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TITLE **Quercetin's Influence On Exercise-Induced Changes In Plasma Cytokines And Muscle and Leukocyte Cytokine mRNA**

RUNNING HEAD: Quercetin, Exercise, and Cytokines

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ABSTRACT: Trained male cyclists (N=40) ingested quercetin (Q, N=20) (1000 mg/day) or placebo (P, N=20) supplements under randomized, double blinded methods for 3 wks prior to and during a 3-d period in which subjects cycled for 3 h/day at ~57% $Watts_{max}$. Blood samples were collected before and after each exercise session and assayed for plasma IL-6, IL-10, IL-1ra, IL-8, TNF-α, and MCP-1, and leukocyte IL-10, IL-8, and IL-1ra mRNA. Muscle biopsies were obtained before and after the first and third exercise sessions and assayed for NFκB, and cyclooxygenase-2 (COX-2), IL-6, IL-8, IL-1β, and TNF-α mRNA. Post-exercise increases in plasma cytokines did not differ between groups, but the pattern of change over the 3-d exercise period tended to be lower in Q versus P for IL-8 and TNF-α (P=0.094 for both). mRNA increased significantly post-exercise for each cytokine measured in blood leukocyte and muscle samples. Leukocyte IL-8 and IL-10 mRNA was significantly reduced in Q versus P (interaction effects, P=0.019 and 0.012, respectively) with no other leukocyte or muscle mRNA group differences. Muscle NFκB did not increase post-exercise and did not differ between Q and P. Muscle COX-2 mRNA increased significantly post-exercise, but did not differ between Q and P. In summary, one gram/day quercetin supplementation by trained cyclists over a 24-day period diminished post-exercise expression of leukocyte IL-8 and IL-10 mRNA, indicating that elevated plasma quercetin levels exerted some effects within the blood compartment. Quercetin did not, however, influence any of the muscle measures including NFκB content, cytokine mRNA, or COX-2 mRNA expression across a 3-day intensified exercise period.

KEY WORDS: Nuclear factor kappa B, cyclooxygenase-2, immune, inflammation, flavonoids.

INTRODUCTION

Plasma concentrations of pro- and anti-inflammatory cytokines increase during prolonged and heavy exertion including interleukin (IL)-6, IL-10, IL-8, IL-1ra, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 beta (MIP-1β), tumor necrosis factor-alpha (TNF-α), and macrophage migration inhibitory factor (MIF) (21,24-29,32). These cytokines are produced by multiple cell types both within and outside the immune system. Several studies indicate that IL-6, IL-8, IL-1β, and TNF-α mRNA content is increased within post-exercise muscle biopsy samples, with the greatest fold increases measured for IL-6 and IL-8 mRNA (20-30 fold change from rest when rest=1) (10,24,25,31). Blood leukocytes may produce IL-8, IL-10, and IL-1ra during sustained exercise (27).

Several types of nutritional supplements including N3-polyunsaturated fatty acids, glutamine, bovine colostrum, and various types of antioxidants such as vitamins C and E have been studied for their potential effect in countering exercise-induced increases in plasma cytokines (23). None have proven consistently effectual except for carbohydrate supplements. Carbohydrate compared to placebo beverage intake attenuates exercise-induced increases in IL-6, IL-10, and IL-1ra, but not IL-8 (23-25). Carbohydrate may exert these effects through multiple mechanisms including elevation in blood glucose and tissue glucose uptake, decreases in cytokine mRNA expression, reductions in pro-inflammatory signals, and an attenuation of IL-6 release from the working muscle tissue (23-25).

The physiologic effects of dietary flavonoids such as quercetin are of great current interest due to their antioxidative, anti-inflammatory, anti-pathogenic, cardioprotective, and anticarcinogenic activities (8,15,28). Quercetin (3,3',4',5,7-pentahydroxyflavone) is the major

bioflavonoid in the human diet and is sufficiently bioavailable to exert wide ranging physiological effects in humans (8,19). 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 vitamin C (2). Significant food sources include apples, onions, berries, leafy green vegetables, hot peppers, red grapes, and black tea. Several studies indicate that quercetin mediates some of its effects by inhibiting cyclooxygenase-2 (COX-2) and nuclear factor-kappaB (NFκB) (2-5,11,18,20,22). NFκB is activated by pro-inflammatory stimuli such as TNF-α and lipopolysaccharide (LPS), and controls the expression of genes encoding pro-inflammatory cytokines and chemokines such as IL-1β, TNF-α, MCP-1, and MIP-1α/β (7,12,14,16). In unstimulated cells, NFκB is localized in the cytoplasm and complexed with the inhibitory protein I kappa B (IκB). Several stimuli including pro-inflammatory cytokines, reactive oxygen species, pathogen exposure, hormones, DNA damage, and physical stress activate protein kinases that phosphorylate IκB, causing its rapid degradation and translocation of NFκB to the nucleus (16). In the nucleus, NFκB binds to target genes, stimulating transcription of inflammatory mediators such as IL-1β, TNF-α, and IL-6. Quercetin acts by blocking the protein kinase-mediated IκB degradation, thereby preventing NFκB activation (4,11,22). Prostaglandins are also involved in inflammation, and COX-2 is a key enzyme regulating prostaglandin production. COX-2 is an inducible enzyme, is abundant in activated macrophages and other cells at sites of inflammation, and converts the essential fatty acid arachidonic acid to prostaglandin. Quercetin inhibits COX-2, and may thus be one of the mechanisms responsible for quercetin's anti-inflammatory effects (11). However, it should be emphasized that most of these data on quercetin's inhibitory effects on NFκB and COX-2 come from cell culture and animal studies.

The influence of quercetin ingestion on exercise-induced increases in cytokine mRNA expression, plasma cytokine levels, muscle NFκB, and muscle COX-2 mRNA in human endurance athletes is unknown. One study showed that supplementation with mixed tocopherols, flavonoids including quercetin, and docosahexaenoate for two weeks decreased plasma IL-6 levels following eccentric arm curl exercise (30). The authors related the IL-6 lowering effect to the anti-inflammatory properties of the combined supplement.

Given quercetin's potential to block NFκB and COX-2, and thereby reduce transcription of pro-inflammatory mediators including prostaglandins, cytokines, and chemokines, we hypothesized that quercetin compared to placebo ingestion would attenuate cytokine mRNA expression in muscle and blood leukocytes and lower plasma cytokine levels in endurance athletes engaging in repeated and sustained heavy exertion.

METHODS

Subjects. Forty trained male cyclists were recruited 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. Two to three weeks prior to the first test session, subjects reported to the ASU Human Performance Lab for orientation and measurement cardiorespiratory fitness. VO_{2max} was determined using a graded maximal protocol (25 watt increase every two minutes starting at 150 watts) with the subjects using their own bicycles on CompuTrainer™ Pro Model 8001 trainers (RacerMate, Seattle, WA). Oxygen uptake and ventilation were measured using the MedGraphics CPX metabolic system (MedGraphics Corporation, St. Paul, MN). Heart rate was measured using a chest heart rate monitor (Polar Electro Inc., Woodbury, NY). Basic

demographic and training data were obtained through a questionnaire.

