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Effects of 12-Week Dietary Nitrate Supplementation with Resistance Training on Skeletal Muscle and Vascular Outcomes and in Older Adults

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Abstract

Increasing dietary nitrate (**NO_3^-**) through beetroot juice (**BRJ**) supplementation elicits acute ergogenic benefits. However, it is unknown whether chronic NO_3^- supplementation can enhance resistance training (**RT**) adaptations in middle-aged and older individuals. Therefore, we sought to determine whether 12 weeks of combined RT and NO_3^- supplementation enhanced hypertrophic, vascular, strength, and skeletal muscle angiogenesis adaptations in this population. Twenty-eight apparently healthy, untrained men and women (56±7 years old, 29.1±5.3 kg/m^2 body mass index) completed 12 weeks of supervised full-body RT (2x/week) while ingesting either BRJ (140 mL daily, providing 800 mg NO_3^-; n=14 with 7M/7W) or NO_3^--depleted BRJ placebo (PLA; n=14 with 7M/7W). Participants underwent a whole-body dual-energy x-ray absorptiometry scan, right mid-thigh ultrasonography for muscle imaging, right leg popliteal artery flow-mediated dilation (**FMD**) assessments, a biopsy of the right mid-thigh vastus lateralis, and strength testing prior to and following the 12-week intervention. Biopsy analyses included a NO_3^-/nitrite (**NO_x**) fluorometric assay, immunoblotting for proteins involved in angiogenesis, and immunohistochemistry to quantify fiber type-specific capillaries and cross-sectional areas. Muscle NO_x values did not significantly change: +15.4% in BRJ (p=0.073) and +7.8% (p=0.514) in PLA. Both groups significantly improved measures related to muscle hypertrophy, strength, and FMD. However, no significant group × time interactions were observed for whole-body lean mass, mid-thigh muscle cross-sectional area, popliteal artery FMD outcomes, or histological or molecular markers. In conclusion, BRJ supplementation does not enhance RT adaptations in middle-aged and older adults.

Keywords: dietary nitrates; resistance training; older adults; skeletal muscle

1. Introduction

Maintaining or increasing skeletal muscle mass is imperative among middle-aged and older adults for reducing the risk of life-threatening injuries from falls, cardiometabolic and cardiovascular disease, and, ultimately, enhancing their quality of life [1–3]. Although resistance training (**RT**) is a viable strategy to maintain or increase skeletal muscle mass, RT interventions among middle-aged

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and older adults produce inconsistent or attenuated hypertrophic responses, especially when compared to younger adults [4–8]. This phenomenon may be explained by anabolic resistance, which is the impaired ability of skeletal muscle to grow in response to a typically anabolic stimulus such as ingesting dietary protein and/or RT, and which adults may begin to experience around the fifth decade of life [2,9,10]. Age-induced anabolic resistance has been studied extensively [9,11–13], and many lines of research have indicated that this issue is primarily due to dampened skeletal muscle protein synthesis.

Aging is associated with progressive declines in macrovascular (large conduit arteries) and microvascular (arterioles and capillaries) function [9]. Specifically, reductions in macrovascular function begin around the fifth decade of life [14]. There is evidence suggesting that reduced nutrient delivery and blood flow may contribute to anabolic resistance [15,16]. For instance, a negative correlation has been reported between arterial stiffness and skeletal muscle mass among middle-aged and older adults [17]. Similarly, reduced endothelial function is associated with lower grip strength in older women [18]. At the microvascular level, decreased skeletal muscle capillarization is associated with attenuated hypertrophic responses to RT in older adults [19,20]. At the molecular level, exercise-induced alterations in vascular endothelial growth factor (**VEGF**), which mechanistically triggers angiogenesis, are diminished in older compared to younger adults [21–24]. These findings highlight the need for interventions that can improve vascular function among aging populations to enhance skeletal muscle hypertrophic response of RT.

Commercially available dietary supplements are commonly used to enhance health and exercise outcomes [25–27]. Dietary nitrate (**NO_3^-**) exhibits health benefits, particularly in relation to vascular function and blood flow [28–32]. While dietary NO_3^- can be ingested from various foods, it is commonly delivered as a supplement in concentrated beetroot juice (**BRJ**) [28–32]. When consumed as a pre-exercise supplement, certain studies report BRJ provides ergogenic benefits including enhanced muscular endurance, improved strength-endurance performance, and reduced fatigue [29,31,33–35]. The proposed mechanism involves the sequential reduction of ingested NO_3^- to nitrite (**NO_2^-**) by oral bacteria, followed by further reduction to nitric oxide (**NO**) in tissues, ultimately resulting in vascular smooth muscle relaxation, vasodilation, and enhanced blood flow [29,36–38]. Although dietary NO_3^- improves vascular health and acute exercise performance [31–33,39], only one study to our knowledge has examined how chronic NO_3^- supplementation during a period of RT affects functional outcomes [40]. Carter and colleagues assigned a cohort of postmenopausal women to either a BRJ + training group or training-only group whereby training consisted of 24 bouts of circuit style RT and cardiovascular training exercises over eight weeks. Functional tests were performed prior to the intervention and during week 9, after BRJ participants had ceased supplementation for one week. Interestingly, BRJ supplementation significantly improved distance covered during a 6-minute walk test greater than training alone, but neither group improved body composition. Additionally, while not measured, it was posited that type II myofiber adaptations with BRJ supplementation could lead to positive functional alterations given that NO_3^- has been posited to provide more benefit to these muscle fibers [28,41].

Thus, a mechanistic knowledge gap remains regarding whether NO_3^- supplementation can augment RT adaptations in middle-aged and older individuals. Evidence from older adults shows that greater skeletal muscle capillarization is associated with enhanced hypertrophic responses to RT, and some data indicate that NO_3^- may mechanistically enhance angiogenesis [42,43]. However, whether NO_3^- supplementation can enhance RT-induced angiogenesis in a middle-aged and older population has not been examined. Therefore, the purpose of this study was to determine whether NO_3^- supplementation via BRJ during a 12-week RT intervention enhances outcomes related to skeletal muscle hypertrophy, vascular function, myofiber characteristics, proteins involved in angiogenesis, and/or strength in middle-aged and older adults. Based on the literature reviewed above, we hypothesized that BRJ supplementation would enhance skeletal muscle hypertrophy and strength outcomes from RT compared to placebo supplementation, and that this would be accompanied by enhancements in vascular function and skeletal muscle angiogenesis.

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2. Methods

2.1. Ethical Approval, Consent, and Experimental Design

Ethical approval and consent. This study was approved by the Auburn University Institutional Review Board (Protocol # 24-863 MR 2405) and adhered to the standards set by the latest revisions of the Declaration of Helsinki except for being pre-registered as a clinical trial. Thirty men and women between the ages of 40–70 years old were recruited from the local community. Potential participants were excluded if they: i) had prior exercise habits (>1 day/week) for one year prior to enrollment, ii) consumed supplemental BRJ (or other NO_3-rich supplements), L-arginine, L-citrulline, or creatine-containing supplements one month prior to the initiation of the study, iii) had blood clotting issues precluding the collection of skeletal muscle biopsies, were allergic to supplement contents and/or lidocaine, or were diagnosed with cardio-metabolic or orthopedic issues precluding study participation. We aimed to enroll postmenopausal women due to vascular fluctuations that influence flow-mediated dilation, which occur throughout the menstrual cycle in premenopausal women [44–46]. Participants who met the inclusion criteria were informed of the study procedures and provided verbal and written consent to participate.

