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Zero effect of multiple dosage of olive leaf supplements on urinary biomarkers of oxidative stress in healthy humans

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Running title: Effect of olive leaf supplementation on oxidative stress

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Conclusion: Dietary supplementation with olive leaf extract did not alter the oxidative status of healthy young adults. © 2008 Published by Elsevier Inc.

Introduction

The 'natural' way of living, such as the use of organic produce and dietary supplementation, is believed to be conducive to good health and disease prevention (Harnack, Block, Subar & Lane, 1998). Many people are turning away from modern medicine and experimenting with natural therapies. It was Hippocrates who said 'Let food be thy medicine'. In our culture of fast and processed foods, illness and diseases of affluence are common. Due both to consumer interest and the potential health

benefits of alternative therapies and phytochemicals, scientific investigations are examining the potential for traditional medicines and natural food components.

Looking at traditional diets for prevention and solutions to many chronic diseases, the Mediterranean diet seems to be close to ideal. Within this diet, much research has focused on the biophenols present in olive oil, due to their potential antioxidant activity. These compounds appear to possess a protective mechanism for delaying or preventing the development of heart and vascular diseases, as well as some cancers as seen in Mediterranean populations (Serra-Majem, Roman & Estruch, 2006). As an extension of both the interest in natural products, and the apparent health benefits of the Mediterranean diet (Martinez-Gonzalez et al., 2002; Serra-Majem et al., 2006), olive leaf extracts have been studied. Olive leaves contain the same biophenols as contained in the oil and fruit of the olive tree, but they occur in much greater concentrations (Briante et al., 2002).

Olive leaves are believed to have been used in traditional medicine for the treatment of malaria and associated fevers, and have a strong antioxidant activity (Benavente-Garcia, Castillo, Lorente, Ortuno & Del Rio, 2000). They have been found *in vitro* to possess hypoglycaemic, hypotensive, anti-HIV and anti-tumour qualities (Andreadou et al., 2007; Coni et al., 2000; Manna et al., 2004; Petroni et al., 1995; Zarzuelo, Duarte, Jimenez, Gonzalez & Utrilla, 1991). Commercially, olive leaf extracts are available in a powdered capsule form, in liquid tonics and also combined with other theoretically beneficial herbs and vitamins. Olive leaf extract supplements are marketed as being antiviral and as such beneficial for cold and flu relief. Additional benefits promoted include fever relief and the promotion of heart health, supporting

the body to fight free radical attack, increasing the concentration span of children with attention-deficit disorder, and preventing cold sores. However, scientific research supporting these claims is lacking. While there has been a moderate amount published about the major olive oil biophenol, hydroxytyrosol, there is limited literature examining the contribution of olive leaf, or its major biophenol, oleuropein on health outcomes. These studies are limited to *in vitro/ex vivo* (Hamdi & Castellon, 2005) and animal studies (Andreadou et al., 2006; Puel et al., 2006). Limited human trials (Visioli et al., 2003) have been conducted to assess the health effects of supplemental olive leaf, oleuropein or other olive phenols.

The current study was designed as a single centre, randomised, single-blinded, prospective pilot comparison of the effect of differing olive leaf antioxidant supplementation on a cohort of young, healthy people. Emphasis was placed on the impact of supplementation on oxidation status of the participants.

Materials and Methods

Subjects

This study was approved by the Ethics in Human Research Committee of Charles Sturt University. Subjects were recruited from a class of first year health science students from Charles Sturt University, Wagga Wagga (Australia). A brief verbal explanation of the study and request for participants was conducted. Those individuals interested in participating received a written explanation of the study's aims and the commitments expected of their participation. Email addresses were collected from those who wished to attend a more detailed information session. Emails were sent, and times selected for attendance to a more in-depth verbal explanation. A thorough

explanation of the study and expectations of the participants was conducted. All questions of the participants were answered. Those subjects who intended to participate signed an informed consent form.

A Screening Questionnaire was completed by study participants at baseline to gain insight into their current health status. In addition, subjects completed a Health Evaluation both at baseline, and follow-up (Supplement). These were scored on a Likert scale. Subjects were randomly allocated into one of three treatment protocols: control, liquid or capsule. This randomisation was prepared and assigned independently to eliminate any researcher bias in assigning participants to supplement protocols.

Known adverse effects of olive leaf extract supplementation are vague but include headache, fatigue and other flu-like symptoms. Subjects were made aware of this and advised to discontinue the supplementation protocol if symptoms occurred.

Dietary Collection

Participants were advised to maintain their current dietary intake for the duration of the study to prevent changes in dietary intake confounding the study results. Nevertheless, dietary intake was assessed both prior to the study's commencement and at its completion, to assess for any change in dietary intake. Dietary intake was assessed by three-day food diaries and an additional mini-food frequency questionnaire (FFQ). This mini-FFQ was completed by all subjects as part of the initial Screening Questionnaire. Foods known to be naturally high in biophenols

including tea, red wine and fruits and vegetables were included. Participants recorded their consumption of these foods as serves per day, or week.