Subjects agreed to avoid the use of large-dose vitamin/mineral supplements (above 100% of recommended dietary allowances), nutritional supplements, ergogenic aids, herbs, and medications known to affect immune function for three weeks prior to and during a 3-day period of intensified exercise. During orientation, a dietitian instructed the subjects to follow a diet moderate in carbohydrate (using a food list) during the 24-day study period. Subjects recorded food intake in a 3-day food record prior to the first exercise test session. The food records were analyzed using a computerized dietary assessment program (Food Processor, ESHA Research, Salem, Oregon).

The cyclists were randomized to quercetin (N=20) or placebo (N=20) groups. Under double blind procedures, subjects received quercetin (1000 mg/day) or placebo supplements for three weeks prior to and during a 3-day period of intensified exercise. Tang™ powder (Kraft Foods Inc., Northfield, IL) and 500 mg pure quercetin powder (QU995, Quercegen Pharma, Newton, MA) were mixed with powdered food coloring and placed in plastic vials. Subjects mixed the contents of the vials with 8 ounces water in green plastic bottles and consumed the beverage before their first and last meals of each day to achieve an intake of 1000 mg quercetin/day. Subjects returned empty vials to the study dietitian to verify compliance with the supplementation regimen.

Subjects came to the lab for three consecutive days following the 3-week quercetin or placebo supplementation period. Subjects cycled for 3 h at ~57% $Watts_{max}$. Subjects reported to the lab at 2:00 pm not having ingested energy in any form after 12:30 pm. Blood samples were collected ~30 minutes pre-exercise and ~15 minutes post-exercise for each exercise session. Subjects ingested 0.5 to 1.0 liters of water per hour during the 3-h cycling bout, with no other

beverages or food ingested during the test sessions. Muscle biopsies were obtained before and after the first and third exercise sessions.

During the test sessions, experimental subjects cycled using their own bicycles on CompuTrainer™ Pro Model 8001 trainers (RacerMate, Seattle, WA) with the exercise load set at ~57% maximal Watts. Metabolic measurements were made every 30 minutes of cycling using the MedGraphics CPX metabolic system to verify workload.

Blood samples. Blood samples were drawn from an antecubital vein with subjects in the supine position. Routine complete blood counts (CBCs) were performed by our clinical hematology laboratory, and provided hemoglobin and hematocrit for determination of plasma volume change using the method of Dill and Costill (6). Other blood samples were centrifuged in sodium heparin or EDTA tubes, and plasma was aliquoted and then stored at -80°C prior to plasma quercetin and cytokine analysis.

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 (Newton, MA, personal communication). This procedure is similar to that previously published by Ishii, et al. (13). Quercetin conjugates were hydrolyzed by incubating 250-500 uL plasma aliquots with 10 uL 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^{o}C. After incubation, 500 µL of 0.01 M oxalic acid was added, and then each sample was vortexed and centrifuged for 5 min at 10000 rpm. 1 mL of supernatants were then applied to solid phase extraction cartridges (Oasis HLB 1cc (30mg) SPE cartridge (Waters Corporation, Milford Massachusetts) 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. Milford Massachusetts). Cartridges were then washed with 0.5 mL of MeOH x 2. Eluant was collected into 1.5 mL microcentrifuge tubes. 10 µL of 10% DTT solution were added to the combined eluant and the samples were then vortexed for 1 minute and placed into a vacuum concentrator (Savant Speed Vac SC 110 Savant Instruments Inc., Farmindale, NY) until MeOH was completely evaporated. The residue was reconstituted with 150 µL MeOH/dH_2O (1/1). 50 µL injections were used for HPLC analysis.

Chromatographic analysis was performed using a Waters Breeze system (Waters Corporation, Milford Massachusetts) consisting of a Waters 1525 Binary HPLC pump, 2487 UV detector and Symmetry C18 5 µm 4.6 x 150 mm column. The mobile phase, consisting of a mixture of acetonitrile with 0.1% HCOOH (0-15 minutes: 10-90%) and dH2O with 0.1% HCOOH (0-15 minutes: 90-10%) with a gradient elution program at a flow rate of 1 mL/min.

Quantitation of the quercetin peak was based on the standard addition method using both plasma and MeOH with similar results. Both standards and samples were treated in an identical manner.

Plasma Cytokine Measurements. Total plasma concentrations of interleukin-1 receptor antagonist (IL-1ra), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), monocyte chemotactic protein 1 (MCP-1), and tumor necrosis factor alpha (TNF-α) 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 pre-exercise samples of IL-6 and IL-10. The minimum detectable concentration of IL-1ra was <22 $pg \cdot ml^{-1}$, IL-6 <0.70 $pg \cdot ml^{-1}$, IL-6 (high sensitivity) <0.039 $pg \cdot ml^{-1}$, IL-8 <3.5 $pg \cdot ml^{-1}$, IL-10 <3.9 $pg \cdot ml^{-1}$, IL-10 (high sensitivity) <0.5 $pg \cdot ml^{-1}$, MCP-1 <5.0 $pg \cdot ml^{-}$

^{1}, and TNF-α <0.106 pg·ml^{-1}. To improve sensitivity in the detection of IL-8, we employed SOFTmax™ analysis software (Molecular Devices, Sunnyvale, CA). When applicable, pre- and post-exercise samples were analyzed on the same assay plate to decrease inter-kit assay variability.

Skeletal muscle biopsies. Skeletal muscle biopsy samples were acquired 30-60 minutes before and 20-40 minutes after the 3-h cycling bouts on Days 1 and 3. The exact same procedures were utilized for all biopsies in accordance with previous studies (24,25). On Day 1, pre- and post-exercise were incisions made in the same thigh ~3 cm apart. One Day 3, muscle samples were collected from the opposite thigh, with leg order determined randomly. Local anesthesia (1% Xylocaine) was injected subcutaneously and into the vastus lateralis. A muscle biopsy sample was then obtained using the percutaneous needle biopsy procedure modified to include suction (9). Muscle biopsy samples were divided into three pieces and immediately frozen in liquid nitrogen. Samples were stored at -80°C until subsequent analysis.

