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Quercetin Ingestion Does Not Alter Cytokine Changes in Athletes Competing in the Western States Endurance Run

DAVID C. NIEMAN,¹ DRU A. HENSON,¹ J. MARK DAVIS,² CHARLES L. DUMKE,¹ SARAH J. GROSS,¹ D. PAUL JENKINS,¹ E. ANGELA MURPHY,² MARTIN D. CARMICHAEL,² JOHN C. QUINDRY,¹ STEVEN R. McANULTY,¹ LISA S. McANULTY,¹ ALAN C. UTTER,¹ and EUGENE P. MAYER²

ABSTRACT

The purpose of this study was to measure the influence of quercetin on plasma cytokines, leukocyte cytokine mRNA, and related variables in ultramarathoners competing in the 160-km Western States Endurance Run (WSER). Sixty-three runners were randomized to quercetin and placebo groups and under double-blinded methods ingested 1000 mg/day quercetin for 3 weeks before the WSER. Thirty-nine of the 63 subjects ($n = 18$ for quercetin, $n = 21$ for placebo) finished the race and provided blood samples the morning before the race and 15–30 min postrace. Significant prerace to postrace WSER increases were measured for nine proinflammatory and anti-inflammatory plasma cytokines, cortisol (quercetin = 94%, placebo = 96%), serum C-reactive protein (CRP) (mean \pm SE absolute increase, quercetin = 31.8 ± 4.2 , placebo = 38.2 ± 5.0 mg/L), and creatine kinase (CK) (quercetin = $21,575 \pm 3,977$, placebo = $19,455 \pm 3,969$ U/L), with no significant group differences. Interleukin-6 (IL-6) mRNA did not change post-WSER, with a significant decrease measured for leukocyte IL-8 mRNA (0.21 ± 0.03 -fold and 0.25 ± 0.04 -fold change from rest, quercetin and placebo, respectively) and significant increases for IL-1Ra mRNA (1.43 ± 0.18 -fold and 1.40 ± 0.16 -fold change, quercetin and placebo, respectively) and IL-10 mRNA (12.9 ± 3.9 -fold and 17.2 ± 6.1 -fold change, quercetin and placebo, respectively), with no significant differences between groups. In conclusion, quercetin ingestion (1 g/day) by ultramarathon athletes for 3 weeks before a competitive 160-km race significantly increased plasma quercetin levels but failed to attenuate muscle damage, inflammation, increases in plasma cytokine and hormone levels, and alterations in leukocyte cytokine mRNA expression.

INTRODUCTION

INTENSIVE EXERCISE OF LONG DURATION increases blood levels of multiple cytokines, with most of these exerting anti-inflammatory effects.^{1,2} In previous studies of athletes competing in the 160-km Western States Endurance Run (WSER), we have shown that the greatest fold increase is experienced prerace to postrace for interleukin-6 (IL-6) (~130-fold), followed by IL-10 (31-fold), granulocyte colony-stimulating factor (G-CSF) (26-fold), IL-8 (9-fold), IL-1 receptor antagonist (IL-1Ra) (7-fold), monocyte chemoattractant protein-1 (MCP-1) (3-fold), macrophage inflammatory protein-1 β (MIP-1 β) (2-fold), macrophage migration inhibitory factor-1 (MIF-1) (1.5-fold), and

tumor necrosis factor- α (TNF- α) (1.3-fold).^{3–5} Underlying mechanisms are being explored, but we have shown that athletes with the greatest degree of muscle damage (as measured by serum creatine kinase [CK]) experience the highest postrace plasma levels for most of these cytokines.^{4,5} IL-6, IL-8, IL-1 β , and TNF- α mRNA content is increased within postexercise muscle biopsy samples, and blood leukocytes may secrete increased amounts of IL-8, IL-10, and IL-1Ra during sustained exercise.^{6–9}

Primary signaling mechanisms for cytokine gene expression during exercise are poorly understood, but recent data suggest that nitric oxide (NO) production is a key regulator.⁹ Other potential triggers of cytokine release during extreme exercise in-

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clude leakage of endotoxins (lipopolysaccharide [LPS]) from the intestines, elevation in catecholamines and cortisol, high core body temperature, glycogen deficiency and other metabolic demands, and oxidative stress.¹⁻⁹ Carbohydrate feedings during intense and prolonged exercise in the fasted state attenuate increases in plasma stress hormones and IL-6, IL-10, and IL-1Ra but have relatively little effect in athletes consuming prerace meals.^{6,7} Although still debated in regard to benefit vs. risk, a reduction in the inflammatory cytokine response to exercise through nutraceutical or pharmacologic means is a goal being pursued by several research teams worldwide.

Plants contain thousands of phenolic compounds, including more than 5000 flavonoids that exhibit strong antioxidant activity.¹⁰⁻¹² Flavonols are the most widespread flavonoids in foods, and the most prominent is quercetin.¹⁰ The richest food sources of quercetin are onions, apples, blueberries, curly kale, tea, and broccoli.¹¹ Total flavonol intake (with quercetin representing about 75%) varies from 13 to 64 mg/day depending on the study sample and the population studied.¹⁰ Human subjects can absorb significant amounts of quercetin from food or supplements, and elimination is quite slow, with a reported half-life ranging from 11 to 28 h.^{12,13} Long-term feeding of quercetin in rats leads to accumulation in several organs, including the lungs, testes, kidney, heart, liver, thymus, and muscle.¹⁴ Despite earlier concerns, long-term, high-dose quercetin supplementation in rodents and humans has not been linked to any adverse effects.¹⁵ Epidemiologic studies indicate that higher compared with lower quercetin intake is associated with reduced risk for ischemic heart disease, type 2 diabetes, asthma, and various types of cancer, including lung cancer, colorectal cancer, and prostate cancer.¹⁶⁻¹⁹

