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THE EFFECTS OF QUERCETIN ON EXERCISE INDUCED CYTOKINE RESPONSE IN TRAINED CYCLISTS

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THE EFFECTS OF QUERCETIN ON EXERCISE INDUCED CYTOKINE RESPONSE IN TRAINED CYCLISTS

By

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Thesis

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

In Partial Fulfillment

of the Requirements

for the Degree of

Master of Science in Kinesiology

The University of Texas at Austin

December 2012

ACKNOWLEDGMENTS

I would like to first thank to Dr. Edward Coyle for allowing me to have this opportunity in his lab. His experience and guidance has assisted my development as I attempt to become a scientist in this field of study. He guided me to improve step by step toward an independent researcher. Next, I would like to thank to my thesis partner Douglas Van Pelt. He always pushed us forward and stimulated us to work harder. Although we had some stressful and incredibly busy periods during the course of the study, I always felt happy to work with him. Through my three years I have had the pleasure of working with some great people in the Human Performance Lab. I must first thank Kevin Christmas for his passion and involvement in assisting Doug and I to solve technical problems. Sarah Hill was also dedicated and invaluable in her role during almost every time trial and assisted us whenever we requested her assistance. I also want to thank Justin Trombold as a senior lab member helped me develop my critical thinking, lab skills and knowledge in Exercise physiology. Thank you to all other member that I have interacted with over my 2 years in the lab. I really appreciate Dr. Matt Brothers taking time out his very busy schedule to be my second reader on my thesis. His expertise and writing suggestions were a great assistance. Thank you Patty Coffman, Elizabeth Hulsey, Mina Rathbun, and Mary Ann Ortiz for all of their incredible support and patience as Doug and I pestered you about ordering items, paying the subjects, shipping items, and reserving parking passes. Your assistance and support was invaluable in the day to day operation of our study.

ABSTRACT

THE EFFECTS OF QUERCETIN ON EXERCISE INDUCED CYTOKINE RESPONSE IN TRAINED CYCLISTS

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Quercetin is a flavonoid found in commonly consumed fruits and vegetables that has exhibited powerful antioxidant and anti-inflammatory properties in rodents and *in vitro*. In humans, the effect of quercetin on exercise induced oxidative stress and inflammation is still equivocal and need to be further investigated. A powerful antioxidant such as quercetin may inhibit the high levels of oxidative stress and inflammation associated with the high volume and intensity of exercise training seen with endurance-trained individuals.

PURPOSE: To determine the effect of 28 days of daily quercetin supplementation on intensive endurance exercise induced cytokine response.

METHODS: Thirteen trained cyclists (VO_2peak 58.8 ± 3.9 ml/kg/min) were recruited for this study from the University of Texas at Austin and the local Austin, Texas community and participated in this placebo controlled, randomized, crossover designed study. After initial assessment of baseline data (VO_2peak, lactate threshold, and two familiarization time trials), participants began daily supplementation with either an antioxidant supplement containing vitamins and quercetin (Q-VIT: 1000mg quercetin, 820mg Vitamin C, 40mg Vitamin B3) or the same vitamin supplement without quercetin (VIT: 820mg Vitamin C, 40mg Vitamin B3). A simulated time trial using an electromagnetically braked cycle ergometer in which subjects had to complete a set amount of work (kJ) as fast as possible was performed on the last day of supplementation.

Blood collection was performed at three time points of the time trial days: before exercise (PRE), after warm up (MIN 20), and immediately after time trial exercise (POST). Measured plasma markers were Interleukin-6 (IL-6), C-Reactive Protein (CRP), and Interleukin-10 (IL-10).

RESULTS: Q-VIT compared to VIT had no effect on pre, min 20 and post exercise plasma IL-6, CRP, and IL-10 (P= 0.7, 0.08, and 0.32 respectively). However there was a trend that Q-VIT lowered plasma CRP compare to VIT (P = 0.08).

CONCLUSION: Chronic supplementation for 28 days with a quercetin based antioxidant supplement did not affect plasma cytokine before during or after exercise. The results of the current study suggest that chronic supplementation with quercetin does not influence plasma cytokine and exercise induced cytokine response in endurance-trained athletes.

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INTRODUCTION

Regular physical activity is well known to reduce the incidence of or attenuate the detrimental effects of various disease states. However, exercise, particularly intense exercise can lead to increased inflammation [1] and oxidative stress [2] following acute bouts of exercise. Antioxidant supplementation has the potential to attenuate exercise-induced oxidative stress and inflammation [3]. Specifically, there has been a large interest in quercetin, a common flavonoid found in grapes, apples, cherries, onions, citrus fruits, broccoli, and tea [4]. Epidemiologic data indicate reduced rates of cardiovascular disease [5]and various types of cancer [6-8] in groups self-selecting diets high in quercetin. The flavonoid quercetin has been associated with numerous potential health benefits in rodents and in vitro including anti-viral [9], hepatoprotective, antifibrogenic [10], antiatherogenic [11], anti-inflammatory [12], and potent antioxidant capabilities [13]. Several recent quercetin based supplementation studies in human athletes have focused on potential influences as a countermeasure to post-exercise inflammation and oxidative stress, however, the results remain equivocal [14-20]. There are several potential reasons for inconsistent results including the training states of subjects, supplementation durations, the timing of last supplement intake, and the types of exercise and exercise protocol.

Due to the mixed results of the previous studies, the goal of this study is to clarify the effect of chronic quercetin supplementation on exercise induced cytokine response in trained cyclists. We hypothesize that the powerful antioxidant properties of quercetin will reduce plasma cytokine and/or attenuate exercise induced cytokine response in trained cyclists after daily supplementation for 28 days.

REVIEW OF LITEERATURE

The following literature review will discuss the flavonoid quercetin and its associated properties in cell culture, animal, and human experimental trials. Specifically, the review will focus on the antioxidant and anti-inflammatory property of quercetin and its potential role in attenuate exercise induced oxidative stress and inflammation. Studies that have evaluated the effect of quercetin based supplements on exercise induced cytokine response and inflammation will be analyzed.

