Protocol Title: The Effects of 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.
Guillaume Spielmann Ph.D.
Jack Losso Ph.D.

Medical Investigator: Daniel Hsia, MD

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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 following 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 $NO^{\bullet}$ 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 ($NO^{\bullet}$) mediated vasodilation is a critical component of blood pressure regulation, endothelial function, and insulin-mediated glucose disposal (5, 6). $NO^{\bullet}$ 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 $NO^{\bullet}$ synthesis in obesity and type 2 diabetes(8-10). In endothelial cells, $NO^{\bullet}$ is synthesized from L -arginine (precursor) by endothelial-nitric oxide

synthase (eNOS) generating NO• and L –citrulline (products)(11) [*adjacent figure*]. Supplemental L-arginine, at high doses (9-24 g), has been shown to improve postprandial endothelial function in response to a high-fat meal in those with low plasma arginine levels but did not improve postprandial blood flow (12). However, oral L-arginine supplementation is subject to a high degree of first-pass extraction and elevates arginase enzyme activity. Furthermore, chronic L-arginine supplementation has been shown, in some cases, to be ineffective and unsafe for those with cardiovascular disease (13, 14).

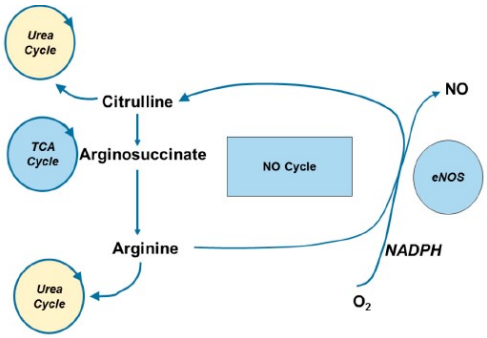
The amino substrate for L-arginine, L-citrulline, is more effective at increases circulating L-arginine than L-arginine supplementation itself (15-17). L-citrulline is rapidly acted on by cytosolic arginosuccinate synthase and converted into arginosuccinate which is then converted into L -arginine by arginosuccinate lyase. L-citrulline or watermelon (the most abundant dietary source of L-citrulline) has been shown to be effective at improving some reactive cardiovascular measurements in normo, pre-, and hypertensive participants (18-20). Preclinical trials have demonstrated potential for L-citrulline to protect against endothelial damage when consuming a high fat/cholesterol diet (21).

Several studies exhibit that hyperglycemia, even in healthy populations, impairs endothelial function and microvascular blood flow by reducing NO• bioavailability (22-25). Increasing the bioavailability and action of NO• by increasing L-arginine levels or reducing the ratio of asymmetric dymethylarginine (ADMA) to L-arginine has the potential to confer protective effects against hyperglycemia-induced endothelial dysfunction. Based on the available data, supplementation with L-citrulline has greater potential, than oral L-arginine supplementation, to increase systemic L-arginine bioavailability. Utilizing a natural food source high in L-citrulline (watermelon juice) to improve the postprandial vascular response to hyperglycemia has not been studied to date. **The purpose of this pilot study is to determine the potential for watermelon juice to attenuate the reduction in postprandial endothelial function associated with reduced skeletal muscle microvascular blood flow (MVBF) during hyperglycemia**.

Inclusion and Exclusion Criteria

Study Population. We will recruit, randomize, and study up to 20 young (18-40 years old), healthy (no metabolic disease), and participants with a normal BMI (18-29.9 kg/m^2) from the LSU community.

Inclusion and Exclusion Criteria



Inclusion Criteria.

1. Capable and willing to give written informed consent and understand inclusion and exclusion criteria.
2. 18-40 years of age
3. BMI between 18-29.9 kg/m^2
4. Willing to allow researchers to draw blood and conduct imaging (DXA) for research purposes.

Exclusion Criteria.

1. Evidence or self-reported history of type 1 or 2 diabetes mellitus
2. Self-reported family history of type 2 diabetes (first degree relative with type 2 diabetes)
3. BMI > 29.9 kg/m^2

4. Evidence or self-report history of deep vein thrombosis, pulmonary embolism, cardiovascular, peripheral vascular, cerebral vascular, pulmonary, or renal disease
5. Allergy to watermelon
6. Use of medication known to influence study outcomes, such as:
    a. Insulin
    b. Anti-diabetics (metaformin)
    c. Corticosteroids
    d. Beta-blockers
    e. Anti-coagulants
7. Use of supplements known to influence study outcomes, such as;
    a. Beta-alanine
    b. L-arginine
    c. L-citrulline
8. Active smoking

Number of Subjects
N=20

Recruitment Methods
We will recruit primarily on LSU campus via word of mouth and flyers.

