

Interactions between vitamins C and E in human subjects

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Despite convincing *in vitro* evidence, a vitamin C–E interaction has not been confirmed *in vivo*. This study was designed to examine the effects of supplementation with either vitamin C or E on their respective plasma concentrations, other antioxidants, lipids and some haemostatic variables. Fasting blood was collected before and after intervention from thirty healthy adults in a double-blinded crossover study. Baselines for measured variables were established after 2 weeks of placebo supplementation, followed by daily supplementation with 73.5 mg *RRR*- α -tocopherol acetate or 500 mg ascorbic acid, and placebo, for 6 weeks. A 2 month washout preceded supplement crossover. Mean values showed that plasma lipid standardised α -tocopherol increased with ascorbic acid supplementation: from 4.09 (SEM 0.51) to 4.53 (SEM 0.66) $\mu\text{mol}/\text{mmol}$ total cholesterol plus triacylglycerol ($P < 0.05$), and plasma ascorbic acid increased from 62.8 (SEM 14.9) to 101.3 (SEM 22.2) $\mu\text{mol}/\text{l}$ ($P < 0.005$). Supplementation with (*RRR*)- α -tocopherol acetate increased plasma α -tocopherol from 26.8 (SEM 3.9) to 32.2 (SEM 3.8) $\mu\text{mol}/\text{l}$ ($P < 0.05$), and lipid-standardised α -tocopherol from 4.12 (SEM 0.48) to 5.38 (SEM 0.52) $\mu\text{mol}/\text{mmol}$ ($P < 0.001$). Mean plasma ascorbic acid also increased with vitamin E supplementation, from 64.4 (SEM 13.3) to 76.4 (SEM 18.4) $\mu\text{mol}/\text{l}$ ($P < 0.05$). Plasma ferric reducing (antioxidant) power and glutathione peroxidase (U/g haemoglobin) increased in both groups, while urate, total cholesterol and triacylglycerol levels decreased ($P < 0.05$ throughout). Results are supportive of an *in vivo* interaction between vitamins C and E.

Antioxidants: Vitamin C: Vitamin E

Vitamins C and E are the main dietary antioxidants. Vitamin C (ascorbic acid) is found in the aqueous, while vitamin E (mainly α -tocopherol) is located in the lipid, compartments of the body (Rock *et al.* 1996). *In vitro* experimental studies have demonstrated that these antioxidants can interact (Strain & Mulholland, 1992; Sharma & Buettner, 1993; Niki *et al.* 1995), but this has not yet been confirmed as occurring *in vivo*. The putative interaction may be direct, via vitamin C ‘sparing’ of vitamin E, where there is an immediate quenching action of vitamin C against aqueous reactive species, thereby preventing the oxidation of vitamin E (Chan, 1993; Constantinecu *et al.* 1993). Alternatively, or in addition, vitamin C may exert a ‘redox recycling’ effect on oxidised vitamin E within lipoproteins and membranes (Frei *et al.* 1989; May *et al.* 1998). Ascorbate has a lower redox potential than α -tocopherol and has been shown to be an efficient co-antioxidant *in vitro* for the regeneration of α -tocopherol from the tocopheroxyl radical (Thomas

et al. 1995). Increased intake of vitamin C, therefore, could lead to improved vitamin E status.

The antioxidant effects of these vitamins may operate within the context of an integrated system (Witting, 1995; Strain & Benzie, 1999). It is possible that the overall antioxidant status is under homeostatic control. Any alteration, therefore, in the status of a single antioxidant vitamin could affect the status of other antioxidant(s). Furthermore, the vitamin C:E ratio may be of central importance in antioxidant protection against the damaging effects of reactive oxygen species (Gey, 1998).

In addition to opposing oxidative stress, increased ascorbic acid has been reported to lower plasma cholesterol (Hallfrisch *et al.* 1994), and both ascorbic acid and α -tocopherol may lower risk of thrombosis by effects on platelet aggregability (Clazada *et al.* 1997) and possible effects on other haemostatic factors such as plasma fibrinogen and factor VII (Yarnell *et al.* 1991; Khaw & Woodhouse,

Abbreviations: FRAP, ferric reducing (antioxidant) power; PTT, partial thromboplastin time.

