Quercetin Supplementation Does not alter Antioxidantstatus in Humans

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Quercetin supplementation does not alter antioxidant status in humans

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Abstract

This study measured the influence of ingesting quercetin on plasma measures for oxidative stress and antioxidant capacity. Male and female subjects ($n = 1002$) varying in age (18–85 years) and body mass index (BMI) (16.7–52.7 kg/m^2) were studied. Subjects were randomized to one of three groups using double-blinded methods: placebo, 500 mg or 1000 mg quercetin/day with 125 mg or 250 mg vitamin C/day, respectively. Pre- and post-study fasting blood samples show that plasma quercetin increased in a dose-responsive manner. The pattern of change in plasma F_2-isoprostanes, oxidized low density lipoprotein, reduced glutathione, ferric reducing ability of plasma (FRAP) and oxygen radical absorbance capacity (ORAC) did not differ between supplementation groups or after adjustment for gender, age, BMI and disease status. In summary, quercetin supplementation over 12 weeks in doses of 500 mg or 1000 mg/day significantly increased plasma quercetin levels, but had no influence on several measures of oxidative stress and antioxidant capacity.

Keywords: *Quercetin, ferric reducing ability of plasma (FRAP), oxygen radical absorbance capacity (ORAC), F_2-isoprostanes, oxidized low density lipoprotein (LDL), glutathione, antioxidant, oxidative stress.*

Introduction

Polyphenols are nutrients present in a wide variety of edible plants [1]. The best defined group of polyphenols in the human diet are the flavonoids, which contain a three-ring structure with two aromatic centres and a central oxygenated heterocycle [2,3]. Flavonols are the most widespread dietary flavonoids, of which quercetin is the most prominent (3,3',4',5,7-pentahydroxyflavone) [3]. Dietary flavonols such as quercetin have been reported to have physiological effects, i.e. antioxidative [4,5], anti-inflammatory [6–8], anti-pathogenic [9,10], anti-viral [11–13], anti-microbial [14], anti-carcinogenic [15], cardioprotective [16] and mitochondrial biogenesis [17] activities and thus provide significant potential in the study of improving human health.

The richest food sources of quercetin are onions, hot peppers, curly kale, blueberries, apples, tea and broccoli [1,2]. Reports of total flavonol intake range from 13–64 mg/day, of which quercetin comprises ~ 75% [2,18]. Humans can absorb substantial amounts of quercetin in a dose-dependent fashion [19] with a reported half-life of 11–28 h [3,20,21]. Notably, chronic, high-dose quercetin ingestion by humans or rodents has not been found to be detrimental [22,23], but an optimal dose has yet to be characterized [24, 25]. In fact, epidemiological studies report that a diet high in quercetin is associated with a decreased risk for ischemic heart disease [26,27] and common types of cancer [15,27].

Quercetin is a powerful antioxidant and free radical scavenger, as demonstrated from *in vitro* studies [28–31]. Animal studies have yielded important information regarding the antioxidant capability of quercetin. For example, quercetin administered to rodents results in increased antioxidant activity [32], decreased lipid peroxidaxtion [5,33–35] and inhibition of LDL

oxidation [36]. A few small-scale human quercetin supplementation studies have produced conflicting results regarding quercetin's potential antioxidant effects. One study of obese subjects found that 6 weeks of quercetin supplementation decreased oxidized LDL [37] but all other human studies report no effect on a variety of measures of antioxidant capacity and oxidative stress [19,38–42].

Several reports indicate that oxidative stress is increased among obese and elderly individuals and those with chronic disease [43–45]. Boots et al. [41] hypothesized that quercetin supplementation may be more efficacious among individuals at risk for oxidative stress. Thus, the primary objective of this study was to measure the influence of two quercetin doses (500 or 1000 mg/day) on plasma oxidative stress and antioxidant capacity in a large community group (n = 1002) varying widely in age, BMI and disease status. We hypothesized that quercetin would have a positive effect on antioxidant status, especially in those presumed to have elevated oxidative stress and depressed plasma antioxidant capacity.

