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Bioavailability and efficiency of rutin as an antioxidant: a human supplementation study

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Objective: To determine the potential antioxidant effect of rutin (quercetin-3-*O*-*β*-rutinoside) supplementation.
Design: A 6-week randomized single-blind placebo controlled trial was conducted; 500 mg rutin supplement was compared to an equivalent amount of glucose placebo. In addition, a pharmacokinetic study was carried out.
Setting: The Rowett Research Institute, Aberdeen, UK.
Subjects: Eighteen healthy non-obese normocholesterolaemic female volunteers in the age range 18–48 y.
Main outcome measures: Plasma flavonoids, ascorbic acid, tocopherols and carotenoids, plasma antioxidant capacity, lymphocyte DNA damage, blood chemistry and haematology, liver function tests, urinary malondialdehyde, 8-hydroxy-2′-deoxyguanosine and 8-iso-prostaglandin $F_{2\alpha}$.
Results: Eighteen volunteers completed the trial. Rutin supplementation did not induce any adverse changes in blood chemistry or indices of liver function. Plasma flavonoids were significantly elevated in the rutin-supplemented group. Endogenous oxidation of pyrimidines was significantly decreased in both rutin- and placebo-treated volunteers. There was no significant change in the level of urinary 8-hydroxy-2′-deoxyguanosine or urinary malondialdehyde in either group. A linear correlation was observed between urinary malondialdehyde and urinary 8-iso-prostaglandin $F_{2\alpha}$ ($R = 0.54$, $P < 0.01$).
Conclusion: Six weeks' rutin supplementation significantly elevated the levels of three plasma flavonoids (quercetin, kaempferol and isorhamnetin) but there was no significant change in plasma antioxidant status. The decrease in the level of endogenous base oxidation in lymphocyte DNA seen in both the placebo- and rutin-supplemented subjects may reflect seasonal changes in other dietary antioxidants.
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Descriptors: flavonoids; bioavailability; plasma antioxidant status; DNA damage; 8-iso-prostaglandin $F_{2\alpha}$
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Introduction

Flavonoids are polyphenolic compounds that occur in most foods of plant origin (Kuhnau, 1976) and are significant though minor constituents of the human diet (Singleton, 1981). The basic structure of flavonoids is 2-phenyl-benzo-γ-pyran and over 4000 different types of compounds have been described (Markham, 1989). Flavonols and flavones usually occur in foods as *O*-glycosides with D-glucose being the most common attached sugar (Herrmann, 1976, 1988). Estimates of the average daily levels of flavonoids in the human diet range from 23 mg/day to 1 g/day (Hertog *et al*, 1993a; Singleton, 1981). Flavonoids are defined as non-nutritive dietary components. Nonetheless there is increasing interest in their potential beneficial health effects.

Flavonoids modulate a number of biological functions such as anti-inflammatory and anti-microbial activities (Middleton & Kandaswami, 1992). Their ability to scavenge free radicals (Robak & Gryglewski, 1988; Sanchez-Moreno *et al*, 1998) and chelate metal ions (Afanas'ev *et al*, 1989) has led to an examination of their activities as antioxidants in *in vitro* systems of lipid peroxidation. This antioxidant activity is related to chemical structure, partition coefficients and rate of reaction with the radicals of interest (van Acker *et al*, 1996; Rice-Evans *et al*, 1996).

In epidemiological studies there is an inverse association between intakes of flavonols and flavones and occurrence of coronary heart disease (Hertog *et al*, 1993b, 1995). The consumption of red wine has also been associated with a reduced coronary heart disease mortality in a population with a relatively high dietary intake of saturated fats (Renaud & Lorgeril, 1992). Thus it has been postulated that the antioxidant properties of the phenolic compounds in red wine may retard atherogenesis. Additionally phenolic compounds in red wine inhibit the *in vitro* oxidation of human low-density lipoprotein (LDL) (Frankel *et al*, 1993, 1995). Whilst there have been numerous reports of the protective effects of polyphenols in altering LDL susceptibility to oxidation *in vitro* (DeWhalley *et al*, 1990; Negre-

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Contributors: ARC was the grant holder and responsible for all stages of the study. All investigators were involved in study design. SPB was the principal investigator and responsible for method development, data collection, laboratory and statistical anlysis and drafting the manuscript. ARC, SJD and SPB were involved in subsequent revision of the manuscript. VLD and SJD were responsible for comet analysis and interpretation respectively. DCH and JAMK were responsible for sample analysis and volunteer management.
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Salvayre & Salvayre, 1992), there have been few reports of a similar *in vivo* activity. The consumption of red wine with meals reduces the propensity of human plasma and LDL to undergo lipid peroxidation (Fuhrman *et al*, 1995). The report of increased resistance of human LDL to *ex vivo* oxidation following 2 weeks supplementation with the flavonoid glycoside, glabridin, suggests that dietary supplementation with flavonoids may offer protection against the onset of atherosclerosis (Fuhrman *et al*, 1997). Further investigations of this type are essential to examine the absorption, metabolism and antioxidant efficiency of flavonoids in human subjects.

