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Effects Of Quercetin And EGCG On Mitochondrial Biogenesis And Immunity

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Abstract:
Purpose: To test the influence of 1000 mg of quercetin (Q) with or without 120 mg of epigallocatechin 3-gallate (EGCG), 400 mg of isoquercetin, and 400 mg of eicosapentaenoic acid and docosahexaenoic acid (Q–EGCG) on exercise performance, muscle mitochondrial biogenesis, and changes in measures of immunity and inflammation before and after a 3-d period of heavy exertion. Methods: Trained cyclists (N = 39) were randomized to placebo (P), Q, or Q–EGCG and ingested supplements in a double-blinded fashion for 2 wk before, during, and 1 wk after a 3-d period in which subjects cycled for 3 hIdj1 at È57% Wmax. Blood, saliva, and muscle biopsy samples were collected before and after 2 wk of supplementation and immediately after the exercise bout on the third day. Blood and saliva samples were also collected 14 h after exercise. Results: Two-week supplementation resulted in a significant increase in plasma quercetin for Q and Q–EGCG and granulocyte oxidative burst activity (GOBA) in Q–EGCG. Immediately after the third exercise bout, significant decreases for C-reactive protein (CRP), and plasma interleukin 6 (IL-6) and interleukin 10 (IL-10) were measured in Q–EGCG compared with P. Granulocyte colony-stimulating factor and CRP were reduced in Q–EGCG 14 h after exercise. No group differences were measured in muscle messenger RNA expression for peroxisome proliferator-activated receptor F coactivator >, citrate synthase, or cytochrome c. Conclusions: Two-week supplementation with Q–EGCG was effective in augmenting GOBA and in countering inflammation after 3 d of heavy exertion in trained cyclists.

The flavonoids are a group of diverse, low-molecular weight polyphenolic substances that occur naturally in plant foods. The structural complexity of flavonoids has led to their subclassification as flavonols, flavones, flavanones, flavan-3-ols (and their oligomers, proanthocyanidins), isoflavones, and anthocyanins (34). They are present in significant amounts in many commonly consumed fruits, vegetables, grains, herbs, and beverages such as tea and fruit juices (7). Flavonoids are strong antioxidants *in vitro*, mainly because of their low redox potential and their capacity to donate several electrons or hydrogen atoms.

Despite the strong antioxidant capacity of flavonoids, their antioxidant efficacy *in vivo* is limited by low absorption, short half-lives, and extensive first-pass metabolism (2,22). In the intestinal mucosa and liver, flavonoids undergo glucuronidation, methylation, and sulfation. This biotransformation alters the physical properties of flavonoids, making them more water soluble with weakened antioxidant and anti-inflammatory activity (2,3,20,33).

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one; molecular mass 302.236 gImol^{j1}) is a flavonol that constitutes the aglycone of the plant glycosides rutin and quercitrin. Of all flavonoids, quercetin is among the most widespread, with broad-spectrum bioactive effects (2). These include anti-inflammatory (3,6,13,20), antipathogenic (9,31,36), and antioxidant activities (2,5,19); immunoregulatory influences (13,33); and central nervous system (1) and muscle mitochondrial biogenesis stimulatory effects (8). Common food sources of quercetin in the United States and in Europe are onions, apples, and various types of berries (7,22). Human subjects can absorb significant amounts of quercetin, with a reported half-life ranging from 3.5 to 28 h (22,25). Long-term high-dose quercetin supplementation in rodents and humans has not been linked to any adverse effects (14,35), and epidemiological studies indicate that higher compared with lower quercetin intake from food is associated with reduced risk for ischemic heart disease, type 2 diabetes, asthma, and various types of cancer, including lung, colorectal, pancreatic, and prostate cancers (2,27).

Endurance athletes undergoing repeated physiologic stress from training and competition may receive benefit

from quercetin supplementation. Animal and human data indicate that quercetin improves endurance performance and lowers infection risk (8,21,31). In two studies with human athletes, however, 3 wk of 1000-mg quercetin·d^{j1} supplementation failed to counter exercise-induced alterations in immunity, inflammation, and oxidative stress (15,23,29–31). One potential weakness of these human studies was that the quercetin supplements were ingested 10–24 h before the completion of exercise, a period that may have been too long given the half-life of quercetin (25). Also, there is increasing support for coingestion of quercetin with other flavonoids and food components to improve and extend quercetin's bioavailability and bioactive effects (4,5,16,17,24,36). These include the flavonoid epigallocatechin 3-gallate (EGCG) from tea (12,24,26), isoquercetin (quercetin-3-glucose or hirsutrin), which is the glycosylated form of quercetin in onions and other foods (2,4,22), N_3-polyunsaturated fatty acids (N_3-PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (4), and the nutrients vitamin C and folate (17,36).

This study extends our previous work in endurance athletes by testing the influence of a quercetin supplement with or without EGCG, isoquercetin, EPA, DHA, vitamin C, and folic acid on chronic and acute measures of immune function, inflammation, and mitochondrial biogenesis before and after a 3-d period of intensified exercise. Supplements were ingested twice daily for 2 wk before, during, and 1 wk after the 3-d period of exercise, with the last daily dose taken 1 h before exercise to ensure high plasma quercetin levels. The outcome measures chosen for this study are similar to those used in previous quercetin projects by our research group to allow comparisons of results (15,29,30).