Materials and methods

Subjects

Male and female subjects (n = 1023), 18–85 years of age, were recruited via mass advertising from the community. Half of the subjects were studied during a 12-week period from January to April 2008 and the second half from August to November 2008. Subjects had to be non-institutionalized and women were excluded if pregnant or lactating. No other exclusion criteria were employed and both diseased and non-diseased subjects were admitted into the study, with monitoring of disease status and medication use. Written informed consent was obtained from each subject and the Appalachian State University institutional review board approved all experimental procedures. During recruitment, subjects were stratified by gender (~ 40% male, 60% female), age (40% young adult (18–40 years of age), 40% middle-aged (41–65) and 20% elderly (65 and over)) and body mass index (BMI) groups (33% normal (18.5–24.9), 33% overweight (25–29.9) and 33% obese (2: 30 kg/m^2)) to ensure representation of these various sub-groups. Subjects agreed to avoid any other supplements containing quercetin; no other restrictions were placed on diet, supplement usage or medications.

Research design

Subjects were randomized to one of three groups: Q-500 (500 mg quercetin/day), Q-1000 (1000 mg quercetin/day) or placebo (PL). Supplements were administered utilizing double blinded procedures. Subjects ingested two soft chew supplements twice daily (upon awakening, and between 2 pm and the last meal of the day) during the 12-week study period. Supplements were prepared by Nutravail Technologies (Chantilly, VA) with Quercegen Pharma (Newton, MA) 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 coloured with FD&C yellow #5 and #6. Placebo supplements were prepared exactly the same way minus the quercetin, ascorbic acid and sodium ascorbate and niacin. Data from Quercegen Pharma (unpublished data, personal communication, Tom Lines) indicate that the bioavailability of quercetin is enhanced with vitamin C and niacin and thus this study tested whether 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 12 weeks. Subjects completed a monthly log to verify adherence to the supplementation regimen, physical activity and diet status, change in disease status and medication use, gastrointestinal (constipation, heartburn, bloating, diarrhea, nausea, vomiting), skin (rash, dryness, flushing), allergy and mental (energy, headache, stress, focus/concentration) symptoms.

Outcome measures

Two weeks prior to the first lab visit for the study, subjects provided demographic and lifestyle habit information via the survey posted on SurveyMonkey.com (Portland, OR). Height was measured with a stadiometer and body mass and body composition determined using a Tanita bioelectrical impedance (BIA) scale (Tanita, Arlington Heights, IL). Blood samples were taken after an overnight fast in the morning (7–9 am) before and after the 12-week supplementation period. Once separated, plasma samples were imme- diately flash-frozen in liquid nitrogent, stored at –80°C and analyzed for the outcome measures described below. Unless specified otherwise all chemicals were purchased from Sigma Aldrich (St Louis, MO).

Plasma quercetin. Plasma quercetin was measured as previously described [25]. Briefly, total plasma quercetin (quercetin and its primary conjugates) from heparin treated blood was measured following solid- phase extraction via reversed–phase HPLC with UV detection. Quercetin conjugates were hydrolysed 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/arylsulphatase and crude extract from Helix pomatia (Roche Diagnostics Corporation, Indianapolis, IN) for 2 h at

37°C. Chromatographic analysis was performed using the Ultimate 3000 HPLC-PDA system (Dionex Corporation, Sunnyvale, CA) with a Gemini C18 column (Phenomenex, Torrance, CA).

Oxidative stress and antioxidant capacity. Plasma F_2-isoprostanes were determined using gas chromatography mass spectrometry (GC-MS) [46,47]. Plasma was collected from heparinized blood, immediately flash- frozen in liquid nitrogen and stored at –80°C [47]. Immediately prior to assay plasma samples were thawed. The samples were used to extract free F_2-isoprostanes with deuterated [$^2H_4$] prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) added as an internal standard. The mixture was then added to a C18 Sep Pak column, followed by silica solid phase extractions. F_2-isoprostanes were converted to pentafluorobenzyl esters, subjected to thin layer chromatography and converted to trimethylsilyl ether derivatives. Samples were analysed by a negative ion chemical ionization GC-MS using an Agilent 6890N gas chromatography interfaced to an Agilent 5975B inert MSD mass spectrometer (Agilent Technologies, Inc. Santa Clara, CA).