Overview of PRE/POST testing, intervention, and supplementation. The intervention consisted of 12 weeks of supervised full-body RE while supplementing daily with either BRJ in the form of commercially available Beet-It (n=14 participants; 140 mL concentrated providing 800 mg NO_3^-) or NO_3^--depleted BRJ PLA (n=14 participants; 140 mL providing less than 50 mg NO_3^-); both supplements (termed BRJ or PLA herein) were purchased from James White Drinks, Ipswich, UK. Prior to (**PRE**) and following the 12-week intervention (POST), participants underwent flow-mediated dilation (**FMD**) of the right leg popliteal artery, a full body dual-energy x-ray absorptiometry (**DXA**) scan, an ultrasound of the right mid-thigh for vastus lateralis (**VL**) muscle cross sectional area (**mCSA**) assessments, a biopsy of the right mid-thigh VL, and various measures of functional and strength testing. For logistical purposes, PRE testing assessments consisted of two days which were 1-5 days apart (day 1: FMD; day 2: DXA scan, mid-thigh ultrasound, VL biopsy, and strength testing). After completing all PRE testing, participants were allocated into the BRJ or PLA groups by a single investigator (ADF), who performed block randomization according to age, sex, and PRE DXA-derived whole-body lean tissue mass.

Approximately 2-3 days following day 2 of the PRE assessments, participants began the 12-week training intervention. During the first training day, participants were provided with a one-week supply of their respective supplement and were instructed to consume 140 mL 2.5 hours prior to workouts on exercise days and in the morning on non-exercise days, per previous recommendations [47,48]. Throughout the entirety of the study, participants were provided with weekly allocations of their respective supplements and adherence was documented based on verbal confirmation. Data collection and analysis (chiefly by MCM and DTB) was completed in a double-bind fashion throughout the entirety of the study.

The participants' last training session was approximately 72-96 hours prior to POST testing and participants were instructed to consume their last supplement 24 hours prior to POST testing. POST testing included FMD, DXA scan, mid-thigh ultrasound, VL biopsy, and strength testing (within a two-hour period), identical to day 2 of PRE testing. Participants were asked to abstain from the use of alcohol-based mouthwash throughout the study to avoid altering oral microbiota that facilitate NO_3^- reduction to NO_2^- [49,50]. A schematic of the study design is presented in **Figure 1** below, and more in-depth details about testing procedures follow.

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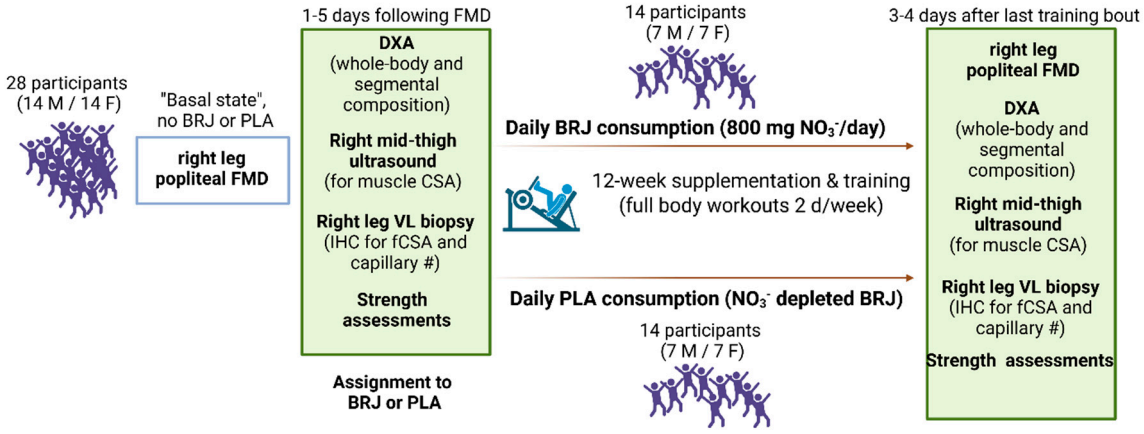

Figure 1. Study design. A schematic of the study design of the 12-week intervention. Abbreviations: BRJ, nitrate-containing beetroot juice; PLA, placebo; FMD, flow-mediated dilation; VL, vastus lateralis; DXA, dual-energy x-ray absorptiometry.

2.2. PRE- and POST-Intervention Blood Pressure and Right Leg Popliteal Artery FMD

Twenty-four hours prior to PRE and POST FMD assessments, participants were instructed to avoid a list of high NO_3^- foods and caffeine intake [45,51,52]. Participants arrived at the School of Kinesiology at Auburn University following an overnight fast for FMD assessments (matched for time of day at PRE and POST ± 1 hour). After ~15-minute dark room supine rest on an athletic training table, blood pressure was obtained in triplicate using an automated oscillometer blood pressure monitor with the cuff placed on the right arm (OMRON model BP785N, Lake Forest, IL, USA). Participants were then repositioned in a prone position on an athletic training table and a high-resolution B-mode and doppler ultrasound (Logiq S7 R2 Expert; General Electric, Fairfield, CT USA) was used to assess right leg popliteal artery blood flow with a multifrequency linear-array transducer (10-12 MHz scanning frequency) for three minutes by a single investigator (DTB). Following baseline artery diameter and blood velocity readings, a pre-placed cuff (Rapid Cuff Inflation System, D.E. Hokanson, Inc., Bellevue, WA, USA) was rapidly inflated and remained inflated for five minutes to 225 mmHg (cuff location was on the proximal right calf, 2-4 cm below the popliteal fossa). Three minutes into occlusion the ultrasound probe was placed in the same position to record the arterial response prior to deflation and up to five minutes post-occlusion.

Image analysis was performed using specialized software (Cardiovascular Suite, QUIPU srl, Pisa, Italy) by a single investigator (DTB). Popliteal artery diameters were determined by measuring the distance between the near and far wall of the intima, and blood velocity was determined via selection of a region of interest around the Doppler waveform. Popliteal FMD (%) was expressed as a percentage increase from baseline diameter to peak diameter following cuff deflation. FMD measurements were also normalized to the mean shear rate calculated from the first 10 seconds following cuff deflation (normalized FMD expressed as FMD(%) ÷ sec^{-1}).

2.3. Pre- and Post-Intervention DXA Scans for Body Composition

Participants arrived at our laboratory following an overnight fast for PRE and POST DXA scans. Prior to DXA scans, participants provided a ≥5ml urine sample that was assessed for urine specific gravity using a handheld refractometer (ATAGO; Bellevue, WA, USA). All participants' urine specific gravity was <1.020, which was used as a threshold of sufficient hydration to continue testing. Height and body mass were measured using a digital column scale (Seca 769; Hanover, MD, USA). Participants were then asked to lie in a supine position for five minutes on the DXA scanner (Lunar Prodigy; GE Corporation, Fairfield, CT, USA) prior to the assessment. Following scans, whole-body lean tissue and fat mass were automatically segmented by the software and are reported in kilograms (kg). Estimated total body skeletal muscle mass was also calculated using appendicular lean mass

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per the equation provided by Kim et al. [53]. Though test-retest DXA scans were not performed on these participants, prior data from our laboratory on test-calibrate-immediate retest on 10 participants produced an intraclass correlation coefficient of 0.998 for total body lean tissue mass [54].

