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The Effects of Watermelon Juice Supplementation on Tissue Oxygen Saturation During Hyperglycemia

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by

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Introduction

Underlying health conditions, including obesity, atherosclerosis, diabetes mellitus, dyslipidemia, hypertension, and endothelial dysfunction have been linked to the greatest risk for the contraction of severe illnesses, like cardiovascular disease (CVD), viruses, and cancer [8]. As of March 2020, data collect indicated individuals diagnosed with chronic lung disease, diabetes mellitus, and/or CVD, are at triple the risk for contracting life-threatening disease [8]. Examples of CVDs that influence the increased risk of early mortality are coronary artery disease, cerebrovascular disease, and peripheral arterial disease [28]. These conditions compromise the blood vessels supplying the heart muscle, brain, and periphery [28]. Furthermore, CVD is the leading cause of death world-wide, annually killing 17.9 million people leading to 31% of all deaths globally [35]. Cardiovascular diseases and severe underlying health conditions both share strong vascular endothelial elements, serving as a target for treatment that could lead to an overall reduction of the frequency and magnitude of premature mortality [26]. Vascular function is related to arginine (Arg) and nitric oxide's (NO) bioavailability, as these two molecules are responsible for blood vessel vasodilation [20].

The vascular endothelium encompasses homeostatic functions and plays a critical role in blood flow regulation. The vascular endothelium is a monolayer of cells lining the entire circulatory system [36]. These endothelial cells are located between the vessel lumen and vascular smooth muscle cells and act as barrier between the blood and other tissues [36]. Endothelium-dependent regulatory systems alter vascular tone of vessels in an effort to regulate the blood flow to a specific area and are mediated by the bioavailability of nitric oxide (NO) [36].

Dysfunction of the endothelium-dependent regulatory systems combined with limited NO bioavailability are linked to the vascular contraction and progression of serious health conditions, including hypertension, atherosclerosis, and CVD [26]. In this review, the relationship between vascular endothelial function and NO bioavailability, and the damaging effects associated with hyperglycemia will be examined. Citrulline supplementation utilized for attenuating the negative impact on NO bioavailability will also be presented with a focus on watermelon juice helping to remediate the vascular endothelial system dysfunction caused by acute hyperglycemia.

Nitric Oxide

Nitric oxide is an inorganic, free radical gas with a major cell-signaling role in the nervous, cardiovascular, and immune systems [37, 38]. The endothelial enzyme, Nitric oxide synthase (eNOS), catalyzes the oxidation of L-arginine (Arg) for the purpose of synthesizing NO [36]. L-arginine, a nonessential amino acid, undergoes oxidation by absorbing five electrons inside the endothelial cell [38]. The three main NOS enzyme families are responsible for the NO-producing reaction in their respective cell type and differ in expression level; nNOS is present in neural cells, eNOS is in endothelial cells, and iNOS is in macrophages [13]. The eNOS reaction yields NO with L-citrulline as a byproduct [15]. Endothelium-derived NO (eNO) is a potent signaling molecule for vasodilation and regulating vascular tone [15]. Once synthesized, NO diffuses across the endothelial cell membrane and enters the smooth muscle cell, activating guanylate cyclase (sGC). The activation of sGC leads to an increase in intracellular concentrations of cyclic guanosine-3’,5-monophosphate (cGMP), a second messenger that mediates NO’s biological effects [36]. cGMP is responsible for signaling the initiation of vasodilation within the endothelium, stimulating vascular tone, smooth muscle relaxation of blood vessels, and blood coagulation while controlling transportation volume and speed

throughout the body [15, 36, 37]. Inefficient eNOS function or enzyme uncoupling can cause eNO depletion [15]. Insufficient levels of luminal NO can hinder vascular conductance, contributing to the development of ischemia, atherosclerosis, and stroke [36].

