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## Acute mitochondrial antioxidant intake improves endothelial function, antioxidant enzyme activity, and exercise tolerance in patients with peripheral artery disease

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# Acute mitochondrial antioxidant intake improves endothelial function, antioxidant enzyme activity, and exercise tolerance in patients with peripheral artery disease

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Peripheral artery disease (PAD) is a manifestation of atherosclerosis in the leg arteries, which causes claudication. This may be in part due to vascular mitochondrial dysfunction and excessive reactive oxygen species (ROS) production. A mitochondrial-targeted antioxidant (MitoQ) has been shown to improve vascular mitochondrial function that, in turn, led to improved vascular function in older adults and animal models.

However, the roles of vascular mitochondria in vascular function including endothelial function and arterial stiffness in patients with PAD are unknown; therefore, with the use of acute MitoQ intake, this study examined the roles of vascular mitochondria in endothelial function, arterial stiffness, exercise tolerance, and skeletal muscle function in patients with PAD. Eleven patients with PAD received either MitoQ or placebo in a randomized crossover design. At each visit, blood samples, brachial and popliteal artery flow-mediated dilation (FMD), peripheral and central pulse-wave velocity (PWV), blood pressure (BP), maximal walking capacity, time to claudication (COT), and oxygen utility

capacity were measured pre- and post-MitoQ and placebo. There were significant group by time interactions ( $P < 0.05$ ) for brachial and popliteal FMD that both increased by  $\Delta 2.6$  and  $\Delta 3.3\%$ , respectively, and increases superoxide dismutase ( $\Delta 0.03$  U/mL), maximal walking time ( $\Delta 73.8$  s), maximal walking distance ( $\Delta 49.3$  m), and COT ( $\Delta 44.2$  s). There were no changes in resting heart rate, BP, malondialdehyde, total antioxidant capacity, PWV, or oxygen utility capacity ( $P > 0.05$ ). MitoQ intake may be an effective strategy for targeting the vascular mitochondrial environment, which may be useful for restoring endothelial function, leg pain, and walking time in patients with PAD.

**NEW & NOTEWORTHY** The results of this study reveal for the first time that acute oral intake of mitochondrial-targeted antioxidant (MitoQ, 80 mg) is effective for improving vascular endothelial function and superoxide dismutase in patients with peripheral artery disease (PAD). Acute MitoQ intake is also effective for improving maximal walking capacity and delaying the onset of claudication in patients with PAD. These findings suggest that the acute oral intake of MitoQ-mediated improvements in vascular mitochondria play a pivotal role for improving endothelial function, the redox environment, and skeletal muscle performance in PAD.

mitochondrial-targeted antioxidant; MitoQ; pulse-wave velocity; vascular function; walking capacity

## INTRODUCTION

Peripheral artery disease (PAD) is a common cardiovascular disease in which atherosclerotic plaque forms in the leg arteries, which causes attenuated blood flow and reduced perfusion in the lower extremity. PAD affects nearly 200 million people worldwide, with over 20% of individuals over 80 yr old being affected by this disease (47). Symptoms often include claudication (leg pain during walking) and foot ulcers, which in the severe form may require revascularization procedures or leg amputation (16). Additionally, these symptoms significantly reduce quality of life and can also cause

depression (29, 43). Identification of therapeutic targets to improve vascular function and symptoms in this population is a top priority in the both translational and clinical research fields.

One of the proposed mechanisms underlying accelerated atherosclerosis and skeletal muscle oxidative stress damage in PAD is increased reactive oxygen species (ROS) (21, 41). NADPH oxidase (NOX) and mitochondria are the major players for ROS production in the vasculature (7, 55). Patients with PAD demonstrate increased NOX2 activity compared with controls (26). Since NOX enzyme-derived ROS production may be classified as non-mitochondrial-derived ROS, it is plausible to expect that general antioxidants may adequately scavenge this excessive NOX-derived ROS in PAD. However, studies that use general antioxidants to scavenge ROS in PAD and atherosclerotic disease have not produced consistent results (23, 27, 58), and long-term supplementation of antioxidants (vitamins E and C, flavonoids,  $\alpha$ -lipoic acid, and resveratrol) for 3–6 mo has demonstrated no improvements in brachial flow-mediated dilation (12, 23), smooth muscle function (23), oxidative stress markers (23, 58), inflammation (58), or maximal walking time and/or walking performance (4, 12, 30), albeit trends for improved distance to claudication were noted (12). These results may suggest that there may be a more specific source of ROS production. Mitochondria have been suggested as a key player for ROS production in PAD, and recent evidence suggests that mitochondria play an important role in the vasculature cell energy production and calcium signaling (55). We previously demonstrated that vascular smooth muscle mitochondrial function is attenuated across age and that this age-associated mitochondrial dysfunction is also significantly associated with reduced endothelial function that was assessed by flow-mediated vasodilation (35). Endothelial dysfunction has been reported in patients with PAD (2, 3). Additionally, skeletal muscle mitochondrial dysfunction and oxidative damage have been reported in PAD (41, 42). Dys-functional vascular mitochondria can augment the production of mitochondrial-derived ROS that scavenges endogenously produced nitric oxide (NO), a potent vasodilator, which is critical for endothelial function (38, 39). Therefore, intact function of the mitochondria in the vasculature is required for regulation of ROS production and NO bioavailability, and the mitochondria may be a novel therapeutic target to improve

vascular function and oxygen transfer and utility capacity in the lower extremity, ultimately reducing leg pain and improving quality of life in patients with PAD.

MitoQ, a mitochondria-targeted antioxidant, includes the antioxidant ubiquinol attached to triphenylphosphonium (33), giving it the ability to cross the cell membrane and accumulate within the mitochondria, which makes MitoQ ideally positioned to scavenge mitochondrial-derived ROS and improve respiratory function (33, 45, 48). Therefore, the use of MitoQ in PAD may be a viable intervention for reducing mitochondrial-derived ROS and improving vascular mitochondrial function, which may further elucidate the roles of vascular mitochondria in PAD. MitoQ has demonstrated great success in a clinical trial involving healthy middle-aged and older adults, which produced reductions in arterial stiffness, oxidative stress, and improvements in endothelial function (46). We have also previously demonstrated that acute *ex vivo* treatment with MitoQ can restore age-related endothelial dysfunction in skeletal muscle feed arteries from older adults ( $72 \pm 5$  yr) (38) and that this age-related impairment is likely augmented by increased mitochondrial ROS production (36). However, to our knowledge, the impacts of MitoQ on vascular function in patients with PAD *in vivo* have not been previously investigated. The purpose of this study was to determine the roles of the vascular mitochondria to improve vascular function *in vivo*. It was hypothesized that acute MitoQ intake (1 dose of 80 mg) would improve endothelial function and the redox environment, which in turn would reduce blood pressure (BP) and arterial stiffness while improving walking capacity and oxygen utility capacity in patients with PAD.

## **METHODS**

*Participants.* Patients with PAD ( $n = 11$ , 5 men and 6 women; age,  $66.1 \pm 10.6$  yr; and body mass index,  $30 \pm 6.8$  kg/m<sup>2</sup>) were recruited using flyers and physician referrals. All participants had a positive history of chronic claudication, an ankle-brachial index of  $<0.90$  (Fontaine classification, stage II and III), and stable management of their blood pressure (BP) and blood lipids for at least 6 wk before study participation. All participating women were classified as post-menopausal (cessation of menses for at least 12 mo). Exclusion criteria included pain at rest (severe claudication) and/or tissue

loss due to PAD, limited walking capacity due to other conditions or ailments, and/or kidney disease. All procedures and laboratory protocols were approved by the Institutional Review Board and performed according with the Declaration of Helsinki. All study participants provided written informed consent before study participation. This study was registered with <http://clinicaltrials.gov/> (NCT03506633).

*Design.* This study consisted of a randomized, placebo-controlled, crossover study design (Fig. 1). Participants were randomly assigned to receive MitoQ (MQ, 80 mg, MitoQ Ltd., Auckland, NZ) or the placebo (PL) for their first visit and received the opposite in their second visit after a 14-day washout period. In a previous study, MitoQ demonstrated pharmacokinetic behavior at doses of 80 mg (~1 mg/kg) and resulted in a maximal concentration of 33.15 ng/mL in the plasma, which was deemed to be a safe level for humans (31). Baseline measurements were performed at each visit after an overnight fast at the same time of day ( $\pm 1$  h). Height, weight, body mass, body mass index (BMI), and body fat percentage were obtained as descriptive characteristics. After characteristics measurements, base-line measurements were taken, including resting heart rate (HR), BP, blood sampling, brachial artery FMD, popliteal artery FMD, arterial stiffness, exercise tolerance, and oxygen utility capacity. Participants then consumed either MQ or PL, and all baseline measures were repeated as experimental measures 40 min after MQ or PL intake. The 40-min digestion period was chosen since oral intake of MitoQ has been shown to reach peak levels in the plasma after ~1 h following ingestion (31); therefore, our primary outcome measurements (brachial and popliteal artery FMD) occurred at ~1 h. This allowed for our team to have the proper time to prepare the participant for the measurement (attach electrodes, move equipment, and have the participant rest on the exam table for ~20 min before the FMD measurements).