2.4. Pre- and Post-Intervention Right Mid-Thigh Ultrasonography Assessment

Following DXA scans, real-time B-mode ultrasonography (NextGen LOGIQe R8, GE Healthcare) with a multifrequency linear-array transducer (L4-12T, 4–12 MHz, GE Healthcare) was used to capture right leg mid-thigh VL images in the transverse plane for measurement of mCSA values. The panoramic function of the device (LogicView, GE Healthcare) was used to capture the entire image of the musculature. All ultrasound settings were held constant across participants and laboratory visits (frequency: 10 MHz, gain: 50 dB, dynamic range: 75). Images were obtained and analyzed by a single investigator who was blinded to group allocations (DLP), and mCSA values were calculated by manual tracing of the border of the VL along the fascia using the polygon function in ImageJ software (National Institutes of Health, Bethesda, MD, USA). DLP's previously determined test-retest reliability for VL mCSA in 10 non-involved participants yielded an intraclass correlation coefficient of 0.984.

2.5. Pre- and Post-Intervention Right Mid-Thigh VL Biopsies

VL biopsies were obtained from the mid-belly of the right leg in the same plane as ultrasound assessments. Following ultrasound assessments, the participants remained in a supine position on an athletic training table while the upper thigh was shaved and cleaned with 70% isopropanol. A subcutaneous injection of 1% lidocaine (0.8 mL) was administered, and the area was cleaned with chlorhexidine solution after a 5-minute wait period. Thereafter, a 7-mm pilot incision was made through the dermis with a sterile No. 11 surgical blade (AD Surgical; Sunnyvale, CA., USA.) A 5-gauge biopsy needle was inserted into the pilot incision, through the muscle fascia, and ~2 cm into the muscle (for a total depth of ~4-7 cm) where a 40-80 mg sample was collected while applying suction. Following the biopsy, the tissue was rapidly teased of blood and connective tissue. A portion of tissue (~50 mg) was preserved in freezing media (OCT; Tissue-Tek, Sakura Finetek Inc; Torrence, CA, USA), slowly frozen in liquid nitrogen-cooled isopentane, placed in a box floating atop liquid nitrogen for ~1-3 hours, and subsequently stored at −80°C for immunohistochemical analysis. Another portion of tissue (~30 mg) was placed in prelabeled foil, placed directly in liquid nitrogen for 1-3 hours, and subsequently stored at −80°C for molecular analysis. This tissue triage process (performed by JMM) took ~2 minutes per participant.

Strength and strength-endurance outcomes At PRE and POST, each participant's estimated one-repetition maximum (est. 1-RM) was assessed using a 5-repetition maximum test for the hex bar deadlift exercise based on National Strength and Conditioning Association testing guidelines [55] (testing performed by KH, BMP, and DAA). Briefly, participants were escorted to an exercise training room following VL biopsies. Participants were then instructed to perform a series of dynamic stretches of the lower body musculature, which took approximately 5 minutes. Following 10 minutes of gait assessments (not reported herein), participants were coached through the form of the hex bar deadlift by study staff, a warm-up set of 10 repetitions was performed prior to 5-RM attempts, and up to five 5-RM attempts were performed with each attempt being separated by 2-3 minutes. Following this assessment, participants rested for 3-5 minutes. A hex bar deadlift repetitions-to failure test was then performed using 60% of each participant's est. 1-RM results. During this test, participants completed as many hex bar deadlift repetitions as possible with proper exercise technique. The primary outcome was volume-load (total weight lifted), calculated by multiplying load by number of repetitions performed.

2.6. Resistance Training Program

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Full-body RT sessions were performed on non-consecutive days at the same time-of-day in a dedicated research training facility (two non-consecutive days/week for 12 weeks) under the supervision of a National Strength and Conditioning Association certified strength and conditioning specialist (MCM) and other research staff using progressive overload that emphasized skeletal muscle hypertrophy. Each training session lasted ~60 minutes beginning with ~10-minute standardized warm-up and warm-up sets of the allocated exercises. Each session included hex bar deadlifts, pin-loaded chest press machine, 45º plate-loaded leg press, pin-loaded cable pulldown machine, pin-loaded leg extensor machine, and pin-loaded prone leg curl machine.

Intensity was monitored using repetitions in reserve (**RIR**) as described by Zourdos et al. [56]. Participant RIR was recorded after every set of each exercise and adjustments in load were made to achieve an RIR of 0-2. If RIR was >2, the load was increased. If participants were unable to complete the prescribed number of repetitions, the load was decreased. The training program utilized a linear progression where load was increased and volume was decreased throughout the 12-week period. Note that no participant was excluded from the study due to exercise adherence, which was predetermined to be ≥4 workouts over the 12-week intervention. For the first four weeks, load was assigned with the intent of participants performing 3 sets of ~12-15 repetitions per exercise. For weeks 5-8, load was assigned with the intent of participants performing 3 sets of 10-12 repetitions for each exercise. For the final four weeks, the load was assigned with the intent of participants performing 3 sets of 8-10 repetitions for each exercise. Following the conclusion of the study, total intervention volume-load (total sets x total repetitions x load per set) as well as lower body volume-load were calculated for each participant and expressed in kg.

2.7. Analyses on Biopsy Specimens

Immunohistochemistry for type I and II fCSA and capillary quantification. Biopsy samples preserved in OCT were sectioned at a thickness of 7-12 µm at -21°C using a cryotome (Leica Biosystems; Buffalo Grove, IL, USA). Sections were adhered to positively charged glass slides (VWR; Radnor, PA, USA) and stored at -80°C until batch processing for immunohistochemical analyses to detect capillaries, type I fibers and type II fibers, and dystrophin. PRE and POST samples from the same participant were placed on the same slide to limit slide-to-slide variation. During batch-processing, sections were removed from -80ºC storage, air-dried at room temperature for ≥2 hours and fixed with acetone at -20°C for five minutes. Slides were then incubated with 3% hydrogen peroxide for 10 minutes at room temperature, followed by one-minute incubation with autofluorescence quenching reagent (TrueBlack, Cat. No. 23007; Biotium, Fremont, CA, USA) and blocked for one hour in 2.5% horse serum in phosphate-buffered saline (PBS) at room temperature. After blocking, slides were incubated overnight at 4°C with a primary antibody cocktail in PBS containing 2.5% horse serum and 1:100 v/v dilutions of anti-PECAM-1, anti-type I myosin heavy chain, and anti-dystrophin antibodies listed in Table 1. The following day, sections were washed with PBS for 3x5 minutes and incubated for 60 minutes in a secondary antibody cocktail containing 1:250 v/v dilutions of the fluorophore-conjugated secondary antibodies listed in Table 1. Slides were then mounted with glass coverslips using 1:1 PBS and glycerol as mounting medium and stored in the dark at 4°C until imaging was conducted. Multiple digital 10× images per sample were captured to visualize the entire section, then stitched together using the "Acquisition" function, selecting "Tiles", then selecting multiple "Support Points" setting with a fluorescence microscope and motorized stage (Zeiss Axio Imager.M2) [57]. All images were captured in 10×. Approximately 388 myofibers were analyzed per sample. Type I and II fiber cross-sectional area (fCSA) quantification was conducted using Myovision [58], and analyzed images were manually checked to remove faulty myofibers (e.g., oblong or freeze-fractured) from the final analysis. Capillary contacts to type I and type II fibers were manually quantified by a single investigator (MCM) using a tally counter in the Zeiss Axio Imager.M2 software under the "Points" and "Events" function [59]. Capillaries that contacted multiple fibers were tallied for each fiber. Capillary number was reported as capillary per fiber type (type I or type II) as previously reported [59].

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Table 1. Antibodies used for immunoblotting and immunohistochemistry.