NFκB Muscle Analysis Frozen skeletal muscle tissue (~10mg) was homogenized in 0.5mL of lysis buffer (Active Motif, Carlsbad, CA) containing 10mM dithiothreitol (DTT) and a protease inhibitor cocktail using a polytron. The homogenized samples were incubated on ice for 30 minutes. Samples were transferred to pre-chilled microcentrifuge tubes and centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant was transferred to a new pre-chilled microcentrifuge tube and centrifuged again to obtain a clarified lysate. Total soluble protein of the lysate was determined via bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). NFκB was determined using a TransAM ELISA based technique designed to determine the p65 subunit of NFκB (Active Motif, Carlsbad, CA). Briefly, 30µL of binding buffer containing DTT and herring sperm DNA was added to each well, followed by 20µL of sample containing

2µg. The plate was sealed and incubated for 1 hour at room temperature with mild agitation (100rpm on a plate shaker). Following the incubation the plate was washed 3 times with 200µL of wash buffer. Primary NFκB antibody (100µL) was added to each well; the plate was covered and incubated for 1hour at room temperature. The wash step was repeated and 100µL of HRP antibody was added to each well followed by a 1 hour incubation period. The plate was washed 4 times before 100µL of developing solution was added to each well. The plate was incubated in the dark for 5 minutes, followed by addition of 100µL of stop solution. The plate was read on a microplate reader within 5 minutes at 450nm with a reference wavelength of 655nm.

Leukocyte mRNA Extraction and cDNA Synthesis. The QIAampRNA Blood Mini Kit Protocol (Qiagen, Valencia CA, Catalog #52304) was utilized to extract mRNA. From each subject, two 1.5 ml aliquots of whole blood collected in EDTA were purified for RNA. Briefly, erythrocytes were selectively lysed and leukocytes 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 of RNase-free water.

The extracted RNA (7.5 µl of sample) was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically 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 10X RT Buffer, 11 µl 25 mM MgCl2, 10 µl deoxyNTPs mixture, 2.5 µl random hexamers, 1 µl RNase inhibitor, and 1.25 µl multiscribe reverse transcriptase (50U/ µl). Reverse transcription was performed at 25°C for 10 minutes, 37°C for 60 minutes, and 95°C for 5 minutes, followed by quick chilling on ice and stored at -20°C until subsequent amplification.

Quantitative real-time PCR analysis. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) analysis was done as per manufacturer's instructions (Applied Biosystems) using TaqMan® Gene Expression Assays. DNA amplification was carried out in 12.5 Taqman Universal PCR Master Mix (AmpliTaq 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, Foster City, CA). After 2 minutes at 50°C and 10 minutes at 95°C, plates were coamplified by 50 repeated cycles of which one cycle consisted of 15 s denaturing step at 95EC and 1 minute annealing/extending step at 60°C. Data was analyzed by ABI software using the CT, cycle threshold, 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) (28), 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 (24,25). Samples were run in duplicate and the intra-assay and inter-assay CVs were determined to be 1.69% and 1.65% for the ΔCTs, respectively.

Muscle Total RNA isolation and cDNA synthesis. Procedures for RNA isolation were in accordance with previous publications (24,25). Briefly, skeletal muscle tissue was homogenized under liquid nitrogen with a polytron, and total RNA was extracted using the guanidine thiocynate method with TRIzol Reagent (Life Technologies, GibcoBRL). The extracted RNA (2.5 µl of sample) was dissolved in diethylpyrocarbonate-treated water and quantified

spectrophotometrically at 260-nm wavelength. RNA was reverse transcribed into cDNA in a 50 µl reaction volume containing 19.25 µl RNA (1.5 µg) in RNase-free water, 5 µl 10X RT Buffer, 11 µl 25 mM MgCl2, 10 µl deoxyNTPs mixture, 2.5 µl random hexamers, 1 µl RNase inhibitor, and 1.25 µl multiscribe reverse transcriptase (50U/µl). Reverse transcription was performed at 25°C for 10 minutes, 37°C for 60 minutes, and 95°C for 5 minutes, followed by quick chilling on ice and stored at -20°C until subsequent amplification.

Calculations for relative quantification. Quantification of mRNA expression for leukocyte IL-8, IL-10, and IL-1ra, and muscle COX-2, IL-6, IL-8, TNF-α , and IL-1β was calculated using the ΔΔCT method as described by Livak and Schmittgen (17). 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 as the standard curve method (33). Briefly, ΔCT (CT(FAM) - CT(VIC)) was calculated for each sample and calibrator. ΔΔCT (ΔCT(calibrator) - ΔCT(sample)) was then calculated for each sample and relative quantification was calculated as 2ΔΔCT. Initial exclusion criteria consisted of FAM CT ≥40 and VIC CT ≥ 23.

Statistical analysis. Data are expressed as mean±SE. Data in Table 1 were compared between groups using student's t-tests. Data in Table 2 and figures 1 through 10 were analyzed using a 2 (groups) x 4 (muscle) or 6 (plasma) (time points) repeated measures ANOVA. For certain variables of interest, an overall "day" effect was calculated that compared changes across the three days of measurement. 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 (Pillais trace). When interaction effects were significant ($P \leq 0.05$), pre- to post-exercise changes were calculated and compared between quercetin and placebo groups using student's t-

tests, with significance set at $P \leq 0.05$.

RESULTS

Subject characteristics and performance data for the 40 cyclists randomized to quercetin and placebo groups are summarized in Table 1. No significant differences were found between groups for age or maximal performance measures. Subjects in the quercetin and placebo groups came into the study averaging 242±27 and 270±29 km/wk, and 1.5±0.2 and 1.6±0.2 h cycling per training bout, respectively. Thus the 3-day intensified exercise period (9 h exercise) represented nearly a doubling of their normal exercise workload. Subjects in quercetin and placebo groups were able to maintain a mean power output of ~57% $watts_{max}$ at an oxygen consumption of ~68% VO_{2max} and a cadence slightly above 80 revolutions per minute during the 9 h of exercise (Table 1).

Plasma volume change did not differ between groups during the three exercise bouts and averaged less than 1% due to ingestion of 0.5-1.0 liters water per hour of exercise (data not shown). After three weeks of supplementation, a 9.2-fold difference in plasma quercetin levels was measured between quercetin and placebo groups ($P<0.001$) (Figure 1).

Three-day food records prior to the 3-day exercise period revealed no significant group differences in energy or macronutrient intake (data not shown), and for all subjects combined, energy intake was 11.2±0.5 MJ/day (2684±117 kcal/day) with carbohydrate representing 56.6±2.5%, protein 16.2±0.8%, and fat 27.2±1.8%. A post-study questionnaire revealed that 9 subjects in each group guessed they were on quercetin, 4 in each group guessed placebo, and 7 in each group were uncertain (thus no significant group differences).

Significant main time effects were measured for each of the six plasma cytokines in Table 2, but the pattern of increase during each 3-h cycling bout did not differ significantly

between quercetin and placebo groups. The patterns of change for plasma IL-8 and TNF-α tended to be lower in the quercetin compared to placebo group (P=0.094 for both). The exercise-induced change in plasma IL-8 was 29% lower in the quercetin compared to placebo group on Day 1 (P=0.161) but was not markedly different on Days 2 and 3. The magnitude of the pre-to-post exercise increases in plasma cytokine levels decreased over the 3-day period for all six cytokines (day x time effect, P<0.05).

