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Regular Dark Chocolate Consumption's Reduction of Oxidative Stress and Increase of Free-Fatty-Acid Mobilization in Response to Prolonged Cycling

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This study investigated the effects of regular consumption of dark chocolate (DC), rich in cocoa polyphenols, on plasma metabolites, hormones, and markers of oxidative stress after prolonged exhaustive exercise. Twenty active men cycled at 60% maximal oxygen uptake (VO_{2max}) for 1.5 hr, with the intensity increased to 90% VO_{2max} for a 30-s period every 10 min, followed by a ride to exhaustion at 90% VO_{2max}. In the 2 wk before exercise participants consumed 40 g of DC or an isocarbohydrate-fat control cocoa liquor–free chocolate (CON) twice daily and once 2 hr before exercise in a randomized, counterbalanced, crossover design. Venous blood samples were taken immediately before exercise, postexercise (fixed duration), postexhaustion, and after 1 hr of recovery. F_2-isoprostanes were significantly lower (post hoc tests: $p < .001$) at exhaustion and after 1 hr of recovery with DC. Oxidized low-density lipoproteins were significantly lower with DC ($p < .001$) both before and after exercise and at exhaustion. DC was also associated with ~21% greater rises in free fatty acids during exercise (main effect: $p < .05$). Changes in circulating glucose, insulin, glucagon, cortisol, and interleukin (IL)-6, IL-10, and IL-1ra were unaffected by treatment. Time to exhaustion at 90% VO_{2max} was not significantly different between trials (398 ± 204 and 374 ± 194 s for DC and CON, respectively). These results suggest that regular DC intake is associated with reduced oxidative-stress markers and increased mobilization of free fatty acids after exercise but has no observed effect on exercise performance.

Keywords: polyphenols, antioxidants, exercise, cocoa

Physical activity results in increased production of free radicals and other reactive oxygen species (ROS), primarily arising from elevated oxidative phosphorylation in the mitochondria of contracting skeletal muscle (Davies, Quintanilha, Brooks, & Packer, 1982; Jackson, Pyne, & Palomero, 2007). Other potential sources include plasma, blood cells such as erythrocytes and leukocytes, smooth-muscle cells, and endothelial cells (Nikolaidis & Jamurtas, 2009). ROS can induce lipid and protein peroxidation, leading to oxidative stress, acute-phase inflammatory responses, and possible acceleration of muscle-fatigue development (Powers, Deruisseau, Quindry, & Hamilton, 2004). Muscle cells can be protected from these ROS by a cooperative system of endogenous defense mechanisms in which antioxidants may play a role.

Supplementation with antioxidants has been proven effective in a number of studies at reducing the magnitude of the oxidative stress and inflammatory response that can occur after prolonged intense exercise, as well as limiting the degree of immunosuppression, although a large part of that research was limited to vitamins C and E (Peake, Suzuki, & Coombes, 2007; Powers et al., 2004). Prolonged (4 weeks) supplementation with a combination of vitamin C and vitamin E attenuated the increase in F_2-isoprostanes (a marker of lipid peroxidation) and was associated with a lower plasma interleukin-6 (IL-6) and cortisol response to prolonged exercise (Fischer et al., 2004). Furthermore, Davison and Gleeson (2007) demonstrated that acute vitamin C supplementation is associated with a reduction in the magnitude of exercise-induced neutrophil-function depression. However, to date, evidence to show that antioxidant supplementation can improve exercise performance and delay fatigue is still limited (Davis, Carlstedt, Chen, Carmichael, & Murphy, 2010; McKenna et al., 2006; Medved et al., 2004).

There has been growing interest in recent research in the potential beneficial effects of cocoa because of the potent antioxidant properties of its constituent polyphenols. In two studies, when a high-phenolic-content antioxidant beverage (Morillas-Ruiz et al., 2005) or a polyphenol-containing sports drink (Morillas-Ruiz, Villegas Garcia, Lopez, Vidal-Guevara, & Zafrilla, 2006) was consumed shortly before 15 min of submaximal aerobic exercise, the magnitude of exercise-induced oxidative stress was significantly reduced. Furthermore, short-term consumption of black currant extract, abundant in polyphenols, reduced exercise-induced oxidative stress and inflammatory responses (lipopolysaccharide inflamma-

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tory responses) to 30 min of rowing (Lyall et al., 2009), and green tea extract consumed acutely has been shown to stimulate lipid catabolism and increase fat oxidation after 30 min of cycling (Venables, Hulston, Cox, & Jeukendrup, 2008). More recently, 7 days supplementation with quercetin, a natural polyphenol flavonoid, was reported to increase endurance capacity in untrained participants, possibly via increased mitochondrial biogenesis (Davis et al., 2010). Dark chocolate is one of the richest dietary sources of antioxidant polyphenols. Cocoa polyphenols, most notably the monomers catechin and epicatechin, can exist in both lipid- and water-based environments (amphipathic), meaning they can spare both lipophilic and hydrophilic vitamins. Studies have shown beneficial effects of chronic cocoa or dark chocolate consumption (Cooper, Donovan, Waterhouse, & Williamson, 2008) in healthy participants (usually ~100 g of dark chocolate per day for 2 weeks or more) on arterial and endothelial function (Vlachopoulos et al., 2005), resting blood pressure and insulin sensitivity (Grassi, Lippi, Necozione, Desideri, & Ferri, 2005), or cholesterol profile (increased high-density lipoprotein [HDL] concentration; Mursu et al., 2004). However, there have been few studies investigating the potential of cocoa-containing foods to modulate exercise-induced oxidative stress and performance. Dark chocolate also contains caffeine and theobromine, which may also have potential ergogenic effects. Caffeine ingestion has been shown to increase fatty-acid mobilization from stores and spare glycogen while also stimulating the central nervous system and increasing muscle excitability, resulting in enhanced performance (Burke, 2008). Theobromine may also increase free-fatty-acid mobilization, although its effects during exercise are unclear (Eteng & Ettarh, 2000).