Oxidative stress also contributes to decreasing eNO bioavailability and occurs with an imbalance between reactive oxygen species (ROS) formation and cellular detoxification [39, 41]. High concentrations of ROS are deleterious to cellular DNA, causing severe damage to tissues and the uncoupling of eNOS [39, 40]. This uncoupling provokes eNOS to produce superoxide free radicals rather than NO, leading to an overall reduction in eNOS activity [42]. Superoxide anions then react with available luminal NO to form peroxynitrite and undergoes further oxidation, resulting in a product unable to be recycled back to useable NO, causing diminished levels of NO synthesis [42]. The oxidative stress associated with the accumulation of ROS mainly acts as an initiator of atherosclerosis, which compromises endothelial function [39]. Chronic oxidative stress, excess superoxide, and/or depleted NO bioavailability may be responsible for the development of CVDs, including hypertension, congestive heart failure, ischemia, cardiomyopathy, and cardiac hypertrophy [39].

Near-Infrared Spectroscopy

Vascular reactivity and the distribution of blood flow is regulated by the endothelium's ability to produce NO [43]. Near-Infrared Spectroscopy is a non-invasive technique used to examine microvascular responsiveness after arterial occlusion [43]. Dysfunction of microvasculature, resistance arterioles and arteries, has been an established antecedent for more critical CVD-related complications [46], which emphasizes the importance of accurately assessing vascular responsiveness [43]. Microvascular changes in endothelial tissues associated with arterial occlusion are most accurately measured through near-infrared spectroscopy (NIRS)

[43]. The NIRS method assesses downstream microvascular responsiveness through the observation of changes in oxygen saturation signal (StO2) of muscle tissue post-occlusion [30]. Flow-mediated dilation (FMD) is another non-invasive assessment of vascular conductance that measures macrovascular changes in the vasculature [44]. The NIRS and FMD techniques have recently been used together in an attempt to investigate downstream microvascular function (forearm NIRS) and its relationship with the upstream arterial FMD (brachial artery) [30].

The ischemia caused by arterial occlusion produces downstream vasodilator metabolites and relaxation of smooth muscle cells, therefore inducing downstream-forearm vasculature dilation [45]. Following the deflation of the cuff, the vasodilation causes rapid blood flow from the upstream brachial artery down to the forearm resistance arterioles and arteries (dilated microvasculature), thus intensifying shear stress [45]. The shear stress signals a boost in NO production and consequently upstream arterial endothelium-dependent vasodilation (FMD) [10, 45]. The perfusion slope for StO2 immediately following arterial occlusion provides reliable and repeatable vascular vasodilation assessment [30].

Hyperglycemia

Hyperglycemia is defined as high concentrations of glucose in the blood [48]. Individuals diagnosed with Type II Diabetes Mellitus (T2D) are at the highest risk for microvascular complications caused by hyperglycemia [48]. Significant periods of hyperglycemia have been seen to precipitate injurious effects to vascular endothelial function [19]. Endothelial vascular vasodilatory function is diminished by postprandial hyperglycemia and has been shown to further contribute to the development of CVD [23]. Healthy individuals are equally subjected to hyperglycemic-related endothelium damage as individuals with a history of endothelial dysfunction [23, 32]. This graded-dose response to postprandial hyperglycemia is suggested to

predict future CVD mortality risks more accurately than compared to previous fasting glucose methods [23]. Recent research has used FMD and NIRS simultaneously for the purpose of demonstrating the direct association between the rapid reduction of the brachial artery's endothelium-dependent vasodilation and glucose loading [19]. The associated decrease in FMD may be the result of lower NO synthesis and the increased uncoupling of eNOS caused by high levels of oxidative stress [19].

Hyperglycemia-induced oxidative stress decreases the activity of the enzyme responsible for denaturing a competitive inhibitor of eNOS, asymmetric dimethylarginine (ADMA) [24, 25]. Consequently, high ADMA:arginine ratios have been linked to an increased risk for CVD by inhibiting arginine from binding to eNOS, leading to a depletion in NO biosynthesis [24]. Acute hyperglycemia suppresses vascular function by escalating lipid peroxidation autonomous to inflammation, causing eNOS inactivity, endothelial cell apoptosis, and compromising the vascular endothelium's integrity [24].