Muscle NFκB content did not differ between quercetin and placebo groups, and was unaltered by exercise (Figure 2) (interaction effect, P=0.662; time effect, P=0.872). Muscle COX-2 mRNA expression increased significantly post-exercise on Days 1 and 3 (P<0.025), but did not differ between quercetin and placebo groups (Figure 3) (interaction effect, P=0.944). Significant main time effects were measured for post-exercise increases in blood leukocyte IL-8, IL-10, and IL-1ra mRNA (P<0.001 for each) (Figure 4 to 6). The overall pattern of change over time was significantly lower for quercetin compared to placebo groups for leukocyte IL-8 and IL-10, but not IL-1ra mRNA (P=0.019, 0.012, and 0.256, respectively). Post-exercise leukocyte IL-8 mRNA averaged 2.8±0.2 and 4.2±0.2 (fold difference from rest) during the 3-day exercise period for quercetin and placebo groups, respectively (33% difference) (P=0.004). Leukocyte IL-10 mRNA expression increased post-exercise on Day 1 (36% less for the quercetin compared to placebo group), but was unaltered on Days 2 and 3 (day x time effect, P<0.001).

Muscle cytokine mRNA expression increased significantly post-exercise for IL-6, IL-8, IL-1β, and TNF-α (main time effects, P<0.001 for each), but the pattern of change did not differ between quercetin and placebo groups (interaction effects, P=0.595, 0.913, 0.910, and 0.930, respectively) (Figure 7 to 10). The magnitude of increase in muscle cytokine mRNA expression decreased across the 3-day period (day x time effects, P<0.001 for each).

DISCUSSION

Quercetin (1,000 mg/day) compared to placebo ingestion by cyclists during a 24-day period did not alter muscle NFκB content or exercise-induced increases in muscle COX-2 mRNA or mRNA expression for IL-6, IL-8, IL-1β, and TNF-α. Quercetin supplementation did attenuate increases in post-exercise blood leukocyte IL-8 and IL-10 mRNA, and tended to lower plasma levels of IL-8 and TNF-α.

Few studies have measured pre- and post-exercise levels for plasma cytokine levels while also providing data on cytokine mRNA expression in muscle samples and blood leukocytes. Exercise-induced increases in these measures confirm previous findings in our laboratory and reports from other investigators (10,24-27,31). Of the various cytokines measured in this study, IL-8 changes with exercise were most strongly influenced by quercetin supplementation. IL-8 mRNA expression in blood leukocytes but not muscle biopsy samples was reduced 33% post-exercise in the quercetin compared to placebo group, with a tendency towards lowered plasma IL-8 levels (29% lower on Day 1).

IL-8 is produced by multiple cell types including endothelial cells, fat cells, mononuclear cells in blood, macrophages, mast cells, and cells in bone, skin, and muscle (10). IL-8 is an important mediator of inflammatory disorders, and stimulates polymorphonuclear cell adherence, degranulation, and respiratory burst activity. IL-8 is rapidly induced by many stimuli including TNF-α, IL-1, and bacterial agents. *In vitro* data indicate that quercetin at concentrations within the range achieved by the subjects in this study inhibits TNF-α production and gene expression via modulation of NFκB and IκB in human peripheral blood mononuclear (PBM) cells (22). Other *in vitro* data with mast cells demonstrate that quercetin strongly inhibits secretion of TNF-

α and IL-8 (15). These two *in vitro* studies, however, used quercetin in agylcone form, and application to the conjugated quercetin found *in vivo* is currently unknown.

Quercetin also significantly reduced blood leukocyte IL-10 mRNA expression after the first day of exercise, but without any measurable effect on plasma IL-10 levels. *In vitro* data from bone marrow-derived macrophages indicate that quercetin stimulates the expression of IL-10, a finding consistent with quercetin's anti-inflammatory effects (3). Thus our finding of an increased IL-10 mRNA expression in blood leukocytes is difficult to interpret, but this effect occurred only after the first day of exercise (3). Most cytokine measures in this study showed diminished post-exercise levels by the third compared to the first day of exercise suggesting adaptation via the hypothalamic-pituitary-adrenal axis as discussed elsewhere (28). These data indicated that trained cyclists adapted quickly to the physiologic stress of repeated bouts of intense and prolonged exercise, and by the second and third bout experienced lower levels of stress hormones and diminished immune perturbations.

Contrary to our hypothesis, neither exercise nor quercetin had an effect on muscle NFκB content, and exercise-induced increases in muscle COX-2 mRNA were similar in quercetin and placebo groups. Prolonged and intensive exercise increased both muscle and leukocyte mRNA cytokine expression and plasma cytokine levels, suggesting that these effects occurred without muscle NFκB regulation. Steensberg et al. (31) also reported exercise-induced increases in muscle IL-6 and IL-8 mRNA without muscle NFκB involvement. Primary signaling mechanisms and transcription factors for cytokine gene expression during exercise are poorly understood, but nitric oxide production, calcineurin nuclear factor of activated T cells (NFAT), mitogen activated protein kinases (MAPKs), and other pathways are being explored (31). NFκB regulates the expression of many target genes within cells of the immune system, including those encoding

cytokines, immune receptors, cell adhesion molecules, apoptosis, and acute phase proteins (22). *In vitro* studies with a variety of cells including macrophages indicate that inhibition of COX-2 by quercetin may contribute to anti-inflammatory effects via mechanisms involving a decrease in NFκB activation (11,18). However, our data do not suggest a linkage between oral quercetin ingestion, muscle NFκB content, and muscle COX-2 mRNA expression during intensified exercise training.

The physiology of NFκB in cells outside the immune system is not well understood. Chronic NFκB activation in muscle leads to muscle wasting as seen in cancer, AIDS, and bed rest (7,16). Rodent studies indicate that the NFκB signaling pathway is activated in skeletal muscle during exercise, with reactive oxygen species generated during exercise serving as critical messengers to activate upstream kinases of the NFκB cascade (1,12,14,16). A high alpha-tocopherol diet in rats blunts exercise-induced increases in muscle NFκB and myeloperoxidase, indicating underlying redox-sensitive pathways (1). Studies with human subjects, however, do not support these findings. Durham et al. (7) showed a 50% decrease in NFκB activity in thigh muscle samples taken from human subjects who engaged in 45 minutes of fatiguing leg squat and extension exercise. The decrease in NFκB was transient with a full recovery by one-hour post-exercise. Male subjects engaging in knee extensor exercise for two hours did not experience an increase in NFκB activity from thigh muscle biopsy samples (31). Plasma F_2-isoprostanes increased significantly in our subjects (data being presented elsewhere) after each exercise bout indicating significant oxidative stress without muscle NFκB activation. Thus currently available data do not support muscle NFκB activation in humans during exercise or a regulatory role in muscle or blood cytokine mRNA expression. We used a whole-cell extract procedure to extract NFκB from homogenized muscle tissue and measured the P65

subunit of NFκB using an antibody ELISA method that is specific for the activated form of p65. Other investigators have measured DNA binding activity of NFκB from muscle cell nuclear extracts (7,14,16) or muscle content of IκBα using Western blot (31). Thus some caution is urged in comparing results across studies. We did not measure NFκB activation in blood PBM, but quercetin's influence in decreasing blood leukocyte IL-8 and IL-10 mRNA expression suggests some level of immune cell NFκB regulation during exercise.