METHODS

Subjects. Thirty-nine trained male ($n = 32$) and female ($n = 7$) cyclists were recruited and tested as experimental subjects through local and collegiate cycling clubs. Written informed consent was obtained from each subject, and the experimental procedures were approved by the institutional review board of Appalachian State University.

Research design. Within 1 wk before the start of supplementation, subjects reported to the university's Human Performance Lab for orientation and measurement of cardiorespiratory fitness. V̇O$_{2max}$ was determined using a graded maximal protocol (25-W increase every 2 min starting at 150 W), with the subjects using their own bicycles on CompuTraineri Pro Model 8001 trainers (RacerMate, Seattle, WA). Oxygen uptake and ventilation were measured using the MedGraphics CPX metabolic system (MedGraphics Corporation, St. Paul, MN). HR was measured using a chest HR monitor (Polar Electro, Inc, Woodbury, NY). Basic demographic and training data were obtained through a questionnaire. Body composition was measured using hydrostatic weighing as previously described (31).

Subjects agreed to avoid the use of large-dose vitamin/mineral supplements (more than 100% of recommended dietary allowances), herbs, and medications known to affect immune function during the entire study. During orientation, a dietitian instructed the subjects to follow a diet moderate in carbohydrate (using a food list) during the weekend before and the 3-d intensified exercise period. At 12:00–12:30 p.m. before each 3-h cycling bout, subjects ingested a standardized liquid meal (Boost Plus; Mead Johnson Nutritionals, Evansville, IN) at an energy level of 63 kJ·kg^{j1} (15 kcal·kg^{j1}). Boost Plus is a nutritionally complete, high-energy oral supplement with an energy density of 6.4 kJ·mL^{j1} (1.52 kcal·mL^{j1}) and 16% of energy as protein, 34% as fat, and 50% as carbohydrate. In quantities of 1000 mL, Boost Plus exceeds daily value recommendations for all major vitamins and minerals. No other food and beverage (other than water) was consumed from this meal until the end of the cycling bout. Subjects ingested 0.5 to 1 L of water per hour of cycling.

The cyclists were randomized to quercetin (Q), quercetin–EGCG (Q–EGCG), or placebo (P) groups. Under double-blind procedures, subjects ingested four P, Q, or Q–EGCG soft chews per day (two in the morning at 7:00–8:00 a.m. and two in the afternoon at 2:00 p.m.) for 14 d before, during a 3-d period of intensified exercise, and 7 d after the training period (thus, a total of 24 d). The soft chew supplements were designed by Quercegen Pharma (Newton, MA) and prepared by Nutravail Technologies (Chantilly, VA). Each placebo soft chew contained brown rice syrup, evaporated cane juice, carnuba wax, natural flavors, gelatin, soy lecithin, palm oil, glycerin, xylitol, monoglyceride and diglyceride, corn starch, carrageenan, sucralose, and 20-kcal energy with citric acid to substitute for taste of vitamin C and FD&C yellow no. 5 and FD&C blue no. 1 to substitute for the quercetin color. Each Q-chew contained all P ingredients with 250 mg of quercetin, 250 mg of vitamin C, 10 mg of niacinamide, and 200 Kg of folic acid. These nutrients were included in the Q-chew to facilitate quercetin absorption in the small intestine (personal communication; Quercegen Pharma). Each Q–EGCG chew contained all Q-chew ingredients with 30 mg of EGCG from green tea extract, 100 mg of isoquercetin, and 100 mg of N_3-PUFA (55 mg of EPA and 45 mg DHA) from fish oil. These food components were included to improve quercetin bioavailability and extend its bioactive effects, as determined by experiments conducted by Quercegen Pharma (personal communication).

Before and after the first 14 d of supplementation, subjects provided blood and saliva samples at 8:00 a.m. after an overnight fast and having avoided exercise training for at least 12 h and before having ingested supplements. Subjects then came to the laboratory for three consecutive days and cycled from 3:00 to 6:00 p.m. at È57% W$_{max}$. During the test sessions, experimental subjects cycled using their own bicycles on CompuTraineri Pro Model 8001 trainers (RacerMate) with the exercise load set at È57% W$_{max}$, as in a previous study conducted within our

laboratory (31). Metabolic measurements were made every 30 min of cycling using the MedGraphics CPX metabolic system (MedGraphics Corporation) to verify workload. Time trials (5, 10, and 20 km on days 1, 2, and 3, respectively) were inserted at the end of each cycling bout, with distances and workload monitored using CompuTraineri MultiRider software (v.3.0; RacerMate). Blood and saliva samples were obtained immediately after completing the third 3-h exercise bout (6:00 p.m.) and then again 14 h later (8:00 a.m., overnight fasted, and having avoided supplements since 2:00 p.m. the previous day). Subjects continued ingestion of the supplements for seven additional days while training normally and being monitored for delayed onset of muscle soreness (DOMS). Subjects recorded muscle soreness during the 3 d of cycling and for a week afterward using a 10-point Likert scale (32). Cyclists were asked to supply a number that best described any general feeling of painful, sore, aching leg muscles using this scale: 1 (no soreness), 2.5 (dull, vague ache), 4 (slight soreness), 5.5 (more than slight soreness), 7 (sore), 8.5 (very sore), and 10 (unbearably sore).