Two three-day food diaries were collected from all subjects, one at baseline (that is, prior to initial supplementation) and one at follow-up (that is at the completion of the supplemental protocol). Subjects were instructed to record all food and beverages consumed within a consecutive three-day period, with the inclusion of two weekdays and one weekend day. Instructions for this task were given both verbally, and in print. An example food diary was also supplied to the study participants. The dietary intake of the study participants was assessed using FoodWorks Professional version 5 software package (Xyris software, Highgate Hill, QLD) which is based on AUSNUT the Australian nutrition database.

Olive Leaf Supplementation

The capsule and liquid olive leaf extract supplements were obtained from commercial suppliers. The capsules contained a dried leaf preparation. Empty bottles for the placebo were supplied by the same manufacturer as the liquid supplement. Bottles were identical to those containing the active liquid olive leaf extract supplement; the only difference was the appearance of the supplied measuring cups. The placebo was prepared by a pharmacist (Sally Day) to match the liquid supplement as closely as possible in look, feel and taste. The placebo consisted of a 1:9.5:15 mix of vinegar, water and glycerol, with yellow, red and green food colour in a 3:1:1.5 mix. As the placebo was concocted from glycerol, no preservatives were required to stabilise the liquid. The avoidance of preservatives was essential to eliminate confounding due to a potential 'antioxidant' effect of preservatives. Subjects were instructed to consume

one supplement (one capsule, or one 5 mL measure of liquid) three times per day, for 28 days.

Compliance

All participants were required to return the supplement bottles at the end of the 28 day period. Compliance was measured by the allotted number of daily doses dispensed minus the number of daily doses returned.

Urine Samples

A spot urine sample was supplied by each subject both at baseline and follow-up in sterile 70 mL containers. These were couriered to the laboratory within 30 min of voiding, and stored in a freezer at -20° C.

Reagents and Standards

Reagents used without further purification: Folin-Ciocalteu reagent from Sigma-Aldrich (Steinheim, Germany); acetonitrile anhydrous, UNICHROME (Sydney, Australia). Water used in all analytical work in this thesis was purified by a Modulab Analytical model (Continental Water Systems Corporation, Sydney, Australia) water system. Phenolic standards were used without further purification: gallic acid from Sigma-Aldrich (Steinheim, Germany) and oleuropein from Extrasynthese (Genay, France).

Procedures for Oxidation Markers

Creatinine

Creatinine was determined using a QuantiChrom Creatinine Assay Kit (DICT 500) that was purchased from ProSciTech (Thuringowa, Qld, Australia). Urine or standards (5 µL of 50 mg/100 mL) were pipetted along with the working reagents (50 µL of each Reagent A and Reagent B and 100 µL of water) into a 96-well plate. Plates were read in a Versamax Tunable (Molecular Devices, Sunnyvale, USA), automated microplate reader at 510 nm at both 1 and 5 min. Analyses were performed in duplicate.

Isoprostanes

Isoprostanes were determined by a competitive enzyme-linked immunoassay (ELISA) kit purchased from Oxford Biomedical Research (Oxford, MI, USA; Urinary Isoprostane Assay Kit, EA 85). Reagents and urine or standards (1 µg/mL 15-isoprostane F2t) were added to a 15-isoprostane F2t antibody coated 96-well microplate. Standard or urine (100 µL) diluted 1:5 with 'Enhanced Dilution Buffer' and diluted 15-isoprostane F2t-horse radish peroxidase conjugate was added to each well, omitting the reagent blank. This was incubated for 2 hours. The wells were then washed. 'Wash Buffer' (300 µL) was pipetted into the wells, let stand for 2 min, and then removed via inversion and the plate blotted on lint free paper towel. This procedure was repeated twice. After the 'Substrate' (200 µL) was pipetted into the wells, the plate was incubated until a blue colour was seen (30 min). Sulfuric acid (3M, 50 µL) was used to stop the reaction. The plate was read in a Versamax Tunable (Molecular Devices, Sunnyvale, USA), automated microplate reader at 450 nm.

8-Hydroxy-2'-deoxyguanosine

A competitive 8-hydroxy-2'-deoxyguanosine (8-OHdG) ELISA kit was purchased from the Japan Institute for the Control of Aging (JaICA, Fukuroi, Shizuoka, Japan). After centrifugation, urine (50 µL) or standard 8-OHdG solution (0.125, 0.25, 0.5, 1, 4, 10 ng/mL; 50 µ L) was added to each well of a precoated 8-OHdG 96-well plate along with reconstituted primary antibody (reconstitute of monoclonal antibody specific for 8-OHdG and phosphate buffered saline; 50 µL). The plate was covered with an adhesive strip, and incubated at 4°C over night. After pouring the contents out of the well, 250 µL of washing solution (5x concentrated phosphate buffered saline) was added to each well. The plate was thoroughly agitated, and the washing solution poured off. The plate was then blotted on lint free paper towel to remove residual washing buffer. This procedure was repeated twice more. Constituted secondary antibody (reconstitute of HRP-conjugated antibody and phosphate buffered saline; 100 µL) was added to each well, and agitated. An adhesive strip was used to cover the plate, and the plate was left to incubate for 1 h at room temperature. After this time, the wash procedure was repeated as before. Reconstituted enzyme substrate (3,3`,5,5`-tetramethylbenzidine and hydrogen peroxide/citrate-phosphate buffered enzyme in a ratio of 1:100; 100 µL) was added to each well, agitated, and let incubate in the dark for 15 min at room temperature. Reaction termination solution (1 M phosphoric acid; 100 µL) was added and the plate agitated. The plate was then read at 450 nm on a Versamax Tunable (Molecular Devices, Sunnyvale, USA), automated microplate reader.