In summary, 1,000 mg/day quercetin supplementation by trained cyclists over a 24-day period did not have widespread effects in countering exercise-induced increases in muscle cytokine or COX-2 mRNA expression or plasma cytokine levels across a 3-day intensified exercise period. Quercetin compared to placebo ingestion did diminish post-exercise expression for leukocyte IL-8 and IL-10 mRNA, suggesting that elevated plasma quercetin levels exerted some effects within the blood compartment. Quercetin ingestion had no influence on any of our muscle outcome measures including muscle NFκB content, and cytokine and COX-2 mRNA, suggesting that quercetin may not accumulate in muscle tissue, at least within the context of this study. Other data from this study published elsewhere indicate that the cyclists experienced a markedly lower incidence of upper respiratory tract infection symptoms during the 2-week period following intensified exercise (28). Group differences in illness rates occurred despite no influence of quercetin on several measure of immune function. Thus quercetin may have reduced illness rates using direct anti-pathogenic pathways as shown *in vitro*. Quercetin is conjugated with glucuronic acid, sulfate, and methyl groups in human plasma, and additional research is needed to discover which metabolites influence blood leukocyte IL-8 and IL-10 mRNA expression and exert anti-pathogenic activity.

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Figure Legends

Figure 1 Plasma quercetin levels after 3-weeks supplementation and prior to the 3-day intensified exercise period in quercetin (N=20) and placebo (N=20) groups (P=0.002).

Figure 2 Muscle NFκB content pre- and post-3h cycling at 57% $watts_{max}$ in quercetin (N=20) and placebo (N=20) groups on days 1 and 3. Main time effect, P=0.872; group x time interaction effect, P=0.662.

Figure 3 Muscle COX-2 mRNA pre- and post-3h cycling at 57% $watts_{max}$ in quercetin (N=20) and placebo (N=20) groups on days 1 and 3. Main time effect, P<0.05; group x time interaction effect, P=0.944.

Figure 4 Blood leukocyte IL-8 mRNA pre- and post-3h cycling at 57% $watts_{max}$ in quercetin (N=20) and placebo (N=20) groups during three consecutive days. Main time effect, P<0.001; group x time interaction effect, P=0.019. P-values for group comparisons of pre- to post-exercise changes were 0.089, 0.010, and 0.148 for Days 1 to 3, respectively.

Figure 5 Blood leukocyte IL-10 mRNA pre- and post-3h cycling at 57% $watts_{max}$ in quercetin (N=20) and placebo (N=20) groups during three consecutive days. Main time effect, P<0.001; group x time interaction effect, P=0.012. P-values for group comparisons of pre- to post-exercise changes were 0.074, 0.855, and 0.298 for Days 1 to 3, respectively.

Figure 6 Blood leukocyte IL-1ra mRNA pre- and post-3h cycling at 57% $watts_{max}$ in quercetin (N=20) and placebo (N=20) groups during three consecutive days. Main time effect, P<0.001; group x time interaction effect, P=0.558.

Figure 7 Muscle IL-6 mRNA content pre- and post-3h cycling at 57% $watts_{max}$ in quercetin (N=20) and placebo (N=20) groups on days 1 and 3. Main time effect, P<0.001; group x time interaction effect, P=0.595.

Figure 8 Muscle IL-8 mRNA content pre- and post-3h cycling at 57% $watts_{max}$ in quercetin (N=20) and placebo (N=20) groups on days 1 and 3. Main time effect, P<0.001; group x time interaction effect, P=0.913.

Figure 9 Muscle IL-1β mRNA content pre- and post-3h cycling at 57% $watts_{max}$ in quercetin (N=20) and placebo (N=20) groups on days 1 and 3. Main time effect, P<0.001; group x time interaction effect, P=0.910.

Figure 10 Muscle TNF-α mRNA content pre- and post-3h cycling at 57% $watts_{max}$ in quercetin (N=20) and placebo (N=20) groups on days 1 and 3. Main time effect, P<0.001; group x time interaction effect, P=0.930.

Table 1 Subject characteristics measured at baseline, and performance data averaged during 3-h cycling bouts over three days (mean±SE).

Variable	Cyclists (N=20) Quercetin	Cyclists (N=20) Placebo	P-value
Baseline Measures			
Age (yrs)	26.1±1.8	29.1±2.4	0.321
Weight (kg)	74.7±0.2	74.2±1.4	0.846
VO_{2max} ($mL \cdot kg^{-1} \cdot min^{-1}$)	53.2±1.2	54.7±1.1	0.365
$Power_{max}$ (watts)	314±9	320±69	0.554
HR_{max} (beats/min)	188±1	190±2	0.328
Performance Measures			
Mean power (watts)	178±5	181±4	0.626
Power (% $watts_{max}$)	56.8±0.2	56.5±0.3	0.486
Mean heart rate (beats/min)	149±2	149±3	0.897
Heart rate (%HR_{max})	79.4±0.8	78.1±1.0	0.300
Mean VO_2 ($mL \cdot kg^{-1} \cdot min^{-1}$)	36.0±0.9	36.9±0.8	0.458
VO_2 (%VO_{2max})	67.8±0.8	67.6±1.1	0.919
Cadence (revolutions/min)	80.8±2.2	83.7±2.2	0.349

VO_2 = volume of oxygen consumption; HR = heart rate.

Table 2 Pre-to-post exercise (3-h cycling bouts at ~57% $watts_{max}$) changes in plasma cytokine levels in quercetin compared to placebo groups over a 3-day period (mean±SE).