Blood samples, C-reactive protein, and creatine kinase. Blood samples were drawn from an antecubital vein with subjects in the supine position. Routine complete blood counts were performed by our clinical hematology laboratory using a Coulter STKS instrument (Coulter Electronics, Hialeah, FL) and provided hemoglobin and hematocrit for the determination of plasma volume change using the method of Dill and Costill (10). Other blood samples were centrifuged in sodium heparin or Ethylene diamine Tetra-acetic Acid (EDTA) tubes, and plasma was aliquoted and then stored at $j80$-C before plasma cytokine analysis. Serum C-reactive protein (CRP), and creatine kinase (CK) were measured in a clinical laboratory using an LX-20 clinical analyzer (Beckman, Brea, CA).

Plasma quercetin. Total plasma quercetin (quercetin and its primary conjugates) was measured after solid-phase extraction via reversed-phase high performance liquid chromatography (HPLC) with UV detection as previously described (29 – 31). Quercetin conjugates were hydrolyzed by incubating 500 KL of plasma aliquots with 10 KL of 10% DL-dithiothreitol solution, 50 KL of 0.58 M acetic acid, and 50 KL of a mixture of A-glucuronidase/arylsulfatase and crude extract from *Helix pomatia* (Roche Diagnostics Corporation, Indianapolis, IN) for 2 h at 37-C. Chromatographic analysis was performed using the Ultimate 3000 HPLC-PDA system (Dionex Corporation, Sunnyvale, CA) with a Gemini C18 column (Phenomenex, Torrance, CA). On the basis of the duplicate analysis of three quality control samples, with human plasma samples spiked with quercetin at concentrations of 1.0, 1.5, and 3.0 $KmolIL^{j1}$, the intra-assay coefficient of variation (CV) was 12.5%.

Plasma cytokine, heat shock protein 70, and myeloperoxidase measurements. Enzyme-linked immunosorbent assays were used, in accordance with the manufacturer protocol, to measure total plasma con-

centrations of interleukin 1 receptor antagonist (IL-1RA), interleukin 6 (IL-6 and IL-6 high sensitivity), granulocyte colony-stimulating factor (G-CSF and G-CSF high sensitivity), granulocyte monocyte colony-stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP-1), myeloperoxidase (MPO), tumor necrosis factor > (TNF->), IL-10 (R&D Systems, Inc., Minneapolis, MN), IL-10 ultrasensitive (BioSource International, Inc., Camarillo, CA), and heat shock protein 70 (HSP-70; Assay Designs, Inc, Ann Arbor, MI). All samples and provided standards were analyzed in duplicate. High-sensitivity kits were used to analyze all TNF-> and preexercise and recovery IL-6, IL-10, and G-CSF samples. The minimum detectable concentrations of IL-1RA, IL-6, IL-6 (high sensitivity), IL-10, IL-10 (high sensitivity), G-CSF, G-CSF (high sensitivity), MCP-1, TNF->, GM-CSF, MPO, cortisol, and HSP-70 were $G2.2$ $pgImL^{j1}$, $G0.7$ $pgImL^{j1}$, $G0.039$ $pgImL^{j1}$, $G3.9$ $pgImL^{j1}$, $G0.2$ $pgImL^{j1}$, $G20$ $pgImL^{j1}$, $G0.80$ $pgImL^{j1}$, $G5.0$ $pgImL^{j1}$, $G0.106$ $pgImL^{j1}$, $G0.26$ $pgImL^{j1}$, 0.100 $ngImL^{j1}$, 0.1 $KgIdL^{j1}$, and 0.09 $ngImL^{j1}$, respectively. Preexercise and postexercise samples were analyzed on the same assay plate to decrease interkit assay variability, and the intra-assay CV for all variables was less than 10%. Data were analyzed with SOFTmax software (Molecular Devices, Sunnyvale, CA).

Whole-blood polymorphonuclear oxidative burst activity assay. Polymorphonuclear (PMN) oxidative burst activity was measured in whole blood according to the procedures previously published by Nieman et al. (31). The mean fluorescence number was determined, and the PMN oxidative burst activity was calculated by subtracting the mean fluorescence intensity of the endogenous respiratory burst activity from the mean fluorescence intensity of the stimulated respiratory burst activity. The intra-assay CV was less than 15% for each time point.

Salivary immunoglobulin A. Saliva was collected and stored, and volume and total protein were measured as previously described (31). Salivary immunoglobulin A (sIgA) concentrations were measured using a quantitative indirect competitive immunoassay provided by Salimetrics, LLC (State College, PA). All samples and standards were analyzed in duplicate, and the intra-assay CV was less than 10% for sIgA concentration across all time points. The minimum detectable concentration for the assay was $G2.5$ $KgImL^{j1}$. Data are expressed as concentration of sIgA relative to total protein concentration ($KgImg^{j1}$).

