

## Dietary vitamin E supplementation inhibits thrombin-induced platelet aggregation, but not monocyte adhesiveness, in patients with hypercholesterolaemia

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**Summary.** Several recent studies have indicated the possible beneficial effects of antioxidants, specifically vitamin E, in primary and secondary coronary prevention. These studies suggest that a diet enriched in vitamin E is insufficient to have a significant protective effect, whereas supplements, in excess of 200 international units (IU) per day, are efficacious in preventing coronary disease in both men and women. The mechanisms by which vitamin E may exert its protection are uncertain, but, vitamin E is lipophilic and has been shown to inhibit the oxidative modification of low density lipoprotein (LDL), a process thought to be of crucial importance in atherogenesis. We have also previously shown that  $\alpha$ -tocopherol (the biologically most potent isomer of vitamin E) has important direct effects on vascular endothelial and smooth muscle cells. In the present study we have investigated the effects of oral supplements of vitamin E (400 IU per day) on platelet and mononuclear cell function in patients with hypercholesterolaemia. We found that although vitamin E supplementation had no significant effect on mononuclear cell adhesion *ex vivo*, it had a significant effect on the thrombin-induced platelet aggregation ( $P < 0.01$ ; ANOVA): 6 weeks after starting the vitamin E supplements, the mean  $EC_{50}$  for thrombin-induced aggregation increased 132% ( $P < 0.05$ ; paired *t*-test) compared to treatment with placebo. The effects of vitamin E on platelet function may, in part, explain its anti-atherogenic properties.

**Keywords:** Vitamin E, hypercholesterolaemia, platelet aggregation, monocyte adhesion

Platelets play an essential role in the pathogenesis of athero-thrombotic disease (Packham & Mustard 1986;

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Ross 1993). Healthy vascular endothelium is antithrombogenic due to its elaboration of mediators such as nitric oxide and prostacyclin which inhibit platelet adhesion (Tschopp & Baumgartner 1981; Radomski *et al.* 1987). The endothelium may become dysfunctional and hence pro-thrombotic following exposure to cigarette smoke, high levels of blood cholesterol, hypertension or diabetes

mellitus. Hypercholesterolaemia is an important and common risk factor for coronary heart disease. It is often accompanied by high levels of platelet associated cholesterol levels (Chetty & Naran 1992), and enhanced platelet responses to aggregatory factors (Carvalho *et al.* 1974). Hypercholesterolaemia may also be associated with increased levels of lipid peroxides which may cause platelet membrane damage by oxidizing its lipid constituents (Stringer *et al.* 1989).

Leucocyte-endothelial cell interactions are an important feature of immune surveillance, and are likely to play a pivotal role in several pathophysiological processes including atherogenesis (Ross 1993). The adhesion of leucocytes to the vascular endothelium is mediated by pairs of regulatable, plasma-membrane associated, adhesion molecules; one member of each pair is present on the leucocyte, the other on the endothelial cell (Springer 1990). Adhesion may be modulated by altering the expression, or activation state of these molecules. *In vitro* treatment of endothelial cells with cytokines such as interleukin-1 (IL-1) or tumour necrosis factor (TNF) stimulates adhesion molecule upregulation (Poher *et al.* 1988). These *in vitro* findings may partially explain the observations of Poston & Johnson-Tidey (1996) who have reported increased adhesion molecule expression in regions of endothelium overlying atherosclerotic lesions. In contrast, the endothelium derived factor, nitric oxide, appears to inhibit monocyte adherence (Bath *et al.* 1991). The ability of the endothelium to produce bioactive nitric oxide has been shown to be rapidly compromised following the induction of hypercholesterolaemia in the rabbit (Stewart-Lee *et al.* 1994, 1995; Andersson *et al.* 1994) and this could be prevented, or reversed by antioxidant treatment (Stewart-Lee *et al.* 1994).

Supplements of vitamin E, a potent lipid-soluble antioxidant, have been shown to be associated with reduced risk of coronary heart disease in men and women (Rimm *et al.* 1993; Stampfer *et al.* 1993). Vitamin E has also been shown to reduce mortality in patients with established coronary heart disease (Stephens *et al.* 1996). The mechanism by which vitamin E exerts its effects is unclear, but it has been shown to inhibit platelet aggregation (Steiner 1983; Kakishita *et al.* 1990; Violi *et al.* 1990, Salonen *et al.* 1991) and adhesion (Jandak *et al.* 1988, Steiner 1983). We have recently shown that another lipid soluble antioxidant, probucol, reduces mononuclear cell adhesion in an animal model of atherogenesis (Ferns *et al.* 1993). In this present study we have investigated the effects of vitamin E supplementation on platelet aggregation and mononuclear cell adhesion *ex vivo*, in

patients with primary hypercholesterolaemia, using a placebo-controlled experimental design.

