Title: Montmorency cherry supplementation attenuates vascular dysfunction induced by prolonged forearm occlusion in overweight middle-aged men.

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Running title: Cherry and vascular function

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ABSTRACT

Flavonoid supplementation improves brachial artery flow-mediated dilatation (FMD), but it is not known whether flavonoids protect against vascular dysfunction induced by ischemia-reperfusion (IR) injury and associated respiratory burst. In a randomized, double-blind, placebo-controlled, crossover study, we investigated whether 4-weeks supplementation with freeze-dried Montmorency cherry (MC) attenuated suppression of FMD after IR induced by prolonged forearm occlusion. Twelve physically inactive overweight middle-aged men (52.8±5.8y, BMI: 28.1±5.3kg.m^{-2}) consumed MC (235mg.d^{-1} anthocyanins) or placebo capsules for 4-weeks, with supplementation blocks separated by 4-weeks washout. Pre and post each supplementation block, FMD responses and plasma nitrate and nitrite ([NO_2^-]) concentrations were measured at baseline and 15, 30, and 45min after prolonged (20-min) forearm occlusion. FMD response was significantly depressed by the prolonged occlusion ($p<0.001$). After 45min reperfusion, FMD was restored to baseline levels after MC (ΔFMD pre: -30.5±8.4%, post: -0.6±9.5%) but not placebo supplementation (ΔFMD pre: -11.6±10.6, post: -25.4±4.0%; condition x supplement interaction: $p=0.038$). Plasma [NO_2^-] decreased after prolonged occlusion but recovered faster after MC compared to placebo (Δ45min to baseline; MC: pre: -15.3±9.6, post: -6.2±8.1; Placebo: pre: -16.3±5.9, post: -27.7±11.1 nmol.L^{-1}; condition x supplement x time interaction: $p=0.033$). Plasma peroxiredoxin concentration ([Prx2]) was significantly higher after MC (pre: 22.8±1.4, post: 28.0±2.4 ng.mL^{-1}, $p=0.029$) but not after placebo supplementation (pre: 22.1±2.2, post: 23.7±1.5 ng.mL^{-1}). In conclusion, 4-weeks MC supplementation enhanced recovery of endothelium-dependent vasodilatation after IR, in parallel with faster recovery of plasma [NO_2^-], suggesting NO dependency. These protective effects seem to be related to increased plasma [Prx2], presumably conferring protection against the respiratory burst during reperfusion.

NEW & NOTEWORTHY This is the first study to demonstrate that four weeks of MC powder supplementation exerted protective effects on endothelium-dependent vasodilation after transient IR injury in overweight, physically inactive, non-medicated hypertensive middle-aged men. These effects seem to be due to increased NO availability as evidenced by higher plasma nitrite concentration and peak arterial diameter during the FMD measurement. This may be a consequence of increased concentration of peroxiredoxin and other anti-oxidant systems, and hence reduced ROS exposure.

KEYWORDS Montmorency cherry**;** forearm occlusion; endothelial function; nitrite/nitrate; peroxiredoxin-2

INTRODUCTION

Cardiovascular diseases (CVD) remain the leading worldwide cause of death with an estimated 17.3 million deaths in 2008, which is projected to increase to 23 million by 2030 (27). Higher consumption of fruit and vegetables is associated with a lower risk of all-cause mortality, particularly cardiovascular disease mortality (43). Epidemiological data suggest that polyphenols within fruits and vegetables may contribute to this observed reduction in CVD risk (4, 13). Specifically, a systematic review of prospective cohort studies found that the dietary intake of different classes of flavonoids including flavonols, flavones, flavanones, flavan-3-ols, anthocyanidins and proanthocyanidins significantly decreased the risk of CVD (44). Risk factors for CVD including hypertension, hypercholesterolemia and diabetes mellitus are associated with enhanced production of reactive oxygen species (ROS), particularly superoxide, generated by enzymes such as xanthine oxidase and NADPH oxidase (NOX) and via the electron transport chain, which induce oxidative stress in the vascular wall, and endothelial dysfunction (29). Ischaemia reperfusion injuries are relatively common vascular events during general surgical procedures, and present a significant problem for organ transplant. For instance, myocardial injury was experienced by 19% of individuals aged over 45y after non-cardiac surgery in vascular surgical patients, with the majority of these events clinically silent (7). Despite this incidence level, and the significant tissue damage resulting from ischaemia reperfusion leading to irreversible pathophysiology such as myocardial infarction and stroke, the availability/efficacy of therapeutic strategies is still poor.

Rodriguez-Mateos et al. (38) found that acute blueberry supplementation (766 mg of total polyphenols) increased FMD in healthy young men, peaking 1 and 6 hours post-consumption, which paralleled the reduction in neutrophil NOX activity. Acute Montmorency cherry (MC) consumption (74 mg anthocyanin, 179 mg total polyphenols) significantly lowered systolic blood pressure in tandem with increases in circulating phenolic acids (22). A number of studies have also found improvements in endothelial dependent vasodilatation after chronic supplementation