Quercetin

Flavonoids occur naturally in fruits, vegetables and beverages such as tea and wine. Quercetin is the major flavonoid which belongs to the class called flavonols. Quercetin is found in many common foods including apples, tea, onions, nuts, berries, cauliflower, cabbage and many other foods. Quercetin has been shown to be an excellent in vitro antioxidant. Within the flavonoid family, quercetin is the most potent scavenger of reactive oxygen species (ROS) [21, 22], and reactive nitrogen species (RNS) [23, 24]and these antioxidative capacities of quercetin are attributed to the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging, i.e. the catechol group in the B ring and the OH group at position 3 of the AC ring [25]. Moreover, quercetin is suggested to substantially empower the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity which is 6.24 times higher than the reference antioxidant trolox, whereas for example the contribution of both vitamin C and uric acid virtually equals that of trolox [26]. Quercetin is also known to possess strong anti-inflammatory capacities [27, 28]. Several in vitro studies using different cellines have shown that the flavonoid is capable of inhibiting lipopolysaccharide (LPS) induced cytokine production. For instance, quercetin inhibits LPS

induced tumor necrosis factor – alpha (TNFα) production in macrophages [29] and LPS-induced interleukin-8 (IL-8) production in lung cells [30] Moreover, in glial cells it was even shown that quercetin can inhibit LPS-induced mRNA levels of two cytokines, i.e. TNFα and interleukin-1 alpha (IL-1α) [31]. A possible explanation for these anti-inflammatory effects of quercetin may be found in the interplay between oxidative stress and inflammation. ROS are not only involved in the occurrence of oxidative stress, but also in the promotion of inflammatory processes via activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein (AP)-1 which induce the production of cytokines like interleukin-6 (IL-6) [12, 32]. Consequently, scavenging ROS would not only prevent the occurrence of oxidative stress but also help mitigate inflammation. Indeed, it has already been shown that quercetin can inhibit the production as well as the gene expression of TNFα via modulation of NF-κB in human peripheral blood mononuclear cells [33]. A possible mechanism behind this modulation was reported to be the inhibition of the degradation of the inhibitory part (IκBα) of this transcription factor [34]. Therefore, quercetin can potentially attenuate cytokines production by directly neutralizing ROS and/or inhibiting the activity of redox-sensitive signal transduction pathways.

Bioavailability of Quercetin

Because the beneficial effects of quercetin are largely dependent on its bioavailability after oral administration, the absorption, distribution, metabolism, and excretion of quercetin have been studied extensively in both laboratory animals and humans. Although initial reports indicated that bioavailability of quercetin was limited, recent evidence suggests otherwise [35]. Large scale epidemiological studies support the availability of quercetin in human plasma and serum when volunteers were supplemented with doses ranging from 50-1000mg/day. Quercetin

serum and plasma levels rose in a direct dose dependent manner [36, 37]. However, the levels of quercetin within the plasma varied greatly among the population with no relationship to demographic or lifestyle factors [37]. Jin et al. [37] reported that the net increase in blood was 332 and 516 micrograms/L, respectively, for the 500 and 1,000 mg/d dose after twelve weeks of quercetin supplementation. Data from their study also showed that the dose response increase in plasma quercetin was achieved within the first month and maintained for the rest of the study, which supports the rationale that choosing 28 days as supplementation duration in our study. Literature indicates that isoquercetin (glycosylated quercetin) 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 of quercetin [38-40]. A possible explanation for this increased absorption, resulting in a higher plasma peak concentration and an increased bio-availability, is the facilitation of glycoside absorption by either deglycosylation [41, 42] or carrier-mediated transport [43, 44]. For example, 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 [45]. Two or more flavonoids ingest together may also 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 [40]. It has been shown that the half live of the quercetin metabolites are rather high, i.e. 11 to 28 h. This indicates that, upon repeated quercetin supplementation, they could attain a considerable plasma level [39, 46]. Furthermore, Davis et al. [35] reported that quercetin can be detected in plasma within 15-30 min after ingestion of a 250 or 500 mg quercetin chew preparation, reaching a peak concentration at

approximately 120-180 min. This supports the rationale that subjects ingested quercetin supplement two hours before starting of exercise in our study.

Exercise and Oxidative Stress and Inflammation

In response to endurance exercise, oxygen (O2) consumption increases 10- to 20-fold systemically and as much as 100- to 200-fold at the level of the skeletal muscle, resulting in substantially increased mitochondrial electron flux. ROS ‘‘leaking’’ from the mitochondria during exercise are considered a main source of oxidative stress [47]. Other potential sources of ROS during exercise include enhanced purine oxidation, damage to iron-containing proteins, disruption of Ca2+ homeostasis [48], and Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [49]. These exercise-induced ROS are also thought to modulate acute-phase inflammatory responses [50]. Exercise-induced tissue damage and/or increased reactive oxygen species (ROS) production stimulate cytokine production, upregulating the inflammatory cascade [50-52]. Initially, pro-inflammatory cytokines, TNFα and IL-1α are produced, stimulating IL-6 production [51]. IL-6, the primary mediator of the acute-phase reaction, stimulates production of acute-phase proteins, including C-reactive protein (CRP), and restricts the extent of the inflammatory response by enhancing production of anti-inflammatory cytokines such as interleukin-10 (IL-10) [52, 53]. Neutrophils, monocytes, and lymphocytes recruited to the site of inflammation also produce ROS and proteolytic enzymes to clear and repair damaged tissue [50, 51, 53].

However, post exercise cytokine response may be independent of oxidative stress and muscle damage induced by exercise. In recently years, it became clear that contracting human skeletal muscle releases significant amounts of interleukin (IL)-6 into the circulation during prolonged single-limb exercise [54]. This increase is followed by the appearance of IL-1 receptor

antagonist (IL-1ra) and the anti-inflammatory cytokine IL-10. Of note, the cytokine response to exercise and sepsis differs with regard to TNFα. Thus, the cytokine response to exercise is not preceded by an increase in plasma TNFα. It appears that, unlike IL-6 signaling in macrophages, which seems to be dependent upon the activation of the NF-κB signaling pathway, intramuscular IL-6 expression is regulated by a network of signaling cascades that among other pathways are likely to involve crosstalk between the Ca2+/nuclear factor of activated T-cells (NFAT) and glycogen/p38 mitogen-activated protein kinase (MAPK) pathways. Thus, when IL-6 is produced by macrophages, it leads to an inflammatory response, whereas muscle cells produce and release IL-6 without activating classical pro-inflammatory pathways[55].

Quercetin and Exercise Induced Inflammation

Chronic Supplementation

The strong anti-inflammatory capacities of quercetin in vitro have led several human studies to investigate the influence of quercetin supplementation on cytokines response to exercise. A study [14]used 40 trained male cyclists in a double-blind, randomized, cross sectional, placebo controlled investigation. Subjects ingested quercetin (pure quercetin 1,000 mg/day mixed in orange Tang powder with powdered food coloring) or placebe for three weeks before and during a 3-day period in which subjects cycled for 3 h/day at 57% maximal work rate to test the influence of quercetin on exercise-induced changes in plasma cytokines and muscle and leukocyte mRNA. Quercetin supplementation significantly increased plasma quercetin levels, however, quercetin only diminished postexercise expression of leukocyte IL-8 and IL-10 mRNA, and exhibited no influence on postexercise increases in plasma cytokines and any of muscle measure, including NF-KB content, cytokine mRNA, or COX-2 mRNA expression. In the same

study, McAnulty et al. [15] also showed that quercetin did not affect oxidative stress, and plasma antioxidant capacity.