The *in vitro* antioxidant activity of flavonoids suggests that they may offer some protection against cancer, but experimental evidence is equivocal. Several polyphenols inhibit cancer development in animal models (Taj & Nagarajam, 1996; Katiyar *et al* 1997) while a quercetin dose-dependent enhancement of tumour development in an animal model for colon carcinogenesis has been reported (Pereira *et al*, 1996). In epidemiological studies there is an inverse correlation between flavonoid intake and incidence of all sites of cancer (Knekt *et al*, 1997) and of stomach cancer (Dorant *et al*, 1996).

The complex and tissue-specific nature of carcinogenesis, however, indicates that further investigations into the role of flavonoids in this process are required. This role will depend not only on their bioavailability but also on their metabolism and tissue distribution within the subject.

To elucidate the mechanism of action of flavonoids in humans, we have carried out *in vivo* investigations—a pharmacokinetic study with a single dose of quercetin-3-*O*-β-rutinoside (a commercially available supplement) and a longer term supplementation trial. Indicators of oxidative stress including urinary 8-iso-prostaglandin $F_{2\alpha}$ formation, malondialdehyde (MDA), DNA strand breaks and oxidized bases, total plasma antioxidant capacity and susceptibility of biomolecules to oxidation were examined for influence of supplementation on antioxidant or prooxidant activity.

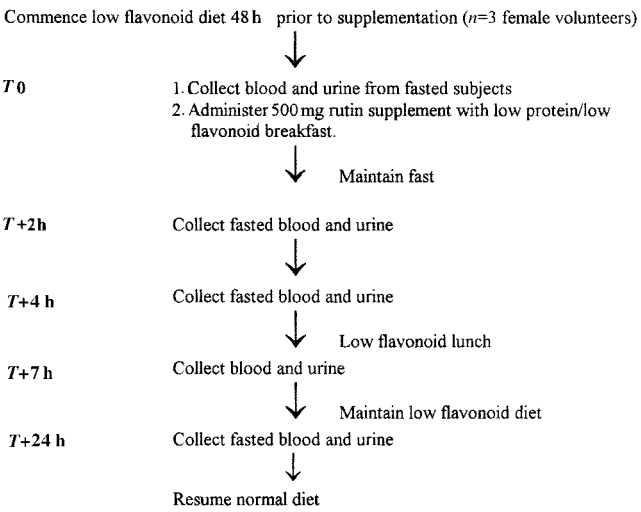
Subjects and methods

Subjects
Female volunteers were recruited (age range 18–48 y). All were considered healthy on the basis of routine haematological and biochemical measurements on blood and urine. Ethical approval for the protocol was obtained from the Joint Ethical Committee of Grampian Health Board and the University of Aberdeen and details of the study were fully explained to the subjects who gave informed consent.

Low flavonoid diet
Volunteers were given a list of fruits and vegetables containing more than 15 mg quercetin/kg and of beverages with more than 4 mg quercetin/L which they avoided during the 48 h prior to rutin ingestion. To avoid any binding of the flavonoid to protein, the breakfasts were low in protein, consisting of protein-free crisp bread, jams made from quercetin-free fruits, coffee without milk, and water.

Rutin pharmacokinetic study
Following a 48 h washout on a low flavonoid diet, three subjects were given a 500 mg rutin tablet with a low protein/low flavonoid breakfast. Blood and urine were collected at intervals over the 24 h post-supplementation period as indicated in the protocol.

Protocol



Study design of 6 week supplementation trial
Age-matched female subjects (range 18–48 y) were allocated to receive either rutin supplement or placebo (eight in each group). Early morning blood and urine samples were collected from fasted volunteers at the start of the trial and then weekly for 6 weeks. Placebo-treated subjects ingested one *VEGICAP* containing glucose monohydrate (300 mg) on a daily basis with their breakfast whilst supplement-treated subjects ingested 500 mg rutin. Volunteers were instructed to follow their normal diet and record details of their intake of 'flavonoid-rich' foods in diaries provided.

Collection of samples
Venous blood samples (40 ml) were collected into vacuum tubes containing EDTA. Samples were immediately taken for assay of DNA damage. Plasma was subsequently separated by centrifugation for 15 min at 3500 *g* at 4°C, and aliquots snap-frozen in liquid N_2 and stored at −80°C. Plasma for ascorbic acid determination was mixed with an equal volume of 10% w/v metaphosphoric acid before storage at −80°C. Urine samples were collected in 25 ml plastic universals and aliquots for malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8OHdG) stabilized with butylated hydroxytoluene (3.2 mM final concentration) or HCl (40 μM final concentration) respectively before storage at −80°C.

Statistical analysis
Paired *t*-tests were carried out to test for differences in plasma flavonoid concentrations and markers of oxidative stress between week 0 and week 6 for each group (supplement and placebo) and between the two groups at week 6. Plasma tocopherol and carotenoid levels were analysed by analysis of variance (ANOVA) with subject treatment and time as blocking factors and treatment and time as factors.