with fruit polyphenols especially amongst study populations with impaired cardiovascular function. Grape juice supplements have been shown to improve FMD in patients with coronary artery disease (56 days, Chou 2011) and patients with hypercholesterolaemia (14 days, Coimbra 2005). Red grapes (72 g/day for 21 days) reduced the suppression of FMD induced by a high fat meal (Chaves et al, 2009), grape polyphenols improved FMD in patients with metabolic syndrome (267 mg polyphenols for 30 days, Barona et al, 2012) and grape seed extract (2 g/day for 4 weeks) improved FMD in patients with increased CVD risk (Clifton 2004). Chokeberry juice supplementation (1g polyphenols/day) for 6 weeks also increased FMD in patients with hypercholesterolaemia (Poreba et al 2009), and Khan et al. (24) found that blackcurrant polyphenol supplementation for 6 weeks (815 mg polyphenols including 143 mg anthocyanins per day) improved FMD in healthy adults consuming 2 or fewer portions of fruit and vegetables per day. Consumption of blueberry, cherry and blackcurrant polyphenols appear to enhance vascular function (22, 24, 38), most likely through reduced ROS exposure and hence increased NO bioavailability (40, 47). Although, not all studies have shown favourable effects, for example chronic supplementation (2 – 6 weeks) with grape seed extract (0.6 – 1g/day) had no effect on FMD in patients with Type 2 diabetes (Kar et al, 2009), participants with elevated CVD risk (although baseline brachial artery diameter was increased, Mellen et al, 2010) and healthy young men (van Mierlo et al 2010).

To date, only one study has investigated whether polyphenol supplementation preserved endothelium-dependent vasodilatation after exposure to prolonged forearm occlusion in healthy adults (39). Schreuder et al. (39) found that after a 7-day period consuming 3 cups of black tea per day (~900 mg total polyphenols) FMD was enhanced 90 min after consumption of ~600 mg black tea polyphenols, but FMD was not preserved 20 min after prolonged forearm occlusion. However, prior chronic supplementation with both blueberry polyphenols (2) and cherry polyphenols (5) reduced IR-induced infarct size, better preserved cardiac function and protected against post-ischemic cardiac remodeling in isolated rat hearts. Collectively, these data suggest that a longer period of cherry or berry polyphenol supplementation may be necessary to provide vaso-protection against IR injury, or that effects may be more likely to be observed in a study population with elevated cardiovascular risk factors. We hypothesized therefore that 4 weeks of Montmorency cherry supplementation would increase endogenous antioxidant capacity and thus improve FMD and protect against the impairment in endothelial-dependent vasodilation induced

by ischemia reperfusion. We adopted an experimental model of a prolonged (20-min) occlusion of the forearm that induces a short-lasting impairment to flow mediated dilatation (FMD) (14, 31), and provides an *in vivo* model of transient IR injury. We recruited a non-medicated, physically inactive, overweight, hypertensive, middle aged male population to take part in the study.

MATERIALS AND METHODS

Participants. Twelve physically inactive, middle-aged male participants (age: 52.8±5.8 y, BMI: 28.1±5.3 $kg.m^{-2}$, Table 1) completed the trials, from a total of fourteen participants recruited from local residents in Devon, United Kingdom by Aboo-Bakkar. All participants were non-smokers, with no known history of diabetes, cardiovascular diseases, bone or joint problems, and were not taking any drugs or nutritional supplements as assessed by questionnaires. Habitual physical activity was assessed using the International Physical Activity Questionnaire, and if completing <150 min moderate or vigorous activity were deemed to meet the physically inactive inclusion criterion. The study was approved by the University of Exeter Research Ethics Committee (2013/409) and conformed with the Declaration of Helsinki. All participants provided their written informed consent before taking part. Two participants withdrew from the study after baseline measurements were completed, but prior to commencing supplementation.

Study design. The study used a double-blind, randomized crossover design with 2 experimental arms and a washout period of ≥ 4 weeks (Figure 1). Each of the participants was randomly assigned by Bowtell using a sealed opaque envelope system to receive either MC capsule in the first supplementation block followed by placebo or vice versa and were scheduled to attend the laboratory for 4 trial visits. Participants and the research team involved in performing the data analysis remained blind to treatment until all analysis was completed. Baseline measurements prior to each supplementation block were made during visits 1 and 3, followed by the 4-week supplementation blocks and post supplementation measurements were made in visits 2 and 4. The participants consumed either 1.7 g MC powder per day for 28 days in the form of 6 capsules (226 mg anthocyanins and 456 mg total phenolics measures as gallic acid equivalents, CherryActive Ltd, Sunbury, UK) or placebo capsules (glucose). Three capsules were consumed in the morning and three in the evening, with the last dose taken the evening

prior to the post-supplementation measures. In line with previous studies (10, 21, 42) there was at least a 4-week washout period between trials.

Experimental Protocol. Participants reported to the laboratory at 08:00 a.m. on each visit, preceded by an overnight fast (≥10 h) and having abstained from alcohol and caffeine for more than 24 h. Participants were asked to avoid food and beverages containing high phenolic compounds and record their diet for 2 consecutive days immediately prior to their first laboratory visit, and to replicate this for 2 days prior to subsequent visits. Diet diaries were analysed using Nutritics software and data presented in (Table 2). Upon arrival, a cannula was placed in the antecubital vein of the left arm; the same arm upon which FMD measurements were made. Trials were conducted in a quiet room with dim light and room temperature set at 24 °C. Participants rested in a supine position for 10 min, before baseline FMD was assessed in the left arm with the cuff placed just below the elbow. Fifteen min after the baseline FMD measurement, the cuff was inflated to 200 mmHg (Hokanson, Bellevue, WA) for 20 min to induce transient ischemia, and then FMD measures were repeated after 15, 30 and 45 min of reperfusion. Venous blood samples were obtained at baseline, immediately, 2, 4, 15, 30 and 45 min after the 20-min occlusion (Figure 2). Blood samples at 15, 30 and 45 min were collected immediately after cuff release for FMD measurement. Samples were centrifuged at 4,000 rpm and 4°C for 10 min, within 1 min of collection. Plasma was subsequently extracted and immediately frozen at −80°C for later analysis for nitrite (NO_2^-), nitrate (NO_3^-), interleukin 6 (IL6), C reactive protein (CRP), protein carbonyl (PC), peroxiredoxin 2 (Prx2) content and catalase (CAT) activity.