There is limited information on the effects of ingesting dark chocolate, rich in cocoa polyphenols, on oxidative stress and immunoendocrine responses after prolonged (>1.5 hr duration) exercise and how this relates to exercise performance. Therefore, the aims of the current study were to examine the effects of a 2-week period of regular dark chocolate consumption on the responses of selected plasma markers of oxidative stress, hormones, and cytokines to a single bout of prolonged exhaustive exercise.

Methods

Participants

After we received university ethical committee approval, 20 healthy men (age 22 ± 4 years, body mass 74.6 ± 8 kg, maximal oxygen uptake [VO_{2max}] 53.1 ± 7.0 ml · kg^{-1} · min^{-1}, power output at VO_{2max} 300 ± 30 W; *M* ± *SD*) participated in the study. They were all familiar with cycling and actively engaged in regular physical activity for a minimum of 2 hr, three times per week, as determined by a physical activity and health questionnaire. They were informed of the experimental procedures (written and verbally) before signing consent forms. Participants meeting one or more of the following criteria were excluded from the study: smoking, regular consumption of dark chocolate or similar high-polyphenol products or vitamin supplements, any sports supplements, medication, and alcohol. All participants completed four exercise bouts: two preliminary trials (VO_{2max} determination and familiarization) and two main trials. Trials were separated by 1 week, and there was a 2-week washout period between the main trials.

Design and Protocol

Preliminary Measurements. Participants completed a continuous incremental exercise test to volitional exhaustion on an electronically braked cycle ergometer (Lode Excalibur, Holland). After a 3-min warm-up at 95 W they cycled with 35-W increments every 3 min until volitional exhaustion. Expired-gas samples were collected in Douglas bags during the final minute of each stage. Heart rate (Polar A1, Polar Electro, Kempele, Finland) and rating of perceived exertion (Borg scale) were collected during this period. The expired gas was analyzed by an oxygen–carbon dioxide analyzer (Servomex 1400, Crowbridge, UK), and volume was measured by a dry gas meter (Harvard Apparatus, Edenbridge, UK) to determine ventilation, oxygen uptake, and carbon dioxide production. From the VO_2–work rate relationship, the work rates equivalent to 60% and 90% VO_{2max} were interpolated. On a second occasion participants completed a familiarization ride. The purpose of this was to familiarize them with the exercise protocol and to check that the correct relative intensity exercise was being performed. Participants cycled for 1.5 hr at a work rate equivalent to 60% VO_{2max}, and every 10 min the exercise intensity was increased to 90% VO_{2max} for 30 s. Expired-gas samples were collected over a 1-min period into Douglas bags after 10 min and 30 min of exercise and then every 30 min thereafter; heart rate and rating of perceived exertion were measured every 15 min.

Main Trials. For the main trials, participants exercised under two different conditions—a DC (dark chocolate) trial or CON (control cocoa liquor–free chocolate) trial—in a single-blind, randomized, and counterbalanced design. They arrived at the laboratory at 9:30 a.m. on the days of the main trials after an overnight fast (>10 hr) followed by a breakfast at 8 a.m. that had consisted of chocolate (40 g DC or 30.4 g isocaloric CON) and 500 ml water only. On arrival, participants were asked to empty their bladder, after which body mass was measured in minimal clothing. The exercise bouts began at 10 a.m. Water (2.5 ml/kg body mass) was consumed at the onset, every 15 min during, and on completion of the exercise bout. Exercise consisted of steady-state cycling at 60% VO_{2max} for 1.5 hr; every 10 min the exercise intensity was increased to 90% VO_{2max} for 30 s. The exercise protocol was chosen to simulate a race situation with occasional bursts of higher intensity work while still having a standardized exercise protocol. It also served to familiarize the subjects with the effort required to exercise at 90% VO_{2max} before they performed the ride

to exhaustion at the end of the trial. At the end of the 1.5 hr of exercise, subjects were given a 5-min break, immediately followed by a time-to-exhaustion trial (at 90% VO_{2max}). They were instructed to maintain a pedal cadence of more than 50 rpm while cycling, to remain seated at all times, and to attempt to cycle for as long as possible. No external encouragement was given during the trial, and no information about elapsed time was given during the ride to exhaustion. Expired gas was collected into Douglas bags (1-min sample) during the 25th, 55th, and 75th minutes of exercise, for analysis of VO_2 and respiratory-exchange ratio (RER). Heart rate and rating of perceived exertion were recorded after 15 min of exercise and every 10 min thereafter. Heart rate alone was recorded unobtrusively every 5 min during the ride to exhaustion. After completion of the exhaustion trial, participants were weighed again.