*Anthropometrics.* Height, total body mass, and body composition were measured for each participant. Participants were asked to remove their shoes. Height was measured with a standard stadiometer (nearest 0.5 cm), and total body mass was measured using a standard scale (nearest 0.1 kg). The BMI was calculated using the total body mass divided by the square of height ( $\text{kg}/\text{m}^2$ ). Body composition was assessed with a handheld bioelectrical impedance analysis (BIA) meter (HBF-306C,

Omron Healthcare, Lake Forest, IL) in duplicate to the nearest 0.1%. The average of the two measurements was used as the body fat percentage.

*Resting heart rate and blood pressure.* Resting HR and BP were assessed in duplicate after 5 min of quiet rest in a seated position with an automated sphygmomanometer (HEM-FL 31, Omron Healthcare, Lake Forest, IL). Five minutes separated the two measurements, and the average of the two was recorded as the resting values.

*Blood sampling and analysis.* Blood samples (10 mL) were drawn from an antecubital vein by a trained phlebotomist with EDTA tubes before and after MitoQ and placebo intake. Blood samples were centrifuged at 3,500 rpm at 4°C for 10 min. Plasma samples were stored at -30°C for later analysis of total antioxidant capacity (TAC), superoxide dismutase (SOD), and malondialdehyde (MDA). TAC was measured with a commercially available Total Antioxidant Capacity Assay Kit (Cat. No. ab65329, Abcam, Cambridge, UK) according to the manufacturer's instructions. After plasma samples were incubated at 23°C, absorbances were assessed at 570 nm using a microplate reader. SOD was assessed using SOD Assay Kit-WST (Cayman Chemical, Ann Arbor, MI). Samples were mixed with WST working solution and enzyme working solution. The samples were then incubated at 37°C for 20 min. The absorbances of the incubated samples were assessed at 450 nm with a microplate reader. SOD activity was measured by a Cobas Mira chemistry analyzer (Roche, Basel, Switzerland) (25). MDA was assessed by a commercially available Lipid Peroxidation (MDA) Assay Kit (Sigma Aldrich, St. Louis, MO).

Samples were incubated with a reagent diluent for 5 min at 23°C. The incubated samples were stimulated by Ran-Cell total antioxidant control (Randox, Crumlin, County Antrim, UK) and were analyzed at 340 nm by a microplate reader (25). The mean intra-assay variability measured as the coefficient of variation for each sample was 2.9, 3.8, and 3.3% for SOD, TAC, and MDA, respectively.

*Endothelial function.* Endothelial function of the brachial and popliteal arteries was determined using flow-mediated dilation (FMD) assessment with a Doppler ultrasound system (Terason uSmart 3300, Terason Division Teratech Corporation, Burlington, MA) and a three-lead electrocardiogram (EKG) system (7700 Series Trigger



Monitor, IvyBiomedical Systems, Inc., Branford, CT).

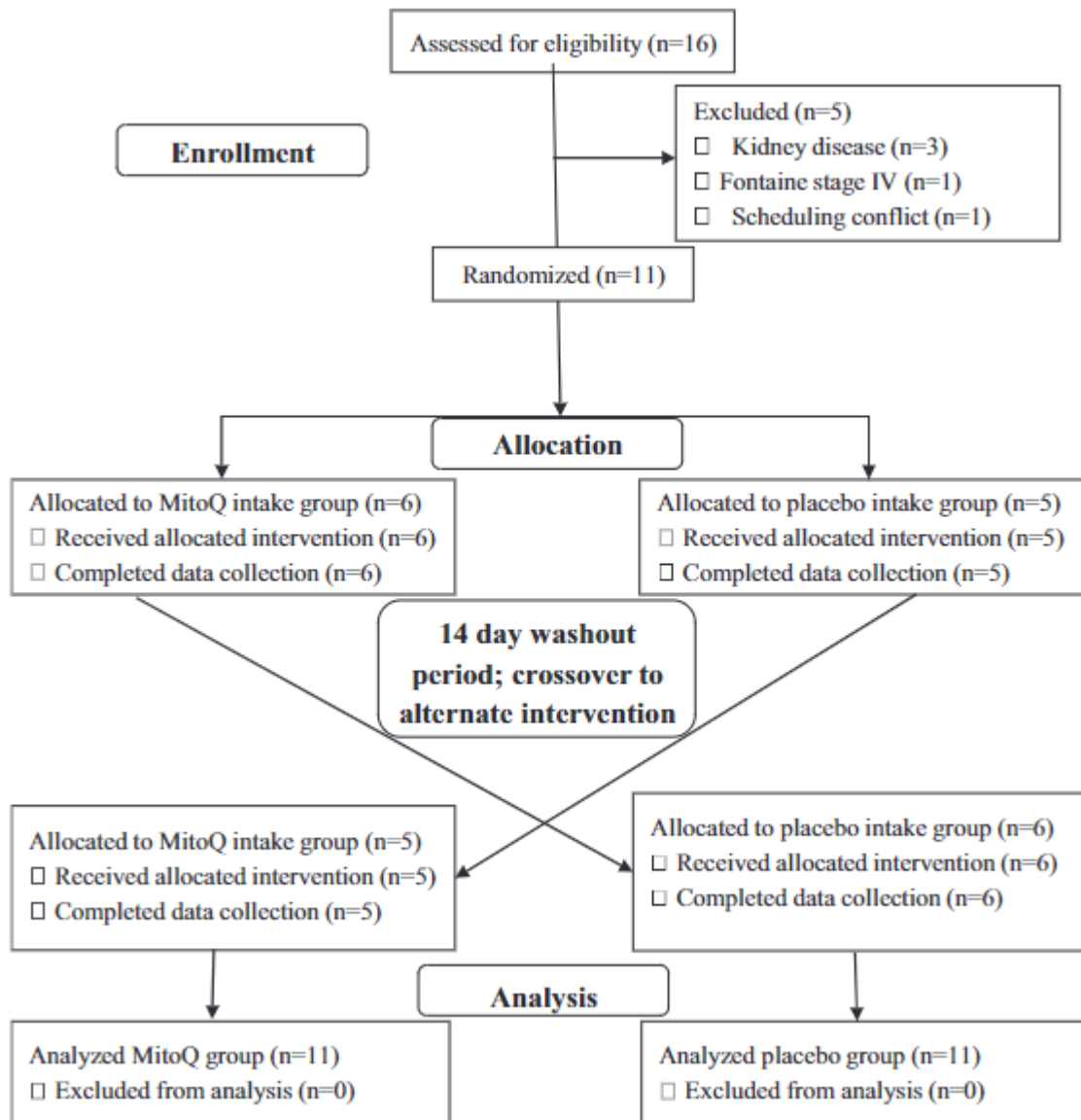


Fig. 1. Randomized, placebo-controlled, crossover design, participant allocation, and analysis.

Following the electrode placement, popliteal artery FMD was assessed with participants in the prone position on a padded table. The prone position was used for participant comfort, which has been previously used in both healthy and PAD populations (17, 34). A rapid-inflation BP cuff was secured just distal to the popliteal fossa (E20 Rapid Cuff and cuff model SC5, D. E. Hokanson, Bellevue, WA) and resting baseline popliteal artery diameter was recorded 1 to 2 centimeters proximal to the cuff for 5 min with the ultrasound probe. The cuff was inflated to 250 mmHg following the

baseline measurement, and the cuff remained inflated for 5 min. The cuff was then released to 0 mmHg, and popliteal diameter was recorded continuously for 5 min (reactive hyperemia) on R-wave trigger. Brachial artery FMD was measured with participants resting in the supine position with the arm extended and supinated. The rapid-inflation cuff was placed just distal to the antecubital fossa, and resting brachial artery diameter was recorded 1 to 2 centimeters proximal to the cuff for 5 min. The cuff was inflated to 250 mmHg for 5 min, and post-hyperemic response was recorded on R-wave trigger for 5 min. Recorded data were saved for later analysis using an image-capturing and -automated edge-detection software (Vascular Imager, Vascular Research Tools 6, Medical Imaging Applications, Coralville, IA). The most stable 30–60 s of the baseline measurement, which includes at least 10 cardiac cycles and is averaged as the resting baseline arterial diameter (15). The percent changes in arterial diameter were calculated as follows:  $[(\text{mean peak post-hyperemic arterial diameter} - \text{average resting baseline arterial diameter}) / \text{mean baseline arterial diameter}] \times 100$ .