Endothelial dysfunction is a predominant feature of diabetic vascular disease and is a known complication associated with aging, menopause, and cardiometabolic disease [27]. Bioavailability of NO has been designated a biomarker of vascular health. Citrulline supplementation, in the form of watermelon juice, has shown promising effects for an increase of baseline StO2 measures in post-ischemic and healthy individuals [29, 16].

Citrulline

Citrulline is a non-essential amino acid that exhibits promising results for increasing NO bioavailability through the increased production of L-arginine, a precursor of NO [21]. Citrulline plays an important part in bodily processes and has been used as a biomarker for renal and small bowl function [12, 21]. Watermelon rind and seeds are known to have high concentrations of

citrulline, making this fruit the main food source for citrulline [28]. One whole watermelon contains 1.6-3.5 g/kg of citrulline [11]. Citrulline is equally effective at both low, 1g/day, and high, 10g/day, doses without any known toxicity [11, 28]. With potential endothelial advantages, citrulline-rich watermelon poses as a natural supplement that will aid in increasing NO bioavailability [20].

Oral supplementation of citrulline has shown higher efficiency for increasing NO concentrations and circulating L-Arg than compared to an equivalent dose of supplemental arginine [1,3]. eNOS catalyzes the oxidation of L-Arg to produce NO and citrulline [13]; however, ingested L-Arg undergoes first-pass extraction by the liver [3]. Approximately 40% is catabolized by intestinal bacteria and then another 10-15% is metabolized by liver enzymes, known as arginases, yielding urea and ornithine [3]. Conversely, ingested citrulline is not metabolized by intestinal bacteria nor liver enzymes but is extracted by the kidneys and can recycled back into Arg for further NO production [3]. Citrulline is acted on by the enzymes, argininosuccinate synthase and lyase in the kidneys, in order to be converted into argininosuccinate, then subsequently into arginine [3]. The mechanisms affecting ingested citrulline produce additional circulating arginine in order to facilitate more NO production by eNOS.

Purpose

The purpose of this study is to investigate if citrulline-rich watermelon juice supplementation will attenuate the reduction in vascular endothelial function associated with hyperglycemia. We hypothesize the watermelon juice supplementation will cause an increase in muscle baseline oxygen saturation, measured through NIRS, during hyperglycemia.

Methods

Participants

Seventeen healthy participants, five male and twelve females, with an average age of 23±3 years (y) and with a BMI average 23.48±3.22 kg/kg/m^2 were recruited for this study. Participants completed an informed consent, health screening questionnaire, and Physical Activity Readiness Questionnaire (PAR-Q) to determine study eligibility. Volunteers were excluded from participation if any of the following were present: immediate family history of type 1 or type 2 diabetes, active smoker, any history of cardiovascular disorders, allergic to watermelon, currently on any medication capable of altering data, or currently taking any arginine, citrulline, or beta-alanine supplementation. Following the review of the participant's documentation and all the inclusion criteria were met, the participant was scheduled for the first screening visit followed by two supplement periods and follow-up testing separated by a washout period.

Experimental Design

Each participant underwent 2 supplementation periods, in a randomized, crossover, counterbalanced design separated by a 2-week washout period. Participants were required to ingest a minimum 10 doses of supplement during the supplementation periods, 1 dose per day. All supplements were distributed by the LSU Ag Center's Food Science Laboratory in a double-blinded design. The 2 supplements were 16 oz of fresh homogenized watermelon juice (WMJ) or placebo developed to mimic the taste and texture of the WMJ. At the end of each supplementation period, participants had a post-supplementation oral glucose tolerance test (OGTT) after an overnight fast. After the post-supplementation OGTT, a minimum 14-day

washout period was required prior to initiating the second supplementation period. A similar fasting OGTT was conducted at the end of the second supplementation period.

Screening Visit

Prior to the screening visit, participants were required to be fasted from food and drink, with exception of water, for at least 10 hours and did not ingest alcohol or engage in strenuous exercise for at least 48-72 hours. First, the participant's body composition was measured by dual x-ray absorptiometry (DXA). Females were required to take a pregnancy test before the DXA scan. All participants were informed of possible risks associated with the DXA, then were instructed to remove all shoes, metal, and baggy clothing for the scan. Following the DXA, a baseline blood draw was conducted to measure baseline glucose levels. Participants were then randomized and sent to LSU Ag Center's Food Science Laboratory in order to schedule the supplement pick-up.