Venous blood samples were taken at rest immediately before exercise (Pre-Ex), immediately after completing the 1.5-hr exercise bout (Post-Ex), immediately after completing an exercise bout to exhaustion (Post-Exh), and after 1 hr of resting recovery (1 hr Post-Exh). On each occasion 25 ml blood was obtained from an antecubital vein with a 21-g butterfly needle and syringe. Blood was then dispensed into five Vacutainer tubes (Becton Dickinson, Oxford, UK), four K_3EDTA tubes, and one heparin tube.

Participants completed a food diary for the 48 hr before the first trial (familiarization) and were required to follow the same diet during the 48 hr before each main trial. They were required to abstain from alcohol, caffeine, polyphenol-containing products (other than the prescribed chocolate), and heavy exercise for 48 hr before each trial and to have a rest day on the day immediately before each trial. It was also stipulated that participants should not take any mineral or vitamin supplement or any other antioxidant supplements during and for the 4 weeks before the study. They were provided with a food-exclusion list to ensure that they avoided high-polyphenol- and -caffeine-containing foods for the 48 hr before each main trial.

Treatments

Participants consumed the total daily amount of prescribed chocolate (80 g for DC, 56.8 g for CON) in two equal portions with their breakfast and evening meals every day for 2 weeks. They also consumed a portion of the same chocolate on the day of the main trials. To prevent changes in daily energy intake, participants were asked to replace their normal snacks (e.g., cookies, candy) or desserts with the chocolate but not to reduce their intake of fruit and vegetables.

The dark chocolate was 40.0 g Nestlé Noir™ 70% chocolate containing cocoa liquor, sugar, cocoa butter, milk fat, lecithin, and vanilla. The control chocolate (30.4 g, which was 71% of the mass of the DC) was matched in terms of fat (18%) and carbohydrate (77%) content and total energy (2.1 MJ) and contained all the same ingredients except the cocoa liquor (0%). (See Table 1 for more detailed composition.)

Table 1 Chocolate Composition

	DC (absolute amount per 40 g)	CON (absolute amount per 40 g)	CON (absolute amount per 30.4 g)
Ash (g)	0.84	0.0	0.0
Protein (g)	2.84	0.0	0.0
Fat (OICC; g)	17.4	25.1	17.8
Fiber (g)	4.3	<0.2	<0.2
Energy (kJ)	932	1,182	839
Sucrose (g)	10.3	14.6	10.4
Caffeine (mg)	41.6	9.6	6.8
Theobromine (g)	267	6.4	4.5
Polyphenols (mg)			
catechin	15.6	0	0
epicatechin	38.7	0	0
Dimer B2	23.4	0	0
Dimer B5	2.9	0	0
Trimer C	13.9	0	0
Tetramer D	4.2	0	0

Note. DC = dark chocolate; CON = control.

Blood Analysis

K_3EDTA blood was used for hematological analysis including hemoglobin, hematocrit, and total and differential leukocyte counts using an automated hematology analyzer (A^C•T 5diff analyzer, Beckman Coulter, UK). Plasma volume changes were calculated according to Dill and Costill (1974). The remaining K_3EDTA and heparinized whole blood was spun at 1,500 *g* for 10 min in a refrigerated centrifuge at 4 °C within 10 min of sampling. The plasma obtained was immediately stored at −80 °C until analysis. K_3EDTA plasma was used to determine changes in the plasma concentrations of selected metabolites, hormones, cytokines, and markers of oxidative stress. Heparinized plasma was used for the analysis of triglycerides, vitamin C, uric acid, and total antioxidant capacity.

Plasma Antioxidants. Plasma Trolox-equivalent antioxidant capacity (TEAC) was determined using an automated analyzer (Cobas-Mira Plus, Roche, Basle, Switzerland) and a commercially available kit (Randox, County Antrim, UK) for measuring the capacity of plasma to scavenge the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation. Trolox was used as the standard, so this measure of total antioxidant status was expressed in Trolox-equivalent antioxidant units (mM). Plasma vitamin C concentration was determined according to Liu, Chin, Kiser, and Bigler (1982) using a specific spectrophotometric ascorbate oxidase (E 1.10.3.3) assay. Plasma uric acid concentration was determined according to a spectrophotometric method using a commercially available kit (Randox, County Antrim, UK).

Plasma Markers of Oxidative Stress. Plasma free F_2-isoprostane concentration and the plasma concentrations of oxidized low-density lipids (LDLs) were determined using commercially available ELISA kits according to the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI, and Mercodia AB, Uppsala, Sweden, respectively).

Plasma Hormone and Cytokine Concentrations. Commercially available ELISA kits were used to determine the concentrations of cortisol (DRG Diagnostics, Marburg/Lahn, Germany), adrenocorticotropic hormone (Biomercia, Irvine, CA), insulin (ultrasensitive kit, DRG Diagnostics), glucagon (Cosmobio, Japan), IL-10 (high-sensitivity kit, Diaclone, Besançon, France), IL-1ra (R&D systems, Minneapolis, MN), and IL-6 (high-sensitivity kit, Diaclone).

