EFFECTS OF QUERCETIN SUPPLEMENTATION ON INNATE IMMUNE FUNCTION AND INFLAMMATION IN FEMALE HUMAN SUBJECTS

A Thesis

by

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ABSTRACT

EFFECTS OF QUERCETIN SUPPLEMENTATION ON INNATE IMMUNE FUNCTION AND INFLAMMATION IN FEMALE HUMAN SUBJECTS

(May 2010)

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PURPOSE: Quercetin, a flavonoid found in fruits and vegetables, is a strong antioxidant with anti-inflammatory, antimicrobial, and immune-modulating properties. The purpose of this study was to investigate the effects of long-term quercetin supplementation on innate immune function and inflammation in human subjects.

STUDY DESIGN: Female subjects (N=120, ages 30-79 years) were recruited from the community and randomized to one of three groups, with supplements administered using double-blinded procedures: Q-500 (500 mg/day quercetin, N=38), Q-1000 (1000 mg/day quercetin, N=40), or placebo (N=42). Subjects ingested two soft chew supplements twice daily during the twelve-week study period. Fasting blood samples were obtained pre- and post-study and were analyzed for plasma quercetin, interleukin (IL)-6, tumor necrosis factor (TNF)-α, and leukocyte subset cell counts. Natural killer cell activity (NKCA) and lymphocyte subsets were assessed on a subset of seventy-four subjects. Granulocyte

oxidative burst activity (GOBA) and phagocytosis were assessed on sixty-four subjects. Eighteen subjects had overlapping data.

RESULTS: Quercetin supplementation at 500 and 1000 mg/day increased plasma quercetin (interaction effect, $P<0.001$) compared to placebo but had no significant influence on blood leukocyte or lymphocyte subset concentration, plasma IL-6 or TNF-α concentration, NKCA, GOBA, or granulocyte phagocytosis. NKCA was inversely correlated with BMI ($r=-0.25$, $P=0.035$) and body fat percentage ($r=-0.38$, $P=0.001$), and positively correlated with self-reported physical fitness level ($r=0.24$, $P=0.032$).

CONCLUSIONS: Results from this double-blinded, placebo-controlled, randomized trial indicate that quercetin supplementation at 500 and 1000 mg/day for twelve weeks significantly increased plasma quercetin levels but had no influence on measures of innate immune function or inflammation in community-dwelling adult females.

DEDICATION

This master's thesis is dedicated to my parents, Virgil and Deidre Edwards, and to my husband, William Heinz, who witnessed my dream in its infancy and who have supported me throughout its unfolding.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Polyphenolic compounds are found in a wide variety of human foods, including fruits, vegetables, nuts, seeds, flowers, tea, and honey (1). Flavonoids are a large and complex group of polyphenols that share a basic three-ring chemical structure, with two aromatic centers and a central oxygenated heterocyclic ring (2). The most prominent flavonoids in fruits and vegetables are flavonols, and of these, quercetin (3,3',4',5,7-pentahydroxyflavone) is the most commonly consumed in the human diet (2). Total dietary flavonol intake estimates for US adults range from 13 to 22 mg/day, with quercetin representing about 75% (3, 4).

Following absorption from food or supplements, elimination of quercetin is slow, with a reported half-life ranging from 11 to 28 hours (2). Quercetin conjugates are widely distributed in the organ tissues of rats after supplementation, where they may be biologically active (5). The physiologic effects of quercetin and other dietary flavonols are of relevance to human health because of their anti-oxidative (6, 7), anti-inflammatory (8, 9), and anti-pathogenic (10, 11) properties. The present study focuses on the potential beneficial health effects of supplementation with quercetin with regard to inflammation and innate immune function.

Inflammation

The human body reacts defensively to pathogenic invasion with an inflammatory response characterized by pain, redness, heat and swelling. Blood vessels dilate at the site of infection, resulting in localized swelling and the accumulation of defensive blood proteins and complement components, while endothelial cells lining the nearby blood vessels are stimulated to express cell adhesion molecules that facilitate the attachment and extravasation of white blood cells such as lymphocytes and monocytes (12). This inflammatory response is mediated by various signaling molecules, including prostaglandins and cytokines. Some cytokines function as chemoattractants (chemokines) that recruit white blood cells to the site of an infection; other cytokines trigger a fever that simultaneously inhibits pathogenic activity and optimizes conditions for host defense (12).

While highly effective against acute infections, inflammatory responses can result in disease states if the inflammation is inappropriate, excessive or chronic. For example, pro-inflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, and IL-6 are known to play critical roles in the development of rheumatoid arthritis via their inflammatory effects on endothelial cells in synovial tissue (13). Chronically elevated levels of the inflammatory marker C-reactive protein (CRP) and the pro-inflammatory cytokine IL-6 have been strongly correlated with increased risk of coronary heart disease (14-16). Because of the potentially deleterious effects of chronic inflammation, the discovery of dietary supplements that could mitigate chronic inflammatory conditions and reduce risk factors for debilitating illnesses is of great current interest.

The advantage of using flavonoids to control inflammation lies in their safety and potential for providing other health benefits with long-term use. Contrary to earlier concerns

regarding quercetin's potential *in vivo* genotoxicity, high-dose supplementation with quercetin (acute dosages of up to 2000 mg/kg body weight and long-term doses of up to 500 mg/kg for two years) has been shown to lack a mutagenic or genotoxic effect in rodents (17, 18). Furthermore, epidemiological studies have correlated numerous health benefits with regular flavonoid consumption by humans, including decreased incidence of coronary heart disease (19), decreased risk of common human cancers (20), and lowered markers of inflammation (21-24).

Innate immune function

In vitro and animal studies also indicate that quercetin supplementation has the potential to exhibit multiple immunomodulatory effects including augmentation of neutrophil chemotaxis and oxidative burst activity (25), macrophage function (26, 27), and natural killer cell lytic activity (27, 28). Natural killer (NK) cells and neutrophils are dominant players in early host defense against pathogens, and their potential augmentation by quercetin could have important health benefits.

NK cells, which comprise 10-15% of circulating lymphocytes in humans, play a central role in initial host resistance to viral, bacterial, and parasitic infections due to their ability to kill target cells without prior sensitization (29, 30). NK cells express both activating and inhibitory receptors on their surfaces, and the killing of potential target cells by NK cells depends on the balance between activating and inhibitory signals. Granules containing granzymes and perforin are released into the immunological synapse between an NK cells and its potential target only if the activation signal dominates. When inhibitory receptors such as killer Ig-like receptors (KIRs) bind to the MHC class I proteins on the potential

target, molecules involved in the NK cell activation pathway are dephosphorylated (31). Thus, if an NK cell encounters a cell expressing normal levels of MHC class I proteins on its surface, the binding of inhibitory receptors leads to intracellular events that override the activation signal and the granules are not released. Because transformed and infected cells often have low MHC class I expression, they are vulnerable to attack by NK cells. The importance of NK cells to host defense against pathogens is underscored by the increased susceptibility to repeat infections experienced by patients with NK cell deficiency conditions (32).

Also critical to optimal cellular immune function are the phagocytic and microbicidal activities of polymorphonuclear leukocytes, or neutrophils, which comprise 50-70% of white blood cells (33). Neutrophils are granulocytes that migrate along chemoattractant gradients to infection sites, where they engulf pathogens via phagocytosis. Pathogens thus ingested by phagocytes can be killed through oxygen-dependent or oxygen-independent mechanisms. In the oxygen-dependent pathway, NADPH oxidase complexes on the phagolysosomal membrane catalyze the oxidation of cytosolic NADPH, a reaction which is coupled to the reduction of oxygen molecules in the phagolysosome (33). The resultant superoxide anions are dismutated by superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2), which generates other reactive oxygen species such as hypochloride (ClO^-), hydroxyl radicals ($OH\cdot$), and singlet oxygen ($\cdot O_2$). These highly toxic compounds are responsible for the oxygen-dependent killing of phagocytosed microbes, and their production causes a transient increase in oxygen consumption by the phagocyte, commonly referred to as the respiratory or oxidative burst.