Another three weeks quercetin supplementation was done by Neiman et al. [16]. This study was a double-blind, cross sectional, placebo controlled study with ultramarathon runners at the 160-km Western States Endurance Run (WSER). Sixty-three runners ingested 1,000 mg/day quercetin or placebo each day for three weeks and just prior to WSER, but did not ingest additional supplements until after post-race 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 post-race. There were no significant groups differences in proinflammatory and anti-inflammatory plasma cytokines, cortisol, serum CRP, and creatine kinase (CK) even through there were significant prerace to postrace increases in there measures.

Abbey al el. [19] performed a crossover, randomized, double-blind, placebo controlled study with fifteen recreationally activity young adult men to test the influence of one week quercetin supplementation on repeated-sprint performance, xanthine oxidase (XO) and inflammation. The supplementation was a 6% carbohydrate commercial drink for placebo, or that drink with 500 mg of quercetin-3-glucoside for quercetin group, consumed twice a day. Quercetin did not improve repeated-sprint performance nor attenuate XO activity and IL-6 response after sprint exercise.

Mixed with other flavonoids

There is increasing support for coingestion of quercetin with other flavonoids and food components to improve and extend quercetin's bioavailability and bioactive effects. Nieman et al. [17] employed the same exercise protocol as previously described to investigate quercetin's influence on exercise induced acute inflammatory response and markers of mitochondrial

biogenesis. Thirty-nine trained cyclists supplemented for 2 weeks on placebo, quercetin, or quercetin with epigallocatechin 3-gallate (EGCG). Subjects ingested four soft chews per day, each quercetin chew contained with 250 mg of quercetin, 250 mg of vitamin C, 10 mg of niacinamide, and 200 mg of folic acid. These nutrients were included in the quercetin group to facilitate quercetin absorption in the small intestine. Each Q–EGCG chew contained all Quercetin group ingredients with 30 mg of EGCG from green tea extract, 100 mg of isoquercetin, and 100 mg of N3-polyunsaturated fatty acids (N3- PUFA) (55 mg of eicosapentaenoic acid (EPA) and 45 mg docosahexaenoic acid (DHA)) from fish oil. These food components were included to improve quercetin bioavailability and extend its bioactive effects. No markers of mitochondrial biogenesis were elevated, however, there were significantly lower levels of CRP, IL-6, and IL-10 after exercise in the quercetin with EGCG group immediately following exercise.

A more recent study performed by McAnulty et al. [18] mixed quercetin with other compounds. This study examined the effects of 1,000 mg quercetin + 1,000 mg vitamin C (QC); 1,000 mg quercetin, 1,000 mg vitamin C, 400 mg isoquercetin, 30 mg epigallocatechin gallate, and 400 mg n-3 fatty acids (QFO); or placebo (P), taken each day for 2 week before and during 3 d of cycling at 57% Wmax for 3 hr on plasma oxidative stress (F2-isoprostanes) and antioxidant capacity. QC and QFC effectively reduced postexercise increase in F2-isoprostanes with no changes of antioxidant capacity between groups. This result indicated that the effect of QC and QFC on F2-isoprostanes independent to changes in plasma antioxidant capacity.

The acute effect of quercetin base supplementation has also been done. Konrad et al. [56] examined the anti-inflammatory and immune-modulating influence of a single dose of a quercetin-base supplement consumed by endurance athletes 15 minutes before a 2-hour run at 70-75% VO2max. In contrast to the two weeks study with similar supplementation [17], a

mixture of 1000mg quercetin, 120mg EGCG, and other vitamins provided no benefit to the post-exercise immune changes or inflammation.

METHODS

Subject

Endurance-trained male (n = 11) and female (n = 2) cyclists (age 30.1 ± 7.1 y, body mass 67.8 ± 10.81 kg, height 173.41 ± 9.15 cm, VO_2max 58.83 ± 3.93 ml/kg/min) (Table 1) were recruited for this study from the University of Texas at Austin and local Austin, Texas community. A trained cyclist was defined as an individual that regularly competes in cycling racing and competitions and had a minimum VO_2max of 53 ml/kg/min for men and 45 ml/kg/min for women on the cycle ergometer. Subjects had to be training a minimum of 7 hours per week on the bike and between 18 and 40 years old. During the entire duration of the study, the subjects were required to maintain their current volume and intensity of cycling training. Any potential participants that were planning on tapering or vastly increasing volume or intensity of training during the 12 weeks following the screening appointment were not included in the study. Subjects completed a questionnaire prior to participation to assure they met the inclusion criteria, which included the following: must be healthy, non-smoking, and have no history of chronic disease. Consumption of anti-inflammatory (Advil, Motrin, etc.) or anti-oxidant supplements (supplemental vitamins/minerals, etc.) could not be consumed during the entire testing period. Subjects must have no history of hypertension, history of or current antihypertensive drugs use, or use of lipid-lowering, or anti-inflammatory steroid medication; no active weight loss > 5 kg in the prior 3 months, and no history of kidney dysfunction. Subjects must not use antidepressant drugs such as Selective Serotonin Reuptake Inhibitors (SSRI) during the testing period. The study was conducted under a protocol approved by the University of Texas at Austin Institutional Review Board, and each subject provided written informed consent.

Study Design

This study was a double blind, randomized, placebo controlled, crossover experiment. There were two testing periods lasting 28 days each with a 1 week washout between them. Preliminary testing for determination of each subject's maximal oxygen consumption (VO2max) and lactate threshold (LT) along with two familiarization time trials (FAM1, FAM2) were performed in a 2 week period prior to the start of the testing period. During each testing period, the subject would be randomly assigned to ingestion of a daily quercetin based vitamin supplement (Q-VIT) or a vitamin supplement without quercetin (VIT). On the last day of each testing period (Day 28) the subject returned to the lab to perform a simulated time trial on a cycle ergometer. Combined with the preliminary testing, there were a total of six visits to the Human Performance Laboratory for each subject. The preliminary phase of testing consisted of the first four visits: VO2max testing, LT testing, FAM1, and FAM2. The testing period consisted of two visits to the lab in which the subjects would perform the simulated time trial on Day 28 of supplementation (TT1 and TT2). Blood draws were collected before exercise(PRE), after warm up(MIN 20), and after time trial (POST) in each the time trials for analyzing plasma concentrations of interleukin-6, C-reactive protein (CRP), and interluukin-10.

