WATERMELON SUPPLEMENTATION TO IMPROVE SYSTEMIC ATHEROSCLEROTIC RISK FACTOR BIOMARKERS IN OVERWEIGHT POST-MENOPAUSAL WOMEN IN A COMMUNITY SETTING

A Thesis
by
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Abstract

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The purpose of this study was to determine the degree to which six select markers of atherosclerotic risk were affected by a six-week watermelon consumption intervention in overweight post-menopausal women in a community setting. METHODS: Forty five subjects met the criteria and completed the pre- and post- study visit after an overnight fast. Height, weight, percentage of body fat, and a blood sample were taken during both visits. During the pre-study visit, the subjects were randomly assigned to either the control (no intervention) group or the watermelon treatment group. The watermelon group consumed 710 mL (1.88 grams of L-citrulline/ 0.39 grams of L-arginine) of watermelon puree per day for the six-week period. The plasma concentration of sVCAM-1, ADAMTS 13, GDF-15, sICAM-1, and sP-selectin were measured pre- and post-treatment period. RESULTS: There was a significant ($p = 0.005$) 8.4% increase in the plasma concentration of L-arginine that occurred in the watermelon group between the pre- and post-treatment period. Also, there was a significant decrease ($p = 0.003$) in plasma VCAM-1 concentrations that occurred in the

watermelon group between the pre- and post-treatment. The pattern of change in the plasma concentration of sICAM-1, ADAMTS 13, GDF-15, sP-selectin, and L-citrulline did not differ between treatment groups (all, $p > 0.05$). The plasma concentration of fibrinogen was above detection limits and therefore, not included in the statistical analysis. CONCLUSION: The six-week watermelon supplementation treatment resulted in a small but significant increase in VCAM-1, but no changes in the other atherosclerotic biomarkers measured. Additional studies are needed to determine if a longer treatment period and/or greater dose of watermelon puree is required to decrease atherosclerotic risk in overweight post-menopausal women in a community setting.

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Introduction

Cardiovascular diseases (CVDs) are the leading cause of death worldwide (50, 65). Menopause, an absence of a menstrual period for one year due to aging, increases a woman's risks for developing CVDs, specifically atherosclerosis, due to estrogen depletion. Furthermore, menopause increases the risk for oxidative stress (2), which can increase the risk for hypertension and arterial stiffness (20, 58). Estrogen plays a protective role in a woman's body by increasing vasodilation through nitric oxide (NO) via endothelium nitric oxide synthase (eNOS) (57). This indirectly protects women from oxidative stress and is done through a receptor mediated system where estrogen binds to the estrogen receptor ERα. During menopause, there is a decrease in circulating estrogen levels (38, 58). This decrease in estrogen levels leaves post-menopausal women more at risk for developing oxidative stress and endothelial dysfunction, increasing their risk for atherosclerosis (32).

Atherosclerosis is the buildup and hardening of plaque on the artery walls, which narrows the cross-sectional area of arteries and consequently decreases blood flow. Atherosclerosis is caused by an increase in oxidative stress and decrease in NO bioavailability leading to endothelial dysfunction (32). Early stages of atherosclerosis include recruitment and buildup of leukocytes mediated by cellular adhesion molecules causing inflammation of the arteries (7). This ultimately leads to a cascade effect causing an increase in endothelial dysfunction, vasoconstriction, and risks for other CVDs.

NO is a reactive molecule that plays an important role in healthy vascular maintenance and vasodilation. There are three nitric oxide synthase (NOS) isoforms that

produce NO. However, eNOS isoform derived NO production results in vasodilation and decreases atherosclerosis risk factors by decreasing platelet activation and platelet adhesion to the endothelium (32). NO production occurs via eNOS, cofactors for eNOS, and the citrulline-NO cycle (24). Oral consumption of L-citrulline and L-arginine modulate NO production (23).

Watermelon is high in free water, has many nutrients, and may decrease overweight post-menopausal women's risk for atherosclerosis. Consumption of watermelon, a rich source of L-citrulline and L-arginine (54), can increase plasma L-citrulline and L-arginine (13, 47) and allow L-citrulline and L-arginine to be readily available for NO production (13). The extracellular availability of L-arginine is a rate-limiting factor in NO production (24). The citrulline-NO cycle plays a more important role in the production of L-arginine for NO production compared to oral supplementation of L-arginine (24). However, oral supplementation of L-arginine and L-citrulline helps in NO production via eNOS (23).

Bloodborne biomarkers can be used to gauge the inflammatory response that leads to atherosclerosis. Circulating levels of inflammatory proteins including soluble vascular cell adhesion molecule-1 (sVCAM-1) (5), soluble intercellular adhesion molecule-1 (sICAM-1) (5), soluble P-selectin (sP-selectin) (5), fibrinogen (5), and growth differentiation factor-15 (GDF-15) (41) increase when atherosclerotic risk factors increase (5, 41). ADAM metallopeptidase with thrombospondin type 1 motif, 13 (ADAMTS 13) circulatory levels decrease as the risk of atherosclerosis risk increases (8). Each biomarker has a different affect in the body; however, each can be used to gauge the amount of inflammation due to endothelial damage.