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1997). The potential benefits of supplementation with vitamin C and/or vitamin E may, therefore, extend beyond simple antioxidant effects.

The aim of this study was to investigate these potential beneficial effects of supplementation with either vitamin C, as ascorbic acid, or vitamin E, as α -tocopherol acetate, on the plasma concentrations of each vitamin, as well as on other measures of antioxidant status, on plasma lipids and on selected haemostatic variables.

Subjects, methods and materials

Subjects

A total of thirty-two apparently healthy consenting adults (sixteen male, sixteen female) were recruited for this double-blinded placebo-controlled supplementation trial. All of the subjects were non-smoking and non-supplement users. One male and one female subject were unable to commit fully to the study regimen, and data presented are from the thirty subjects who completed the study.

Ethical approval for the study was obtained from the Research Ethical Committee of the University of Ulster and written informed consent was obtained from each participant. All procedures involving the human subjects complied with the Declaration of Helsinki, as revised in 1989.

Study protocol

A diet history (Livingstone *et al.* 1992) was taken and personal details were recorded (Table 1). Subjects were randomised to receive either vitamin C or vitamin E (six males and nine females were given vitamin C first and nine males and six females were given vitamin E first) in a double-blinded crossover study. Subjects were instructed to take daily supplements (from Larkhall Natural Health Ltd, London, UK) providing 73.5 mg *RRR*- α -tocopherol acetate, or 500 mg ascorbic acid, as well as a visually identical vitamin C or E placebo. The order of administration of each supplement was randomised. The choice of dose in each

case was based on previous supplement citations and a pilot study (Rimm *et al.* 1993; Benzie & Strain, 1997a, 1999a). There was a 2-week double placebo-controlled run-in period, followed by 6 weeks of active vitamin supplementation. This was followed by 8 weeks of washout, during which no supplement or placebo was taken. After a further 2-week run-in period, subjects then crossed over onto the other active vitamin supplementation scheme for the final 6 weeks of the study.

Sample collection

Fasting blood samples were taken immediately prior to and at the end of each run-in and supplementation period, i.e. six blood samples (two at the beginning and end of each run-in, and one at the end of each supplementation period) from each subject were collected over the course of the study. Subjects were contacted regularly and questioned to ensure compliance during this time. Subjects were instructed not to take supplements on the days of sampling until after blood had been drawn.

Fasting venous blood samples (22 ml per individual) were collected into evacuated anticoagulant collection tubes. Blood samples were kept chilled and in the dark until centrifugation (performed at 717g, for 10 min, at 10°C) within 1 h of collection. An aliquot of heparinised whole blood was stored at -70°C for haemoglobin and glutathione peroxidase measurement. Aliquots of heparinised plasma were stored at -70°C for α - and γ -tocopherol, retinol, lutein, α - and β -carotene, α - and β -cryptoxanthin, lycopene, and for total cholesterol, triacylglycerol and urate concentrations. Citrated plasma, stored at -70°C, was used to measure partial thromboplastin time (PTT), fibrinogen concentration and factor VII activity. Frozen samples were thawed once prior to measurement, and sample aliquots (*n* 6 per subject) were batched and analysed together. EDTA blood was used on the day of collection to obtain a full blood picture (including packed cell volume, haemoglobin, red blood cell count, mean cell volume, mean cell haemoglobin content, white cell count and white cell differential count), and

Table 1. Subject characteristics and estimated daily nutrient intakes at baseline
(Mean values with standard errors of the mean for thirty subjects)