Materials
Chemicals. Rutin tablets (500 mg) and *VEGICAPS* were purchased from Solgar Vitamins Ltd, Herts, UK. Glucose monohydrate conforming to *European Pharmacopoeia* (1997) and *British Pharamacopoeia* (1993) was obtained

from Roquette UK Ltd., Tunbridge Wells, UK. Apigenin, isorhamnetin, luteolin, quercitrin, quercetin and quercetin-3-glucoside were purchased from Apin Chemicals Ltd, Oxon, UK. European pharmacopoeial ascorbic acid, United States pharmacopeial ascorbic acid and fat-soluble-vitamins (National Institute of Standards and Technology) were purchased from Promochem Ltd, Welwyn Garden City, UK. Echinone was kindly donated by F Hoffman La Roche AG, Basle, Switzerland. Genesis C_{18}. 4 μm (150 × 4.6 mm i.d.) and Nucleosil ODS 5 μm (250 × 4.6 mm i.d.) columns were purchased from Jones Chromatography, Mid Glamorgan, UK. 5 ODS2 (C_{18}) columns (250 × 4.6 mm i.d.) were from Hichrom Ltd, Reading, Berks, UK. Quick-Seal centrifuge tubes and Ultrasphere 5 ODS (250 × 4.6 mm i.d.) were purchased from Beckman Instruments Inc, Spinco Division, CA, USA and Vectaspin units (0.2 μm) from Whatman International Ltd, Maidstone, Kent, UK. All other chemicals were supplied by Sigma Chemical Co., Poole, Dorset, UK and were >95% pure.

Analytical methods

A reverse-phase HPLC technique using combined UV and fluorimetric detection was employed for flavonoid determination in plasma (Hollman *et al*, 1996a). Acid hydrolysis of rutin (to yield quercetin) facilitated post-column derivatization with aluminium nitrate enabling fluorimetric detection which afforded significantly enhanced assay sensititivity (Hollman *et al*, 1996a). Acid hydrolysis of plasma was performed in 50% methanol containing 20 mmol/l diethyldithiocarbamate and 1.2 M HCl (Aziz *et al*, 1998). Plasma ascorbic acid levels in metaphosphoric acid-treated samples were determined by reverse-phase HPLC with UV detection at 263 nm (Ross, 1994) Determination of plasma retinol, α-tocopherol, γ-tocopherol and six carotenoids was by reverse-phase HPLC with simultaneous UV and fluorimetric detection (Hess *et al*, 1991). Extraction efficiency was determined by use of an echinone internal standard.

Plasma Antioxidant Capacity

The total phenolic content of urine and plasma was determined spectrophotometrically by a modified Folin Ciocalteu assay (Serafini *et al*, 1998). The total antioxidant activity of plasma was quantified by the ferric reducing ability of plasma (FRAP) assay (Benzie and Strain, 1996). Oxidized glutathione (GSSG) was quantified by measuring the total glutathione present after the derivatization of reduced glutathione (GSH) with 2-vinyl pyridine (Allen & Arthur, 1987).

Malondialdehyde determination

The malondialdehyde (MDA) content of urine and plasma was determined by resolution of the TBA-MDA adduct using isocratic reverse-phase HPLC with fluorimetric detection. A standard curve of malondialdehyde was prepared using 1, 1, 3, 3-tetraethoxypropane (Yagi, 1987).

Spectrophotometric determinations

Plasma cholesterol and high density lipoprotein (HDL) were determined spectrophotometrically at 510 nm (Allain *et al*, 1974). Urinary creatinine was reacted with picrate under alkaline conditions to form a chromogen and the absorbance at 510 nm measured (Larsen, 1972). Whole blood haemoglobin was determined spectrophotometrically at 540 nm with reference to a standard from Sigma Chemical Co., Poole, Dorset.

Liver function tests were carried out under contract by the Department of Clinical Biochemistry, Aberdeen Royal Infirmary, Foresterhill, Aberdeen. A 5 ml aliquot of whole blood was collected into plain tubes at week 0 and week 6 from rutin-treated volunteers. The tubes were transferred on ice and the levels of alkaline phosphatase, lactate dehydrogenase, γ-glutamyl transferase and alanine aspartame transaminase determined using standard enzymatic determinations.

Erythrocyte catalase activity was determined spectrophotometrically (Aebi, 1985).

Analysis of DNA damage (comet assay)

Endogenous oxidative DNA damage was measured in lymphocytes using a modified comet assay (alkaline single cell gel electrophoresis), which estimates both strand breakage and oxidized pyrimidines (Collins *et al*, 1993). In brief, cells were embedded in a thin layer of agarose on a microscope slide, lysed for 1 h in 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris−HCl, pH 10, 1% Triton X-100, washed with 0.1 M KCl, 0.5 M Na_2EDTA, 40 mM HEPES-KOH, 0.2 mg/ml bovine serum albumin, pH 8, and incubated with endonuclease III in this buffer at 37°C to convert oxidized bases to strand breaks. Control slides were incubated with buffer but no enzyme. Resistance of lymphocyte DNA to oxidative damage was also determined using the comet assay to measure DNA strand breaks induced by exposure to hydrogen peroxide (H_2O_2, 50 and 200 μM) *ex vivo* (Duthie *et al*, 1996). Comet scoring was by visual classification using a method which has been validated by comparison with selected comets using computerized image analysis (Komet 3.0, Kinetic Imaging Ltd, Liverpool, UK) (Collins *et al*, 1997).

Quantitation of urinary 8-iso prostaglandin $F_{2\alpha}$

Urinary 8-iso prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) levels were determined by a competitive *in vitro* enzyme-linked immunoassay kit supplied by Assay Designs Inc., MI, USA.

Quantitation of urinary 11-dehydro-thromboxane (TX)B_2

Urinary thromboxane B_2 (TXB_2), a non-invasive index of *in vivo* platelet activation, was determined by a kit purchased from Assay Designs Inc., MI, USA.