Previous research has demonstrated the efficacy of daily watermelon consumption to increase the bioavailability of L-citrulline and L-arginine to increase fasting plasma levels of L-arginine (13) thus increasing NO via eNOS. This can enhance in vitro NO-dependent aortic ring vasoreactivity (66) and may decrease inflammation associated with the risk for atherosclerosis. Previous research has also demonstrated that the selected inflammation biomarkers can be used to gauge a person's risk for atherosclerosis (5, 8, 41). This study tested the hypothesis that sICAM-1, sVCAM-1, sP-selectin, GDF-15, and fibrinogen plasma concentrations will decrease, and ADAMTS 13 plasma concentrations will increase after six weeks of watermelon supplementation in overweight post-menopausal women. Therefore, the purpose of this study was to determine the degree to which six select markers of atherosclerotic risk were affected by the six week watermelon consumption intervention in overweight post-menopausal women in a community setting.

Literature Review

Cardiovascular diseases (CVDs), specifically atherosclerosis, pose a serious problem for overweight post-menopausal women (20, 29, 58, 62). There are many risk factors for CVD that increase when a woman becomes post-menopausal, but some risk factors can be diminished (59, 68). Watermelon supplementation may decrease CVD risk factors by increasing plasma L-arginine and L-citrulline levels, which can lead to the production of nitric oxide (NO) (13, 23, 47).

Previous research has demonstrated the efficacy of daily watermelon consumption to increase the plasma levels of L-arginine and L-citrulline (13, 47). In Zucker diabetic fatty (ZDF) rats, watermelon pomace juice supplementation increases tetrahydrobiopterin (BH4) synthesis and bioavailability by increasing GTP cyclohydrolase-1 activity (the first and rate limiting enzyme in the de novo production of BH4) while increasing endothelium nitric oxide synthase (eNOS) activity and NO production (66). Increased circulating NO enhances vasodilation and may decrease inflammation associated with the risk for CVD. Previous research has also demonstrated that the selected inflammation biomarkers can be used to gauge a person's risk for atherosclerosis (5, 8, 41). This study tested the hypothesis that sICAM-1, sVCAM-1, sP-selectin, GDF-15, and fibrinogen plasma concentrations will decrease, and ADAMTS 13 plasma concentrations will increase after six weeks of watermelon supplementation in overweight post-menopausal women. Therefore, the purpose of this study was to determine the degree to which six select markers of atherosclerotic risk were affected by the six week watermelon consumption intervention in overweight post-menopausal women in a community setting.

Cardiovascular Disease and Post-Menopausal Women

Cardiovascular diseases affect the heart and blood vessels and are the leading cause of death worldwide (50, 65). Cardiovascular diseases have many modifiable and non-modifiable risk factors. Non-modifiable risk factors that cannot be prevented include heredity, sex, ethnicity, and age; while modifiable risk factors include hypertension, high cholesterol, obesity, smoking and a sedentary lifestyle (21, 22). These are also some of the main risk factors for developing atherosclerosis.

Atherosclerosis is the buildup and hardening of plaque on the artery wall. This narrows the lumenal area of arteries and consequently decreases blood flow. Atherosclerosis is caused by an increase in oxidative stress and a decrease in NO bioavailability leading to endothelial dysfunction (32). Early stages of atherosclerosis includes a recruitment and buildup of leukocytes mediated by cellular adhesion molecules causing inflammation of the arteries (7). This ultimately leads to a cascade effect causing an increase in vasoconstriction, endothelial dysfunction, and risks for other CVDs.

Menopause is an absence of a menstrual period for one year due to aging. It is unavoidable for aging women and is accompanied by many health and lifestyle changes. Menopause can increase a woman's risk for hypertension and arterial stiffness (20), which can increase oxidative stress and endothelial dysfunction (2, 32). This is due to physical changes in hormone levels and lifestyle of women during the aging process. During post-menopause, there is a decrease in circulating estrogen and other sex hormone levels (38, 58). This is harmful for aging women, because estrogen plays a protective role in the woman's body, and through estrogen depletion, there is a decrease in NO bioavailability leading to an

increased risk for multiple chronic diseases in women (38, 57). Specifically, the decrease in estrogen levels leaves post-menopausal women more at risk for oxidative stress and endothelial dysfunction increasing their risk for atherosclerosis (32).

Estrogen protects women from atherosclerosis while growth factors and cytokines play important roles in mediating atherosclerosis (37). Kamada et al. (37) examined fifteen cytokines from 97 post-menopausal women who were either receiving hormone replacement treatment or no treatment. It was concluded that hormone replacement therapy can increase the production of macrophage colony-stimulating factor, which can decrease serum cholesterol levels (37). This may prevent atherosclerosis and provides evidence that estrogen is a protective hormone for women.

Estrogen can increase endothelial NO production through a receptor-mediated system and the estrogen receptor ERα (11, 31, 44, 57). Estrogen and ERα activate mitogen-activated protein (MAP) kinase and tyrosine kinase, which causes intracellular Ca^{2+} and NO to increase (11). This is important because estrogen can increase vasodilation and protect women from atherosclerosis. It has been observed in monkeys and rabbits that short-term and long-term estrogen therapy can improve endothelium-dependent vasodilation (44) through a protein kinase B (Akt)-dependent mechanism (35). When women reach post-menopause, NO production decreases due to estrogen depletion; however, therapeutic strategies to increase endothelial NO production have been sought.