	Quercetin (N=20)		**Placebo (N=20)**		**P-values : Time;**
Variable	**Pre-**	**Post-**	**Pre-**	**Post-**	**2 x 6 interaction**
IL-6 (pg/ml)					
Day 1	1.1±0.3	35.7±4.5	0.6±0.1	28.3±6.0	<0.001; 0.279
Day 2	1.4±0.4	23.1±4.7	1.2±0.4	14.1±1.9	Day P<0.001
Day 3	1.4±0.4	15.7±1.9	0.7±0.1	11.0±1.7	
IL-10 (pg/ml)					
Day 1	4.6±0.7	80.9±19.0	5.0±1.0	95.0±23.9	<0.001; 0.924
Day 2	5.0±0.7	22.8±4.0	6.0±1.0	23.3±4.2	Day P<0.001
Day 3	4.5±0.6	25.8±5.4	5.2±1.0	19.7±5.3	
IL-8 (pg/ml)					
Day 1	4.2±1.0	10.7±1.4	5.4±1.0	14.6±1.5	<0.001; 0.094
Day 2	4.3±0.9	8.9±1.3	5.6±1.1	10.6±0.8	Day P<0.001
Day 3	4.1±1.0	8.6±1.2	5.4±0.4	8.2±1.1	
IL-1ra (pg/ml)					
Day 1	135±16	871±373	135±10	1016±446	<0.001; 0.755
Day 2	174±21	768±366	180±22	376±52	Day P=0.051
Day 3	152±18	484±130	150±13	305±38	
TNF-α (pg/ml)					
Day 1	1.09±0.10	1.26±0.10	1.08±0.09	1.31±0.07	<0.001; 0.094
Day 2	0.99±0.07	1.21±0.07	1.11±0.08	1.39±0.10	Day P<0.001
Day 3	0.97±0.06	1.06±0.08	1.15±0.08	1.20±0.09	
MCP-1 (pg/ml)					
Day 1	123±7	310±27	127±9	291±23	<0.001; 0.680
Day 2	131±11	247±16	129±15	231±16	Day P<0.001
Day 3	122±6	237±14	132±11	231±16	

Figure 1

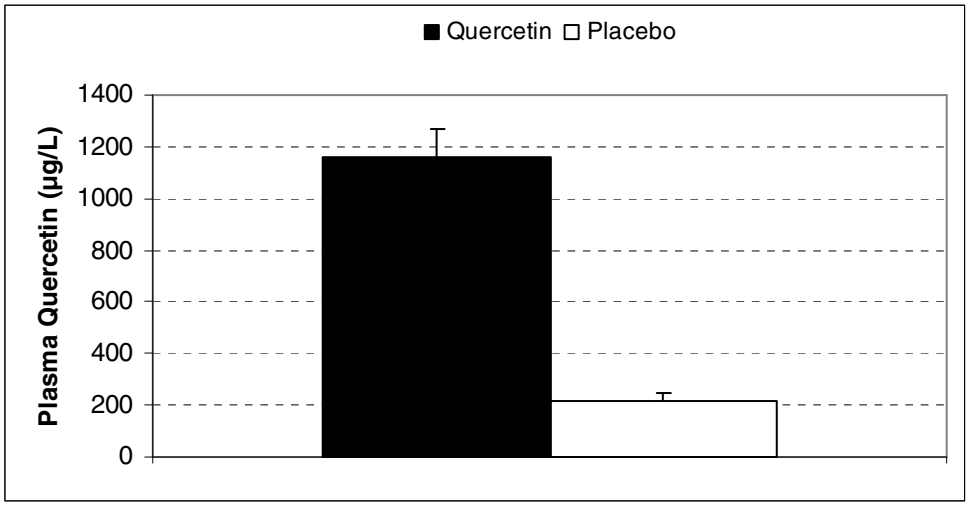

Quercetin
Placebo
Plasma Quercetin (µg/L)
1400
1200
1000
800
600
400
200
0

Figure 2

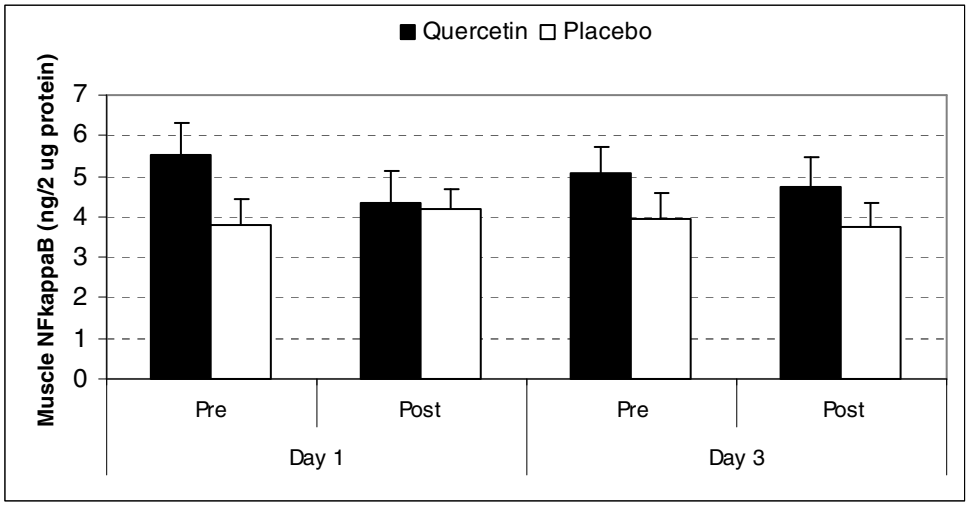

Quercetin
Placebo
Muscle NFkappaB (ng/2 ug protein)
7
6
5
4
3
2
1
0
Pre
Post
Pre
Post
Day 1
Day 3

Figure 3

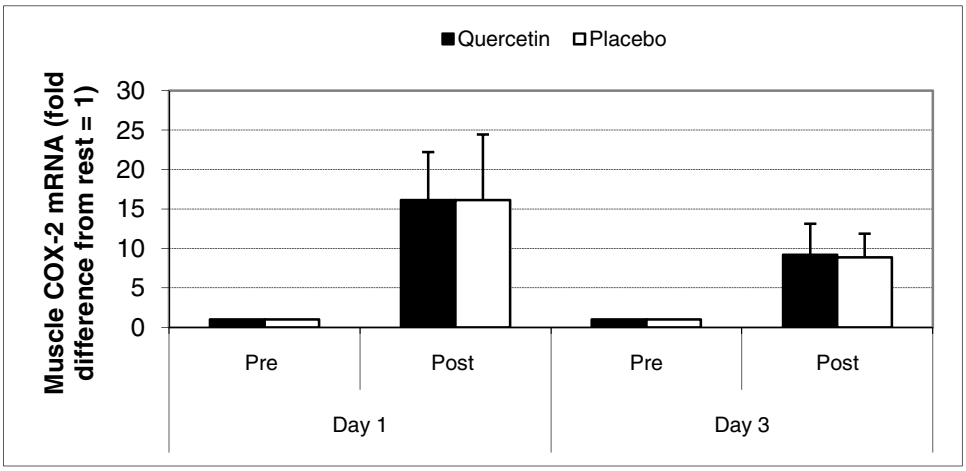

Quercetin
Placebo
Muscle COX-2 mRNA (fold difference from rest = 1)
30
25
20
15
10
5
0
Pre
Post
Pre
Post
Day 1
Day 3

