Short Communication

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

Ammu K. Radhakrishnan1*, Ai-Ling Lee1, Pak-Fong Wong2, Jagmohni Kaur3, Htin Aung3 and Kalanithi Nesaretnam4

1Department of Pathology, International Medical University, 126, Jalan 19/155B, Bukit Jalil, 57000 Kuala Lumpur, Malaysia
2Department of Nursing, International Medical University, 126, Jalan 19/155B, Bukit Jalil, 57000 Kuala Lumpur, Malaysia
3Clinical Skills Unit, Faculty of Medicine, Pharmacy and Health Sciences, International Medical University, 126, Jalan 19/155B, Bukit Jalil, 57000 Kuala Lumpur, Malaysia
4Department of Nutrition, Malaysian Palm Oil Board, 6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor Darul Ehsan, Malaysia

(Received 8 January 2008 – Revised 4 June 2008 – Accepted 19 June 2008 – First published online 15 August 2008)

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>0.05). 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 palm oil 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. Both forms of vitamin E are powerful lipid-soluble antioxidants that can prevent lipid peroxidation, scavenge peroxyl radicals and protect thalassaemic erythrocytes from undergoing premature lysis due to oxidative stress. However, both groups also differ in their pharmacokinetics and biological properties. Recent reports show that tocotrienols may have more potent biological effects than α-tocopherol as these are more readily transferred between cell membranes and incorporated into the cell membranes, underlining its importance to the immune system. The present scientific literature provides a body of evidence to support the idea that vitamin E plays an important role in human immunity. 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.

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. To date, no studies on the effect of tocotrienols on human immune modulation have been published.

Abbreviations: IFN-γ, interferon-γ; TRF, Tocotrienol-rich fraction.
* Corresponding author: Dr Ammu Kutty Radhakrishnan, fax +60 3 86567229, email ammu_radhakrishnan@imu.edu.my
**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; not taking placebo tablets. These doses were chosen based on previous(27), a normal-phase HPLC system equipped with a silica column using Shimadzu LC-10AT (Shimadzu Corporation, Kyoto, 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 leukocytes 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 × 10⁵ cells/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
British Journal of Nutrition

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

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Day</th>
<th>α-Tocopherol (µg/ml)</th>
<th>Tocotrienol (µg/ml)</th>
<th>Total vitamin E (µg/ml)</th>
<th>$P$ value for within-subjects effects†</th>
<th>$F$ value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>17</td>
<td>0</td>
<td>7.10 3.67</td>
<td>0.72 0.44</td>
<td>8.48 4.12</td>
<td>0.195</td>
<td>1.553</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>9.30 2.94</td>
<td>0.44 0.44</td>
<td>10.11 2.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>0</td>
<td>10.09 3.17</td>
<td>0.38 0.22</td>
<td>10.88 3.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>15</td>
<td>0</td>
<td>7.87 3.97</td>
<td>0.48 0.31</td>
<td>8.87 4.23</td>
<td>&lt;0.001</td>
<td>6.415</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>15.82 5.89</td>
<td>0.77 0.74</td>
<td>16.77 6.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>0</td>
<td>17.92 6.59</td>
<td>0.60 0.33</td>
<td>18.71 6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRF</td>
<td>16</td>
<td>0</td>
<td>6.68 2.91</td>
<td>0.58 0.38</td>
<td>7.62 3.39</td>
<td>&lt;0.001</td>
<td>7.7074</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>10.70 2.80</td>
<td>0.72 0.63</td>
<td>11.75 3.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>0</td>
<td>12.09 2.85</td>
<td>0.58 0.39</td>
<td>13.12 3.14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.
Flow-cytometry analysis showed that neither TRF nor \( \alpha \)-tocopherol supplementation had a statistically significant effect on the percentages of different blood leucocytes (lymphocytes, T-cells, T-helper 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 \( \alpha \)-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 \( \alpha \)-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

### 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 \( \alpha \)-tocopherol (\( \alpha \)-T), a tocotrienol-rich fraction (TRF) or placebo for 56 d*

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>0</td>
<td>37.88</td>
<td>4.78</td>
<td>28.88</td>
<td>5.12</td>
<td>1.31</td>
<td>0.39</td>
<td>10.59</td>
<td>2.79</td>
<td>14.72</td>
<td>7.77</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>37.39</td>
<td>6.83</td>
<td>29.12</td>
<td>5.94</td>
<td>1.33</td>
<td>0.43</td>
<td>11.13</td>
<td>2.45</td>
<td>12.00</td>
<td>4.62</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>36.25</td>
<td>5.92</td>
<td>29.76</td>
<td>6.55</td>
<td>1.25</td>
<td>0.41</td>
<td>11.94</td>
<td>3.63</td>
<td>14.13</td>
<td>6.47</td>
</tr>
<tr>
<td>( \alpha )-T</td>
<td>0</td>
<td>38.36</td>
<td>4.92</td>
<td>27.60</td>
<td>6.79</td>
<td>1.45</td>
<td>0.57</td>
<td>11.00</td>
<td>4.03</td>
<td>14.19</td>
<td>7.99</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>38.46</td>
<td>4.35</td>
<td>26.21</td>
<td>5.10</td>
<td>1.49</td>
<td>0.48</td>
<td>12.21</td>
<td>3.51</td>
<td>13.71</td>
<td>7.88</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>36.71</td>
<td>4.76</td>
<td>26.71</td>
<td>5.93</td>
<td>1.37</td>
<td>0.42</td>
<td>13.43</td>
<td>3.27</td>
<td>14.13</td>
<td>7.46</td>
</tr>
<tr>
<td>TRF</td>
<td>0</td>
<td>36.74</td>
<td>7.73</td>
<td>30.78</td>
<td>6.63</td>
<td>1.25</td>
<td>0.46</td>
<td>10.78</td>
<td>3.54</td>
<td>13.89</td>
<td>5.66</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>35.83</td>
<td>7.24</td>
<td>30.29</td>
<td>6.42</td>
<td>1.24</td>
<td>0.47</td>
<td>12.53</td>
<td>3.83</td>
<td>13.76</td>
<td>4.22</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>38.25</td>
<td>6.30</td>
<td>29.87</td>
<td>6.70</td>
<td>1.34</td>
<td>0.48</td>
<td>11.94</td>
<td>3.68</td>
<td>11.73</td>
<td>4.65</td>
</tr>
</tbody>
</table>

* 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\(^+\).

Discussion

The analysis of plasma levels of total vitamin E, \( \alpha \)-tocopherol and TRF by HPLC in the volunteers showed that there was compliance amongst the volunteers in taking their supplements. The highest plasma \( \alpha \)-tocopherol levels were observed in the \( \alpha \)-tocopherol-supplemented volunteers (6.58 pg/ml on day 0 to 11.66 pg/ml on day 56). There was a modest increase in the plasma levels of \( \alpha \)-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 \( \alpha \)-tocopherol test subjects. 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
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 \( \alpha \)-tocopherol or a TRF.

Acknowledgements

The authors thank Dr Jasbir Singh Dhaliwal for allowing us to use the flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) located at the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia, Mr Ghazali of the Malaysian Palm Oil Board (MPOB) for his technical assistance with the HPLC analysis and Dr R. Krishnan of the International Medical University for her help with blood-taking from the volunteers. The present study was supported by research grants from the International Medical University and the MPOB. The soft-gel TRF and placebo capsules were a gift of Tocovid SupraBio, Hovid Sdn. Bhd., Ipoh, Malaysia.

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.

References