L-Citrulline, L-Arginine, and Nitric Oxide Relationship

Nitric oxide plays an important role in vasodilation and is a component of healthy vasculature maintenance. There are three isoforms of nitric oxide synthase (NOS). Two are

constitutively expressed (NOS1 or neuronal NOS and NOS3 or endothelial NOS) and the third is inducible (NOS2 or inducible NOS). eNOS is the isoform that is involved in the endothelium response activating vasodilation through the L-citrulline-NO cycle (24). eNOS is regulated and activated by Ca^{2+} and calmodulin (1, 25) and has cofactors including: BH4, nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide/flavin adenine dinucleotide (FMN/FAD) (56). eNOS is also the NOS isoform that plays an important role in decreasing atherosclerosis risk. Atherosclerosis is increased when there is a dysfunction with endothelial cells, decreased bioavailability of L-arginine and cofactors, and decreased expression of eNOS (10, 32, 34). Endothelial cell dysfunction increases lesion formation (30). This increases inflammation and hypertrophy of smooth muscle cells, which contributes to blood clotting and vasoconstriction (6, 55). A component of endothelial cell dysfunction is decreased bioavailability of L-arginine.

L-arginine has the ability to reduce atherosclerosis risk factors including adipose tissue, hypertension, and aortic stiffness (23, 48, 66). L-arginine is a cell signaling molecule to NO, glutamate, and agmatine (decarboxylated arginine) (67). Extracellular L-arginine is a rate-limiting factor in NO production; however, the majority of L-arginine availability is not utilized in NO production (24). While oral L-arginine supplementation can increase NO levels (23, 66), the L-citrulline-NO cycle plays a more important role in the production of L-arginine for NO production (24).

L-citrulline is a non-essential amino acid that can be used in the synthesis of L-arginine and thus NO (24, 54). Endogenous synthesis of L-arginine from L-citrulline occurs when L-citrulline is released by the small intestine into the blood stream where it is extracted

by the kidneys (67). L-citrulline is then converted into L-argininosuccinate through argininosuccinate synthase, then to L-arginine through argininosucinate lyase, and finally L-arginine is converted to NO through eNOS and eNOS cofactors (24, 42, 49, 67). This is known as the citrulline-NO cycle (24, 67). Nitric oxide can also be increased by L-arginine through intracellular tetrahydrobioprterin (BH-4)-dependent NO synthase (33, 66).

Watermelon and the L-Citrulline-Nitric Oxide Cycle

Consumption of watermelon, a rich source of L-citrulline and L-arginine (54), increases fasting plasma L-citrulline and L-arginine (13). Wu et al. (66) compared L-arginine availability during watermelon pomace juice supplementation (which included L-citrulline and L-arginine) in Zucker diabetic fatty (ZDF) rats. The ZDF rats were deficient in GTP cyclohydrolase-1, thus, impairing eNOS. GTP cyclohydrolase-1 is the rate-limiting enzyme for BH4 synthesis (66). In ZDF rats, higher L-arginine concentrations increased GTP cyclohydrolase-1 activity, thus increasing the synthases and bioavailability of BH4 (66). Wu et al. (66) reported that GTP cyclohydrolase-1 activity increased 39% and BH4 concentrations increased about 36% with watermelon pomace juice supplementation. Additionally, serum concentrations of L-arginine, nitrite and nitrate, and oxidation products of NO, were increased through watermelon supplementation (66). Wu et al. concluded that L-arginine synthesis occurred via L-citrulline and is then converted into NO via eNOS (66). This data supports the notion that L-citrulline can increase circulating L-arginine levels, and thus, increase the eNOS cofactor BH4 and NO production.

Collins et al. (13) compared the fasting plasma concentration of L-arginine and L-citrulline through watermelon juice consumption in healthy adults. Twelve males and eleven

females, ages 36-69 years old, consumed one of three different amounts of watermelon juice for three-weeks each. The low watermelon condition consisted of three containers each day of 0.78kg of juice to deliver 1.025g of L-citrulline plus 0.228g of L-arginine per day. The high watermelon condition consisted of six containers each day of 1.56kg of juice to deliver 2.051g of L-citrulline plus 0.455g of L-arginine per day. Meals were prepared by a registered dietitian to control L-citrulline and L-arginine intake and to maintain body mass throughout treatment. The authors concluded that fasting plasma L-arginine concentrations increased in the high (22%) and low (11%) watermelon groups during the three-week supplementation period, while fasting L-citrulline plasma concentrations had no significant changes. They concluded that L-citrulline is bioavailable and is converted into L-arginine, and thus increases plasma L-arginine levels (13).

Figueroa et al. (23) examined the effect of watermelon supplementation on aortic blood pressure, wave reflection, and pulse wave velocity on pre-hypertensive middle-aged individuals (ages ranging between 51-57 years). Four males and five females, who were all pre-hypertensive, were recruited for the study and randomly divided into two groups. Each group concluded the six-week watermelon supplementation intervention and the six-week placebo intervention with a four-week washout period in between interventions. During the six-week watermelon supplementation period, subjects consumed 1.35g of L-citrulline and 0.65g of L-arginine two times per day to deliver 2.7g of L-citrulline and 1.3g of L-arginine per day. An overnight fast was conducted before and after each intervention, and blood pressure, wave reflection, and pulse wave velocity were taken during each visit. The authors concluded that aortic blood pressure, wave reflection, and aortic and brachial pulse pressure decreased significantly after the six-week watermelon supplementation period. This study

indicates that a six-week watermelon intervention of L-citrulline and L-arginine can have beneficial effects on pulse pressure, which can decrease hypertension (a risk factor for atherosclerosis) (23).