*Arterial stiffness.* Arterial stiffness was assessed with participants in the supine position. Carotid-to-femoral pulse-wave velocity (cf-PWV), augmentation index (AIx), and augmentation index adjusted to 75 beats/min (AIx@75) were measured using applanation tonometry (SphygmoCor XCEL, AtCor Medical, Sydney, Australia). To assess AIx, a BP cuff placed on the upper arm, which assessed pressure waveforms to estimate central aortic pressure. AIx was calculated as the ratio of wave reflection amplitude to the central pulse pressure. For cfPWV, the carotid artery pulse was assessed using applanation tonometry, and femoral artery pulse waves were measured with a BP cuff on the upper leg. The ratio of the distance between the carotid and femoral pulse sites to the transit time between the pulse waves was used to calculate cfPWV.

Carotid-to-radial pulse-wave velocity (crPWV) and carotid-to-ankle pulse-wave velocity (caPWV) were measured with participants in the supine position using applanation tonometry (Complior Analyze, Alam Medical, Saint-Quentin-Fallavier, France). crPWV is calculated by dividing the carotid-to-radial distance (mm) by the transit time (s), and caPWV is calculated by dividing the carotid-to-ankle (posterior tibial

artery) distance (mm) by the transit time (s). The Complior device uses the carotid waveforms to calculate central aortic pressure and calibrates the carotid waveform with the mean arterial pressure. Pulse waveforms for crPWV and caPWV were recorded for 30 – 60 s. Central systolic and diastolic BP, deceleration time (DT), augmentation pressure (AP), peripheral pulse pressure (PP), and central PP were calculated by the system in the central pressure analysis.

Table 1. *Participant characteristics at the placebo and MitoQ visits*

	<b>Placebo</b>	<b>MiltoQ</b>
<i>n</i>	11	11
Age, yr	66.1 ± 10.6	66.1 ± 10.6
Height, cm	163.5 ± 13.8	163.5 ± 13.8
Body mass, kg	80.1 ± 19.7	79.7 ± 19.3
BMI, kg/m <sup>2</sup>	30.0 ± 6.8	29.9 ± 6.7
Body fat, %	37.4 ± 8.5	37.6 ± 8.6

Values are means ± SD. BMI, body mass index.

*Exercise tolerance and skeletal muscle tissue oxygen transfer and utility capacity.* A maximal walking capacity assessment was used to assess exercise tolerance on a standard treadmill (Lode, B.V., Groningen, The Netherlands) using the modified Gardner protocol (11). Participants were told to inform the researchers when they felt the onset of leg pain [time to onset of claudication (COT)] and then to continue walking until they could not continue (maximal walking time). An EKG (Datex-Ohmeda S/5 Light Patient Monitor, GE Healthcare, Chicago, IL) was used during the test to monitor cardiac activity.

Tissue oxygen utility capacity of the gastrocnemius was assessed before, during, and after the walking test with a commercially available near-infrared spectroscopy (NIRS) unit (Artinis PortaMon, Einsteinweg, The Netherlands) and Oxysoft software (v. 3.0.103.3, Einsteinweg, The Netherlands). Before the walking test, participants were asked which leg experiences the most pain while walking and the approximate area of the calf, and this is where the NIRS device was placed and secured (22) with a double-sided adhesive and wrapped in black light-absorbing cloth to prevent extraneous light from reaching the device. Data were continuously collected at a frequency of 10 Hz and

used to quantify the tissue saturation (StO<sub>2</sub>), deoxygenated hemoglobin concentration ([HHb]), and oxygenated hemoglobin concentration ([O<sub>2</sub>Hb]) at baseline, throughout the walking test, and recovery. Baseline oxygenation was assessed in a resting standing position, and participants were asked to shift their weight to the opposite limb of the NIRS device. Following baseline, the walking test began and oxygen utility data were collected continuously throughout the test. After the walking test, the participant stood in a position similar to the baseline oxygenation measurement with a thigh rapid inflation cuff (cuff model SC10, D. E. Hokanson, Bellevue, WA) placed (~250 mmHg for 5 min) 3 to 4 cm proximal to the knee. The cuff pressure was released, and the peak hyperemic response was used to determine the 100% tissue oxygenation level and thus to calculate normalized [HHb]. The NIRS data were reduced to 1 Hz and exported to Microsoft Excel for later analysis.

*Statistical analysis.* The Shapiro-Wilk test was used to determine normality of the data. Differences at baseline between the MQ and PL visits were evaluated using Student's *t*-tests. Dependent variables were assessed using a two-way repeated measures ANOVA [group (MQ and PL) X time (before and after supplement intake)] to determine differences between pre- and post-MQ and PL intake. If a significant effect was found, paired *t*-tests were used for post hoc comparisons. All statistical analyses were performed with SPSS 26.0 (IBM, Armonk, NY). Data are presented as means ± SD, unless noted otherwise. Statistical significance was set to  $P < 0.05$ . A power analysis calculation determined that a minimum sample size of 20 (10 in each group) would allow for the observation of a difference of 3–5% between groups (PL vs. MitoQ) on the measure of PWV with a power of 80% (49). We also performed an effect size analysis using Cohen's *d* and interpreted 0.2, 0.5, and 0.8 as small, medium, and large effect sizes, respectively (50).

## **RESULTS**

The acute dose of MitoQ (80 mg) was well tolerated by all participants (Table 1), and no unfavorable or adverse side effects were reported. Additionally, participants reported little discomfort during assessments that included tingling and numbness sensations during the brachial artery and popliteal artery FMD assessments, which were all relieved with cuff release. Participant comorbidities included hypertension,

dyslipidemia, prediabetes, and arthritis, and participants were on medications for these respective conditions (Table 2). Brachial FMD and popliteal FMD increased (from  $3.8 \pm 0.5$  to  $6.4 \pm 0.7\%$ ,  $P < 0.001$ ,  $d = 1.4$ , and from  $2.7 \pm 0.6$  to  $6.0 \pm 0.6\%$ ,  $P = 0.001$ ,  $d = 1.8$ , respectively) after MitoQ intake (Fig. 2). There were also increases in maximal walking time (from  $442.7 \pm 52.6$  to  $516.5 \pm 47.1$  s,  $P < 0.001$ ,  $d = 0.6$ ), maximal walking distance (from  $438.5 \pm 69.0$  to  $487.8 \pm 69.1$  m,  $P = 0.012$ ,  $d = 0.3$ ), and COT (from  $202.3 \pm 28.8$  to  $246.5 \pm 48.3$  s,  $P = 0.046$ ,  $d = 0.5$ ) after MitoQ intake compared with placebo (Fig. 3). SOD concentration significantly increased after MitoQ intake (from 0.37 to 0.40 U/mL,  $P = 0.01$ ,  $d = 1.2$ ) (Fig. 4A). There were trends for reduced diastolic BP (from  $82.9 \pm 2.5$  to  $79.8 \pm 2.7$  mmHg,  $P = 0.08$ ,  $d = 0.2$ ) (Table 3), carotid-to-ankle PWV (from  $10.3 \pm 0.5$  to  $9.5 \pm 0.6$  m,  $P = 0.054$ ,  $d = 0.6$ ) (Fig. 5C), and MDA concentration (from  $35.7 \pm 1.0$  to  $29.5 \pm 1.6$  1Jmol/mL,  $P = 0.07$ ,  $d = 1.3$ ) (Fig. 4C). There were no changes in resting HR, systolic BP, central BP, DT, maximum first derivative of pressure ( $dP/dt_{max}$ ), carotid-to-radial PWV, carotid-to-femoral PWV, Alx, Alx@75, TAC, StO<sub>2</sub>, [HbO<sub>2</sub>], or normalized [HHb] ( $P > 0.05$ ) following acute MitoQ intake (Table 3; Figs. 4 – 6).

## DISCUSSION

The present study was conducted to understand the roles of the vascular mitochondria in PAD in vivo by examining the impacts of acute MitoQ intake on endothelial function, BP, arterial stiffness, walking capacity, and oxygen utility capacity in patients with PAD. There were several novel findings that further probe the relevance of the vascular mitochondria and findings that may be clinically significant for patients with PAD. The results of our study revealed, for the first time, that acute MitoQ intake significantly improved brachial artery endothelial function and popliteal artery endothelial function, suggesting that the vascular mitochondria play an important role in endothelial function in patients with PAD. SOD, the first line of the endogenous antioxidant defense system, significantly increased following MitoQ intake. There were also improvements in the physical functional capacity in the patients with PAD, such as significantly increased maximal walking time, maximal walking distance, and delayed COT; however, there were no significant changes in the oxygen utility capacity measurements of StO<sub>2</sub>,

[HbO<sub>2</sub>], or [HHb] during walking, as indicated by NIRS following MitoQ intake. These improvements in endothelial function and functional capacity are likely mediated, in part, by both improved mitochondrial function, reduced mitochondrial-derived ROS in the vasculature, and improvements in blood SOD levels. These findings provide evidence to support the concept that the mitochondria in the vasculature are important in terms of regulating endothelial function and functional capacity. Additionally, MitoQ intake may be a beneficial drug therapy for improving endothelial function by targeting mitochondrial-derived ROS. Therefore, vascular mitochondria may be a potential therapeutic target for improving endothelial function, the redox environment, and walking capacity in patients with PAD.