Characteristics	Males		Females	
	Mean	SEM	Mean	SEM
Age (years)	40	2.3	30	2.2
Blood type (<i>n</i>)				
A	6		4	
B	1		4	
O	8		7	
Height (m)	1.76	0.05	1.63	0.05
Weight (kg)	75.85	1.27	62.93	1.93
BMI (kg/m ²)	24.52	0.56	23.81	0.89
Energy intake (kJ)	9996	750	8519	426
Energy intake (kcal)	2388	182	2024	103
Protein intake (% of energy)	15.6	1.6	14.3	1.2
Total fat intake (% of energy)	35.5	2.0	35.1	1.6
Total carbohydrate intake (% of energy)	48.9	2.3	50.7	2.4
Vitamin C intake (mg)	117	14	136	20
Vitamin E intake (mg)	10.9	1.1	8.6	0.7

freshly separated heparinized plasma was used for ferric reducing (antioxidant) power (FRAP) and ascorbic acid measurements. All measurements were performed in duplicate and average values calculated. Commercially available serum and controls supplied with the respective assay kits were used throughout. The average values for the two blood samples taken at each run-in period were taken as the baseline values.

Methods of analysis

Simultaneous FRAP value and ascorbic acid measurements were performed using a modification of the FRAP assay (FRASC; Benzie & Strain, 1997b, 1999b) (US patent pending). The assay was performed using a Cobas Fara centrifugal analyser (Roche Diagnostic Systems, Basel, Switzerland). The concentrations of fat-soluble vitamins and carotenoids were measured simultaneously, using α -tocopherol acetate as an internal standard, on a Waters Wisp HPLC system (Waters Ltd, Watford, Herts., UK) (Thurnham *et al.* 1988).

Total cholesterol, triacylglycerol and urate concentrations were measured using commercial kits (Randox Laboratories, Crumlin, Co. Antrim, Northern Ireland, UK), on a Cobas Fara (Roche Diagnostic Systems). Total cholesterol and triacylglycerol values were used to lipid-standardize α -tocopherol concentrations (Thurnham *et al.* 1986; Gey & Puska, 1989).

Activity of whole blood glutathione peroxidase, expressed as U/g haemoglobin, was measured using a commercially available kit method (Randox Laboratories) following the manufacturer's instructions and run on a Cobas Fara (Roche Diagnostic Systems). Haemoglobin concentrations were measured using a haemoglobinometer (Coulter Electronics, Luton, Beds., UK) and a standard protocol (Bain, 1995).

Fibrinogen concentration and PTT, determined simultaneously, and factor VII activity were measured using

commercial kits and following the manufacturer's instructions on an ACL-100 (Instrumentation Laboratories, Warrington, Ches., UK).

Statistical analyses

Mean, standard error of the mean and median values for the variables were calculated at baseline, that is, the measurements were recorded at the end of the run-in period, and at the end of each supplementation period. Pre- and post-supplementation results were compared using the paired *t* test. Possible carry-over and period effects were examined by independent *t* tests. Placebo differences were examined using one-way ANOVA. The two-tailed *P* values are shown, and statistical significance was sought at the 0.05 level. All statistical analyses were performed using a Microsoft Excel package (Windows '97).

Results

Results showed that supplementation with either ascorbic acid or α -tocopherol was associated with increased fasting ascorbic acid and lipid-standardized α -tocopherol concentrations, decreased total cholesterol and triacylglycerol concentrations, increased FRAP values, increased glutathione peroxidase activities, increased PTT, and decreased urate concentration (Table 2). Supplementation with ascorbic acid was associated with more marked changes in FRAP values, ascorbic acid and urate concentrations than was α -tocopherol supplementation. Supplementation with α -tocopherol was associated with more marked changes in plasma total and lipid standardized α -tocopherol than ascorbic acid supplementation. No change was seen in fibrinogen levels after supplementation with either vitamin C or E. However, PTT was slightly increased after both

Table 2. Baseline compared with post-supplementation (73.5 mg α -tocopherol/d or 100 mg ascorbic acid/d for 6 weeks) data for the variables measured†

(Mean values with standard errors of the means for thirty subjects)