Quercetin, which has been shown in animal and *in vitro* studies to augment NK cell cytolytic activity and neutrophil function (25, 27, 28), could potentially be taken as a supplement to improve innate immune function, thereby reducing risk of illness.

Previous work

Numerous *in vitro* studies that have examined the effects of quercetin on pro-inflammatory cytokine production in a wide variety of cells have shown that quercetin exerts strong anti-inflammatory effects via suppression of nuclear factor-kappa B (NF-κB) activation (9, 34, 35). Homo- and hetero-dimers of NF-κB transcription factors are located in the cytoplasm of unstimulated immune cells, where they are tightly bound to IκB inhibitory proteins (9). When immune cells are stimulated, the IκB proteins are phosphorylated, ubiquitylated, and degraded, freeing the NF-κB dimers for translocation to the nucleus, where they bind to the promoter regions of genes involved in the inflammatory response. Inhibition of IκB phosphorylation represents one mechanism by which quercetin could suppress the expression of numerous inflammatory cytokines.

Cho *et al*. (34) used RAW 264.7 cells, a line of murine macrophages, to study quercetin's anti-inflammatory actions. Macrophages are white blood cells that are important mediators of inflammatory response due to their production of cytokines and nitric oxide (NO) following stimulation by an endotoxin expressed on the cell walls of Gram-negative bacteria, lipopolysaccharide (LPS). Cho *et al.* found that nitrate generation, iNOS mRNA production, and iNOS protein expression were inhibited by quercetin in a dose-dependent manner, as were TNF-α, IL-1β, and IL-6 mRNA production. Their results indicated that incubation with quercetin inhibited the phosphorylation of ERK and p38 MAPK and also

inhibited the phosphorylation and degradation of IκBα, thereby stabilizing the NF-κB/IκB complex and suppressing the activation of NF-κB.

Similar experiments were performed by Nair *et al*. (9) and Min et al. (35). Nair *et al.* (9) studied TNF-α production and regulation in peripheral blood mononuclear cells (PBMCs), which are agranular immune cells that include lymphocytes (B cells, T cells, and NK cells) and monocytes. Their results indicated that *in vitro* quercetin treatment down-regulated expression of TNF-α and NF-κB mRNA, suppressed exogenous and induced TNF-α production in PBMCs, and inhibited the activation of NF-κB via suppression of IκBα and IκBβ phosphorylation. Min et al. (35) examined quercetin's effects on pro-inflammatory cytokine expression in the human mast cell line, HMC-1. Mast cells are key mediators of allergic inflammatory responses. Once activated, mast cells produce numerous chemotactic and pro-inflammatory cytokines, including IL-6 and TNF-α. Min et al. found that quercetin treatment decreased TNF-α, IL-1β, IL-6, and IL-8 gene expression and production in stimulated mast cells via the inhibition of NF-κB and p38 MAPK activation.

As described above, quercetin has been shown to exert *in vitro* anti-inflammatory effects through inhibition of IκB phosphorylation and the consequent suppression of NF-κB activation. Binding sites for NF-κB proteins have been found in the promoter regions of the genes that code for both TNF-α and IL-6, and studies have shown that IL-6 mRNA production does not occur in the absence of activated NF-κB (36, 37). It might be expected, therefore, that quercetin supplementation would decrease circulating levels of IL-6 and TNF-α. However, most of the existing data regarding quercetin's anti-inflammatory properties have come from *in vitro* study designs, and relatively few *in vivo* studies have been performed.

Choi et al. (38) fed mice either vitamin E-deficient or control diets for nine weeks and supplemented subgroups of the mice with 0.5% quercetin. After four weeks, they induced arthritis in the animals by immunization with collagen. Choi et al. found that the increase in joint tissue TNF-α and IL-1β mRNA expression associated with the vitamin-E deficient diet was reduced to control levels in the quercetin-supplemented group at nine weeks; however, no differences in cytokine mRNA expression were found between the quercetin-supplemented and control mice on a normal diet. Mamani-Matsuda et al. (39) found that oral supplementation with quercetin (30 mg every two days) for ten days significantly reduced clinical signs of arthritis in adjuvant-induced arthritic Lewis rats, and showed that quercetin inhibited murine macrophage activation and TNF-α production *ex vivo* and *in vitro*.

Stewart et al. (40) fed C57BL/6J mice a high-fat diet with or without 0.8% quercetin (approximately 0.8 mg/gram body weight per day) and found that circulating markers of inflammation INF-γ, IL-1α, and IL-4 were significantly lower in the quercetin-fed mice compared to controls after eight weeks. These changes in inflammatory status were independent of adiposity, which was itself unaffected by quercetin consumption, suggesting that quercetin supplementation might be used to mitigate the chronic low-grade inflammation associated with obesity.

Few studies have examined the effects of quercetin supplementation on chronic inflammation in humans, however. A recent study by Egert et al. investigated whether 150 mg/day quercetin for six weeks would decrease plasma TNF-α in overweight and obese subjects (BMI 25-35 kg/m^2), but no such effect was found (41). The authors postulated that the dosage of quercetin used in their study was insufficient to exert significant anti-inflammatory effects in the blood compartment. Beyond this, the limited human studies to

date have largely focused on the acute effects of quercetin in athletes following exercise stress. Longer-term supplementation studies are needed to assess the effects of quercetin consumption on circulating markers of inflammation.

As with inflammation, the body of evidence indicating that quercetin augments innate immunity including natural killer cell and neutrophil function is comprised primarily of data from animal and *in vitro* studies (25, 27, 28). Exon et al. (28) showed that rats fed 100 mg/kg quercetin dihydrate daily for seven weeks had significantly elevated natural killer cell activity (NKCA) compared to controls. This was in contrast to their *in vitro* results, which showed that splenic NK cells exposed to quercetin had decreased cytotoxicity (28). Exon et al. postulated that this discrepancy could be due to an indirect enhancement of NKCA by quercetin *in vivo*, perhaps through enhancement of interferon (IFN) effects. Quercetin has been shown to upregulate IFN-γ gene expression and production when cultured with PBMCs at concentrations from 10-50 μM (42). IFN-γ, in turn, regulates NK cell function (43) and may mediate the potential immunostimulatory properties of quercetin *in vivo*.

A recent study by Yu et al. (27) found that the cytotoxic activity of NK cells was increased in BALB/c mice treated with 2 and 4 mg/kg quercetin daily for three weeks following injection with WEHI-3 leukemia cells compared to mice that did not receive quercetin. The phagocytic activity of peritoneal macrophages was also increased in mice treated with 2 mg/kg quercetin compared to the untreated group, but macrophages isolated from peripheral blood did not show a similar response to quercetin. Akbay et al. (25) found that incubation with a quercetin glycoside (quercetin-3-*O*-rutinoside) increased the oxidative burst activity of human neutrophils *in vitro*, with maximum ROS production measured in neutrophils that were incubated with 4μg/mL. These studies indicate that quercetin may exert

immunomodulatory effects through the enhancement of NKCA and granulocyte oxidative burst activity (GOBA).