Table 2. *Participant comorbidities and medications*

n	11
Comorbidity or condition	
Hypertension	7
Dyslipidemia	9
Prediabetes	2
Arthritis	6
Medications	
Angiotensin-converting enzyme inhibitors	2
Angiotensin II receptor blockers	4
Diabetic medication (metformin)	2
β-Blockers	4
Calcium channel blockers	1
Anticoagulants	1
Diuretics	1
Statins	8
Nonsteroidal anti-inflammatory medication	8
Sleep aids	4

*Endothelial function.* Mitochondrial dysfunction and mitochondrial-derived ROS production have been suggested as key players in the development of vascular dysfunction and cardiovascular diseases (52). ROS act as signaling molecules that are critical for moderating cell function (9) and are produced at several sites in the mitochondria, including complexes I, II, and III of the electron transport chain (38).

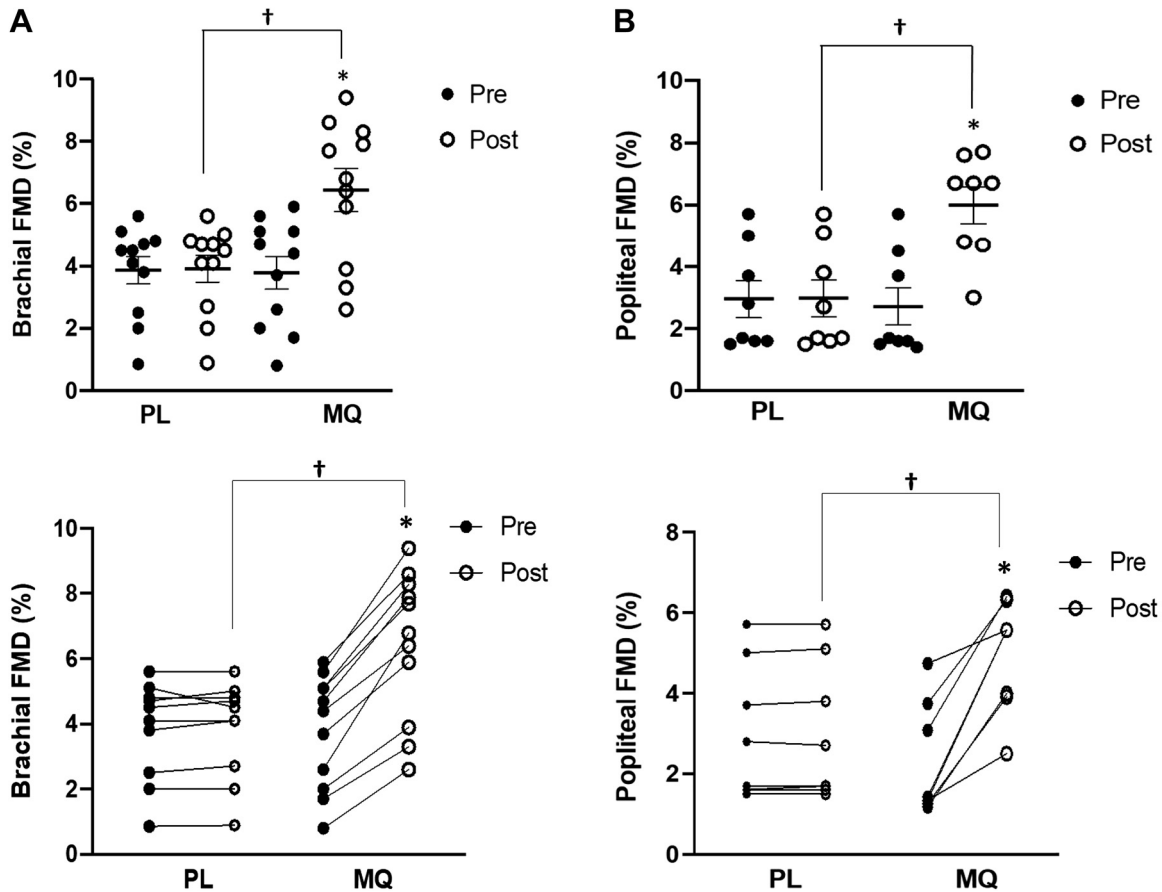


Fig. 2. Flow-mediated dilation (FMD, %) in the brachial and popliteal arteries pre- and post-placebo (PL) and MitoQ (MQ) intake. **A:** percent brachial artery FMD significantly increased post-MQ and was significantly greater than post-PL ( $n = 11$ ). ANOVA main effect,  $<0.001$ ; Cohen's  $d$ , 1.4. **B:** percent popliteal artery FMD significantly increased post-MQ and was significantly greater than post-PL ( $n = 8$ ). ANOVA main effect, 0.001; Cohen's  $d$ , 1.8. Values are means  $\pm$  SE. \* $P < 0.01$  vs. Pre. † $P < 0.01$  vs. PL.

However, if this excessive ROS production is increased beyond the endogenous antioxidant defense system, this can become oxidative stress and can lead to mitochondrial dysfunction, or vice versa, which results in a vicious cycle that impairs the mitochondrial environment (61). Mitochondrial-derived ROS accumulation has specifically been reported to contribute to endothelial dysfunction (14), although mechanisms by which mitochondrial-derived ROS induce endothelial dysfunction are complex and not well investigated. A commonly proposed mechanism is the scavenging of NO by mitochondrial-produced superoxide and uncoupling of endothelial nitric oxide synthase (eNOS). When we consider that mitochondrial defects, elevated levels of

oxidative stress, and mitochondrial dysfunction are present in patients with PAD (41, 42), scavenging this mitochondrial-derived superoxide may be a critical component to restore vascular endothelial function in this population (41). In fact, the primary antioxidant enzyme responsible for scavenging mitochondrial-derived superoxide, MnSOD, has been reported to be attenuated in patients with PAD (41), giving evidence that the primary line of defense may not be sufficient to reduce excessive mitochondrial-derived ROS. Therefore, a mitochondrial-targeted antioxidant therapy may be useful for scavenging this mitochondrial-derived ROS in PAD.

