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Short Communication

Daily supplementation of tocotrienol-rich fraction or α-tocopherol did not induce immunomodulatory changes in healthy human volunteers

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Vitamin E is divided into two subgroups; tocopherols and tocotrienols. Both have protective roles in biological systems. The present study was conducted to compare the effect of short-term supplementation at 200 mg/d of either α-tocopherol or a tocotrienol-rich fraction (TRF) from palm oil on immune modulation and plasma vitamin E levels in normal healthy Asian volunteers. In a randomised, double-blind placebo-controlled trial conducted, fifty-three healthy volunteers aged 20–50 years were recruited based on the study's inclusion and exclusion criteria. They were randomly assigned into three groups, i.e. two experimental groups that received daily supplementation at 200 mg of either α-tocopherol or the TRF, and the control group that received a placebo. Blood was drawn on days 0, 28 and 56 for several laboratory analyses. Differences in the production of IL-4 or interferon-γ by concanavalin A-stimulated lymphocytes isolated from these volunteers were not significant ($P > 0{\cdot}05$). There were no significant differences observed in immune parameters between the healthy volunteers who received daily supplementation with either α-tocopherol or the TRF. As these observations were made in the absence of any immunogenic challenge, we feel it would be of benefit to study if there would be any differences observed when an immunogenic challenge such as vaccination were introduced.

Vitamin E: α-Tocopherol: Tocotrienol-rich fraction: Cytokines: Immune system

Vitamin E is a generic term that refers to a class of compounds that are divided into two subgroups called tocopherols and tocotrienols. The major sources of dietary tocopherols are plant oils such as wheat-germ oil, safflower-seed oil, maize oil and soyabean oil as well as nuts, for example, almonds, groundnuts and pistachios, while plant oils such as palm oil, rice bran oil, palm kernel oil, coconut oil, and cereal grains such as oats, barley, and rye are the main sources of tocotrienols[1]. Both forms of vitamin E are powerful lipid-soluble antioxidants[2] that can prevent lipid peroxidation[3], scavenge peroxyl radicals[4,5] and protect thalassaemic erythrocytes from undergoing premature lysis due to oxidative stress[6]. However, both groups also differ in their pharmacokinetics[7] and biological properties[8,9]. Recent reports show that tocotrienols may have more potent biological effects than α-tocopherol as these are more readily transferred between and incorporated into the cell membranes[10–12]. In addition, only tocotrienols could exert direct inhibitory and apoptotic effects on *in vitro* human breast cancer cell lines[13–15] and *in vivo* animal models[16,17]. The present scientific literature provides a body of evidence to support the idea that vitamin E plays an important role in human immunity[3,18–20]. Recently, it has been shown that the vitamin E concentration in lymphocytes is ten-times higher than in erythrocytes, underlining its importance to the immune system[21].

Most of the clinical studies on the effect of vitamin E supplementation on the human immune system have been using α-tocopherol. The present study is a pilot study aimed at comparing the effect of short-term supplementation of α-tocopherol or a tocotrienol-rich fraction (TRF) on immune modulation in healthy young Asian subjects. Currently, data regarding immune modulation by vitamin E on Asian populations are scarce. Studies based on Asians and ethnicity are also needed, as various ethnic populations have been reported to show differences in their immune subsets[22–24]. To date, no studies on the effect of tocotrienols on human immune modulation *in vivo* have been published.

Abbreviations: IFN-γ, interferon-γ; TRF, Tocotrienol-rich fraction.

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Subjects and methods

Study design and protocol

The present study is a double-blinded, randomised and placebo-controlled trial that was conducted in accordance with good clinical practice guidelines. Ethical approval for the study was obtained from the Ethics Committee of the International Medical University. Informed written consent was obtained from all volunteers participating in the study.