Antibody	Host species (isotype)	Company (Cat. No.)
Western blotting		
VEGF	Rb	Cell Signaling (65373)
VEGFR2	Rb	Cell Signaling (9698)
TSP-1	Rb	Cell Signaling (37879)
TFEB	Rb	Cell Signaling (83010)
eNOS	Rb	Cell Signaling (32027)
Phospho-eNOS (Ser1177)	Rb	Cell Signaling (9507)
Anti-Rb IgG, HRP-conjugated	G	Cell Signaling (7074)
Immunohistochemistry		
CD31/PECAM-1	M (IgG1)	DSHB (P2B1)
Type I myosin heavy chain	M (IgG2b)	DSHB (BA-D5)
Dystrophin	Rb (IgG)	Abcam (ab218198)
Anti-M IgG1, AF555-conjugated	G	Thermo Fisher (A-21127)
Anti-M IgG2b, AF488-conjugated	G	Thermo Fisher (A-21141)
Anti-Rb IgG, AF647-conjugated	G	Thermo Fisher (A-21245)

Abbreviations: DSHB, Developmental Studies Hybridoma Bank (Iowa City, IA, USA); G, goat; M, mouse; Rb, rabbit. Other note: protein acronyms are defined in text.

Immunoblotting. Per a recent publication from our laboratory [59], the immunoblotting targets of interest were related to increased and inhibited angiogenesis and responsiveness. Specifically, primary targets included vascular endothelial growth factor (**VEGF**), VEGF receptor 2 (**VEGFR2**), and thrombospondin-1 (**TSP-1**), an inhibitor of angiogenesis [60]. Transcription factor EB (**TFEB**) was assayed given its role in regulating angiogenesis [61]. Finally, total endothelial nitric oxide synthase (eNOS) and bioactive phosphorylated eNOS (**p-eNOS**, Ser1177) were assayed given the interaction of this enzyme with NO_3^- in regulating nitric oxide production [62].

Muscle tissue (~20 mg) was lysed using a general cell/tissue lysis buffer (Cell Signaling Technology, Danvers, MA, USA; Cat. No. 9803) and tight-fitting pestles. Lysates were then centrifuged at 500 g for five minutes and supernatants were placed into new 1.7 mL tubes. A commercially available BCA protein assay kit (Thermo Fisher, Waltham, MA, USA; Cat. No. A55864) was used to determine supernatant protein concentrations. Thereafter, supernatants were prepared for western blotting at equal protein concentrations (1 µg/µL) using 4x Laemmli buffer and deionized water. Western blot preps (15 µL) were pipetted onto SDS gels (4-15% Criterion TGX Stain-free gels, Bio-Rad Laboratories; Hercules, CA, USA), and proteins were separated by electrophoresis at 180 V for 50 minutes. Proteins were then transferred to methanol-preactivated PVDF membranes (Bio-Rad Laboratories) for 2 hours at 200 mA. Following transfers, the membranes were Ponceau stained for 10 minutes, washed with distilled water for ~30 seconds, dried, and digitally imaged (ChemiDoc Touch, Bio-Rad). Following Ponceau imaging, membranes were reactivated in methanol, blocked with 5% non-fat bovine milk in tris-buffered saline with Tween-20 (TBST) for one hour, and washed 3x5 minutes in TBST. Membranes were then incubated with primary antibodies diluted 1:1000 in TBST containing 5% bovine serum albumin (BSA) on a rocker overnight at 4°C (antibodies listed in Table 1).

Following overnight primary antibody incubations, antibody solutions were decanted, and membranes were washed for 3x5 minutes in TBST. The membranes were then incubated at room temperature for 60 minutes in TBST containing 5% BSA and a 1:2000 v/v dilution of HRP-conjugated antibody against the host species of the primary antibody (antibodies listed in Table 1). The secondary antibody solution was decanted, and the membranes were washed for 3x5 minutes in TBST. The membranes were then developed in a gel documentation system (ChemiDoc Touch, Bio-Rad) with enhanced chemiluminescent reagent (Luminata Forte HRP substrate; Millipore Sigma, Burlington, MA, USA), and band densitometry was performed using associated software. For non-

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phosphorylated targets, target band densities were obtained and divided by Ponceau densitometry values and fold-change values were derived by dividing Ponceau-normalized band density values by the aggregate PRE mean value of the PLA group. Band density values for phosphorylated eNOS were divided by corresponding pan band density values, and again fold-change values were derived by dividing these values by the aggregate PRE mean value of the PLA group.

Skeletal muscle nitrate/nitrite measurement. General skeletal muscle lysates were assayed in duplicate using a commercially available fluorometric assay kit (Cayman Chemical; Nitrate/Nitrite Fluorometric Assay Kit; Item No. 780051, Ann Arbor, MI, USA) according to manufacturer's instructions. First, lysates were spun in 30 kD molecular weight cutoff spin filter tubes at 12,000 g for 10 minutes. Filtrates (10 µL) were then placed in a white 96-well plate; 90 µL of kit assay buffer was added to each well, and 20 µL of nitrate reductase/enzyme co-factor solution was added to each well. Following a 120-minute room temperature incubation in the dark, 10 µL of 2,3-diaminonaphthalene solution was added to each well. The plate was then incubated at room temperature for 10 minutes and 10 µL of sodium hydroxide provided in the kit was added to each well. The plate was then read using a microplate fluorometer (BioTek Synergy H1, Winooski, VT, USA) with an excitation wavelength of 360 nm and emission wavelength of 430 nm. Sample NO_x concentrations were extrapolated using a standard curve, and these data were normalized to lysate protein concentrations yielding nmol NO_x/milligram of protein values.

2.8. Statistical Analyses

Statistical analyses were completed using GraphPad Prism (Version 10.4.2). Except for 12-week training volume-load and adherence data (which were compared between groups using independent samples t-tests), outcome variables were analyzed using two-way mixed ANOVA (supplement group × time) with repeated measures on the time factor. Additional exploratory analyses were performed using jamovi v2.3.28 and included examining potential sex interactions for FMD and other key outcome variables, which was performed using three-way (supplement group × sex × time) repeated measures ANOVAs. Data are presented as mean ± standard deviation values, and statistical significance was set at $p<0.05$ throughout.

3. Results

3.1. Participants, Protocol Adherence, and Missing Data

Of the 30 participants initially enrolled in the study, 28 (14 male; 14 female) participants completed the intervention. Two participants did not return following PRE assessments due to time constraints and, thus, were not randomized into groups. Table 2 contains pre-intervention participant characteristics for the 28 completers; notably, no significant pre-intervention differences existed for age, height, body mass, body mass index, or hex bar deadlift strength between groups.

Supplementation adherence was excellent, with BRJ participants reporting 98±2% and PLA participants 99±2% compliance ($p=0.313$). Exercise adherence was also excellent as twenty-six of the 28 participants completed all 24 training sessions; one BRJ participant missed three sessions, and one PLA participant missed one session. Due to poor image quality, complete DXA data was obtained for 13 PLA and 13 BRJ participants, and complete muscle ultrasound data was obtained for 14 PLA and 12 BRJ participants. Technical issues with biopsies or tissue processing (low protein yield or damaged sections) resulted in varying sample sizes: 13 PLA and 13 BRJ for immunoblotting, 11 PLA and 13 BRJ for fCSA, 10 PLA and 11 BRJ for capillary data, and 12 PLA and 13 BRJ for muscle NO_x data. Additionally, 10 PLA and 11 BRJ participants provided adequate quality images for FMD. No data were manually excluded from any analysis.

Table 2. Pre-intervention characteristics between supplementation groups.