Study Timelines

Based on the project notification date of March 15th, 2019 we plan to proceed with study recruitment in April of 2019 with a study approximate study start at the end of May 2019. The total study commitment for each participant is 42 days. We anticipate completing the study in the early fall (September/October) of 2019. Given these target dates are met we plan to complete the preliminary analysis by December 2019.

Study Endpoints
The primary endpoints for the study are changes in postprandial flow-mediated dilation and microvascular blood flow. Secondary endpoints will include plasma biomarkers of nitric oxide active and oxidative stress. We will also explore changes in heart rate variability as a secondary outcome.

For the oral glucose tolerance test participants arriving with fasting blood glucose $\geq$100 mg/dl will not proceed with testing and will be referred to the student health center for follow up after consulting with the study MI. Any blood glucose $\geq$ 200 mg/dl during the OGTT will result in test termination and referral to the student health center after consulting with study MI.

Procedures Involved

There will be 1 screening visit, and 2 outpatient visits. Participants will consume watermelon juice or placebo for 2 weeks. The washout period will last 2 weeks prior to the cross-over to the opposite condition. Participant visits will be conducted at the Vascular and Resting Metabolism Lab located in the Department of Kinesiology at LSU.

Screening Visit

Participants will undergo consenting and fasting blood draws to measure glucose, lipids, and CBC. Vital signs (blood pressure, body weight, body composition (DXA), heart rate, etc) will be measured. For DXA procedure participants will undergo a whole body scan lasting approximately 10 minutes. Participants will remove all metal objects from their body and lie down on the table. The legs will be secured together using two Velcro straps. The participant will be instructed to remain completely still during the scan. Randomization will be performed to allocate the study participant for their initial group assignment to either the watermelon juice group or placebo. Group assignments will be generated by randomization software (http://www.graphpad.com/quickcalcs/index.cfm) third-party faculty member not associated with the study.

Daily Juice Drop-in visits

Since the required supplementation duration is 14 days participants will be provided with 2 days (Saturday and Sunday) worth of watermelon juice or placebo to account for days when the LSU AG Center will not be open. Participants will also be provided with juice or placebo for anticipated instances where they cannot be on campus. In order to monitor compliance participants will be asked to return the juice container for the days where juice was consumed off-premises.

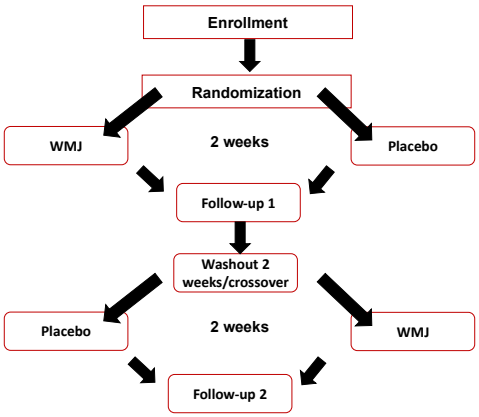


Figure 1. Experimental Design

Visit 2 and 3: Oral Glucose Challenge – Postprandial FMD and MVBF

Participants will arrive in morning at 6:00am fasted for 10-hours. Body composition will be measured by DXA. Next, participants will rest for 30 minutes in the supine positing while wearing a heart rate monitor (Zephyr, Bioharness) to measure heart rate and heart rate variability. The resting metabolic rate will be measured via indirect calorimetry for 20 minutes. Then fasting measures of blood glucose, FMD (ultrasound), and MVBF (near-infrared spectroscopy) will be taken followed by ingestion of 75 g of glucose (glucola). An IV line will be placed in the participant's arm vein for blood draw purposes and will remain there throughout the testing. A blood sample will be drawn, and then the participant will drink a sugar solution consisting of 75 grams of glucose. Blood will be drawn at specific times after you consume the drink. Each blood sample will be about 1 tablespoon. (6 tablespoons total for the test). **During the IV procedure, a small amount of the participant's own blood (less than 1 teaspoon) will immediately be returned into your vein through the IV after each specimen is collected.** Blood will be drawn at minutes 15, 30, 60, 90, and 120 minutes. Postprandial measurements of FMD and MVBF will be taken at 30, 60, 90, and 120 minutes.