Healthy men (*n* 19) and women (*n* 34) aged 20–50 years were recruited. The subjects were informed of the objective and design of the study and the level of commitment required. All subjects had to give signed informed consent. The inclusion criteria for volunteer selection included age between 20–50 years; to be healthy and free of diseases; not to have organ failures; not on any treatment/medication; not taking vitamin A, C or E supplements in the last 3 months; non-smoker; not pregnant. The exclusion criteria included age below 20 years or above 50 years; to have health-related problems; have organ failures; on some treatment or medication; currently on vitamin A, C or E supplements; to be pregnant; have smoked within the past 1 month. Individuals whose blood pressure and BMI exceeded 120/80 mmHg and 30 kg/m^2, respectively, were also excluded from the study. These criteria are important as it has been reported that lymphocyte subsets, degenerative diseases and oxidative DNA damage can be influenced by smoking, sex differences, BMI and stress[25].

The subjects were randomly assigned to three groups. Overnight fasting blood samples were obtained from all subjects to establish pre-supplementation baseline levels of all the parameters that were to be tested in the present study. Subjects in one experimental group received 200 mg TRF/d (Tocovid SupraBio; Hovid Sdn Bhd, Ipoh, Malaysia) while the subjects in the second experimental group were given 200 mg α-tocopherol/d (Natural vitamin E; 21st Century HealthCare, Inc., Tempe, AZ, USA). The TRF contains 70 % tocotrienol (113 mg α-tocotrienol/g, 91 mg γ-tocotrienol/g, 36 mg δ-tocotrienol/g, 10 mg β-tocotrienol/g) and 30 % tocopherols (78 mg α-tocopherol/g, 0·5 mg β-tocopherol/g). The third group received placebo tablets. These doses were chosen based on a literature review on the safety of these supplements[26] and also on the availability of drugs that have been approved by the National Drug Control Authority of Malaysia. All subjects were asked to take one capsule daily after dinner for 56 d. Overnight fasting blood samples were also drawn from all subjects after 28 and 56 d of supplementation for investigations related to the present study.

Sample collection and analysis

Overnight fasting blood samples from all subjects who had fasted overnight were collected and processed within 2 h on days 0, 28 and 56. On days 0 and 56, 10 ml was withdrawn while only 5 ml was taken on day 28. Half of the blood drawn on days 0 and 56 was sent to an accredited pathology laboratory (PATHLAB, Kuala Lumpur, Malaysia) for various biochemical tests such as full blood count, Hb, blood cholesterol, blood lipids, blood sugar, liver function test and renal function test. The remaining 5 ml of blood collected in heparinised tubes were used for lymphocyte culture, flow-cytometry and plasma vitamin E analysis.

High-performance liquid chromatography

About 4·5 ml of the heparinised blood were centrifuged at 1500 rpm (10 min, 4°C) to separate plasma and cellular components. Plasma was stored at −30°C before analysis. About 1 ml of plasma taken on days 0, 28 and 56 was analysed by HPLC to determine plasma levels of α-tocopherol, tocotrienol and total vitamin E. Plasma levels of other isomers of vitamin E were also determined (data not shown). As described previously[27], a normal-phase HPLC system equipped with a silica column using Shimadzu LC-10AT (Shimadzu Corporation, Kyota, Japan) HPLC equipment was used.

Immunophenotyping by flow-cytometry analysis

About 500 μl of fresh heparinised blood were used for staining with the TriTEST reagents (2054; Becton Dickinson, NJ, USA) as recommended by the manufacturers. The percentages of lymphocyte subsets (total T-lymphocytes ($CD3^+$), $CD4^+$ and $CD8^+$T-lymphocytes, B-lymphocytes ($CD19^+$), natural killer cells ($CD16^+$ and/or $CD56^+$)) were obtained using a FACSCalibur flow cytometer located at the Department of Immunology, Institute for Medical Research, Kuala Lumpur. Data were analysed using the Multiset program that is used for this equipment.

Lymphocyte isolation and culture

The cellular component of blood obtained from centrifugation was treated with G-Dex™ II RBC Lysis Buffer (iNtRON Biotechnology, Seoul, South Korea) as recommended by the manufacturers to remove the erythrocytes. The leucocytes recovered by centrifugation (1000 rpm × 10 min; 4°C) were cultured in ninety-six-well flat-bottomed MICROTEST™ tissue culture plates (Falcon 3075; Becton Dickinson, NJ, USA) at 5×10^5 cells/per well in the presence of concanavalin A (Sigma-Aldrich, Inc., St Louis, MO, USA), a mitogen, at 50 μg/well for 72 h. Cultured cells were frozen (−30°C) until the analysis of cytokine production by ELISA was performed on the culture supernatant fraction.