Variable (fasting level)	Vitamin C (500 mg/d)				Vitamin E (73.5 mg/d)			
	Baseline		Post-supplement		Baseline		Post-supplement	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Ascorbate ($\mu\text{mol/l}$)	62.8	3.25	101.3***	5.42	64.4	3.18	76.3*	4.20
α -Tocopherol ($\mu\text{mol/l}$)	26.1	0.99	26.8	1.24	26.8	1.03	32.3*	0.92
γ -Tocopherol ($\mu\text{mol/l}$)	1.84	0.14	1.86	0.13	1.75	0.13	1.20***	0.10
LS α -tocopherol ($\mu\text{mol/mmol}$)‡	4.09	0.12	4.53*	0.16	4.12	0.11	5.38**	0.12
Total cholesterol (mmol/l)	5.36	0.13	5.03*	0.17	5.47	0.16	5.18*	0.13
Triacylglycerol (mmol/l)	1.06	0.08	0.97*	0.08	1.04	0.08	0.85*	0.06
Urate ($\mu\text{mol/l}$)	256	14.1	230***	14.7	262	13.9	246*	12.6
GPx (U/g Hb)	50.0	2.79	53.1*	2.58	48.8	2.39	53.5*	2.90
FRAP ($\mu\text{mol/l}$)	1103	30.3	1158**	30.9	1125	30.1	1161**	27.7
α -Carotene ($\mu\text{mol/l}$)	0.14	0.01	0.13	0.01	0.15	0.01	0.13*	0.01
α -Cryptoxanthin ($\mu\text{mol/l}$)	0.08	0.01	0.08	0.01	0.08	0.01	0.07*	0.01
PTT (s)	14.4	0.17	14.7*	0.21	14.3	0.20	14.6*	0.20
Factor VII (% activity)	103	4.3	98*	4.2	105	4.1	102	3.6
Fibrinogen (mg/dl)	3.21	0.13	3.12	0.13	3.08	0.13	3.06	0.10

GPx, glutathione peroxidase; Hb, haemoglobin; FRAP, ferric reducing (antioxidant) power; PTT, partial thromboplastin time.

Mean values were significantly different from baseline values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

† For details of subjects and procedures see Table 1 and pp. 262–263.

‡ LS, lipid-standardised α -tocopherol is the plasma α -tocopherol concentration expressed per mmol cholesterol + triacylglycerol.

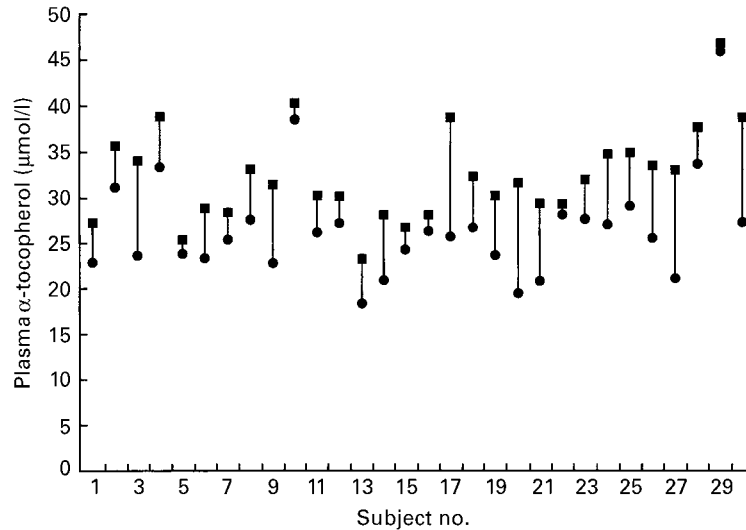


Fig. 1. Intra-subject variation in total plasma α -tocopherol concentration response to 73.5 mg α -tocopherol acetate/d given over 6 weeks. (■), Pre-supplementation; (●), post-supplementation. Approximately half of the subjects showed a marked response compared with the remainder, who demonstrated only a slight increase in plasma α -tocopherol concentration. For details of subjects and procedures see Table 1 and pp. 262–263.

supplementation periods and factor VII activity slightly decreased after ascorbic acid supplementation.