Quercetin is a powerful antioxidant, and *in vitro* data show that quercetin in aglycone form has an antioxidant potency that is approximately 5-fold greater than that of vitamin C.²⁰ *In vitro* cell culture studies indicate that quercetin also exerts anti-inflammatory effects and inhibits nuclear factor- κ B (NF- κ B) signaling in a variety of cells, including macrophages and peripheral blood mononuclear cells (PBMCs).²⁰⁻²⁷ Quercetin acts by blocking the protein kinase-mediated I κ B degradation, thereby preventing NF- κ B activation.²⁸ NF- κ B is a central mediator of immune responses and regulates the expression of genes encoding many cytokines, such as IL-1 β , TNF- α , MCP-1, and MIP-1 α/β .²⁹ NF- κ B is activated by proinflammatory stimuli, such as TNF- α and LPS, and various stress stimuli, including elevated hormones, physical stress, and oxidative stress.^{24,29} Prostaglandin and NO are also involved in inflammation, and inducible isoforms of nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) are responsible for the production of large amounts of these proinflammatory mediators.²⁸ Several *in vitro* studies indicate that quercetin inhibits NO production and iNOS expression.^{23,25,28} Thus, inhibition of iNOS by quercetin may be one of the mechanisms responsible for its anti-inflammatory effects and may be an important regulatory pathway during exercise, as shown by Steensberg et al.⁹

Given quercetin's antioxidant, anti-inflammatory, and NF- κ B and iNOS inhibitory effects, we hypothesized that quercetin compared with placebo supplementation would attenuate leukocyte mRNA expression for cytokines and diminish postrace plasma cytokine levels in endurance athletes competing in the 160-km WSER. We designed a randomized, double-blinded,

placebo-controlled study whereby runners registered for the 2006 160-km WSER ingested soft-chew supplements with or without 1000 mg quercetin for 3 weeks prior to the race event. Blood samples were collected 1 day prerace and immediately after completion of the race and analyzed for nine cytokines, leukocyte cytokine mRNA expression, cortisol, C-reactive protein (CRP), CK, and various diagnostic chemistries.

MATERIALS AND METHODS

Subjects and race description

Sixty-three experienced male and female ultramarathoners from the 2006 160-km WSER were recruited and randomized to quercetin and placebo groups. Thirty-nine of the 63 subjects ($n = 18$ for quercetin with 14 males and 4 females, $n = 21$ for placebo with 18 males and 3 females) finished the race and provided blood samples prerace and postrace. Prior to testing, informed consent was obtained from each subject, and the experimental procedures were approved by the institutional review board of Appalachian State University. To enter the study, subjects must have qualified for the 2006 160-km WSER (qualification criteria: completed a 160-km trail race or three 80-km races within the cutoff times or raced 80-km in under 11 h or 100-km in under 14 h).

The 160-km WSER is a point-to-point trail run in the Sierra Nevada Mountains of northern California and is regarded as one of the most arduous organized running events in the United States. The race starts at Squaw Valley, California (1890 m altitude), and finishes at Auburn, California (366 m). The trail race course ascends 777 m to Emigrant Pass (2668 m, the highest point) within the first 7 km and then passes through remote and rugged territory to Auburn. The total altitude gain and loss during the race is 5500 m and 6700 m, respectively. The race starts at 5:00 AM, and runners must reach the finish line within 30 h to be eligible for an award.

Research design

Following recruitment and randomization, subjects ingested soft chews with or without 1000 mg/day quercetin using double-blinded procedures for 3 weeks before and the morning of the WSER. This quercetin dosage was based on unpublished animal data (J. Mark Davis, personal communication). Subjects in the quercetin group ingested four soft, individually wrapped chews each day (two prior to breakfast and two prior to dinner) that contained 250 mg quercetin, 250 mg vitamin C, 20 mg niacin, and 20 kcal sugars in a carnauba wax and soy lecithin base colored with FD&C yellow 6 (Nutravail Technologies Inc., Chantilly, VA, and Quercegen Pharma, Newton, MA). Placebo supplements were prepared exactly the same way minus the quercetin, vitamin C, and niacin. Data from Quercegen Pharma indicate that the bioavailability of quercetin is enhanced with vitamin C and niacin, and thus this study tested whether or not soft chews with or without the combination of quercetin, vitamin C, and niacin had an influence on the outcome measures. On race day, subjects ingested all four chews prior to the 5:00 AM start time. During the prerace supplementation period, subjects were instructed to ingest their normal intake of food, beverages, and supplements while avoiding any other supplements

containing quercetin, with verification by a dietitian following the race. A 3-day food record was obtained during the 3-week prerace supplementation period and analyzed using the computerized nutrition software program, Food Processor (ESHA Research, Salem, OR).

Subjects provided blood samples during registration in Squaw Valley, held the morning before the race, and 15–30 min posttrace in Auburn. Plasma aliquots were frozen immediately, transported on dry ice, and then stored at -80°C until analysis. During race registration, body mass was measured, and subjects filled in a questionnaire on basic demographics and training history. On race day, body mass was measured at the 90-km aid station (Michigan Bluff, 1220 m) and within 5–15 min posttrace at Auburn. Subjects completed a posttrace questionnaire indicating adherence to the research design. Subjects consumed food and beverages *ad libitum* during the race.