## Materials and methods

### Materials

Eahy cells were a gift from Dr Cora-Jean Edgell, University of North Carolina, USA (Edgell *et al.* 1983). Vitamin E and placebo capsules were kindly donated by Dr Rupert Mason of Bioglan Ltd, Herts, UK. RPMI-1640, Dulbecco's modified Eagle's medium and foetal calf serum were purchased from Gibco (Uxbridge, UK). All other reagents were from Sigma Chemical Co. (Dorset, UK).

### Subjects

Patients were recruited from those newly referred to the Lipid Clinic at Glenfield General Hospital, Leicester, with a diagnosis of primary hypercholesterolaemia (serum cholesterol >5.7 mmol/l, but <10 mmol/l and serum triglycerides <3.3 mmol/l). Exclusion criteria included: a positive recent smoking habit, diabetes mellitus or hypothyroidism. Patients taking antihypertensive or diuretic therapy, steroids or vitamin supplements were also excluded. Ethical approval was sought from the Local Ethics Committee and was granted prior to the start of the trial. Patients were asked to read an information sheet, briefly explaining the experimental protocol, at the same time they were told they would be taking either the active vitamin E capsules or 'dummy' capsules for six weeks, at which point the capsules would be switched.

### Vitamin supplements and blood sampling

Blood samples for baseline lipid levels (total cholesterol, triglycerides and HDL cholesterol) and antioxidant vitamin ( $\alpha$ -tocopherol, retinol and ascorbate) concentrations were taken following a 12 hour, overnight fast. Patients were then given a supply of placebo capsules (containing soybean oil) for six weeks. This was followed by a six week period taking vitamin E at a dose of 400 IU per day. Blood samples were taken every three weeks for the duration of the study, for the assessment of the parameters listed above.

### Lipid profiles

Plasma was obtained from fasting blood samples collected into lithium-heparin tubes (1.5 IU heparin/ml) and centrifuged at 1500×g for 10 min at 4 °C. Measurements

of plasma total- and HDL-cholesterol, and triglycerides were made using a Kodak Ektachem 700XR Analyser C series (Eastman Kodak Company, Rochester, USA). Plasma LDL cholesterol concentrations were estimated using the Friedwald formula (Friedwald *et al.* 1972).

#### *Plasma antioxidant vitamin levels*

Plasma samples were obtained from lithium heparin blood samples as described above. Plasma  $\alpha$ -tocopherol and retinol were determined simultaneously by HPLC using the method of Bieri *et al.* (1979).

Ascorbate levels were measured using plasma from samples of EDTA anticoagulated blood (1 mg/ml). Samples were centrifuged at  $1500\times g$  for 10 min at  $4^\circ\text{C}$ . Immediately after harvesting the plasma, ascorbic acid was stabilized by the addition of an equal quantity of 10% (w/v) metaphosphoric acid. Samples were vortex mixed, centrifuged at  $1500\times g$  for 10 min at  $4^\circ\text{C}$  and the supernatants collected for storage at  $-70^\circ\text{C}$  for up to one month. Ascorbic acid was measured by HPLC using a modification of the method of Lunec & Blake (1985).

#### *Platelet isolation*

Whole blood anticoagulated with 1/10th volume of 3.8% (w/v) trisodium citrate was centrifuged at  $200\times g$  for 20 min and the upper platelet-rich layer was collected. Apyrase (final concentration  $10\ \mu\text{g/ml}$ ) and prostacyclin (final concentration  $0.33\ \mu\text{g/ml}$ ) were added to this platelet-rich plasma to prevent premature platelet activation, this was centrifuged at  $800\times g$  for 15 min. The resulting platelet pellet was resuspended in  $\text{Ca}^{2+}$ -free Tyrodes buffer (10 mmol/l HEPES, 145 mmol/l NaCl, 2.7 mmol/l KCl, 1.8 mmol/l  $\text{MgCl}_2$ , 5.55 mmol/l glucose, 5.95 mmol/l  $\text{NaHCO}_3$ , 0.42 mmol/l sodium dihydrogen phosphate, pH 7.4) containing bovine serum albumin (2 mg/ml) at a concentration of  $300\times 10^9/l$ . Platelets were allowed to equilibrate at ambient temperature for 1 hour prior to use.

