishing the synthesis of NO [28]. In healthy individuals, BH_4 maintains the function of eNOS by being oxidized into trihydropterin radicals (BH_3H^+) which are then recycled into BH_4 by reduction [28].

1.5 Flow-Mediated Dilation and Near-Infrared Spectroscopy

Changes in the vascular and endothelial tissues are best measured by using flow-mediated dilation (FMD) of the brachial artery. Near-infrared spectroscopy (NIRS) is another method to measure microvascular responses post-ischemia and can be used in conjunction with FMD.

FMD and NIRS have been used in conjunction to measure the vascular and microvascular response to hyperglycemia. FMD is a non-invasive measure believed to reflect endothelium-dependent NO-mediated arterial function and vascular health [41]. NIRS has been proven to be a valid measure to detect changes in oxidative metabolism as well as microvascular responsiveness during an oral glucose challenge [42, 43]. FMD and NIRS have recently been used in conjunction to support the relationship of down (brachial artery FMD) and upstream (forearm NIRS) vascular reactivity [44, 45, 46]. Soares et al. (2019) showed a significant correlation ($r=0.66$; $P = 0.001$) between the NIRS reperfusion slope and FMD % in healthy active individuals [44].

1.6 Type 2 Diabetes

T2D involves many complications with the vascular and endothelial systems. Vascular disease, which originates from endothelial dysfunction, is the primary cause of morbidity and mortality in individuals with T2D [34, 35, 36]. Insulin-stimulated NO production via increasing NOS stimulation, thus insulin-resistant individuals have a reduced NO-mediated vasodilatory response [48]. Individuals with T2D have diminished endothelium-derived vasodilatory in response to NO as well as impairments to the sympathetic nervous system compounding further vascular dysfunction [48]. Endothelial dysfunction is likely due to chronic hyperglycemia, the eNOS inhibitor asymmetric dimethylarginine (ADMA), and ROS in individuals with T2D. Chronically elevated glucose levels can inactivate the NOS cofactors located in the kidney and endothelium, causing NOS uncoupling and therefore reductions in citrulline concentrations [37, 38]. Individuals with T2D typically have elevated circulating free fatty acid levels. The elevated levels of free fatty acids activate lipoprotein lipase to metabolize the high levels of free fatty acids circulating and decrease insulin sensitivity. The elevated levels of circulating free fatty

acids could be a contributing factor to reduced NOS activity thus a decreased NO bioavailability in insulin-resistant individuals [48].

1.7 Hyperglycemia

Postprandial hyperglycemia in which the vascular recovery of the endothelium is compromised after ingesting glucose or a mixed meal has been shown to contribute to CVD. Recent evidence demonstrates that acute or postprandial hyperglycemia shows impairments in vascular function of individuals with a history of vascular endothelial dysfunction as well as healthy individuals [8, 9, 10, 29]. The severity of postprandial hyperglycemia has been shown to be a better predictor of future CVD mortality compared with fasting glucose in both diabetic and normal individuals [8]. Numerous other studies have observed a graded relationship between increasing fasting or postprandial glucose levels and the risk of cardiovascular events in nondiabetic populations [9]. Kawano et al. (1999) showed that glucose loading suppresses endothelium-dependent vasodilation in healthy individuals as well as impaired glucose tolerance groups, measured by FMD [10]. This decrease in FMD could be due to decreases in NO synthesis, increased uncoupling of eNOS, and/or greater consumption of NO by reactive oxygen species [10]. Oxidative stress induced by hyperglycemia decreases the activity of dimethylarginine dimethylaminohydrolase (DDHA) which degrades ADMA, whose role is to inhibit eNOS (19). Higher levels of ADMA relative to arginine have been related to an increased CVD risk by decreasing the biosynthesis of NO due to inhibiting the binding of arginine to eNOS (19). Acute hyperglycemia induces lipid peroxidation and increases ADMA: arginine independent of any changes in inflammation leading to the suppressed vascular function (18).

Endothelial dysfunction has been linked to individuals with diabetes as well as individuals with chronic hyperglycemia which are often seen as the cause of endothelial function [10]. These diseases contribute to abnormalities in the vascular and smooth muscle cells contributing to vascular disease. Since eNOS is constitutively active, the bioavailability of NO is considered a marker of vascular health. The uncoupling of eNOS is thought to be a major contributor to the endothelial dysfunction associated with aging, cardiometabolic disease, and menopause. The bioavailability of NO is a reflection of the balance between its production from NOS and its degradation, particularly by the ROS superoxide [11].

NADPH oxidases transfer electrons across biological membranes, with oxygen often being the acceptor of the electron [47]. The free radical superoxide has been shown to react with NO to produce peroxynitrite which decreases NO bioavailability [21]. NO and superoxide have opposite effects on the vasculature. NADPH-dependent ROS generation causes the depletion of NO and promotes the uncoupling of NOS [47]. This NOX activation leads to an increased production of superoxide leading to a breakdown of NO [47]. Notably, this relationship between NO and NADPH derived superoxide act as a reversible signaling mechanism [47]. Furthermore, superoxide can be removed by superoxide dismutase to reduce the breakdown of NO [49].

Citrulline has been shown to increase levels of arginine, nitrate/nitrite (NO_x), and cGMP activities after citrulline supplementation in healthy young adults [3, 12]. While other studies have seen similar increases in arginine availability and increased urinary NO_x levels, endothelial function is measured by brachial artery FMD, but acute supplementation with citrulline (~7 days) did not show an increase in FMD [3, 13].

Acute (~7 days) citrulline supplementation has shown an increase in exercise performance and prolongs time to fatigue in mice [17]. In humans, acute ingestion (6g/day for

14 days) of citrulline has shown a significant increase to blood flow and plasma arginine levels in older men and women despite not showing an increase in NO_x during exercise [14]. Other studies have shown an increase in endurance exercise performance with citrulline supplementation, including an increase in plasma nitrate-nitrite for both the citrulline (21%) and arginine (28%) groups. This increase in plasma nitrate-nitrite in both groups, despite the significantly improved tissue oxygen index and power outputs for the citrulline group, put into question to what extent does plasma nitrate-nitrite reflect skeletal muscle NOS activity (12).

To summarize, vascular and endothelial function plays a pivotal role in the health and wellbeing of individuals with NO and its bioavailability at the foundation. There is strong evidence that oral consumption of watermelon juice could be beneficial in attenuating the decrement seen in endothelial function during hyperglycemia.

1.8 Purpose

The purpose of this pilot study is to determine the potential for citrulline-rich watermelon juice to attenuate the reduction in postprandial endothelial function associated with reduced skeletal muscle microvascular blood flow (MVBF) during hyperglycemia. We hypothesize that the watermelon juice supplementation will attenuate the reduction in endothelial dysfunction and microvascular blood flow during an oral glucose challenge. We also hypothesize that watermelon juice will increase L-arginine bioavailability during hyperglycemia and correlate with improved vascular response.