Figure 4

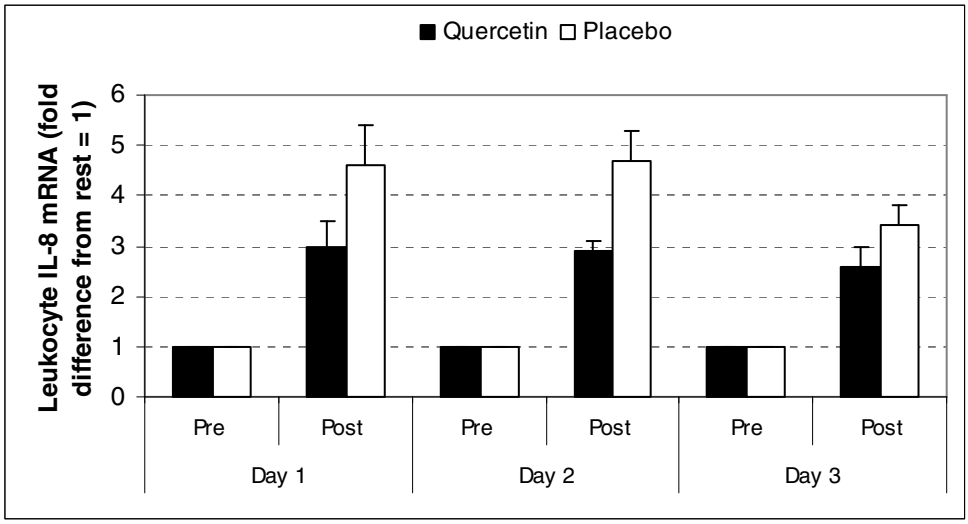

Quercetin
Placebo
Leukocyte IL-8 mRNA (fold difference from rest = 1)
6
5
4
3
2
1
0
Pre
Post
Pre
Post
Pre
Post
Day 1
Day 2
Day 3

Figure 5

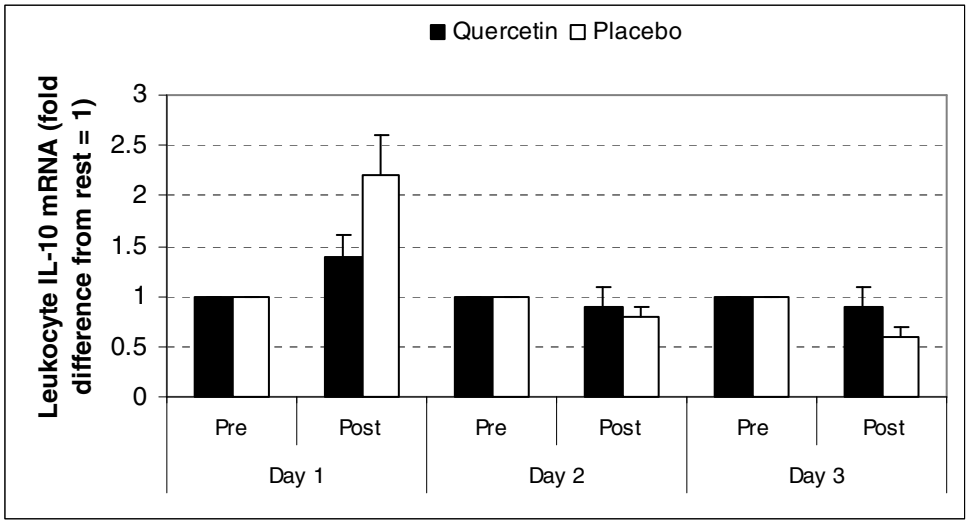

Quercetin
Placebo
Leukocyte IL-10 mRNA (fold difference from rest = 1)
3
2.5
2
1.5
1
0.5
0
Pre
Post
Pre
Post
Pre
Post
Day 1
Day 2
Day 3

Figure 6

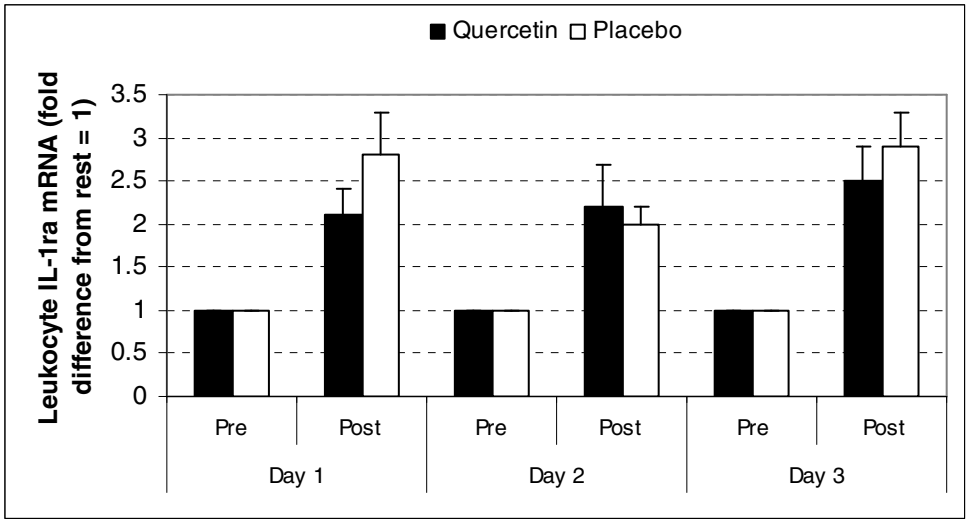

Quercetin
Placebo
Leukocyte IL-1ra mRNA (fold difference from rest = 1)
3.5
3
2.5
2
1.5
1
0.5
0
Pre
Post
Pre
Post
Pre
Post
Day 1
Day 2
Day 3

Figure 7

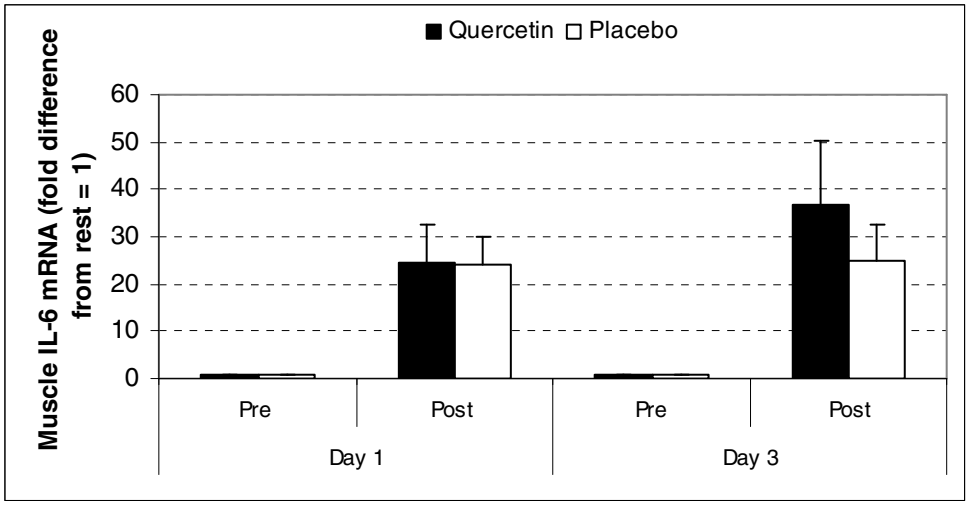

Quercetin
Placebo
Muscle IL-6 mRNA (fold difference from rest = 1)
60
50
40
30
20
10
0
Pre
Post
Pre
Post
Day 1
Day 3