Brachial artery FMD. Brachial artery FMD was examined in the left arm, with the arm extended and positioned at an angle of approximately 80° from the torso. Three electrocardiogram (ECG) electrodes were positioned on the left and right intraclavicular fossa and left iliac crest region respectively. A high-resolution ultrasonography system (Sequoia 512, Acuson; Siemens) with a 13-MHz linear array transducer was used to image the brachial artery in the distal one-third of the upper arm in accordance with the established protocol in our laboratory (8, 9) and recent guidelines (41). To ensure arm stability and transducer placement, a customized arm rest and transducer holder device cradled the arm and locked the transducer in the position providing the optimal image of the brachial artery. Ultrasound parameters were set to optimize the longitudinal B-mode images of the lumen–arterial wall interface and were then held stable for a 1-min baseline recording of the image and Doppler velocity. Continuous

Doppler velocity assessment was collected using a 60° insonation angle, which did not vary during each study. The occlusion cuffs were inflated to 250 mm Hg to completely block the arterial inflow for 5 min. Diameter and flow recordings resumed 30 s before cuff deflation and continued for 3 min thereafter. All FMD analyses were performed by the same investigator who was blind to condition. Ultrasound images triggered at the beginning of the R-wave that marks the end of the diastolic phase were captured and then analyzed off-line using a validated ECG-gating software (Brachial Analyzer, 5 vascular devices, Medical Imaging Applications, Coralville, IA). The region of interest was carefully defined and frame-by-frame analysis of artery diameter (mm) and blood flow velocity (m/s) was performed by the same analyst. Baseline diameter was calculated as the mean of the artery diameter from a 1-min baseline recording before each cuff inflation. Peak diameter was determined as the highest artery diameter measurements after each cuff deflation. Endothelium-dependent vasodilation was calculated as the percentage increase in arterial diameter after a 5-min ischemic stimulus. The total shear rate under the curve (SR AUC) was calculated as the area under the shear curve versus time immediately after cuff deflation until peak arterial diameter (41).

Plasma nitrite and nitrate concentration. All glassware, utensils, and surfaces were rinsed with deionized water to remove residual [NO_2^-] and [NO_3^-] before blood analyses. The undiluted (nondeproteinized) and deproteinized plasma was used to analyze [NO_2^-] and [NO_3^-] respectively using a Sievers gas-phase chemiluminescence NO analyzer (NOA; Sievers NOA 280i; Analytix, Durham, UK) as described in previous studies (45, 46).

Plasma Prx2 concentration and CAT activity. Plasma was diluted 16 fold and analyzed for [Prx2] using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Cloud-Clone Corp, Texas, USA). For CAT activity, plasma was diluted 5 fold and analyzed using a commercial ELISA kit according to the manufacturer's instructions (Cayman Chemical, Michigan, USA).

Plasma CRP and IL6 concentration. Plasma [CRP] was determined using a turbidometric assay on a Siemens Advia 2400 autoanalyser and using commercially available reagents (PZ Cormay, Lublin, Poland). Plasma [IL6] concentration was assessed using a commercially available ELISA kit (IBL International, Hamburg, Denmark).

Plasma PC concentration. Protein content in all plasma samples were measured using NanoDrop Lite (Thermo Fisher Scientific Inc, Delaware, USA). Each plasma sample was diluted to 10 μg/ml of protein in 1X phosphate buffer saline. The OxiSelect Protein Carbonyl ELISA kit (STA-310, Cell Biolabs Inc, California, USA) was used to measure protein carbonyls. Briefly, samples were allowed to adsorb to wells of a 96-well plate and then reacted with dinitrophenol hydrazine. The protein carbonyls derivatised to dinitrophenyl hydrazone (DNP) were then probed with an anti-DNP antibody. The standard curve was prepared from commercially prepared reduced and oxidised BSA standards as provided.

Statistical Analysis. Sample size was based on the power curves for the use of FMD in cross-over designs, reported by Donald et al. (16) and the effect size reported by Khan et al (24) after polyphenol supplementation. With 80% power, 5% significance and an effect size of 0.8 for increase in FMD (24), 12 participants are required in each group. All data are presented as mean ± standard error of the mean (SEM). FMD, baseline and peak brachial artery diameter, SR AUC, plasma [NO_2^-] and [NO_3^-] were analyzed by 3 way repeated measures ANOVA: condition (MC versus placebo) by supplement (pre- versus post-supplementation) by time (FMD measures: baseline, 15, 30 and 45 min post-occlusion; plasma nitrate and nitrite measures: 0, 2, 4, 15, 30 and 45 min post-occlusion). Targeted post hoc two way repeated measures ANOVA were completed where significant 3 way interaction effects were identified. Baseline plasma [CRP], [IL6], [PC], [Prx2] and CAT activity before and after each supplementation block were analysed by 2 way repeated measures ANOVA. Mauchly's sphericity test was used to check the homogeneity of variance for all ANOVA analyses; when necessary, violations of the assumption were corrected with the use of the Greenhouse-Geisser adjustment. Significant main effects were followed up with the use of least significant difference post hoc analysis. Data were analyzed using statistical software (SPSS Version 19; IBM Corporation, New York, USA), with significance accepted as $p \leq 0.05$.