As recommended the outer wells of the plate were not used. These were filled with distilled water to a uniform volume of that in the other wells, to eliminate high readings due to evaporation of the reagents.

Total Phenols Using Folin-Ciocalteu (FC) reagent

A modified version of Singleton and Rossi's (Singleton & Rossi, 1965) method was developed. Urine (5 µL) was added to a 96-well microplate containing 145 µL water. FC reagent (60 µL) was added and after 1 min, aqueous sodium carbonate solution (10% w/v; 90 µL) was added. The microplate was shaken for 1 h at ambient temperature to mix the contents thoroughly. The absorbance was read at 760 nm. Results are expressed by reference to a six-point regression curve as milligrams of gallic acid equivalents per litre of urine.

RESULTS

Forty five male and female subjects started the study, and thirty six adequately completed the study (nineteen female and seventeen male participants). No subjects dropped out during the study protocol. All subjects reported compliance with no significant difference for supplement consumption (Chi² p= 0.443 for the capsule group; p= 0.692 for the liquid group and p= 0.756 for the control group). However, from measurement of supplement doses, mean compliance overall was moderate at 82%. Mean compliance in the capsule group was high at 97% versus 79% and 70% in the liquid and control groups, respectively. Nine subjects were excluded from analysis as they were deemed to have been non-compliant.

Baseline characteristics are outlined in Table 1. Inclusion criteria to participate in the study included being aged over 18 years or independent, in generally good health, non-smoker and suffer from no known food allergies/intolerances. Exclusion criteria included being pregnant or breastfeeding, suffer from any major chronic disease, wear

a pacemaker, smoke cigarettes regularly or suffer from food allergies/intolerances. Eleven (31%) of the study participants were using vitamin/mineral supplements at baseline. Twelve (63.2%) of the female study participants reported using the contraceptive pill. Responses to questions regarding general health and diet at baseline and follow-up were analysed using simple Chi² statistics. There was no significant difference in the responses of the three groups at baseline (p=0.05) indicating that overall health and energy levels between the groups did not significantly differ at baseline. Additionally, no significant difference was found from baseline to follow-up (p=0.05) for any of the three groups. This indicates that overall health and energy levels did not change over the experimental protocol.

Repeated measures ANOVAs were performed on dietary data (Table 2) including energy intake, macronutrients including alcohol and dietary fibre, and the micronutrients β-carotene and vitamin A equivalents to assess for a time, interact, and group effect. All parameters were non-significant (p<0.05) with the exception of a time effect for carbohydrate (p=0.041), indicating that there was a change in carbohydrate intake from baseline to follow-up for the groups.

The oxidative status of subjects was assessed by measurement of urinary $F_{2\alpha}$-isoprostane (8-isoPGF$_{2\alpha}$), 8-OHdG and Folin-Ciocalteu Total Phenols. Data are presented for creatinine (Figure 1) and for the markers normalised to creatinine (Figures 2 – 4). For the 8-OHdG assay, measurement was restricted to 18 samples because of the cost of the reagent kit. Associations between baseline markers of oxidative status and intake of foods known to be high in phenolic compounds was

assessed using a two-tailed Spearman's correlation. At the p<0.05 level of significance, no correlation was observed with any of the baseline markers and foods.

Data were tested for outliers using the Shapiro-Wilk test for normality. For all variables, the test statistic was significant, indicating possible outliers. These values have been included as valid data points in subsequent statistical analyses as removal of outliers can cause serious bias and a deviation from protocol (Simon, 2001). Therefore, non-parametric statistics were used. The continuous variable data were analysed by repeated measures ANOVA using the Wilks Lambda general linear model test (Table 3). There was no significant interaction in any of the tests indicating that there was no significant difference in the change for the different groups.

DISCUSSION

This study demonstrated no significant effects of olive leaf extract consumption on oxidative markers when compared with control.

Oxidative Status

There are numerous markers of oxidative status. The biomarkers used in this study were 8-isoPGF$_{2\alpha}$ for the determination of lipid peroxidation, 8-OHdG for the measurement of DNA oxidation, and Folin-Ciocalteu for 'total phenol' quantification. These tests were carried out on urine, and therefore the results represent whole body oxidative status (Hermans et al., 2007). Mean baseline values of 8-isoPGF$_{2\alpha}$, 8-OHdG, and Folin-Ciocalteu total phenols were: 6.4 ± 3.8, 9.5 ± 1.1, and 7.5 ± 5.2 µg/L; 0.09 ± 0.13, 0.07 ± 0.07, and 0.05 ± 0.04 µg/L; and 1.06 ± 0.74, 1.33 ± 0.46, and 1.08 ± 0.65 g/L for control, liquid and capsule groups, respectively. There was no