Cytokine production by enzyme-linked immunosorbent assay

The frozen tissue culture plates were thawed and the contents transferred into sterile 1·5 ml microfuge tubes. The tubes were centrifuged at 1000 rpm for 10 min at 4°C. The supernatant fraction was transferred to fresh microfuge tubes. The pellet was discarded. The supernatant fraction was analysed to quantify the amount of IL-4 and interferon-γ (IFN-γ) using commercial ELISA kits for human IL-4 and IFN-γ (eBioscience, San Diego, CA, USA) as recommended by the manufacturers. The limit of sensitivity for the detection of IFN-γ and IL-4 was 8 and 4 pg/ml respectively.

Statistical analysis

Data are presented as the mean values and standard deviations. All statistical analyses were carried out using SPSS *v.* 11.5 for

Windows software (SPSS Inc., Chicago, IL, USA). Data were first entered into Microsoft® Excel (Microsoft Corp., Berkeley, CA, USA) and the database was converted into SPSS files for treatment (removal) of outliers, assumptions testing (for example, for normality, homogeneity of inter-correlations, sphericity of data) and descriptive statistical analyses. Finally, either split-plot ANOVA or one-way ANOVA was used to analyse the data for differences between the three experimental groups (TRF, α-tocopherol or placebo).

Results

Effect of vitamin E supplementation on plasma levels of α-tocopherol, tocotrienol and total vitamin E

The baseline levels of total plasma vitamin E and plasma α-tocopherol amongst the volunteers, though high, were similar (Table 1). There was a significantly higher ($P<0·001$) increase in the total vitamin E levels in the plasma of volunteers who took either α-tocopherol or TRF supplementation when compared with those taking the placebo. The highest total plasma vitamin E levels were found to be in the volunteers supplemented with the TRF (Table 1). Plasma α-tocopherol levels also significantly ($P<0·001$) increased from day 0 to day 56 in the volunteers taking the α-tocopherol supplementation (Table 1). The group that was taking the TRF had higher levels of α-tocopherol compared with those taking the placebo. There was a gradual decline of plasma tocotrienol levels in the placebo-supplemented volunteers. In contrast, plasma levels of tocotrienol in the volunteers who took either α-tocopherol or TRF supplementation increased when the blood was analysed on day 28. In both these groups, plasma levels of tocotrienol dropped slightly (from 0·62 to 0·5 pg/ml) on day 56. *Post hoc* testing for differences in plasma total vitamin E between experimental groups showed that there was a significant ($P<0·001$) difference between the placebo and the two vitamin E groups. However, there was no significant ($P>0·05$) difference between the two experimental groups. There was a significant ($P<0·05$) difference in plasma α-tocopherol levels between the α-tocopherol supplementation and placebo ($P<0·001$) and TRF-supplemented group ($P>0·05$). In contrast, there was no significant difference in plasma tocotrienol between the two experimental groups.

Effect of vitamin E supplementation on T-lymphocytes, B-lymphocyte and natural killer cell count

There was no measurable difference in the CD4, CD8, B-cell or natural killer cell count between volunteers who received placebo, α-tocopherol or TRF supplementation (Table 2). When the percentages of these cells on days 0, 28 and 56 were compared, again there was no measurable difference. The standard deviation amongst the groups is rather wide, making it difficult to draw useful conclusions from the data. There was no difference in the ratio of CD4:CD8 cells amongst all the three groups. It should be noted here that normality tests performed on the data showed that the distribution of lymphocytes (T-, B-, $CD4^+$ or $CD8^+$) as a percentage of leucocytes data were normal (AK Radhakrishnan, AL Lee and K Nesaretnam, unpublished results). Skewness and kurtosis of the data were within the normal range of -1 and 1. In addition, Box's M had a P value that was not significant ($P=0·283$), therefore there was homogeneity of data between the groups.