Although these *in vitro* and animal studies have yielded promising results with regard to quercetin's potential effects on innate immune function and inflammation, previous human studies have been limited. The majority of quercetin supplementation trials to date have been short-term studies focused on quercetin's acute effects on post-exercise inflammatory and immune function perturbations in athletes.

Acute heavy exertion is known to cause temporary changes in host immunity due to its suppression of NK cell function, T and B cell activity, and granulocyte oxidative burst activity (44). Prolonged high-intensity exercise is also correlated with increased plasma concentrations of pro- and anti-inflammatory cytokines. The period of impaired immunity after intense exercise results in an "open window" during which an athlete is more susceptible to infection by viral and bacterial pathogens. Much of the research on quercetin to date has focused on its effects on subjects following heavy exertion to determine whether quercetin exhibits immunoprotective properties that might counter this post-exercise increase in illness susceptibility.

Davis et al. (45) investigated the effects of quercetin on infection rates in mice using a viral-challenge study design. The data from this study indicated that 12.5 mg/kg quercetin feedings for seven days prior to inoculation with an LD50 dose of A/Puerto Rico/8/34 (H1N1) influenza virus significantly reduced the increase in morbidity, symptom severity, and mortality in mice exercised to exhaustion for three consecutive days. This was the first *in vivo* experiment to provide evidence for the immunoprotective effects of quercetin ingestion in a placebo-controlled viral challenge model. However, the Davis et al. study did not

examine measures of immune function, so it was unclear whether this effect was due to direct anti-viral activities or to augmentation of immune function by quercetin.

A double-blind placebo-controlled study of forty trained cyclists previously performed in our lab found that supplementation with 1000 mg/day aglycone quercetin for three weeks before, during, and two weeks after a three-day period of intense exercise significantly reduced incidence of self-reported upper respiratory tract infection (URTI) following the exercise period (46). In this study, measures of immune function such as inflammatory markers, NKCA, and GOBA were examined, but were not found to be altered by short-term quercetin supplementation. Another study in trained cyclists found that supplementation with 1000 mg/day quercetin for three weeks before and during a three-day period of intensified training attenuated the post-exercise increase in leukocyte IL-8 and IL-10 mRNA expression compared to placebo, providing evidence for immune-modulating effects of quercetin through suppression of cytokine gene expression *in vivo* (47). Another short-term quercetin supplementation study in human athletes found that three weeks supplementation with 1000 mg/day quercetin did not offset the post-exercise decrease in GOBA experienced by runners who completed a 160-km ultramarathon; nor was quercetin supplementation effective at counteracting exercise-induced perturbations in leukocyte subset counts (48). However, it should be noted that plasma quercetin levels in the experimental group dropped significantly over the 20 to 30 hours it took for the athletes to complete the race; it is unknown whether consistently-maintained high plasma quercetin concentrations could have attenuated the immune perturbations experienced by these ultramarathon runners.

In summary, most of the existing evidence supporting quercetin's anti-inflammatory and immunomodulatory properties has come from *in vitro* and animal studies. Furthermore,

in vivo data regarding the effects of quercetin on human subjects have primarily come from short-term studies focused on quercetin's acute effects on post-exercise immune function perturbations in highly-trained athletes, and as such are limited in their application to non-athletes. It is currently unknown whether long-term quercetin supplementation could have beneficial health effects in member of the general community.

Purpose of study

The present study will examine the chronic effects of quercetin consumption on innate immune function and inflammation in female subjects recruited from the general population. It is currently unknown whether long-term quercetin supplementation exhibits an immunomodulatory effect in humans, or whether these potential effects are dose-dependent. The objective of this study was to measure the influence of twelve-week quercetin supplementation in two doses (500 and 1000 mg/day) on measures of innate immune function (NKCA, GOBA, and granulocyte phagocytosis) and markers of inflammation (plasma IL-6 and TNF-α) in middle-aged, community-dwelling female subjects. Dosages and supplementation periods used were chosen based on animal studies and prior work in our laboratory (47, 49).

METHODS

Subjects

One hundred twenty females, 30-79 years of age, were recruited via mass advertising. Subjects had to be healthy and noninstitutionalized, and women were excluded if pregnant or lactating. Subjects agreed to avoid any other supplements containing quercetin; no other restrictions were placed on diet, supplement usage, or medications. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the institutional review board of Appalachian State University. Written informed consent was obtained from all subjects.

Research design

Subjects were randomized to one of three groups: Q-500 (500 mg/day quercetin, N=38), Q-1000 (1000 mg/day quercetin, N=40), or placebo (N=42). Supplements were administered utilizing double blinded procedures. Subjects ingested two soft chew supplements twice daily (upon awakening, and between 14:00 hours and the last meal of the day) during the twelve-week study period. Supplements were prepared by Nutravail Technologies (Chantilly, VA USA) with Quercegen Pharma (Newton, MA USA), and were soft, individually-wrapped chews (5.3 g/piece) that contained either 125 or 250 mg quercetin, 125 or 250 mg vitamin C (ascorbic acid and sodium ascorbate), 5 or 10 mg niacin, and 20 kilocalories of sugars in a carnauba wax, soy lecithin, corn starch, glycerine, and palm oil

base colored with FD&C yellow #5 and #6. A series of HPLC measurements was conducted to determine that the amount of quercetin in each chew was stable and accurate. Placebo supplements were prepared in the same way minus the quercetin, vitamin C, and niacin. Data from Quercegen Pharma indicate that bioavailability of quercetin is enhanced with vitamin C and niacin; hence, this study tested whether or not soft chews with or without the combination of quercetin, vitamin C, and niacin had an influence on the outcome measures. Subjects started supplementing after the first blood sample and continued for twelve weeks. Two weeks prior to the first lab visit for the study, subjects provided demographic and lifestyle habit information via a survey posted on the web using SurveyMonkey.com (Portland, OR USA). Information on dietary patterns was obtained through a semi-quantitative food frequency questionnaire for food groups including fruit, vegetables, cereals, meat, dairy, and fat. Exercise habits were assessed through answers to categorical questions dealing with both leisure-time and work activities. Physical fitness levels were self-reported using a ten-point Likert scale, with one corresponding to low fitness and ten to high fitness. Subject height was measured with a stadiometer, and BMI and body composition were determined using a Tanita bioelectrical impedance scale (Tanita, Arlington Heights, IL USA). Before and after the twelve-week supplementation period, subjects came to the lab in the morning (7:00 - 9:00 hours) after an overnight fast to donate blood samples. Blood samples were taken from an antecubital vein with subjects in a seated position. Plasma quercetin levels and leukocyte subset cell counts were analyzed for all samples. NKCA and lymphocyte subsets were analyzed for seventy-four subjects, and GOBA and phagocytosis were assessed on sixty-four subjects. There was some overlap between the cohorts: eighteen subjects were tested for both NKCA and GOBA/phagocytosis.

Plasma quercetin

Total plasma quercetin (quercetin and its primary metabolites) was measured following solid-phase extraction via reversed-phase HPLC with UV detection as previously described (50, 51). Quercetin conjugates were hydrolyzed by incubating 500 µl plasma aliquots with 10 µl 10% DL-Dithiothreitol solution, 50 µl 0.58 M acetic acid, 50 µl of a mixture of β-glucuronidase/arylsulfatase and crude extract from *Helix pomatia* (Roche Diagnostics Corporation, Indianapolis, IN USA) for 2 hours at 37°C. Chromatographic analysis was performed using the Ultimate 3000 HPLC-PDA system (Dionex Corporation, Sunnyvale, CA USA) with a Gemini C18 column (Phenomenex, Torrance, CA USA). Three quality control samples, using human plasma samples spiked with quercetin at concentrations of 1.0, 1.5, and 3.0 µmol/l, were assayed in duplicate, with an intra-assay coefficient of variation (CV) of 12.5%.