Quantification of oxidized low density lipoproteins (oxidized LDL) in a sub-group of subjects was performed as previously described [48,49] using standard protocols for a competitive ELISA kit (Mercodia Oxidized LDL Competitive Enzyme-Linked Immunosorbent Assay, Mercodia Inc., Sweden). Plasma from EDTA treated blood was used accordingto the commercially available protocol and absorbance was read at 450 nm.

Red blood cell reduced glutathione (GSH) was assayed in a sub-group of subjects using the Cayman Chemical GSH assay kit (#703002, Ann Arbor, MI). This assay utilizes an optimized enzymatic recycling method, using glutathione reductase to quantify the amount of GSH in red blood cells collected from heparinized blood. The samples were read at 405 nm and reported as μM of reduced glutathione per gram of haemoglobin [50].

Total plasma antioxidant power was determined by the ferric reducing ability of plasma (FRAP) assay [46,51], a single electron transfer reaction. This assay utilizes water-soluble antioxidants native to the plasma collected from EDTA treated blood to reduce ferric iron to the ferrous form subsequently producing a chromogen identifiable at 593 nm. Samples and standards are expressed as ascorbate equivalents based on an ascorbate standard curve. Intra-assay and inter-assay coefficients of variation were less than 5% and 7%, respectively.

Oxygen radical absorbance capacity (ORAC) was measured in a sub-group of subjects using methods described previously [52]. Serial dilutions of 50 μM, 25 μM, 12.5 μM and 6.25 μM of Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were made using phosphate buffer solution and used as standards. Fluorescien solution was made by diluting 800 μL of stock solution into 50 mL phosphate buffer. This solution was incubated completely, at 37°C, prior to use. AAPH solution was made by dissolving 0.108 g of AAPH (Wako Chemicals, Richmond, VA) in 5 mL of phosphate buffer solution (incubated at 37°C prior to use with AAPH) and 20 μL (1.6 μmol) of AAPH solution was then added to each well immediately before reading. The microplate was loaded in a 'forward-then-reverse' order, with edge wells loaded only with phosphate buffer to avoid edge effects. Blanks, Trolox standards and samples (human plasma from ETDA treated blood) were loaded into appro- priate wells, followed by 200 μL of fluoroscien work- ing solution. The plate was then covered and incubated at 37°C for at least 20 min, followed by the addition of 20 μL AAPH working solution. ORAC values were calculated by the plate reader (Spectra Max Gemini XPS, Molecular Devices) (area under the curve). Excitation wavelength was 485 nm and emission wavelength was 520 nm.

Statistical procedures

Data were analysed using a 3 (group) X 2 (time) repeated measures ANOVA, between groups design, with *post-hoc* analysis using Bonferroni adjusted independent *t*-tests that contrasted pre- to post-supplementation changes of Q-500 and Q-1000 with PL ($p<0.0125$). Additional repeated measures ANOVAs were conducted by adding categorical covariates to the model to test for the influence of gender (male, female), BMI (normal<25 kg/m^2, overweight 25–29.9 kg/m^2, and obese 2: 30 kg/m^2), age (< 40, 40–59, 2: 60 years) and chronic disease status (with or without). Data are expressed as means ± SE.

Results

Of the 1023 subjects recruited into the study, 1002 completed all phases of the study. Among the 21 drop-outs (seven from the PL group, six from Q-500 and eight from Q-1000), 12 failed to take the supplement and/or adhere to testing procedures and nine reported adverse symptoms from taking the supplement. Follow-up revealed no consistent pattern of symptoms that could be ascribed to taking the quercetin supplements. Table I shows that subject characteristics did not differ significantly between groups. Subjects were 60% female and 40% male, ranged widely in age, BMI and body composition and were predominately Caucasian. Thirty-seven per cent of subjects reported past or current history for one or more chronic diseases: hypertension (19%), arthritis (16%), cancer (6%), cardiovascular disease (4%), diabetes (4%). For all subjects combined, F_2-isoprostanes were significantly

Table I. Subject characteristics (n = 1002) (mean ± SE).