simple relationship between outliers (Table 4) and dietary intakes or health evaluations. A normal range of 0.0 to 49.0 μg/L has been reported for 8-OHdG (Cutler & Rodriguez, 2003) whereas values of approximately 6 μg/L were found for 8-OHdG in ship engine room personnel (Nilsson et al., 2004). Tomey et al. (Tomey et al., 2007) reported 8-isoPGF$_{2\alpha}$ values for a female cohort of 0.4 μg/L and 0.9 μg/L for non-smokers and smokers, respectively. However, as individuals do not produce the same volume of urine everyday, or at the same time everyday, direct comparison of biomarkers by concentration units expressed as mass per unit volume is problematic. Normalisation to creatinine is used to overcome this limitation. This is based on the assumption that creatinine excretion is relatively constant from day to day as observed in the present study with the exception of six subjects (Figure 1). One draw back to normalisation is the requirement that creatinine, 8-isoPGF$_{2\alpha}$, 8-OHdG, and total phenols must involve the same excretory mechanism (Que Hee, 1993).

Normal values of these oxidation markers have not been established. The baseline values of 8-isoPGF$_{2\alpha}$ collected in the present study were 0.24 ± 0.13, 0.29 ± 0.23 and 0.21 ± 0.13 μg/g creatinine for control, liquid and capsule groups, respectively. Reported mean values in urine of healthy volunteers differ greatly (e.g. from 0.25 to 1.11 μg/g creatinine) (Haschke et al., 2007) while values of 0.81 ± 0.60 μg/g creatinine were found in patients with severe heart failure (Baxter, 2000; Cracowski et al., 2000). The latter study utilised ELISA techniques but the kit brand was different from that used in the present study, and hence comparison between these values are of limited benefit. The baseline values of 8-OHdG collected in the present study were 9.16 ± 2.94, 6.74 ± 1.64 and 6.29 ± 1.77 μg/g creatinine for control, liquid and capsule groups, respectively. Baseline measures for 8-OHdG of 106.7 ± 37.7 μg/g

creatinine and 65.8 ± 24.7 μg/g creatinine have been reported using the same ELISA kit for 8-OHdG as utilised in the present study (Boyle et al., 2000). The variation is possibly because the cohort used in the earlier study consisted of females aged 18-48 years, whereas the cohort used in the present study had an average age of approximately 20 years and comprised both male and female participants. 8-OHdG levels have been shown to be different at baseline between males (29.6 ± 24.5 μg/g creatinine) and females (43.9 ± 42.1 μg/g creatinine) (Wu, Chiou, Chang & Wu, 2004).

The variability in results emphasises the need to establish acceptable baseline levels of oxidation markers for commonly utilised methods. As limited data exist for a young, healthy Australian population, our values contribute significantly to the data set of normal baseline levels of oxidation. Results obtained from different biomarker methods are not equivalent (Peoples & Karnes, 2005; Proudfoot et al., 1999; Shimoi, Kasai, Yokota, Toyokuni & Kinae, 2002). Current thinking is that HPLC methodology to analyse 8-OHdG overestimates oxidative stress, but that enzymic methodology underestimates it (Moller & Loft, 2006). However, various authors (Hu et al., 2004; Shimoi et al., 2002) found that HPLC and ELISA methods were well correlated, but the ELISA results were two-fold higher than the HPLC results. The situation for isoprostane measurements is similar and the accuracy of ELISA kits for 8-isoPGF$_{2\alpha}$ quantification has been questioned (Halliwell & Whiteman, 2004; Hwang & Kim, 2007; Hwang & Kim, 2007; Proudfoot et al., 1999). GC-MS is possibly a more accurate measure of 8-isoPGF$_{2\alpha}$, and while Proudfoot et al. (1999) warned against comparing results obtained from GC-MS and ELISA methods since the results yielded are not equivalent, the two methods showed significant correlation. The

interested reader is referred to papers elsewhere that examine the reasons for the different measurements between methods (Callewaert, Mcgowen, Godshalk & Gupta, 2006; Li et al., 1999; Sasaki et al., ; Sircar & Subbaiah, 2007; Wood, Gibson & Garg, 2006). In the present study, ELISA was used as a comparative tool and various studies (Shimoi et al., 2002) have concluded that this is valid in studies involving comparison of baseline and follow-up data where precision is the goal rather than accuracy.

The antioxidant status of the urine of participants was also determined by using the generalised Folin-Ciocalteu procedure. The baseline values of Folin-Ciocalteu total phenols collected in the present study were 424.9 ± 121.4, 645.1 ± 655.1, and 476.1 ± 238.9 mgGAE/g creatinine for the control, liquid and capsule groups, respectively. Whilst values reported from this method are often reported as total phenols the values are more accurately termed the total reducing capacity of the urine samples (Huang, Ou & Prior, 2005).

Baseline oxidative status biomarkers for the study population showed no correlation to responses from the mini-FFQ. This indicates that baseline dietary intake was not a confounder for the oxidation markers, and reduces the possibility of dietary confounding. The use of simple and complex statistical analyses found that there was not a significant increase or decrease in any of the markers for oxidative status with olive leaf extract supplementation between the groups from baseline to follow-up; that is, supplementation or supplement dose form, did not change oxidative stress measures from baseline (Figures 2-4). Reasons for the lack of change could be that the biomarkers used were not the best markers of oxidative status in this cohort. Alternatively, individuals may have responded more or less favourably to the

antioxidant dosing regime, indicating an individual variation effect with olive leaf extract supplementation. It must be noted that the standard deviations for these biomarkers were relatively large, indicating a highly individual response to the treatments in all arms. This suggests the possibility of methodological errors with the assays although this is not supported by the precision of the assays (coefficient of variation for samples and standards were less than 10%).