Quantitation of urinary 8-hydroxy-2′-deoxyguanosine

A competitive *in vitro* enzyme-linked immunoassay kit produced by the Japan Institute for The Control of Aging, Fukori City, Japan was used for the quantitation of urinary 8-hydroxy-2′-deoxyguanosine (8OHdG). Urinary determinations were undertaken in morning void samples which had been stabilized with HCl (40 μM final concentration) before storage at −40°C. Data from within the laboratory (unpublished results) suggests a linear relationship ($r = 0.87$; $P = 0.001$) between the levels of urinary 8-hydroxy-2′-deoxyguanosine in 24 h collection and morning void urine samples.

Results

This work reports the pharmacokinetics of a single dose of the flavonoid glycoside, quercetin-3-O-β-rutinoside (rutin),

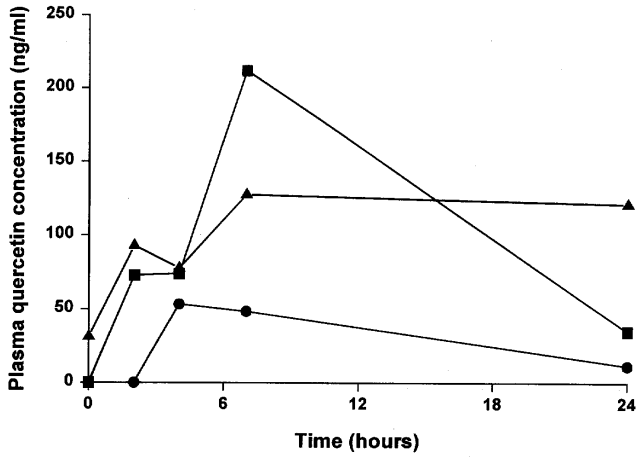


Figure 1 Variations in plasma quercetin level in three volunteers following a single dose of 500 mg rutin. Quercetin was measured after acid hydrolysis of plasma. Data are the means of duplicate determinations.

in female volunteers and examines the effect of longer term supplementation in a 6 week placebo-controlled trial.

Pharmacokinetics of rutin absorption

Plasma flavonoids were determined following acid hydrolysis. Following supplementation with rutin, the principal flavonoid found in all three volunteers' plasma was quercetin with trace amounts of kaempferol and isorhamnetin also apparent in some samples. Subjects show differing kinetics of uptake and clearance with two subjects having a maximal plasma concentration of quercetin at 7 h and the third subject achieving a maximal plasma concentration at 4 h (Figure 1). There was inter-individual variation in the extent of absorption (range 40–220 ng/ml) and the rate of clearance of this single dose was also variable between subjects (Figure 1). No effect was seen on the level of endogenous DNA damage (strand breaks or oxidized bases) or resistance to oxidative damage (H_2O_2 treatment) in lymphocytes (Figure 2) and there was no significant change in either the plasma antioxidant capacity or excretion of urinary MDA (data not shown). The slow clearance of the dose in some individuals suggested that repeated dosing may lead to an accumulation of quercetin in some individuals. It is also possible that the response to supplements might change with time. Therefore a 6 week placebo-controlled trial was undertaken.

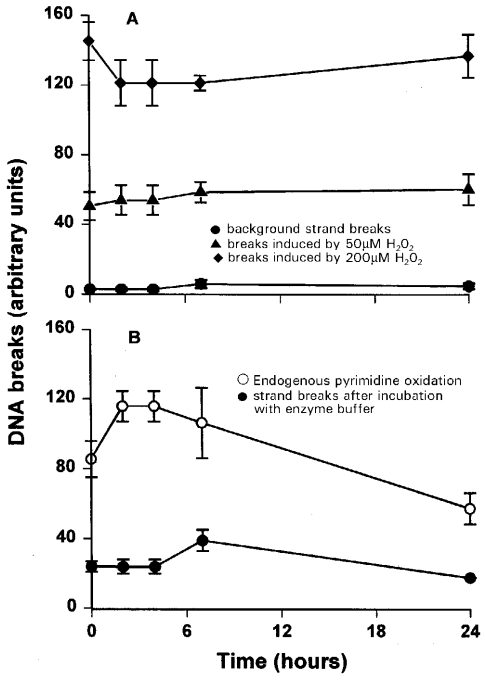


Figure 2 DNA damage (measured with the comet assay) in lymphocytes after a single dose of 500 mg of rutin. (A) Background strand breaks and breaks induced by treatment of cells with H_2O_2, at 50 μM or 200 μM. (B) Endogenous pyrimidine oxidation detected by incubating lymphocyte DNA (without H_2O_2 treatment) with endonuclease III before alkaline electrophoresis. (The strand breaks detected after incubation with enzyme buffer alone are also shown; it is normal for the incubation to cause some increase in 'background' breakage compared with the unincubated counterpart in A.)