Leukocyte differential

A complete blood count (CBC) with leukocyte differential was analyzed in the clinical lab of the Watauga Medical Center (Boone, NC USA) using standard clinical laboratory equipment and quality standards.

Plasma IL-6 and TNF-α

High-sensitivity enzyme-linked immunosorbent assay kits were used to measure total plasma concentrations of IL-6 and TNF-α in accordance with manufacturer protocol (R&D Systems, Inc., Minneapolis, MN USA). All samples and provided standards were analyzed in duplicate. The minimum detectable concentrations of IL-6 and TNF-α were <0.039 pg/ml

and <0.106 pg/ml, respectively. Pre- and post-supplementation samples were analyzed on the same assay plate to decrease interkit assay variability, and the intra-assay CV for all variables was less than 10%. Data were analyzed with SOFTmax software (Molecular Devices, Sunnyvale, CA USA).

GOBA and phagocytosis

Simultaneous measurement of granulocyte oxidative burst activity and phagocytosis was performed using a modified flow cytometric assay (52). For each sample, 100 µl heparinized whole blood was dispensed into two 15 x 75 mm (5-ml) tubes. To each tube, 10 µl hydroethidine (HE) working solution (10 µg/ml HE in PBS-glucose; Invitrogen Corporation, Carlsbad, CA USA) was added. The tubes were vortexed briefly, incubated in a 37ºC water bath for 15 minutes, and cooled in a 4 ºC ice-water bath for 12 minutes. After the HE-loaded blood samples were cooled, 20 µl of working bacteria-FITC solution (*Staphylococcus aureus* labeled with FITC, diluted in PBS to 1.33 x 10^8 particles/ml; Invitrogen Corporation, Carlsbad, CA USA) was added to both tubes and vortexed briefly. Tube 2 (test) was transferred to a 37ºC water bath, and tube 1 (control) was left in the ice. The tubes were incubated for 20 minutes, placed in an ice water bath, and 100 µl ice-cold Quench Solution (0.025% Trypan blue in 0.1 M Citrate buffer, pH 4.0) was added to each tube. The tubes were vortexed for 10 seconds and incubated for 1 minute to quench the FITC fluorescence of any non-internalized bacteria, after which the cells were washed twice with ice-cold PBS and resuspended in 50 µl cold fetal bovine serum. Samples were processed on a Q-Prep™ Workstation (Beckman Coulter, Inc., Fullerton, CA USA), which lysed the erythrocytes and stabilized and fixed the white blood cells. Tubes were stored at room

temperature in the dark until flow cytometric analysis, which was performed within 24 hours of blood collection for all samples.

Analysis of samples was performed using a Beckman Coulter FC-500 flow cytometer with CXP software (Fullerton, CA USA). FITC emits a green fluorescence that can be detected on fluorescence channel 1 (FL1); therefore, cells that phagocytose the FITC-labeled bacteria are FL1-positive, and the strength of the fluorescent signal detected on that channel is proportionate to the number of bacteria that have been phagocytosed by the cells. Similarly, cells thus stimulated by the bacteria will undergo an oxidative burst, oxidizing the non-fluorescent HE to ethidium bromide, which emits a red fluorescence that can be detected on fluorescence channel 3 (FL3). After gating on the granulocyte cell population (predominantly comprised of neutrophils) using forward scatter and side scatter, the mean fluorescence intensity (x-mean) for each channel was determined and shifts in x-mean were calculated by subtracting the control (4°C) x-mean from the test (37°C) x-mean for FL1 and FL3. Typically, 5000 granulocytes were counted for each reaction tube. The mean intra-assay CV was <7% for phagocytosis and <10.5% for GOBA.

Lymphocyte subsets

Lymphocyte subset data (%NK, %B, and %T) was also obtained by flow cytometry. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood by density gradient centrifugation with Fico/Lite. The cells were washed twice and resuspended to 1 ml in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin, streptomycin, and L-glutamine (complete-RPMI). A sample (100 µl) of cells was removed and stained for 15 minutes with 10 µl CYTO-STAT® tetraCHROME™ CD45-

FITC/CD56-RD1/CD19-ECD/CD3-PC5 monoclonal antibody reagent (Beckman Coulter, Fullerton, CA USA). CYTOSTAT® tetraCHROME™ is a combination of four murine monoclonal antibodies, each conjugated to a specific fluorochrome, used to differentiate lymphocyte subsets based on their cell surface markers. The lymphocytes were gated using intracellular complexity (side scatter) and FITC fluorescence intensity (CD45+), and flow cytometric dot plots of CD19-ECD vs. CD3-PC5 and CD56-PE vs. CD3-PC5 were produced. In this manner, the percentage of lymphocytes that were NK cells (CD56+ CD3-), B-cells (CD19+ CD3-), and T-cells (CD3+ CD19-) was determined for each subject. Absolute numbers of each cell type were then calculated using the CBC data to allow group comparison of circulating cell counts.

NK cell activity

NK cell cytotoxic activity was assessed using a modification of a flow cytometric assay (46, 53). PBMC (effector cells) were isolated from heparinized blood by density gradient centrifugation with Fico/Lite, washed twice, and resuspended to 3.75×10^6 cells/ml. K562 cells in log phase (target cells, 1×10^6 cells/ml) were labeled for 20 minutes at 37°C in 5% CO_2 with 10 µl 3 mM DiO solution [3,3'-dioctadecyloxacarbocyanine perchlorate (DiO; Sigma Chemical Co., St. Louis, MO USA) in DMSO] per 1 ml cell suspension. After labeling, the target cells were washed and resuspended in complete-RPMI to a concentration of 5×10^5 cells/ml. Effector and target cells were combined in four 15 x 75 mm (5-ml) tubes to yield effector:target (E:T) ratios of 60:1, 30:1, 15:1, and 7.5:1 with final volumes of 0.9 ml. Given the proven reproducibility, high sensitivity, and large number of cells used in this flow cytometric assay, a single assay tube was used for each E:T ratio (53). Control tubes

received target cells with no effectors. All tubes received 0.1 ml of a 500 µg/ml solution of propidium iodide (PI; Sigma Chemical Co., St. Louis, MO USA) in RPMI, after which the tubes were vortexed, pelleted by centrifugation for 2 minutes at 1200 rpm, and incubated for 2 hours at 37°C in 5% CO_2. Following incubation, the tubes were vortexed briefly, placed in an ice-water bath, and analyzed by flow cytometry within 45 minutes.

Analyses of samples were performed using a Beckman Coulter FC-500 flow cytometer with CXP software (Fullerton, CA USA). DiO-labeled target cells emit a green fluorescence that can be detected on FL1 and compromised cells that have taken up PI emit a red fluorescence that can be detected on FL3. The percentage of target cells that were compromised was determined for each tube. The results were acceptable if the spontaneous lysis of target cells (percent non-viable target cells in the control tube) was less than 5%. NK cell-induced killing of target cells was determined by subtracting the spontaneous lysis of target cells from the percent non-viable target cells in tubes containing both effectors and targets at each E:T ratio. Results were normalized by conversion to lytic units, calculated as the number of effector cells required to kill 20% of 5000 target cells, and reported as the number of lytic units contained in 10^7 cells (54).