#### *Platelet aggregometry*

Platelet aggregation was measured using the method of Frantantoni & Poindexter (1990). Briefly, the time course of platelet aggregation was monitored in 96 well microtitre plates by following the change in optical density using an automated Anthos HTIII plate reader (Labtech International, East Sussex, U.K.). Increasing concentrations of thrombin were added to quadruplicate wells at a

final concentration ranging from 0 to 2000 Units/l. Calcium chloride was added to each well at a final concentration of  $0.033\ \mu\text{mol/l}$ . Platelet suspension ( $135\ \mu\text{l}$ ) was then added to each well giving a final total volume per well of  $150\ \mu\text{l}$ . The initial absorbance at 405 nm of each well was measured, and subsequent readings taken at one minute intervals for 20 min. The microtitre plate was maintained at  $37^\circ\text{C}$  throughout the experiment, and shaken between readings at the maximum speed setting. The time taken for the optical density of each well to reach 0.200 was calculated using the BIOLISE software package (Labtech International). Using this information a dose response curve was plotted, and the  $\text{EC}_{50}$  Units/litre (U/l) determined.

#### *Mononuclear cell isolation*

Whole blood anticoagulated with 1/10th volume of 3.8% (w/v) trisodium citrate was centrifuged at  $200\times g$  for 20 min and the upper platelet-rich layer was removed without disturbing the 'buffy-coat'. The blood was restored to its original volume with PBS, and 5 ml aliquots overlaid onto Ficoll-Paque. Blood mononuclear cells were prepared as previously described (Bøyum 1968). In brief the tubes were centrifuged at  $400\times g$  for 30 min, the mononuclear cell layer was recovered and washed twice in 5 volumes of PBS. Immediately prior to use, the cells were resuspended in serum-free RPMI 1640 medium at a concentration of  $6\times 10^9$  cells/l. Prior to use, cell viability was assessed by trypan blue exclusion, and on all occasions viability exceeded 95%. The content of contaminating platelets was low, with a ratio of platelets: mononuclear cells being  $<1$ .

#### *EAhy 926 cell culture*

The endothelial cells were maintained in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, 10% foetal calf serum and  $100\ \mu\text{mol/l}$  hypoxanthine, 0.4 mol/l aminopterin and  $16\ \mu\text{mol/l}$  thymidine. Cells were used between passages 30–40.

#### *Measurement of monocyte adhesion*

Mononuclear cell suspensions in serum-free RPMI were added to endothelial monolayers ( $6\times 10^5$  MNCs per well), or cell-free wells ( $6\times 10^5$  MNCs per well) in 96 well plates. The plates were incubated at  $37^\circ\text{C}$  for 30 min, and nonadherent cells removed by washing twice with PBS. Monocyte specific adherence was determined by a modification of the method described by Bath *et al.* (1989). This method relies on conversion of a

colourless substrate tetramethyl benzidine (TMB) to a blue product by the action of monocyte-specific myeloperoxidase activity. The cells contained in each well were lysed in 100  $\mu$ l hexadecyltrimethyl-ammonium bromide (0.5% in PBS; pH 5.0) at 37 °C for 60 min. A fresh solution of TMB (0.1 mg/ml in 0.05 M phosphate citrate buffer (pH 6.0) containing 0.03% sodium perborate) was added to each well and the plate incubated for 10 min at room temperature. The reaction was stopped by the addition of 2.5 M sulphuric acid and the absorbance was measured at 450 nm using an Anthos HTIII microplate reader. A standard curve of cell number vs absorbance was constructed for each batch of MNCs and absolute adhesion calculated by reference to this curve.

### Statistical analysis

Results are expressed as mean  $\pm$  SEM. Significance was assessed using ANOVA, and paired *t*-tests using Instat software (GraphPAD Inc, USA) on a PC.

## Results

### Patient characteristics

A total of 28 subjects (male: female ratio 15: 13); mean age 55.2  $\pm$  2.14 years (range 33–73 years) were recruited into the study. Their clinical characteristics are summarized in Table 1. Of the patients recruited 15

**Table 1**—Basal characteristics of the hypercholesterolaemic subjects.

<i>n</i>	28	
mean age	55.2	$\pm$ 2.1
M:F ratio	15:13	
Pure hypercholesterolaemia	15	
Mixed hyperlipidaemia	13	
Lipoprotein profile		
mean total serum cholesterol (mmol/l)	7.08	$\pm$ 0.15
mean HDL cholesterol (mmol/l)	1.29	$\pm$ 0.05
mean LDL cholesterol (mmol/l)	4.93	$\pm$ 0.15
mean total triglycerides (mmol/l)	1.98	$\pm$ 0.15
Plasma antioxidant concentrations		
Vitamin A (mg/l)	0.71	$\pm$ 0.02
Vitamin E (mg/l)	19.39	$\pm$ 0.95
Vitamin C ( $\mu$ mol/l)	60.81	$\pm$ 3.76
Dehydroascorbate ( $\mu$ mol/l)	5.85	$\pm$ 0.55
Cell Function		
Monocyte adhesion to ECs (%)	12.15	$\pm$ 1.36
Monocyte adhesion to plastic (%)	70.99	$\pm$ 3.16
Platelet aggregability to Thrombin (EC <sub>50</sub> ) (U/l)	286	$\pm$ 33

had pure hypercholesterolaemia, 13 had a mixed hyperlipaemia.