Muscle biopsies. Muscle biopsies were obtained from the vastus lateralis before and after 2 wk of supplementation. The postsupplementation biopsy was obtained È2 cm proximal from the presupplementation biopsy site. Muscle samples were collected from the opposite leg after exercise, with leg order randomly determined. Local anesthesia (2% xylocaine) was injected subcutaneously and intramuscularly. After a small incision (È0.5 cm), a muscle biopsy sample (È100 mg) was obtained using the percutaneous needle biopsy procedure modified to include suction (11).

Variable	P ($n = 12$)	Q ($n = 13$)	Q–EGCG ($n = 14$)	P
Baseline measures				
Age (yr)	26.3 ± 1.7	26.8 ± 2.6	28.1 ± 2.8	0.876
Body mass (kg)	72.0 ± 4.0	72.6 ± 3.8	71.8 ± 2.9	0.987
$\dot{V}O_{2max}$ (mL·kg^{-1}·min^{-1})	60.2 ± 1.8	61.4 ± 2.3	60.3 ± 2.2	0.906
Power$_{max}$ (W)	308 ± 12.2	301 ± 16.9	321 ± 16.5	0.628
HR$_{max}$ (beats·min^{-1})	189 ± 2.7	187 ± 2.4	184 ± 2.0	0.261
Body composition (% fat)	15.0 ± 1.7	16.2 ± 1.0	14.3 ± 1.5	0.630
Performance measures				
Mean power (W)	174 ± 7.2	173 ± 9.9	182 ± 8.7	0.720
Power (% W$_{max}$)	56.5 ± 0.5	57.5 ± 0.6	56.6 ± 0.6	0.458
Mean HR (beats·min^{-1})	147 ± 3.8	145 ± 1.4	144 ± 2.2	0.576
HR (% HR$_{max}$)	78.0 ± 1.1	77.7 ± 0.9	78.6 ± 1.1	0.818
Mean $\dot{V}O_2$ (mL·min^{-1})	2670 ± 92	2661 ± 134	2721 ± 133	0.931
$\dot{V}O_2$ (% $\dot{V}O_{2max}$)	62.3 ± 1.7	60.7 ± 2.3	62.8 ± 1.1	0.679
Time trials (min)	43.8 ± 1.0	43.4 ± 0.9	42.9 ± 1.5	0.860

Performance measures represent the mean of three exercise sessions.
$\dot{V}O_2$, volume of oxygen consumption.

Muscle was trimmed of connective tissue and fat and was immediately frozen in liquid nitrogen. Samples were stored at j80-C until subsequent analysis.

Muscle RNA isolation and complementary DNA synthesis. Skeletal muscle was homogenized under liquid nitrogen with a micropestle, and total RNA was extracted by using the guanidine thiocyanate method with TRIzol (Invitrogen, Carlsbad, CA). The extracted RNA was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at 260 nm. Intact RNA was confirmed by denaturing agarose gel (1%) electrophoresis. RNA was reverse transcribed using the high-capacity complementary DNA (cDNA) reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

Quantitative real-time polymerase chain reaction analysis. Briefly, 25 ng of cDNA was used in each reaction (TaqMan Gene Expression Assays; Applied Biosystems) as per manufacturer's instructions. Relative expression levels of citrate synthase, cytochrome *c* oxidase I, peroxisome proliferator-activated receptor F coactivator > (PGC-1>), and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were determined with predesigned, preoptimized gene-specific probe sets provided by Applied Biosystems (TaqMan Gene Expression Assays). Samples were loaded in a MicroAmp 96-well reaction plate and run in triplicate. Quantitative real-time polymerase chain reaction was performed on an ABI 7300 sequence detector. Data were analyzed using Applied Biosystems 7300 sequence detection software v1.3.1. Relative messenger RNA (mRNA) levels were determined using the $\$\$C_t$ method, with *GAPDH* serving as the as the endogenous control (18).

Statistical analysis. Data are expressed as mean ± SE. Data in Tables 1 and 4 were compared between groups using one-way ANOVA. Data in Tables 2 and 3 and in Figures 1 to 4 were analyzed using a 3 (groups) × 4 (time points) repeated-measures ANOVA. The DOMS data in Figure 4 were analyzed using a 3 × 10 repeated-measures ANOVA. When Box's *M* suggested that the assumptions necessary for the univariate approach were not tenable, the multivariate approach to repeated-measures ANOVA was used (Pillai's trace). When interaction effects were P G 0.30 (with this level chosen because of a mixture of chronic and acute changes across the four time points), the changes before and after at a given time point were calculated and compared between groups using Student's *t*-tests, with significance set at P e 0.05. This data analysis allowed group comparisons for three different effects: 2-wk chronic supplementation (before and after supplementation, 8:00 a.m., overnight-fasted), acute postexercise changes, and 14 h postexercise recovery.

RESULTS

Subject characteristics and performance data for the 39 cyclists randomized to P ($n = 10$ males, 2 females), Q ($n = 11$ males, 2 females), and Q – EGCG ($n = 11$ males, 3 females) groups and completing all phases of the study are summarized in Table 1. Outcome data for male and female cyclists did not differ significantly and are presented together for each group.

TABLE 2. Blood leukocyte counts, CK, sIgA–protein ratio, and MPO.