Plasma quercetin

Total plasma quercetin (quercetin and its primary conjugates) was measured following solid-phase extraction via reverse-phase HPLC with UV detection as described by Quercegen Pharma. This procedure is similar to that previously published by Ishii et al.³⁰ Quercetin conjugates were hydrolyzed by incubating 250–500 μL plasma aliquots with 10 μL 10% DL-dithiothreitol (DTT) solution, 50 μL 0.58 M acetic acid, 50 μL of a mixture of β -glucuronidase and arylsulfatase, and crude extract from *Helix pomatia* (Roche Diagnostics GmbH, Mannheim, Germany) for 40 min at 37°C . After incubation, 500 μL of 0.01M oxalic acid was added, and each sample was vortexed and centrifuged for 5 min at 10,000 rpm. Supernatants (1 mL) were then applied to solid-phase extraction cartridges (Oasis HLB 1cc, 30 mg, SPE cartridge) (Waters Corp., Milford, MA) that were preconditioned with 1 mL methanol (MeOH), 0.5 mL 0.01M oxalic acid, and 1 mL dH_2O and drawn through at a rate of 0.5 mL/min using a vacuum manifold (Waters Corp). Cartridges were washed with 0.5 mL $\text{MeOH} \times 2$. Eluant was collected into 1.5-mL microcentrifuge tubes. DTT (10% solution, 10 μL) was added to the combined eluant, and the samples were vortexed for 1 min and placed in a vacuum concentrator (Savant Speed Vac SC 110, Savant Instruments Inc., Farmingdale, NY) until MeOH was completely evaporated. The residue was reconstituted with 150 μL $\text{MeOH}/\text{dH}_2\text{O}$ (1:1). Injections (50 μL) were used for HPLC analysis.

Chromatographic analysis was performed using a Waters Breeze system (Waters Corp.) consisting of a Waters 1525 Binary HPLC pump, 2487 UV detector, and Symmetry C18 5 μm

$4.6 \times 150\text{-mm}$ column. The analysis data were acquired and processed using Breeze software (v3.02). A mixture of acetonitrile with 0.1% formic acid (A) and dH_2O with 0.1% formic acid (B) was used as the mobile phase. The gradient elution was programmed as follows: 0–1.5 min, 10/90% A/B; 1.5–9.5 min, 40/60% A/B; 9.5–11.5 min, 90/10% A/B; 11.5–15 min, 10/90% A/B. The column temperature was maintained at 30°C . The flow rate was 1.0 mL/min. Quantification of the quercetin peak was based on the standard addition method using both plasma and MeOH, with similar results. Standards and samples were treated in an identical manner.

Cytokine measurements

Total plasma concentrations of IL-1Ra, IL-6, IL-8, IL-10, G-CSF, MCP-1, MIP-1 β , TNF- α , and MIF-1 were determined using quantitative sandwich ELISA kits provided by R&D Systems, Inc. (Minneapolis, MN). All samples and provided standards were analyzed in duplicate. High sensitivity kits were used to analyze TNF- α and prerace samples of IL-6, IL-10, and G-CSF. The minimum detectable concentration of IL-1Ra was $<22 \text{ pg} \cdot \text{mL}^{-1}$, IL-6 $<0.70 \text{ pg} \cdot \text{mL}^{-1}$, IL-6 (high sensitivity) $<0.039 \text{ pg} \cdot \text{mL}^{-1}$, IL-8 $<3.5 \text{ pg} \cdot \text{mL}^{-1}$, IL-10 $<3.9 \text{ pg} \cdot \text{mL}^{-1}$, IL-10 (high sensitivity) $<0.50 \text{ pg} \cdot \text{mL}^{-1}$, G-CSF $<20.0 \text{ pg} \cdot \text{mL}^{-1}$, G-CSF (high sensitivity) $<0.80 \text{ pg} \cdot \text{mL}^{-1}$, MCP-1 $<5.0 \text{ pg} \cdot \text{mL}^{-1}$, MIP-1 β $<11.0 \text{ pg} \cdot \text{mL}^{-1}$, TNF- α $<0.106 \text{ pg} \cdot \text{mL}^{-1}$, and MIF-1 $<0.017 \text{ ng} \cdot \text{mL}^{-1}$. To improve sensitivity in the detection of IL-8, we employed SOFTmax analysis software (Molecular Devices, Sunnyvale, CA). Preexercise and postexercise samples for the cytokines were analyzed on the same assay plate to decrease interkit assay variability.

Leukocyte mRNA extraction and cDNA synthesis

The QIAampRNA Blood Mini Kit Protocol (Qiagen, Valencia CA) was used to extract mRNA. From each subject, 3-mL aliquots of whole blood collected in EDTA were purified for RNA. Briefly, erythrocytes were selectively lysed, and leukocytes were recovered by centrifugation. Samples were briefly centrifuged through a QIAshredder spin column, ethanol was added to adjust binding conditions, and the sample was applied to a QIAamp spin column. RNA was bound to the silica gel membrane during a brief centrifugation step. Contaminants were washed away, and total RNA was eluted in 30 μL RNase-free water.

The extracted RNA (7.5 μL of sample) was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically.