Variable	BRJ (n=14)	PLA (n=14)	Ind. t-test p-value

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Age (years)	56±6	56±7	1.000
Sex	7 M / 7 F	7 M / 7 F	1.000
Height (cm)	174±8	172±13	0.685
Body mass (kg)	87.3±16.9	85.1±25.1	0.788
BMI (kg/m^2)	29.4±5.6	28.9±5.1	0.788
Hex bar est. 1-RM	70.0±22.8	75.2±53.7	0.741

Legend: independent samples t-tests were used to compare data between groups except for a Chi-square test being used to compare sex differences. Abbreviations: BMI, body mass index; Hex bar est. 1-RM, estimated maximal strength assessed using a five-repetition test for the hex bar exercise.

3.2. Whole Body and Mid-Thigh Skeletal Muscle Hypertrophic Outcomes

Main effects of time, but no significant group × time interactions (p>0.05), were observed for whole-body lean/soft tissue mass (time: p=0.002; Figure 2a), estimated total body skeletal muscle mass (time: p=0.001; Figure 2b), and VL mCSA values (time: p<0.001; Figure 2c). Each of these measures was significantly greater at POST than PRE.

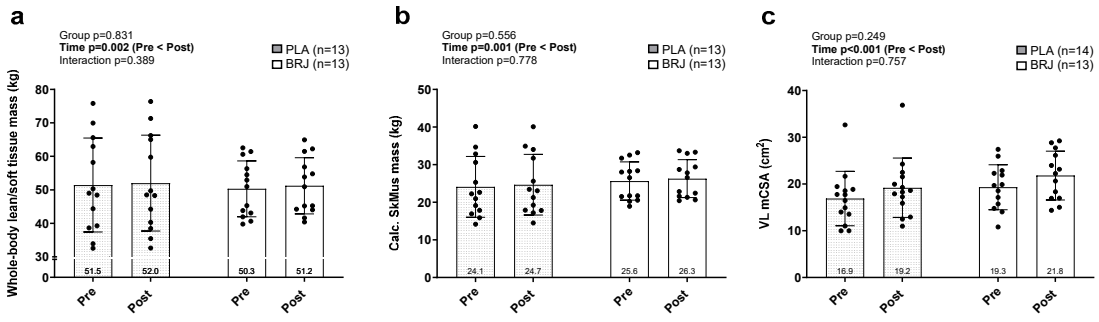

Figure 2. DXA and mid-thigh ultrasound outcomes. Legend: Data for whole-body lean/soft tissue mass (a), calculated skeletal muscle (SkMus) mass when only considering dual-arm and dual-leg lean/soft tissue mass values (b), and vastus lateralis (VL) muscle cross-sectional area (mCSA, c) prior to (PRE) and following the 12-week intervention (POST). Bar graphs depict mean ± standard deviation bars, individual respondent data are superimposed on bar graphs, and mean values are shown at the bottom of each bar.

3.3. Strength and Strength-Endurance Outcomes

An independent t-test determined no statistical differences between groups for 12-week total training volume-load (p=0.294) and 12-week lower body training volume-load (*p*=0.268) between PLA and BRJ (Figure 3a). Main effects of time were observed for hex bar estimated 1-RM (p<0.001; Figure 3b) and hex bar strength-endurance test volume-load completed (p<0.001; Figure 3c) whereby POST values were significantly greater than PRE, but no significant group × time interaction was found for either measure (p>0.05).

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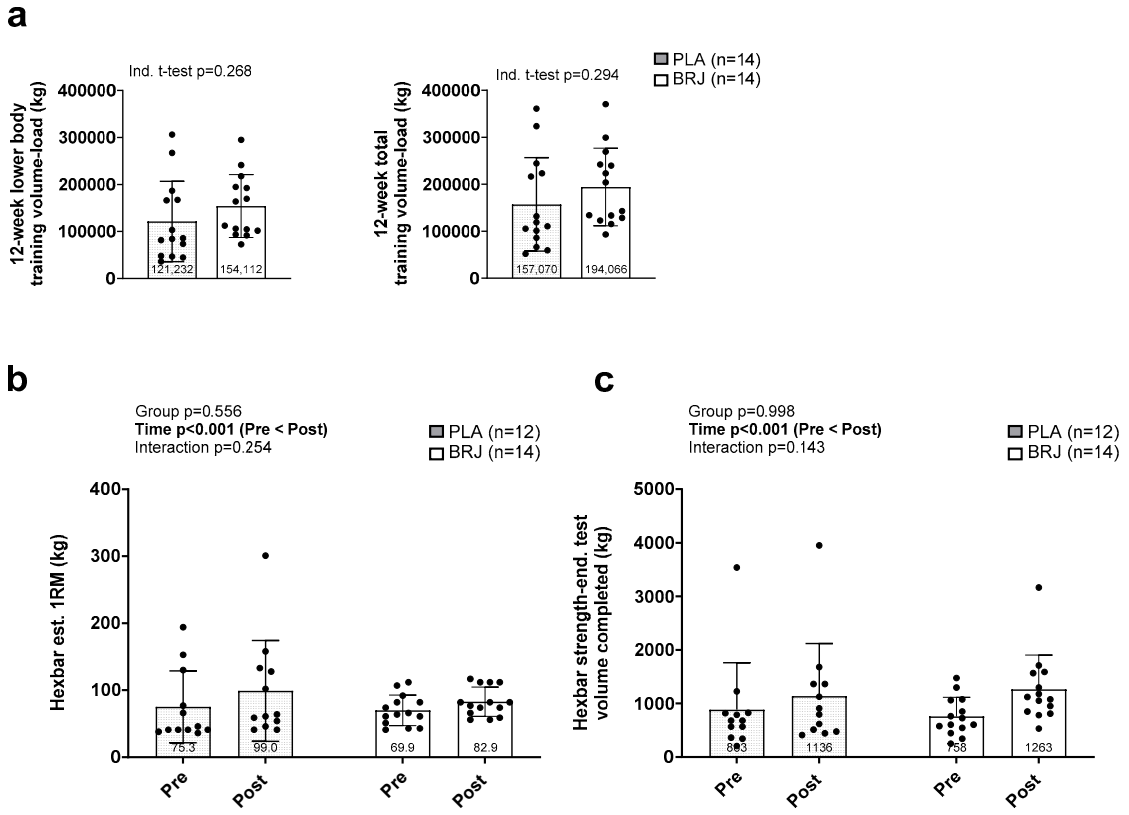

Figure 3. Training volume and strength outcomes. Legend: Data for 12-week lower-body and total volume-load (a), hex bar estimated one repetition maximal lifts (est. 1-RM, b), volume performed based on 60% of hex bar est. 1-RM (c) prior to (PRE) and following the 12-week intervention (POST). Bar graphs depict mean ± standard deviation bars, individual respondent data are superimposed on bar graphs, and mean values are shown at the bottom of each bar.

3.4. Popliteal Artery FMD Outcomes

Main effects of time were observed for relative FMD ($p<0.001$; Figure 4a) and normalized FMD ($p<0.001$; Figure 4b) whereby POST values were significantly greater than PRE, but no significant group × time interactions were present ($p>0.05$). Table 3 has all FMD-related variables (including Figure 4 data) and three-way ANOVA statistics.