Statistical analysis

All statistical analyses were performed using SPSS PC v.16.0 software (SPSS Inc., Chicago, IL USA). Data are expressed as mean ± SEM. Data were analyzed using a 3 (group) x 2 (time) repeated measures ANOVA, between groups design, with post-hoc analysis using independent t-tests that contrasted pre-to-post-supplementation changes of Q-500 and Q-1000 with placebo. Self-reported fitness levels, BMI, body composition and

lifestyle data were correlated with NKCA using Pearson correlations. Subject characteristics were contrasted between groups using one-way ANOVA. For all tests, $P<0.05$ was considered significant.

RESULTS

Subject characteristics

Subjects were Caucasian females, ranging in age from 30 to 79 years, with a large variance in body mass and composition (Table 1). No changes in body mass and composition were noted over the course of the study and interaction effects indicated no group differences. With all subjects combined, no significant correlations were found between subject age and any of the pre-study measures of innate immune function (all $P>0.4$) or inflammation (all $P>0.2$).

Plasma quercetin

Plasma quercetin increased significantly above placebo levels after twelve-week supplementation with 500 or 1000 mg/day aglycone quercetin (Group x time interaction effect, $P<0.001$) (Fig. 1). No significant correlations were found between plasma quercetin levels and dietary variables.

Leukocyte subsets

The pattern of change over time was not significantly different between groups for total leukocytes ($P=0.306$), total lymphocytes ($P=0.867$) or neutrophil counts ($P=0.193$) (Table 2).

Plasma IL-6 and TNF-α

Twelve-week quercetin supplementation at 500 and 1000 mg/day had no effect on plasma concentrations of IL-6 (P=0.812) or TNF-α (P=0.208) (Table 2).

GOBA and phagocytosis

The pattern of change over time for granulocyte phagocytosis (P=0.990) (Fig. 2) or oxidative burst activity (P=0.602) (Fig. 3) was not significantly different between groups. No correlations were found between any outcome measures and GOBA or phagocytosis. For all subjects combined, GOBA increased pre- to post-supplementation (P<0.001).

Lymphocyte subsets

Circulating counts of NK, B, or T cells were not influenced by twelve-week supplementation with quercetin at 500 or 1000 mg/day (Table 3).

NK cell activity

Pre- and post-supplementation data for killing of K562 target cells by NK cells showed no significant differences between groups across all E:T ratios (Table 4). The pattern of change over time for total NK cell cytotoxic activity was not different between treatment groups (P=0.696) (Fig. 4). Although NKCA tended to increase pre- to post-supplementation for all subjects combined, this apparent time effect did not reach significance (P=0.163). When pre- and post-study measures were averaged for all subjects combined (N=74), NKCA was correlated positively with self-reported physical fitness level (r=0.24, P=0.032) and negatively with BMI (r=-0.25, P=0.035) and body fat percentage (r=-0.38, P=0.001) (Fig. 5).

DISCUSSION

This is the first human clinical trial to investigate the long-term effects of aglycone quercetin supplementation on measures of innate immune function and inflammation in subjects recruited from the community. Subjects tended to be overweight individuals (mean BMI greater than 25.0 kg/m^2) with mean pre-study plasma IL-6 and TNF-α concentrations that were slightly elevated compared to values reported for lean, healthy women in other studies (55, 56). The data indicate that twelve-week supplementation with 500 or 1000 mg/day quercetin was not associated with changes in NKCA, GOBA, phagocytosis, or plasma concentration of IL-6 and TNF-α in this population.

Quercetin is a powerful antioxidant (6) that exerts *in vitro* anti-inflammatory effects through the inhibition of NF-κB signaling in a variety of cells including macrophages and peripheral blood mononuclear cells (8, 9). It was expected, therefore, that long-term quercetin supplementation would decrease circulating levels of IL-6 and TNF-α; however, quercetin had no effect on these markers of inflammation in the present study. Likewise, quercetin supplementation had no effect on NKCA, GOBA, or phagocytosis. A moderate time effect was noted for GOBA, highlighting the importance of seasonal effects on measures of immune function. The post-supplementation blood draw was in November, twelve weeks after the August pre-supplementation blood draw. Similar circannual rhythms in immune function have been observed in previous studies performed by our lab (57, 58).

Nonetheless, the pattern of change over time was not significantly different between treatment groups for any of the outcome measures in this study.

With regard to NKCA, GOBA, and granulocyte phagocytosis, the present findings are consistent with studies previously conducted in our lab investigating the effects of short-term quercetin supplementation on similar measures of innate immune function in athletes (46, 48), but they are contrary to the results of quercetin studies utilizing rodent models and *in vitro* study designs (25, 27, 28). However, these human and animal studies may not be comparable for a number of reasons. For example, the Exon et al. (28) study used a larger dosage of quercetin (100 mg/kg) compared to the present study, which supplemented with average daily doses of 7 and 14 mg/kg quercetin for Q-500 and Q-1000. The dosages used in the Yu et al. (27) study were smaller (2 and 4 mg/kg), but the relevance of their results is confounded by the lack of a quercetin-treated group not injected with WEHI-3 cells. Possible species-dependent variations in phase II metabolism call into question the applicability of these animal study data to humans (59); furthermore, because of its metabolic transformation, quercetin circulates in the body and accumulates in tissues in forms that are quite different from those used in most *in vitro* studies (60).

Aglycone quercetin supplementation may lack an anti-inflammatory or immunomodulatory effect in humans due to either its reduced bioavailability or its metabolic transformation *in vivo*. Quercetin is found in fruits and vegetables in a water-soluble form in which the quercetin molecule is conjugated to a sugar moiety (2). The *in vitro* study conducted by Akbay et al. showed that neutrophil oxidative burst was increased by incubation with quercetin in such a glycosylated form (25). However, when quercetin is consumed, the sugar is cleaved off in the small intestine, allowing the lipid-soluble aglycone

form of quercetin to diffuse into the cells lining the intestinal wall (61). Quercetin in this aglycone form was used in the anti-inflammatory *in vitro* studies performed by Cho et al. (34), Nair et al. (9), and Min et al (35). During phase II metabolism in the liver, however, aglycone quercetin is either methylated, sulphated, or glucuronidated. Quercetin predominately circulates in the bloodstream in these conjugate forms, which differ not only from aglycone quercetin, but also from the glycosylated forms found in plant foods. Relatively few studies have examined the bioactivity of quercetin conjugates (60) and further work in this area is indicated.

Recent evidence in a murine model suggests that variation in apoE genotype may alter responsiveness to flavonoid supplementation. Boesch-Saadatmandi et al. (62) fed apoE3 and apoE4 transgenic mice 400 mg/kg quercetin per day for six weeks and found that the quercetin-associated reduction in TNF-α production was significantly greater in mice with the apoE3 genotype. The Boesch-Saadatmandi et al. study assessed TNF-α production in whole blood stimulated *ex vivo* with lipopolysaccharide; thus their results are indicative of the effects of quercetin supplementation on inflammatory responses rather than on basal expression of TNF-α. Data regarding the effects of apoE genotype on responsiveness to quercetin supplementation in humans are limited. Egert et al. (63) assessed plasma TNF-α concentration after six weeks' supplementation with 150 mg/day quercetin in obese and overweight human subjects, and found quercetin's influence on serum HDL concentration and systolic blood pressure were dependent on apoE genotype, although no effect with TNF-α was found. Future studies correlating apoE genotype with subject responsiveness to quercetin supplementation would be useful.