Chapter 2. Methods

2.1 Participants

Seventeen participants (5 Male, 12 Females) were recruited through word of mouth and flyers from the LSU campus. Participants were healthy males and females 18 – 40 years of age with a BMI between 18-29.9 kg/m². Participants were excluded if they or a first-degree relative had type 1 or 2 diabetes, were an active smoker, allergic to watermelon, have a history of vascular disorders, on any known medication that could influence the study outcomes, or taking supplements including arginine, citrulline, or beta-alanine.

Participants interested in participation filled out a health screening, PAR-Q, as well as the informed consent for the study. After these documents were reviewed, if participants met all of the inclusion/exclusion criteria, they were scheduled for a screening visit then a follow-up OGTT visit. The study was a double blinded, randomized, counterbalanced design.

2.2 Screening Visit

During the screening visit, participants were required to come in fasted from food and drink (except water) for at least 10 hours, 48-72 hours without alcohol or strenuous exercise. A fasting baseline blood draw was taken to measure glucose, lipids, and CBC. Then participants had body composition measured by dual x-ray absorptiometry (DXA). Women were required to take a pregnancy test before the DXA. Participants were briefed on the risks of the DXA, then prepared by removing all metal and shoes for the full-body scan. Once the DXA was completed the participants were randomized and put into contact with the distributor at the LSU AG Center to schedule out their visits for receiving the supplement or placebo.

2.3 Supplement Visits

Participants were required to have a minimum of 10 and a maximum of 17 doses of supplementation (average 12.8, 10-16 days). The participants were asked to receive the juice or placebo from the food science laboratory at LSU Agricultural Center where they would take one dose per day. On weekends participants were provided with two doses of juice or placebo to account for the closure of the AG Center. Participants were also provided with juice or placebo for anticipated instances where they could not make it to the AG Center. Both the participants as well as the researchers administering the OGTT were blinded to the supplementation.

2.4 Washout Period

Before starting phase 2 of supplementation, the participants were required to wash out a minimum of 14 days following the day of the OGTT. Immediately before the initiation of phase 2, participants underwent a post-washout fasted blood draw. Following the blood draw, the participant would be again put into contact with the AG Center and start receiving the condition they did not receive prior.

2.5 Visit 2 & 3: Oral Glucose Tolerance Test – Postprandial FMD and MVBF

Participants arrived in the morning fasted from food and drink (except water) for at least 10 hours, 48-72 hours without alcohol or strenuous exercise. Body composition was measured by DXA and pregnancy test was administered for females before testing. Next, participants rested for 30 minutes in the supine position while wearing a heart rate monitor (Zephyr, Bioharness) to measure heart rate and heart rate variability. Resting metabolic rate was measured via indirect calorimetry for 20 minutes. MVBF (near-infrared spectroscopy) was measured for 3 rounds of 10 seconds on 2 minutes off then brachial artery FMD (ultrasound) was taken following a 5-minute occlusion. An intravenous catheter was inserted in the patient's left

arm and a baseline blood sample was taken. The participant then had 5 minutes to drink the 75-gram glucose solution, once the participant was finished, a timer was started. Blood was drawn from the IV at 15, 30, 60, 90, and 120 minutes post glucose ingestion. Postprandial measurements of FMD and MVBF were taken at 30, 60, and 90 minutes post glucose ingestion.

2.6 Flow-Mediated Dilation

FMD measurements were taken according to the standard procedures supported by the American Heart Association [40]. With permission from the participant, A blood pressure cuff (Delphi) was placed on the upper forearm distal to the ultrasound probe and proximal to the NIRS optodes. The participant was asked to stay still through the duration of the measurement with their right arm out at 80 degrees and supinated. The brachial artery of the right arm was identified using color and Doppler modes and scanned using B-mode on the ultrasound (Hologic). Following at least 2 minutes post microvascular occlusion, baseline images were recorded of the brachial artery pre-occlusion. Each image attained was ~17 seconds in duration. The ultrasound probe was held in place by using an ultrasound adjustable clamp (QUIPU, Italy) to ensure image quality and reproducibility. The cuff was inflated using a rapid cuff inflation system (Hokanson E20, Bellevue WA) controlled using a custom-built control system Labview Software algorithms (developed by Pedro J Chacon Dominguez, Jin-Woo Choi, and Brian A Irving). Inflation was continuous at 250mmHg for 5 minutes. Before the release of cuff inflation, the participant was reminded to remain still and videos of the brachial artery were attained immediately post-deflation, 1, 3, and 5 minutes post deflation. FMD videos were analyzed by Brachial Analyzer for research software (mia-llc, Coralville, Iowa). Then FMD was measured using the following equation:

$$FMD = (Peak\ diameter - Baseline\ diameter) \div Baseline\ diameter$$

2.7 Microvascular Blood Flow-Near Infrared Spectroscopy (NIRS)

The NIRS system was calibrated prior to the subject arrived for each visit. The NIRS optodes (Oxymon MKIII, Artinis Medical Systems) were placed longitudinally on the flexor digitalis superficialis (forearm) and measured to ensure reproducibility. The optodes were secured in place with two-sided adhesive tape and further anchored in place with additional medical tape. The rapid inflation cuff was placed proximal to the optodes. After ensuring correct probe placement the participants laid their supine at 80 degrees and were instructed to remain still for the course of the measurements. The cuff was rapidly inflated to 60mmHg for 10 seconds and rapidly deflated for 2 minutes for 3 rounds. NIRS signals for oxygenated (O₂Hb/O₂Mb), deoxygenated (HHb/HMb), total (tHb/tMb) hemoglobin/myoglobin, and tissue O₂ Saturation (TSI) (%) were continuously monitored through the MVBF as well as FMD.

The microvascular blood flow response was measured by calculating the slope of the linear increase total hemoglobin (tHb): $BF = ((DtHb \times 60) / ((([Hb] \times 1000) / 4)) \times 1000 / 10$ in ml-1 •min•100ml-1. Baseline TSI was calculated from the 2 minutes prior to ischemic occlusion. The ischemic-reperfusion slope was calculated from the slope of linear increase at the point of cuff release to 10-seconds post-reperfusion.

Paired t-test and repeated measures and mixed mold two-way ANOVA were utilized to analyze data where appropriate (Prism, Graphpad). Main effects were defined as “time” (between individual time points within a visit) and “treatment” (PLA vs. WMJ). Multiple comparisons were made by Holm-Sidak’s test. Statistical significance was declared at P<0.05. All data were reported as mean ±SEM. Calculations of the incremental area under the curve (AUC) were made by the trapezoidal method.

