Title: Effects of Blueberry Consumption on Cardiometabolic Parameters in Men with Type 2 Diabetes.

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1. RATIONALE

Diabetes mellitus and its sequelae are a major and growing public health problem. The prevalence of diabetes worldwide is 194 million persons, or 5.1% of the population, and is projected to increase to 333 million, or 6.3% of the population, by 2025 (1). The number of persons with impaired glucose tolerance is estimated to increase as well. Diabetes is related to obesity, inactivity, population growth, and aging. In addition, diabetes is recognized as a group of metabolic disorders characterized by hyperglycemia and glucose intolerance due to insulin deficiency, impaired effectiveness of insulin action, or both (2).

Lifestyle strategies that include dietary modification, such as consumption of a plant-based diet, are well recognized in disease prevention and may improve type 2 diabetes (3,4). Various components of a plant-based diet may contribute to its beneficial health effects, but there has been keen interest in the possibility that plant polyphenols may have a role. Blueberries are dietary sources of polyphenols, specifically anthocyanins. Anthocyanins consist of approximately 35 – 74% of total phenolic compounds in blueberries, followed by hydroxycinnamic acid derivatives, flavonols, and flavanols (5).

There is encouraging pre-clinical evidence that berries may improve glucose management. Bilberry extract ameliorated hyperglycemia and insulin sensitivity in type 2 diabetic mice (6). Blueberry extract also reduced plasma glucose 6 h after ingestion in mice (7). Blueberry polyphenols stabilized in soybean flour reduced glucose intolerance in mice and decreased glucose production in rat hepatocytes (8). Further, lowbush blueberry extracts were found to induce beta-cell proliferation in vitro (9).

Several promising human studies have also been conducted. In healthy males, a meal with lingonberry powder plus yogurt produced an equivalent post-prandial glucose response as a meal with only yogurt, despite the extra sugar contained in the lingonberry powder containing meal (10). In another study, various berries, including strawberries, bilberries, lingonberries, and chokeberries, reduced insulin response after a meal containing berries plus bread (11). In the same study, strawberries and mixed berries also reduced glucose response after the berry-bread meal (11). In a third study, a mix of bilberries, blackcurrants, cranberries, and strawberries decreased the post-prandial glucose response in healthy adults (12).

More broadly, to date there are few clinical trials evaluating the beneficial health effects of blueberries in populations with type 2 diabetes. Four randomized parallel design studies have reported that consumption of blueberries may beneficially affect early biomarkers of cardiovascular disease and diabetes, such as altering blood pressure, insulin sensitivity and oxidative stress (13-16). A systematic review has also concluded that there is a lack of clinical trials evaluating blueberry consumption in humans with type 2 diabetes (17).

2. OBJECTIVE

A. Research question to be addressed (Objective)

How does highbush blueberry polyphenol consumption affect cardiometabolic parameters in males with type 2 diabetes?

3. STUDY DESIGN:

Sixty males (BMI >25 kg/m^2) with type 2 diabetes will be recruited to enroll in a placebo-controlled, double-blind, randomized parallel arm study with 2 treatment groups for a total of 8 weeks.

4. METHODS:

A. Procedures

Treatment. Participants will be randomly assigned to one of two treatment groups; either 1) 22 g of a control blueberry placebo treatment (matched in energy and carbohydrate content to the blueberry treatment; ingredients are maltodextrin, fructose, artificial blueberry flavoring and coloring, citric acid and silica dioxide) along with their free-living diet per day, 2) 22 g of a freeze-dried whole blueberry powder treatment along with their free-living diet per day. One cup of fresh blueberries is equivalent to approximately 22 g of freeze-dried whole blueberry powder. This amount was chosen as it is a reasonable amount of fruit for subjects with type 2 diabetes to consume. Subjects will be asked to consume 11 g of freeze dried blueberry powder or placebo, reconstituted with 240 ml water, with their morning and evening meals for 8 weeks. Participants will be counseled by a registered dietitian as to how the treatment is to be consumed, and compliance will be monitored by a daily questionnaire. In addition, the number of used and unused treatment packets will be tracked. Subjects will receive a 4 week supply of these products during a visit to the Stratton VA Medical Center. Treatments will be blinded to the study investigators and subjects (e.g., investigators and subjects will not know the composition of the treatments until after the study is complete). The US Highbush Blueberry Council will hold the code to the study treatments.