### Effect of vitamin E on plasma lipid and antioxidant vitamin levels

There was no significant change in mean plasma total-, calculated LDL-, or HDL-cholesterol, triglycerides, or ascorbic acid and retinol during treatment with placebo or vitamin E supplementation (Figure 1c-f). Although plasma  $\alpha$ -tocopherol levels were not affected by treatment with placebo, they increased significantly following 3 weeks treatment with vitamin E ( $P < 0.0001$ , 38.38  $\pm$  1.68 mg/l vs. 20.36  $\pm$  0.99 mg/l), by six weeks of supplementation the plasma  $\alpha$ -tocopherol levels had plateaued ( $P < 0.0001$ , 36.42  $\pm$  1.7 mg/l vs. 20.36  $\pm$  0.99 mg/l) (Figure 1a). Over the experimental period there was a substantial increase in plasma  $\alpha$ -tocopherol levels in most of the subjects (range 3–188%) (Figure 1b).

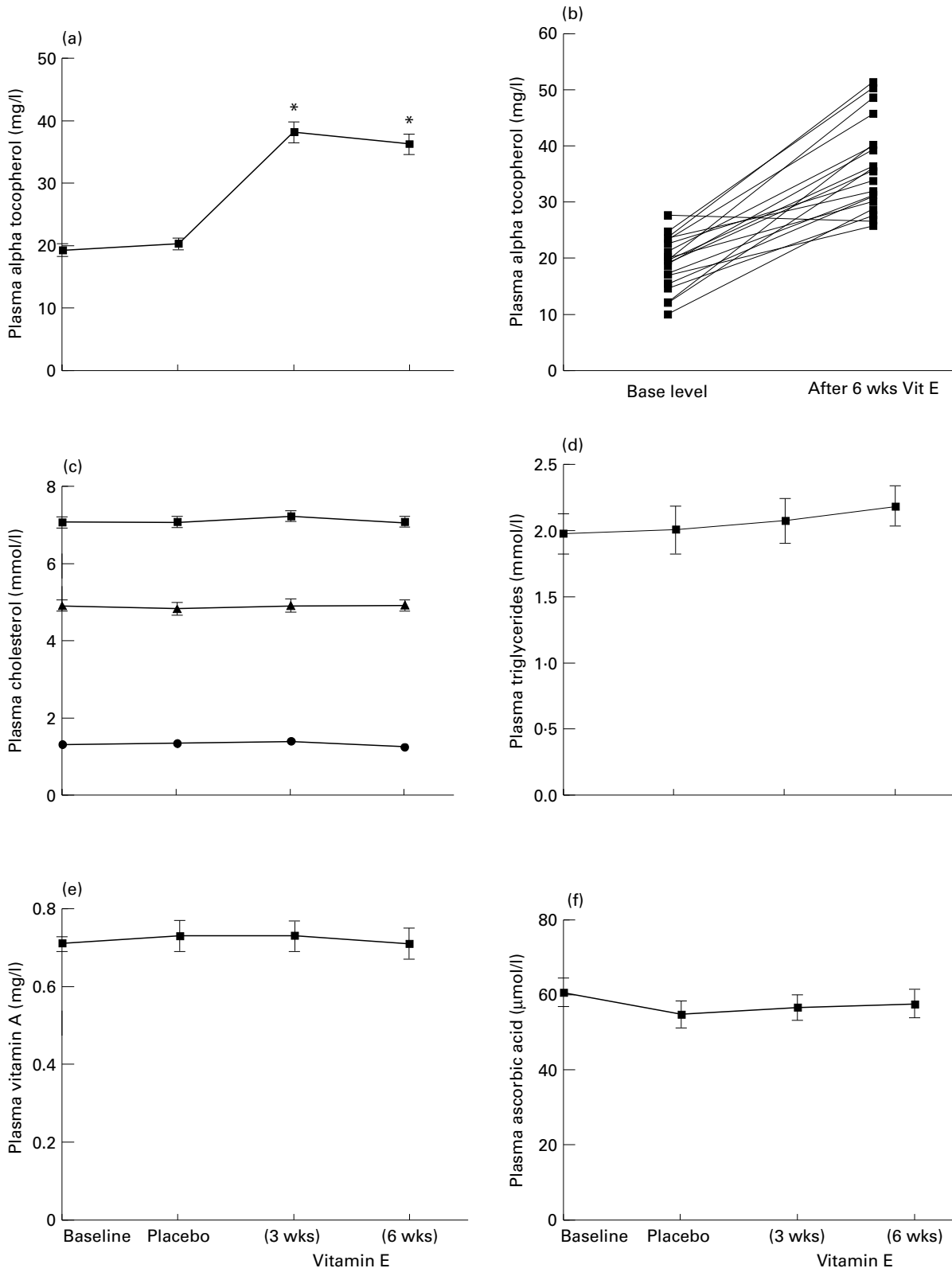
### Effects of vitamin E supplementation on platelet aggregation ex vivo