Chapter 3. Results

3.1 Participant Characteristics

All individuals were non-obese (%Fat Mass= $27.72 \pm 7.89\%$), young adults (Age= 23 ± 3 y), with normal or overweight BMI (BMI= $23.48 \pm 3.22 \text{ kg/m}^2$) with 17 total participants (12 female, 5 male). Two of the female participants completed only 1 of the visits and were removed from repeated measures analysis. The participant characteristics did not differ from baseline to their last visit as shown in **Table 1**. The average time of supplementation for WMJ trial supplemented was 13 days (Range 10-15) and the average time for the placebo was 13 days (Range: 10-16). The washout period lasted for 16 days (Range: 14-21) days. Due to technical issues with the production of the WMJ supplement, the first 5 participants were given WMJ in the first trial, the following 5 participants were given PLA as the first trial to match this, then the remaining participants were randomized to the order of their trials. However, the double-blinded, cross-over, counterbalanced nature of the study design was maintained throughout the study.

Table 1. Participant Characteristics

	BL	PLA	WMJ
N	17	17	16
Age (y)	23 ± 3	23 ± 3	23 ± 3
Bodyweight (kg)	66.71 ± 12.77	66.55 ± 12.48	67.28 ± 12.36
Height (cm)	168.08 ± 8.31	168.08 ± 8.10	168.68 ± 7.97
BMI (kg/m ²)	23.48 ± 3.22	23.43 ± 3.20	23.54 ± 3.27
DXA DATA			(N=11)
FM (kg)	18.77 ± 6.92	18.91 ± 6.87	19.97 ± 7.90
FFM (kg)	46.60 ± 10.21	46.37 ± 10.24	48.35 ± 11.94
%FM	27.72 ± 7.89	27.96 ± 7.87	28.26 ± 8.78
%FFM	69.90 ± 7.96	69.72 ± 7.68	69.68 ± 8.73
VFAT (kg)	0.249 ± 0.113	0.243 ± 0.101	0.251 ± 0.127
Table 1. Participant characteristics. BMI, body mass index. FM, fat mass. FFM, fat free mass. %FM, % fat mass. %FFM, % fat free mass. VFAT, visceral fat. BL, baseline. PLA, placebo. WMJ, watermelon juice. Statistics are shown as Mean ± SD.			

3.2 Oral Glucose Tolerance Test

Participants baseline blood glucose concentrations increased from ($5.20 \pm 0.37 \text{ mmol} \cdot \text{L}^{-1}$) at baseline to ($6.73 \pm 0.93 \text{ mmol} \cdot \text{L}^{-1}$) at 15 minutes and peaking at ($8.06 \pm 1.38 \text{ mmol} \cdot \text{L}^{-1}$) at 30 minutes, then ($7.52 \pm 2.10 \text{ mmol} \cdot \text{L}^{-1}$) at 60 minutes, to ($6.10 \pm 1.52 \text{ mmol} \cdot \text{L}^{-1}$) at 90 and finally (5.53 ± 1.60) at 120 minutes post glucose ingestion. **Figure 1** depicts a significant time effect in glucose levels ($p < 0.0001$) with no significant treatment ($p = 0.18$) or treatment by time effect ($p = 0.74$) in WMJ or PLA groups. Glucose AUC was not significant throughout the trail ($p = 0.43$). RER at baseline was 0.787 then 0.841 at 60 minutes and 0.826 at 120 minutes post glucose ingestion with a significant time effect ($p < 0.0001$) with no treatment effect ($p = 0.46$).

Figure 1.

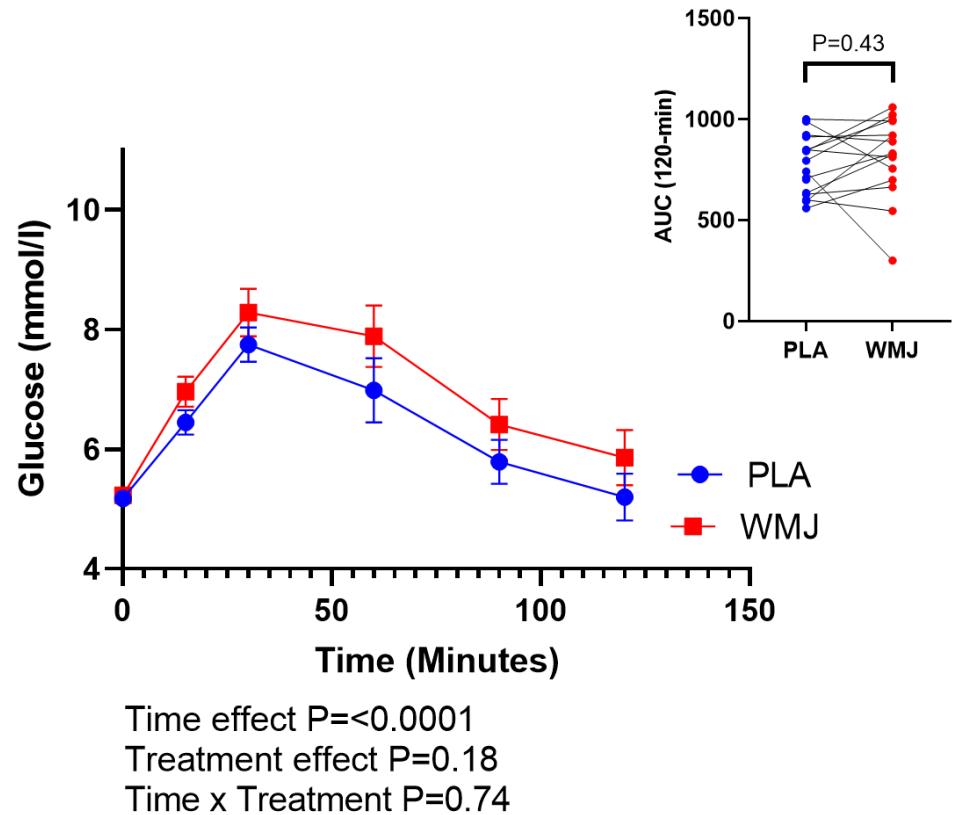


Figure 1. Plasma glucose from baseline to 120 min post glucose ingestion with a significant time effect. Glucose AUC in WMJ vs PLA with no significance. AUC, area under the curve. PLA, placebo. WMJ, watermelon juice

3.3 Flow-Mediated Dilation

Table 2 displays the baseline and peak brachial artery diameter at 0 (pre glucose ingestion) 30, 60, and 90 minutes following the oral glucose ingestion. **Figure 2b** depicts the percent change in FMD between WMJ and PLA groups following the oral glucose challenge with a significant treatment effect ($p=0.038$). Percent change in 60-second post occlusion FMD has a time effect trending towards significance ($p=0.057$) in the WMJ group demonstrated in **Figure 2a**. A higher percentage of participants demonstrated a longer time to peak artery diameter at 30 minutes post glucose ingestion represented in **Figure 3**.