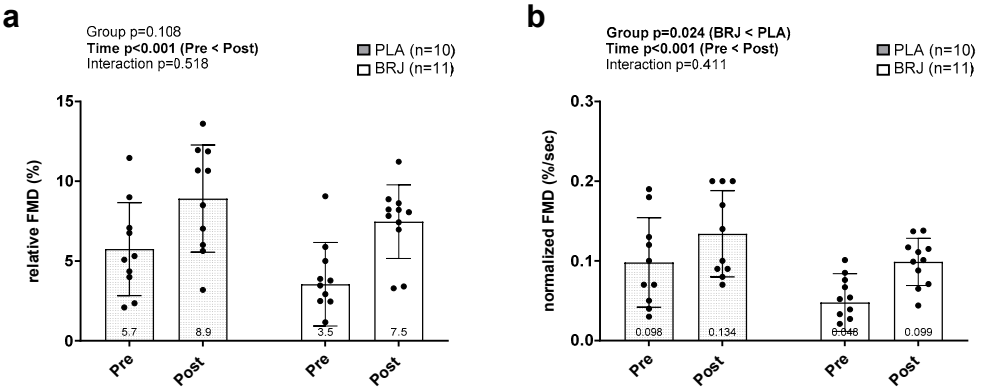

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Figure 4. Popliteal artery flow-mediated dilation outcomes. Legend: Data for relative flow-mediated dilation (FMD, a), and FMD normalized to shear stress (b) prior to (PRE) and following the 12-week intervention (POST). Bar graphs depict mean ± standard deviation bars, individual respondent data are superimposed on bar graphs, and mean values are shown at the bottom of each bar.

Table 3. Exploratory group × sex × time statistics for popliteal artery flow-mediated dilation variables.

Variable	BRJ	PLA	p-values	
Heart rate (bpm)	Pre: 61 ± 6 Post: 66 ± 9	Pre: 65 ± 7 Post: 73 ± 25	Time Sex × time Supplement × time Sex × supplement × time	0.061 0.472 0.587 0.317
SBP (mmHg)	Pre: 126 ± 13 Post: 131 ± 17	Pre: 128 ± 19 Post: 128 ± 19	Time Sex × time Supplement × time Sex × supplement × time	0.400 0.460 0.420 0.795
DBP (mmHg)	Pre: 80 ± 6 Post: 81 ± 8	Pre: 83 ± 10 Post: 83 ± 13	Time Sex × time Supplement × time Sex × supplement × time	0.681 0.082 0.506 0.918
MAP (mmHg)	Pre: 111 ± 10 Post: 114 ± 13	Pre: 113 ± 16 Post: 113 ± 17	Time Sex × time Supplement × time Sex × supplement × time	0.424 0.356 0.442 0.837
Baseline Diam. (mm)	Pre: 6.28 ± 1.31 Post: 6.25 ± 1.31	Pre: 6.19 ± 1.42 Post: 5.82 ± 1.49	Time Sex × time Supplement × time Sex × supplement × time	0.723 0.738 0.488 0.476
Average Shear Rate (s^{-1})	Pre: 44.4 ± 19.0 Post: 39.1 ± 17.6	Pre: 49.4 ± 22.8 Post: 44.3 ± 17.3	Time Sex × time Supplement × time Sex × supplement × time	0.210 0.950 0.805 0.944
Peak Shear Rate (s^{-1})	Pre: 67.1 ± 22.1 Post: 77.8 ± 22.8	Pre: 62.8 ± 9.7 Post: 65.9 ± 13.8	Time Sex × time Supplement × time Sex × supplement × time	**0.004** 0.659 0.599 0.762
Shear Rate Area Under the Curve (AU)	Pre: 6275 ± 2046 Post: 7000 ± 2054	Pre: 5228 ± 1858 Post: 5929 ± 1238	Time Sex × time Supplement × time Sex × supplement × time	**0.005** 0.959 0.987 0.494
FMD (%)	Data presented in Figure 4		Time Sex × time Supplement × time Sex × supplement × time	**0.001** 0.844 0.327 0.151
Normalized FMD (%)	Data presented in Figure 4		Time Sex × time Supplement × time Sex × supplement × time	**0.001** 0.699 0.279 0.211

Legend: bold p-values indicate significance. Abbreviations: BRJ, nitrate-rich beetroot juice group; PLA, placebo group; F, females; M, males; MAP, mean arterial pressure; Diam., vessel diameter; FMD, flow-mediated dilation; AU, arbitrary units.

3.5. Fiber Cross-Sectional, Fiber Type, and Skeletal Muscle Capillarization Outcomes

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Type I fCSA displayed a significant main effect of time (p=0.017; Figure 5a) as PRE was greater than POST, but no significant group × time interaction existed. No significant main effects of time or group × time interactions (p>0.05) existed for type IIA/X fCSA (Figure 5b), type IIA/X percentage (Figure 5c), type I myofiber capillary contacts per fiber (Figure 5d), or type II myofiber capillary contacts per fiber (Figure 5e).