Another possibility for the lack of change in oxidative status is that the investigated cohort consisted of young, healthy individuals. Therefore, the participant's baseline level of oxidative damage may have been such that additional protection, imparted from olive leaf extract supplementation, would be of little clinical significance (Hercberg, Czernichow & Galan, 2006). One study has demonstrated that response to dietary antioxidants was not uniform but rather was conditional based on a participant's oxidation status at the time of commencing the study (Thompson et al., 2006). In our study, all participants in the treatment arms with a baseline 8-isoPGF$_{2\alpha}$ above 0.3 µg/g creatinine showed a reduction in marker level at follow-up. Participants with baseline levels below this value showed a variable response to supplementation. Does this reflect a pro-oxidant/antioxidant conflict in cases where oxidative status is not compromised?

Other reasons for a lack of change in the measured biomarkers include the inability of olive leaf extract supplements to affect the physiological stress in the body. However, there are many reasons why this pessimistic view may be challenged. This was a healthy and young population that may have well regulated endogenous antioxidant systems, to which the addition of an exogenous antioxidant may be of little assistance.

Therefore, one cannot rule out that in a diseased, or immune compromised cohort, olive leaf extract supplement antioxidants could be beneficial. Another explanation is that of a poorly designed urine collection protocol. It is reported that excretion rates of biophenol metabolites are greatest at 2 hours (Scalbert & Williamson, 2000). It could be that any antioxidant effect occurs within this initial 2 hour period after supplementation, and therefore a change in the markers would not have been seen unless urine samples were examined within this time frame. In the current study, participants were not supplemented with an acute dose; rather supplementation lasted for 28 days. Levels of the olive leaf extract biophenols may have accumulated in the body and reached a steady state level over the experimental period. Hence, this proposed 2 hour excretion time may not hold true for chronic supplementation. No data were identified to explain this possibility, and therefore is an area that requires further investigation.

Another potential confounder for this study is that of varied compliance. Of particular interest are the results between the two dosage forms. No significant differences were seen between the capsule and liquid groups for the selected biomarkers, even though compliance was much greater for the capsule group. It may be that the capsule dosage form was less bioavailable or that this supplement was physiologically less active as presented in this form. As compliance was poorer for the liquid supplement, yet showed the same results as the capsule, the reverse may be said: the liquid supplement could be more bioavailable and physiologically active. If this liquid olive leaf extract supplement was taken at the recommended dosage, a change in oxidative status may have been observed. Nevertheless the dosage used was that recommended by the

manufacturers. It could be that the dosage was not high enough for an appreciable level of change to occur.

The interpretation of changes in biomarker levels is also crucial in the analysis of results. For example, an increase in 8-OHdG following antioxidant supplementation is presently interpreted as an increase in oxidative stress. However, it could be that the antioxidant is in fact stimulating repair of the DNA, in reality decreasing oxidative stress, and therefore more 8-OHdG is excreted and observed in the urine. The reverse is also true: a decrease in urinary 8-OHdG might indicate a decrease in antioxidant defence, and therefore an increase in oxidative stress. This could be interpreted as a decrease in oxidative stress, when the reverse is true (Halliwell, 2000).

Biophenols may exert a local antioxidant action in the gastrointestinal tract (Covas et al., 2006). The actual mechanism of antioxidant action is pH dependent and thus may differ between parts of the body (Lemanska et al., 2001). Indeed, mistaking correlation for causation is common (Halliwell, 1999) and it is possible that the beneficial effects of biophenols may involve mechanisms outside antioxidant action. Any health benefits and antioxidant action could be correlated with the real protective effect and the need to look beyond the antioxidant action of biophenols for their bioactivity has been highlighted (Nurmi et al., 2006; Yang, Kong & Zhang, 2007).

Method related issues
Confounders and compliance to experimental protocol are major hurdles in nutritional studies and this trial was no exception. Confounders were carefully documented and

controlled during analysis to prevent compromising the results, as was compliance carefully documented.

Study participants were screened and found to be free of all major diseases, food intolerances and pregnancy. Participants were all over the age of 17 years and age did not pose a significant difference in the participants' characteristics between the groups. Weight and body mass index were found to be significant between the groups (Table 1). This was due to one obese individual, and therefore is unlikely to compromise the study results. All study participants reported as non-smokers.

Study participants responded positively to the Health Evaluation completed at baseline and were therefore deemed satisfied with their health. Responses to stimuli regarding general energy and health, as measured on a Likert scale, were found not to be significantly different between the groups either at baseline or follow-up.