Plasma Metabolite Concentrations. Plasma glucose concentration was determined on an automated analyzer (Cobas Mira Plus, Roche, Basel, Switzerland) using a colorimetric glucose oxidase PAP kit (Randox, County Antrim, UK). Plasma lactate concentration was determined on a spectrophotometer at 340 nm using a standard enzymatic assay with lactate dehydrogenase and nicotinamide adenine dinucleotide (Sigma, Poole, UK). Analysis of free fatty acids (FFAs) was performed using the Wako 999–75406 NEFA-C kit, and triglyceride analysis was performed using the triglyceride Infinity reagent kit (Alpha Laboratories, Eastleigh, UK).

Data Analysis

Data are presented as means and standard deviations. To compare the two different trials, DC and CON, a two-way repeated-measures ANOVA (Trial × Time) was used. Data that were not normally distributed were normalized with log transformation. Post hoc analysis was carried out, when appropriate, using paired-samples *t* tests with the Holm–Bonferroni correction. When the two-way ANOVA showed a significant Trial × Time interaction (indicating different temporal responses depending on trial), one-way repeated-measures ANOVA was also used to determine the temporal response in each trial independently. The Greenhouse–Geisser correction was applied to all ANOVA *p* values.

Results

The effects of DC on the physiological demands of exercise are presented in Table 2. There were no significant differences between trials in overall exercise intensity during the steady-state exercise. Mean heart rates were similar between the two conditions, as were mean ratings of perceived exertion. RER did not differ significantly between trials ($p = .08$). Times to exhaustion at 90% VO_{2max} were 398 ± 204 and 374 ± 194 s for the DC and CON trials, respectively. There was no significant difference between treatments.

Markers of Antioxidant Capacity

Plasma uric acid concentration increased Post-Exh and 1 hr Post-Exh compared with Preex in both trials (main effect of time, $p < .001$). Vitamin C increased with time in both trials (main effect of time, $p < .001$). Total antioxidant status was higher Post-Exh than Pre-Ex (main effect of time, $p < .001$). These temporal responses were not significantly influenced by DC supplementation (Table 3).

Markers of Oxidative Stress

There was a significant main Trial × Time interaction effect ($p = .045$) and main effect of time ($p < .01$) and trial ($p < .001$) for plasma free F_2-isoprostane concentra-

Table 2 Heart Rate, Rating of Perceived Exertion, Percent of Maximal Oxygen Uptake, and Respiratory-Exchange Ratio During Exercise, *M* (*SD*)

	25 min	55 min	75 min	Main-effects *p* values, trial; time; Trial × Time[a]
Heart rate (beats/min)				>.1; <.001; >.1
dark chocolate trial	144 (11)	152 (13)	155 (12)	
control trial	145 (10)	149 (12)	152 (10)	
Rating of perceived exertion				>.1; <.001; >.1
dark chocolate trial	11 (1)	12 (1)	12 (1)	
control trial	10 (1)	12 (1)	12 (1)	
Percent of maximal oxygen uptake				>.1; <.001; >.1
dark chocolate trial	62.5 (4.1)	64.5 (4.1)	65.0 (4.9)	
control trial	61.5 (6.3)	63.2 (6.4)	64.6 (6.8)	
Respiratory-exchange ratio				.08; <.001; >.1
dark chocolate trial	0.90 (0.05)	0.88 (0.04)	0.87 (0.04)	
control trial	0.92 (0.03)	0.90 (0.04)	0.90 (0.03)	

[a]Results from two-way ANOVA.

tion. Values increased Post-Exh in both CON ($p < .001$) and DC ($p < .001$) but were significantly lower in DC at Post-Exh ($p < .001$) and 1 hr Post-Exh ($p < .05$) than in CON (Figure 1). There was a significant main Trial × Time interaction effect ($p = .027$) and main effect of time ($p < .001$) and trial ($p = .001$) for plasma oxidized LDLs (Figure 2). Values were significantly lower in DC at Pre-Ex, Post-Ex, and Post-Exh (all $p < .001$) than in CON.

Plasma Hormones

Plasma insulin concentration decreased Post-Ex (main effect of time, $p < .001$), plasma glucagon increased Post-Ex (main effect of time, $p < .001$), and adrenocorticotropic hormone concentration increased Post-Ex and Post-Exh (main effect of time, $p < .001$) compared with Pre-Ex. The overall temporal responses on these values were the same between trials (Table 4). Plasma cortisol concentration was not significantly affected by exercise or by trial (Table 4).