Table 2. Baseline Brachial Artery Vessel Diameter (mm)				
	0-min	30-min	60-min	90-min
PLA	3.31 ± 0.65	3.44 ± 0.70	3.46 ± 0.74	3.56 ± 0.74
WMJ	3.53 ± 0.71	3.61 ± 0.73	3.41 ± 0.69	3.53 ± 0.64
Peak Brachial Artery Vessel Diameter (mm)				
	0-min	30-min	60-min	90-min
PLA	3.57 ± 0.58	3.65 ± 0.73	3.81 ± 0.74	3.78 ± 0.75
WMJ	3.86 ± 0.85	3.91 ± 0.87	3.80 ± 0.77	3.74 ± 0.72
Data are mean ± SD; PLA, Placebo; WMJ, Watermelon Juice				

Figure 2.

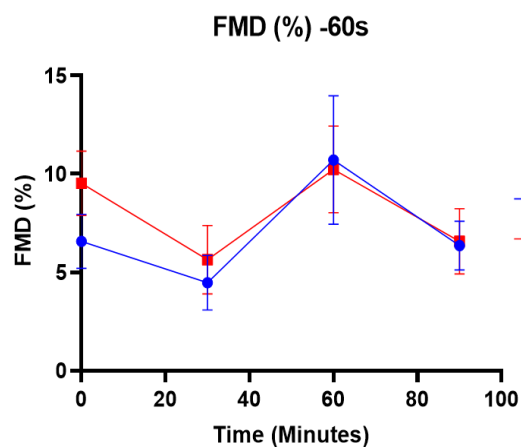


Figure 2a. Percentage FMD at 60 seconds post occlusion for baseline, 30 minutes, 60 minutes, 90 minutes following glucose ingestion in PLA and WMJ groups. FMD, flow-mediated dilation. PLA, placebo. WMJ, watermelon juice

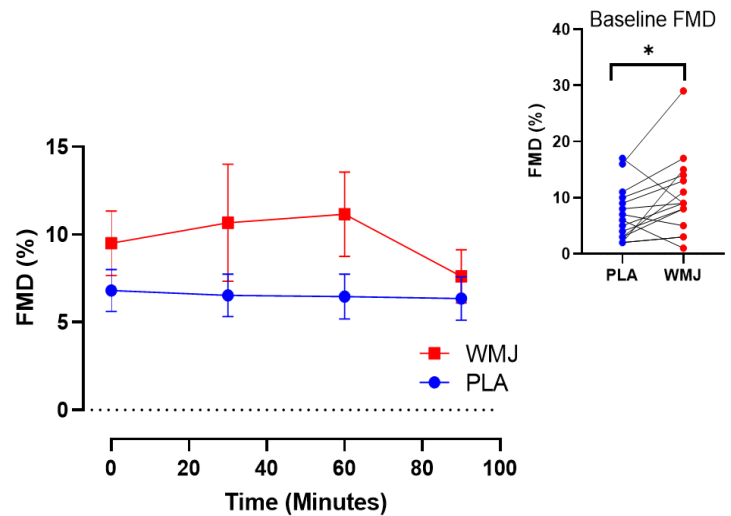


Figure 2b. Peak percent FMD at baseline, 30 minutes, 60 minutes, 90 minutes following glucose ingestion in PLA and WMJ groups. Baseline FMD was significantly different in WMJ vs Placebo. FMD, flow-mediated dilation. PLA, placebo. WMJ, watermelon juice.

Figure 3.

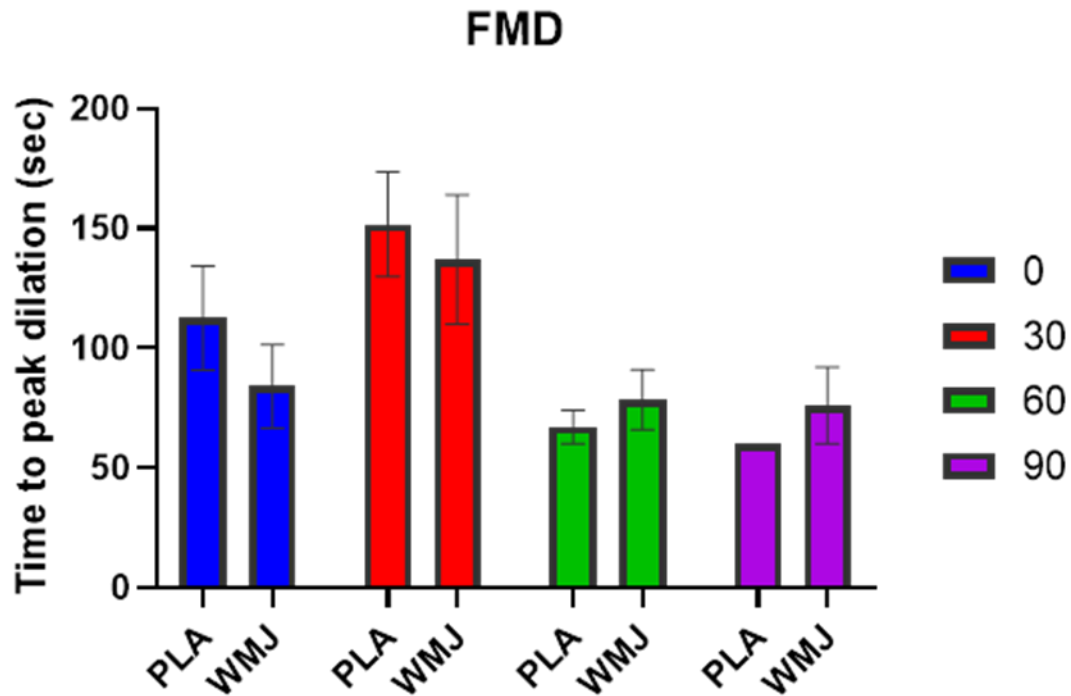


Figure 3. Time to peak dilation by timepoint and treatment. PLA, placebo. WMJ, watermelon juice

3.4 Near-Infrared Spectroscopy

At 30 minutes post glucose ingestion, plasma glucose increases while oxygen saturation (StO₂) decreases in both WMJ and PLA groups as shown in **Figure 4**. Baseline StO₂ (average StO₂ 180 seconds prior to cuff occlusion) was significantly higher in WMJ vs PLA ($p=0.031$) and the baseline StO₂ area under the curve (AUC) was significantly greater in WMJ vs PLA ($p=0.03$) as depicted in **Figure 5**. Hyperglycemia has been cited to reduce microvascular endothelial function [54]. Slope 2 (reperfusion slope) is inversely correlated with plasma glucose ($R^2=0.17$; $p=0.0002$) depicted in **Figure 6**. **Figure 7** displays a significant time effect ($p=0.03$) and time by treatment effect trending towards significance ($p=0.072$) in WMJ vs PLA

reperfusion slope. Reperfusion AUC (8-minute) shows a significant time effect ($p<0.0001$) and trends towards a significant treatment effect ($p=0.13$) as displayed in **Figure 8a**. The mean reperfusion AUC (8-minute) trends towards a significant increase in WMJ vs PLA group ($p=0.086$) as exhibited in **Figure 8b**. **Figure 9** depicts the microvascular blood flow from 0-minute (pre-glucose) to 90 minutes post glucose ingestion showing a significant time effect ($p=0.023$) with no significant treatment effect. No significant differences were observed in mean blood flow or baseline blood flow in WMJ vs PLA despite baseline blood flow trending toward significance ($p=0.15$) as exhibited in **Figure 10**.