Biomarkers for Atherosclerotic Risk Factors

Cell Adhesion Molecules

Cell adhesion molecules (CAMs) are proteins on the cell surface that interact with ligands, which allow for cell to cell or cell to matrix interactions (39). There are many different types of CAMs; some of which are linked to inflammation and immune responses (39). CAMs have been used as CVD and atherosclerosis biomarkers because, inflammation is related to atherosclerosis risk factors, and higher expressions of CAMs are associated with high endothelial inflammation (4, 43). Inflammation markers are significant predictors of CVD events and atherosclerosis in post-menopausal women (53). Soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), and soluble P-selectin (sP-selectin) are proteins of the endothelial cell surface that interact with extracellular matrix components to attract immune cells to damaged endothelial areas. Specifically, sP-selectin draws leukocytes from the circulation to the damaged endothelium by weakly binding to carbohydrate ligands on the surface of the leukocytes. The weak binding will cause the leukocyte to decelerate and roll across the endothelium surface, where sVCAM-1 and sICAM-1 create a strong attachment to the leukocyte and are involved with transendothelial migration (18, 36). While the effects of these selected CAMs are mainly on cell surfaces, their soluble forms found in plasma are indicative of their membrane bound expression levels (45).

Elevated sICAM-1 is an independent risk factor for myocardial infarction (18) and allows for leukocytes to form an adhesion to damaged or inflamed endothelium (51). sICAM-1 allows for leukocytes to form an adhesion to the endothelium through binding of leukocyte intergrins, leukocyte function associated antigen-1 (LFA-1) and Mac-1(51). Ridker et al. (53) examined 28,263 post-menopausal women's plasma sICAM-1 levels over a three-year time period. The data indicates that sICAM-1 was a significant predictor for adverse cardiovascular events, i.e., higher plasma levels of sICAM-1 are correlated with atherosclerosis risk factors and CVD events (53).

sVCAM-1 is a cell adhesion molecule that can have a weaker expression during CVD compared to other CAMs; however, sVCAM-1 has a higher expression during the early stages of atherogenesis (36). sVCAM-1 interacts with the very late antigen 4 to allow the activation and initiation of atherosclerosis (7). There is evidence that NO can limit the expression of sVCAM-1 (43); therefore, increases in NO may result in lower sVCAM-1 expression.

sP-selectin is a cell adhesion molecule involved with leukocyte attachment to damaged endothelium sites (9, 51). Carter et al. (9) compared the relationship of plasma sP-selectin levels to coronary artery disease in 249 coronary artery patients and 252 healthy patients. sP-selectin levels were significantly higher in patients with higher cardiovascular risk factors and become more apparent when there are signs of atherosclerosis (9).

A study conducted by Demerath et al. (18) compared the relationship of sICAM-1, sVCAM-1, sP-selectin, and E-selectin to CVD and atherosclerosis risk factors in 281 males and 311 females. Other CVD lifestyle risk factors were measured. The results indicated that

soluble adhesion molecule concentrations increase as CVD risk factors increase (18). This supports the use of measuring plasma levels of sVCAM-1, sICAM, and sP-selectin as biomarkers for atherosclerosis.

ADAMTS 13

ADAM metallopeptidase with thrombospondin type 1 motif, 13 (ADAMTS 13) is a metalloprotease that decreases inflammation during atherosclerosis by cleaving highly active Von Willebrand Factor (VWF) thus making less active VWF (8, 27). VWF proteins are glycoproteins that are part of the blood clotting cascade and are involved in the recruiting and adhesion of platelets to atherosclerotic sites (27). The ratio between ADAMTS 13 and VWF are significant predictors of CVD risks when there is a low ADAMTS 13 and high VWF:Ag, also ADAMTS 13 alone is significantly correlated to endothelial dysfunction (28).

ADAMTS 13 levels are lower and VWF levels are high in young individuals (<45 years males, <55 years females) with higher risk of CVD than in subjects of the same age range with low risk of CVD (8). Subjects with lower levels of ADAMTS 13 were five times more at risk for CVD than subjects with normal levels of ADAMTS 13 (8). This data supports the use of ADAMTS 13 as a marker of CVD risk.

Growth Differentiation Factor – 15

Growth differentiation Factor – 15 (GDF-15) is a stress responsive cytokine used as a biomarker to identify endothelial dysfunction and cardiovascular dysfunction (41, 46). Increased levels of GDF-15 are linked to increased risk of CVD and atherosclerosis (64). GDF-15 is weakly expressed in healthy individuals; however, its expression increases

sharply during tissue injury or inflammation (46). GDF-15 is not a cardiac specific cytokine, but it could affect other tissues that are injured or inflamed. However, in rats and humans GDF-15 expression is increased in the heart after a myocardial infarction as a defensive mechanism (40). GDF-15 expression is increased during oxidative stress, antiangiogenic stress, and in response to increased pro-inflammatory cytokines, increased oxidized low density lipoproteins, and mechanical stress (41); all risk factors for atherosclerosis. A decrease in GDF-15 suggests a decreased risk for CVD.