During a supplemental protocol, monitoring of food intake during the study period is essential to ensure that any effects, or changes from baseline observed, are in fact caused by the supplement and not from a change in dietary intake (Kendall, Batterham, Prenzler, Robards & Ryan., 2008). As such, three day food diaries were used at baseline and follow-up to assess the subject's average nutrient consumption. These were used to compare intakes between subjects within a group, and also to compare intakes between groups over the study period. While dietary collection techniques do suffer from factors such as seasonal variation and participant bias (Bingham, 1991; Bingham et al., 1994; Blake, Guthrie & Smiciklaswright, 1989; Callewaert et al., 2006; Haschke et al., 2007; Ma et al., 2006; Macdiarmid &

Blundell, 1997; Rebro, Patterson, Kristal & Cheney, 1998; Sasaki et al., ; Sircar & Subbaiah, 2007; Wood et al., 2006), the three day food diary was considered the most appropriate tool due to its ability to assess intake over a number of days, allow specification of amount consumed and food preparation technique, as well as documentation of weekend eating patterns. The mini food frequency questionnaire (Supplement) that was administered at baseline, supplemented the three day food diary by allowing participants to report their usual intake of foods and beverages known to contain high levels of phenolic compounds.

Analysis of the three day food diaries revealed a statistically significant time effect for carbohydrate consumption over the study period (Table 2); however, no other noteworthy differences in intake were found. The change in carbohydrate consumption was approximately 30 g which equates to the amount of carbohydrate in a bread roll and is unlikely to be of clinical significance. Additionally, the result should be viewed in the context of the raw data undergoing multiple statistical tests during analysis, leading to a higher risk of a type 1 error. These results indicate that study participants consumed a reasonably similar diet over the study period; as many of the subjects were in their first year of university, and lived in catered on-campus accommodation, similar diets may be expected. Also, subjects were close in age and therefore were likely to recall eating habits socially acceptable for this age group (Scagliusi, Polacow, Artioli, Benatti & Lancha, 2003).

Compliance to the supplemental protocol was monitored by both subjective participant recall and by counting the number of doses consumed. Participant compliance to the supplementation protocol over the course of 28 days varied. Those

participants randomised to the capsule group showed a very high compliance at 97% indicating that participants closely adhered to the supplementation of one capsule three times per day. With a protocol of 5 mL three times per day, the liquid supplement and control groups however were not as compliant at 79% and 70%, respectively. This may be due to the characteristics of the individuals randomised to these protocols, though many participants reported the taste and texture (or mouth-feel) of the liquid supplement and placebo as challenging. In fact, a number of participants verbally reported that they would have preferred to be randomised to the capsule supplement, and believed they would have been more compliant taking a capsule supplement. As this trial did not employ a cross-over design, it cannot be known if a higher compliance to the capsule, and a lower adherence to the liquid/control supplements, is universally the case, or just applicable to this population (Peat, Mellis, Williams, & Xuan, 2001).

Another reason why compliance in the liquid and control groups was comparatively lower could have been related to the identification of the placebo. While care was taken to ensure the control and liquid supplements had a similar appearance, texture and taste, the study participants reported a difference between the two treatments. As the cohort enrolled in this study were a class of university students, and a 'tight knit' group, talking about the supplements was inevitable. While this may have been an advantage in increasing compliance due to a peer pressure effect, it may also have been a disadvantage in the discovery of the placebo and therefore a poorer compliance due to lesser perceived gain experienced by the control group. Therefore, in the future, more careful consideration of cohorts is necessary.

Acknowledgements

Table 1: Baseline characteristics of participants (n=36)

Parameter	Normal range	Mean ± SD of group	Control (n= 10)	Capsule (n= 14)	Liquid (n= 12)	P value, 3 groups (ANOVA)
Age (years)		20.2± 3.0	19.7 ± 2.1	19.4± 1.7	21.6± 4.4	0.159
Weight (kg)		70.5 ± 16.8	62.3± 8.8	80.1± 20.4	66.2± 11.7	0.015
BMI (kg/m²)	20-25	23.2± 4.4	21.2± 2.6	25.8± 5.5	21.9± 2.5	0.015*

* BMI of the capsule group was skewed due to one individual (BMI: 41.0 kg/m²)

Table 2: Mean Dietary Intake for Subjects (n=36) at baseline and follow-up

		NRV*	Control (n=10)	Capsule (n=14)	Liquid (n=12)
Baseline	Energy (kJ)		10373±2818	11589±2584	11902±3060
	CHO^ (g)		306±72	326±105	352±92
	Fat (g)		89±34	98±25	96±27
	Protein (g)	45-65 g/day	91±36	100±24	95±31
	Alcohol (g)		10±12	23±28	24±37
	Dietary fibre (g)	22-30 g/day	21±7	22±7	24±8
Follow-up	Energy (kJ)		9956±2662	10620±3683	11151±4040
	CHO (g)		270±76	305±126	324±117
	Fat (g)		88±28	84±36	85±25
	Protein (g)	45-65 g/day	95±31	90±36	93±27
	Alcohol (g)		11±16	26±28	30±43
	Dietary fibre (g)	22-30 g/day	38±57	21±9	22±7

* NRV: Nutrient Reference Value

^ CHO: Carbohydrate

Table 3: Repeated measures analysis of variance using Wilks Lambda

Measure	Change control	Change liquid	Change tablet	Time (P)	Group (P)	Time x group interaction (P)
8-isoPGF$_{2\alpha}$ creatinine	-0.021±0.192	0.074±0.324	0.010±0.174	0.605	0.049	0.632
8-OHdG	-2.29±1.88	2.95±8.49	0.85±2.51	0.688	0.744	0.249
Folin-Ciocalteu	-80±218	-216±610	-10±302	0.153	0.390	0.457