Figure 4.

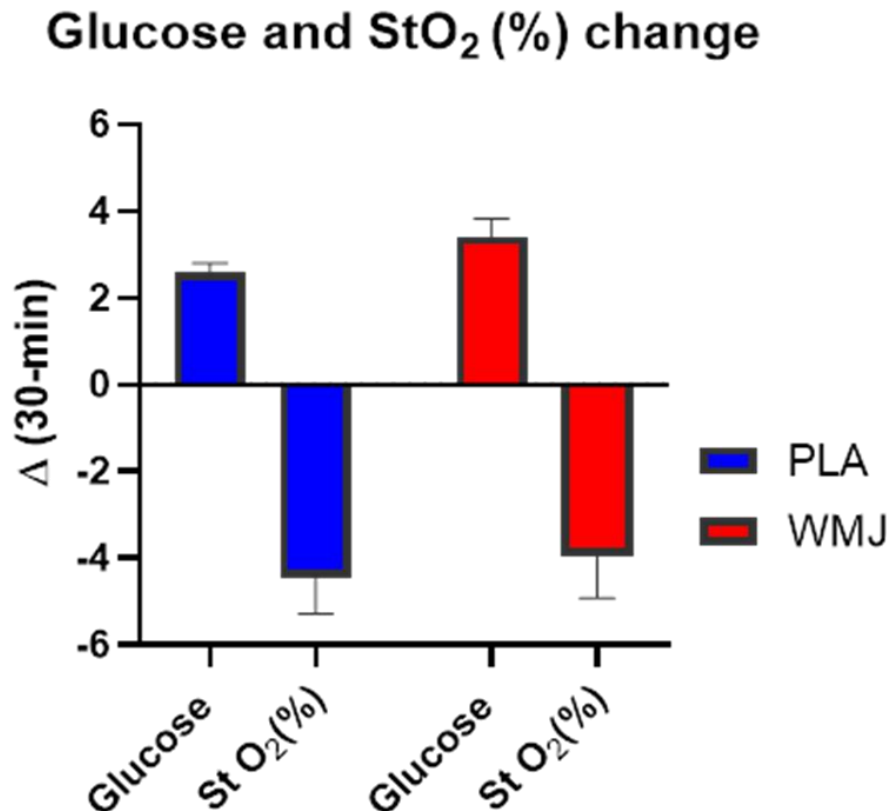


Figure 4. Change in plasma glucose and oxygen saturation at 30 minutes in PLA and WMJ groups. Δ , delta. StO₂, oxygen saturation. PLA, placebo. WMJ, watermelon juice

Figure 5.

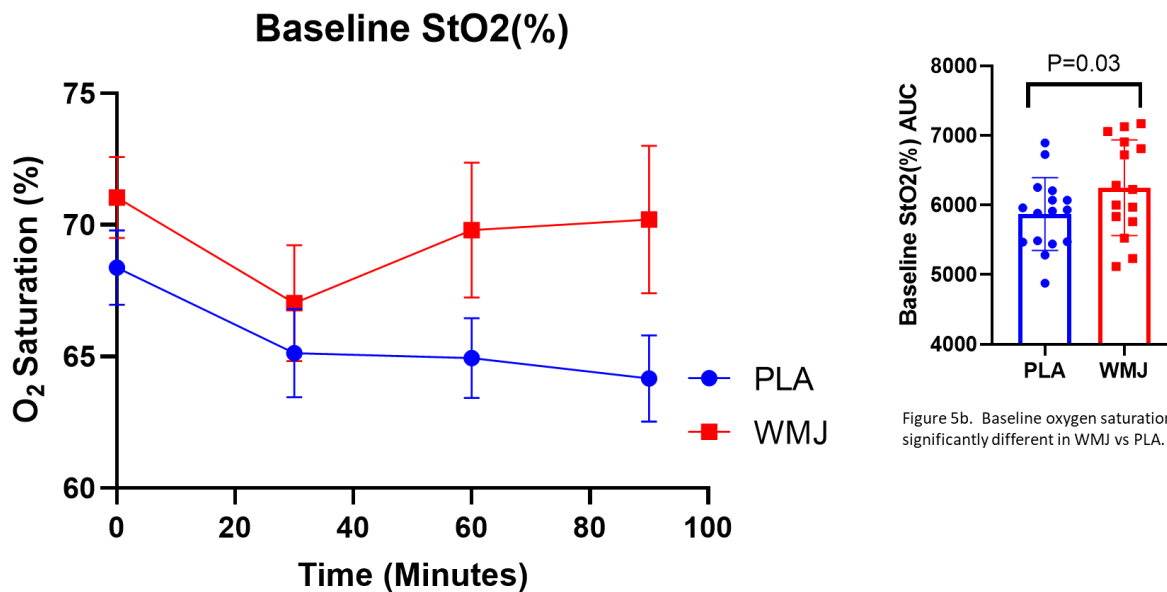


Figure 6.

Hyperglycemia impairs skeletal muscle reperfusion

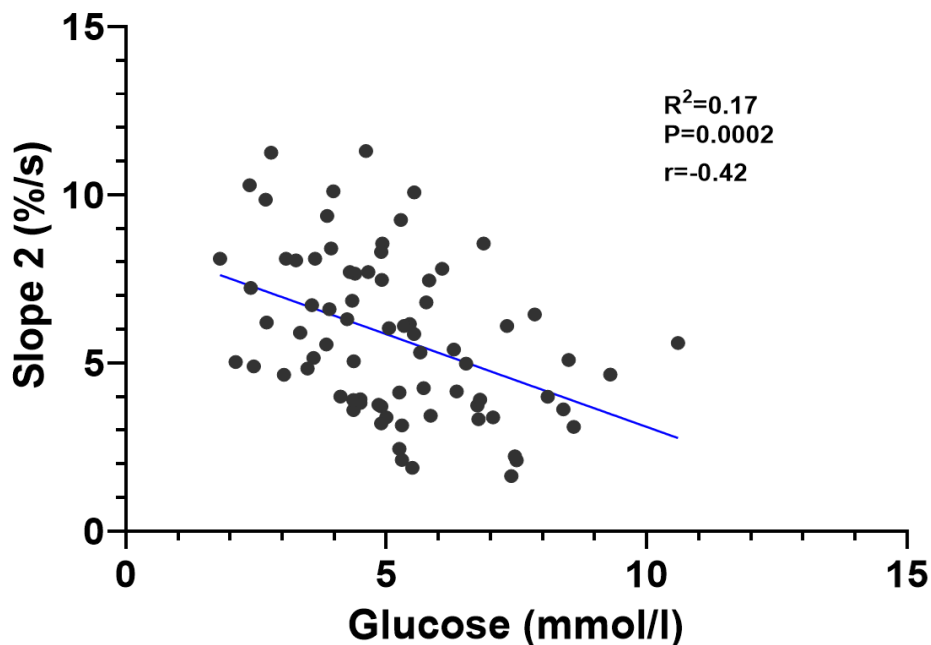


Figure 6. Significant inverse relationship in NIRS reperfusion slope post occlusion release and plasma glucose levels. Slope 2, reperfusion slope.