Variable	Before Supplementation	After Supplementation	After Exercise	14 h after Exercise	P
Total blood leukocytes (10^9 L^{-1})					
P	5.25 ± 0.22	5.61 ± 0.28	12.30 ± 1.16	6.18 ± 0.30	0.110
Q	5.29 ± 0.30	5.23 ± 0.32	10.22 ± 0.62	5.44 ± 0.25*	
Q–EGCG	5.51 ± 0.31	5.42 ± 0.37	10.26 ± 0.67	5.42 ± 0.34*	
CK (U·L^{-1})					
P	150 ± 28.4	119 ± 14.5	347 ± 111	333 ± 109	0.672
Q	161 ± 33.3	129 ± 19.5	209 ± 39.9	189 ± 28.9	
Q–EGCG	149 ± 18.4	156 ± 28.2	379 ± 157	323 ± 101	
sIgA–protein (Kg·mg^{-1})					
P	278 ± 35.4	201 ± 39.7	127 ± 22.1	290 ± 35.8	0.694
Q	244 ± 36.3	199 ± 36.6	96.2 ± 11.9	263 ± 33.7	
Q–EGCG	272 ± 33.8	280 ± 31.1	172 ± 24.6	301 ± 31.4	
MPO (ng·mL^{-1})					
P	95.1 ± 18.2	69.7 ± 7.9	178 ± 20.4	90.1 ± 10.9	0.225
Q	90.0 ± 8.8	80.3 ± 9.9	132 ± 16.3	96.3 ± 20.8	
Q–EGCG	112 ± 19.9	58.5 ± 8.3	141 ± 33.8	86.6 ± 10.2	

P value represents group (3) × time (4) interaction. Significant time effects were shown for all variables listed (P G 0.001).
* P G 0.05, change from before supplementation compared with placebo.

TABLE 3. Plasma cytokine and HSP-70 levels before and after 2 wk of supplementation and immediately and 14 h after exercise.

Variable	Before Supplementation	After Supplementation	After Exercise	14 h after Exercise	P
G-CSF (pgImL^{j1})					
P	25.2 T 1.4	26.1 T 2.6	36.3 T 4.1	39.2 T 5.1	0.016
Q	25.2 T 1.2	26.6 T 1.7	34.2 T 3.4	27.1 T 1.8*	
Q–EGCG	26.1 T 2.7	29.0 T 4.4	27.2 T 3.5	24.0 T 1.8*	
IL-10 (pgImL^{j1})					
P	13.6 T 2.9	19.6 T 4.5	66.3 T 18.6	17.6 T 4.3	0.025
Q	15.1 T 4.4	11.2 T 2.0	31.5 T 4.4*	22.3 T 6.1	
Q–EGCG	19.4 T 5.4	22.1 T 6.7	33.6 T 5.6*	16.1 T 4.3	
MCP (pgImL^{j1})					
P	280 T 24.8	289 T 22.2	435 T 40.9	303 T 23.7	0.174
Q	309 T 16.8	282 T 21.1	411 T 26.2	296 T 19.4	
Q–EGCG	284 T 18.0	290 T 13.5	370 T 24.2	293 T 13.1	
IL-1RA (pgImL^{j1})					
P	183 T 10.7	167 T 11.2	569 T 169	276 T 47.2	0.630
Q	173 T 10.1	179 T 15.8	774 T 273	233 T 29.2	
Q–EGCG	191 T 27.8	226 T 61.9	458 T 161	181 T 15.2	
TNF-> (pgImL^{j1})					
P	3.38 T 0.58	2.61 T 0.26	2.37 T 0.40	2.65 T 0.41	0.427
Q	4.40 T 0.58	3.47 T 0.39	2.21 T 0.24	2.36 T 0.31	
Q–EGCG	4.21 T 0.81	3.44 T 0.53	2.76 T 0.42	2.84 T 0.42	
HSP-70 (ngImL^{j1})					
P	1.89 T 0.47	1.68 T 0.31	2.77 T 0.37	1.85 T 0.43	0.734
Q	1.88 T 0.37	1.99 T 0.44	2.35 T 0.28	1.76 T 0.32	
Q–EGCG	2.03 T 0.40	2.31 T 0.40	3.01 T 0.40	1.77 T 0.32	

P value represents group (3) ✕ time (4) interaction. Significant time effects were shown for all variables listed (P G 0.001).

* P G 0.05, change from before supplementation compared with placebo.

No significant differences were found among groups for age, body composition, or maximal performance measures. Subjects (all combined) averaged 260 T 30.4 kmIwk^{j1} of cycling during the previous year and 0.65 T 0.04 h of training per day during the previous 18 d (off-season training). Thus, the 3-d intensified exercise period (9-h exercise) represented a 3.6-fold increase in training duration per bout at this time of the year and a substantial increase overall in exercise workload and intensity. Subjects in all groups were able to maintain a mean power output of 56.9 T 0.6% W_{max} at an oxygen consumption of È62% $\dot{V}$ O_{2max} during the exercise bouts (Table 1). Total time trial duration (sum of 5-, 10-, and 20-km trials conducted during the last portion of each 3-h bout) did not differ among groups (Table 1). Plasma volume change did not differ among groups after

exercise on the third day and averaged less than 2% because of ingestion of 0.5 – 1.0 L of water per day of exercise (data not shown).