TABLE 1. SUBJECT CHARACTERISTICS (MEAN \pm SE)

Variable	Quercetin (n = 18)	Placebo (n = 21)	p value
Age, years	44.2 \pm 2.0	46.0 \pm 2.3	0.575
Running experience, years	13.0 \pm 2.3	13.3 \pm 1.7	0.780
Ultramarathons raced	39.6 \pm 10.6	37.9 \pm 6.0	0.988
Training distance, $\text{km} \cdot \text{wk}^{-1}$	83.4 \pm 5.0	75.8 \pm 7.1	0.398
Race time, h	26.4 \pm 0.7	27.5 \pm 0.6	0.237
Body mass, prerace, kg	68.9 \pm 2.7	73.9 \pm 2.7	0.168
Body mass, midrace, kg	68.9 \pm 2.3	73.5 \pm 2.7	0.169
Body mass, postrace, kg	68.0 \pm 2.3	73.0 \pm 2.7	0.140

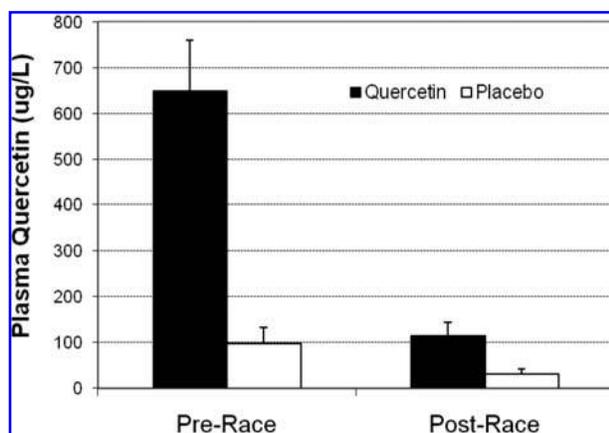


FIG. 1. Plasma quercetin in runners before and after a 160-km race in quercetin ($n = 18$) and placebo ($n = 20$) groups. Time effect, $p < 0.001$; quercetin/placebo group \times time effect, $p < 0.001$.

metrically at 260-nm wavelength. RNA was reverse transcribed into cDNA in a 50 μL reaction volume containing 19.25 μL RNA in RNase-free water, 5 μL 10 \times RT buffer, 11 μL 25 mM MgCl_2 , 10 μL deoxyNTPs mixture, 2.5 μL random hexamers, 1 μL RNase inhibitor, and 1.25 μL multiscribe reverse transcriptase (50 U/ μL). Reverse transcription was performed at 25 $^\circ\text{C}$ for 10 min, 37 $^\circ\text{C}$ for 60 mins, and 95 $^\circ\text{C}$ for 5 min, followed by quick chilling on ice, and stored at -20°C until subsequent amplification.

Quantitative real-time PCR analysis

Quantitative RT-PCR analysis was done as per manufacturer's instructions (Applied Biosystems, Foster City, CA) us-

ing TaqMan Gene Expression Assays. DNA amplification was carried out in 12.5 Taqman Universal PCR Master Mix (AmpliQ Gold DNA Polymerase, Passive Reference 1, Buffer, dNTPs, AmpErase UNG), 1 μL cDNA, 9 μL RNase-free water, and 1.25 μL 18S primer (VIC) and 1.25 μL primer (FAM) (for endogenous reference and target cytokine) in a final volume of 25 μL /well. Human control RNA (calibrator RNA) was also used and served as a calibrator for each plate. Samples were loaded in a MicroAmp 96-well reaction plate. Plates were run using ABI Sequence Detection System (Applied Biosystems). After 2 min at 50 $^\circ\text{C}$ and 10 min at 95 $^\circ\text{C}$, plates were coamplified by 50 repeated cycles, of which one cycle consisted of a 15-sec denaturing step at 95 $^\circ\text{C}$ and 1-min annealing/extension step at 60 $^\circ\text{C}$. Data was analyzed by ABI software using the cycle threshold (CT), which is the value calculated and based on the time (measured by PCR cycle number) at which the reporter fluorescent emission increases beyond a threshold level (based on the background fluorescence of the system),³¹ and it reflects the cycle number at which the cDNA amplification is first detected. We have previously reported detailed methodology concerning the dual amplification technique.^{6,7} Samples were run in duplicate, and the intraassay and interassay coefficients of variation (CVs) were determined to be 1.69% and 1.65% for the ΔCTs , respectively.

Calculations for relative quantification

Quantification of cytokine gene expression for IL-8, IL-10, IL-1Ra, and IL-6 was calculated using the $\Delta\Delta\text{CT}$ method as described by Livak and Schmittgen.³² This method uses a single sample, the calibrator sample, for comparison of every unknown sample's gene expression. This method of analysis and quantification has been shown to give similar results to the standard curve method.³¹ Briefly, ΔCT ($\text{CT}(\text{FAM}) - \text{CT}(\text{VIC})$) was calculated for each sample and calibrator. $\Delta\Delta\text{CT}$ ($\Delta\text{CT}(\text{cal}$ -

TABLE 2. PRERACE AND POSTRACE (160 KM) SERUM GLUCOSE, CORTISOL, C-REACTIVE PROTEIN, CREATINE KINASE, AND DIAGNOSTIC CHEMISTRIES IN QUERCETIN ($n = 18$) AND PLACEBO ($n = 21$) GROUPS (MEAN \pm SE)