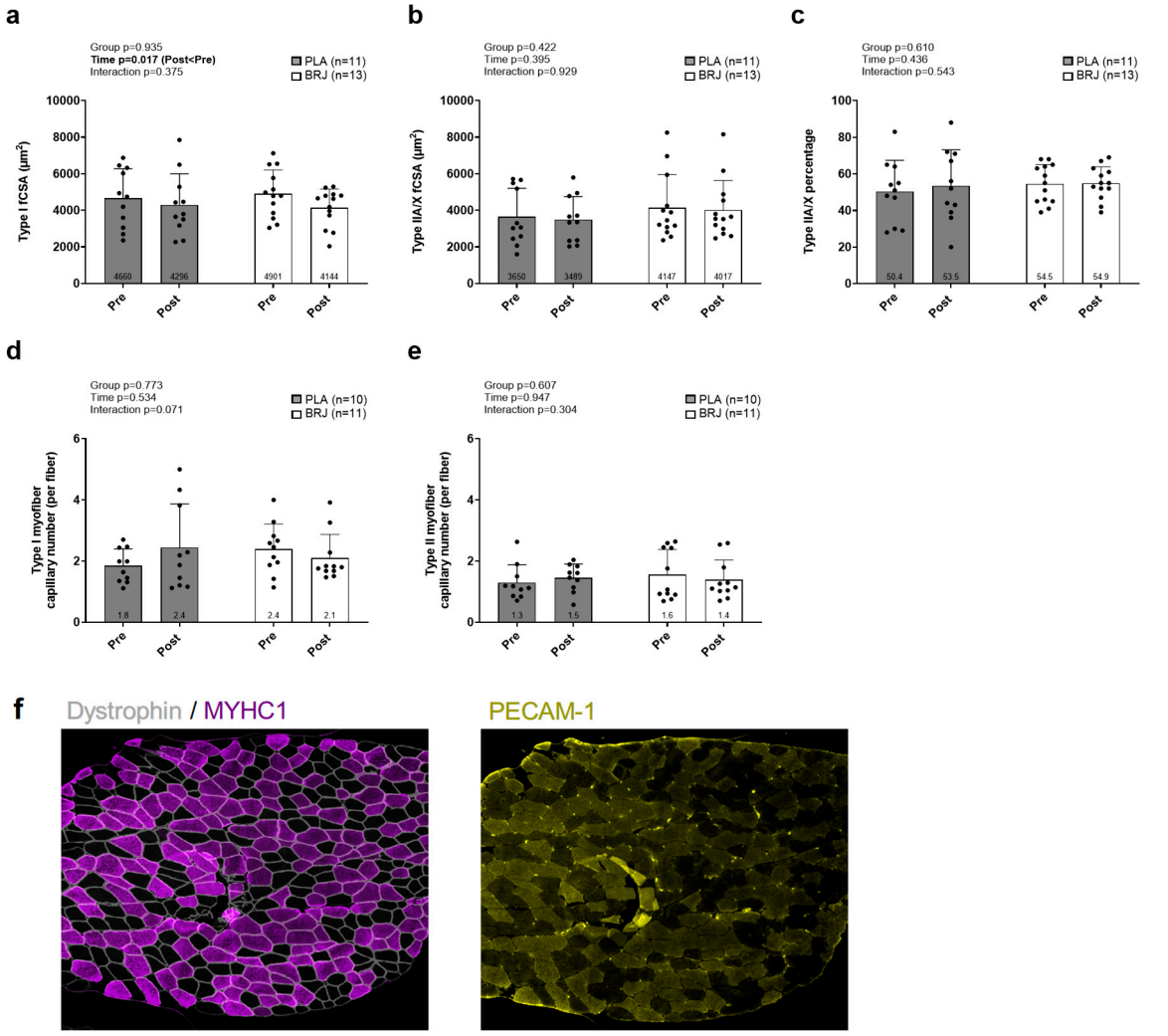

Figure 5. Vastus lateralis immunohistochemistry outcomes. Legend: Data for type I fiber cross-sectional area (a), type IIA/X fiber cross-sectional area (b), fiber type percentage (c), capillary contacts with type I fibers (d), and capillary contacts with type II fibers (e) prior to (PRE) and following the 12-week intervention (POST). Panel f displays representative 10x microscopy images; note, images are pseudo-colored from original fluorescence colors for enhanced clarity (dystrophin: gray, MYHC1: purple, capillaries: yellow). Bar graphs depict mean ± standard deviation bars, individual respondent data are superimposed on bar graphs, and mean values are shown at the bottom of each bar.

3.6. Immunoblotting Markers and Muscle NO_x

TSP-1 protein content displayed a significant main effect of time (POST>PRE, p=0.031; Figure 6b), but no significant group × time interaction was present. VEGF protein content (Figure 6a), VEGFR2 protein content (Figure 6c), TFEB protein content (Figure 6d), pan eNOS protein content (Figure 6e), and phosphorylated-eNOS protein content (Figure 6f) did not display significant main effects of time or group × time interactions. VL muscle NO_x (*not graphed*) did not display a significant main effect of time (p=0.141) or a group × time interaction (p=0.741). Levels non-significantly increased 15.4% in the BRJ group from PRE to POST (7.7±2.1 to 8.9±1.6 ng/mg protein, p=0.073) and

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7.8% in the PLA group from PRE to POST (9.6±2.8 to 10.4±3.4 ng/mg protein, p=0.514) though these changes were not statistically significant.

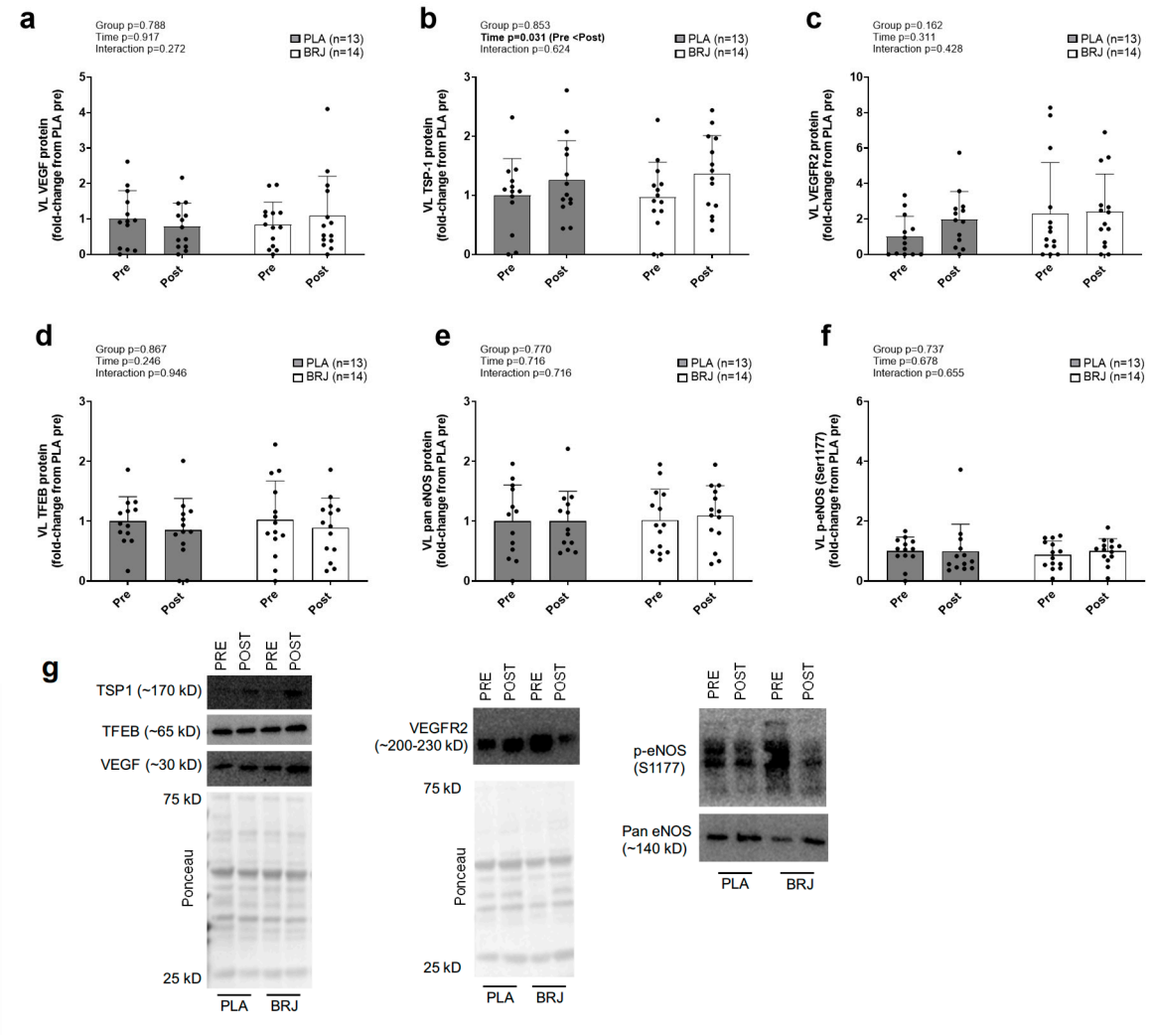

Figure 6. Vastus lateralis immunoblotting outcomes for markers of angiogenesis. Legend: Protein levels for vascular endothelial growth factor (VEGF, a), Thrombospondin-1 (TSP-1, b), VEGF receptor 2 (VEGFR2, c), Transcription Factor EB (TFEB, d), endothelial nitric oxide synthase (eNOS, e), and phosphorylated eNOS (Ser1177, f) prior to (PRE) and following the 12-week intervention (POST). Panel g displays representative immunoblots. Bar graphs depict mean ± standard deviation bars, individual respondent data are superimposed on bar graphs, and mean values are shown at the bottom of each bar.

3.7. Exploratory Analysis of Sex Effects for Non-FMD Variables

Table 4 contains group × sex × time interaction p-values for the other (non-FMD) outcome variables demonstrating significant training effects. Three-way ANOVA analyses revealed no significant group × sex × time interactions for RT-responsive outcome variables, suggesting that supplementation effects were comparable between males and females.

Table 4. Exploratory group × sex × time statistics for non-FMD variables demonstrating training effects.

Variable	BRJ & PLA F/M	Stats
DXA lean tissue mass	BRJ: 7F/6M PLA: 7F/6M	Sex **p<0.001** (M>F) Sex × time p=0.478 Sex × supplement × time p = 0.615

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VL mCSA	BRJ: 6F/7M PLA: 7F/7M	Sex **p=0.021** (M>F) Sex × time p=0.074 Sex × supplement × time p = 0.456
Hex bar est. 1-RM	BRJ: 6F/7M PLA: 7F/7M	Sex **p<0.001** (M>F) Sex × time p=0.119 Sex × supplement × time p = 0.106
Hex bar strength-end. test	BRJ: 6F/7M PLA: 7F/5M	Sex **p<0.001** (M>F) Sex × time p=0.176 Sex × supplement × time p = 0.492

Legend: data are from three-way repeated measures ANOVAs on outcomes demonstrating significant training effects. Although sex differences were noted (M>F, $p<0.05$), no significant group × sex × time interactions were observed. Abbreviations: BRJ, nitrate-rich beetroot juice group; PLA, placebo group; F, females; M, males; DXA, dual-energy x-ray absorptiometry; VL mCSA, vastus lateralis muscle cross-sectional area; est. 1-RM, estimated maximal strength.