After 2 wk of consuming two soft chew supplements at 8:00 a.m. and 2:00 p.m. each day, overnight-fasted plasma quercetin levels were 36% and 62% greater than the presupplementation levels in the Q and Q – EGCG groups, respectively, compared with a 24% decrease in P (Fig. 1). After the third day of exercise, plasma quercetin levels at 6:00 p.m. (i.e., 4 h after the last supplement dose) were 221% and 364% greater than the presupplementation levels in the Q and Q – EGCG groups, respectively. At 14 h after exercise, a significant 111% increase in plasma quercetin levels was measured in the Q – EGCG group.

Total blood leukocytes tended to be lower after exercise in the Q and Q – EGCG groups compared with P (both
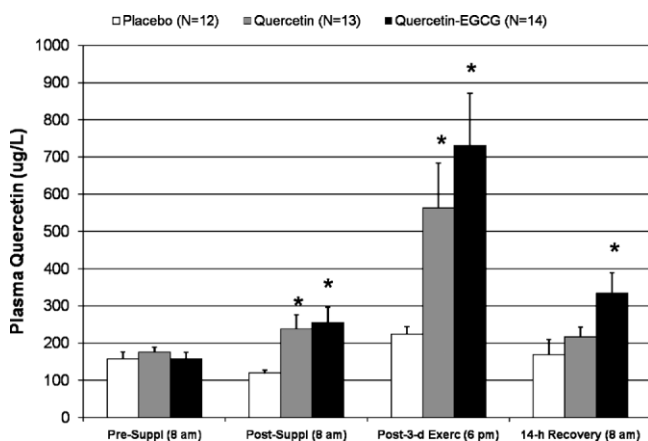

FIGURE 1—Plasma quercetin before and after 2 wk of supplementation, immediately after exercise after 3 d of cycling (3 hId^{j1}), and 14 h after exercise. Group ✕ time interaction P value = 0.008. *P G 0.05, change from before supplementation compared with placebo.
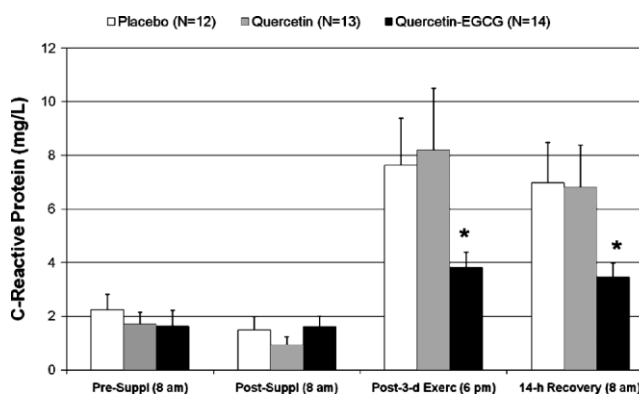

FIGURE 2—Serum CRP before and after 2 wk of supplementation and immediately and 14 h after exercise. Group ✕ time interaction P value = 0.023. *P G 0.05, change from before supplementation compared with placebo.
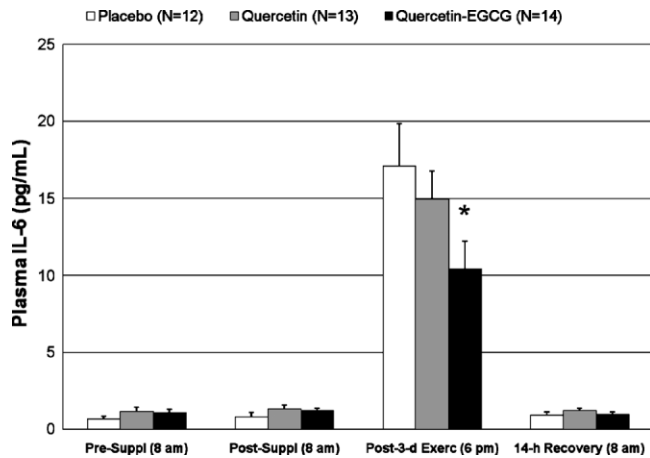
FIGURE 3—Plasma IL-6 before and after 2 wk of supplementation and immediately and 14 h after exercise. Group ✕ time interaction P value = 0.021. *P G 0.05, change from before supplementation compared with placebo.

P = 0.094), with significantly lower levels 14 h after exercise (Table 2). No significant group differences were found after 2 wk of supplementation or after exercise for CK, the ratio of sIgA to protein, or MPO.