Fibrinogen

Fibrinogen is a glycoprotein involved in the blood clotting cascade and is an independent risk factor for CVD (60). Fibrinogen is increased when inflammation is increased and has negative effects by increasing the blood clotting cascade (4, 17, 60). Fibrinogen increases platelet aggregation, increases plasma viscosity, and promotes fibrin formation (60). Increases in fibrinogen are correlated with obesity, age, smoking, diabetes mellitus and total cholesterol increases (60).

Many studies report that increases in atherosclerosis risk factors are associated with increased circulatory levels of inflammatory proteins including sVCAM-1, sICAM-1, sP-selectin, and Fibrinogen (5) and can increase GDF-15 (41). These risk factors include obesity, smoking, diabetes, cholesterol, triglycerides, and shear stress (the frictional force of blood that acts against the endothelium) (5, 9, 18, 36). However, studies have also reported that decreases in risk factors are associated with decreases in inflammatory proteins (5) and higher high density lipoprotein levels are associated with lower sVCAM-1 and sICAM-1 (36). ADAMTS 13 determines atherosclerosis risk factors differently than other biomarkers

where decreased a level of ADAMTS 13 is correlated with increases in atherosclerotic risk (8). Each biomarker reflects a different biological process; however, each can be used to gauge a person's risk for atherosclerosis.

Conclusion

Atherosclerosis and cardiovascular disease risk is increased during post-menopause due to a decrease in both estrogen and vasodilation. Previous research has demonstrated the efficacy of daily watermelon consumption to increase the bioavailability of L-citrulline to increase the fasting plasma level of L-arginine, thus increasing NO via eNOS and eNOS cofactors. This can increase vasodilation and may decrease inflammation associated with the risk for atherosclerosis. Previous research has also demonstrated that the selected inflammation biomarkers can be used to gauge a person's risk for atherosclerosis. This study tested the hypothesis that sICAM-1, sVCAM-1, sP-selectin, GDF-15, and fibrinogen plasma concentrations will decrease, and ADAMTS 13 plasma concentrations will increase after a six-week watermelon supplementation in overweight post-menopausal women. Therefore, the purpose of this study was to determine the degree to which six select markers of atherosclerotic risk where affected by a six-week watermelon consumption intervention in overweight post-menopausal women in a community setting.

Methods

Subjects

Sixty overweight (BMI ≥25kg/m^2) post-menopausal women with ages ranging between 50-75 years were recruited. After completing a voluntary informed consent form, subjects were required to complete a medical health questionnaire; both forms were approved by Appalachian State University's Institutional Review Board (14-0092). A medical health questionnaire was used to verify medical history and lifestyle habits of each subject and was compared to the exclusion criteria. Subjects were nonsmokers with no diagnosed history of chronic diseases including: coronary heart disease, stroke, cancer, type 1 or 2 diabetes mellitus, or rheumatoid arthritis. Subjects that did not meet the inclusion criteria were not enrolled in the study.

Experimental Design

The study consisted of two visits: pre- and post-study, six weeks apart at Appalachian State University's Vascular Biology and Autonomic Studies Laboratory. Each subject completed an overnight fast prior to the pre- and post-visits. Overnight fast included no food or beverage, other than water, nine or more hours prior to the visits. During both visits, height, weight, and percentage of body fat were measured in addition to a blood sample being taken. Blood samples, ~40mL, and were obtained by a trained phlebotomist from the antecubital vein of each subject while the subject was in a seated position. Blood samples were then centrifuged, the plasma was aliquotted, and stored at -80°C until further analysis.

During the pre-study visit, the subjects were randomly assigned either the control (no intervention) group or the watermelon treatment group. Each group was required to maintain their normal diet and physical activity level, and were not allowed to make any formal attempt to lose weight or take any weight-loss medications throughout the six-week period. The watermelon group consumed 710 mL (2.27 grams of L-citrulline/L-arginine) of watermelon puree per day (between breakfast and dinner time), while the control group consumed no watermelon.

Watermelon Supplementation

Millionaire variety seedless Sunripe watermelons (Falls Church, VA, USA) were used to make the watermelon puree, and were provided by a pilot plant at the U.S. Department of Agriculture Citrus and Subtropical Products Laboratory (Winterhaven, FL, USA). Watermelon was cold pressured pasteurized and then analyzed for citrulline and arginine concentration (Dr. Penelope Perkins-Veazie – Plants for Human Health Institute, North Carolina State University).

Watermelon puree was held at -20°C before use. Subjects were instructed to thaw each bottle of watermelon puree before consumption by running the bottle under hot water. Each 710 mL. of watermelon puree provided 1.88 grams of L-citrulline and 0.39 grams of L-arginine equaling 2.27 grams L-citrulline/L-arginine per day.

Analysis of Blood

After the pre- and post-study blood samples were collected, the blood samples were centrifuged for 10 minutes at 1000 RCF and 4°C. The plasma was aliquotted and stored at -

80°C until analysis. After the study was completed, all of the plasma samples were analyzed for the select cardiovascular disease biomarkers. Commercial bead-based multiplex assay kits were used to measure the plasma concentration of the following cardiovascular disease markers: ADAMTS 13, GDF-15, sVCAM-1, sICAM-1, sP-Selectin, and fibrinogen (MILLIPLEX® Map Kit – Human Cardiovascular Disease Magnetic Bead Kit Panel 2 and Panel 3; Millipore, Billerica, MA). Analysis of the cardiovascular disease markers was conducted according to the manufacturer's specifications using the MAGPIX® instrument and xPONENT® analysis software (Luminex, Austin, TX).