Variable	Prerace	Postrace	Time; interaction effects, p value
Glucose, $\text{mmol} \cdot \text{L}^{-1}$			
Quercetin	5.17 \pm 0.17	6.49 \pm 0.33	<0.001; 0.355
Placebo	5.54 \pm 0.20	6.38 \pm 0.28	
Cortisol, $\text{nmol} \cdot \text{L}^{-1}$			
Quercetin	434 \pm 34	835 \pm 80	<0.001; 0.956
Placebo	412 \pm 29	806 \pm 97	
C-reactive protein, $\text{mg} \cdot \text{L}^{-1}$			
Quercetin	0.4 \pm 0.1	32.2 \pm 4.3	<0.001; 0.346
Placebo	0.8 \pm 0.2	39.0 \pm 5.1	
Creatine kinase, $\text{U} \cdot \text{L}^{-1}$			
Quercetin	159 \pm 25	21733 \pm 3980	<0.001; 0.710
Placebo	171 \pm 27	19626 \pm 3981	
Alanine aminotransferase, $\text{U} \cdot \text{L}^{-1}$			
Quercetin	26.1 \pm 1.1	132 \pm 20	<0.001; 0.598
Placebo	26.1 \pm 1.9	118 \pm 18	
Aspartate aminotransferase, $\text{U} \cdot \text{L}^{-1}$			
Quercetin	28.9 \pm 1.3	528 \pm 75	<0.001; 0.937
Placebo	29.6 \pm 1.6	519 \pm 95	
Blood urea nitrogen, $\text{mmol} \cdot \text{L}^{-1}$			
Quercetin	4.7 \pm 0.3	11.0 \pm 1.0	<0.001; 0.888
Placebo	4.8 \pm 0.4	10.9 \pm 1.0	

TABLE 3. PRERACE AND POSTRACE (160 KM) PLASMA CYTOKINE LEVELS IN QUERCETIN ($n = 18$) AND PLACEBO ($n = 21$) GROUPS (MEAN \pm SE)

Variable	Prerace	Postrace	Time; interaction effects, p value
IL-6, pg · ml ⁻¹			
Quercetin	0.9 \pm 0.1	90.4 \pm 21.0	<0.001; 0.340
Placebo	1.2 \pm 0.4	66.5 \pm 14.6	
IL-10, pg · ml ⁻¹			
Quercetin	9.5 \pm 0.5	132.1 \pm 28.6	<0.001; 0.228
Placebo	9.2 \pm 0.3	89.3 \pm 20.8	
G-CSF, pg · ml ⁻¹			
Quercetin	34.6 \pm 3.1	264.2 \pm 68.8	0.001; 0.908
Placebo	30.8 \pm 2.8	246.7 \pm 90.3	
IL-1Ra, pg · ml ⁻¹			
Quercetin	137 \pm 6	1037 \pm 228	<0.001; 0.769
Placebo	123 \pm 9	919 \pm 261	
IL-8, pg · ml ⁻¹			
Quercetin	4.5 \pm 0.2	25.3 \pm 3.7	<0.001; 0.415
Placebo	5.1 \pm 0.3	21.6 \pm 3.7	
MCP-1, pg · ml ⁻¹			
Quercetin	129 \pm 8	529 \pm 52	<0.001; 0.423
Placebo	122 \pm 6	473 \pm 36	
MIP-1 β , pg · ml ⁻¹			
Quercetin	35.4 \pm 2.8	73.1 \pm 10.4	<0.001; 0.300
Placebo	33.5 \pm 2.8	99.0 \pm 22.6	
TNF- α , pg · ml ⁻¹			
Quercetin	0.93 \pm 0.11	1.62 \pm 0.12	<0.001; 0.287
Placebo	1.15 \pm 0.11	2.34 \pm 0.42	
MIF-1, ng · ml ⁻¹			
Quercetin	5.4 \pm 0.5	12.0 \pm 1.5	<0.001; 0.667
Placebo	4.7 \pm 0.3	12.3 \pm 1.8	

ibrator) - Δ CT(sample)) was then calculated for each sample, and relative quantification was calculated as $2^{\Delta\Delta$ CT. Initial exclusion criteria consisted of FAM CT \geq 40 and VIC CT \geq 23.

Hemoglobin, hematocrit, diagnostic chemistries, CRP, CK cortisol

Complete blood counts for hemoglobin and hematocrit were measured using a Coulter STKS instrument (Coulter Electronics, Inc., Hialeah, FL). Plasma volume changes were estimated using the method of Dill and Costill.³³ The comprehensive diagnostic chemistry panel, CRP, and CK were measured in a clinical laboratory using an LX-20 clinical analyzer (Beckman, Brea, CA).

Plasma concentrations of cortisol were determined using the competitive solid-phase 125I radioimmunoassay (RIA) technique (Diagnostic Products Corporation, Los Angeles, CA) with cortisol-specific antibody (Ab) coated tubes (Coat-A-Count tubes). Intraassay (CV_{intra}) and interassay (CV_{inter}) CVs were 4.5% and 5%, respectively. Assay sensitivity was 5.5 nmol/L (0.2 μ g/dL).

Delayed onset of muscle soreness (DOMS)

Subjects recorded muscle soreness after the race and during the week following the race using a 10-point Likert scale.³⁴ Runners 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 sore-

ness), 5.5 (more than slight soreness), 7 (sore), 8.5 (very sore), and 10 (unbearably sore).

Statistical analysis

Data are expressed as mean \pm SE, and were analyzed using a 2 (groups) \times 2 (time points) repeated measures ANOVA.

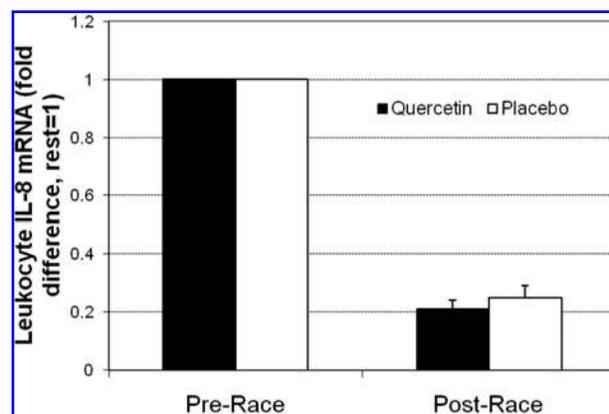


FIG. 2. IL-8 mRNA expression in blood leukocytes from runners before and after a 160-km race in quercetin ($n = 18$) and placebo ($n = 20$) groups. Values are expressed as a fold change from rest, with rest equal to 1. Time effect, $p < 0.001$; quercetin/placebo group \times time effect, $p = 0.437$.