RESULTS

Brachial artery FMD. There were no significant differences in baseline FMD values for the 4 measurement visits. The FMD response was significantly depressed by the prolonged occlusion in all visits (main effect of time: $P<0.001$; Figure 3) with a significant reduction in

FMD 15 and 30 min post-occlusion, but FMD was not significantly different to baseline 45 minutes after prolonged occlusion. There was a significant condition x supplementation x time interaction (P=0.022), whereby 45 min after prolonged occlusion, the FMD response was reduced after placebo supplementation but better preserved after MC supplementation (Figure 3). Specifically, the magnitude of FMD suppression 45 min after the prolonged occlusion (change from baseline FMD to 45 min post occlusion) was significantly attenuated after MC (pre: -30.5±8.4% vs post: -0.6±9.5%) compared to placebo (pre: -11.6±10.6 vs post: -25.4±4.0) supplementation (condition x supplementation interaction: P=0.038). Similarly, there was a reduction in SR AUC (main time effect: P<0.001; Table 3) after prolonged occlusion, but there was no condition or supplementation effect. Normalization of the FMD data to SR AUC (FMD:SR AUC) did not affect the outcome of the analysis (Table 3). Peak brachial artery diameter was significantly suppressed after prolonged occlusion (main time effect, P<0.001). There was a condition x supplementation x time interaction (P=0.011; Figure 3), whereby peak diameter was higher following prolonged occlusion post versus pre MC supplementation but not post versus pre placebo supplementation. In contrast, basal diameter of the brachial artery (immediately prior to occlusion for FMD measurement) was increased after the prolonged occlusion (main time effect, P<0.001; Figure 3), but there was no condition or supplementation effect.

Plasma analytes. Plasma [NO_2^-] and [NO_3^-] were significantly depressed by the prolonged occlusion in all visits (main effects of time, P<0.001; Table 4). Plasma [NO_2^-] returned towards baseline during the 45-min recovery after prolonged occlusion more quickly after MC supplementation compared to placebo (condition x supplement x time interaction, P=0.033, Figure 4). There was no significant effect of supplementation on plasma [NO_3^-]. Basal plasma [Prx2] was higher after MC (P=0.029) but not after placebo supplementation (Figure 5). However, there was no effect of supplementation on basal plasma CAT activity (P>0.05, Figure 5), or plasma [CRP], [IL6] and [PC] (P>0.05, Table 5).

DISCUSSION

Four weeks MC supplementation enhanced recovery of endothelium-dependent vasodilation in overweight, physically inactive, non-medicated hypertensive, middle-aged men

after the transient ischemia-reperfusion injury induced by prolonged forearm occlusion. Plasma nitrite concentration was higher after MC supplementation during the recovery period after ischemia. Moreover, plasma peroxiredoxin 2 concentration was increased after 4 weeks MC supplementation indicating increased capacity for reduction of hydrogen peroxide and therefore superoxide quenching. This supports the notion that MC supplementation enhanced NO bioavailability and vascular function under conditions of increased ROS generation after prolonged blood flow occlusion via increased endogenous antioxidant capacity.

Prolonged forearm occlusion induced transient IR injury as evidenced by attenuated endothelium dependent vasodilation. DeVan et al. (14) reported that FMD in middle-aged sedentary subjects was decreased by 68% by the same occlusion protocol employed in the present study, while Schreuder et al. (39) and Rakobowchuk et al. (37) reported 33% and 36% reductions in FMD 15 minutes after prolonged occlusion in eight healthy women and twenty healthy older adults, respectively. In the present study, the reduction in FMD after prolonged occlusion appears to be reproducible across trials with FMD approximately 53% lower, 15 min after prolonged occlusion when compared to baseline (52±19 %, pre-MC; 58±13 %, pre-placebo; 49±21 %, post-placebo; and 55±17 %, post-MC respectively). The FMD reduction after prolonged occlusion seems to be a consequence of both increased baseline artery diameter (12, 37, 39) and reduced peak artery diameter (25, 37). It has been suggested that this impaired endothelium-dependent vasodilation is due to reduced NO bioavailability as a consequence of increased ROS production during reperfusion (17, 35). Our data are consistent with this hypothesis, since plasma nitrate and nitrite concentrations were lower in the venous blood exiting the forearm subjected to prolonged occlusion. Under normal resting conditions, the rate of superoxide production is lower than the flux of NO and this allows effective scavenging of the low intracellular levels of hydrogen peroxide and superoxide by anti-oxidant systems including PRX2 as well as by NO, resulting in low levels of the cytotoxic derivative, peroxynitrite (18). However, during reperfusion immediately after prolonged occlusion, superoxide production is increased (35, 42) resulting in greater peroxynitrite formation (30), and reduction in NO availability (34, 35). We cannot exclude the possibility that the reduced pH and pO_2 induced by ischemia enhanced degradation of nitrite to NO (32, 33), which may also contribute to the reduction in plasma nitrite concentration observed in the present study.