Variable	Placebo n = 335 M = 123; F = 212	Q-500 n = 334 M = 138; F = 196	Q-1000 n = 333 M = 134; F = 199	Group (All) n = 1002 M = 395; F = 607
Age (years)				
Males	43.8 ± 1.5	45.3 ± 1.2	45.5 ± 1.4	46.0 ± 0.5
Females	47.4 ± 1.1	47.2 ± 1.1	45.2 ± 1.1	(18–85)
Weight (kg)				
Males	84.8 ± 1.4	85.7 ± 1.2	88.1 ± 1.5	77.2 ± 0.6
Females	71.2 ± 1.1	71.6 ± 1.2	71.4 ± 1.3	(42.7–157.5)
Height (m)				
Males	1.77 ± 0.06	1.78 ± 0.04	1.77 ± 0.06	1.70 ± 0.03
Females	1.64 ± 0.05	1.65 ± 0.05	1.64 ± 0.04	(1.39–2.02)
BMI (kg/m^2)				
Males	27.0 ± 0.4	26.9 ± 0.4	28.1 ± 0.4	26.7±0.2
Females	26.4 ± 0.4	26.2 ± 0.4	26.4 ± 0.5	(16.7–52.7)
Body composition (% fat)				
Males	22.1 ± 0.8	22.1 ± 0.7	24.9 ± 0.8	30.0 ± 0.4
Females	34.8 ± 0.7	34.8 ± 0.7	33.8 ± 0.7	(3.4–59.5)
Education (years)	15.5 ± 0.2	15.5 ± 0.2	15.8 ± 0.2	15.6 ± 0.1
Marital status	34% single 53% married 13% other	32% single 60% married 8% other	33% single 55% married 12% other	33% single 56% married 11% other
Race	93% white 2% black 5% other	93% white 2% black 5% other	92% white 4% black 4% other	93% white 3% black 4% other

elevated: 16.3% in the obese (n = 244) (48.4 ± 1.1 pg/mL) vs those with normal weight (n = 435) (41.6 ± 0.6 pg/mL) (p<0.001), but no differences were found when accounting for gender, age and disease status. FRAP differed significantly between obese (+ 13.7%) (614 ± 10.2 μmol/L) and normal weight (540 ± 6.5 μmol/L) subjects (p<0.001). Additionally, FRAP was elevated in males (+17.5%) and in subjects who were older (2: 60 vs< 40 years, +14.9%) or diseased (+11.4%) (all p<0.001).

Plasma quercetin (overnight fasted) in both quercetin groups increased significantly above PL levels in a dose-responsive manner (interaction effect, p<0.001) (Figure 1) and monthly measures (data not shown) indicated that the increase peaked during the first month and was maintained for 12 weeks. Individuals varied widely in their plasma quercetin response to supplementation, but increases were not related to gender, BMI, age or chronic disease status.

The pattern of change over time between groups did not differ significantly for plasma F_2-isoprostanes (interaction effect, p = 0.280) (Figure 2), oxidized LDL (interaction effect, p = 0.831) (Figure 3), GSH (interaction effect, p = 0.800) (Figure 4), FRAP (interaction effect, p = 0.528) (Figure 5) or ORAC (interaction effect, p = 0.525) (Figure 6). Separate analyses by gender, BMI, age and chronic disease status failed to find significant differences in the outcome measures across Q-500, Q-1000 and PL groups. For the sub-group for which oxidized LDL was measured, no significant interaction effects were shown for LDL-cholesterol or HDL-cholesterol (data not shown).