Serum CRP was 50% less than P in the Q – EGCG group both immediately after exercise on the third day and 14 h after exercise (interaction P value = 0.023; Fig. 2). Plasma IL-6 was 39% less than P in the Q – EGCG group immediately after exercise (interaction P value = 0.021; Fig. 3). Plasma G-CSF was 25% less than P in the Q – EGCG group immediately after exercise (P = 0.087), with significant decreases measured in Q (31%) and Q – EGCG (39%) 14 h after exercise (Table 3). Plasma IL-10 was significantly less than P levels immediately after exercise in both Q and Q – EGCG groups (Table 3). Plasma MCP tended to be lower than P immediately after exercise in the Q – EGCG group (15%,
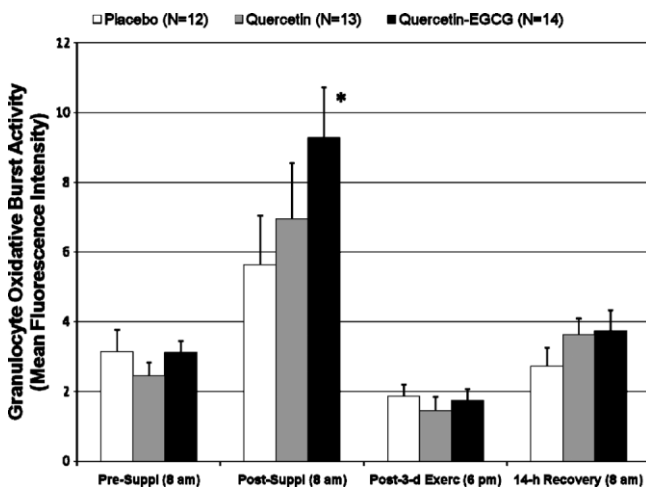
TABLE 4. Muscle mRNA expression for PGC-1>, cytochrome c oxidase, and citrate synthase.

Variable	Before Supplementation	After Supplementation	P
PGC-1>			
P	1	1.01 T 0.05	0.337
Q	1	1.00 T 0.02	
Q–EGCG	1	0.94 T 0.03	
Cytochrome c			
P	1	1.13 T 0.08	0.335
Q	1	1.08 T 0.05	
Q–EGCG	1	1.29 T 0.15	
Citrate synthase			
P	1	1.01 T 0.08	0.440
Q	1	1.06 T 0.06	
Q–EGCG	1	0.93 T 0.05	

P value represents one-way ANOVA F probability comparing after supplementation. Data are expressed as fold change from before supplementation (set as 1); n = 6 for each group.

P = 0.060). Groups did not differ at any time points for plasma IL-1RA, TNF->, and HSP-70 (Table 3). Figure 4 shows that granulocyte oxidative burst activity (GOBA) was significantly elevated greater than that in P after 2 wk of supplementation in the Q – EGCG group, with no group differences in measures after exercise (interaction P value = 0.168).

Table 4 summarizes skeletal muscle mRNA expression data for three genes related to mitochondrial biogenesis (n = 6 per group), with no differences measured between groups after 2 wk of supplementation or after exercise. Figure 5 shows that DOMS scores were significantly reduced 2 and 3 d after the 3-d cycling sessions in Q, with scores tending to be lower in the Q – EGCG group on these same 2 d (P = 0.158 and P = 0.104, respectively).

DISCUSSION

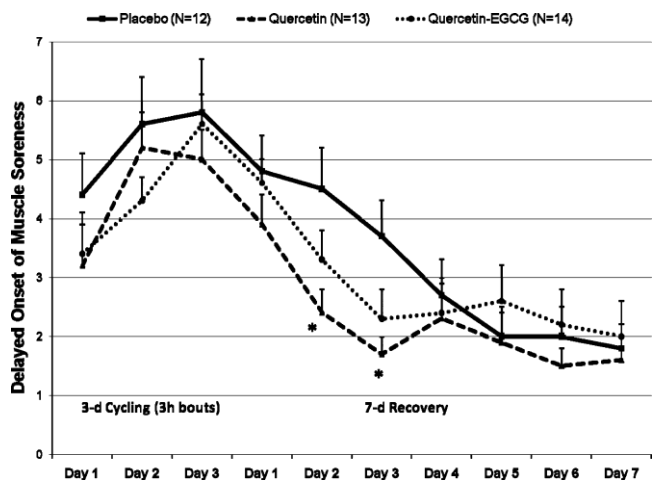The exercise-induced changes in immune and inflammation outcome measures reported in this study are similar to

FIGURE 4—GOBA before and after 2 wk of supplementation and immediately and 14 h after exercise. Group ✕ time interaction P value = 0.168. *P G 0.05, change from before supplementation compared with placebo.

FIGURE 5—DOMS at the end of the third 3-h cycling bout and during 1 wk of follow-up. Group ✕ time interaction P value = 0.252. *P G 0.05, change from before supplementation compared with placebo.

what we have previously reported under similar conditions (28,30,31). The data from this study indicate that a quercetin supplement combined with EGCG, isoquercetin, EPA, and DHA is more effective than a placebo or quercetin supplement without these added components in elevating plasma quercetin and countering exercise-induced inflammation. The Q‒EGCG supplement also increased GOBA after 2 wk of supplementation relative to placebo but did not counter the exercise-induced drop in this measure after 3 d of 3-h cycling bouts. Cycling time trial performance at the end of the 3-h cycling bouts or mRNA expression for genes related to skeletal muscle mitochondrial biogenesis did not differ between groups. DOMS 2 and 3 d after exercise tended to be lower in both quercetin groups compared with placebo.