Subjects will be responsible for their own meals and will continue to follow their typical pattern of food intake (food consumption will be monitored periodically as described below).

General Physiological Variables. Body weight will be determined following an 8-h fast. Height will be measured with participants positioned in the Frankfort frontal plane. Each subject's height without shoes will be measured to the nearest 0.1 cm with a wall-mounted stadiometer. Waist and hip circumferences will be collected according to standard protocols. Body weight and composition, and waist/hip circumference will be obtained at baseline, at 4 weeks, then after 8 weeks of the study. These measurements require 30 minutes for the entire study.

Blood pressure will be collected according to a standard protocol. Subjects will be taken to a quiet room with subdued lighting. The appropriate size cuff will be attached to the upper right arm according to the manufacturer's instructions. Blood pressure will be measured three times with a Dinamap Compact Blood Pressure Monitor, which will be programmed to take an initial reading and then a reading after 5 minutes and after 10 minutes. The arm will be adjusted so that the height of the upper arm is level with the heart. Subjects will have both feet flat on the floor. Subjects

will be instructed to refrain from consuming coffee for at least 30 minutes prior to the measurement and will be advised to empty their bladder prior to the measurement. Blood pressure will be obtained at baseline, at 4 weeks, then after 8 weeks of the study. Each measure requires 20 minutes (20 minutes x 3) = 1 hour for the entire study.

Blood Collection. Twice during the study, at baseline and after the 8-week treatment period, 25 ml of blood will be collected from the antecubital veins of subjects following an overnight fast. A total of 50 ml of blood will be collected. Plasma and serum will be collected then analyzed. Plasma and serum will be used for analysis of health indices and biomarkers of cardiovascular disease and diabetes (cardiometabolic parameters). Blood will be analyzed for a complete metabolic panel, blood lipids, lipoproteins and inflammation (triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, and high sensitivity CRP) along with markers of glucoregulatory mechanisms (hemoglobin A1C, fructosamine, insulin, and glucose). Each blood draw requires 20 minutes, (20 minutes x 3) = 1 hour for the entire study.

Urine Collection. Twice during the study, at baseline and after the 8-week treatment period subjects will provide a urine sample of at least 10 ml. Urine will be collected in plastic containers and will be stored on ice during collection and until samples can be delivered to the laboratory. Each urine collection requires 5 minutes, (5 minutes x 2) = 10 minutes for the entire study. Urine will be analyzed for urine microalbumin to creatinine ratio.

Questionnaires. Participants will be asked to complete a daily questionnaire regarding their general health; if they consumed any over the counter or prescription medications and their general feeling of well-being.

Study Application (administered once) – 30 minutes- to determine eligibility
Health History (administered once) – 30 minutes- to determine eligibility
Three-Day Food Record (2 weekdays and 1 weekend day; administered 8 times) – 20 minutes per record, (20 minutes x 8) = 2 hours 40 minutes
Daily Questionnaire (administered for 56 days) – 5 minutes per questionnaire (5 minutes x 56) = 4 hours 40 minutes

B. Study sample and recruitment plan

Population: Adult males will be recruited from the Stratton VA Medical Center in Albany, NY. Recruitment will focus on individuals who have type 2 diabetes. Currently, there are over 5000 Veterans receiving care for type 2 diabetes at the Stratton VA Medical Center.

Potential subjects will be required to attend an individual information session and the requirements of participating in the study will be detailed along with the risks and benefits. At the information session, the requirements of the study, the time commitment, and risk and benefits will be described in detail. Following the session, interested subjects will be required to complete and sign a consent form and complete a study application. During this process, a study investigator will be available to answer all questions that may arise.

Subjects will be informed that participation is completely voluntary and they may withdraw from the study at any time. Should they choose to withdraw, they will be provided with a travel and meal stipend commensurate with the level of study that they have completed.

Interested volunteers will undergo a medical evaluation by a physician or nurse practitioner. The physician or nurse practitioner will review the volunteer's medical history, previous clinical laboratory tests, including a profile of hematological and biochemical parameters along with a hemoglobin A1C. The physician or nurse practitioner and research investigators will use the clinical screening and evaluation results to identify individuals who qualify for the study.