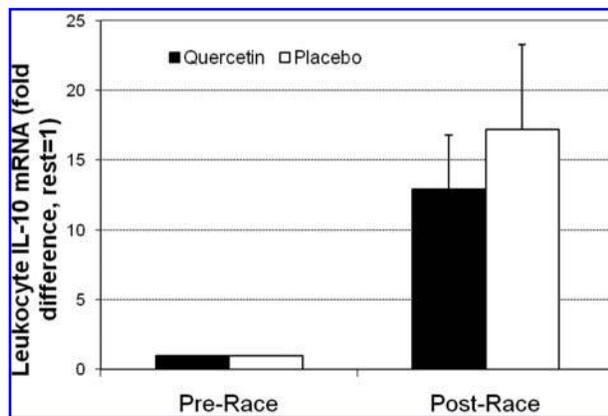


FIG. 3. IL-10 mRNA expression in blood leukocytes from runners before and after a 160-km race in quercetin ($n = 18$) and placebo ($n = 20$) groups. Values are expressed as a fold change from rest, with rest equal to 1. Time effect, $p = 0.001$; quercetin/placebo group \times time effect, $p = 0.568$.

When the interaction effect was significant ($p \leq 0.05$), prerace to posttrace changes were calculated and compared between quercetin and placebo groups using Student's t -tests, with significance set at $p \leq 0.05$. DOMS data were analyzed using a 2×8 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).

RESULTS

Thirty-nine of 63 subjects ($n = 18$ for quercetin, $n = 21$ for placebo) completed the 160-km race event and adhered to all aspects of the study design. A poststudy questionnaire revealed a successful implementation of the double-blinded supplementation (of quercetin subjects, 22% indicated they were on quercetin, 17% on placebo, and 61% were not sure, compared with 38%, 29%, and 33%, respectively, of placebo subjects).

Air temperature was 22°C at the start of the race at 5:00 AM (Squaw Valley, CA), 38°C by 2:00 PM (Michigan Bluff), 27°C by 12:00 AM (race finish, Auburn, CA), and 31°C by 11:00 AM. The humidity ranged from 25% to 58% during the last half of the race. There were no significant differences in subject characteristics between the quercetin and placebo groups (Table 1). Male ($n = 32$) and female ($n = 7$) runners did not differ significantly in race time (27.0 ± 0.5 h vs. 27.0 ± 1.39 h, respectively, $p = 0.992$) or change in any of the other variables measured in this study except for those related to body mass and composition. Thus, male and female runner data were combined for this analysis.

Plasma volume did not change appreciably and did not differ significantly between groups ($-0.3 \pm 0.5\%$ and $-0.3 \pm 0.4\%$, respectively, $p = 0.928$), and body mass was maintained near prerace levels for both groups (Table 1). Three-day food records prior to the race event indicated no significant differences in macronutrient intakes between groups, with a mean energy intake for all subjects combined of 11.0 ± 0.8 MJ \cdot day $^{-1}$ (2620 ± 184 kcal \cdot day $^{-1}$) and percent of energy intakes

of $51.1 \pm 2.3\%$ carbohydrate, $31.5 \pm 1.8\%$ fat, and $18.0 \pm 1.2\%$ protein.

After 3 weeks of supplementation, plasma quercetin levels were 6.6-fold higher in the quercetin compared with the placebo group and 3.1-fold higher postrace (interaction effect, $p < 0.001$) (Fig. 1). Prerace to postrace increases in serum glucose, cortisol, CRP, CK, alanine and aspartate aminotransferase, and blood urea nitrogen did not differ significantly between quercetin and placebo groups (Table 2). Prerace to postrace increases in nine cytokines listed in Table 3 did not differ significantly between quercetin and placebo groups.

Figures 2, 3, 4, and 5 summarize the leukocyte cytokine mRNA data. Leukocyte IL-8 mRNA decreased significantly postrace, with no differences between quercetin and placebo groups (Fig. 2). Leukocyte IL-10 mRNA (Fig. 3) and IL-1Ra mRNA (Fig. 4) increased significantly postrace, with no group differences. Leukocyte IL-6 mRNA did not change postrace, with no group differences (Fig. 5).

Quercetin and placebo groups did not differ in ratings for DOMS on race day or the week after the race (interaction effect, $p = 0.986$) (Fig. 6).

DISCUSSION

High-dose quercetin ingestion (1 g/day) by ultramarathon athletes for 3 weeks significantly increased plasma quercetin levels but failed to attenuate muscle damage, DOMS, inflammation, substantial increases in plasma cytokine and hormone levels, and alterations in leukocyte cytokine mRNA expression following the 160-km WSER.