Fruit derived polyphenols improve endothelium dependent vasodilatation, for instance, FMD response peaked between 1 to 2 hours, and 6 hours after consuming 766 mg blueberry polyphenols (310 mg anthocyanins, (38). Keane et al. (22) showed that systolic blood pressure (SBP) was significantly lowered in hypertensive (SBP ≥ 130 mmHg) men 2 h after ingestion of 60 mL of MC concentrate (74 mg cyanidin-3-glucoside/L, 179 mean gallic acid equivalent/L). Chronic supplementation interventions of 4 to 6-weeks with fruit juice or powder (32 – 143 mg anthocyanins/day) increased FMD response in men with mild hypercholesterolemia (36) and the metabolic syndrome (6), as well as men and women with low fruits and vegetables intake (<2 portion/day) (24). However, in the present study, 4 weeks of MC powder supplementation providing 256 $mg.d^{-1}$ anthocyanins (split into 2 doses, morning and evening) did not improve FMD response in overnight fasted, overweight, physically inactive, non-medicated hypertensive middle aged men. Collectively our findings and previous literature suggest that resting vascular function is only improved after chronic fruit based supplement consumption in those with clinical conditions or low habitual polyphenol intake, but not in those with elevated CVD risk factors. However, MC supplementation was able to protect the vasculature from the decline in endothelium-dependent vasodilatation induced by ischaemia reperfusion in the present study. Polyphenols are suggested to increase calcium-dependent endothelium nitric oxide synthase (eNOS) phosphorylation through the phosphatidylinositol 3-kinase-Akt pathway (3) thus enhancing NO production. Phenol metabolites such as vanillic acid have also been shown *in vivo* to inhibit NOX activity in neutrophils (38). The resulting reduction in superoxide production and hence reduced NO degradation to peroxynitrite will also improve NO bioavailability. Anthocyanins and their metabolites have been shown to interact with cysteine residues present in Kelch-like ECH-associated protein 1 (Keap1), resulting in nuclear translocation and phosphorylation of Nrf2 (20, 26, 28) with subsequent signaling through the antioxidant response element pathway to increase production of downstream phase II antioxidant enzymes such as the peroxiredoxins (1). The increased plasma PRX2 concentration observed after MC supplementation is consistent with this mechanism, and suggests that enhanced capability to quench ROS may contribute to the improved NO bioavailability evident in the present study.

Prolonged forearm occlusion resulted in elevated baseline diameter in all trials in this and previous studies (12, 37, 39). This basal dilation might be related to increased endothelium-derived hyperpolarizing factor (EDHF) production since this pathway is upregulated during

eNOS dysfunction, and results in arterial vasodilation via vascular smooth muscle hyperpolarization (11). In contrast to the favorable effects of MC supplementation on the NO dependent measures (peak brachial artery diameter and plasma nitrite concentration), MC supplementation did not influence this NO independent effect on basal brachial artery diameter.

In conclusion, the results of this randomized placebo-controlled, cross-over study demonstrate that four weeks of MC powder supplementation exerted protective effects on endothelium-dependent vasodilation after transient IR injury in overweight, physically inactive, non-medicated hypertensive middle-aged men. These effects seem to be due to increased NO availability as evidenced by higher plasma nitrite concentration and peak arterial diameter during the FMD measurement. This may be a consequence of increased concentration of peroxiredoxin and other anti-oxidant systems, and hence reduced ROS exposure.

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DISCLOSURES

The authors have no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

JF, PEG, SR, AMJ, JB designed research; ZAB, JF, PEG, SR, BB, JB conducted research; ZAB, BB, SR, JB analyzed data; ZAB, JF, PEG, BB, AMJ, JB wrote the paper; JB had primary responsibility for final content. All authors read and approved the final manuscript.

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Table 1. *Participant characteristics.*

Variables	
Age, y	54±1
Height, cm	179±1
Weight, kg	91.5±4.0
Body Mass Index, $kg.m^{-2}$	28.2±1.6
Resting Heart Rate, beat/min	66±0
Systolic Blood Pressure, mmHg	142±6
Diastolic Blood Pressure, mmHg	88±3

Values are expressed as mean ± SEM.

Table 2. *Average dietary composition*

Dietary composition	
Energy ($kcal.d^{-1}$)	1883 ± 115
Protein ($g.d^{-1}$)	74.6 ± 4.1
Protein ($g.kg^{-1}.d^{-1}$)	0.9 ± 0.1
Carbohydrate ($g.d^{-1}$)	237.8 ± 29.9
Fat ($g.d^{-1}$)	66.9 ± 5.5
Total cholesterol ($mg.d^{-1}$)	328.5 ± 48.0
Saturated fat ($g.d^{-1}$)	23.2 ± 3.1
Dietary fiber ($g.d^{-1}$)	16.3 ± 2.2
Sugars ($g.d^{-1}$)	77.5 ± 22.4

Average dietary composition for the 48-h period prior to each trial. Values are expressed as mean ± SEM.

Table 3. *Shear rate area under the curve and FMD normalized to SR AUC*

	Time			
	Baseline	15 min post	30 min post	45 min post
SR AUC*				
Placebo				
Pre	663±91	623±63	594±75	629±68
Post	683±101	533±60	550±52	579±85
Montmorency cherry				
Pre	669±85	525±58	524±47	577±64
Post	632±63	456±54	444±57	544±63
FMD:SR AUC*				
Placebo				
Pre	0.99±0.16	0.46±0.06	0.66±0.12	0.85±0.12
Post	1.00±0.13	0.67±0.13	0.75±0.10	0.92±0.15
Montmorency cherry				
Pre	0.90±0.09	0.49±0.07	0.65±0.10	0.70±0.11
Post	1.06±0.22	0.61±0.09	1.11±0.29	1.15±0.20

Shear rate area under the curve (SR AUC) and FMD normalized to SR AUC (FMD:SR AUC) measured 15 min before 20-min occlusion (baseline), 15, 30 and 45 min after reperfusion, before and after each supplementation block. Values are expressed as mean ± SEM, *-main effect of time $p< 0.001$.