Inclusion Criteria:

- Male
- Ages 45 to 75 years at beginning of study
- BMI >25 kg/m^2
- HbA1C > 6.5 and < 9
- Medical diagnosis of type 2 diabetes for at least 6 months

Exclusion Criteria:

- Use of insulin
- Presence of chronic kidney disease (GFR $\leq$ 45 mL/min), liver cirrhosis, gastrointestinal disease, pancreatic disease, or malabsorption syndromes
- Weight loss of >10% of body weight over the last 6 months; routine participation in heavy exercise
- Heavy smokers (>20 cigarettes per day)
- Unable or unwilling to give informed consent or communicate with study staff
- Other medical, psychiatric, or behavioral factors that in the judgment of the Principal Investigators may interfere with study participation or the ability to follow the intervention protocol
- Allergies to blueberries or blueberry products

C. Data management

Health and other personal information given in participation of this research is confidential. All information will be kept in strictest confidence. Each subject will be assigned a code number for the study. This number will be assigned at the initial visit and will be used to identify samples and data. Names or other identifiers will only be used on screening documents or other procedures where person-to-person communication is required. Subjects will not be personally identified in any of the reports of research. Subjects, and their physicians with written subject consent, will have access to subjects' records. Records will be reviewed with each subject with one of the investigators, if requested, to provide an explanation of the findings. Whenever research in which subjects have participated is published, they will be given a copy of the report, if requested. All biologic samples will be labeled only with ID numbers. All data will be de-identified and stored under ID numbers to diminish risk of disclosure. Key containing the subjects names, social security number and their study specific ID

number will be stored in a separate folder on the T-drive:\ Al-Research\PI\Wilson. Only the study investigators will have access to files containing subjects' names. The research data will be securely stored on the T: Drive (T:\Research\PI\Wilson), at the Stratton VA Medical Center, during and after the study. The study investigators, Margaret Wilson and Kim Stote, will have access to the data. Any hardcopies of individual data will be stored in a secure (locked) file cabinet in room 602Core.

Data from the study will be stored indefinitely or according to Records Control Schedule Requirements. In addition, any event of loss of data, breach of confidentiality or serious adverse event will be reported to the Information Security Office and Privacy Officer within 24 hours of learning of the event and will be reported to the Institutional Review Board.

Every effort will be made to protect the confidentiality of the information provided insofar as it is legally possible.

Quality control of data management will incorporate software routines that identify and flag missing and out-of-range values, double entry of data into the computer, and checking of typed data for a random subset.

D. Data analysis and sample size justification

Data analysis:

The data will be analyzed using a mixed model in order to partition variance from random and fixed effects. The model will account for age, BMI and the significance for other terms will be tested to determine if they are to remain in the model. Some terms may be used as covariates. Split-plot and split-split plot analysis will be used to partition variances from measurements for which there are repeated measures. Contrasts statements or appropriate multiple comparisons of means will be completed using least-square means.

Sample size:

Sixty males with type 2 diabetes will be recruited for the study. The sample size for this study is based on a standard deviation from a previous study conducted by Basu et al (13), which showed that 50 g of freeze-dried blueberry powder lowered systolic and diastolic blood pressure. A sample size calculation was estimated for a 1-factor ANOVA for a parallel-arm study with > 80% power to detect a significant difference. Twenty-four participants per group are needed, and 30 per group are determined based on a 20% drop out rate.

5. RISK/BENEFIT ASSESSMENT:

This study has a minimal level of risk. Blood collection may cause a small amount of pain when the needle is inserted into the vein. Sometimes, after blood is taken, a small bruise can appear at the site. This bruise, however, does not cause any long-term problems. Aside from the discomfort of the needle puncture, there is nothing in this procedure that can cause any problem. There is also a remote chance of fainting, light-headedness, or infection. Blood will be drawn from a forearm vein by a trained

professional using sterile, disposable material.

To minimize the risk of bruising and pain, skilled and experienced phlebotomists will collect blood samples. If during the study, a participant ever experiences vertigo, he will thereafter have his blood drawn while reclining in a suitable chair (during subject selection, participants are asked if they have or had a history of fainting while giving blood).

6. STUDY POPLUATION – GENDER/ETHNIC INCLUSION:

Rationale for research subject selection – The population from which participants will be recruited consists of approximately 5000 individuals receiving care for type 2 diabetes living within commuting distance of the study site at the Stratton VA Medical Center, Albany, NY.

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