The athletes consuming quercetin for 3 weeks achieved plasma quercetin levels that were 6.6-fold greater than those of athletes randomized to placebo ingestion. On the day of the race, athletes in the quercetin group consumed 1 g between 4 and 5 AM but did not consume additional quercetin supplements during the race. Plasma quercetin levels in the quercetin group fell to 115 μ g/L after the 160-km race, a concentration similar to prerace levels in the placebo group. The half-life of quercetin

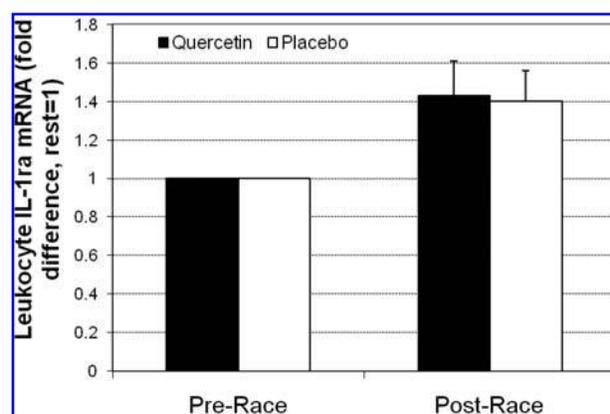


FIG. 4. IL-1Ra mRNA expression in blood leukocytes from runners before and after a 160-km race in quercetin ($n = 18$) and placebo ($n = 20$) groups. Values are expressed as a fold change from rest, with rest equal to 1. Time effect, $p = 0.001$; quercetin/placebo group \times time effect, $p = 0.888$.

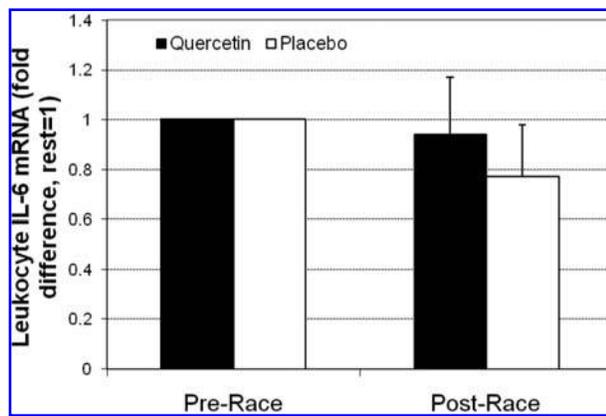


FIG. 5. IL-6 mRNA expression in blood leukocytes from runners before and after a 160-km race in quercetin ($n = 18$) and placebo ($n = 20$) groups. Values are expressed as a fold change from rest, with rest equal to 1. Time effect, $p = 0.351$; quercetin/placebo group \times time effect, $p = 0.577$.

has been reported to be 11–28 h, and we hypothesized that plasma quercetin levels would remain high during the race after 3 weeks of supplementation and a full 1 g quercetin dose just prior to the start of the race. This is the first study of human athletes using high-dose quercetin supplements prior to

ultramarathon competition, and we learned that the plasma quercetin level fell substantially more than expected. Ingestion of quercetin supplements throughout the race would have maintained plasma quercetin at a higher level, with perhaps different outcomes.

The results of this study, however, are consistent with those of most other investigations that have failed to show that ingestion of antioxidants, such as vitamins E and C, has meaningful effects on exercise-induced inflammation, muscle damage, increases in plasma cytokines, and immune perturbations.^{2,35,36} In contrast to our previous findings showing the importance of muscle damage, we have been unable to demonstrate that oxidative stress is strongly associated with inflammation or cytokine changes in athletes competing in ultramarathons.^{3–5} We had hypothesized that a stronger antioxidant, such as quercetin, would prove more useful than vitamin C or vitamin E as a nutritional countermeasure to exercise-induced cytokine changes for endurance athletes, but our findings were not supportive.²²

In vitro/cell culture data indicate that quercetin in aglycone form exerts impressive antioxidant and anti-inflammatory effects and inhibits proinflammatory cytokine production and gene expression through modulation of NF- κ B.^{20–23,28} Available data demonstrate, however, that quercetin undergoes considerable chemical modification during digestion and absorption and is metabolized to methylated, glucurono-sulfated derivatives.^{12,13,22} The quercetin conjugates may have altered bi-