Discussion

We found that 12-weeks supplementation with 500 or 1000 mg quercetin per day significantly increased overnight fasted plasma quercetin. However, contrary to our hypothesis, the increase in plasma quercetin was not associated with significant alterations in plasma measures of oxidative stress or antioxidant

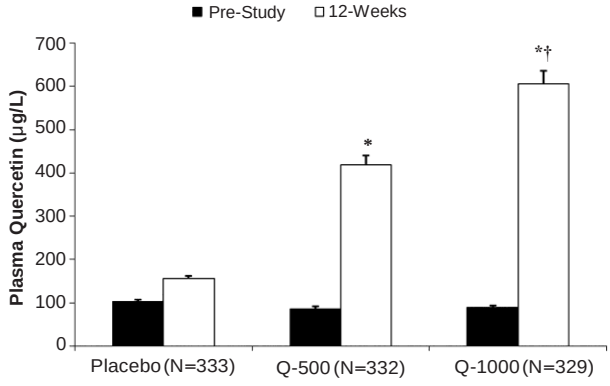

Figure 1. Plasma quercetin concentration was significantly increased by 500 mg/d (Q-500) or 1000 mg/d (Q-1000) for 12 weeks. Plasma quercetin concentration is expressed as μg/L.
*p < 0.05 compared to Placebo; †p < 0.05 compared to Q-500.

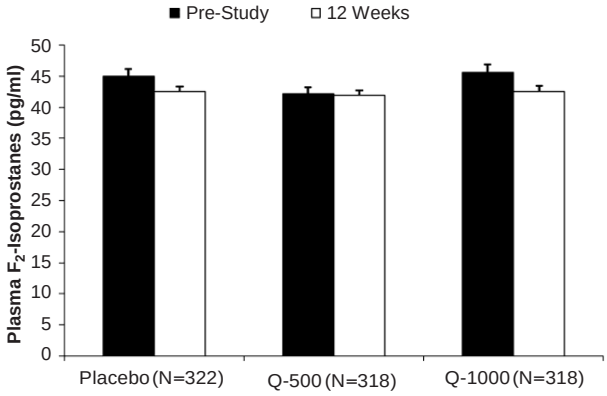

Figure 2. Plasma F_2-isoprostanes, a measure of oxidative stress, were measured before and after 12 weeks of quercetin supplementation, 500 mg/d (Q-500) or 1000 mg/d (Q-1000). Plasma F_2-isoprostane concentration is expressed as pg/ml.

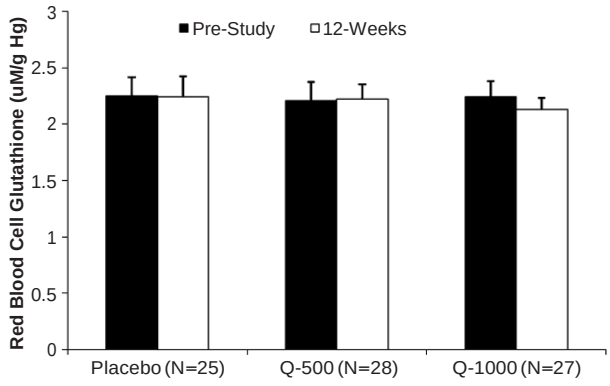

Figure 4. Reduced glutathione (GSH) was determined in red blood cells as a measure of antioxidant capacity in a sub-group of subjects. GSH was measured before and after 12 weeks of quercetin supplementation, 500 mg/d (Q-500) or 1000 mg/d (Q-1000). Data are expressed as μM of reduced glutathione per gram of haemoglobin (Hg).

capacity in community-dwelling adults. Our study included a relatively large number of subjects with multiple sub-groups. We employed two different doses of quercetin and the oxidative stress and antioxidant capacity results are in agreement with the handful of human studies previously conducted.

Plasma F_2-isoprostane, formed as a result of arachidonic acid peroxidation, was unchanged with 12-weeks of quercetin supplementation; a finding in agreement
with short-term studies conducted by our group [40,46] and others [53]. Contrary to our hypothesis, plasma F_2-isoprostanes were not significantly elevated in the older subjects or in those with chronic disease. However, plasma F_2-isoprostanes were significantly elevated in obese subjects, as previously reported by others [43,44].