Visits 2 & 3: OGTT – MVBF-NIRS

After a minimum of 10 days of supplementation, participants came in fasted (same requirements as baseline blood draws) and a DXA scan was conducted to measure body composition. Females were again required to take a pregnancy test before the start of the scan. Afterward, participants put on a heart rate monitor (Zephyr, Bioharness) and rested in a supine position for 30 minutes. Heart rate and heart rate variability (HRV) were measured during this resting period. Next, resting metabolic rate (RMR) was measured via indirect calorimetry for 20 minutes, then microvascular blood flow-near infrared spectroscopy (MVBF-NIRS) collected 3 rounds of occlusion alternating between 10 seconds on, 2 minutes off. An intravenous catheter was inserted into the participants left arm to collect a baseline blood sample. Then, the participant was given 5 minutes to drink the 75g glucose solution (Glucola) in its entirety.

Postprandial MVBF-NIRS measurements were taken again 30, 60, and 90-minutes after ingestion of the glucose drink and blood samples were collected from the IV 15, 30, 60, 90, and 120-minutes post-glucose ingestion.

Microvascular Blood Flow-Near Infrared Spectroscopy (MVBF-NIRS)

Prior to each participants' visits, the NIRS system was calibrated. The optodes (Oxymon MKIII, Artinis Medical Systems) of the NIRS system were longitudinally situated on the anterior side of the right forearm. This aided in establishment of reproducibility for the measurements. The optodes were fixed in place with both double-sided adhesive tape and medical tape. Following correct probe placement, participants laid in supine with their right arm abducted 80 degrees and were told to remain still for the duration of the test. The accelerated inflation cuff was proximally located to the probes and was rapidly inflated to 60mmHg for 10 seconds. Then the cuff was quickly deflated for 2 minutes. The inflation and deflation alternated for a total of 3 rounds. Throughout the MVBF, NIRS signal for tissue O2 saturation % (TSI) were consistently observed.

Microvascular responsiveness was reflected by calculating the Baseline TSI from the 2-minute mark prior to forearm occlusion. Then the reperfusion slope was determined from the slope of linear increase between the point of cuff deflation and the 10-seconds post-reperfusion. Repeated measures within a mixed-model will be employed for the purpose of accurately assessing pre-oral glucose vs. postprandial variations in MVBF-NIRS.

Statistical Analyses

All statistics were performed using JMP statistical software (SAS Inc, Cary, NC, version 14.2). Demographic and screening data were examined using independent and paired t-tests where appropriate to examine the differences between sex at baseline and baseline measures for

fasting glucose and muscle oxygen saturation (StO2). Two-way (supplement x time) repeated measures analysis of variance (2way-RM-ANOVA) within a mixed-model was employed for the purpose of accurately assessing postprandial variations in plasma glucose and MVBF-NIRS StO2. Post hoc analyses using Student t-test was used to examine supplement differences across time. All data are reported as Mean±SD unless described below. Statistical significance was determined as $\alpha<0.05$.

Results

Population Characteristics

The population of interest utilized 17, young-adult, non-obese, volunteers. The participants' average age was 23±3 years (y), percent fat mass averaged at 27.72±7.89%, and BMI averaged at 23.48±3.22 kg/kg/m^2. There were five male and twelve female participants, however, two females only finished one OGTT visit and were excluded from measurement analysis. There were no significant differences in participant characteristics from baseline to last visit, as displayed in **Table 1**. The duration for supplementation, watermelon juice (WMJ) and placebo (PLA), averaged at 12.8 WMJ's range of 10-15 days and placebo being 10-16 days. The washout period between OGTT visits averaged at 16.0 with a minimum of 14 days and a high of 21 days. Technical complications arose with the WMJ supplement production, resulting in the first five volunteers receiving WMJ for the first phase and the second five volunteers receiving PLA for their first

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.

phase for the purpose of equalization. The remaining volunteers were randomly assigned the supplement order for each phase. The study maintained the double-blind and counterbalanced structure for its entirety.