Weeks 1 - 2	Weeks 3 - 6	TT	Week 7	Weeks 8 - 11	TT
VO2max	Supplementation 1 (Q or P)		Washout	Supplementation 2 (Q or P)	
LT	TT on last day			TT on last day	
2 Fam TT					

Activity and Diet Control

Subjects were asked to refrain from any major changes in exercise training for the duration of the study. Subjects recorded daily training during the first supplementation periods that had to be closely matched during the second treatment period. All subjects were required to refrain from exercise the day before their time trial.

Diet was recorded during the three days leading up to the time trial during the first treatment period. This diet had to be replicated three days prior to the time trial during the second treatment period. A minimum of 2 hours was required between the time trial and the subject's last meal.

Supplementation

Subjects supplemented twice daily with a chewable flavored tablets. Two tablets were ingested in the morning and two were ingested in the evening with 12 hours part. The Q-VIT tablets each contained 250 mg quercetin, 205 mg Vitmain C, and 10 mg Vitamin B3 for a total of 1000 mg/day quercetin, 820 mg/day Vitamin C, and 40 mg/day Vitamin B3. The placebo chews (VIT) contained identical vitamin ingredients minus the quercetin. The placebo chews had a similar external appearance and flavor. On the final day of supplementation, subjects were instructed to ingest all four of the daily chews two hours before the time trial.

Exercise Trial

Upon arrival to the laboratory, subjects were seated and rested for five minutes before initiation of any measures or activity. Before and after a 20 minute warmup ride on the cycle ergometer at 70% VO2max, subjects were seated and blood was drawn via venipuncture of an antecubital vein. After the second blood draw, the subjects began the exercise time trial on the cycle ergometer. The goal of the time trial was to complete a set amount of work as quickly as possible.

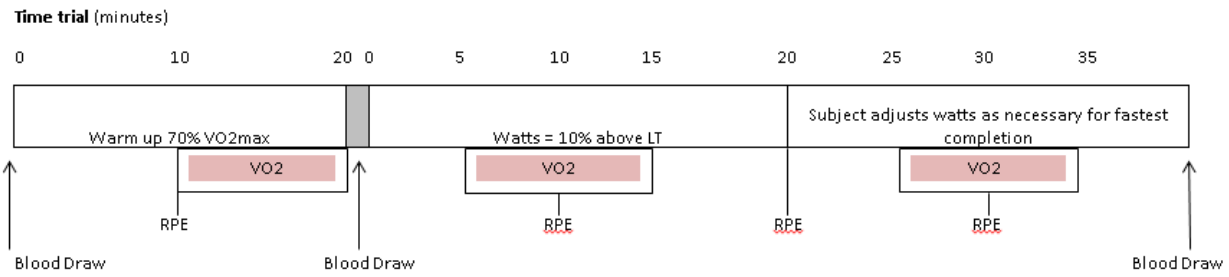
The required Kilojoules that the participant needs to complete during the time trial was calculated from the equation below:

• Ttworkrate = watts = Joules/sec

• Joules/sec X 2400sec = Joules

• Joules/1000 = Time Trial kJ (TT kJ)

The first 20 minutes of the time trial were set at a fixed workrate equal to the aforementioned Ttwokrate. Computer software (Lode Ergometer Manager V 9.1, Lode; Gronigen, Netherlands) was used to program the cycle ergometer to have a fixed power (fixed power mode) independent of cadence during the first 20minutes. After the first 20 minutes, the cycle ergometer would change to a cadence dependent mode (linear mode). In this mode, the subjects could increase or decrease power output (watts) by increasing or decreasing cadence from a previously established preferred cadence or by requesting the investigators to raise the Ttworkrate. Workrate was raised or lowered in pre-determined increments equal to 5% of their Ttworkrate. The subjects were told when they had completed 60, 70, 80, 90, and 95 percent of the work they must complete and the approximate time left until completion at their current power output. Subjects were verbally encouraged at these intervals to complete the time trial as fast as possible. Time to completion was recorded as the total time to complete TT kJ. The subject would immediately move off of the cycle ergometer to a seated position for the final blood draw. Aside from the pacing information, the subjects were blinded from all performance information until participation in the study was complete.



Blood Collection

Blood was drawn via venipuncture from an antecubital vein before exercise (PRE), after the warm up (MIN 20), and after completion of the time trial (POST) for both VIT and Q –VIT treatments. All blood samples collected were immediately transferred to K2 EDTA collection tubes (BD Vacutainer, Franklin Lakes, NJ), centrifuged at 2,000 g for 15 minutes at 4°C, and plasma was aliquoted and then stored in at -80°C until later analysis. All samples for each subject were performed in duplicate and within the same plate to decrease interkit assay variability. Enzyme-linked immunosorbent assays (ELISA) were used, in accordance with the manufacturer protocol, to measure total plasma concentrations of interleukin-6 (IL-6 high sensitivity), C-reactive protein (CRP), and interluukin-10 (IL-10 high sensitivity) (R&D Systems, Inc., Minneapolis, MN).

Interleukin-6

Interluekin-6 was measured in duplicate using the enzyme-linked immunosorbent method. Samples of plasma were pipetted into antibody, specific to IL-6, pre-coated wells and any IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked antibody specific for IL-6 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. After an incubation period, an amplifier solution was added to the wells and color developed in

proportion to the amount of IL-6 bound in the initial step. The color development was stopped and intensity of the color was measured using a spectrophotometer plate reader at 490nm and 650nm. Subtracted reading at 650nm from the reading at 490nm. This subtraction was to correct for optical imperfections in the plate. The minimum detectable concentration was 0.039 pg/mL. The intra-assay coefficient of variation was 4.0%.

C-reactive protein

C-reactive protein was measured in duplicate using the enzyme-linked immunosorbent method. Samples of plasma were pipetted into antibody, specific to CRP, pre-coated wells and any CRP present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked antibody specific for CRP was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of IL-6 bound in the initial step. The color development was stopped and intensity of the color was measured using a spectrophotometer plate reader at 450nm and 540nm. Subtracted reading at 650nm from the reading at 450nm. This subtraction was to correct for optical imperfections in the plate. The minimum detectable concentration was 0.010 ng/mL. The intra-assay coefficient of variation was 4.6%.

Interluukin-10

Interluekin-10 was measured in duplicate using the enzyme-linked immunosorbent method. Samples of plasma were pipetted into antibody, specific to IL-10, pre-coated wells and any IL-10 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked antibody specific for IL-10 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. After an incubation period, an amplifier solution was added to the wells and color developed in

proportion to the amount of IL-10 bound in the initial step. The color development was stopped and intensity of the color was measured using a spectrophotometer plate reader at 490nm and 690nm. Subtracted reading at 690nm from the reading at 490nm. This subtraction was to correct for optical imperfections in the plate. The minimum detectable concentration was 0.09 pg/mL. The intra-assay coefficient of variation was 6.7%.