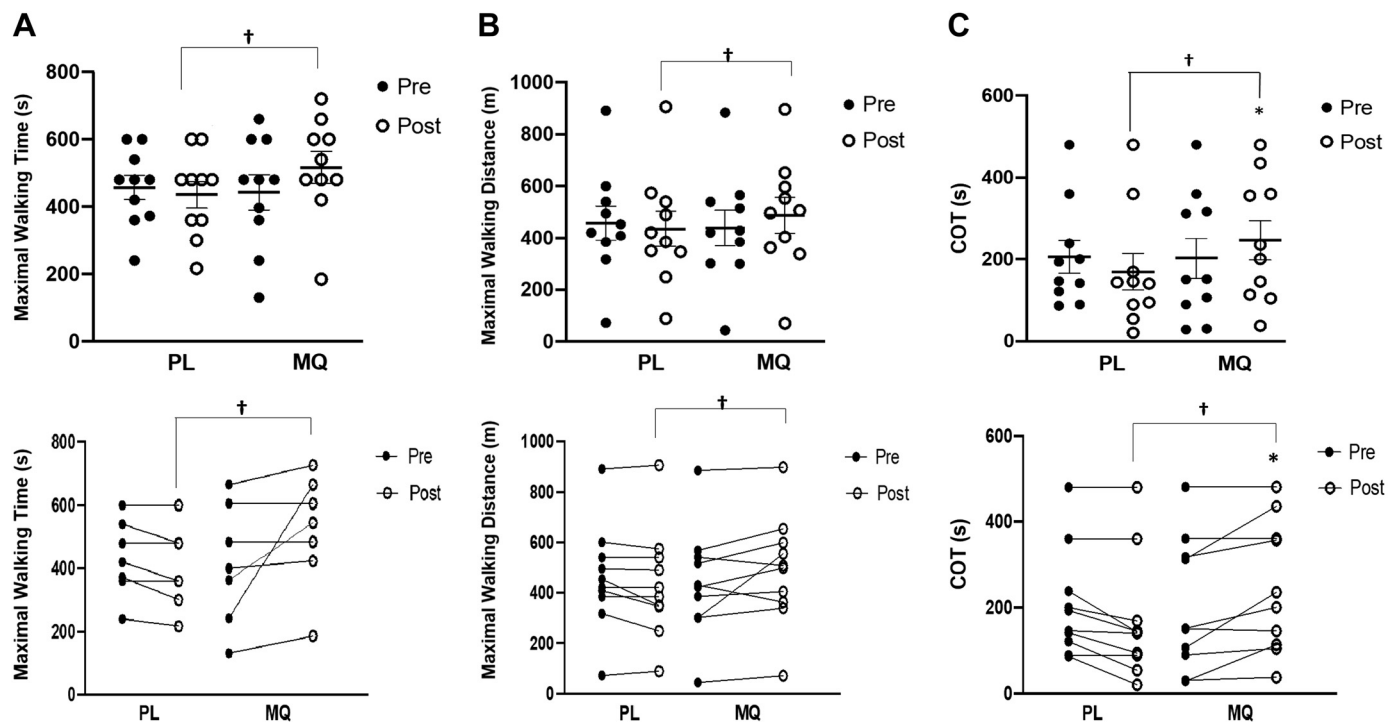


Fig. 3. Maximal walking time (s), maximal walking distance (m), and time to onset of claudication (COT, s) pre- and post-placebo (PL) and MitoQ (MQ) intake. A: maximal walking time in post-MQ was significantly greater than post-PL ( $n = 10$ ). ANOVA main effect, 0.042; Cohen's  $d$ , 0.6. B: maximal walking distance in post-MQ was significantly greater than post-PL ( $n = 10$ ). ANOVA main effect, 0.028; Cohen's  $d$ , 0.3. C: COT significantly increased post-MQ and was significantly greater than post-PL ( $n = 10$ ). ANOVA main effect, 0.001; Cohen's  $d$ , 0.5. Values are means  $\pm$  SE. \* $P < 0.05$  vs. Pre.

† $P < 0.05$  vs. PL.



It has been previously shown that 80 mg of oral MitoQ intake is pharmacokinetically active in the plasma and can reduce ROS and oxidative stress levels (31). MitoQ supplementation improves the age-related decline in endothelial-mediated dilation in older mice and improves endothelial function (brachial artery FMD) in healthy middle-aged and older adults (14, 46), and we previously reported that acute MitoQ treatment improves endothelial-mediated dilation in skeletal muscle feed arteries biopsied from older adults (38). These improvements in endothelial function in these previous studies were thought to be partially mediated by the attenuation of ROS accumulation in the vasculature (14, 38, 46) and the preservation MnSOD in the vasculature (38). In support of these previous findings with MitoQ utilization, the present study revealed that the brachial artery ( $\Delta 2.6\%$ ,  $d = 1.4$ ) and popliteal artery ( $\Delta 3.3\%$ ,  $d = 1.8$ ) endothelial-mediated vasodilation improved after acute MitoQ intake (Fig. 2). These results suggest that the endothelial mitochondria play a key role in endothelium-dependent vasodilatory function both systemically and locally in patients with PAD.

A potential mechanism underlying these improvements may be due, in part, to an increased antioxidant capacity after MitoQ intake (14, 38, 46). SODs specifically have been identified as the first line of defense against ROS-induced injury (10). Our results indicated that SOD concentration significantly improved following MitoQ intake ( $\Delta 0.03$  U/mL,  $P = 0.01$ ,  $d = 1.2$ ) (Fig. 4). Our study demonstrates that MitoQ may play a role in upregulating SODs and the antioxidant defense system (33, 45, 48). This is in support of previous *ex vivo* work, as it has been hypothesized that scavenging of mitochondrial-derived ROS may also help support upregulating MnSOD (38), which has been reported to improve mitochondrial function and biogenesis (14, 38). Therefore, direct reduction in mitochondrial-specific ROS by MitoQ may help improve mitochondrial function and MnSOD activity. Although it was not statistically significant, we found a trend that MDA was reduced ( $P = 0.07$ ,  $d = 1.3$ ) (Fig. 4). These results may be because oxidative stress is a complicated and multifaceted process where a prooxidative environment prevails and induces irreversible damage; the process likely cannot be significantly improved following an acute intervention. Taken together, these results may ultimately improve vascular endothelial mitochondrial function in patients with PAD (14, 41, 42). In

addition to evaluation of redox balance and oxidative stress in the blood, future investigations should include measurements of SOD and MDA levels in the skeletal muscle and vascular smooth muscle tissues to give a more comprehensive assessment of the redox environment and how this may affect endothelial function in PAD.

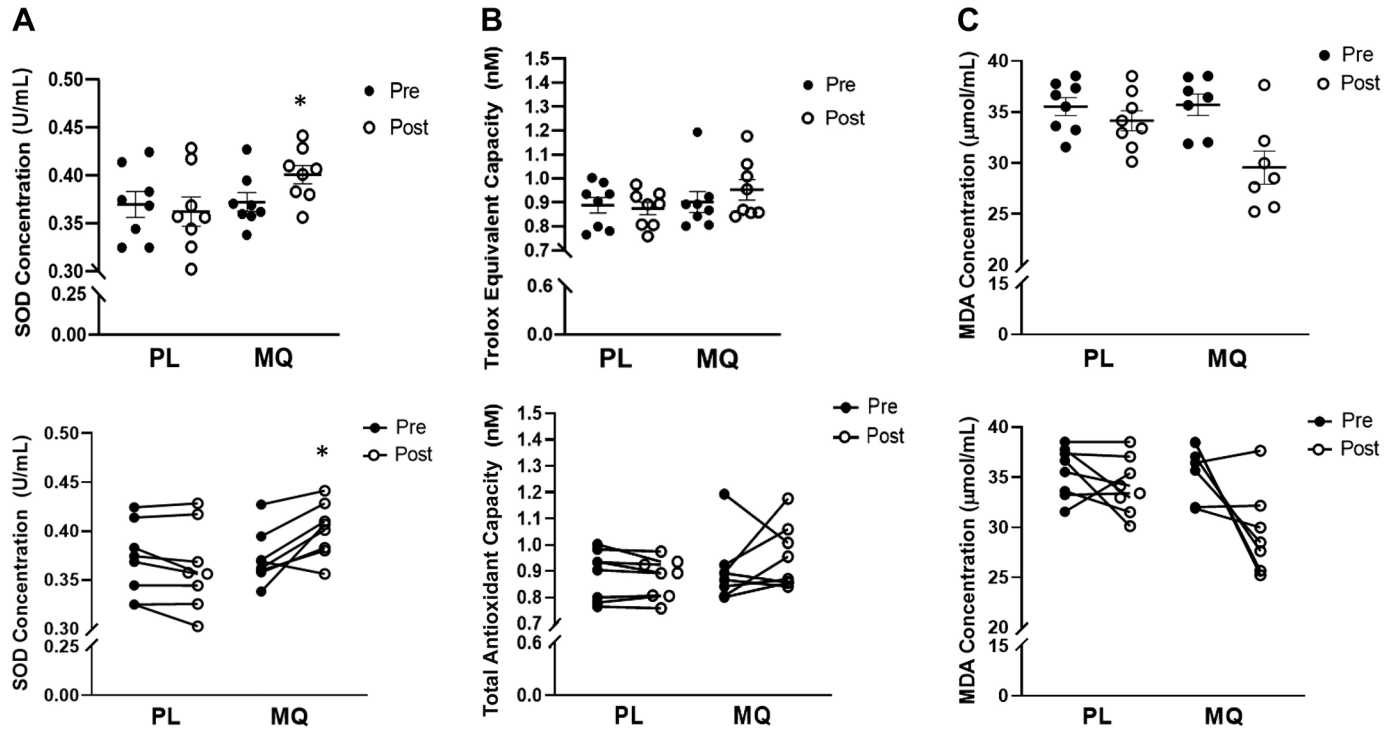


Fig. 4. Superoxide dismutase (SOD), total antioxidant capacity (TAC), and malondialdehyde (MDA) concentrations pre- and post-placebo (PL) and MitoQ (MQ) intake. A: SOD (U/mL) was significantly greater post-MQ ( $n = 8$ ) compared with pre-MQ ( $n = 8$ ). ANOVA main effect, 0.02; Cohen's  $d$ , 1.2. B: no significant changes in TAC (nM) pre- and post-PL ( $n = 8$ ) or MQ intake ( $n = 8$ ). ANOVA main effect, 0.225; Cohen's  $d$ , 0.8. C: no significant changes in MDA (1Jmol/mL) pre- and post-PL ( $n = 8$ ) or MQ intake ( $n = 7$ ) ANOVA main effect, 0.07; Cohen's  $d$ , 1.3. Values are means  $\pm$  SE. \* $P < 0.05$  vs. Pre.