Table 1 Variation in plasma antioxidant content (μg/ml)

	Rutin group		*Placebo group*	
Parameter	*Week 0*	*Week 6*	*Week 0*	*Week 6*
Vitamin C	84.7 ± 18.1	91.4 ± 9.8	77.6 ± 17.2	74.8 ± 15.0
Retinol	0.63 ± 0.15	0.58 ± 0.09*	0.67 ± 0.12	0.58 ± 0.14*
γ-tocopherol	0.74 ± 0.41	0.63 ± 0.11	0.79 ± 0.35	0.89 ± 0.33
α-tocopherol	9.26 ± 1.37	10.6 ± 0.70*	10.41 ± 1.01	10.59 ± 1.27*
Lutein/zeaxanthin	0.2 ± 0.09	0.18 ± 0.07	0.18 ± 0.03	0.18 ± 0.04
β-cryptoxanthin	0.07 ± 0.02	0.11 ± 0.06*	0.07 ± 0.04	0.1 ± 0.06*
Lycopene	0.39 ± 0.14	0.4 ± 0.08	0.4 ± 0.16	0.45 ± 0.17
α-carotene	0.07 ± 0.03	0.1 ± 0.07	0.05 ± 0.03	0.06 ± 0.04
β-carotene	0.26 ± 0.1	0.3 ± 0.16	0.28 ± 0.16	0.37 ± 0.19

Values represent the mean of eight subjects (triplicate determinations for each assay) ± s.d. Statistical significance (*$P < 0.05$) from week 0 to week 6 determined by ANOVA.

Effects of long-term rutin supplementation

Plasma flavonoids. Following 6 weeks supplementation with rutin, significant changes in the plasma levels of quercetin, kaempferol and isorhamnetin were measured in the rutin-treated volunteers. Increases of 2.5 fold ($P < 0.03$) in plasma quercetin (Figure 3), 3-fold ($P < 0.05$) in plasma kaempferol (Figure 3) and 10 fold ($P < 0.02$) in plasma isorhamnetin (Figure 3) occurred at week 6 compared with week 0 samples.

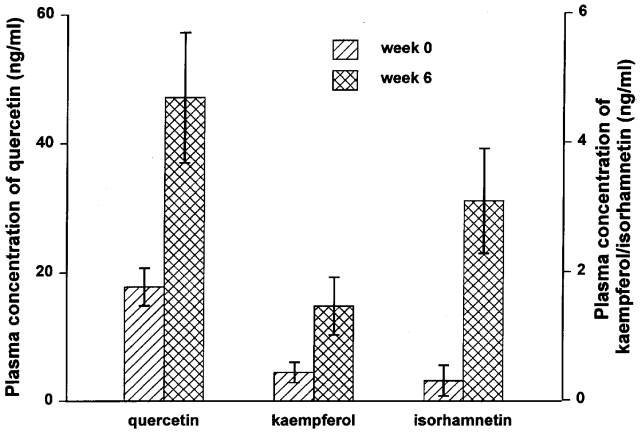


Figure 3 Plasma quercetin, kaempferol and isorhamnetin concentrations before (hatched bars) and after (cross-hatched bars) rutin-supplementation. Fasted blood was collected weekly, with the last sample being taken 24 h after the final supplement. Triplicate analyses were performed on each plasma and the mean concentration determined for each subject at week 0 and week 6. Data show the mean plasma levels in eight volunteers ± s.e.m.

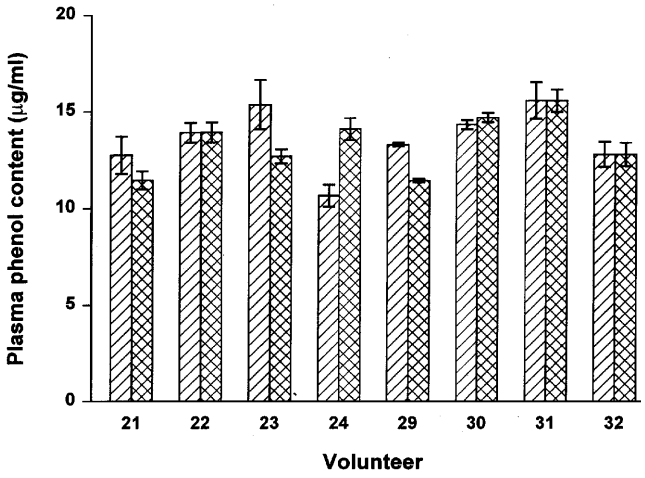


Figure 4 Plasma phenolic content in volunteers receiving rutin. Data are shown for eight volunteers at week 1 (hatched bars) and week 5 (cross-hatched bars) of the trial. The data are the means ± s.e.m. of triplicate determinations.

Plasma antioxidant content. Plasma vitamin C content remained relatively constant throughout the trial and there was no significant change as a result of the rutin treatment (Table 1). There were, however, significant changes in plasma retinol (decreased 11%, $P < 0.05$, $n = 16$), α-tocopherol (increased 8%, $P < 0.05$, $n = 16$) and β-cryptoxanthin (increased 50%, $P < 0.05$, $n = 16$) in both the rutin- and placebo-treated groups.

Plasma antioxidant capacity. Whilst significant changes in the plasma concentration of individual antioxidants were observed, there was no accompanying change in plasma antioxidant capacity as measured by the FRAP assay (data not shown). Plasma MDA concentrations were 1.42 ± 0.35 μmol MDA/litre plasma ($n = 16$ subjects) at week 0 and there was no change following 6 weeks' rutin supplementation, with all values remaining within the range previously reported for normal volunteers (Richard *et al*, 1992).