During the analysis process, plasma samples were diluted according to the manufacturer's specifications, i.e., 1:100 with assay buffer for Panel 2 (ADAMTS 13, GDF-15, sVCAM-1, sICAM-1, and sP-Selectin), and diluted 1:40,000 with assay buffer for Panel 3 (fibrinogen). Seven standards were utilized to create a standard curve and two quality controls were utilized for each analyte. Diluted plasma samples, controls, and standards were added, in duplicate, across four 96-well plates. Pre- and post-study plasma samples from each individual were analyzed on the same plate.

Statistics

All data are expressed as mean ± SE. The biomarker data were analyzed using a 2 (condition) × 2 (time) repeated-measures ANOVA, between-subject design. When significant interaction effects were found ($p \leq 0.05$), changes were compared using two-tailed t-tests with significance set after Bonferroni adjustment at $p \leq 0.0125$. A Cohen's d was used to find the effect size of significant interactions by taking the difference between the two means and dividing it by the standard deviation (12).

Results

Subject Characteristics

A total of 51 subjects volunteered and completed all aspects of this study; however, 6 subjects had a fasting blood glucose >125mg/dL at the pre-study measurement and were excluded from analyses. Subject's age, body mass, body mass index (BMI), and percentage of body fat were compared pre- and post-intervention (Table 1). Significant differences ($p \leq 0.05$) over time occurred in the percent body fat and in the body mass.

Table 1 Subject Characteristics

	Pre-Study	Post-Study	Time; interaction effects, P-Value[a]
Age			
CT	60.1 ± 1.6		
WM	59.5 ± 1.0		
Mass (kg)			
CT	82.6 ± 3.3	82.7 ± 3.3	0.048; 0.297
WM	84.2 ± 3.4	84.8 ± 3.4	
BMI (kg/m^2)			
CT	30.3 ± 1.4	30.4 ± 1.1	0.285; 0.351
WM	29.9 ± 1.2	31.1 ± 0.92	
Body Fat %			
CT	42.6 ± 1.1	43.3 ± 1.2	0.007; 0.259
WM	42.9 ± 0.95	43.2 ± 1.0	

Notes: All data are means ± SE. [a]The first P-value represents the overall time effect; the second P-value represents the condition (control vs. watermelon) × time (2 time points) interaction effects. CT = Control, WM = Watermelon

L-Arginine and L-Citrulline

Pre- and post-study L-arginine and L-citrulline plasma concentrations were compared (Table 2). The pattern of change in the plasma concentration of L-arginine was significantly different between groups ($p = 0.022$). Post-hoc analysis revealed a significant time effect ($p = 0.005$) within the watermelon group, i.e., there was a significant 8.4% increase in the plasma L-arginine concentration that occurred between the pre-watermelon and post-watermelon treatment period. However, the effect size was small (Cohen's $d = -0.3486$). The plasma L-arginine concentration did not change in the control group ($p > 0.05$). The pattern of change in the plasma L-citrulline concentration did not differ ($p > 0.05$) between the control and the watermelon group.

Table 2. L-arginine and L-citrulline Pre- and Post-Study Plasma Concentrations

	Pre-Study	**Post-Study**	**Time; interaction effects, P-Value[a]**
L-arginine			
CT	113 ± 5.9	111 ± 6.3	0.154; 0.022
WM	98.5 ± 5.1	107 ± 5.4 *	
L-citrulline			
CT	45.0 ± 3.3	44.2 ± 2.9	0.881; 0.577
WM	37.9 ± 2.9	38.4 ± 2.3	

See Table 1 for notes. * $p = 0.005$, within treatment group.

Biomarkers

Pre- and post-study plasma concentrations for sVCAM-1, ADAMTS 13, GDF-15, sICAM-1, and sP-selectin were compared (Table 3). Analysis of sVCAM-1 plasma concentrations revealed a significant time by treatment effect ($p = 0.023$). Post-hoc analysis revealed a significant pattern of change within the watermelon group, i.e., there was a

significant decrease (p = 0.003) in plasma sVCAM-1 concentration that occurred between the pre-watermelon and post-watermelon period (Table 3); however, the effect size was small (Cohen's d = 0.2388). The pattern of change in the plasma concentration of sVCAM-1 (p > 0.05) did not differ in the control and group. The pattern of change in the plasma concentration of sICAM-1, ADAMTS 13, GDF-15, and sP-selectin did not differ between treatment groups (all, p >0.05). The plasma fibrinogen results during analysis were above detection limits, i.e., the analyte concentrations were higher than the highest point of the standard curve; therefore, fibrinogen analytes could not be accurately assessed in this investigation.

Table 3 CVD Biomarker Pre- and Post-Study Plasma Concentrations

	Pre-Study	**Post-Study**	**Time ; interaction effects, P-Value[a]**
sVCAM-1 (ng/mL)			
CT	7.41 ± 0.40	7.46 ± 0.39	0.082; 0.023
WM	6.86 ± 0.35	6.46 ± 0.33*	
ADAMTS 13 (ng/mL)			
CT	12.5 ± 0.55	12.0 ± 0.54	0.043; 0.864
WM	12.0 ± 0.47	11.5 ± 0.46	
GDF-15 (pg/mL)			
CT	9.35 ± 1.00	9.36 ± 1.00	0.150; 0.147
WM	9.74 ± 1.00	9.04 ± 1.00	
sICAM-1 (ng/mL)			
CT	1.70 ± 0.10	1.75 ± 0.095	0.988; 0.092
WM	1.85 ± 0.09	1.80 ± 0.081	
sP-Selectin (ng/mL)			
CT	1.18 ± 0.11	1.14 ± 0.094	0.245; 0.175
WM	1.19 ± 0.09	0.982 ± 0.08	

See Table 1 for notes. * p= 0.003, within treatment group.