Statistical Analysis

Plasma IL-6, CRP and IL-10 were analyzed with a two-way repeated measures ANOVA to analyze interaction of treatment and time. The criterion for significance was set at $p < 0.05$. All statistics were calculated with IBM SPSS Statistics software, version 19. Results are reported as means ± SEM.

RESULTS

Interleukin-6

Blood was collected before exercise (PRE), after the warm up (MIN 20), and after completion of the time trial (POST). The concentration of plasma interleukin-6 was expressed as picograms per milliliter (pg/mL). In VIT treatment, the plasma IL-6 for PRE, MIN 20, and POST were 3.59 ± 0.34 pg/ml, 4.63 ± 0.37 pg/ml, and 26.52 ± 1.54 pg/ml, respectively. Plasma IL-6 was significantly higher at (POST) compared to both MIN 20 ($p < 0.01$) and PRE ($p < 0.01$). Plasma IL-6 in treatment VIT was also significantly higher at MIN 20 compared to PRE ($p < 0.01$). In Q – VIT treatment, the plasma IL-6 for PRE, MIN 20, and POST were 3.922±0.415 pg/ml, 4.67 ± 0.56 pg/ml, and 24.96 ±1.98 pg/ml, respectively. Plasma IL-6 was significantly higher at POST compared to both MIN 20 ($p < 0.01$), and PRE ($p< 0.01$). Plasma IL-6 in treatment Q – VITwas also significantly higher at MIN20 compared to PRE ($p < 0.01$). There was no significant difference between treatment VIT and Q – VIT. ($p= 0.70$).

C-Reactive Protein

The concentration of plasma C-Reactive Protein was expressed as in nanograms per milliliter (ng/mL). In VIT treatment, the plasma CRP for PRE, MIN 20, and POST were 11.64 ± 2.72 ng/ml, 12.59 ± 2.47 ng/ml, and 12.94 ± 2.86 ng/ml, respectively. In Q – VIT treatment, the plasma CRP for the same data points were 8.36 ± 2.75 ng/ml, 8.84 ± 3.08 ng/ml, and 8.84 ± 3.41 ng/ml, respectively. There was an overall time effect of exercise on plasma CRP concentration ($p < 0.01$). Plasma CRP concentration was significantly higher at MIN 20 compared to PRE ($p <$

0.01). There was no significant difference between treatments VIT and Q – VIT, although there was a trend that VIT was higher than Q – VIT ($p = 0.08$).

Interleukin-10

The concentration of plasma interleukin-10 was expressed as picograms per milliliter (pg/mL).In VIT treatment, the plasma IL-10 for PRE, MIN 20, and POST were 0.38 ± 0.11 pg/ml, 0.36 ± 0.09 pg/ml, and 0.59 ± 0.10 pg/ml, respectively. Plasma IL-10 was significantly higher at POST compared to MIN 20 ($p = 0.03$). In Q – VIT treatment, the plasma IL-10 for the same data points were 0.273±0.036pg/ml, 0.266±0.027pg/ml, and 0.52 ± 0.12 pg/ml, respectively. Plasma IL-10 was significantly higher at POST compared to MIN 20 ($p = 0.046$). There was no significan difference between treatment VIT and Q – VIT ($p = 0.32$).

Plasma Quercetin

Supplementation with Q-VIT was successful in significantly raising plasma quercetin concentration when compared to the VIT treatment. Q-VIT quercetin plasma concentration was 2197 ± 409 ng/mL and VIT quercetin plasma concentration was 27 ± 9.69 ng/ml ($p < 0.01$).

Table 1 – Characteristic of research participates

Variable	Mean ± SE
n	11
Age (y)	30.1 ± 7.1
Height (cm)	173.4 ± 9.1
Weight (kg)	67.8 ± 10.8
BMI	22.42 ± 1.98
VO2max (ml/kg/min)	58.83 ± 3.93
VO2max (l/min)	3.98 ± 0.62

Table 1: Characteristics of research participates. Presented as MEAN ± SEM. (n=11)

Abbreviations: BMI, body mass index; VO2max, Maximal oxygen consumption.

Figure 1

Interleukin-6

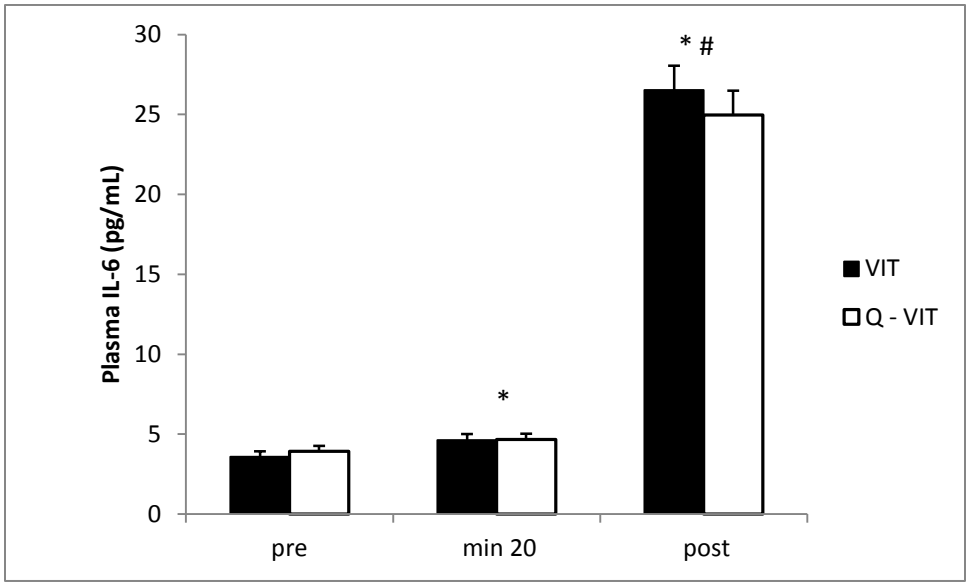


Figure 1: Plasma Interleukin-6 concentration reported in picograms per milliliter (pg/mL). Treatments were a vitamin supplement (VIT) and a vitamin supplement with quercetin (Q-VIT). Presented as MEAN ± SEM. (n=11). * VIT and Q - VIT were significantly greater than PRE ($p < 0.01$). # VIT and Q - VIT was significantly greater than minute 20 ($p < 0.01$). There were no significant differences between the treatments ($P = 0.70$).