Effect of vitamin E supplementation on the production of cytokines by mitogen-stimulated lymphocytes in culture

Lymphocytes isolated from the peripheral blood of all volunteers on days 0, 28 and 56 were cultured in the presence of concanavalin A for 72 h as described in the Subjects and methods section. The lymphocytes were cultured for 72 h based on results from preliminary studies to determine the optimal duration for culture (AK Radhakrishnan, AL Lee and K Nesaretnam, unpublished results). There was a reduction in IL-4 production by the concanavalin A-stimulated lymphocytes taken from the volunteers of all three groups (Fig. 1 (a)). This reduction was maintained even at day 56. In contrast there was a sharp increase in the levels of IL-10

Table 1. Plasma levels of α-tocopherol, tocotrienol and vitamin E before and after vitamin E supplementation determined using HPLC*
(Mean values and standard deviations)

			α-Tocopherol (μg/ml)		Tocotrienol (μg/ml)		Total vitamin E (μg/ml)			
Group	*n*	Day	Mean	SD	Mean	SD	Mean	SD	*P* value for within-subjects effects†	*F* value†
Placebo	17	0	7·10	3·67	0·72	0·44	8·48	4·12	0·195	1·553
		28	9·30	2·94	0·44	0·44	10·11	2·89		
		56	10·09	3·17	0·38	0·22	10·88	3·37		
α-tocopherol	15	0	7·87	3·97	0·48	0·31	8·87	4·23	<0·001	6·415
		28	15·82	5·69	0·77	0·74	16·77	6·1		
		56	17·92	6·59	0·60	0·33	18·71	6·8		
TRF	16	0	6·68	2·91	0·58	0·38	7·62	3·39	<0·001	7·7074
		28	10·70	2·80	0·72	0·63	11·75	3·12		
		56	12·09	2·85	0·58	0·39	13·12	3·14		

TRF, tocotrienol-rich fraction.
* Mauchly's test tests the null hypothesis that the error covariance matrix of the orthonormalised transformed dependent variables is proportional to an identity matrix. The sphericity assumption is not violated if the within-subjects effect of time is not significant ($P>0·05$).
† Results were considered significant if $F>1$ and if $P<0·05$. The P value for the between-groups effect is $>0·05$, i.e. statistically not significant.

British Journal of Nutrition

Table 2. Comparison of the number (%) of T-helper lymphocytes ($CD4^+$) and cytotoxic T-lymphocytes ($CD8^+$), B-lymphocytes ($CD19^+$) and natural killer (NK) cells in the peripheral blood of human volunteers who received α-tocopherol (α-T), a tocotrienol-rich fraction (TRF) or placebo for 56 d*
(Mean values and standard deviations)

		Immune cells									
		T-helper		Cytotoxic T-lymphocytes		CD4:CD8 ratio		B-lymphocytes		NK cells†	
Group	Day	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Placebo	0	37·88	4·78	28·88	5·12	1·31	0·39	10·59	2·79	14·72	7·77
	28	37·39	6·83	29·12	5·94	1·33	0·43	11·13	2·45	12·00	4·62
	58	36·25	5·92	29·76	6·55	1·25	0·41	11·94	3·63	14·13	6·47
α-T	0	38·36	4·92	27·60	6·79	1·45	0·57	11·00	4·03	14·19	7·99
	28	38·46	4·35	26·21	5·10	1·49	0·48	12·21	3·51	13·71	7·88
	58	36·71	4·76	26·71	5·93	1·37	0·42	13·43	3·27	14·13	7·46
TRF	0	36·74	7·73	30·78	6·63	1·25	0·46	10·78	3·54	13·89	5·66
	28	35·83	7·24	30·29	6·42	1·24	0·47	12·53	3·83	13·76	4·22
	58	38·25	6·30	29·87	6·70	1·34	0·48	11·94	3·68	11·73	4·65

* Analysis was performed on heparinised blood taken on days 0, 28 and 56, using the FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using the Multiset program.
† $CD56^+$ and/or $CD16^+$.