Table 4. *Plasma nitrite [NO_2^-] and nitrate [NO_3^-] concentrations*

	Time						
	Baseline	0 min post	2 min post	4 min post	15 min post	30 min post	45 min post
[NO_2^-]*# ($nmol.l^{-1}$)							
Placebo							
Pre	87.3±7.6	63.5±4.2	70.9±3.4	68.3±4.8	72.8±10.8	69.0±5.7	71.0±7.4
Post	104.2±13.5	91.3±9.7	86.2±9.1	83.8±11.3	88.7±7.6	82.3±6.5	76.5±6.9
MC							
Pre	82.1±10.0	80.7±11.1	74.2±11.3	71.9±10.1	68.2±14.9	64.2±11.8	66.8±10.1
Post	83.2±10.5	65.6±7.3	77.7±8.6	70.2±6.4	81.1±7.3	74.2±9.4	77.0±10.7
[NO_3^-]* ($\mu mol.l^{-1}$)							
Placebo							
Pre	32.8±3.9	29.7±3.6	28.6±3.5	28.7±3.3	27.7±2.9	27.9±2.9	27.2±3.0
Post	27.3±2.6	25.7±3.0	26.8±3.3	25.9±2.4	25.7±2.7	24.4±2.8	25.8±2.7
MC							
Pre	27.6±4.9	24.8±4.4	26.2±4.7	25.8±4.8	25.6±4.6	25.0±4.5	24.7±4.1
Post	29.5±5.2	28.2±4.9	29.3±4.9	27.9±4.9	26.8±4.7	26.6±4.4	26.3±4.4

Plasma [NO_2^-] and [NO_3^-] measured 15 min before 20-min occlusion (baseline), and immediately, 2, 4, 15, 30 and 45 min after reperfusion, before and after placebo or Montmorency cherry (MC) supplementation. Values are expressed as mean ± SEM, *-main effect of time $p< 0.001$, #-3 way interaction effect.

Table 5. *Plasma biomarkers*

	Placebo		Montmorency Cherry	
	Pre	Post	Pre	Post
Interleukin-6 ($pg.ml^{-1}$)	0.06 ± 0.04	0.06 ± 0.06	0.06 ± 0.03	0.07 ± 0.09
C Reactive Protein ($mg.l^{-1}$)	1.01 ± 0.33	3.90 ± 2.69	1.90 ± 0.40	3.40 ± 1.27
Protein Carbonyl ($nmol.mg^{-1}$)	0.86 ± 0.11	0.69 ± 0.18	0.93 ± 0.17	0.71 ± 0.10

Baseline plasma biomarkers before and after 4 weeks of Montmorency cherry and placebo supplementation. Values are expressed as mean ± SEM.

Figure Legends

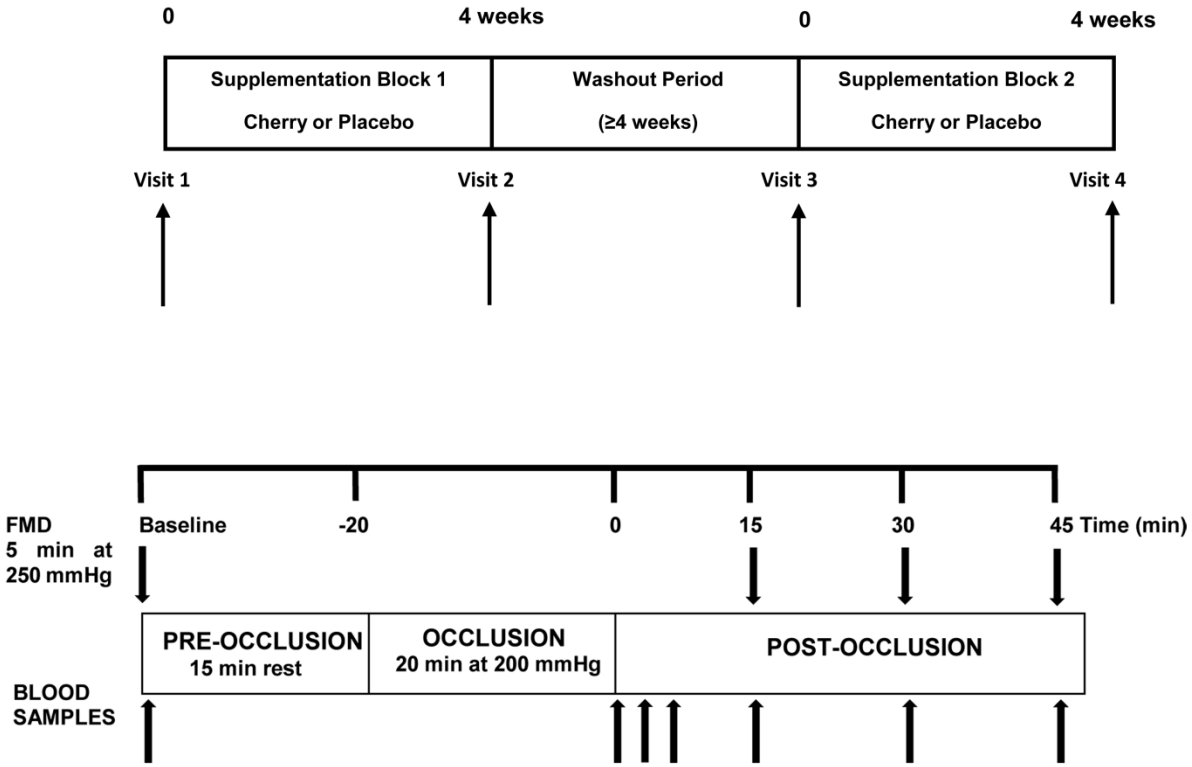
Fig. 1. Schematic diagram of study design.