Figure 2

C-Reactive Protein

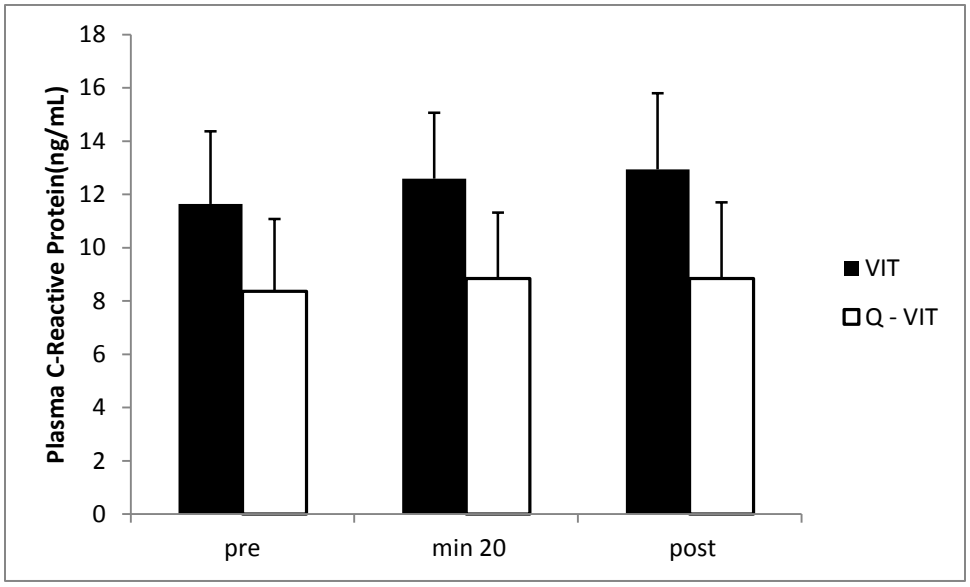


Figure 2: Plasma C-Reactive Protein concentration reported in nanograms per milliliter (ng/mL). Treatments were a vitamin supplement (VIT) and a vitamin supplement with quercetin (Q-VIT). Data presented as MEAN ± SEM. (n=11) Overall time ($p < 0.01$). * MIN 20 was significantly higher than PRE ($p < 0.01$). There were no significant differences between the treatments ($P = 0.08$)

Figure 3

Interleukin-10

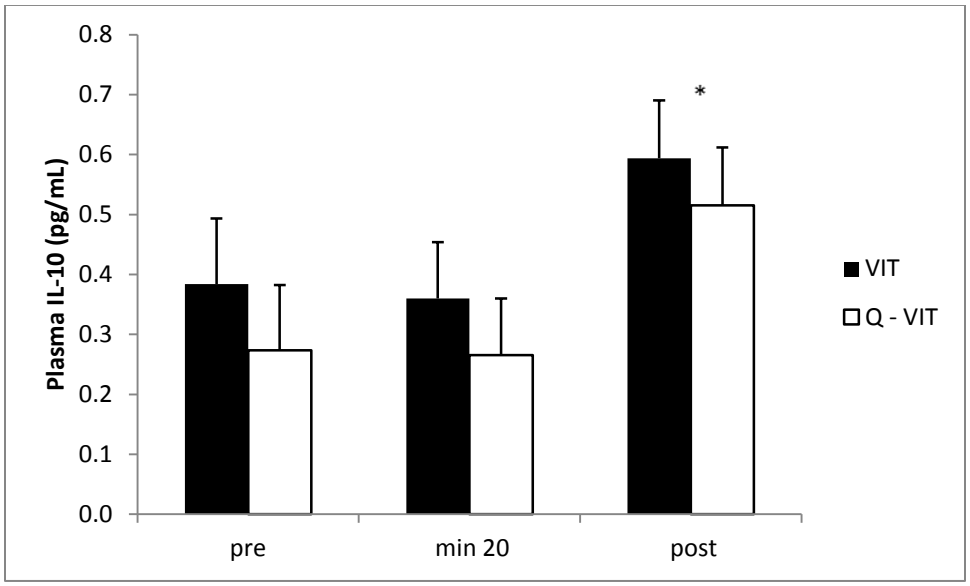


Figure 3: Plasma Interleukin-10 concentration reported in picograms per milliliter (pg/mL). Treatments were a vitamin supplement (VIT) and a vitamin supplement with quercetin (Q-VIT). Data presented as MEAN ± SEM. (n=11). * VIT and Q - VIT significantly greater than MIN 20 ($p < 0.05$).). There was no significant difference between treatments ($p = 0.32$).

DISCUSSION

The primary finding of this study was that there was a time effect of exercise on plasma Interleukin-6 (IL-6), C-reactive protein (CRP), and Interleukin-10 (IL-10) concentrations for both VIT and Q - VIT treatments. Our results show that intense endurance cycling exercise significantly increased both pro-inflammatory (IL-6) and anti-inflammatory cytokines (IL-10) after daily supplementation with VIT and Q –VIT for 28 days in trained cyclists. However, there were no significant differences between treatment VIT and Q - VIT in IL-6, CRP, and IL-10 concentrations at any time points. There was a trend that the plasma CRP concentration for Q-VIT was lower than VIT ($p = 0.08$).

In agreement with our study, several human studies have shown that there was no effect of chronic quercetin supplementation on exercise-induced inflammation. However, the timing of last quercetin supplementation before exercise needs to be carefully considered. Since plasma quercetin concentration peak at 120- 180 min after supplementation[35], performing exercise 2-3 hours after supplementation is more reasonable in studies using quercetin as supplement. However, this is not the case in aforementioned studies. In the first study[14, 15], cyclists were randomized to 1,000 mg/day quercetin or placebo for three weeks and cycled three hours per day three days in a row at 57% watts-max. Subjects ingested only 500 mg before breakfast and performed exercise at 2 pm. Therefore, there was a seven hours gap between quercetin ingestion and the beginning of exercise. A second three weeks quercetin supplementation study with ultramarathon runners at the 160-km Western States Endurance Run also showed no effect of quercetin on exercise induced inflammation[16]. In the study, subjects ingested 1,000 mg quercetin each day for three weeks and then just prior to the 160-km race, but did not ingest

additional supplements throughout the race (an average 27 hour). Plasma quercetin levels dropped to very low levels in the quercetin group and were not different from the placebo group post-race.