With reference to Table 2, it can be seen that after supplementation with ascorbic acid, both total cholesterol and triacylglycerol levels in fasting plasma decreased ($P < 0.05$). Total α -tocopherol levels, however, did not decrease in parallel as might be expected, given that α -tocopherol is transported within plasma lipoproteins. Indeed, owing to the combination of the change in plasma lipids and the constancy (stability) of total α -tocopherol levels, the concentration of lipid-standardised α -tocopherol increased by 10% ($P < 0.05$).

Supplementation with α -tocopherol led to a relatively small but significant overall increase in fasting total α -tocopherol. Large individual variations, however, were observed in response to supplementation. Looking at individual responses (Fig. 1), as opposed to the average change, around half of the subjects taking α -tocopherol showed post-supplementation increases in plasma α -tocopherol of 40–50%, while half showed very little increase. The magnitude of response did not appear to be related to sex, initial baseline concentrations of α -tocopherol, or to the order of supplementation. The combination of increased total ($P < 0.01$) α -tocopherol and decreased ($P < 0.01$) plasma lipids, led to an overall increase ($P < 0.01$) of almost 30% in plasma lipid standardised α -tocopherol.

There was no statistically significant supplementation-related changes in retinol, or the carotenoids (except for α -carotene and α -cryptoxanthin which decreased after α -tocopherol supplementation but these small changes were probably not biologically significant). Only slight changes in the measured haemostatic variables were observed, with increased PTT after supplementation with either vitamin, and decreased factor VII activity after supplementation with ascorbic acid. No change in the full blood picture was observed (data not shown). There was no evidence of carry-over or period effects.

Discussion

The results of the current study show that supplementation with ascorbic acid increased ascorbic acid and lipid-standardised α -tocopherol levels in plasma, and that supplementation with α -tocopherol was associated with increased plasma ascorbic acid concentration, as well as improved vitamin E status. These results are supportive of the putative vitamin C–E interaction demonstrated in many *in vitro* studies (Stoyanovsky *et al.* 1995; Halpner *et al.* 1998) but, to date, unconfirmed in human subjects. Results also show that plasma lipids and urate decreased, and that total antioxidant power and glutathione peroxidase values increased after supplementation with either vitamin C or E.

The increase in fasting plasma ascorbic acid concentration after supplementation with α -tocopherol, together with the increased vitamin E status observed after ascorbic acid supplementation, implies an *in vivo* interaction between these two antioxidant vitamins. The increase in vitamin E status after ascorbic acid supplementation probably indicates a sparing on regeneration of α -tocopherol. The increase in vitamin C status after supplementation with α -tocopherol is more difficult to explain but might be linked to increased absorption and/or decreased plasma clearance of ascorbic acid.

The post-ascorbic acid supplementation decreases in plasma cholesterol and triacylglycerol concentrations shown here are consistent with the reported negative associations between ascorbic acid and plasma cholesterol and triacylglycerol (Samman *et al.* 1997). It has previously been shown that guinea-pigs fed supplementary ascorbic acid had lowered serum and hepatic cholesterol and triacylglycerol levels (Fernandez *et al.* 1997). A study in which guinea-pigs were fed oxidised frying oil, known to deplete vitamin E, but supplemented with vitamin C, reported significantly lower plasma cholesterol and higher tissue concentrations of both vitamins C and E (Lim & Lee, 1998). Conversely,

several studies have shown increases in plasma cholesterol and triacylglycerol concentrations in guinea-pigs fed scorbutic diets (Ginter *et al.* 1987).

The vitamin E associated decrease in plasma lipids may be related, in part, to vitamin C-dependent pathways, as improved ascorbic acid was seen after vitamin E supplementation in this current study. Increased plasma vitamin C has been reported following vitamin E supplementation of scorbutic osteogenic disorder shionogi (ODS) rats (Tanaka *et al.* 1997). Several animal studies have shown that supplementary vitamin E is associated with less dietary fat-induced atheroma formation and lower serum cholesterol (Verlangierii & Bush, 1992; Willingham *et al.* 1993; Chen *et al.* 1995; Schemenke & Behr, 1998).