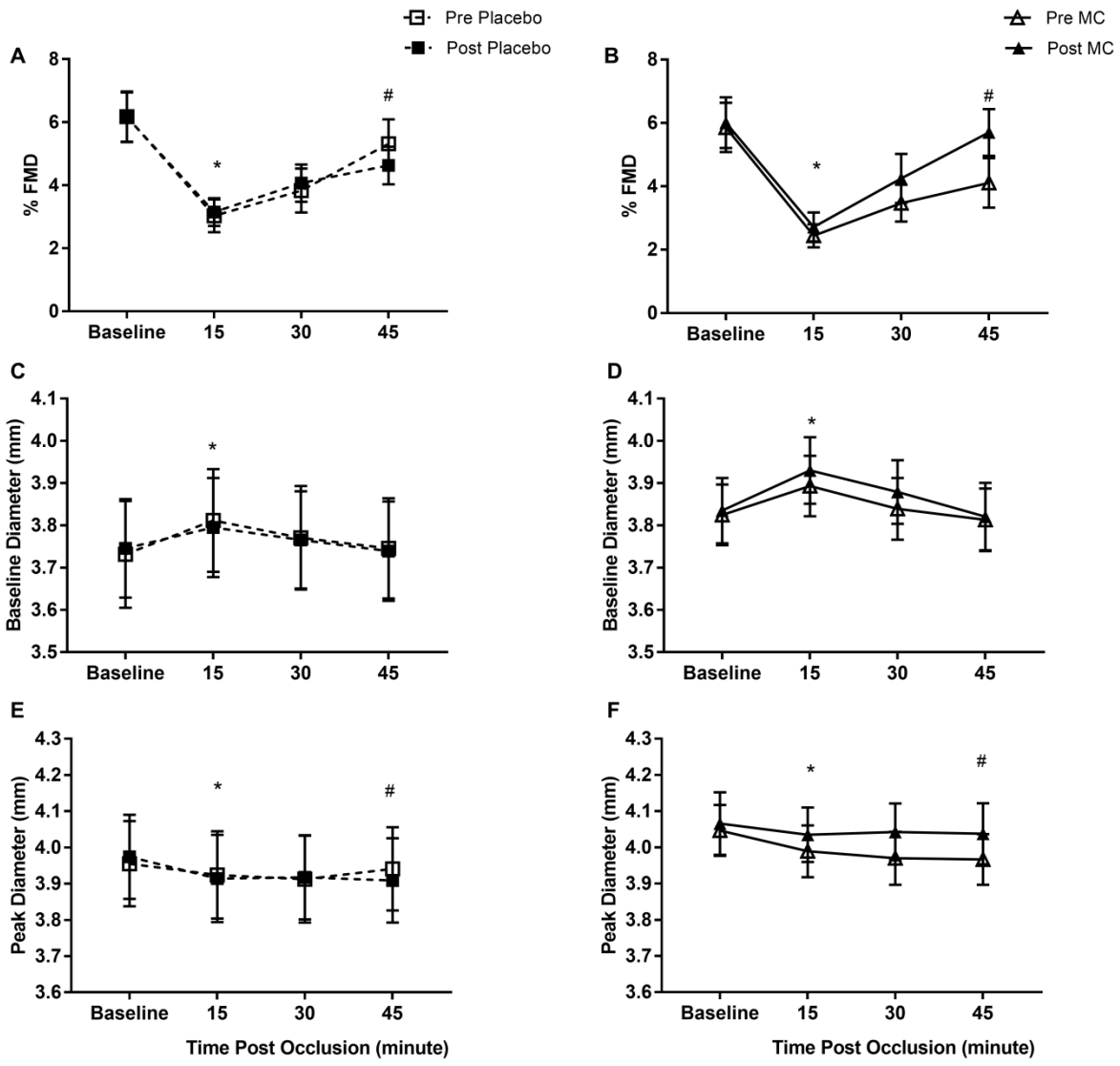
Fig. 2. Brachial artery FMD and venous blood collection before and after prolonged occlusion protocol to induce transient IR injury. Arrows represent FMD measurements and blood samples for plasma analysis.

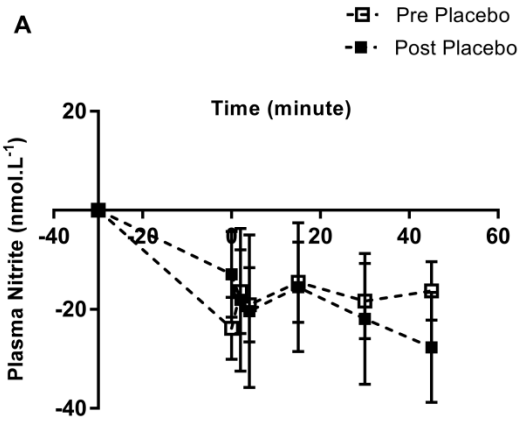
Fig. 3. FMD (A, B), baseline diameter (C, D) and peak diameter (E, F) before and after prolonged occlusion for placebo (A, C and E) and Montmorency cherry (B, D and F) conditions, mean±SEM, n=12. * indicates a significant difference from baseline (main effect for time, $p<0.05$); # indicates a condition vs supplementation vs time interaction ($p<0.05$).