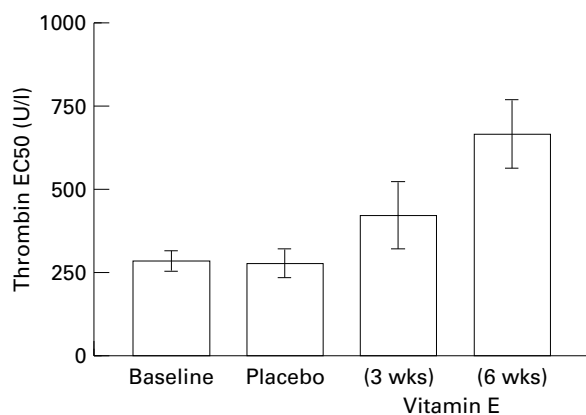
There was no significant change in the mean EC<sub>50</sub> (U/l) following placebo treatment. After 3 weeks of vitamin E supplementation, the EC<sub>50</sub> increased by approximately 47% (420.5  $\pm$  102.9 U/l vs. 278.4  $\pm$  44.1 U/l) but this failed to reach significance. However, after 6 weeks of treatment with vitamin E a further increase in EC<sub>50</sub> (U/l) of 132% was observed (664.0  $\pm$  103 U/l vs 278.4  $\pm$  44.1 U/l,  $P < 0.05$ ) (Figure 2). Over the trial period vitamin E was found to have a significant inhibitory effect on thrombin-induced platelet aggregation ( $P < 0.01$ , ANOVA,  $n = 12$ ).

### Effects of vitamin E supplementation on monocyte adhesion ex vivo

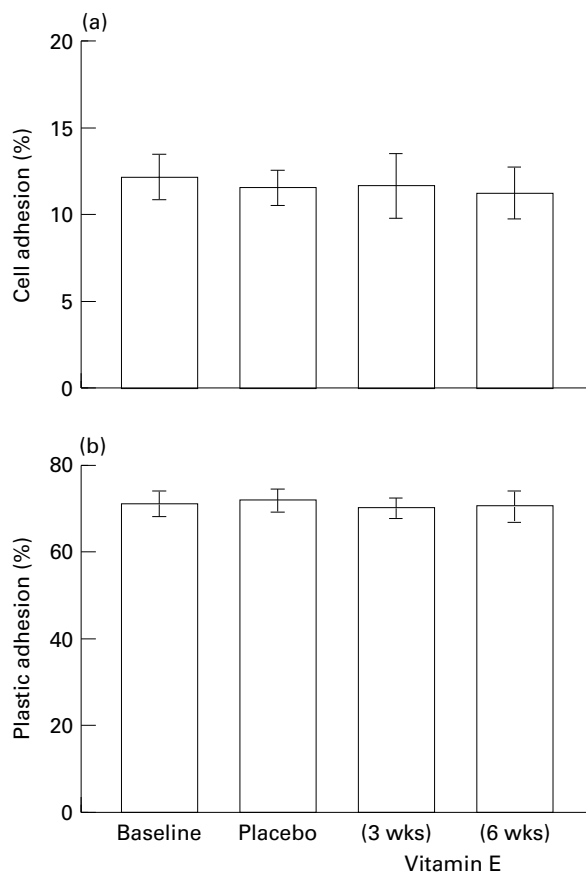
The basal adherence of mononuclear cells to confluent EaHy endothelial cells was approximately 12% and 70% to tissue culture plastic. Neither placebo, nor vitamin E treatment significantly affected mononuclear cell adhesion over the duration of the study (Figure 3).

**Figure 1.** a, Effects of placebo and vitamin E supplementation on mean plasma  $\alpha$ -tocopherol levels (mean  $\pm$  SEM, \*  $P < 0.0001$  vs. baseline or placebo). b, Graph showing the individual  $\alpha$ -tocopherol responses to six weeks supplementation with vitamin E. Effects of vitamin E supplementation on: c, plasma lipoprotein cholesterol levels. ■ total cholesterol, ▲ LDL cholesterol ● HDL cholesterol. d, plasma triglyceride concentrations. e, plasma vitamin A, f, plasma vitamin C levels.





**Figure 2.** Effects of placebo (Soybean oil) and vitamin E treatment on the EC<sub>50</sub> (U/l) of thrombin-induced platelet aggregation in an unselected group of patients with hypercholesterolaemia ( $n = 12$ ).



**Figure 3** Effects of vitamin E supplementation on monocyte adhesion to a, EaHy endothelial cells and b, tissue culture plastic.