Abbey et al. [19] performed a one week quercetin supplementation study with 1,000 mg/d quercetin-3-glucoside also showed no effect on repeated-sprint performance, plasma xanthine oxidase activity, and IL-6. However, the supplementation duration was only one week, which is not enough for quercetin to accumulate in plasma at high level. Jin et al. [37] reported that it took four weeks for quercetin supplement to reach plateau in plasma. In addition, plasma quercetin concentration reaches peak level two to three hours after ingestion. However, subjects took their last quercetin supplement 15 minutes before exercise and the exercise trial lasted less than 30 minutes. Due to the duration of the supplementation and the timing of the last quercetin ingestion, we expected the plasma quercetin concentration were not high in their study and they did not report the data of plasma quercetin concentration.

Unlike most previous studies and present study found no effect of quercetin supplementation on markers of oxidative stress and inflammation[14-16, 19, 20]. Nieman et al. [17] showed that two weeks of quercetin combined with epigallocatechin 3-gallate (EGCG) supplementation resulted in significantly reduced post-exercise measures for both inflammation and oxidative stress. McAnulty et al. [18] also revealed that two weeks of quercetin combined with vitamin C, EGCG and n-3 fatty acids supplementation did not exhibit a significant increase in post-exercise oxidative stress. One potential reason of the inconsistent results in these study compares to our study is that 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 liver and via inhibiting efflux transporters [40], therefore

extend its bioactive effects. Another potential reason of inconsistent finding is that, in our study, there was no increase in plasma CRP level after exercise and plasma CRP concentration was very low at level around 0.012 mg/l. In previous study [17], plasma CRP increased to 8 mg/L in the placebo group after the third day of three hours per cycling suggests that the exercise load in our study was not enough to increase plasma CRP level and therefore less opportunity for quercetin to exert anti-inflammatory effect.

. We chose quercetin without combination with flavonoids because our goal is to examine the effect of single flavonoids, quercetin, on exercise induced cytokine response. Even though previous study did not show any effect of quercetin alone without other flavonoids on exercise induced inflammation, the study designs were not optimal in terms of the duration of supplementation, the timing of last dose supplement, and the reality of the exercise protocol. Therefore, our goal was to investigate the effect of chronic supplementation of single flavonoids, quercetin, on exercise induced cytokine response in trained cyclists with better study design. In our study, the supplementation duration was four weeks, which has been shown enough for quercetin to accumulate in plasma and reach plateau[37], and to our knowledge, this is the first study using four weeks as supplementation duration and is longer than all the studies examining the effect of quercetin supplementation on exercise induced oxidative stress and inflammation. While MacRae et al. [57] found six weeks of quercetin base supplementation increase performance on a cycling time trial in trained cyclists, the supplementation contained caffeine which is generally known as a ergogenic aid in sport and they did not measure any markers of inflammation and oxidative stress. In terms of the timing of last dose supplement, the subjects in our study ingested 1,000 mg quercetin 2 hours before exercise which we expected the plasma quercetin concentration reached to peak level. In addition, the exercise duration in our study was

approximately an hour, therefore the plasma quercetin concentration was maintained at high level throughout the exercise trial. Finally, our exercise protocol was a time trial very similar to a 30 kilometer time trial that competitive cyclists frequently perform.

We hypothesize that chronic quercetin supplementation will reduce exercise induced cytokine responses due to the anti-oxidative and anti-inflammatory capacities. The possible mechanism is that quercetin can neutralize ROS induced by intense aerobic cycling exercise, as during endurance exercise, oxygen consumption increases 10- to 20-fold systemically and as much as 100- to 200-fold at the level of the skeletal muscle, resulting in substantially increased mitochondrial electron flux. ROS ‘‘leaking’’ from the mitochondria during exercise are considered a main source of oxidative stress [47]. Quercetin is the most potent scavenger of ROS among flavonoid family [21, 22], and these antioxidant capacities of quercetin are attributed to the presence of two antioxidant pharmacophores within the molecule that has the optimal configuration for free radical scavenging. Since ROS can promote inflammatory processes via activation of transcription factors such as NF-κB which induce the production of cytokines like IL-6. Consequently, scavenging ROS by quercetin might not only prevent the occurrence of oxidative stress but also help mitigate inflammation.

In addition to ROS scavenging property of quercetin that exerts anti-oxidative and anti-inflammatory effect, quercetin can directly regulate NF-κB without involving in ROS. It has already been shown that quercetin can inhibit the degradation of the inhibitory part (IκBα) of NF-κB [34]. By decreasing the activation of NF-κB and as a result of decreasing the production of cytokines and inflammation. Therefore, quercetin can attenuate cytokines production by directly neutralizing ROS and/or inhibiting the activity of redox-sensitive signal transduction pathways such as NF-κB pathway. However, it should be emphasized that most data on

quercetin's anti-oxidative and anti-inflammatory effect come from cell culture and animal studies.

Even with better study design in terms of supplementation duration and timing of last dose supplement ingestion, the result of our study, however, were consistent with previous human studies that failed to show that quercetin supplementation has effect on exercise induced cytokine response. There were no significant difference between treatment VIT and Q - VIT in IL-6, CRP, and IL-10 concentrations at any time points. One possible explanation is that the evidence presented from in vitro studies and rodents does not translate into in vivo human study with exercise induced oxidative stress and inflammation. In vitro/cell culture data indicate that quercetin in the aglycone form exerts impressive antioxidant and anti-inflammatory effect and inhibits cytokines production and gene expression through modulation NF-κB. However, quercetin undergoes considerable chemical modification during digestion and absorption and is metabolized to methylated, glucurono-sulfated derivate[39]. The quercetin conjugates may have altered biologic properties and potencies. Another factor to consider is that high quercetin concentrations used in cell culture studies may not apply to the low plasma concentrations achievable through oral supplementation in human studies.

Another explanation for lack effect of quercetin supplementation on exercise induced cytokine response in our study is that quercetin may have in vivo antioxidant function, but lack of linkage between oxidative stress and inflammation. ROS generated during exercise serve as critical messengers to activate the NF-κB which induce the production of cytokines such as IL-6. However, Nieman et al.[14] showed that exercise-induced increase in muscle cytokine mRNA without muscle NF-κB involvement. In fact, Pedersen[55] suggested that unlike IL-6 signalling in macrophages, which seems to be dependent upon the activation of the NF-κB signalling

pathway, intramuscular IL-6 expression is regulated by a network of signaling cascades that among other pathways are likely to involve crosstalk between the Ca2+/nuclear factor of activated T-cells (NFAT) and glycogen/p38 mitogen-activated protein kinase (MAPK) pathways. In addition, contracting skeletal muscle *per se* is the main source of the IL- 6 in the circulation in response to exercise[54]. Therefore, quercetin may preserve its antioxidant function and scavenge ROS generated during exercise. However, contracting skeletal muscles produced Il-6 by different pathway independent of ROS regulation of NF-κB signalling pathway. As a result, both VIT and Q-VIT showed significant increase in plasma IL-6 concentration immediately after exercise (POST) to the same extent. Since IL-6 induced production of anti-inflammatory cytokine IL-10, the plasma IL-10 also increased to the same extent in both VIT and Q-VIT without treatment effect between them.