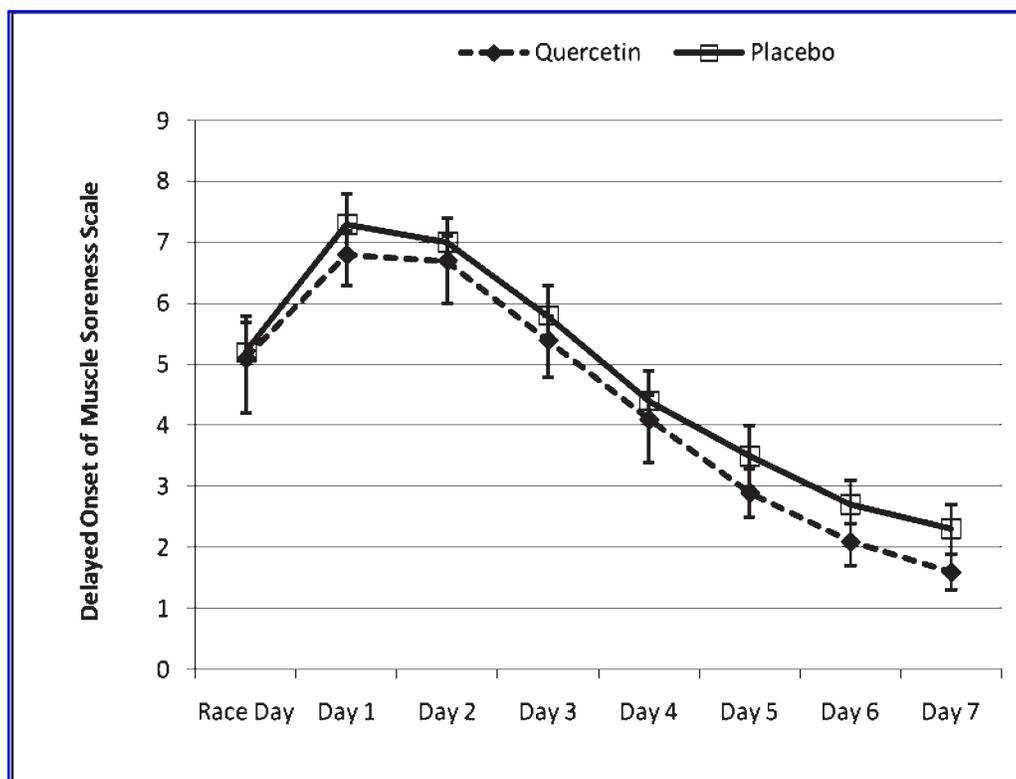


FIG. 6. Delayed onset of muscle soreness (DOMS) in runners on race day and the week following a 160-km race in quercetin ($n = 18$) and placebo ($n = 21$) groups. Time effect, $p < 0.001$; quercetin/placebo group \times time effect, $p = 0.986$. DOMS scale: 1 (no soreness), 2.5 (dull, vague ache), 4 (slight soreness), 5.5 (more than slight soreness), 7 (sore), 8.5 (very sore), and 10 (unbearably sore).

ologic properties and potencies when compared with *in vitro* quercetin aglycone experiments, but few human data are available.^{22,27} Chen et al.²² showed that in contrast to quercetin in aglycone form, quercetin-3'-sulfate had no antioxidant or inhibitory effects on LPS-induced and interferon- γ (IFN- γ)-induced iNOS gene expression in mouse BV-2 microglia cell cultures. Another factor to consider is that the high quercetin concentrations used in many cell culture studies may not apply to the lower plasma concentrations achievable through oral quercetin supplementation. Nonetheless, epidemiologic studies indicate that individuals ingesting quercetin from fruits and vegetables experience a reduced risk for lung cancer and ischemic heart disease, with these benefits apparently related to quercetin's antioxidative and anti-inflammatory capacities. The high-dose quercetin ingestion in this study and a previous study in our laboratory failed to counter exercise-induced perturbations in inflammation, plasma cytokines, and oxidative stress.³⁵ It can be argued that the degree of oxidative, inflammatory, and physiologic stress acutely induced by prolonged and intensive exertion overwhelms attempts to apply nutritional countermeasures and that quercetin ingestion is more effective within the context of countering the inflammation and oxidative stress related to chronic disease.

Race time was about 1 h (4%) shorter in the quercetin compared with the placebo group, but this did not achieve statistical significance. MacRae and Mefferd³⁷ reported that 6 weeks of antioxidant supplement with quercetin (600 mg/day) by 11 elite male cyclists decreased the time to complete a 30-km time trial by 3.1%. These authors speculated that the performance improvement could have been related to quercetin's effects on skeletal muscle redox state, reductions in muscle inflammation and protection of skeletal muscle protein, and facilitation of motor unit recruitment. Environmental conditions during the 2006 WSER were severe, and other confounding factors during this difficult and long trail race create a context that is less than ideal to test quercetin's potential effects on performance.

Plasma cytokine levels following the 160-km race rose to levels similar to what we have previously reported.³⁻⁵ In a previous laboratory study during which 12 athletes cycled intensively for 2 h, blood leukocyte mRNA expression for IL-10 increased 2.7-fold, IL-1Ra 2.2-fold, and IL-8 2.4-fold, with little change in IL-6.⁸ Following the 160-km WSER, changes in blood leukocyte mRNA expression were comparable to the laboratory study for IL-1Ra and IL-6, with a much higher and lower fold change recorded for IL-10 and IL-8, respectively. These data are difficult to interpret because of the lack of congruence between plasma cytokine levels and blood leukocyte cytokine mRNA expression.⁸ Nonetheless, the marked decrease in leukocyte IL-8 and increase in IL-10 mRNA expression are notable findings and suggest an attempt by the immune system to limit polymorphonuclear cell adherence, degranulation, respiratory burst activity, and inflammation when the athlete is already experiencing significant muscle cell damage and oxidative stress.

In conclusion, using randomized, double-blinded procedures, 1 g/day quercetin ingestion for 3 weeks prior to the 160-km WSER did not counter changes in blood leukocyte cytokine mRNA or the large increases in inflammation, muscle soreness, and plasma cytokines experienced by the athletes. We suggest that quercetin's failure to exert effects in our human athletes

anticipated from *in vitro* studies may have been due to several factors, including quercetin conjugation following ingestion, the large drop in plasma quercetin levels during the race event, the overwhelming level of oxidative stress and inflammation induced by running a competitive 160-km trail race, or the inherent lack of association between oxidative stress and exercise-induced cytokine production within the context of extreme exertion. In a previous study, we showed that quercetin ingestion lowered the incidence of upper respiratory tract infection in athletes following a period of intensified exercise.³⁵ Quercetin's pronounced antiviral capacities have been linked *in vitro* at least in part to quercetin methylated conjugates. These are common quercetin metabolites in humans, thus providing a physiologic rationale for our finding.³⁸ Only a few *in vitro* studies have tested quercetin's impressive antioxidant and anti-inflammatory effects using physiologically relevant metabolites, and this research is needed to test the value of quercetin supplementation for endurance athletes beyond the disease reduction benefits shown by epidemiologic research.

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