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Impacts of mitochondrial-targeted antioxidant on leg function, leg blood flow and skeletal muscle mitochondrial function in peripheral artery disease patients: full study protocol and statistical analysis plan

NCT# 03506633

Study protocol

Participants

Patients with PAD (n = 13, 7 males and 6 females) were recruited for study participation. Disease staging, claudication history, and ankle-brachial index of <0.90 were determined by both self-report and by physician diagnosis, and all participants were classified as Fontaine stage IIa and IIb (16). Participants were also required to have a stable BP, lipid, and/or diabetes regimen for ≥ 6 wk prior to participation, and all females were postmenopausal (no menses for ≥ 12 consecutive months). Participants were excluded from the study if they had a history of pain at rest (Fontaine stage III) and/or tissue loss due to PAD (Fontaine stage IV), limited walking capacity due to conditions other than PAD, or renal disease. Participants were asked to withhold medications (at least 12 h prior to the visits) for ≥ 24 h (17). Participants were also asked to track what they consumed the day before their first visit so they could consume the same foods prior to their second visit. In addition, participants were requested to not alter their dietary habits during the 2 wk washout period. All procedures were performed according with the protocols approved by the Institutional Review Board and performed in accordance with the Declaration of Helsinki. All participants provided written, informed consent prior to beginning the study. This study was registered with https://clinicaltrials.gov/ (NCT03506633).

Design

This study used a randomized, double-blinded, placebo-controlled, crossover design with a washout period of 14 days between the 2 study visits **(Fig. 1)**. Participants were randomly assigned to receive either 80 mg of MitoQ or placebo. In a previous study, MitoQ showed pharmacokinetic behavior at doses of 80 mg (1mg/1kg), resulting in plasma maximal concentration of 33.15 ng.ml, which is safe for humans. Additionally, administration of 20 mg/1kg of mitoQ was shown to have no toxicity in previous research. The dose in this study was considerably lower than this amount. These findings led us to believe the recommended

80 mg dose is ideal for this study. The PL consisted of tapioca powder capsules that possessed no nitrate or antioxidant properties. Participants were informed that we were researching the effects of commercially available juice and pills on their vascular function and walking capacity; therefore, they were not aware that the capsules were PL. All assessments were performed after an overnight fast and at the same time of day (9:00 AM ± 1 h). Anthropometric measurements were obtained upon arrival at both visits. Following anthropometrics, baseline experimental measurements were conducted, after which the MitoQ supplement or PL were consumed. All experimental measurements were repeated ~ 1 h following ingestion. All data analyses were performed in a blinded manner.

Anthropometrics

Anthropometric measurements included height, body mass, and body composition. Height was measured using a stadiometer (nearest 0.5 cm). Body mass was measured using a standard scale (nearest 0.1 kg), and body mass index was calculated by taking the body mass divided by height squared (kg/m^2). Body fat percentage was assessed using bioelectrical impedance analysis in duplicate to the nearest 0.1% (HBF – 306C, Omron Healthcare, Lake Forest, IL), and the average of the 2 measures was recorded as the body fat percentage.

Resting heart rate and blood pressure

Participants sat for 15 min in a quiet and temperature-controlled room. Resting heart rate and BP were assessed in duplicate using an automated sphygmomanometer (HEM – FL 31, Omron Healthcare, Lake Forest, IL). The two measurements were taken with 5 min separating each measurement, and the average of the 2 was recorded as the resting heart rate and BP.

Endothelial function

Endothelial function was assessed using brachial artery flow-mediated dilation (FMD) and popliteal artery FMD using a Doppler ultrasound (Terason uSmart 3300, Terason Division Teratech Corporation, Burlington, MA), rapid inflation cuff system (E20 Rapid Cuff and cuff model SC5, D.E. Hokanson, Bellevue, WA), and a 3-lead electrocardiogram system (7700 Series

Trigger Monitor, IvyBiomedical Systems Inc., Branford, CT) as previously described (22). Briefly, for both brachial and popliteal assessments, the rapid inflation cuff was placed just distal to the antecubital fossa and just distal to the popliteal fossa, respectively (22). For both assessments, resting arterial diameter was recorded with the ultrasound probe ~ 1-2 cm proximal to the rapid-inflation cuff for 5 min (22). The cuff was inflated to a pressure of 250 mmHg after baseline diameter was obtained and remained inflated for 5 min (22). The cuff was then deflated and the reactive hyperemic arterial response was recorded continuously on R-wave trigger for 5 min (22). Data were analyzed using an image capturing and automated edge-detection software (Vascular Imager, Vascular Research Tools 6, Medical Imaging Applications, Coralville, IA) as previously described (22). The relative artery diameter changes were calculated as previously described (22).