Figure 7.

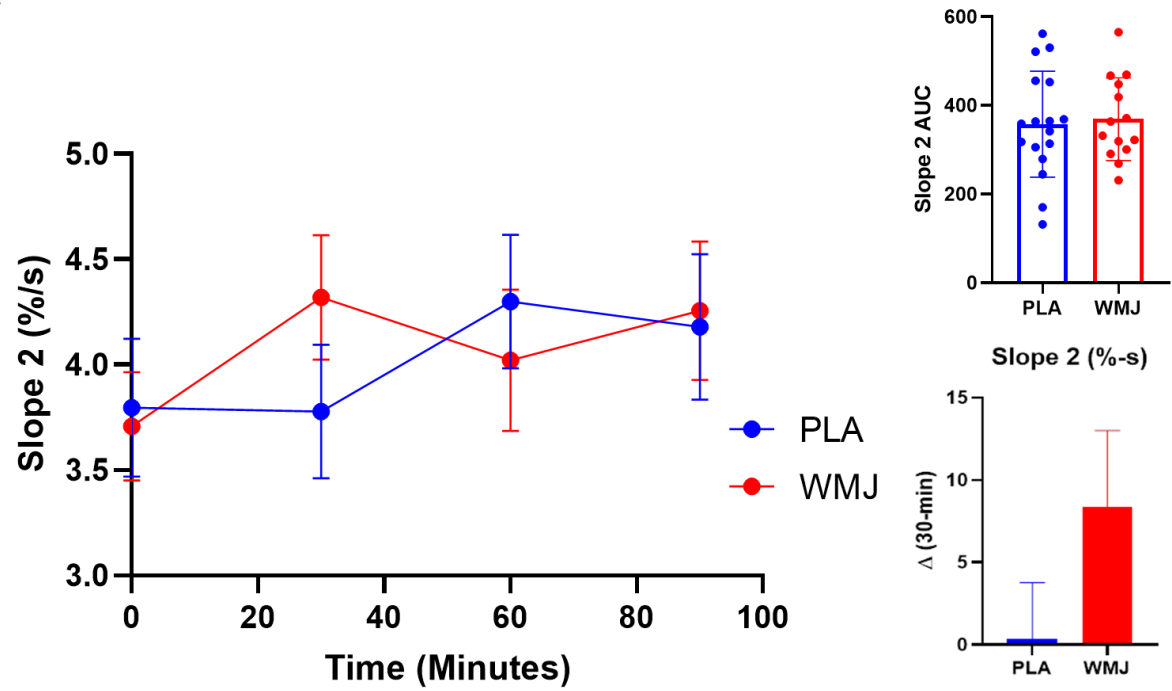


Figure 7. Reperfusion slope (%/second) at baseline, 30 minutes, 60 minutes, 90 minutes in WMJ and PLA groups. Slope 2, reperfusion slope. AUC, area under the curve. PLA, placebo. WMJ, watermelon juice. Δ, delta.

Figure 8.

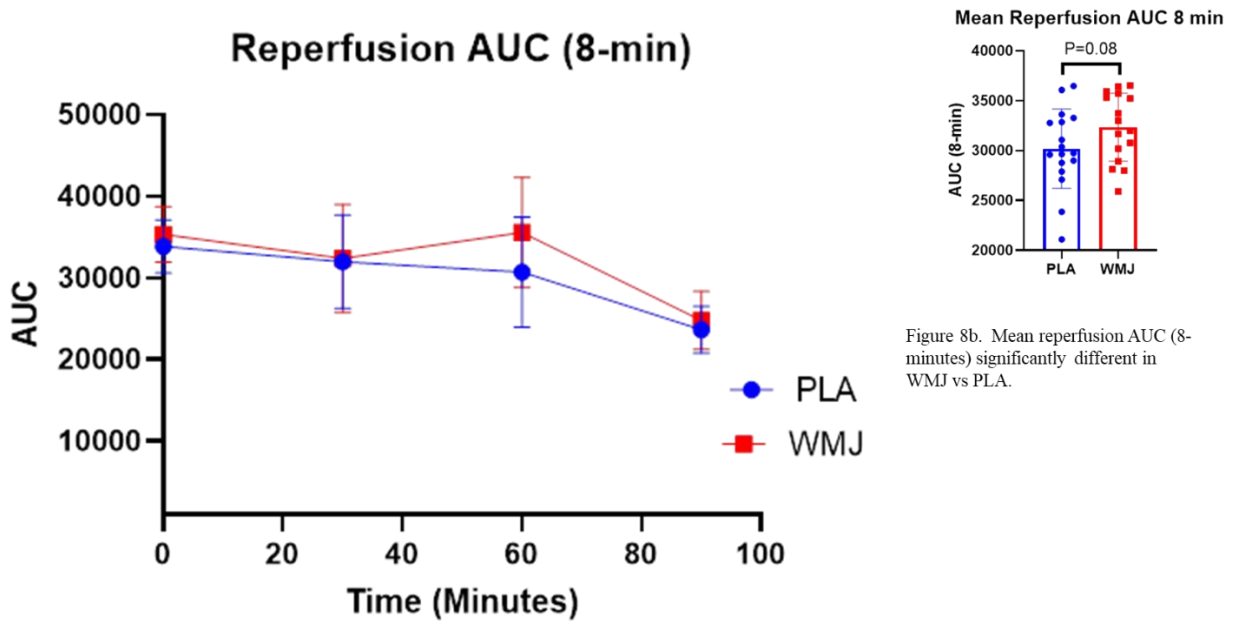


Figure 8a. Reperfusion AUC (8-minutes) from the time baseline is achieved post occlusion to 8 minutes out with a significant time effect. AUC, area under the curve. PLA, placebo. WMJ, watermelon juice.

Figure 8b. Mean reperfusion AUC (8-minutes) significantly different in WMJ vs PLA.

Figure 9.