Although quercetin supplementation has been found to attenuate the *ex vivo* oxidation of LDL [24], our *in vivo* results suggest that long-term quercetin supplementation does not alter the levels of oxidized LDL in humans, as also reported by Egert et al. [19]. However, another study of overweight subjects using a cross-over design with a 5-week washout period found a 14% decrease in oxidized LDL following 6 weeks of supplementation with 150 mg quercetin per day compared to a 7% decrease during the placebo condition [37].

We measured the antioxidant capacity of plasma using three different methods; GSH, FRAP and ORAC. GSH, a non-enzymatic free radical scavenger, was unaffected by quercetin supplementation, as previously reported by Boots et al. [41]. FRAP assessed the reducing capacity of plasma while ORAC assessed the radical-scavenging capacity of plasma. Our results indicate that quercetin supplementation relative to PL does not alter FRAP or ORAC, as previously reported by Egert et al. [19] in healthy adults, and by our research team in studies of endurance athletes [40,46]. FRAP and ORAC as well as other antioxi-

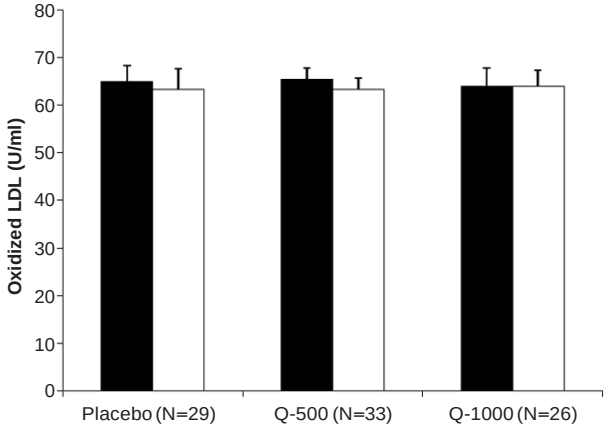

Figure 3. Oxidized low density lipoproteins (oxidized LDL), a measure of oxidative stress, was measured before and after 12 weeks of quercetin supplementation, 500 mg/d (Q-500) or 1000 mg/d (Q-1000) in a sub-group of subjects. Data are expressed as units (U) of oxidized LDL/ml.

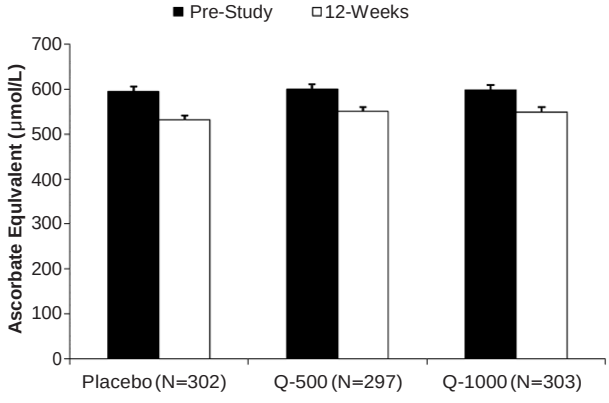

Figure 5. The ferric reducing ability of plasma (FRAP) assay was used as a measure of total plasma antioxidant power. FRAP was measured before and after 12 weeks of quercetin supplementation, 500 mg/d (Q-500) or 1000 mg/d (Q-1000). Data are expressed as ascorbate equivalents (μmol/L).

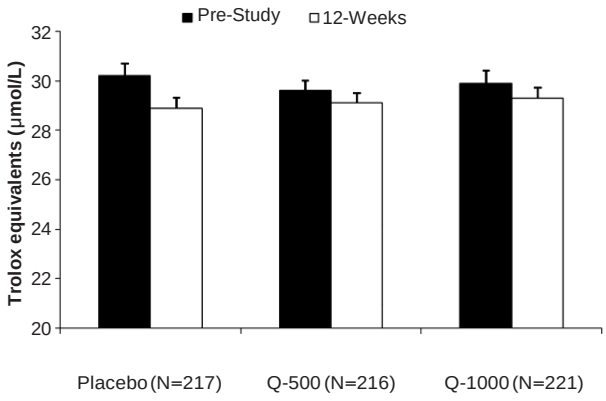

Figure 6. The oxygen radical absorbance capacity (ORAC) assay was used as a measure of total antioxidant power in a sub-group of subjects. ORAC was measured before and after 12 weeks of quercetin supplementation, 500 mg/d (Q-500) or 1000 mg/d (Q-1000). Data are expressed as Trolox equivalents (µmol/L).