Despite its known anti-inflammatory properties, the present study did not find a quercetin-related effect on circulating levels of IL-6 and TNF-α. It should be noted that, although the mean plasma TNF-α and IL-6 levels of subjects in this study were slightly elevated compared to those reported for lean females in other studies (55, 56), they fell within the normal ranges for adult females (64). It is possible that the lack of quercetin-related effect on inflammation reported in the present study may be because these subjects were already within normal limits with regard to the outcome measures. Future studies comparing the effects of quercetin supplementation on inflammatory status in normal, overweight, and obese subjects would be of value.

While supplementation with aglycone quercetin appears to have no significant effect on inflammation, basal NKCA, or granulocyte function in humans, quercetin may exhibit an immunomodulatory and anti-inflammatory effect when ingested in other forms or in combination with other flavonoids. A recent human study found that four weeks supplementation with a fermented food rich in quercetin and other flavonoids significantly increased activation of NK cell cytotoxicity in response to IL-2 stimulation, but did not influence basal (non-stimulated) NKCA (65). Supplementation with polyphenol-rich cereal fractions has been shown to increase basal NKCA in prematurely aging mice, in addition to augmenting macrophage phagocytosis and ROS production (66). Our lab recently found that a two-week supplementation with a combination of quercetin, epigallocatechin 3-gallate (EGCG), isoquercetin, and omega-3 polyunsaturated fatty acids (N3-PUFAs) increased GOBA and decreased post-exercise plasma concentration of IL-6 (51). Future research will examine the combined supplements' potential effects on NKCA and granulocyte phagocytosis. It may also be helpful to investigate whether IL-2-stimulated NK cell cytotoxic

activity in humans is affected by quercetin supplementation, alone or in combination with other flavonoids. In addition to studying quercetin's effects on circulating levels of inflammatory markers, future studies may also assess inflammatory responsiveness using *ex vivo* stimulation of cells.

In the present study, we found that total NK cell cytotoxic activity was related inversely to BMI and body fat percentage, and positively with self-reported fitness level. These findings are consistent with previous studies linking NK cell function with physical fitness and healthy lifestyles (67, 68). A cross-sectional comparison between marathon runners and sedentary controls found that the marathoners had significantly greater NKCA than the controls, and that percent body fat was negatively correlated with NKCA for all subjects combined (67). In addition to physical exercise, other lifestyle factors such as not smoking and eating a balanced diet have been associated with elevated numbers of NK cells and enhanced NKCA, possibly due to an increased percentage of NK cells expressing perforin, granulysin, and granzymes A and B in subjects with good health practices (68). This suggests that optimization of NK cell function can be achieved through the maintenance of a healthy lifestyle.

In summary, twelve-week aglycone quercetin supplementation in doses of 500 and 1000 mg/day had no effect on leukocyte subset counts, plasma IL-6 or TNF-α concentration, NKCA, GOBA, or granulocyte phagocytosis relative to placebo. A growing body of evidence indicates that a mixed flavonoid approach to modifying innate immunity is more effective than supplementation with a pure flavonoid such as aglycone quercetin. In athletes, a quercetin supplement combined with EGCG, isoquercetin, and N3-PUFAs significantly reduced post-exercise inflammation and augmented neutrophil function (51). Future research

will determine if the immunomodulatory effects of quercetin can be enhanced through the addition of other flavonoids (e.g., EGCG) or food components.

TABLE 1. Subject characteristics (N=120, females, ages 30-79 years)[1].

	Placebo (N=42)	Q-500 (N=38)	Q-1000 (N=40)	F-Probability
Age (y)	47.0 ± 1.1	45.4 ± 1.1	47.4 ± 1.3	0.459
Height (m)	1.65 ± 0.01	1.64 ± 0.01	1.64 ± 0.01	0.940
Weight (kg)	72.2 ± 2.9	71.3 ± 2.5	71.7 ± 3.4	0.979
BMI (kg/m^2)	26.6 ± 1.0	26.4 ± 0.9	26.6 ± 1.2	0.994
Body fat (%)	34.5 ± 1.7	35.3 ± 1.5	34.3 ± 1.6	0.896

[1] Data are presented as mean ± SEM.

TABLE 2. Blood leukocyte subset cell counts and plasma inflammatory markers, at baseline and after twelve-week supplementation with quercetin at 500 or 1000 mg/day compared to placebo[1, 2].

	Placebo (N=42)	Q-500 (N=38)	Q-1000 (N=40)	Interaction P-value
Leukocytes (10^9/l)				
Pre-study	5.91 ± 0.34	5.80 ± 0.23	5.78 ± 0.26	0.306
Post-study	5.75 ± 0.29	5.94 ± 0.25	5.98 ± 0.31	
Neutrophils (10^9/l)				
Pre-study	3.41 ± 0.24	3.50 ± 0.19	3.21 ± 0.20	0.193
Post-study	3.28 ± 0.21	3.63 ± 0.20	3.48 ± 0.24	
Lymphocytes (10^9/l)				
Pre-study	1.81 ± 0.09	1.71 ± 0.07	1.87 ± 0.09	0.867
Post-study	1.81 ± 0.09	1.71 ± 0.08	1.84 ± 0.10	
IL-6 (pg/ml)				
Pre-study	1.72 ± 0.18	1.64 ± 0.19	2.38 ± 0.78	0.812
Post-study	1.76 ± 0.20	1.84 ± 0.25	2.58 ± 0.96	
TNF-α (pg/ml)				
Pre-study	1.19 ± 0.10	1.59 ± 0.22	1.22 ± 0.17	0.208
Post-study	1.24 ± 0.10	1.39 ± 0.19	1.24 ± 0.17	

[1] Pre- and post-study blood samples were obtained from all subjects (N=120) after an eight-hour fast.
[2] Data are presented as mean ± SEM.

TABLE 3. Blood lymphocyte subset cell counts for subjects with NKCA data (N=74), at baseline and after twelve-week supplementation with quercetin at 500 or 1000 mg/day compared to placebo[1,2].

	Placebo (N=26)	Q-500 (N=24)	Q-1000 (N=24)	Interaction P-value
T cells (10^9/l)				
Pre-study	1.42 ± 0.08	1.33 ± 0.09	1.47 ± 0.08	0.999
Post-study	1.41 ± 0.09	1.32 ± 0.09	1.46 ± 0.10	
B cells (10^9/l)				
Pre-study	0.24 ± 0.03	0.22 ± 0.02	0.23 ± 0.03	0.603
Post-study	0.25 ± 0.02	0.22 ± 0.02	0.25 ± 0.04	
NK cells (10^9/l)				
Pre-study	0.20 ± 0.02	0.16 ± 0.02	0.18 ± 0.02	0.885
Post-study	0.19 ± 0.02	0.15 ± 0.02	0.18 ± 0.02	

[1] Pre- and post-study blood samples were analyzed for lymphocyte subsets using flow cytometry.
[2] Data are presented as mean ± SEM.

TABLE 4. NK cell activity, pre- and post- twelve-week supplementation with 500 or 1000 mg/day quercetin compared to placebo, expressed as percent non-viable (%NV) target cells at four effector-to-target (E:T) cell ratios[1].

E:T ratio	Placebo (N=26)	Q-500 (N=24)	Q-1000 (N=24)	Interaction P-value
60:1 (%NV)				
Pre-study	43.5 ± 3.4	36.6 ± 3.3	40.8 ± 2.8	0.582
Post-study	47.2 ± 3.1	44.0 ± 3.6	49.1 ± 3.2	
30:1 (%NV)				
Pre-study	37.9 ± 3.0	32.3 ± 3.3	35.6 ± 2.8	0.659
Post-study	42.7 ± 3.0	37.5 ± 3.4	43.6 ± 3.3	
15:1 (%NV)				
Pre-study	29.2 ± 2.5	24.3 ± 3.0	28.3 ± 2.9	0.943
Post-study	32.6 ± 2.6	27.8 ± 2.8	32.6 ± 3.0	
7.5:1 (%NV)				
Pre-study	19.9 ± 1.8	17.0 ± 2.3	20.1 ± 2.5	0.950
Post-study	22.6 ± 1.9	19.8 ± 2.2	22.2 ± 2.4	

[1] NK cells (effectors) were incubated with K562 cells (targets) and analyzed by flow cytometry for 74 subjects; the percentage of target cells that were killed due to NKCA at each E:T ratio is presented here as mean ± SEM.