Plasma Metabolites

Plasma glucose concentration decreased at 1 hr Post-Exh compared with Pre-Ex (main effect of time, $p < .001$), and plasma lactate concentration increased Post-Ex and Post-Exh (main effect of time, $p < .001$). The temporal responses were not different between trials (Table 5). Plasma FFA concentration increased Post-Ex and Post-Exh compared with Pre-Ex in both trials. There was also a significant main effect of trial ($p = .038$), with higher FFA levels in DC than in CON (Figure 3). Plasma triglyc-

Table 3 Markers of Antioxidant Status Before and After Exercise, *M* (*SD*)

	Pre-Ex	Post-Ex	Post-Exh	1 hr Post-Exh	Main-effects *p* values, trial; time; Trial × Time[a]
Vitamin C (μM)					>.1; <.001; >.1
dark chocolate trial	54.8 (5.0)	58.3 (5.7)	62.9 (5.6)	61.9 (5.9)	
control trial	54.2 (6.1)	56.7 (6.8)	61.8 (6.2)	62.3 (5.6)	
Uric acid (μM)					>.1; <.001; >.1
dark chocolate trial	352 (63)	375 (73)	417 (110)	424 (83)	
control trial	345 (55)	351 (53)	407 (80)	400 (72)	
Total antioxidant status (mM)					.07; <.001; >.1
dark chocolate trial	1.45 (0.09)	1.54 (0.14)	1.62 (0.15)	1.50 (0.11)	
control trial	1.35 (0.30)	1.48 (0.06)	1.58 (0.20)	1.45 (0.14)	

Note. Ex = exercise; Exh = exhaustion.

[a]Results from two-way ANOVA.

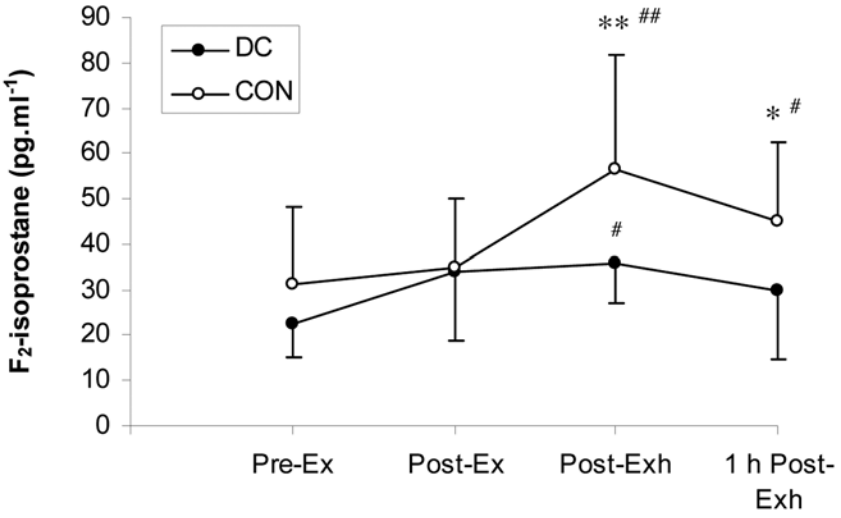

Figure 1 — F_2-isoprostane levels before and after exercise, *M* (*SD*). DC = dark chocolate; CON = control; Pre-Ex = preexercise; Post-Ex = postexercise; Post-Exh = postexhaustion. *Significantly different from CON, $p < .05$. **Significantly different from CON, $p < .001$. #Significantly higher than resting values within trial, $p < .05$. ##Significantly higher than resting values within trial, $p < .001$.

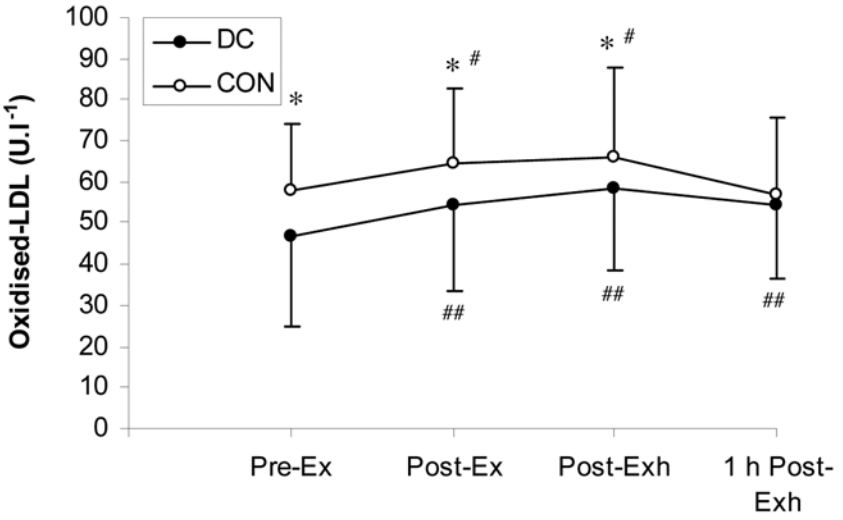

Figure 2 — Oxidized low-density-lipid (LDL) levels before and after exercise, *M* (*SD*). DC = dark chocolate; CON = control; Pre-Ex = preexercise; Post-Ex = postexercise; Post-Exh = postexhaustion. *Significantly different from CON, $p < .001$. #Significantly higher than resting values within trial, $p < .05$. ##Significantly higher than resting values within trial, $p < .001$.