dant capacity assays (e.g. trolox equivalent antioxidant capacity (TEAC) and total radical-trapping antioxidant parameter (TRAP)) measure the contribution of all reducing and antioxidant substances and thus lack specificity [54]. However, our chosen marker of oxidative stress and the three measures of antioxidant capacity used in this study were not affected by quercetin ingestion, suggesting that this formulation of quercetin does not act as an *in vivo* antioxidant. Interestingly, FRAP was significantly increased in obese, male, elderly and diseased subjects, irrespective of quercetin intake. This finding suggests that FRAP is increased in those under oxidative stress, a novel finding that has not been previously reported. The FRAP assay is sensitive to ascorbate levels and accounts for ~ 15% of the plasma FRAP value [51]. ORAC, on the other hand, did not differ between sub-groups within our population and is not sensitive to ascorbate levels [52,54]. Plasma ascorbate levels were not measured in the current study.

The hypothesis that dietary flavonoids play a significant role as antioxidants *in vivo* has been challenged recently because of the growing realization that most flavonoids have low bioavailability and undergo extensive metabolism that reduces antioxidant capacity [39,41,54,55]. After ingestion, quercetin and its methylated derivatives (isorhamnetin, tamarixetin) are not present in aglycone form but only in the conjugated form (mainly glucuronide and sulphate conjugates) in species investigated thus far including humans, pigs and rats [55,56].

The lack of quercetin-related decreases in oxidative stress measures in humans [19,39,40,42,54] is in contrast to findings from *in vitro* experiments [28–31] and animal studies [5,32–36] that indicate strong antioxidant influences. Loke et al. [55] conducted an eloquent *in vitro* study demonstrating that free quercetin provides greater protection from oxidative stress than its conjugated metabolites found in the plasma. While quercetin supplementation studies demonstrate little evidence of protection from oxidative stress, many studies indicate a decrease in disease risk and improved antioxidant capacity when foods rich in quercetin are consumed in the diet [54].

There is increasing support that co-ingestion of quercetin with other flavonoids and food components improve and extend quercetin's bioavailability and bioactive effects [10,57]. These inclue the flavonoid epigallocatechin 3-gallate (EGCG) from tea [58], iso- quercetin (quercetin-3-glucose or hirsutrin) which is the glycosylated form of quercetin in onions and other foods, n3-polyunsaturated fatty acids (n3-PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [59] and the nutrients vitamins C, E and folate [10,60]. In a recent experiment we found that 2-weeks ingestion of a mixed flavonoid-fish oil supplement significantly decreased acute exercise-induced oxidative stress (20% decrease in plasma F_2-isoprostanes) and inflammation (50% decrease in C-reactive protein (CRP), 39% decrease in IL-6) compared to quercetin alone or placebo [25]. We are currently investigating whether the supplement effects found in exercised athletes [25] extends to a group of middle-aged and overweight/obese women with chronic inflammation and oxidative stress.

In summary, quercetin supplementation in doses of 500 mg or 1000 mg/day did not improve antioxidant capacity or decrease oxidative stress in a large population of subjects ranging widely in age, BMI and disease state. Future research will determine if the bioavailability of quercetin and its bioactive effects can be augmented by adding other flavonoids (e.g. EGCG) and food components [10,58,61].This mixed flavonoid supplement approach may be efficacious in at risk populations such as obese, older adults with chronic oxidative stress and inflammation.

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Declaration of interest: D. C. Nieman is a board member of Quercegen Pharma, the remaining authors have no conflict of interest.

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