In a previous study conducted by our research team using a similar exercise regimen, quercetin (1000 mgId^{j1} in a Tang beverage, 8:00 a.m. and 6:00 p.m. dosing) ingestion by cyclists for 3 wk did not alter exercise-induced increases in measures of inflammation and oxidative stress or alterations in immune outcomes (23,30,31). A second 3-wk quercetin supplementation study with ultramarathon runners at the 160-km Western States Endurance Run (WSER) produced similar null results when compared with placebo (15,29). At the WSER, subjects ingested 1000 mg of quercetin or placebo supplements just before the 160-km race (5:00 a.m.) but did not ingest additional supplements until after postrace blood samples were acquired (an average of 27 h later). Plasma quercetin levels dropped to very low levels in the quercetin group and were not much different from the placebo group after the race.

These results led us to reevaluate the literature on the bioavailability and half-life of quercetin. A recent quercetin pharmacokinetics study suggests that the average terminal half-life of quercetin may be as low as 3.5 h (25), but most other studies indicate a range of 11‒28 h (22). Additional literature indicates that isoquercetin is more completely absorbed than is quercetin in aglycone form and that the simultaneous ingestion of quercetin with vitamin C, folate, and additional flavonoids improves bioavailability (14,22,24). The glucose molecule in isoquercetin may favor the use of the sodium-dependent glucose transport pathway of the intestinal brush border membrane, improving absorption rates when compared with the pure aglycone form of quercetin (37). Two or more flavonoids ingested together may increase bioavailability and decrease elimination via competitive inhibition of glucuronide and sulfate conjugation in both the intestine and the liver and via inhibition of efflux transporters such as P-glycoprotein, breast cancer resistance protein, and multidrug resistance protein 2 (24). Quercetin's anti-inflammatory effects may be augmented by coingestion of N_3-PUFA (4), vitamin C, and EGCG (16). For example, the concurrent administration of EPA, DHA, and quercetin resulted in a synergistic anti-inflammatory effect in rats with intestinal inflammatory disorders (4). *In vitro* data indicate that quercetin exhibits

antiviral activity only when protected against oxidative degradation by ascorbate (36).

The Q‒EGCG supplement was formulated to improve the bioavailability and bioactive effects of quercetin, and the research design was changed to emphasize ingestion of half the daily dose 1 h before heavy exertion. The quercetin supplement (without added components) was related to some effects that we previously did not find, including a decrease in total blood leukocytes and G-CSF the morning after 3 d of heavy exertion, a reduction in plasma IL-10 after exercise, and a lowered DOMS 2 and 3 d afterward. These effects may have been related to the 8:00 a.m. and 2:00 p.m. dosing regimen (compared with 8:00 a.m. and 6:00 p.m. or immediately after exercise in our previous study) (23,30,31). The Q‒EGCG supplement produced more widespread effects on inflammation (in particular, reduced CRP and IL-6). These effects may have been related to the coingestion of quercetin with isoquercetin, EGCG, EPA, and DHA (4,16,24). The morning after the 3-d period of intensified exercise, plasma quercetin levels in Q‒EGCG were elevated 98% greater than that in placebo and 54% ($P = 0.06$) greater than that in Q, thus supporting the concept of a more prolonged quercetin effect from ingestion of the quercetin cocktail supplement.

No group differences were measured for cycling time trial performance (after a substantial exercise preload) or mRNA expression for three genes related to muscle mitochondrial biogenesis. One study of 11 elite male cyclists reported a 1.7% 30-km time trial performance enhancement greater than placebo after 6 wk of quercetin supplementation (21). This study used a randomized, double-blinded, crossover design, and the exercise regimen used a mountainous terrain format without an exercise preload. Another study using sedentary mice showed that 7 d of quercetin feeding (both 12.5 and 25 mgIkg^{j1}) increased cytochrome c concentration and citrate synthase activity in soleus muscle by 20%‒30% (8). Quercetin feedings also increased treadmill run time to fatigue by approximately 30%, with both quercetin doses equally effective. Together, these data suggest that quercetin may have a larger effect on untrained muscle, which has a lower mitochondrial density than in trained muscle. The data from available studies suggest that if a muscle mitochondrial biogenesis and performance effect exists in endurance athletes, quercetin supplementation may have to be 6 wk or longer, and the magnitude may be well below what could be measured in untrained subjects.

In summary, a quercetin supplement combined with EGCG, isoquercetin, and N_3-PUFA was more effective than quercetin without these added components in partially countering exercise-induced inflammation but without effects on cycling time trial performance or muscle mitochondrial biogenesis compared with placebo. These data add to the growing literature support for the concept that quercetin's anti-inflammatory effects are amplified when coingested with other flavonoids, food components,

and micronutrients. Additional research is needed to determine whether the proportions and amounts used in this study (i.e., 1000 mg of aglycone quercetin, 400 mg of isoquercetin, 120 mg of EGCG, 400 mg of N_3-PUFA) are optimal and whether additional food components might add to the effects measured in this study. The duration of supplementation used in this study (2 wk before intensified exercise) was based in part on quercetin pharmacokinetic data and findings from animal studies (8,22,24), but Q–EGCG's countermeasure effects may benefit from a more prolonged supplementation period. Cycling induces less muscle damage, inflammation, and oxidative stress than running, and findings reported in this study on cyclists may differ in magnitude from those measured in competitive marathon runners.

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