Plasma total phenolic content. Plasma total phenolic content was determined in rutin-treated and placebo-treated volunteers. Ethical considerations limited the amount of blood which could be taken from volunteers in any one sampling day and so aliquots of week 0 and week 6 plasmas were not available for this analysis. Week 1 and week 5 plasma samples were used. There was no significant difference in the plasma phenolic content of treated and placebo subjects at week 1 (mean plasma phenolic content = $13.8 \mu g/ml \pm 2.0$ for $n = 16$) and this was not significantly altered following rutin supplementation (Figure 4).

Effect of rutin supplementation on liver function. Alkaline phosphatase, lactate dehydrogenase, γ-glutamyl transferase and alanine aspartame transaminase were determined as indicators of liver function in treated volunteers. All values were within the normal range throughout the supplementation period (data not shown).

Effect of rutin supplementation on lymphocyte DNA damage. Six weeks' rutin supplementation had no effect on endogenous DNA strand breakage nor on resistance of lymphocytes to H_2O_2-induced damage (Figure 5). However, a large decrease in the level of endogenously oxidized pyrimidines was observed in both the placebo- and rutin-treated volunteers (Figure 5).

Urinary markers of oxidative stress. There was no treatment effect on the levels of urinary MDA or on the level of urinary 8-iso-$PGF_{2\alpha}$ (Table 2) with concentrations remaining within the normal physiological range of healthy volunteers (Davi *et al*, 1997). However, a significant positive correlation ($P = 0.01$) was found between the urinary excretion of 8-iso-$PGF_{2\alpha}$ and malondialdehyde (Figure 6). There was no significant change in the level of urinary TXB_2 in rutin-treated subjects (data not shown) and no correlation between the urinary excretion of TXB_2 and 8-iso-$PGF_{2\alpha}$ (Figure 7). Levels of urinary 8OHdG were comparable in rutin-treated and placebo-treated volunteers at week 0. No significant change in the level of 8OHdG occurred following the 6 week trial period (Table 2).

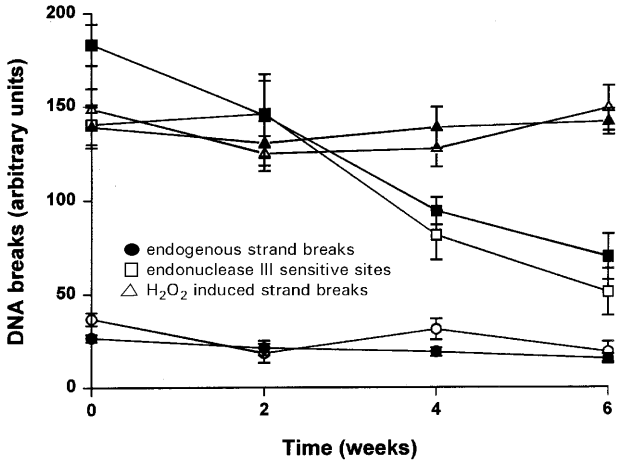


Figure 5 Oxidative DNA damage in lymphocytes from rutin-supplemented (solid symbols) and placebo-treated volunteers (open symbols), mean values ± s.e.m. are shown (duplicate determinations for each treatment).

Table 2 Urinary markers of oxidative stress

	Week 0		*Week 6*	
Parameter	*Placebo-treated*	*Rutin-treated*	*Placebo-treated*	*Rutin-treated*
MDA (nmol/μmol creatine)	0.215 ± 0.038	0.157 ± 0.023	0.224 ± 0.065	0.25 ± 0.043
8-iso-$PGF_{2\alpha}$ (pg/mg creatine)	114.7 ± 28.5	85.3 ± 9.1	160.7 ± 31.7	150.9 ± 23.6
8OHdG (ng/mg creatine)	106.7 ± 37.7	65.8 ± 24.7	235.9 ± 210.8	130.5 ± 46.5

Assays were performed in triplicate and the mean urinary concentration determined for each subject. Data show the mean ± s.e.m. for the placebo- and rutin-treated subjects (eight subjects in each group).

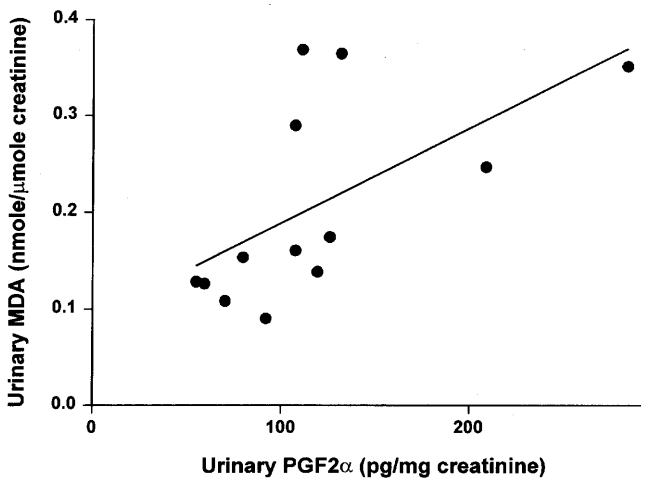


Figure 6 Correlation between urinary 8-iso-$PGF_{2\alpha}$ and urinary MDA in human subjects.