The oral glucose challenge will be conducted by Drs. Allerton, Johannsen, Spielmann, and Irving under the medical direction of Dr. Hsia.

Research Methods

To measure postprandial FMD and MVBF we will use a standard oral glucose tolerance test format with periodic measurement of substrate oxidation (indirect calorimetry).

Flow Mediated Dilation

FMD will be measured according to standard procedures supported by the American Heart Association. A blood pressure occlusion cuff will be placed on the forearm distal to the ultrasound probe in preparation for measurement. The brachial artery of the right arm will be identified and scanned using B-mode ultrasound after a 10-minute resting period. Continuous cross-sectional images will be recorded for 3-minutes at rest, during cuff inflation (250 mmHg for 5 minutes) and flowing release of cuff inflation. Fasting measurement will take place -30-mintutes, whereas, postprandial measurement will take place +30, +60, +90, and +120 minutes. Vascular images will be acquired using a 10mHz linear array transducer (8L-RS).
FMD will be calculated as follows:

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

Raw values for vessel diameter and shear stress will also be collected and reported.

Microvascular Blood Flow-Near Infrared Spectroscopy (NIRS)

The NIRS optodes (Oxymon MKIII, Artinis Medical Systems) will be placed longitudinally on the *flexor digitalis superficialis (forearm)*. The optodes will be secured in place with two-sided adhesive tape and further anchored in place with additional medical tape. A blood pressure cuff (Hokanson, Bellevue WA) will be placed proximal to the optodes, distal to the FMD ultrasound probe and connected to a rapid cuff inflation system (Hokanson E20, Bellevue WA) controlled using a custom-built control system and Labview Software algorithms (developed by Pedro J Chacon Dominguez, Jin-Woo Choi, and Brian A Irving). NIRS signals for oxygenated (O_2Hb/O_2Mb), deoxygenated (HHb/HMb), and total (tHb/tMb) hemoglobin/myoglobin will be continuously monitored (29).

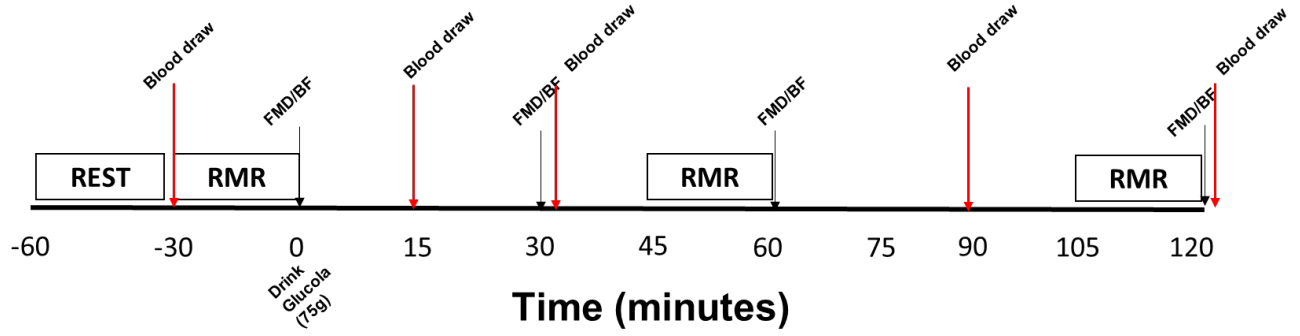
Table 1.