Figure 8

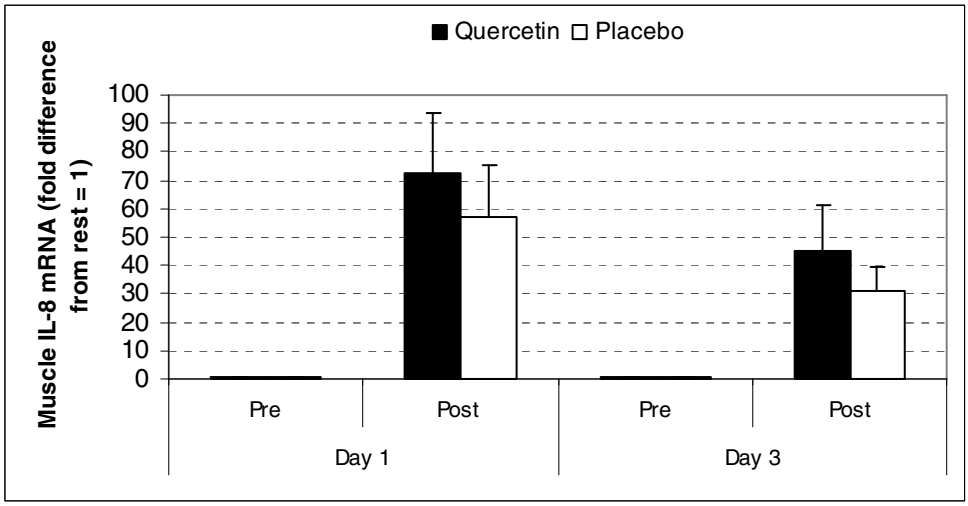

Quercetin
Placebo
Muscle IL-8 mRNA (fold difference from rest = 1)
100
90
80
70
60
50
40
30
20
10
0
Pre
Post
Pre
Post
Day 1
Day 3

Figure 9

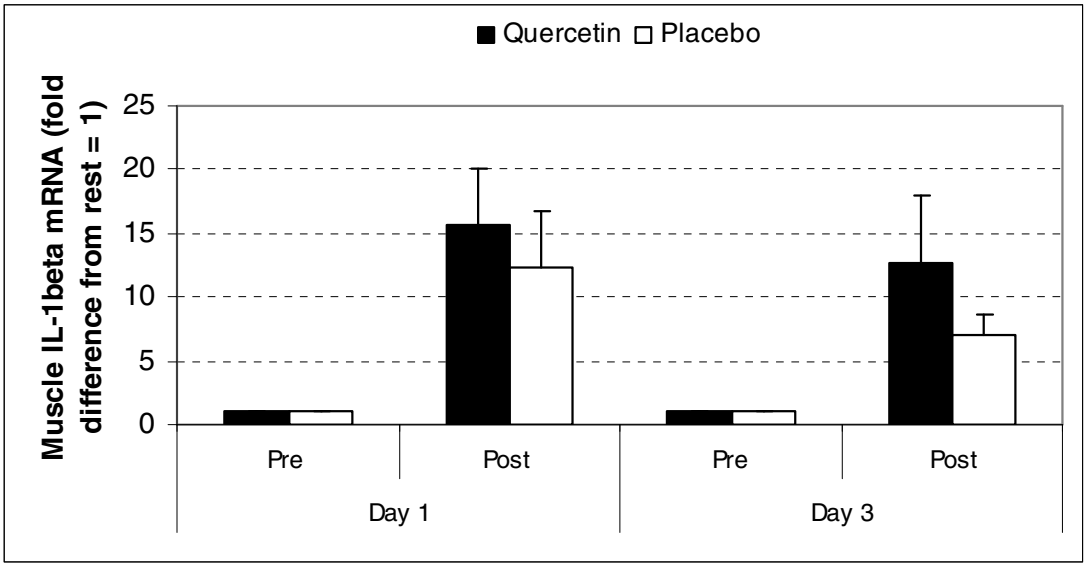
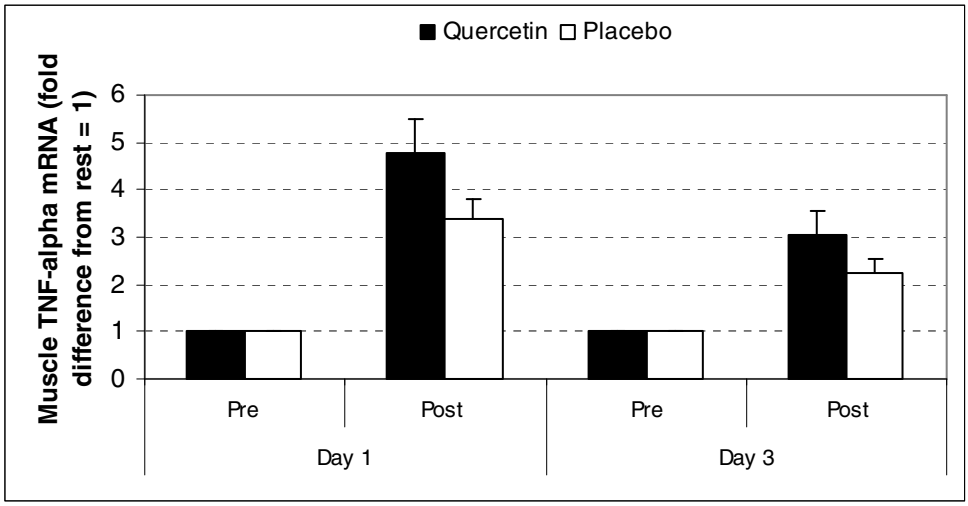

Quercetin
Placebo
Muscle IL-1beta mRNA (fold difference from rest = 1)
25
20
15
10
5
0
Pre
Post
Pre
Post
Day 1
Day 3

Figure 10


Quercetin
Placebo
Muscle TNF-alpha mRNA (fold difference from rest = 1)
6
5
4
3
2
1
0
Pre
Post
Pre
Post
Day 1
Day 3