(Fig. 1 (b)) and IFN-γ (Fig. 1 (c)) produced by the concanavalin A-stimulated lymphocytes following 72 h of culture between days 0 and 28. However, the levels of both cytokines decreased on day 56. It was of interest to note that the amount of IFN-γ produced by the mitogen-stimulated lymphocytes from the volunteers supplemented with just the placebo also rose on day 28 and dropped on day 56.

Discussion

The analysis of plasma levels of total vitamin E, α-tocopherol and TRF by HPLC in the volunteers showed that there was compliance amongst the volunteers in taking their supplements. The highest plasma α-tocopherol levels were observed in the α-tocopherol-supplemented volunteers (6·58 pg/ml on day 0 to 16·53 pg/ml on day 56). There was a modest increase in the plasma levels of α-tocopherol in the volunteers who took TRF supplementation (6·41 pg/ml on day 0 to 11·66 pg/ml on day 56). Similar observations have been previously reported[27,28]. The analysis also showed that time had a significant effect, with the levels in the plasma increasing over time following supplementation, which were further confirmed by the highly significant ($P<0·05$) interaction effects. Thus there was compliance in the α-tocopherol test subjects. As the TRF supplements also contained a substantial proportion of α-tocopherol (30 %), a rise in plasma α-tocopherol was also observed in this group. Hence, it was suggestive that there was high compliance amongst the TRF group. The increase in plasma tocotrienol levels in the TRF-supplemented volunteers was not statistically significant ($P>0·05$). This finding was in agreement with a report by Mustad *et al.*[29] but in contrast with the findings of Mensink *et al.*[28] who reported an increased tocotrienol concentrations following supplementation. Tocotrienols have been reported to have a short elimination half-life and low bioavailability[7]. So, it is not surprising that we did not observe increased levels of plasma tocotrienols. Studies in the rat have shown that there is a preferential deposition of tocotrienols in the tissues, especially adipose tissues[30].

Flow-cytometry analysis showed that neither TRF nor α-tocopherol supplementation had a statistically significant effect on the percentages of different blood leucocytes (lymphocytes, T-cells, T-helper cells, CD8 T-cells, B-cells and natural killer cells). These results were not in agreement with the findings reported by Lee & Wan[21] and Penn *et al.*[20], who both showed that α-tocopherol supplementation enhanced the proliferation of total T-cells, T-helper cells, as well as the CD4:CD8 ratio but in agreement with Meydani *et al.*[12], who reported that enhancement of the immune response following α-tocopherol supplementation could only demonstrated in the elderly population. So, it was not surprising that the present results differed from Penn *et al.*[20] as the volunteers in the present study were young healthy volunteers. As our sample size was similar to Lee & Wan[21], the difference in results could be due to different population or differences in diet or immune status.

Further analysis of the data using split-plot ANOVA suggested that the type of supplementation did influence the proportion of lymphocytes in the leucocytes; but this difference was not significant statistically. The split-plot ANOVA analyses also showed that time had a very strong effect on all three cytokines, influencing their concentrations whereas the effects of any interaction between the experimental group variable and time were all not significant.

Taken together, these results show that vitamin E did not have any significant effect on immune modulation in healthy young subjects, unlike as reported in the Lee & Wan[21] study. The sample number used in the present study is similar, if not higher, than that used in the Lee & Wan[21] study. However, the lack of statistical significance could still be due to sample size, differences in the population or nutrition amongst the volunteers. In addition, each volunteer may be exposed to different types of foreign substances and this makes it difficult to observe statistically significant changes to the immune system, as in the current model there is no common specific antigens to which specific immune responses could be measured. For future studies, we propose that a specific immunogenic challenge such as a vaccine should be