	Screening Visit	2 weeks (WMJ or PLA)	Study Visit 1	Washout- 2 weeks	2 weeks WMJ or PLA	Study Visit 2
Informed Consent	X					
Randomization	X					
Height/Weight/BMI	X					
Vital Signs (blood pressure, heart rate)	X					
Questionnaires	X					
DXA	x		x			x
Oral Glucose Challenge			x			x
RMR			x			x
FMD/MVBF			x			x
Blood Draws	x		x			x
Daily Trips to Drink Juice or Placebo			x			x

Resting muscle microvascular blood flow will be measured -30 minutes in triplicate using venous occlusions by inflating the blood pressure cuff to (50-60 mmHg) for 20 seconds with at least 120 seconds between each measurement (29). The same procedure will be conducted +30, +60, +90, and +120 minutes post-glucose challenge. We will measure blood flow by calculating the slope of the linear increase total hemoglobin (tHb): BF= (((ΔtHb x 60)/ ((([Hb] x1000)/4))x1000/10 in ml^{-1}•min•$100ml^{-1}$ (29). **NOTE: FMD and MVBF measurement times will be staggered 3 minutes after blood draws to avoid confounding factors*.

Lab Samples: Measurement of glucose, insulin and free fatty acids will be measured as a part of the basic metabolic profile for the oral glucose challenge. Fasting and postprandial measurements of arginine, citrulline, and ADMA will be measured to compare the impact of watermelon juice consumption on postprandial amino acid concentrations. Plasma markers of endothelial function (vascular adhesion molecule-1 (VCAM-1), E-selectin, plasminogen activator inhibitor-1 (PAI-1), and CRP) and oxidative stress (peroxinitrite, reduced gluthatione, oxidized LDL) will also be measured.

Figure 2.



Risks Associated with Procedures

Blood Draws: There is the possibility of infection and/or pain and bruising at the vein on the arm where the needle is inserted. Aseptic (sterile) technique and trained personnel minimize these risks

OGTT: There is a possibility of pain, bruising, or infection at the site of the needle insertion for the IV line. Trained personnel minimize this risk. The drink may make cause nausea, vomiting, abdominal bloating, or a headache.

Whole Body Scan GE iDXA (Dual energy x-ray absorptiometry)
The amount of radiation used for this procedure is very small. The radiation dose for this scan is equivalent to the radiation that one would naturally exposed to in the environment in less than one day. Scans will not be performed on any subject who is pregnant. A urine pregnancy test will be performed within 72 hours before the scan on females of child-bearing potential.

Muscle Blood Flow and Metabolism Measurements: Inflation of the blood pressure cuff around the arm may cause some discomfort and pain during the test. The temporary numbness and tingling are similar to the sensation of having the hand "fall asleep". If the discomfort is too severe,the participant may stop the test by notifying the technician to stop and the test will be immediately terminated. There is no known risk from the use of skin folds and/or ultrasound to measure adipose tissue thickness.

Power analysis.
To detect a 30% difference in FMD or MVBF (power =0.80, α=0.05) we will require 18 participants. Assuming a 10% drop out rate this will require the need to enroll 20 participants.

Data and Specimen Management
A mixed model with repeated measures will be employed to assess pre- oral glucose vs. postprandial changes in FMD, MVBF, and plasma markers.

Participants will be provided an identification code not linked to any personal information. Study data will be input into an excel spreadsheet and saved to the department of kinesiology drive and password protected. Only the study PI and co-investigators will have access to the study files. Paper files will be locked in a secure filling cabinet with access only provide to study personnel. Likewise, at LSU, to preserve confidentiality and data integrity, all data collection forms obtained will be secured in locked filing cabinets, or on password protected computers. Access to the primary data collection forms will be under the control of the PI and approved study personnel. Consistent with good clinical practice we will keep hard copy data records for a minimum of 3 years at which time they may be destroyed or kept indefinitely.

Biospecimens will be temporarily stored in secured -80 freezer at LSU kinesiology then will be transported by the study PI (Tim Allerton) in the following manner.

Plasma and serum samples will be transferred from Louisiana State University department of kinesiology to Pennington Biomedical Research Center. Samples will be in clearly labeled, leak-proof primary containers placed in a biohazard labeled, leak-proof secondary container containing absorbent material sufficient to retain the total sample volume. The packaged samples will be placed on dry ice in a Styrofoam transport vessel and transported in a non-passenger compartment of Dr. Allerton's vehicle with a printed sample log or other documentation describing the samples being transported. Dr. Allerton will maintain a biohazard spill kit in the vehicle.

Provisions to Monitor the Data to Ensure the Safety of Subjects
This Human Subjects Research meets the definition of a Clinical Trial. This study does not involve major risk to subjects. Efforts to minimize the potential risks of the assessment methods and outcome variables include frequent monitoring by the investigators to assure that no volunteer suffers any adverse effects from participating in the research.