Elevation of CRP levels in blood is recognized as a marker of inflammation and is associated with an increased risk of cardiac disease [58], as well as the acute exercise response in healthy individuals. Quercetin and resveratrol suppressed cytokine-induced CRP expression in Hep3B cells[59]. Garcia-Mediavilla et al.[60] found that quercetin exhibited anti-inflammatory effects and subsequent reduction of CRP in Chang liver cells. In our study, similar to the plasma IL-6 and IL-10, no effect of quercetin was found in reducing CRP after heavy endurance exercise even though there was a trend that plasma CRP concentration for Q-VIT was lower than VIT ($p = 0.08$).

It should be emphasized that our subject population consisted of highly trained athletes who are known to possess up-regulated antioxidant defenses[61]. The presence of highly elevated antioxidant defense systems may have masked any beneficial effects of quercetin administration. It would be interesting to add another group using untrained individuals and

examine whether the untrained individuals with less endogenous antioxidant defenses will have different responses.

It should be also noticed that although excessive states of oxidative stress are likely detrimental, it is also known that oxidative stress from exercise also play fundamental roles in cell signaling and gene expression [62, 63]. Evidence suggests that antioxidant supplementation may in fact attenuate some of the exercise-induced cellular signals that stimulate adaptations in skeletal muscle and vascular tissue also exist [64-66]. The rationale for antioxidant supplementation in relation to intensive endurance exercise is to inhibit exercise induced inflammation. Inflammation is a double-edge sword, on one hand, inflammation is required in order to combat infection and acute injury and on the other hand, chronic systemic inflammation causes impaired metabolism. Therefore, when using antioxidant supplementation in an attempt to attenuated exercise induced inflammation, one should carefully control antioxidant dosage and exercise stress and cautiously examine actual consequences of antioxidant administration.

In addition, the role of IL-6 induced by exercise may very different from what was generally thought. It was commonly thought that the exercise-induced increase in IL-6 was a consequence of an immune response due to local damage in the working muscles [67], and it was hypothesized that the immune cells were responsible for this increase and leads to an inflammatory response[68]. Today, it is clear that the contracting skeletal muscle cells per se produce and release IL-6 without activating classical pro-inflammatory pathways [55]. In fact, muscle drives important IL-6 mediates autocrine, paracrine and endocrine functions. Thus IL-6 is involved in mediating glucose uptake in muscle, fat oxidation, lipolysis and possesses strong anti-inflammatory properties [69]. Therefore, an attenuate exercise induce cytokines response by

antioxidant supplementation may not actually be beneficial and further studies are needed to investigate the exercise induced cytokine response and inflammation.

There were limitations that must be realized in this study. The placebo (VIT) contained Vitamin C and Vitamin B3 in order to expound the effects of quercetin itself on exercise induced cytokine response, however the effect of the vitamin supplementation itself and the effect of quercetin itself cannot be determined with this design.

In conclusion, daily supplementation with the flavonoid quercetin for 28 days has no effect on intensive endurance induced cytokine response, including IL-6, CRP IL-10. However, there was a trend ($p=0.08$) that daily supplementation quercetin with vitamins for 28 days lowered the plasma CRP both before and after intensive endurance exercise compare to supplementation with vitamins alone. With intensive endurance exercise induced oxidative stress, cytokine response and inflammation are complicated and the intervention of antioxidants adds further complexity. Further research is needed to clarify whether the antioxidants supplementation is beneficial or detrimental.

APPENDIX: DATA TABLES

Interleukin-6

	VIT			Q - VIT		
Subject	pre	min20	post	pre	min20	Post
3	2.927	4.729	28.574	5.090	6.259	28.920
4	3.485	3.759	19.035	3.016	2.440	19.720
5	1.461	2.724	20.414	2.635	3.016	17.211
6	5.108	6.637	33.321	4.570	5.196	25.704
7	3.918	3.997	22.398	4.844	4.835	22.216
8	3.829	5.504	35.287	1.354	1.907	17.420
9	3.600	4.324	22.840	5.028	5.636	25.160
11	5.345	6.110	27.952	5.820	7.926	34.270
12	2.387	3.034	25.782	2.688	3.379	36.623
13	4.279	5.565	27.313	3.379	4.350	19.451
19	3.175	4.606	28.790	4.720	6.382	27.831
AVERAGE	3.592	4.635	26.519	3.922	4.666	24.957
SEM	0.339	0.373	1.536	0.415	0.559	1.976

C-reavtive Protein

	VIT			Q - VIT		
Subject	pre	min20	post	pre	min20	post
3	11.967	16.065	15.194	8.673	7.252	7.190
4	21.240	21.578	19.533	19.191	21.874	17.534
5	5.037	6.631	5.487	7.614	6.866	5.136
6	4.487	8.664	9.376	6.295	5.944	7.517
7	4.601	4.601	4.863	2.470	2.722	2.879
8	19.067	19.648	21.758	2.244	2.484	2.215
9	4.850	5.496	4.858	2.604	2.753	2.661
11	8.276	8.883	7.722	4.036	4.159	4.051
12	13.172	13.738	14.734	1.613	1.775	1.840
13	31.648	29.194	34.551	31.463	34.646	40.297
19	3.700	4.013	4.221	5.742	6.781	5.949
AVERAGE	11.640	12.592	12.936	8.359	8.841	8.843
SEM	2.722	2.472	2.858	2.751	3.077	3.413

Interleukin-10

	VIT			Q - VIT		
Subject	pre	min20	post	pre	min20	post
3	1.444	1.208	1.047	0.234	0.234	0.300
4	0.135	0.124	0.670	0.146	0.146	0.245
5	0.278	0.300	0.442	0.311	0.322	0.289
6	0.267	0.223	0.267	0.256	0.223	0.344
7	0.278	0.157	0.464	0.322	0.289	0.311
8	0.399	0.399	0.703	0.475	0.322	0.344
9	0.209	0.209	0.493	0.190	0.275	0.748
11	0.152	0.123	0.180	0.076	0.123	0.171
12	0.341	0.512	0.275	0.246	0.303	0.360
13	0.313	0.218	0.833	0.294	0.228	1.155
19	0.407	0.483	1.155	0.455	0.455	1.401
AVERAGE	0.384	0.360	0.594	0.273	0.266	0.515
SEM	0.109	0.094	0.097	0.036	0.027	0.123

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