Table 4 Circulating Hormones Before and After Exercise, *M* (*SD*)

	Pre-Ex	Post-Ex	Post-Exh	1 hr Post-Exh	Main-effects *p* values, trial; time; Trial × Time[a]
Insulin (mU/L)					>.1; <.001; .071
dark chocolate trial	7.3 (4.0)	4.3 (4.2)	3.6 (4.3)	1.8 (1.3)	
control trial	4.9 (3.5)	2.6 (2.2)	3.4 (3.1)	1.6 (1.2)	
Glucagon (pg/ml)					>.1; <.001; >.1
dark chocolate trial	337 (190)	411 (249)	415 (237)	352 (207)	
control trial	356 (261)	402 (200)	432 (277)	359 (206)	
Adrenocorticotropic hormone (pg/ml)					>.1; <.001; >.1
dark chocolate trial	34.1 (21.2)	62.0 (41.8)	88.4 (59.4)	22.9 (15.4)	
control trial	35.4 (27.8)	60.9 (37.5)	88.0 (44.6)	19.4 (11.3)	
Cortisol (nM)					>.1; .094; >.1
dark chocolate trial	365 (103)	377 (136)	391 (137)	359 (109)	
control trial	370 (94)	406 (123)	422 (109)	351 (94)	

Note. Ex = exercise; Exh = exhaustion.

[a]Results from two-way ANOVA.

erides were higher at Post-Ex and Post-Exh than Pre-Ex on both trials (main effect of time, $p < .001$) but did not differ significantly between trials (Figure 4).

Circulating Leukocytes and Cytokines

Circulating leukocyte count ($p = .001$), neutrophil count ($p < .001$), plasma IL-6 concentration ($p < .001$), and IL-10 concentration ($p < .001$) all increased after exercise and remained elevated throughout the exercise protocol (Table 6). However, these temporal patterns did not differ between trials. Plasma IL-1ra (Table 6) was elevated at Post-Exh and 1 hr Post-Exh compared with Pre-Ex (main effect of time, $p < .001$), with no difference between trials.

Discussion

The main findings of the study were that chronic consumption of DC daily for 2 weeks before an acute bout of prolonged exhaustive exercise resulted in lower resting and postexercise plasma levels of the oxidative stress markers F_2-isoprostanes and oxidized LDL but had no effect on cytokine responses. Dark chocolate ingestion was associated with greater rises in plasma FFA during exercise and a tendency for a lower RER (suggesting a higher contribution of fat oxidation to energy expenditure). However, regular consumption of DC had no significant effect on time to fatigue compared with isocarbohydrate-fat control.

Table 5 Circulating Glucose and Lactate Before and After Exercise, *M* (*SD*)

	Pre-Ex	Post-Ex	Post-Exh	1 hr Post-Exh	Main-effects *p* values, trial; time; Trial × Time[a]
Glucose (mM)					>.1; <.001; >.1
dark chocolate trial	4.97 (0.50)	4.82 (0.78)	4.85 (0.90)	4.53 (0.78)	
control trial	5.05 (0.40)	4.84 (0.52)	5.06 (0.92)	4.58 (0.44)	
Lactate (mM)					>.1; <.001; >.1
dark chocolate trial	1.2 (0.3)	3.1 (1.2)	8.2 (1.7)	1.2 (0.4)	
control trial	1.2 (0.2)	3.3 (1.1)	8.1 (1.8)	1.2 (0.3)	

Note. Ex = exercise; Exh = exhaustion.

[a]Results from two-way ANOVA.

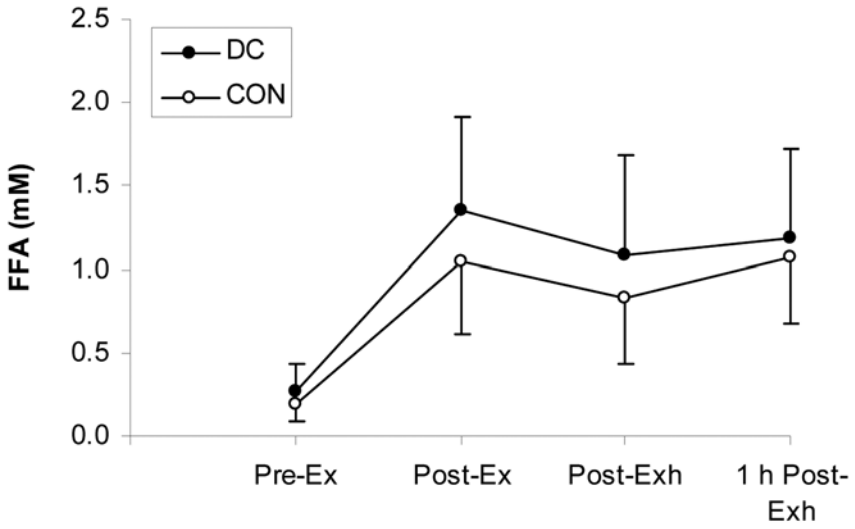

Figure 3 — Free-fatty-acid (FFA) concentration before and after exercise, *M* (*SD*). Significant main effect of trial, $p = .038$. DC = dark chocolate; CON = control; Pre-Ex = preexercise; Post-Ex = postexercise; Post-Exh = postexhaustion.