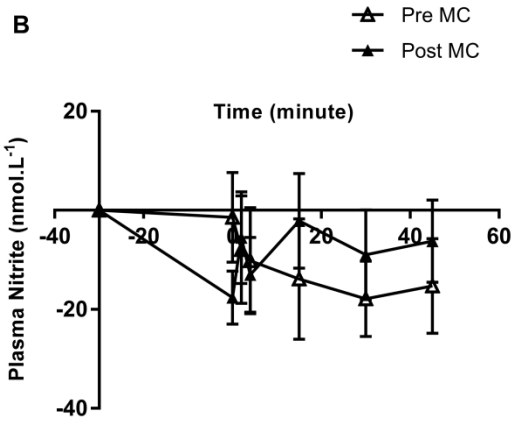
Fig. 4. Change from baseline in plasma nitrite concentrations after prolonged occlusion performed pre and post 4 weeks of placebo (A) and MC (B) supplementation, mean±SEM, n=9, # - condition x supplement x time interaction: $p<0.05$.

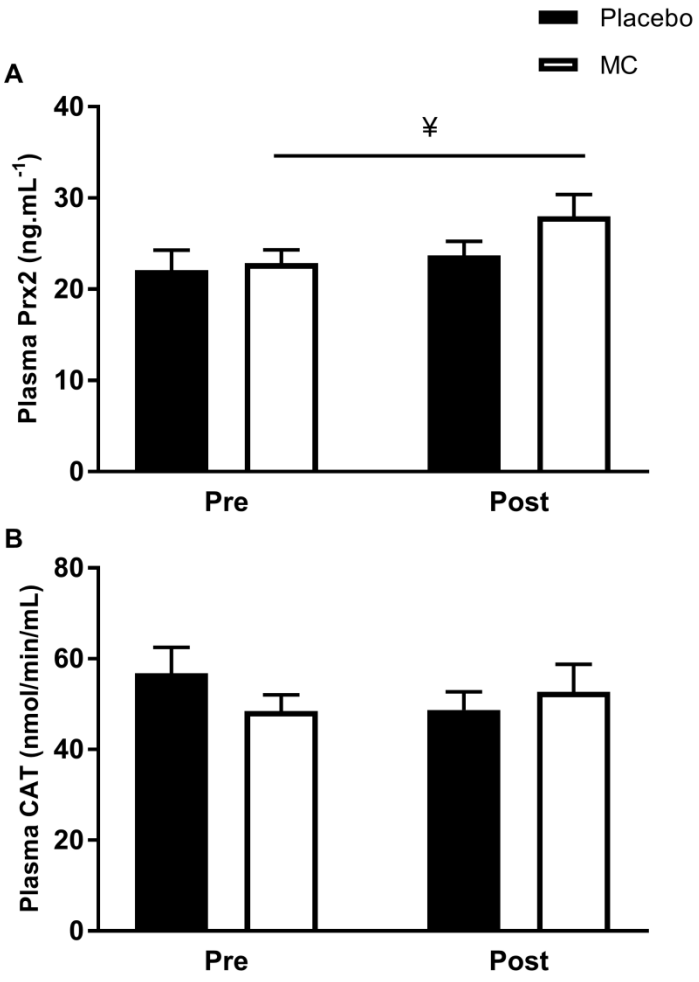
Fig. 5. Baseline plasma [Prx2] (A) and CAT activity (B) before and after prolonged occlusion performed pre and post 4 weeks of placebo and MC supplementation, mean±SEM, n=12, ¥ - supplement effect: $p<0.05$.


0
4 weeks
0
4 weeks
Supplementation Block 1
Cherry or Placebo
Washout Period
(≥4 weeks)
Supplementation Block 2
Cherry or Placebo
Visit 1
Visit 2
Visit 3
Visit 4
FMD
5 min at
250 mmHg
Baseline
-20
0
15
30
45 Time (min)
PRE-OCCLUSION
15 min rest
OCCLUSION
20 min at 200 mmHg
POST-OCCLUSION
BLOOD
SAMPLES


A
Pre Placebo
Post Placebo
% FMD
Baseline
15
30
45
B
Pre MC
Post MC
% FMD
C
Baseline Diameter (mm)
D
Baseline Diameter (mm)
E
Peak Diameter (mm)
Time Post Occlusion (minute)
F
Peak Diameter (mm)
Time Post Occlusion (minute)


A
Pre Placebo
Post Placebo
Time (minute)
Plasma Nitrite (nmol.L-1)
20
-20
-40
-40
-20
0
20
40
60


B
Pre MC
Post MC
Time (minute)
Plasma Nitrite (nmol.L-1)
20
-20
-40
-40
-20
0
20
40
60


Placebo
MC
A
Plasma Prx2 (ng.mL-1)
40
30
20
10
0
¥
Pre
Post
B
Plasma CAT (nmol/min/mL)
80
60
40
20
0
Pre
Post