Oral Glucose Tolerance Test

Blood glucose concentrations increased from baseline, starting at 5.20±0.37 mmol·$L^{-1}$to 6.73±0.93 mmol·L^{-1} at the 15-minute time point. The blood glucose concentrations peaked at the 30-minute mark with a value of 8.06±1.38 mmol·L^{-1}, then decreased to 7.52±2.10 mmol·L^{-1} at the 60-minute mark, then again at 90 minutes to 6.10±1.52 mmol·L^{-1}. Lastly at the 120-minute time point after glucose ingestion, blood glucose concentration decreased to 5.53±1.60 mmol·L^{-1}. Glucose levels demonstrated a significant time-effect with a p-value of p≤0.0001 as seen in **Figure 1**. However, there were no significant differences, for treatment type, p=0.18, nor time effect on treatment, p=0.74, between WMJ and PLA supplement phases. Overall, blood glucose concentration area under the curve (AUC) showed no significance throughout the OGTT time points within the study.

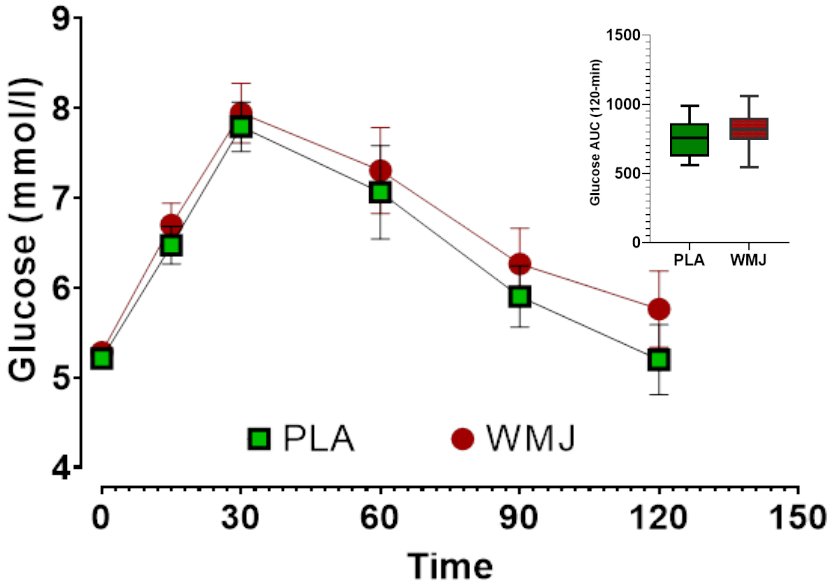

Figure 1. Glucose concentrations from time point baseline (0 min) to 120 min post-glucose ingestion with significant time-effect; No significance in glucose AUC in WMJ vs. PLA.

Near-Infrared Spectroscopy

For both WMJ and PLA treatments, plasma glucose levels increased while oxygen saturation (StO2) decreased (WMJ StO2=69% and PLA StO2=66%) at the 30-minute mark following glucose ingestion, as seen in **Figure 2a**. In baseline StO2, the average StO2% prior to

cuff inflation, there was a significant increase with WMJ vs. PLA (p=0.031). **Figure 2b** displays AUC for baseline StO2 is significantly greater for WMJ vs. PLA (p=0.03).

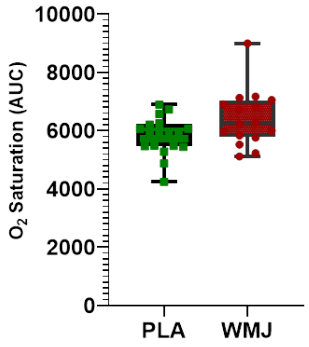

Figure 2a. Baseline oxygen saturation AUC from baseline (0 min) to 90 min post-glucose ingestion for WMJ and PLA groups. PLA, placebo group, WMJ, watermelon juice group.