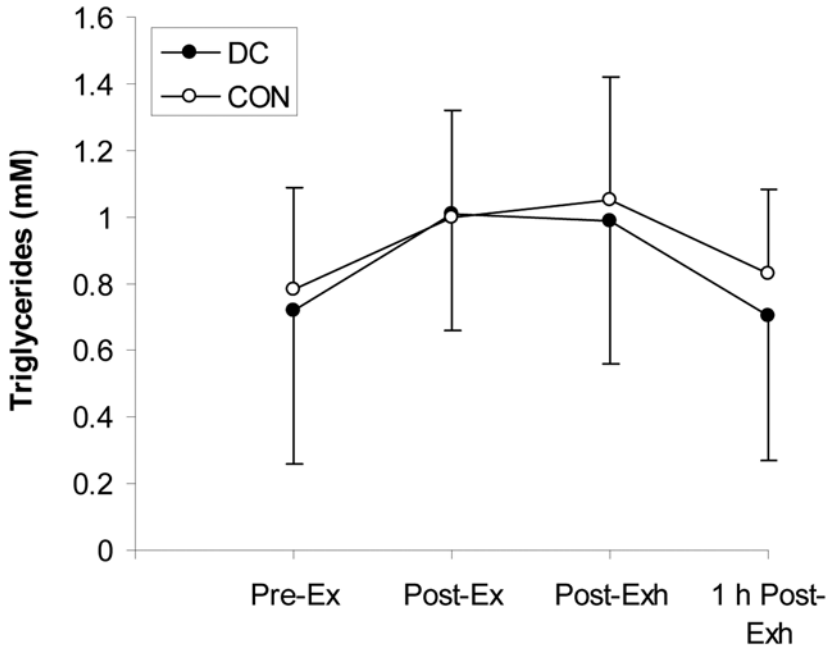

Figure 4 — Triglyceride concentration before and after exercise, *M* (*SD*). DC = dark chocolate; CON = control; Pre-Ex = preexercise; Post-Ex = postexercise; Post-Exh = postexhaustion.

Table 6 Circulating Leukocyte Counts and Plasma Cytokines Before and After Exercise, *M* (*SD*)

	Pre-Ex	Post-Ex	Post-Exh	1 hr Post-Exh	Main-effects *p* values, trial; time; Trial × Time[a]
Leukocyte count ($\times 10^9$/L)					>.1; <.001; >.1
dark chocolate trial	5.8 (1.6)	10.9 (3.9)	13.8 (5.0)	11.4 (4.4)	
control trial	5.5 (1.6)	10.3 (3.7)	13.2 (4.3)	11.3 (3.4)	
Neutrophil count ($\times 10^9$/L)					>.1; <.001; >.1
dark chocolate trial	2.8 (1.2)	6.6 (3.3)	8.3 (4.1)	8.8 (3.9)	
control trial	2.7 (0.9)	6.3 (2.7)	7.9 (3.3)	8.8 (3.0)	
IL-6 (pg/ml)					>.1; <.001; >.1
dark chocolate trial	1.7 (2.3)	3.0 (2.1)	3.9 (1.8)	2.7 (2.2)	
control trial	1.8 (2.2)	3.2 (2.4)	4.2 (2.7)	2.7 (2.0)	
IL-10 (pg/ml)					>.1; .031; .053
dark chocolate trial	1.9 (1.7)	2.7 (1.5)	2.8 (1.9)	3.7 (2.7)	
control trial	1.8 (1.2)	2.3 (1.2)	2.9 (1.5)	2.5 (1.9)	
IL-1ra (pg/ml)					>.1; <.001; >.1
dark chocolate trial	320 (180)	402 (187)	502 (220)	1067 (895)	
control trial	267 (75)	348 (115)	438 (170)	1081 (845)	

Note. Ex = exercise; Exh = exhaustion.

[a]Results from two-way ANOVA.

The current results show a blunting of the oxidative-stress marker F_2-isoprostane in response to prolonged exercise with DC consumption. F_2-isoprostane is considered a reliable and sensitive marker of lipid peroxidation (Roberts & Morrow, 2000). These findings are in line with those of Wiswedel et al. (2004), who observed a lower level of plasma F_2-isoprostane after a high-flavanol (187 mg flavanols/100 ml) cocoa drink than with a low-flavanol cocoa drink (14 mg flavanols/100 ml). However, they observed no effect of exercise on oxidative stress, which was most likely because of the relatively short duration of the exercise bout (29 min). Furthermore, a lower F_2-isoprostane response has been observed with vitamin C and E consumption after 3 hr of knee-extensor exercise at 50% of maximal power output (Fischer et al., 2004) and after a 50-km ultramarathon (Mastaloudis, Morrow, Hopkins, Devaraj, & Traber, 2004). In the current study, the reduced F_2-isoprostane response with DC consumption was paralleled by lower levels of oxidized LDL. Decreased rates of ex vivo LDL oxidation have been shown after consumption at rest of flavanol-rich chocolate (Osakabe et al., 2001; Wan et al., 2001). Although these results indicate a chronic effect of DC on oxidative stress both at rest and in response to exercise, one cannot exclude a possible acute effect of the 40 g of DC ingested 2 hr before exercise.