## Discussion

### *Effect of vitamin E supplementation on plasma lipid and antioxidant vitamin profiles*

Treatment with placebo or vitamin E at a dose of 400 IU per day was not associated with any significant effects on plasma total-, LDL-, or HDL-cholesterol, or triglycerides. These findings are consistent with previous reports (Szczekliik *et al.* 1985; Salonen *et al.* 1991), although others have reported that vitamin E supplements decrease plasma cholesterol levels (Cloarec *et al.* 1987).

Mean plasma  $\alpha$ -tocopherol levels increased by approximately two-fold following vitamin E supplementation at a dose of 400 IU per day, which is similar to that reported by Princen *et al.* (1995) using the same dose. Mean plasma levels of the other antioxidant vitamins (A & C) were not affected by vitamin E supplementation at this dose.

### *Vitamin E supplementation did not affect mononuclear cell adhesion ex vivo*

Over the duration of the present study, vitamin E supplementation at 400 IU per day had no significant effect on monocyte adhesion *ex vivo*. This was so whether examining their adherence to the endothelial cell line, EaHy, or to tissue culture plastic. A recent paper by Devaraj *et al.* (1996) reported that supplementation with 1200 IU per day vitamin E led to a significant decrease in monocyte adhesion to human umbilical vein endothelial cells. This difference could be explained by the use of a much larger dose of vitamin E in this study and the fact that this group were looking at adhesion to primary cultured human umbilical vein endothelial cells as opposed to the cell line used in our study.

### *Vitamin E supplementation inhibits thrombin induced platelet aggregation ex vivo*

Vitamin E supplementation, at a dose of 400 IU per day for six weeks caused a significant increase in the EC<sub>50</sub> of thrombin-induced platelet aggregation, indicative of decreased platelet sensitivity to the effects of this agonist. Srivastava (1986) and Fong (1976) have previously shown that vitamin E inhibits platelet aggregation *in vitro*, whilst the effects of vitamin E supplements on platelet aggregation *ex vivo* are less clear. Although Steiner (1983) have reported a small but significant inhibition of platelet aggregation at high levels of supplementation (up to 1200 IU per day), other groups (Stampfer *et al.* 1988; Salonen *et al.* 1991) failed to

observe any significant inhibitory effect. However, the latter studies were performed on healthy volunteers, whose basal platelet reactivity may be less marked. Another study of the effects of vitamin E supplementation at a dose of 600 IU per day for 14 days in patients with hyperlipidaemia demonstrated that vitamin E inhibited arachidonic acid and adenosine diphosphate-induced aggregation (Szczeklik *et al.* 1985). However, the subjects in their study were more heterogeneous, several having clinically overt arterial disease. Only four of the sample population were asymptomatic, while in our study, none of the patients had evidence of heart disease. We have not established the mode of action by which vitamin E inhibits thrombin induced platelet aggregation or whether this inhibitory effect is observed when other agonists such as collagen, arachidonic acid or adenosine diphosphate are employed, though Szczeklik *et al.* (1985) have reported an inhibition of arachidonic acid and adenosine diphosphate induced aggregation by vitamin E. It is possible that vitamin E has a stabilizing effect on the platelet membrane (Steiner 1981) through its interactions with polyunsaturated fatty acids (Diplock & Lucy 1973). Vitamin E may also alter the activity of cyclo-oxygenase (Ali *et al.* 1980) or lipoxygenase (Mower & Steiner 1983), key enzymes in the aggregatory process. A further mechanism by which vitamin E may inhibit platelet aggregation is via its ability to scavenge reactive oxygen species. Previous reports indicate that these are released during platelet aggregation, and that antioxidants can inhibit aggregation *in vitro* (reviewed by Salvemini & Botting 1990).

#### Vitamin E in coronary prevention

Our data indicate that vitamin E supplementation may be beneficial in coronary prevention due to its capacity to inhibit platelet aggregation. Its effects were observed within weeks of starting supplements, and this may be one explanation for the rapid reduction in myocardial events following vitamin E supplementation observed in the CHAOS trial (Stephens *et al.* 1996). Clearly longer-term benefits may accrue from its effects on LDL oxidation (Steinberg *et al.* 1989) and smooth muscle cell proliferation (Konecny *et al.* 1995).