Table 4: Subject Outliers

| Test	Subject number
8-isoPGF$_{2\alpha}$ Baseline	36
8-isoPGF$_{2\alpha}$ Follow-up	13, 25
8-OHdG Baseline	1, 3, 5, 30
8-OHdG Follow-up	29
Folin-Ciocalteu Baseline	17, 29, 32
Folin-Ciocalteu Follow-up	12

Figure 1: Urinary creatinine for subjects (n= 36) at baseline and follow-up (CV: 1-7%). Participants were assigned to treatments as placebo (Subjects 1-10), capsule (Subjects 11-24) and liquid (subjects 25-36)
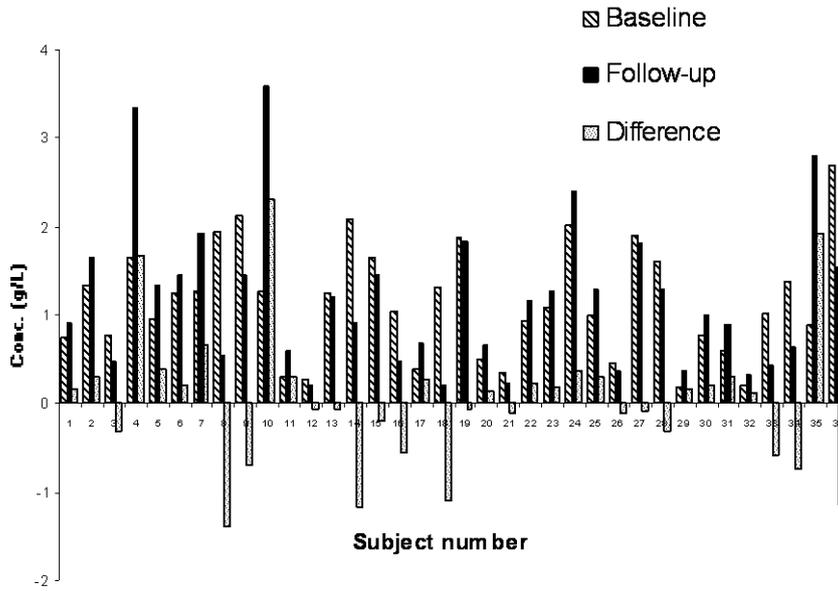
Figure 2: Urinary 8-isoPGF$_{2\alpha}$ normalised to creatinine for subjects (n= 36) at baseline and follow-up. Participants were assigned to treatments as placebo (Subjects 1-10), capsule (Subjects 11-24) and liquid (subjects 25-36)