*Arterial stiffness.* Arterial stiffening is often attributed to changes in structural proteins and changes in responses to vasoactive molecules and metabolites, hence reducing arterial distensibility (59). Furthermore, arterial stiffness is associated with atherosclerotic lesion formation (57), and patients with PAD tend to have elevated levels of arterial stiffness (18). Although mechanisms underlying the manifestation of arterial stiffness have not been fully elucidated, a potential mechanism underlying this process may be increased mitochondrial-derived ROS production and the resultant oxidative stress in both endothelial and smooth muscle cells (5, 56). The role(s) of mitochondrial-derived ROS and oxidative stress in arterial stiffening and atherosclerotic development have gained significant attention over the past several years (1, 5). It has been reported that MnSOD-deficient mice suffer from accelerated age-related arterial stiffening and vascular disease, and this is likely due to decreased scavenging of mitochondrial-derived ROS (5). Additionally, mitochondrial-derived ROS have been reported to upregulate arterial endothelial NF- $\kappa$ B signaling (54), and this ROS-induced inflammation may also contribute to the manifestation of arterial stiffness by other mechanisms such as endothelial dysfunction and atherosclerosis (32). Therefore, directly targeting mitochondrial-derived ROS, and also thereby reducing ROS-induced inflammation, may be effective for both directly and indirectly reducing arterial stiffness.

Rossman and colleagues (46) previously demonstrated that MitoQ supplementation reduced cfPWV in participants who had elevated arterial stiffness, whereas the participants with clinically normal levels of arterial stiffness did not demonstrate any significant change following MitoQ supplementation. Although this study focused on aging-related arterial stiffness and not necessarily arterial stiffness associated with peripheral arterial diseases, the studies suggest that when arterial stiffness levels are elevated, MitoQ may be effective for reducing arterial stiffness.

Targeting reductions in arterial stiffness specific to the lower limb in PAD may be particularly important because previous studies suggested that increased lower extremity arterial stiffness has been shown to attenuate arterial blood flow to the lower extremities (51); that is an important factor for peripheral circulation (24), which can contribute to claudication (53). Our finding in the present study is consistent with these previous studies examining the impacts of MitoQ on arterial stiffness (13, 46). There

were no significant changes in central (cfPWV,  $\Delta$ -0.1 m/s,  $P = 0.7$ ,  $d = 0.1$ ) or systemic arterial stiffness (crPWV,  $\Delta$ 0.15 m/s,  $P = 0.6$ ,  $d = 0.1$ ); however, caPWV showed a trend to be reduced ( $\Delta$ -0.8 m/s,  $P = 0.054$ ,  $d = 0.6$ ) (Fig. 5). This result suggests that MitoQ intake may be more impactful in areas that experience a greater degree of vascular stiffening (13, 46). Albeit, this result is inconclusive and requires further investigation with a larger sample size.

Table 3. Participant HR, BPs, and central PP analyses pre- and post-placebo and MitoQ intake

	Placebo			MitoQ			ANOVA	Cohen's <i>d</i>
	Pre	Post	$\Delta$	Pre	Post	$\Delta$		
<i>n</i>		11			11			
Resting HR, beats/min	68.2 $\pm$ 4.8	67.7 $\pm$ 4.3	-0.5 $\pm$ 0.5	69.3 $\pm$ 4.2	66.7 $\pm$ 3.6	-2.6 $\pm$ 0.6	0.10	0.1
Systolic BP, mmHg	134.6 $\pm$ 5.3	135.1 $\pm$ 5.4	-0.5 $\pm$ 0.5	133.3 $\pm$ 5.3	130.6 $\pm$ 5.0	-2.7 $\pm$ 0.3	0.30	0.3
Diastolic BP, mmHg	80.8 $\pm$ 2.6	81.4 $\pm$ 2.6	0.6 $\pm$ 0	82.9 $\pm$ 0.5	79.8 $\pm$ 2.7	-3.1 $\pm$ 0.2	0.09	0.2
Central systolic BP, mmHg	123.5 $\pm$ 4.3	124.6 $\pm$ 4.4	1.1 $\pm$ 0.1	123.6 $\pm$ 4.9	120.8 $\pm$ 3.9	-2.8 $\pm$ 1.0	0.18	0.4
Central diastolic BP, mmHg	80 $\pm$ 2.5	80.1 $\pm$ 2.4	0.1 $\pm$ 0.1	82.2 $\pm$ 2.6	80.7 $\pm$ 2.9	-1.5 $\pm$ 0.3	0.45	0.1
Resting brachial artery diameter, mm	4.5 $\pm$ 0.03	4.5 $\pm$ 0.03	0.0 $\pm$ 0.0	4.3 $\pm$ 0.03	4.2 $\pm$ 0.03	-0.1 $\pm$ 0.0	0.27	0.3
Resting popliteal artery diameter, mm	6.2 $\pm$ 0.02	6.2 $\pm$ 0.03	0.0 $\pm$ 0.01	6.2 $\pm$ 0.02	6.2 $\pm$ 0.04	0.0 $\pm$ 0.02	0.90	0.1
Deceleration time, ms	685.9 $\pm$ 61.2	675.7 $\pm$ 62.0	-10.2 $\pm$ 0.8	650.8 $\pm$ 77.2	668.2 $\pm$ 65.4	17.4 $\pm$ 11.8	0.53	0.1
dP/dt <sub>max</sub> , mmHg/s	683.0 $\pm$ 43.4	720.9 $\pm$ 67.9	37.9 $\pm$ 5.5	668.2 $\pm$ 65.4	669.4 $\pm$ 49.9	1.2 $\pm$ 15.5	0.64	0.3
Peripheral PP, mmHg	47.4 $\pm$ 4.5	47.5 $\pm$ 4.7	0.1 $\pm$ 0.2	47.9 $\pm$ 4.3	49.9 $\pm$ 4.7	2 $\pm$ 0.4	0.38	0.2
Central PP, mmHg	43.5 $\pm$ 4.3	44.6 $\pm$ 4.4	1.1 $\pm$ 0.1	41.5 $\pm$ 4.0	40.1 $\pm$ 3.9	-1.4 $\pm$ 0.1	0.28	0.4
Ap, mmHg	21.9 $\pm$ 13.6	21.7 $\pm$ 14.1	-0.2 $\pm$ 0.5	20.8 $\pm$ 12.9	21.1 $\pm$ 12.9	0.3 $\pm$ 0.0	0.78	0.1
Alx, %	19.7 $\pm$ 3.5	21.5 $\pm$ 4.5	1.8 $\pm$ 1.0	21.1 $\pm$ 3.5	20.7 $\pm$ 4.8	-0.3 $\pm$ 1.3	0.50	0.2
Aix@75, %	35.6 $\pm$ 4.9	27 $\pm$ 10.1	-8.6 $\pm$ 5.2	29.4 $\pm$ 2.8	25.8 $\pm$ 3.8	-3.6 $\pm$ 1.0	0.53	0.1

Values are means  $\pm$  SE. HR, heart rate; BP, blood pressure; PP, pulse pressure; dP/dt<sub>max</sub>, maximum first derivative of pressure; AP, augmentation pressure; Alx; augmentation index; Aix@75; augmentation index adjusted to 75 beats/min.