Safety Monitoring. In this study, an **adverse event or experience** is defined as any health-related unfavorable or unintended medical occurrence that happens throughout study participation. Examples of adverse events include but are not limited to the following:

- A clinically significant laboratory or clinical test result.
- An event that results in missing a study visit.
- An event that requires a visit to a physician.
- An event that occurs as a result of a study procedure.

Withdrawal of Subjects
Subject could be withdrawn at any time during the study by Dr. Allerton (PI) or Dr. Hsia (study MI) on the grounds that removal from the study would be in the best interest of the subject. Non-compliance to the study protocol could also result in removal from the study. The IRB and or the sponsor may terminate the study. Additionally, study subject may withdraw voluntarily from the study themselves.

Potential Benefits to Subjects
There is no direct known benefit for subjects participating in this study.

Vulnerable Populations
No vulnerable population will be studied in this trial.

Sharing of Results with Subjects

The results of the tests done in the study will be analyzed and published in a medical journal. The results will be summarized and shared with the study subjects. After the study, individual results will be made available to the study subjects and, at their request, with their primary physician

Setting
The study screening visit, and visit 1 and visit 2 will be conducted in the department of kinesiology at LSU Vascular and Resting Metabolism Laboratory. Dr. Tim Allerton will serve as study PI, with Drs. Neil Johannsen, Brian, Irving, and Guillaume Speilmann will serve as study Co-Investigators. Dr. Daniel Hsia will provide medical oversight at study MI>

Resources Available
Vascular and Resting Metabolism Laboratory (VRML) (500 ft^2) is OSHA approved for blood work and consists of five rooms (office, work room, metabolic testing suite, and heat chamber). The VRML is equipped with a near-infrared spectrometer (Oxymon Mk III NIRS system, Artinis), a rapid cuff inflation system (E20, Hokanson), portable vascular ultrasound (LOGIC e, GE), Brachial Analyzer for Research Software (Vascular Research Tools, MIA-LLC), SphygmoCor (AtCor Medical), Caposcope, Laser Doppler (PeriFlux 5000, Perimed), data acquisition system (MP150, Biopac), biochemistry analyzer (GL5, Anolox), cholesterol analyzer (Cholestech LDX, Alere), electrolyte analyzer (EasyLyte, Medica), portable lactate analyzers (Lactate Pro), centrifuge, vapor pressure osmometer (Vapro, Wescor), and a hygrometer (Dew Point Mirror 473).

Prior Approvals

This study will be supported by that National Watermelon Promotional Board.

Compensation
Participants are eligible to be paid $75.00 for completion of the study.

Participants will receive $25 for completing at least one study visit paid upon notification of drop out of study. Participants will not be compensated for completing the screening visit.

Provisions to Protect the Privacy Interests of Subjects
Participants will review the consent form will study personnel in a private room. The signed consent form will be stored in a secure file cabinet. The subject will receive a copy of the study consent. We will explain to the subject that the results will be published in a medical journal, but subjects will not be identified.

Compensation for Research-Related Injury
No form of compensation for medical treatment or for other damages (i.e., lost wages, time lost from work, etc.) is available from the Pennington Biomedical Research Center or LSU. In the event of injury or medical illness resulting from the research procedures in which a subject participates in, the subject will be referred to a treatment facility. Medical treatment may be provided at the subject's expense or at the expense of his/her health care insurer (e.g., Medicare, Medicaid, Blue Cross-Blue Shield, Dental Insurer, etc.) which may or may not provide coverage. The Pennington Biomedical Research Center is a research facility and provides medical treatment only as part of research protocols. Should a subject require ongoing medical treatments, they must be provided by community physicians and hospitals.

Economic Burden to Subjects
We do not anticipate any economic burden as a part of this study.

Consent Process
Written informed consent will be obtained from the subjects taking part in this study. The consent process will be initiated prior to screening by Drs. Allerton, Irving, Spielmann, Johannsen, or IRB approved personnel but the process will continue throughout the study and subjects will be encouraged to ask any questions they may have. If subjects wish to take the consent home for further reflection or to discuss with their family or counselors, this will be permitted.