Increased vitamin C and vitamin E concentrations have been reported to improve insulin action, and this could lead, in turn, to decreased triacylglycerol levels (Paolisso *et al.* 1995). The Seven Countries Study showed an inverse correlation between vitamin C and glucose intolerance, suggesting a role for vitamin C in glucose disposal (Feskens *et al.* 1995). In non-insulin-dependent-diabetes-mellitus (type 2) patients, vitamin C supplementation has been shown to improve lipid and glucose metabolism (Peuchant *et al.* 1997). Supplementation-induced increases in ascorbic acid paralleled the decline in LDL-cholesterol and insulin concentrations in fasting plasma (Armstrong *et al.* 1996). Improved insulin action may lead to lower VLDL and LDL concentration, and to a more favourable LDL subclass distribution, potentially increasing resistance of LDL to oxidation as small, dense LDL subclasses have been shown to be more susceptible to oxidation (Sprenger *et al.* 1998; Austin *et al.* 1999; Jari *et al.* 1999).

In terms of supplementation-induced changes in antioxidant status, ascorbic acid and α -tocopherol produced the expected increase in the total antioxidant power of plasma. Vitamins E and C are reported to contribute 5–10% and 10–15% respectively to the FRAP value of fasting plasma (Benzie & Strain, 1997b; Vasankari *et al.* 1997). In the current study, the mean relative contribution of ascorbic acid plus α -tocopherol to the overall 'total' antioxidant power following ascorbic acid or α -tocopherol supplementation increased from 16.1% to 22.1%, and from 16.2% to 18.7% respectively.

In this present study, urate levels decreased after ascorbic acid and after α -tocopherol supplementation. Urate has been reported to be an important endogenous antioxidant (Benzie & Strain, 1996), but it is also an independent risk factor for IHD (Torun *et al.* 1998; Johnson *et al.* 1999). At elevated concentrations, therefore, the detrimental effects of urate may outweigh any health benefits conferred by its antioxidant properties (Lee *et al.* 1995). Although urate concentrations were not elevated in the current study, results are consistent with previous reports of a negative association between plasma urate and ascorbic acid, and a direct correlation between urate and triacylglycerol (DiSciascio *et al.* 1994). The decrease in urate may have been related to increased renal excretion (not measured in the current study), as ascorbic acid has an uricosuric effect (Schlotte *et al.* 1998). Elevated urate is found along with increased triacylglycerol concentrations in the syndrome of insulin resistance. While the relationship is unclear, the decreased

urate and triacylglycerol concentrations seen in the current study after antioxidant supplementation may be related to improved insulin action (Jacob, 1995; Benzie & Strain, 1996). In addition, it has been suggested that vitamin E has the potential to inhibit xanthine oxidase, the enzyme involved in urate synthesis (Choc, 1991; Shaheen *et al.* 1996).

The slight decreases in carotenoids seen in this study were likely to be a simple reflection of decreased lipids. Of the haemostatic factors measured, two (PTT and the activity of factor VII), showed slight improvement in terms of anti-thrombotic tendency. The determinants of the levels of these haemostatic factors in populations is not well defined (Khaw & Woodhouse, 1997). The finding of lower factor VII activity in association with higher plasma ascorbic acid concentration is consistent with previous findings (Yarnell *et al.* 1991; Khaw & Woodhouse, 1997). No change in fibrinogen concentration, however, was seen, and this, taken with the lack of detectable differences in white blood cell count after supplementation, indicate no change in inflammatory status in these healthy subjects.

In conclusion, the results of the current study are supportive of an *in vivo* interaction between vitamins C and E as indicated by similar changes in plasma vitamin, antioxidant power and lipid concentrations in response to supplementation with pharmacological doses of either vitamin C or vitamin E.

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