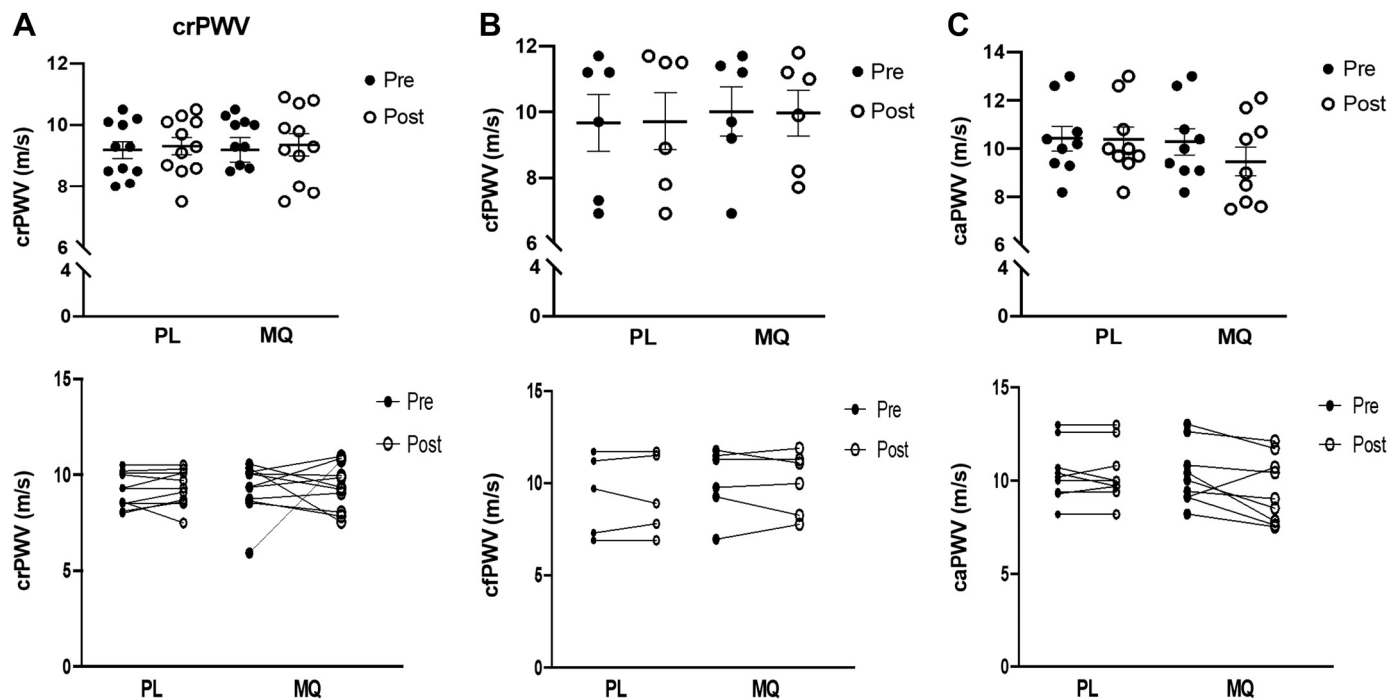


Fig. 5. Carotid-to-radial pulse-wave velocity (crPWV, m/s), carotid-to-femoral pulse-wave velocity (cfPWV, m/s), and carotid-to-ankle pulse-wave velocity (caPWV, m/s) pre- and post-placebo (PL) and MitoQ (MQ) intake. *A*: no significant changes in crPWV pre- and post-PL or MQ intake ( $n = 11$ ). ANOVA main effect, 0.67; Cohen's  $d$ , 0.1. *B*: no significant changes in cfPWV pre- or post-PL and MQ intake ( $n = 6$ ). ANOVA main effect, 0.77; Cohen's  $d$ , 0.1. *C*: no significant changes in caPWV pre- or post-PL and MQ intake ( $n = 9$ ). ANOVA main effect, 0.054; Cohen's  $d$ , 0.6. Values are means  $\pm$  SE.

Mitochondrial-derived ROS have been shown to influence activation of arterial endothelial NF-KB signaling (54), which has been reported to influence arterial stiffness manifestation; therefore, reducing mitochondrial-derived ROS by improving antioxidant enzyme activity may play a role in reducing the inflammation associated arterial stiffness development (32). In particular, the arterial stiffening that patients with PAD experience is likely not just a consequence of natural vascular aging but is likely linked to vascular ischemic stress-induced mitochondrial-derived excessive ROS production, oxidative stress damage, and increased inflammation (19). In the present study, there were significant improvements in SOD, which demonstrates MitoQ improves antioxidant enzyme activity acutely (Fig. 4). However, these improvements could not be directly translated to improvements in MDA ( $P = 0.07$ ,  $d = 1.3$ ) (Fig. 4). Even with the significant improvement in SOD and meaningful effect sizes for reduced MDA ( $d = 1.3$ ) and

increased TAC ( $d = 0.8$ ), it may be inferred that these changes are likely not powerful enough to induce significant reductions in arterial stiffness acutely. Future studies should incorporate MitoQ supplementation and examine changes in the redox environment and inflammation and how these changes may impact arterial stiffness, which may be the next critical step to understanding MitoQ intake and reversing arterial remodeling in PAD (19).

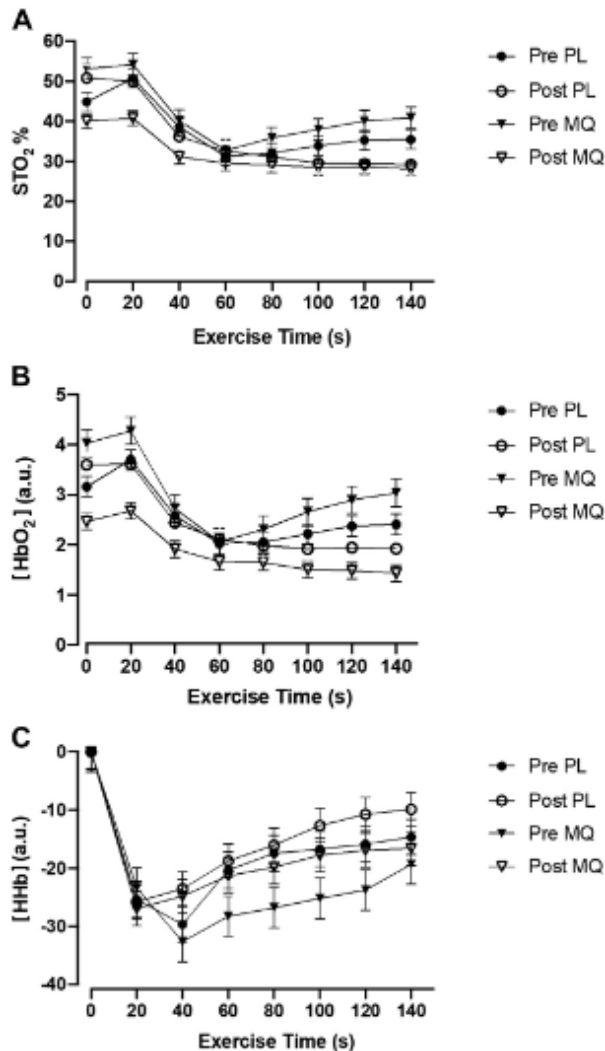


Fig. 6. Group mean changes in tissue saturation index (StO<sub>2</sub>, %), oxygenated hemoglobin {[HbO<sub>2</sub>], arbitrary units (a.u.)}, and normalized deoxygenated hemoglobin ([HHb], a.u.) pre- and post-placebo (PL) and MitoQ (MQ) intake every 20 s during the maximal walking capacity test. A: no significant changes in StO<sub>2</sub> pre- or post-PL and MQ intake ( $n = 10$ ). ANOVA main effect, 0.41; Cohen's  $d$ , 0.3. B: no significant changes in [HbO<sub>2</sub>] pre- and post-PL or MQ intake ( $n = 10$ ). ANOVA main effect, 0.22; Cohen's  $d$ , 0.3. C: no significant changes in normalized [HHb] pre- or post-PL and MQ intake ( $n = 10$ ). ANOVA main effect, 0.29; Cohen's  $d$ , 0.1. Values are means  $\pm$  SE.

*Walking capacity, exercise tolerance, and skeletal muscle oxygen transfer and utility capacity.* Intermittent claudication, which is considered the most common symptom experienced by patients with PAD, reduces walking capacity and negatively impacts health-related quality of life (8). Alleviating leg symptoms and improving walking capacity have been suggested as potential factors to help improve quality of life in patients with PAD (37, 40). In the present study, there were improvements in maximal walking distance (~11%,  $\Delta 49$  m,  $d = 0.3$ ), COT (~22%,  $\Delta 45$  s,  $d = 0.5$ ), and maximal walking time (~17%,  $\Delta 74$  s,  $d = 0.6$ ) following MitoQ intake (Fig. 3). We recently demonstrated that walking distance improved by ~24% and COT by ~41% following 3 mo of heated-water exercise therapy for patients with PAD and that walking distance increased by ~19% and COT by ~30% following land-based walking exercise therapy for 3 mo for patients with PAD (40). Therefore, our data demonstrate that acute MitoQ intake may provide nearly half of the benefits received from 12 wk of supervised exercise therapy for patients with PAD, which may be clinically relevant for this population.