Discussion

The current study investigated the degree to which six select biomarkers of atherosclerotic risk factors were affected by the six-week watermelon consumption intervention in overweight post-menopausal women in a community setting. The major findings of this study were that fasting L-arginine plasma concentrations increased 8.4% through oral consumption of 0.39 grams of L-arginine and 1.88 grams of L-citrulline per day, and that sVCAM-1 plasma concentration significantly decreased over time in the watermelon group.

Watermelon Supplementation

The increase in the fasting plasma concentration of L-arginine over time in the watermelon group was due to the L-arginine and L-citrulline in the watermelon supplement. The fasting plasma concentration of L-citrulline was not increased. L-arginine is used throughout the body. 40% percent is catabolized in the intestine and 10-15% catabolized in the liver (13); however, extracellular L-arginine is the rate-limiting factor in nitric oxide production (24). L-citrulline can be released from the intestines and can bypass the liver to be taken up and converted into L-arginine in the kidneys (67). The fasting plasma concentration of both L-arginine and L-citrulline measured in this study reflected their known metabolic fates.

Collins et al. (13) supports our findings of a significant increase in the fasting L-arginine plasma levels while no significant changes in L-citrulline fasting plasma levels can be found with a 2g L-citrulline/L-arginine per day watermelon supplementation. Collins et al. (13) concluded that fasting L-arginine plasma levels increased 11% after a 3-week

supplementation of 1.253g L-citrulline/L-arginine (1.025g L-citrulline/ 0.228g L-arginine), and fasting L-arginine plasma levels increased 22% after a 3-week supplementation of 2.506g L-citrulline/L-arginine (2.051g L-citrulline/ 0.455g L-arginine) (13). There was a significant 8.4 % increase in fasting plasma L-arginine concentrations during this study. Collins et al. (13) controlled subject diet, while this study was in a community setting and diet was not strictly controlled. The community setting may account for the smaller increase in fasting L-arginine plasma concentration compared to the Collins et al. (13) study.

Figueroa et al. (23) supports the employment of a six-week supplementation period. Figueroa et al. (23) concluded that a six-week watermelon supplementation period can have beneficial results on aortic blood pressure, wave reflection, and aortic and brachial pulse pressure. From these findings, one may predict that six-week supplementation of watermelon could have beneficial effects on CVD biomarkers; however, there were limited significant changes in the biomarkers measured in the current study. There was a smaller amount of L-arginine and L-citrulline supplementation per day (2.27grams of L-citrulline/L-arginine) in this study compared to the Figueroa et al. (23) study (4grams L-citrulline/L-arginine). Thus, supplementation was sufficient to increase plasma L-arginine concentrations, but may not have been sufficient to produce beneficial results on all atherosclerotic biomarkers.

The control group did not make changes to their normal dietary routine. Thus, no changes in L-arginine and L-citrulline plasma concentrations occurred.

Atherosclerotic Risk Factor Biomarkers

While L-arginine plasma concentrations increased through watermelon supplementation, NO production via eNOS may have not increased due to a deficiency in tetrahydrobiopterin (BH4). BH4 is an essential cofactor for eNOS (10, 34, 42). GTP cyclohydrolase-1 is the first and rate-limiting enzyme for the synthesis of BH4, and a deficiency of GTP cyclohydrolase-1 in diabetic rats results in decreased BH4 synthesis, thus impairing NO synthases via eNOS (66). The subjects in our study were not diabetic (i.e., fasting blood glucose <125mg/dL); therefore, low GTP cyclohydrolase-1 activity is not likely. Further research will need to be conducted to examine if GTP cyclohydrolase-1 activity is impaired in this population.

Under oxidative stress, BH4 is oxidized into BH2 and biopterin causing a deficiency in BH4 (10, 34, 42). A deficiency in BH4 will cause eNOS uncoupling and generates superoxide rather than NO (10, 32, 34, 61). Oxidative stress will also decrease the production of NO (63). The uncoupling of eNOS and the decrease in NO bioavailability during oxidative stress can lead to endothelial dysfunction and ultimately atherosclerosis. Measures of oxidative stress were not made in this study; however, previous studies indicate that overweight post-menopausal women are under chronic oxidative stress (2). Thus, providing more substrate, i.e. L-arginine, may have had a limited effect on NO production.

sVCAM-1

sVCAM-1 significantly decreased over time in the watermelon group in this study. The increase in the L-arginine concentration over the six-week supplementation period may

have increased NO through the L-citrulline-NO cycle. NO can limit the expression of sVCAM-1 (16, 43), which may account for the decrease in the plasma concentration of sVCAM-1 in the watermelon group. However, the small Cohen's D effect size of this result may be explained by the uncoupling of eNOS. sVCAM-1 is involved in the recruitment and binding of leukocytes to the endothelial cell during inflammation (39). sVCAM-1 expression is mainly in lesions and lesion-predisposed regions, and is not expressed under healthy conditions (14). sVCAM-1 increases significantly during endothelial dysfunction, atherosclerotic conditions, and early stages of atherogenesis (14). The significant decrease in its plasma level may indicate that there may have been a small regression in atherosclerosis, endothelial dysfunction, and inflammation.