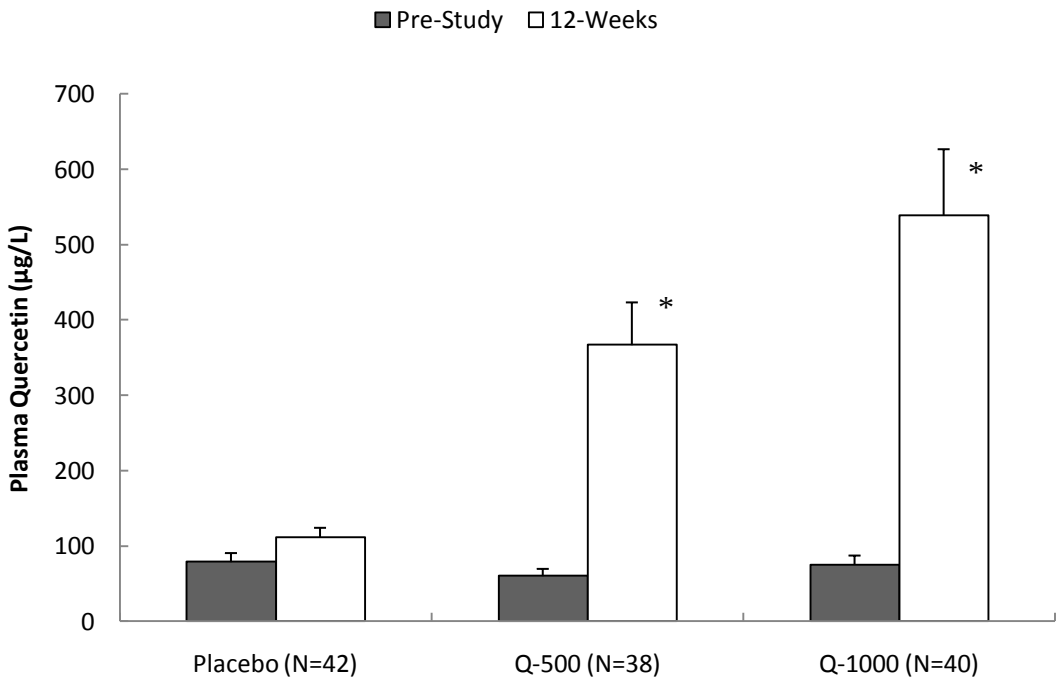

FIGURE 1. Plasma quercetin levels in response to twelve-week supplementation with 500 or 1000 mg/day quercetin compared to placebo (N=120). Group x time interaction $P<0.001$. * $P \leq 0.05$, change from pre-study compared to placebo.

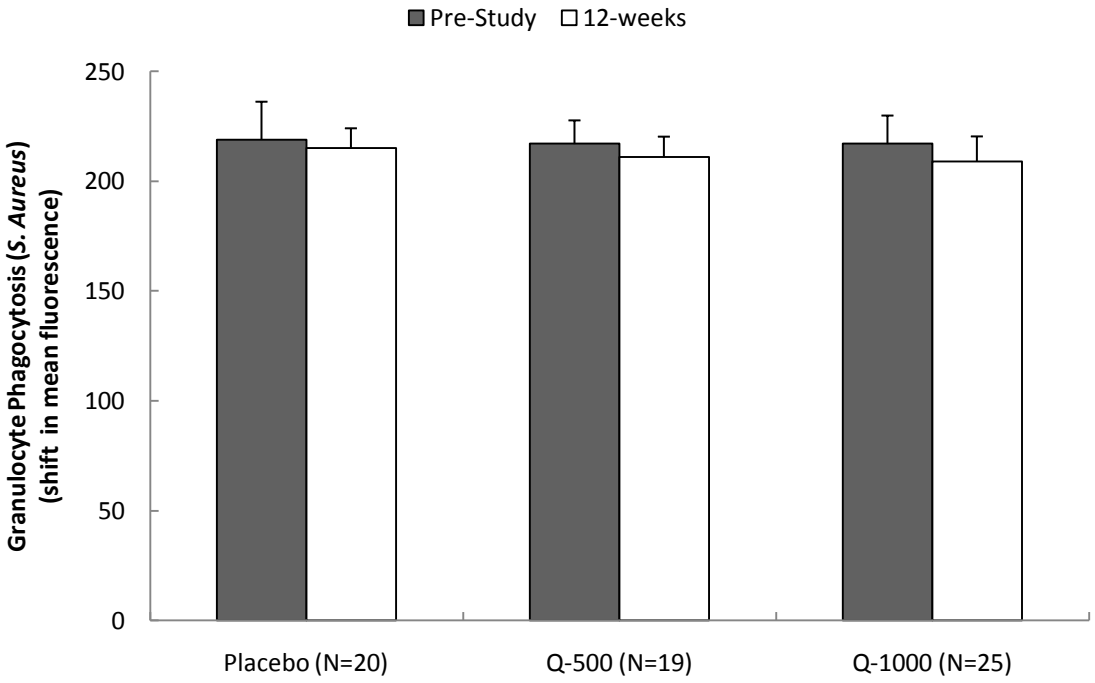

FIGURE 2. Granulocyte phagocytosis of FITC-labeled *S. aureus* pre- and post-twelve week supplementation with 500 or 1000 mg/day quercetin compared to placebo (N=64). Data are expressed as shifts in mean FITC fluorescence from 4°C to 37°C. Group x time interaction P=0.990.