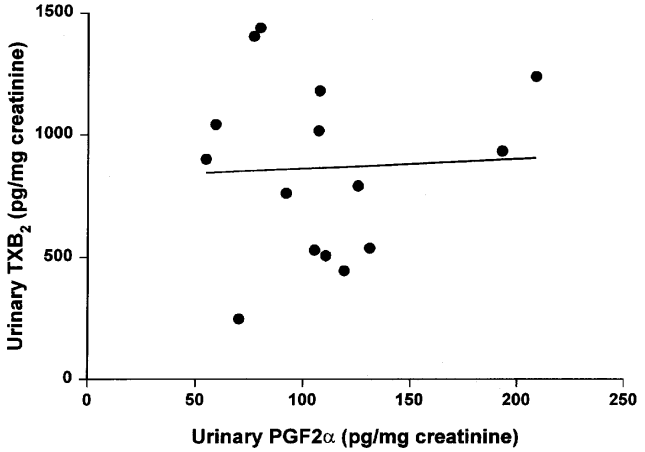


Figure 7 Correlation between urinary TXB_2 and 8-iso-PGF_2 in rutin-treated volunteers.

Erythrocyte enzymes. Erythrocyte catalase activities were not significantly altered during the trial and the ratio of GSSG/GSH in rutin-treated or placebo-treated volunteers remained constant in both groups with no evidence of oxidant stress during the trial period (data not shown).

Discussion

Epidemiological studies have indicated an inverse association between intakes of flavonols and flavones and the occurrence of coronary heart disease (Hertog *et al*, 1993b, 1995). Several studies have provided information on the bioavailability of flavonoids from a food matrix, however they give no indication of the potential antioxidant activity associated with this absorption (Hollman *et al*, 1995, 1996b, 1997a, b; Aziz *et al*, 1998). We have therefore examined the effects of long-term rutin (quercetin-3-O-β-rutinoside) supplementation in human volunteers. For ease of analysis, plasma quercetin was measured in acid-hydrolysed samples as a marker of rutin absorption (Hollman *et al*, 1996c). The effects of glycosylation on quercetin absorption have been studied for quercetin, quercetin glucosides and quercetin-3-rutinoside and uptakes of 24%, 52% and 17% respectively reported (Hollman *et al*, 1995, 1997b). Significantly elevated plasma concentrations of quercetin were found in all three volunteers following dosing with rutin (Figure 1). Inter-individual variation is evident in both the extent of absorption and rate of clearance of the dose with maximal plasma concentrations in the range of 40–220 ng/ml being observed at 7 h in two subjects (Figure 1). The kinetics of quercetin absorption following rutin dosing are similar to those previously reported by Hollman *et al* (1997b). Clearance is not complete in 24 h and so the possibility exists that significant accumulation might occur in the blood after repeated daily supplementation.

Following 6 weeks' supplementation with rutin, significant increases in plasma levels of quercetin (2.5-fold, $P < 0.03$), kaempferol (3-fold, $P < 0.05$) and isorhamnetin (10-fold, $P < 0.02$) were detected relative to week 0 samples. These concentrations were measured on samples taken 16–24 h after the previous day's supplement which is well after the time of maximal concentration resulting from a single dose (Figure 1). The fact that plasma concentrations are still relatively low indicates that little if any accumulation has occurred in the 6 weeks. There was no evidence of a significant change in the level of total dietary phenolics (Figure 4), probably reflecting a lack of specificity of the modified Folin Ciocalteu assay in the determination of flavonoids. Similarly, the relatively large changes in plasma flavonoid content were not accompanied by detectable changes in the antioxidant capacity from volunteers' plasma when assayed by the FRAP assay (data not shown). The lack of an effect using the FRAP assay is not particularly surprising given that approximately 60% of the ferric reducing ability of plasma is attributable to uric acid (Cao & Prior, 1998). These results highlight the need for direct measurement of specific flavonoids rather than estimates of total polyphenolic or total antioxidant activity. Nonetheless in spite of our failure to demonstrate a significant change in *in vitro* plasma antioxidant capacity following rutin supplementation, the possibility that the increased plasma concentration of flavonoids may afford a protective effect by metal chelation still remains. *In vivo* the oxidation of LDL has been shown to be a mediating factor in atherosclerosis development (Aviram, 1983; Palinski *et al*, 1989; Steinberg *et al* 1989) and Fuhrman *et al* (1995) have reported a 20% reduction in the propensity of plasma to undergo oxidation following 2 weeks of supplementation with 400 ml red wine/day. Recently McAnlis *et al*, (1999) have shown that, although dietary flavonoids are extensively absorbed, they do not accumulate within the LDL but are tightly bound to plasma proteins located predominantly within the HDL fraction. In this state, no significant protection can be afforded to LDL but flavonoids or their metabolites may prevent the oxidation of plasma proteins or lipids located within the HDL fraction.

Significant increases in plasma α-tocopherol and β-cryptoxanthin were also found in both the rutin- and placebo-treated groups but there was no evidence of a treatment effect (Table 1). Seasonal variations in plasma antioxidant levels (α tocopherol and β carotene) have been reported (Rautalahti *et al*, 1993; Olmedilla *et al*, 1994) Changes in the plasma flavonoid and antioxidant levels did not significantly alter the susceptibility of plasma lipids to oxidation. A dramatic decrease in oxidized pyrimidines (endogenous oxidative damage) occurred in both the placebo and rutin-treated subjects during the 6 weeks of the trial (Figure 5), suggesting the involvement of some other

dietary or seasonal factors in this protective effect. This effect was not apparent at the level of urinary 8OHdG (Table 2), which is believed by some to be a product of repair of oxidative damage to cellular DNA (Simic, 1992) or to the nucleotide DNA precursor pool (MO *et al*, 1992), or which may simply reflect oxidative stress (Lindahl, 1993). No correlation was found between the level of urinary 8OHdG exereted and the level of lymphocyte DNA damage detected either as endogenous strand breaks oxidized pyrimidine bases or resistance to H_2O_2 challenge.