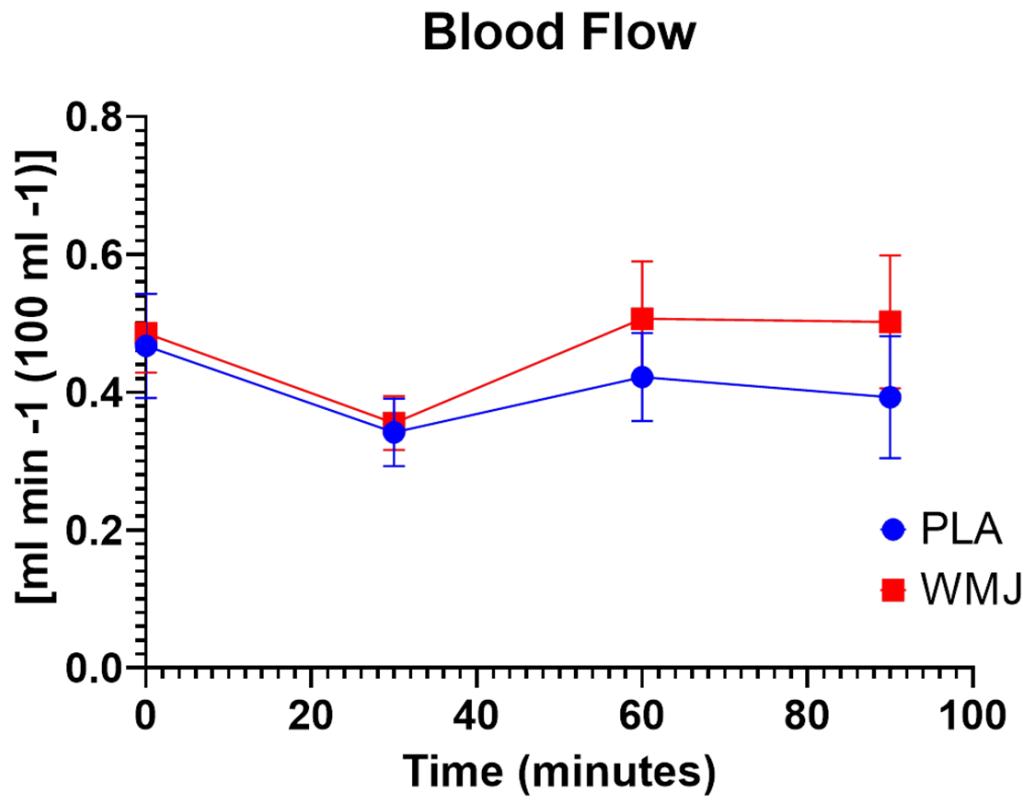


Figure 9. Microvasculature blood flow from baseline to 90 minutes following glucose ingestion in WMJ vs PLA with a significant time effect.
PLA, placebo. WMJ, watermelon juice.

Figure 10.

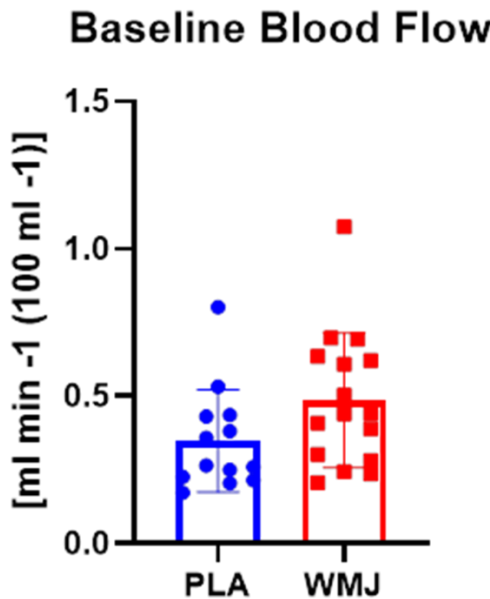


Figure 10a. Baseline microvasculature blood flow in WMJ vs PLA.
PLA, placebo. WMJ, watermelon juice.

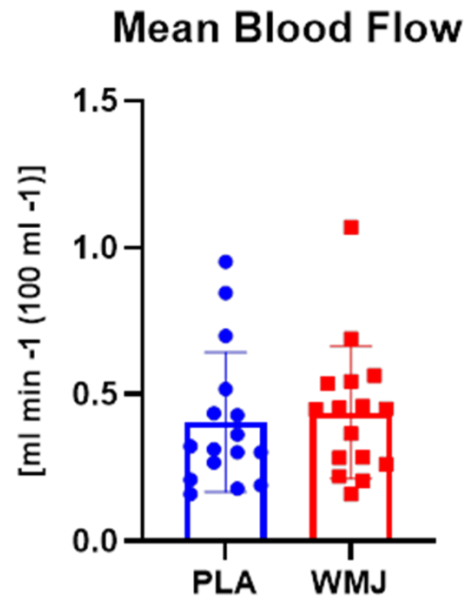


Figure 10b. Mean microvasculature blood flow in WMJ vs PLA.

Chapter 4. Discussion

4.1 Hyperglycemia and Vascular Function

Diseases such as T2D, hypertension, obesity, and CVD all negatively impact health on a global scale. The decrement in quality of life caused by these diseases has been linked to diminished vascular-endothelial function. Hyperglycemia has been shown to cause a reduction in blood flow and vascular responsiveness [8, 43, 51]. We sought to attenuate the reduced endothelial response to postprandial hyperglycemia via supplementation with WMJ which has shown to have promising endothelial effects.

4.2 Oral Glucose Tolerance Test

A 75-gram glucose OGTT was administered to induce a hyperglycemic response which has been shown to increase plasma glucose levels rapidly causing vascular resistance despite the following increase in plasma insulin. Glucose was elevated as expected and peak glucose occurred at the 30-minute time and stayed elevated at 60 minutes in accordance with similar trials in healthy individuals [8,43]. WMJ groups showed no significant differences in hyperglycemia indicating changes in FMD or NIRS were not derived from differences in plasma glucose.

4.3 Flow-Mediated Dilation

We expected to see decrements in FMD% when plasma glucose was at peak indicating the attenuated endothelial response caused by hyperglycemia in response to an OGTT as shown by Russell et al [51]. The change in vessel diameter (FMD%) was assessed at 60 seconds, 180 seconds, and 300 seconds after cuff release. Peak vessel diameter change was reduced 30-minutes post glucose ingestion. Mah et al. reported a similar decrement in FMD but at 60

minutes post glucose ingestion, this could be due to our population including healthy females and males as opposed to solely males [18]. Black et al (2005) reported 58% of peak FMD% were found from 50-70 seconds post cuff release in healthy young individuals [52]. Conversely, 80% of our participants reached peak FMD% at 60 seconds at baseline, but 30 minutes following glucose ingestion the time to peak FMD% increased then recovers similar to baseline. This is likely attributed to the peak in glucose occurring at the same time followed by the vasodilatory response of insulin in the later time points. This interaction could also be linked to peak glucose, further attenuating vasoresponsiveness during hyperglycemia. Baseline FMD% significantly increased with the supplementation of WMJ when compared to PLA. This response can likely be attributed to the prolonged supplementation of citrulline leading to increased NO bioavailability. In conjunction with FMD, the NIRS data also exhibited similar promising trends.