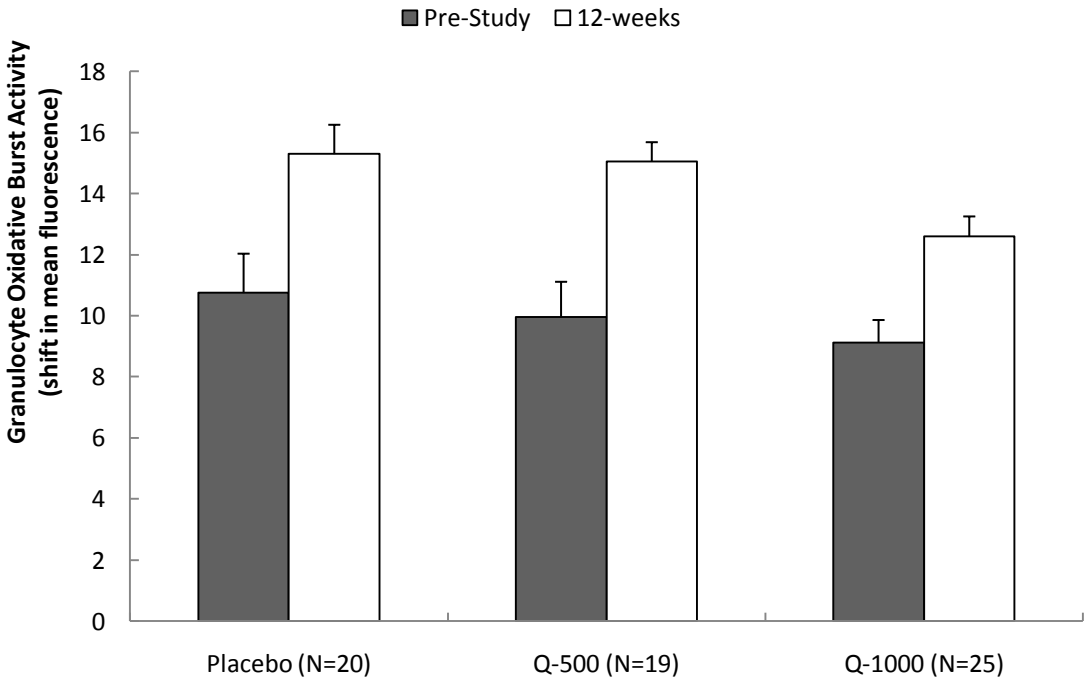

FIGURE 3. Granulocyte oxidative burst activity following incubation with *S. aureus* pre- and post-twelve week supplementation with 500 or 1000 mg/day quercetin compared to placebo (N=64). Data are expressed as shifts in mean ethidium bromide fluorescence from 4°C to 37°C. Group x time interaction P=0.602.

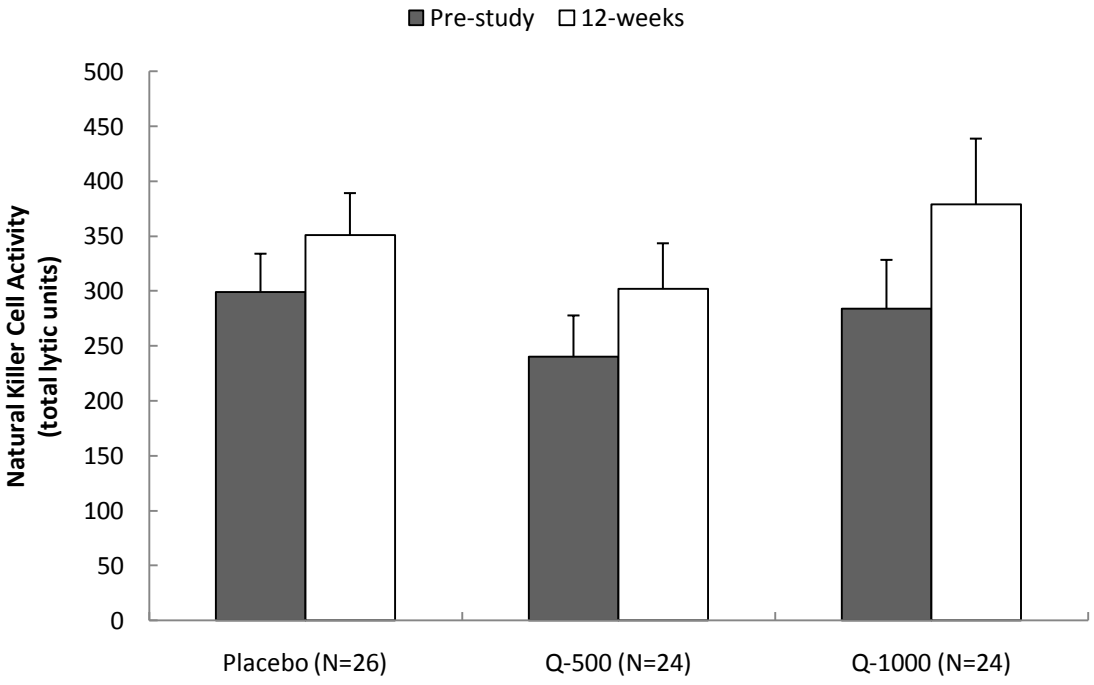

FIGURE 4. NK cell activity pre- and post-twelve week supplementation with 500 or 1000 mg/day quercetin compared to placebo (N=74). NKCA is expressed as the number of lytic units contained in 10^7 cells, where a lytic unit is defined as the number of NK cells required to kill 20% of 5000 K562 target cells. Group x time interaction P=0.696.

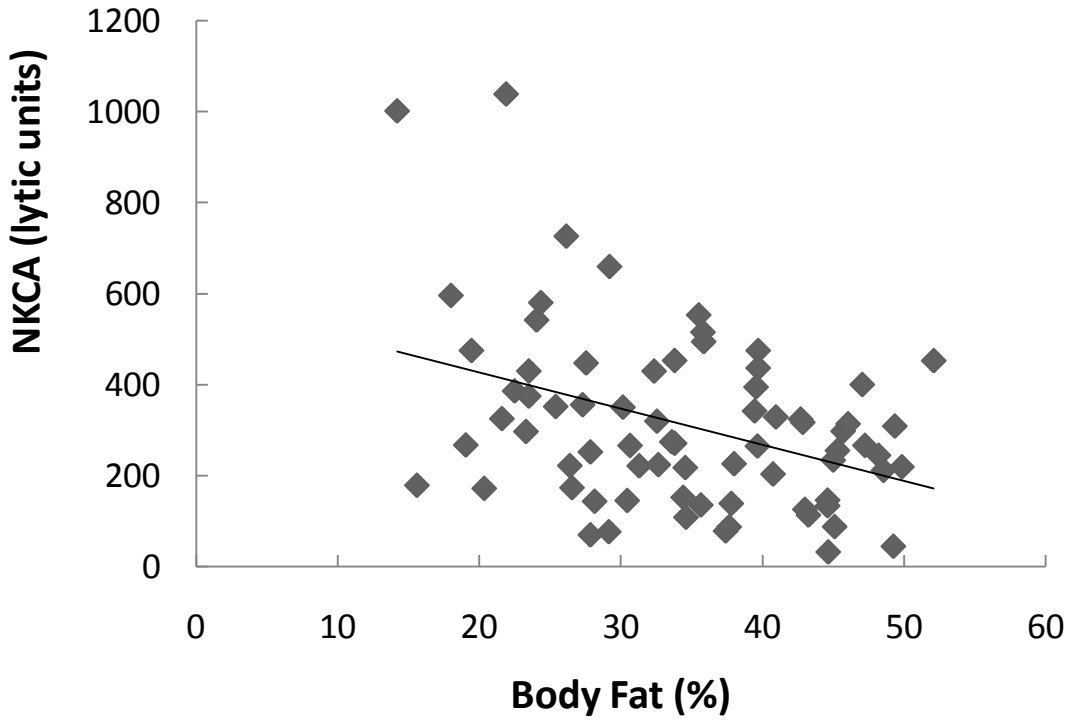

FIGURE 5. Relationship between body fat percentage and NKCA of human subjects (r=-0.38, P=0.001). Data points average pre- and post-study measures for all subjects with NKCA data (N=74).

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BIOGRAPHICAL SKETCH

Serena Ann Heinz was born in New Bern, North Carolina. She is the daughter of Virgil and Deidre Edwards. Serena attended elementary, middle, and high schools in New Bern and began her college career at UNC-Greensboro as a music performance major. Following her freshman year, she transferred to Appalachian State University, where she began her studies as a student of biology. After a several-year hiatus from university work, she returned to Appalachian State University and completed a Bachelor of Science in Biology, graduating *magna cum laude* in December 2007. She began her graduate coursework at Appalachian State University in August 2008. During her second year of graduate work, Serena served as President of the Graduate Student Association Senate, and she was inducted into the Cratis D. Williams Honor Society in April 2010. She received a Master of Science in Biology with a concentration in Cell and Molecular Biology in May 2010. Serena will begin an M.D.-Ph.D. program in June 2010, with the aim to continue her immunological research as a physician scientist.