Previous studies have indicated that obese post-menopausal women are more at risk for oxidative stress (63). Oxidative stress leads to an increase in oxidation of low-density lipoproteins (LDL) (63), and a decrease in NO production (2). Oxidized LDL can induce the transcription of sVCAM-1 gene in endothelial cells (15). The significant decrease in sVCAM-1, with a small Cohen's D effect size, could indicate that there was a small change in oxidized LDL levels and oxidative stress.

sICAM-1

While sVCAM-1 and sICAM-1 are very similar in structure (14), no significant difference was found in the sICAM-1 plasma concentration between treatment groups. sVCAM-1 and sICAM-1 differ in expression timing and location (14). sICAM-1 is present during basal levels (non-inflammatory state), and is expressed in endothelial cells and leukocytes (7). sICAM-1 expression is not limited to mature lesion regions and lesion-

predisposed regions and may also be expressed in lesion protected regions (14). In an atherosclerotic state, oxidized LDL and secreted chemokines promote the expression of sICAM-1 (51). The six-week watermelon treatment period may not have been sufficient to alter oxidized-LDL or chemokine levels enough to affect sICAM-1 expression and thus sICAM-1 plasma concentration.

sP-Selectin

sP-selectin, mainly found on platelets, is stored within α-granules of platelets and Weibel-Palade bodies of endothelial cells, and are recruited to the endothelial cell surface after activation (7, 9, 52). As previously mentioned, oxidative stress increases oxidized LDL (63), and decreases NO production (2). Oxidized LDL stimulates sP-selectin expression on endothelial cells (15). While the small change in sVCAM-1 plasma concentrations suggest that oxidized LDL may have decreased, it might not have been sufficient to affect changes in sP-selectin plasma levels.

Growth Differentiation Factor - 15

GDF-15 expression is increased during oxidative stress, anti-angiogenic stress, and in response to increased pro-inflammatory cytokines, increased oxidized LDL, mechanical stress (41), and in response to endothelial dysfunction (46). Each of these are risk factors for atherosclerosis. Lack of a significant change in GDF-15 plasma concentrations may indicate that there were no beneficial changes to atherosclerotic risk factors or change in endothelial dysfunction. GDF-15 is not exclusively an atherosclerotic risk factor, and its expression and

production can be increased in response to pathological or environmental stress (64). Thus, GDF-15 plasma concentrations may have been maintained by other mechanisms.

ADAMTS 13

ADAMTS 13 decreases inflammation during atherosclerosis by cleaving highly active Von Willebrand Factor (VWF), and thus, making less active VWF (8, 27). VWF is part of the blood clotting cascade, and is involved in the recruitment and adhesion of platelets to activated endothelial cells at atherosclerotic sites (27). While ADAMTS 13 can bind to VWF during static conditions, shear stress may be needed to stretch the endothelial surface to expose VWF and its binding site for ADAMTS 13 (19). Further research including exercise and oral L-arginine and L-citrulline via watermelon puree needs to be conducted to determine if shear stress and L-arginine supplementation can affect ADAMTS 13 plasma concentrations.

Gandhi et al. (26) examined the mechanism by which ADAMTS 13 modulates atherosclerosis and the breakdown of VWF in hypercholesterolemic mice. Gandhi et al. (26) concluded that ADAMTS 13 modulates inflammatory plaque progression through VWF-dependent mechanism. While further research is needed to determine VWF-mechanisms, this may account for the lack of change in ADAMTS 13 plasma concentrations.

Fibrinogen

It was hypothesized that pre- and post-study fibrinogen plasma concentrations would decrease in the watermelon treatment group over time; however, fibrinogen results during analysis were above detection limits and could not be accurately measured or compared.

During analysis, the analyte concentrations were higher than the highest point of the standard curve. This did not allow the analytes to be compared to a known value for that specific analyte. Further research will need to be conducted to examine the effects of a six-week L-arginine and L-citrulline supplementation on plasma fibrinogen concentrations in obese post-menopausal women in a community setting.

Conclusion

In conclusion, the six-week watermelon supplementation treatment resulted in a small but significant decrease in the fasting sVCAM-1 concentration, but no changes in the other atherosclerotic biomarkers measured. Also, the six-week watermelon supplementation treatment resulted in an increase in the fasting L-arginine plasma concentrations.

Further research with exercise and oral L-arginine and L-citrulline via watermelon puree supplementation is required to determine if shear stress and oral L-arginine supplementation together can affect NO mechanisms and inflammation biomarkers in overweight post-menopausal women in a community setting. Shear stress has many beneficial properties to increase NO production, and vasodilation, including its ability to induce and increase BH4 levels (3). This can increase BH4 bioavailability and decrease the uncoupling of eNOS. By increasing BH4 bioavailability through shear stress and increasing plasma levels of L-arginine through consumption of watermelon puree, beneficial inflammation responses to decrease the risk for atherosclerosis and CVD may be obtained in this population. Furthermore, additional studies are needed to determine if a longer treatment period and/or greater dose of watermelon puree is required to decrease atherosclerotic risk in overweight post-menopausal women in a community setting.

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Vita

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