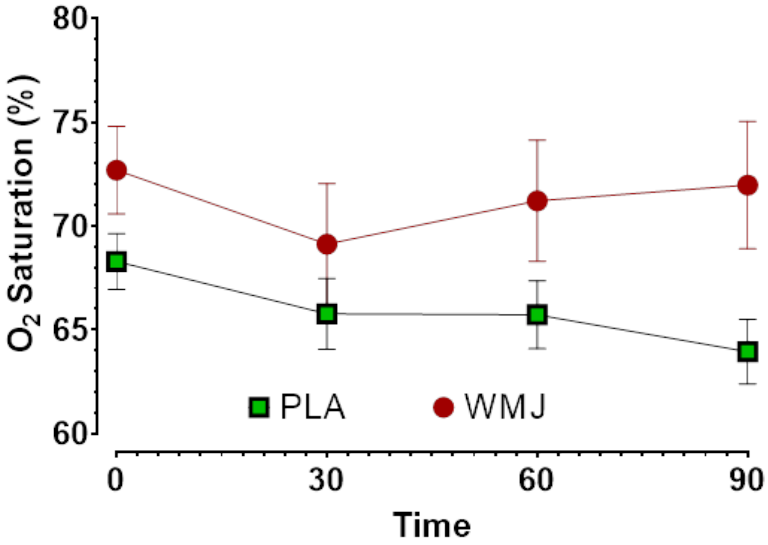

Figure 2b. Oxygen Saturation (%) from time point baseline (0 min) to 120 min post-glucose ingestion with significant supplement effect between WMJ and PLA.

Discussion

The purpose of this study was to examine if citrulline-rich watermelon juice attenuates the reduction of vascular endothelial function caused by hyperglycemia. The results show WMJ supplementation significantly increased tissue baseline StO2% at peak blood glucose concentration, supporting the study's original hypothesis. The baseline StO2 advancements suggest WMJ improves microvascular endothelial responsiveness during hyperglycemia.

During the OGTT, baseline StO2% decreased when peak blood glucose concentration was reached. Both supplement groups displayed the significant decrease at the 30-minute time point. However, the WMJ treatment group experienced significant effects of both time (p=0.03) and treatment (p=0.072) when compared to the PLA group. Previous research proposed NIRS measurements of tissue StO2 accurately reflects microvascular responsiveness [47]. The noticeable improvements of the WMJ group's tissue baseline StO2 indicates a positive correlation between citrulline supplementation and increased vascular endothelial function.

Additionally, WMJ supplementation demonstrated a significant increase in StO2 AUC, presumably due to the long-lasting improvements in endothelial reactivity. Conversely, Soares (2017) found StO_2 AUC decreased at peak blood glucose levels, although their study did not include any supplementation [43]. Soares' (2017) differences in results could be attributed to the lack of a supplement's attenuation for oxidative stress-related endothelial dysfunction caused during hyperglycemia from the OGTT [43]. On account of variations in the current study's and Soares' (2017) results, it is reasonable to suggest watermelon juice improves microvascular endothelial function by attenuating oxidative stress during hyperglycemia [43].

Near-infrared spectroscopy was utilized in this experiment's methodology for the purpose of measuring tissue baseline oxygen saturation to observe the effects watermelon juice has endothelial function. A strength of the current study's design was the population of interest was an accurate representation of healthy-young adults within the general public. A limitation of the study included the lack of cardiovascular fitness tests within the screening-visit. Physical activity-related cardiovascular fitness critically influences vascular endothelial function and overall vascular health. However, physical activity questionnaires were implemented during the pre-screening visit, but this approach does not provide reliable measurements.

Future research could include using NIRS to identify effected vascular reactivity mechanisms during hyperglycemia for high-risk populations, including individuals diagnosed with CVD or T2D. The findings from this study warrant future research for the effectiveness of citrulline supplement intervention on improving cardiovascular fitness and exercise performance.

Conclusion

In conclusion, the present study suggests that citrulline-rich watermelon juice improves hyperglycemic-reductions in baseline tissue oxygen saturation of young-healthy individuals. The

findings additionally suggest that watermelon juice supplementation is an effective method for improving microvascular responsiveness and overall vascular endothelial function.

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