British Journal of Nutrition

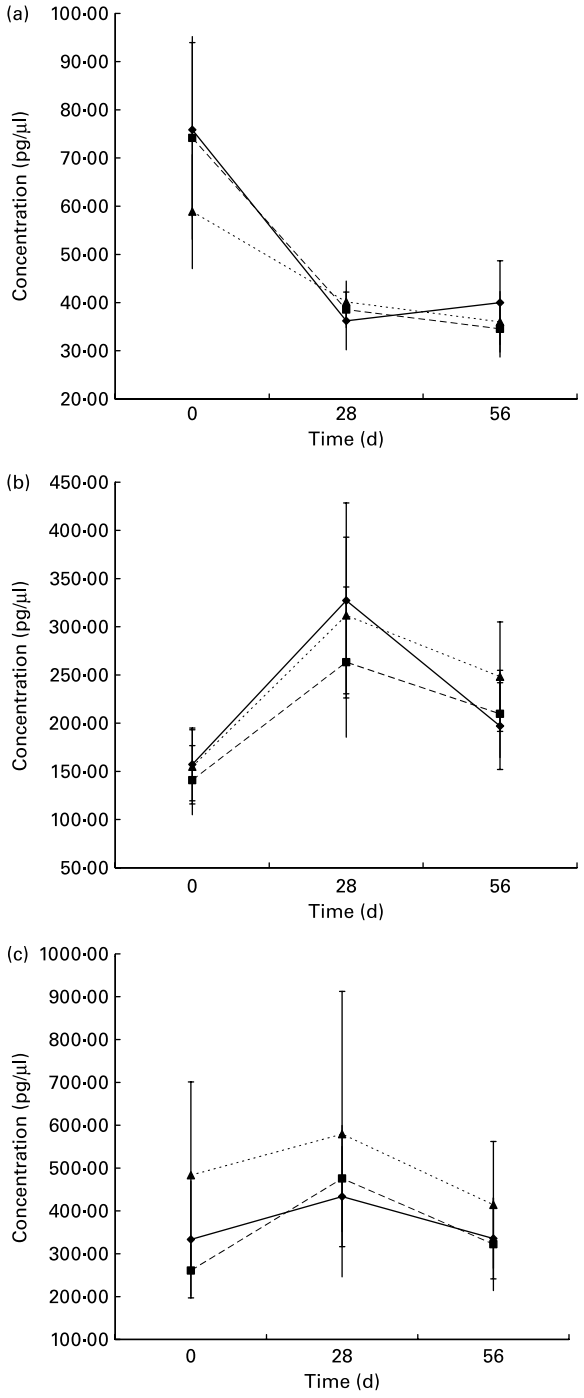

Fig. 1. The levels of (a) IL-4, (b) IL-10 and (c) interferon-γ (IFN-γ) in the culture supernatant fraction obtained from the concanavalin A-stimulated culture of lymphocytes isolated from the peripheral blood of human volunteers who received α-tocopherol (■), a tocotrienol-rich fraction (▲) or a placebo (◆) for 56 d were determined using ELISA. Analysis was performed on the supernatant fraction obtained following 72 h of culture on the lymphocytes isolated from heparinised blood taken on days 0, 28 and 56. Values are means, with 95 % CI represented by vertical bars. Statistical analysis showed that the 'within-subject effect' (time) for IFN-γ, IL-4 and IL-10 production was statistically significant ($P<0·001$) while the 'between-subjects effect' (group) and 'interaction effect' (time *v.* groups) were not statistically significant.

administered to volunteers and the specific immune response to the vaccine be measured. This approach might provide clearer answers to the present research question.

In conclusion, the present study shows that there is no significant difference in immune modulation between healthy Asian volunteers supplemented daily with either α-tocopherol or a TRF.

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A. K. R and K. N. conceived and designed the study while A. L. L., A. K. R., P. F. W., J. K. and H. A. implemented the study. A. R. was involved in project coordination and performed the flow-cytometry analysis; A. L. L., P. F. W. and H. A. were responsible for volunteer screening, i.e. physical examination and blood-taking as well as volunteer enrolment and monitoring; A. L. L. performed most of the lymphocyte culture, ELISA and data analysis; K. N. coordinated measurements of plasma vitamin E levels by HPLC; A. R. supervised the data management; A. L. L. analysed the data; A. R. wrote the manuscript; and K. N. contributed to the writing and editing of the manuscript. None of the authors had any personal or financial conflicts of interest.

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