Levels of urinary MDA in both the rutin-treated and placebo-treated groups were higher at week 6 than at week 0 but the increases did not reach statistical significance (Table 2). Malondialdehyde is one of a number of breakdown products produced during lipid peroxidation (Esterbauer, 1982) and has traditionally been used as a measure of oxidative stress. The specificity of this compound as a marker of lipid peroxidation is somewhat dubious (Knight *et al*, 1988) with a number of other biological process yielding MDA as an end product (McMillan *et al*, 1978). Therefore levels of 8-iso-prostaglandin $F_{2\alpha}$, one of a series of bioactive isoprostanes which is produced from arachidonic acid via the predominantly non-enzymic process of membrane lipid and LDL oxidation (Awad *et al*, 1993; Lynch *et al*, 1994), were also measured. There was no significant change in the level of urinary 8-iso-$PGF_{2\alpha}$ following 6 weeks' rutin supplementation and all levels remained within the normal physiological range (Davi *et al*, 1997). However, a significant positive correlation ($P = 0.01$) was found between the urinary excretion of 8-iso-$PGF_{2\alpha}$ and malondialdehyde (Figure 6). Formation of each of these compounds can arise from either enzymic processes or free radical-mediated events. Thus while malondialdehyde is used as a marker of lipid peroxidation, it can also be produced by cyclooxygenase and thromboxane synthase in intact platelets (McMillan *et al*, 1978). In healthy subjects the formation of 8-iso-$PGF_{2\alpha}$ is predominantly non-enzymic (Morrow *et al*, 1990, Davi *et al*, 1997) but a minor component may be due to the cyclooxygenase activity of either the constitutively expressed platelet PGH synthase-1 (Pratico *et al*, 1995) or the inducible monocyte PGH synthase-2 (Pratico & FitzGerald, 1996). To determine the degree to which the cyclooxygenase pathway contributed to MDA or 8-iso-$PGF_{2\alpha}$ formation, levels of urinary 11-dehydro-thromboxane B_2 were also measured. There was no significant change in the level of urinary TXB_2 in rutin-treated subjects, suggesting no modulation of cyclooxygenase activity as a result of flavonoid supplementation. There was no correlation between the levels of urinary 8-iso-$PGF_{2\alpha}$ and urinary TXB_2 (Figure 7) or between urinary MDA and urinary TXB_2 suggesting that formation of both MDA and 8-iso-$PGF_{2\alpha}$ are cyclooxygenase-independent. The strong correlation between urinary 8-iso-$PGF_{2\alpha}$ and MDA possibly reflects their common origin. It has been suggested that prostaglandin-like compounds such as 8-iso-$PGF_{2\alpha}$, which arise from oxidation of polyunsaturated fatty acids (PUFAs), are a major source of malondialdehyde (Pryor & Stanley, 1975) while 8-iso-$PGF_{2\alpha}$ is a more specific marker of arachidonic acid oxidation which comprises $>25\%$ of the PUFA content of cell membranes such as erythrocytes (Driss *et al*, 1991). Thus free radical-mediated peroxidation of cell membranes results in the formation of non-specific end-products such as malondialdehyde, while the high proportion of arachidonic acid leads to significant levels of 8-iso-$PGF_{2\alpha}$.

While there was no evidence of modulation of cyclooxygenase activity during this human intervention trial there have been reports of altered cyclooxygenase and lipoxygenase activities in various *in vitro* animal or cell culture systems (Landolfi *et al*, 1984; Laughton *et al*, 1991; Kim *et al*, 1998). This may reflect the significantly higher flavonoid concentrations used in *in vitro* studies (10–50 μM; Mower *et al*, 1984) compared with those achieved following 6 weeks' rutin supplementation (mean plasma quercetin concentration 0.14 ± 0.03 μmol/l plasma). It therefore appears that the concentrations of flavonoids necessary for anti-aggregatory activity *in vitro* have not been attained *in vivo* by rutin supplementation at the recommended dose or by dietary intake as previously demonstrated by Janssen *et al* (1998).

Conclusion

In the pharmacokinetic study, a single dose of rutin resulted in peak plasma concentrations at around 6–7 h, with complete clearance in 24 h in two of three subjects. No significant changes were seen in various markers of oxidative stress.

Prolonged supplementation with rutin led to significantly elevated levels of quercetin, kaempferol and isorhamnetin but no significant change in plasma antioxidant status. Compared with a placebo group, rutin-supplemented subjects had the same level of oxidative base damage in lymphocyte DNA. There was no evidence of any supplementation effects on platelet cyclooxygenase activity and thus the concentrations of flavonoids necessary for anti-aggregatory activity *in vitro* are not attainable *in vivo* by rutin supplementation at the recommended dose.

In summary, while flavonoids may act as antioxidants *in vitro*, the flavonoid tested here has no detectable effect on oxidative stress *in vivo*.

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