4.4 Near-Infrared Spectroscopy

Following the trends with FMD, we observed a decrease in baseline StO₂ at 30 minutes in both groups where peak glucose occurred. In the WMJ group, there was a trend toward a significant treatment effect ($p=0.11$) as well as a significant time effect ($p=0.03$) when compared to the placebo. The observed improvement in baseline StO₂ suggests WMJ attenuates the reduction of endothelial function observed during hyperglycemia. Supplementing with WMJ significantly increased baseline StO₂ AUC which is likely related to improved chronic endothelium function. Soares et al. (2017) noted a linear significant increase in slope 2 (reperfusion slope) at 90 minutes post glucose ingestion, however, they did not measure insulin which is a major vasodilator [43]. In our hands, we noted a similar trend in PLA peak slope 2 at 60 minutes, whereas the WMJ group demonstrated peak slope 2 at 30 minutes post glucose

ingestion, despite having a significant time effect ($p=0.043$). McLay et al (2016) suggest that slope 2 is a more repeatable measure of vascular responsiveness than FMD% [53]. We observed a significant inverse relationship ($p=0.0002$) in glucose and slope 2 displaying that hyperglycemia impairs skeletal muscle reperfusion. There was no correlation between reperfusion AUC (8-minute) and slope 2 whereas Soars et al (2017) reported an inverse relationship between the two [43]. We did not detect a significant treatment effect for the reperfusion AUC (8-minute) despite a trend toward improvement with WMJ ($p=0.13$). Likewise, the mean reperfusion AUC (8-minute) was also nearly significant ($p=0.08$). It is reasonable to speculate that WMJ supplementation improves the vascular environment during hyperglycemia based on the sustain higher StO₂ and trend towards improved vascular reperfusion. In T2D as well as hyperglycemia microvascular blood flow is significantly reduced [9,10,18,51]. We observed a significant time effect ($p=0.023$) in microvasculature blood flow with a reduction in blood flow at peak glucose. Baseline microvasculature blood flow trends towards significance in the WMJ group

4.5 Limitations

This study was designed to investigate upstream and downstream vascular responsiveness to 2 weeks of WMJ supplementation shown to provide an additional 3g of Citrulline compared to supplementation with a placebo designed to taste similar to the WMJ. Technical limitations prevented the assessment of simultaneous pulse wave velocity with a high-quality B mode video. Instead, we used NIRS to measure microvascular blood flow. Cardiovascular fitness is a major predictor of endothelial function and vascular health. We did require a physical activity questionnaire and asked participants to abstain from exercise prior to study visits, general physical activity was not accounted for. Last, we cannot confirm the pancreatic responsiveness

to the OGTT with insulin or determine the effectiveness of the WMJ supplementation to raise L-citrulline levels. However, in the future, we will use archival samples from this study to measure serum insulin and L-citrulline levels. At the present time, we do not have serum insulin or circulation markers of NO activity (Nitrate) these analyses will be run in the future.

4.6 Considerations for Future Research

Future research could investigate at a combination supplement of citrulline, arginine, and nitrates in conjunction to possibly elicit a more robust response. The data from this study give merit for a similar study in individuals with decreased vascular responsiveness such as T2D or family history of T2D.

4.7 Conclusion

The main finding of this study confirms the previously reported relationship between hyperglycemia and decreased skeletal muscle microvascular function. We also confirmed decreased endothelial function, in a healthy-young population, during an OGTT. The purpose of this study was to determine the effect of WMJ supplementation on endothelial function and skeletal muscle microvascular function during experimental hyperglycemia. We demonstrated WMJ supplementation is an effective method for improving vascular responsiveness during hyperglycemia via improved baseline oxygen saturation and improved baseline FMD.

Appendix.

IRB Approval Form

Pennington Biomedical IRB FWA 00006218
Approved October 22, 2019

Protocol Title: The Effects Watermelon Juice Supplementation on Postprandial Vascular Endothelial Function and Blood Flow During Hyperglycemia: A Pilot Study

PI Name: Timothy Allerton Ph.D., LCEP

Sub-Investigator's Name(s):

Brian Irving Ph.D.

Neil Johannsen Ph.D.

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Jack Losso Ph.D.

Medical Investigator: Daniel Hsia, MD

Protocol Version Date: 10/15/19

Objectives

The objective of this pilot study is to determine the potential for watermelon juice to attenuate the reduction postprandial endothelial function and skeletal muscle microvascular blood flow (MVBF) experienced during hyperglycemia.

We will attempt to answer the follow hypotheses:

Hypothesis 1: Watermelon juice supplementation will attenuate the reduction in endothelial dysfunction and microvascular blood flow during an oral glucose challenge.

Hypothesis 2: Watermelon juice will increase L-arginine bioavailability during hyperglycemia and correlate with improved vascular response.

Exploratory Aim: The postprandial period is defined by increased sympathetic nervous system activity (vasoconstriction) and NO^{*} mediated vasodilation. Heart rate variability (HRV) is a measurement of the balance of parasympathetic to sympathetic activity. We will measure HRV during the oral glucose challenge to interrogate the possibility that watermelon juice can modulate the balance of blood vessel constriction and relaxation during an oral glucose challenge.

Background

Cardiovascular disease is the primary cause of death in people with obesity and type 2 diabetes (1, 2). Postprandial hyperglycemia, hyperlipidemia, and endothelial dysfunction are strong predictors of future CVD events and death (3, 4). Nitric oxide (NO^{*}) mediated vasodilation is a critical component of blood pressure regulation, endothelial function, and insulin mediated glucose disposal (5, 6). NO^{*} is also essential to insulin-mediated vasodilation of large and microvascular blood vessels in response to a nutrient challenge (mixed-meal, glucose, fat). The capacity of insulin to increase vasodilation and postprandial microvascular blood flow (MVBF) accounts for 40-50% of insulin-stimulated glucose disposal (5, 7). Strategies to increase the vasodilatory actions of insulin would therefore make a significant impact on vascular and metabolic health.

Several studies have documented reduced NO^{*} synthesis in obesity and type 2 diabetes (8-10). In endothelial cells, NO^{*} is synthesized from L-arginine (precursor) by endothelial-nitric oxide

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Vita

Cullen Vincellette was born in Chalmette, LA to Sandra Vincellette and raised in Slidell, LA. Cullen graduated from Pope John Highschool in 2013 and upon graduation began his college career at Louisiana State University. Cullen received his bachelor's degree in Kinesiology in August of 2018 from Louisiana State University. After a short time as an Assistant Sports Performance Coach at Southeastern Louisiana University he began working towards his Master's degree at LSU. He aims to receive his Master's degree in May 2020. After completing his Master's degree, Cullen aims to continue learning, researching, and optimizing human performance while adventuring to new places.