Mechanisms by which walking capacity and COT improved are not entirely clear; however, these improvements may be partially attributed to improved oxygen transfer capacity by the vasculature, which is likely due to reduction in excessive ROS and improved NO synthesis and bioactivity. Previous studies suggested that a prooxidative environment prevails in PAD (19, 21, 41). The excessive ROS in this prooxidative environment can attenuate blood flow by reducing NO bioavailability and bioactivity, which therefore may reduce oxygen transfer capacity and tissue perfusion (6). Improving the redox environment and thus NO bioactivity via MitoQ intake may positively influence blood flow and oxygen utility capacity within the skeletal muscle. In the present study, SOD significantly improved, suggesting an improvement in the redox environment. Additionally, we found an improvement in FMD, which may suggest improved NO synthesis and bioactivity that likely also improves blood flow regulatory potential in the skeletal muscle (44). Therefore, this potential increased blood flow capacity may help increase oxygen delivery and utility, thereby contributing to the walking capacity improvements and delayed claudication in this study (20). However, these potential improvements in blood flow regulatory potential and oxygen transfer

capacity could not be translated to significant improvements in gastrocnemius oxygen utility capacity as assessed by NIRS. There were no significant changes in  $StO_2$ ,  $[HbO_2]$ , or  $[HHb]$  in the skeletal muscle following acute MitoQ intake despite that walking capacity had significantly improved (Fig. 6), which suggests that gastrocnemius tissue saturation and oxygen extraction and utilization were not significantly improved by an acute intake of MitoQ. The lack of significant change in measurements of  $StO_2$ ,  $[HbO_2]$ , and  $[HHb]$  may be explained, at least in part, by our subject population, considering that PAD is a chronic disease condition and that these patients specifically demonstrate oxidative damage within the skeletal muscle. Patients with PAD demonstrate myopathy in the lower extremities, and this is often also characterized by skeletal muscle mitochondrial dysfunction, oxidative damage, fibrosis, and myofiber degeneration (41, 42, 60). Although skeletal muscle myopathy in PAD is multifaceted and complex, this myopathy may ultimately result in poor oxygen extraction and utilization by the affected skeletal muscle in these individuals. However, the extent of the myopathy present in this study sample and how it may have affected leg function and walking performance were not directly investigated in response to MitoQ intake. Future studies regarding the impacts of MitoQ on skeletal muscle mitochondrial function, macro-vascular and microvascular blood flow during exercise, oxygen uptake and utilization, and severity of fibrosis on these measures are warranted.

*Experimental considerations and limitations.* Although this study has revealed several novel findings that are relevant for this area of research, there are some experimental considerations that must be considered for future study designs. First, we did not evaluate smooth muscle function in response to acute MitoQ intake. Our preliminary data using skeletal muscle arterioles from patients with PAD (Fig. 7) demonstrated that skeletal muscle arteriole smooth muscle function (evaluated by sodium nitroprusside) is not significantly different between patients with PAD when compared with age-matched healthy control. Additionally, Maruhashi and colleagues (28) demonstrated that smooth muscle function determined by nitroglycerin-induced vasodilation in patients with PAD is not significantly different compared with that in age-matched healthy subjects. Although, critical limb ischemia, the most advanced form of PAD, demonstrates attenuated smooth muscle function (28). Our study participants are



classified as Fontaine stage II and III, not IV. Therefore, smooth muscle function of our participants was likely normal and would be unchanged in response to MitoQ. Despite this information, it is still important to consider evaluating smooth muscle function in response to oral MitoQ intake in vivo. This would help us either confirm that MitoQ intake in PAD improves endothelial function independent of the vascular smooth muscle or if potential improvements in macrovascular smooth muscle function also contribute to the improvements seen in endothelial function.

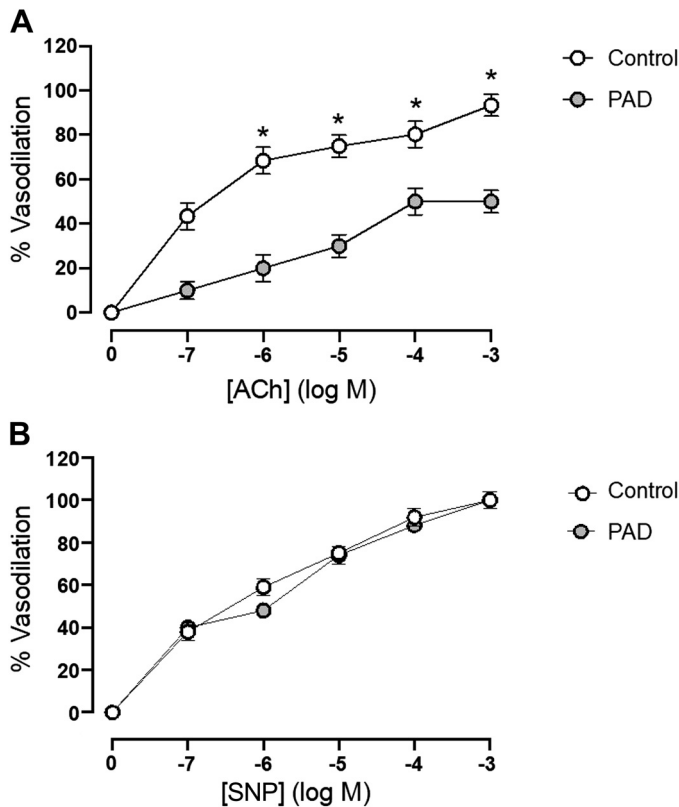


Fig. 7. The vasodilator dose-response curves of skeletal muscle arterioles from patients with peripheral artery disease (PAD;  $n = 8$ ) and age-matched healthy control ( $n = 8$ ) induced by acetylcholine (ACh; A) and sodium nitroprusside (SNP; B). Values are means  $\pm$  SE. \* $P < 0.05$  vs. control.

Second, we assessed SOD, TAC, and MDA but did not assess mitochondrial-specific ROS production before and after MitoQ intake. Although we did not quantify mitochondrial-specific ROS, a previous study demonstrated that the dose used in this study (80 mg) exhibits effective pharmacokinetic behavior in the plasma that can reduce ROS and oxidative stress levels and therefore prevented excessive mitochondrial-derived ROS accumulation in these patients (31). We previously showed that MitoQ

treatment can reduce mitochondrial-specific superoxide levels and can help improve MnSOD protein expression in skeletal muscle feed arteries from old adults (38). However, the extent to which this preclinical finding for mitochondrial-specific ROS may translate to the vascular smooth muscle and even skeletal muscle in patients with PAD in vivo was not investigated in the present study and should be considered in both clinical and preclinical studies involving patients with PAD. Future research should also include investigation of skeletal muscle tissue and vascular smooth muscle tissue in addition to blood samples for a more comprehensive assessment of changes in the redox environment.

Third, we did not assay MitoQ levels at the beginning of each visit to ensure a proper washout of MitoQ. However, a 2-wk washout period was previously deemed sufficient to ameliorate the effects of MitoQ and its presence in the plasma in middle-aged and older adults, which is likely also true for patients with PAD (46). Additionally, MitoQ was delivered by oral intake, and MitoQ may directly affect skeletal muscle mitochondria function and ROS production. This may be a confounder for examining roles of vascular mitochondrial function in terms of oxygen transfer and utilization capacity in the skeletal muscle. Future study warrants intra-arterial infusion of MitoQ to examine the effects of improved endothelial function on muscular oxygen utilization capacity and muscle function in this patient population.

*Perspectives and clinical implications.* We demonstrated, for the first time, that acute MitoQ intake can improve vascular endothelial function, improve endogenous antioxidant enzymes, improve walking capacity, and can delay onset of claudication in patients with PAD. These findings may be clinically relevant for this population to improve their endothelial function and functional walking capacity. Notably, these findings also establish a potential pivotal role of the vascular mitochondria for improving endothelial function in PAD, which may be a critical area for devising new therapeutic treatments for PAD and other vascular diseases. Since this was an acute oral intake study (one dose) and we now know that MitoQ intake can pose several benefits for this population, further research is needed to appropriately devise a supplementation regimen to determine the long-term effects of chronic MitoQ intake in PAD, similar to the study performed by Rossman and colleagues. Additionally, further research should also

include evaluations of vascular mitochondrial function in response to mitoquinol mesylate ex vivo (e.g., mitochondrial complex function, ATP production, calcium signaling, dynamics, etc.) in patients with PAD.

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## **DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

## **AUTHOR CONTRIBUTIONS**

S.-Y.P. conceived and designed research; S.-Y.P., E.J.P., R.J.H. 3rd, W.-M.S., T.K.W., J.S., and S.K.Y. performed experiments; S.-Y.P., E.J.P., R.J.H. 3rd, W.-M.S., T.K.W., J.S., S.K.Y., P.K.M., and G.L. analyzed data; S.-Y.P., E.J.P., G.L., and I.I.P. interpreted results of experiments; E.J.P. and T.K.W. prepared figures; S.-Y.P. and E.J.P. drafted manuscript, S.-Y.P., E.J.P., R.J.H. 3rd, W.-M.S., T.K.W., J.S., and G.L. edited and revised manuscript; S.-Y.P., E.J.P., R.J.H. 3rd, W.-M.S., T.K.W., J.S., S.K.Y., P.K.M., G.L., and I.I.P. approved final version of manuscript.

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Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. *Circulation* 113: e463–e654, 2006.

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