Walking capacity and skeletal muscle oxygen utilization capacity

Maximal walking capacity was assessed on a standard treadmill (Lode, B.V, Groningen, The Netherlands) using the modified Gardner protocol (22, 24). Participants were asked to notify the test administrators about their onset of claudication (the exact moment their leg pain starts) and to walk as long as they could tolerate even if the claudication persisted (maximal walking time and distance). The test was ended once patients could not tolerate their symptoms (i.e., severe claudication).

Gastrocnemius oxygen utilization capacity was measured with a portable near-infrared spectroscopy (NIRS) unit (Artinis PortaMon, Einsteinweg, The Netherlands) and Oxysoft software (v. 3.0.103.3, Einsteinweg, The Netherlands) during the exercise testing as previously described (22). Briefly, the NIRS device was adhered to the gastrocnemius, at the approximate location where the participant felt the most pain, using a commercially available double-sided adhesive and a commercially available black bandage to prevent extraneous light from reaching the device (15, 22). NIRS data were collected continuously at a frequency of 10 Hz and used to

estimate the change in tissue saturation (StO_2), deoxygenated hemoglobin concentration ([HHb]) and oxygenated hemoglobin concentration ([O_2Hb]) at resting baseline and during the walking test. Upon walking test completion, the participant stood in a resting standing position and a thigh rapid inflation cuff (cuff model SC10, D.E. Hokanson, Bellevue, WA) was placed on the thigh ~ 3-4 centimeters proximal to the knee on the limb with the NIRS device. The cuff was inflated to ~ 250- 260 mmHg for 5 min to deoxygenate the tissue with the same rapid inflation cuff system used for FMD (22, 25). The cuff pressure was released after 5 min, and the peak hyperemic response following cuff release was used to determine the 100% oxygenation level for each participant and was used to calculate normalized [HHb] (22, 25). The raw NIRS data were reduced to 1 Hz files and exported to Microsoft Excel files for later analysis.

Autonomic function

Autonomic nervous system function was measured using heart rate variability. Participants wore a simple heart rate monitor (Suunto Smart Sensor, Suunto, Vantaa, Finland). The researcher assisted the participant with putting on the heart rate monitor. The participant was positioned in a supine position on a padded examination table for 5 minutes. The subject was then be assisted with standing upright in front of the table for 5 minutes. Heart rate was recorded during supine and standing positions and used to determine autonomic nervous activity.

Statistical analysis

The Shapiro-Wilk test was used for all dependent variables to determine the normality of the data. Independent *t*-tests were used to determine any differences between subject characteristics at the MitoQ and PL visits. A two-way repeated measures analysis of variance (ANOVA) [group (MitoQ and PL) x time (before and after supplement intake)] was used to compare the changes between MitoQ and PL intake within and between groups on the dependent variables. When a significant main effect or interaction was found, paired *t*-tests were used for *post-hoc* comparisons for the normally distributed variables, and paired samples

Wilcoxon tests were used for non-normally distributed variables. Based on a power calculation from a previous study, a sample size of a minimum of total 14 (n = 7 per group) would allow for 80% power to detect differences between FMD between MitoQ vs. PL (26). Associations between dependent variables were assessed using Pearson's product-moment correlation coefficient. Effect size analyses were conducted using Cohen's *d* and interpreted as 0.2 as a small effect size, 0.5 as a medium effect size, and 0.8 as a large effect size (22, 27). All analyses were performed using SPSS 26.0 (IBM, Armonk, NY). Descriptive characteristics are presented as Mean ± SD, and all other data are presented as Mean ± SE. Statistical significance was set to $p < 0.05$.