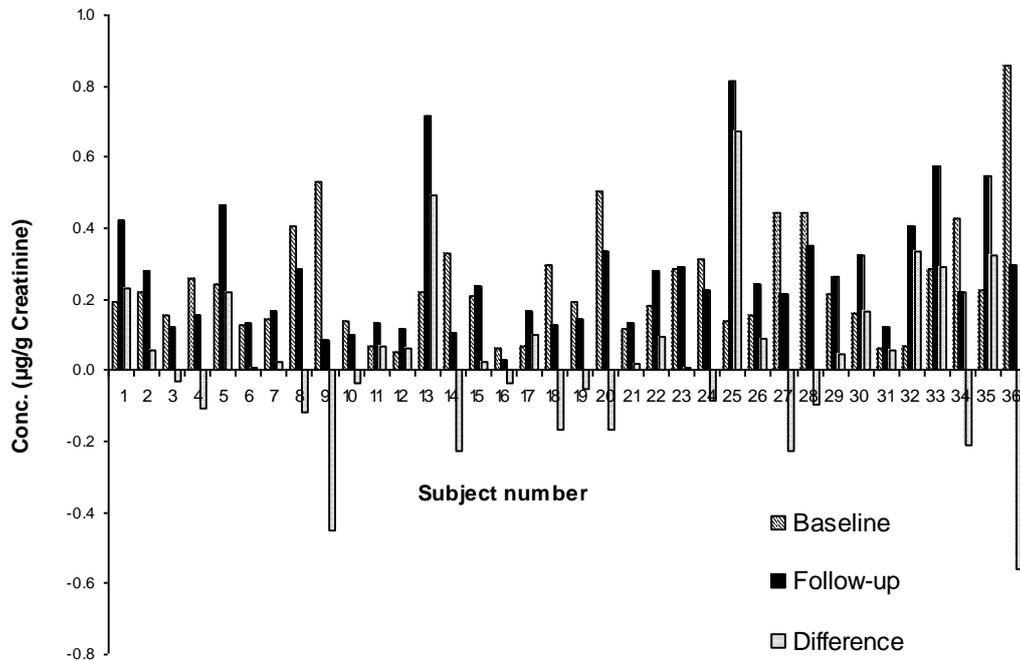
Figure 3: Urinary 8-OHdG normalised to creatinine for subjects (n= 18) at baseline and follow-up. Participants were assigned to treatments as placebo (Subjects 1-6), capsule (Subjects 11-16) and liquid (subjects 25-30)
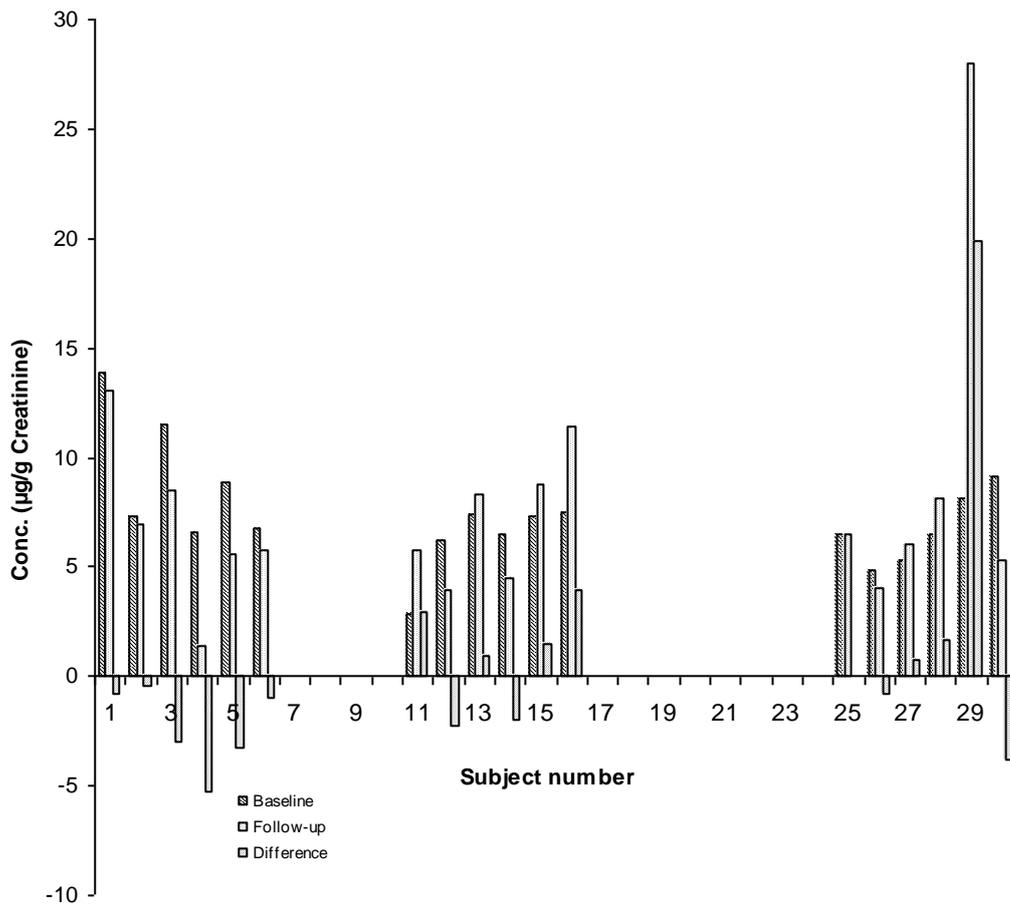
Figure 4: Urinary Folin-Ciocalteu normalised to creatinine for subjects (n= 36) at baseline and follow-up. Participants were assigned to treatments as placebo (Subjects 1-10), capsule (Subjects 11-24) and liquid (subjects 25-36)
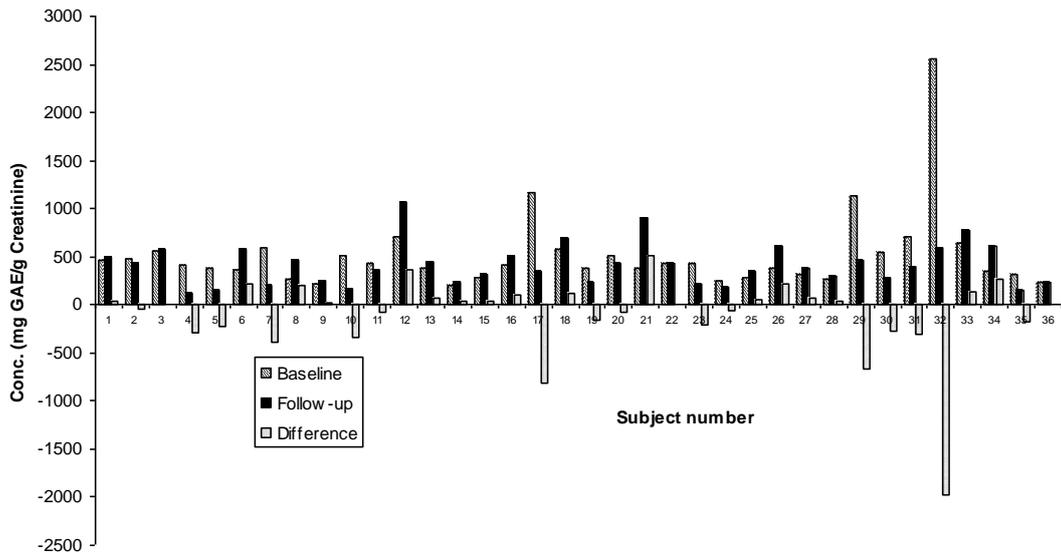
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