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#### References

- ALI M., GUDBRANSON C.G. & MCDONALD J.W.D. (1980) Inhibition of platelet cyclo-oxygenase by alpha-tocopherol. *Prostaglandins Leukotrienes Med* **4**, 79–85.
- ANDERSSON T.C., MATZ J., FERNS G.A.A. & ANGGARD E.E. (1994) Vitamin E reverses cholesterol-induced endothelial dysfunction in the rabbit coronary circulation. *Atherosclerosis* **111**, 39–45.
- BATH P.M.W., BOOTH R.F.G. & HASSELL D.G. (1989) Monocyte-lymphocyte discrimination in a new microtitre based adhesion assay. *Immunol. Meth* **118**, 59–65.
- BATH P.M.W., HASSALL D.G., GLADWIN A.M., PALMER R.M.J. & MARTIN J.F. (1991) Nitric oxide and prostacyclin. Divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium *in vitro*. *Arterioscler and Thromb* **11**, 254–260.
- BIERI J.G., TOLLIVER T.J. & CATIGNANI G.L. (1979) Simultaneous determination of alpha-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am J Clin Nutr* **32**, 2143–2149.
- BØYUM A. (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Invest.* **21**, 77–79.
- CARVALHO A.C.A., COLMAN R.W. & LEES R.S. (1974) Platelet function in hyperlipoproteinaemia. *N Engl J Med* **290**, 434–438.
- CHETTY N. & NARAN N.H. (1992) Platelet hyper-reactivity in hyperlipidaemia with specific reference to platelet lipids and fatty acid composition. *Clin Chim Acta* **213**, 1–13.
- CLOAREC M.J., PERORISSET G.M., LAMBERDIERE F.A., COLAU-BELCOUR J.F., SAUZIERES J.P., NEUFELD H.N. & GOLDBOURT U. (1987) Alpha-tocopherol: effect on plasma lipoproteins in hypercholesterolaemic patients. *Isr J Med Sci* **23**, 869–872.
- DEVARAJ S., L.I.D. & JIALAL I. (1996) The effects of  $\alpha$ -tocopherol supplementation on monocyte function, decreased lipid oxidation, interleukin  $1\beta$  secretion and monocyte adhesion to endothelium. *J Clin Invest* **98**, 756–763.
- DIPLOCK A.T. & LUCY J.A. (1973) The biochemical modes of action of vitamin E and selenium: a hypothesis. *FEBS Lett* **29**, 205–208.
- EDGEALL C.-J., MCDONALD C.C. & GRAHAM J.B. (1983) Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc.Natl.Acad. Sci.,USA* **80**, 734–737.
- FERNS G.A.A., FORSTER L.A., STEWART-LEE A.S., NOUROOZ-ZADEH J. & ANGGARD E.E. (1993) Probuconol inhibits mononuclear cell adhesion to the vascular endothelium in the cholesterol-fed rabbit. *Atherosclerosis* **100**, 171–181.
- FONG J.S. (1976) Alpha tocopherol; its inhibition on human platelet aggregation. *Experimentia* **32**, 639–641.
- FRATANTONI M.D. & POINDEXTER B.J. (1990) Measuring platelet aggregation with microplate reader. *Am J Clin Path* **94**, 613–617.
- FRIEDWALD W.T., LEVY R.J. & FREDRICKSON D.S. (1972) Estimation of low density lipoprotein cholesterol in plasma without the use of preparative ultracentrifugation. *Clin Chim Acta* **18**, 499–502.