4. Discussion

To our knowledge, this is the first study to comprehensively examine how chronic NO_3^- supplementation affects RT adaptations in middle-aged and older adults. The primary finding was that 12 weeks of BRJ supplementation (800 mg NO_3^-/day) did not enhance skeletal muscle hypertrophy, strength, or vascular adaptations compared to placebo during the supervised RT program. Additionally, our three-way ANOVA results did not reveal sex-specific effects with supplementation. These null findings were contrary to our hypotheses and warrant further discussion.

Several factors may explain why BRJ supplementation did not enhance chronic training adaptations despite other literature reporting acute performance benefits. First, while muscle NO_x levels numerically increased in the BRJ group (+15.4% vs +7.8% in placebo), this was not statistically significant. As our hypothesis was predicated on BRJ supplementation increasing muscle nitrate content to elicit beneficial training effects, this may not have been accomplished with the 800 mg/day supplementation protocol. It is also possible that the 24-hour washout period prior to POST testing may have allowed tissue NO_3^- levels to return toward baseline, potentially explaining the lack of significant muscle NO_x accumulation. This aspect of the study design was chosen to ensure that FMD outcomes were not influenced by the consumption of BRJ during the morning of POST testing. However, it is notable that the timing of NO_3^- consumption (e.g., 1-2 hours prior to exercise) likely influences exercise performance outcomes as well as plasma and tissue NO_3^- concentrations [28]. Second, the 800 mg/day dose, while effective for acute performance benefits, may be insufficient for chronic adaptations in this population. In this regard, several acute supplementation studies have reported NO_3- supplementation increases repetitions to failure [33], oxygen and nutrient delivery to skeletal muscle during exercise [34], power-related outcomes [63], and exercise tolerance [29]. Additionally, a study by Benjamin et al. [50] reported six days 800 mg NO_3- supplementation improves cardiovascular parameters in postmenopausal women following acute submaximal exercise, and others have reported four weeks of L-citrulline supplementation (a supplement that also increases nitric oxide bioavailability [64]) improves cardiovascular measures in postmenopausal women [65,66]. However, our null findings agree with other published reports in older adults examining longer-term supplementation paradigms. For instance, Caballero-Garcia et al. [67] reported that six weeks of L-citrulline supplementation did not affect various strength or endurance outcomes in older individuals. Carter et al. [40] reported eight weeks of BRJ (providing the same dose of NO_3- as our study) combined with circuit-style training did not significantly increase knee extensor power in postmenopausal women. Collectively, the disconnect between acute improvements in exercise performance with acute peri-exercise NO_3- ingestion and chronic adaptations with long-term supplementation remains unclear.

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At the molecular level, we posited that BRJ supplementation may also affect markers of angiogenesis (VEGF, VEGFR, TSP-1, and eNOS) or skeletal muscle capillarization since increased nitric oxide can alter vascular function and shear stress to potentially affect these outcomes [68]. However, these markers were also not affected by supplementation. In contrast with other studies reporting RT to increase skeletal muscle VEGF, VEGFR, and skeletal muscle capillarization [19,59,69–71], we also did not observe training effects in spite of participants experiencing increases in outcomes related to skeletal muscle hypertrophy. It is worth noting that findings from several of the aforementioned studies were in younger adults and these adaptations (VEGF expression in particular) are seemingly blunted in older adults [21–24]. Our results also suggest that BRJ supplementation does not augment markers of angiogenesis from RT among middle-aged and older adults. In contrast, we found that RT improved FMD and the shear rate during reactive hyperemia (both peak shear and the shear area under the curve), indicating an improvement in applied measures of microvascular and macrovascular endothelial function, although there was no effect of supplementation. These findings suggest that the RT intervention led to favorable adaptations that could reduce cardiovascular and cardiometabolic disease risk in this population.

A final noteworthy and unanticipated finding unrelated to supplementation was the decrease in type I fCSA from PRE to POST as well as no change in type II fCSA values with training. This contrasts with several studies indicating that RT increases type I and II fCSA values [72]. Both groups experienced significant increases in other skeletal muscle hypertrophy outcomes (e.g., VL mCSA and DXA lean tissue mass metrics) following the 12-week RT intervention. However, this disconnect is not entirely surprising given that our laboratory has previously reported that different measures of skeletal muscle hypertrophy do not always correlate [57,73]. Moreover, our laboratory and others have reported that RT interventions in older individuals do not significantly affect type I or II fCSA outcomes despite other tissue-level hypertrophy variables significantly increasing [4,74]. It is also notable that magnitudes of skeletal muscle hypertrophy, vascular health, and plasma NO_3^- also differ between younger and older adults [6,75,76]. Hence, a younger cohort serving as a comparator group could have provided additional insight into the chronic effects of NO_3^- supplementation, and future studies should consider examining similar outcomes in a younger population.

4.1. Limitations and Future Considerations

Though well-designed and executed, several limitations, in addition to sample size, are noted. There were no measures of skeletal muscle blood flow indicators during training sessions. Future studies assessing skeletal muscle blood flow or proxy measurements such as near-infrared spectroscopy to evaluate blood flow measures during exercise could be insightful [77]. Diet influences hypertrophic outcomes [2], but dietary habits were not recorded and may have affected our results. Another limitation is that we did not assess peri-ingestion plasma NO_x to determine whether the dose of BRJ we provided lead to substantial increases in NO_3^- and NO_2^- within our participants. Achieving large increases in circulating NO_x would presumably be needed to facilitate an increase in muscle NO_x, which we did not observe. Nonetheless, the dose of NO_3^- we administered was consistent with several prior studies.

4.2. Conclusions

Our findings indicate that 12 weeks of NO_3^- rich BRJ supplementation does not enhance various RT adaptations in middle-aged and older adults. Future studies are needed to understand the disconnect between acute ergogenic findings that are widely reported and the null findings regarding chronic NO_3^-supplementation in this and other studies.

Author Contributions: This study served as a dissertation project for MCM. MCM. primarily conceived the study idea with significant input from MDR, DTB, LBG, ATR, and ANK. MCM, MDR, and DTB applied for and were awarded grant funding for the study. MCM and MDR primarily drafted the manuscript and prepared figures. MCM primarily carried out laboratory-based assays. BJM, DRT, DAA, AA-B, GK Jr., DLP, MLM, NJK,

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JMM, KH, BMP, DL, MKC, and HK provided crucial assistance with original data collection procedures and/or training. All co-authors assisted with revising and editing the manuscript, and all co-authors approved the final version.

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Institutional Review Board Statement: This study was not registered as a clinical trial. Study protocols were carried out in accordance with the most recent version of the declaration of Helsinki. All study procedures were approved by Auburn University's Institutional Review Board (approval number: Protocol # 24-863 MR 2405). All participants in this study provided verbal and written consent in accordance with the above IRB approval.

Data Availability Statement: Data for this study are available from one of the co-corresponding authors (mdr0024@auburn.edu) upon reasonable request.

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Conflicts of Interest: The authors declare they have no competing interests in relation to these data.

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