DC resulted in an increase in FFA concentration postexercise, indicating an increase in adipocyte lipolysis. These effects most likely occur via the inhibition of catechol O-methyltransferase, an enzyme that degrades noradrenaline, which may prolong adrenergic drive and increase lipolysis (Dulloo et al., 1999).Venables et al. (2008) demonstrated that three capsules of green tea (890 mg polyphenols and 366 mg epigallocatechin gallate) ingested in the 24-hr period before 30 min of exercise at 60% VO_{2max} resulted in higher levels of plasma FFA with a concomitant increase in fat oxidation. RER values measured during exercise tended to be lower with DC in the current study, suggesting an increase in fat oxidation to energy expenditure. However, because FFA concentration was only measured in the postexercise period, a direct comparison between these two parameters cannot be made. An increase in FFA mobilization during prolonged exercise after dark chocolate consumption could be viewed as beneficial to those who may be exercising as part of a weight-loss program, in addition to athletes attempting to conserve carbohydrate stores. It is worth noting that both caffeine and theobromine can independently promote lipolysis and increase FFA concentration (Durham, Miller, Lindeman, & Lapachet, 1993). Furthermore, catechins are suggested to act synergistically with caffeine to enhance fat oxidation (Dulloo, Seydoux, Girardier, Chantre, & Vandermander, 2000). Because the DC contained low levels of caffeine (41.6 mg) and theobromine (267 mg) that were ingested 2 hr before exercise, it is not possible to determine whether caffeine, theobromine, or polyphenol content, either in isolation or in combination, was responsible for the effect on FFA.

Previous studies have shown enhanced antioxidant capacity after DC consumption (Rein et al., 2000; Serafini et al., 2003; Wang et al., 2000). Several components of plasma contribute to plasma TEAC. Albumin and uric acid are thought to account for two thirds of the plasma TEAC. Many proteins such as ceruloplasmin and transferrin and small antioxidant molecules such as nonprotein thiols and vitamins C and E account for the remainder of the plasma TEAC. Ingestion of polyphenol-containing foods could raise the plasma polyphenol

content, although this may not be enough to affect the TEAC value because of the overwhelming contribution of the other plasma components. However, ingestion of polyphenols or indeed other components in the chocolate may affect the TEAC through changes in the endogenous plasma components. This may explain the lack of significant difference in plasma antioxidant capacity between treatments observed in the current study. Furthermore, the physiological relevance of these tests in plasma is currently controversial (Sies, 2007).

There are currently limited data in human studies to suggest that antioxidant supplementation enhances exercise performance through preventing oxidative mechanisms and muscle fatigue (Powers et al., 2004). N-acetylcysteine supplementation acutely (<1 hr) before exercise and during exercise delayed time to fatigue by ~24% at 91% VO_{2max} after 45 min submaximal cycling at 71% VO_{2max} (McKenna et al. 2006), and 7 days of quercetin supplementation increased VO_{2max} (~3.9%) and cycling time (~13.2%) to fatigue at 75% VO_{2max} in untrained participants (Davis et al., 2010). There was no significant effect of dietary DC intake on exercise performance in the current study. In fact, some evidence suggests that antioxidant supplementation may inhibit some of the exercise-induced cellular signaling that up-regulates adaptations in vascular tissue and skeletal muscle (Sen, 2001), which may limit exercise performance (Malm, Svensson, Ekblom, & Sjodin, 1997; Peake et al., 2007).

Chronic daily ingestion of vitamin C or combined vitamin C and E supplements over several weeks have been shown to decrease the IL-6 and cortisol responses to prolonged exercise (Fischer et al., 2004; Vassilakopoulos et al., 2003). However, Nieman et al. (2004) reported no effect of daily vitamin E supplements over 2 months on cortisol and IL-6 compared with placebo after a competitive triathlon event. Furthermore, Mastaloudis et al. (2004) found no effect of 6 weeks of vitamin C and E supplementation on IL-6 after a 50-km ultramarathon, despite preventing increases in plasma lipid peroxidation. In the current study chronic consumption of DC had no effect on plasma adrenocorticotropic hormone, cortisol, IL-6, or IL-1ra concentrations after prolonged exercise, and in line with Mastaloudis et al., this indicates that oxidative damage and the inflammatory response to exercise operate independently (Nieman et al., 2002).

Oxidative stress has been implicated in the depression of immune-cell function that typically occurs after prolonged exercise, the magnitude of which may be decreased with appropriate preexercise antioxidant supplementation (Davison & Gleeson, 2006; Lee & Wan, 2000; Robson, Bouic, & Myburgh, 2003). However, there was no significant effect of DC consumption on circulating leukocyte or neutrophil numbers at rest or in response to exercise. Leukocytosis is influenced by both cortisol and some cytokines (IL-6), and because there was no effect of DC consumption on these responses in the current study, it is perhaps not surprising that there was no effect on neutrophil numbers. The finding of a reduction in oxidative-stress markers with DC with no effect on leukocytes also suggests that these effects are a result of a direct "antioxidant" effect and not via other indirect mechanisms occurring in blood.

In conclusion, the current study demonstrates that DC consumption daily for 2 weeks and 2 hr before prolonged exercise has significant effects on oxidative-stress markers and the temporal response of plasma FFA. However, there were no effects on the plasma concentration of measured hormones, cytokines, or circulating numbers of total leukocytes and neutrophils or on exercise performance.

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