- JANDAK J., STEINER M. & RICHARDSON P.D. (1989) Alpha-tocopherol an effective inhibitor of platelet adhesion. *Blood* **73**, 141–149.
- KAKISHITA E., SUEHIRO A., OURAY. & NAGAI K. (1990) Inhibitory effect of vitamin E on spontaneous aggregation in whole blood. *Throm Res* **60**, 489–499.
- KONNEH M., RUTHERFORD C., L.I.S.-R.ANGGARD E.E. & FERNS G.A.A. (1995) Vitamin E inhibits the intimal response to balloon catheter injury in the carotid artery of the cholesterol-fed rat. *Atherosclerosis* **113**, 29–39.
- LUNEC J. & BLAKE D.R. (1985) The determination of dehydroascorbic acid and ascorbic acid in the serum and synovial fluid of patients with rheumatoid arthritis. *Free Rad Res Comm* **1**, 31–39.
- MOWER R. & STEINER M. (1983) Biochemical interactions of arachidonic acid and vitamin E in human platelets. *Prostaglandins Leukotrienes Med* **10**, 389–403.
- PACKHAM M.A. & MUSTARD J.F. (1986) The role of platelets in the development and complications of atherosclerosis. *Semin. Haematol* **23**, 8–26.
- POBER J.S. (1988) Cytokine-mediated activation of vascular endothelium. *Amer. J. Path.* **133**, 426–433.
- POSTON R. & JOHNSON-TIDEY R.R. (1996) Localised adhesion of monocytes to human atherosclerotic plaques demonstrated *in vitro*. Implications to atherogenesis. *Am J Path* **149**, 73–80.
- PRINCEN H.M.G., VAN DUYNENVOORDE W., BUYTENHEK R., VAN DER LAARSE A., VAN POPPEL G., GEVERS LEUVEN J.A. & VAN HINSBERGH V.W.M. (1995) Supplementation with low doses of vitamin E protects LDL from lipid peroxidation in men and women. *Arterioscler. Thromb. Vasc. Biol.* **15**, 325–333.
- RADOMSKI M.W., PALMER R.M.J. & MONCADA S. (1987) The role of nitric oxide and cGMP in platelet and vascular endothelium. *Biochem Biophys Res Comm* **148**, 1482–1489.
- RIMM E.B., STAMPFER M.J., ASCHERIO A., GIOVANNISCU E., COLDITZ G.A. & WILLET W.C. (1993) Vitamin E consumption and the risk of coronary heart disease in men. *N Engl J Med* **328**, 1450–1456.
- ROSS R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature* **362**, 801–808.
- SALONEN J.T., SALONEN R., SEPPANEN K., RINTA-KIIKKA S., KUUKKA M., KORPELA H., ALFTHAN G., KANTOLA M. & SCALCH W. (1991) Effects of antioxidant supplementation on platelet function in a randomised pair-matched, placebo-controlled, double-blind trial with men with low antioxidant status. *Am J Clin Nutr* **53**, 1222–1229.
- SALVEMINI D. & BOTTING R. (1990) The effect of free radical scavengers on platelet adhesion and aggregation. *Drug News and Persp.* **3**, 202–212.
- SPRINGER T.A. (1990) Adhesion receptors of the immune system. *Nature* **346**, 425–434.
- SRIVASTAVA K.C. (1986) Vitamin E exerts anti-aggregatory effects without inhibiting the enzymes of the arachidonic acid cascade in platelets. *Prostaglan Leuk & Med* **21**, 177–185.
- STAMPFER M.J., JAKUBOWSKI J.A., FAIGEL D., VAILLANCOURT R. & DEYKIN D. (1988) Vitamin E supplementation effect on human platelet function, arachidonic acid metabolism and plasma prostacyclin levels. *Am. J. Clin. Nutr.* **47**, 700–706.
- STAMPFER M.J., HENNEKENS C.H., MANSON J.E., COLDITZ G.A., ROSNER B. & WILLET W.C. (1993) Vitamin E consumption and the risk of coronary heart disease in women. *N Engl J Med* **328**, 1444–1449.
- STEINBERG D., PARATHASARATHY S., CAREW T.E., KHOO J.C. & WITZTUM J.L. (1989) Beyond cholesterol. Modifications of low density lipoprotein that increases atherogenesis. *N Engl J Med* **20**, 915–924.
- STEINER M. (1981) Vitamin E changes the membrane fluidity of human platelets. *Biochim Biophys Acta* **640**, 100–105.
- STEINER M. (1983) Effects of alpha-tocopherol administration on platelet function in man. *Thromb & Haemostasis* **49**, 73–77.
- STEPHENS N.G., PARSONS A., SCHOFIELD P.M., KELLY F., CHEESEMAN K., MITCHISON M.J. & BROWN M.J. (1996) Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* **347**, 781–786.
- STEWART-LEE A.L., FORSTER L.A., NOUROOZ-ZADEH J., FERNS G.A.A. & ANGGARD E.E. (1994) Vitamin E protects against impairment of endothelium-mediated relaxations in cholesterol-fed rabbit. *Arterioscler.* **14**, 494–499.
- STEWART-LEE A.L., FERNS G.A.A. & ANGGARD E.E. (1995) Differences in the onset of impaired endothelial responses and the effects of vitamin E in the hypercholesterolaemic rabbit carotid and renal arteries. *J Cardiovasc Pharm* **25**, 906–913.
- STRINGER M.D., GOROG P.G., FREEMAN A. & KAKKAR V.V. (1989) Lipid peroxides and atherosclerosis. *Br Med J* **298**, 281–284.
- SZCZEKLIK A., GRYGLEWSKI R.J., DOMAGALA B., DWORSKI R. & BASISTA M. (1985) Dietary supplementation with vitamin E in hyperlipoproteinaemias: Effects on plasma lipid peroxides, antioxidant activity, prostacyclin generation and platelet aggregability. *Thromb Haemostasis* **54**, 425–430.
- TSCHOPE T.B. & BAUMGARTNER H.R. (1981) Platelet adhesion and mural thrombus formation on aortic subendothelium of rats, rabbits and guinea pigs correlate negatively with vascular PGI<sub>2</sub> production. *J Lab Clin Med* **98**, 402–411.
- VIOLIO F., PRACTICO D., GHISELLI A., ALESSANDRI C., IULIANO L., CORDOVA C. & BALSANO F. (1990) Inhibition of cyclo-oxygenase independent platelet aggregation by low vitamin E concentration. *Atherosclerosis* **82**, 247–252.