Palm Oil γ-tocotrienol and α-tocopherol Act as Potent Inducers in the Immune Response of Mouse splenocytes *in vitro*

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ABSTRACT

Vitamin E may have anti carcinogenesis effect in human and animal models via the mechanism of cell cycle arrest and enhancement of immune system. The cell-mediated immune inducing properties of palm oil vitamin E, for example, γ-tocotrienol and α-tocopherol, were investigated by measuring the mitogenesis response of splenocytes, extracted from normal male Mus musculus to splenic T-lymphocytes mitogens, phytohemagglutinin (PHA; 0.25 µg/mL) and concanavalin A (Con A; 1.0 µg/mL); and B-lymphocytes mitogen i.e., lipopolysaccharide (LPS; 1.0 µg/mL). Both γ-tocotrienol and α-tocopherol enhanced the cell proliferation of mitogen untreated splenocytes as determined by 5-Bromo-2'-deoxy-uridine (BrdU) detection method. Both compounds also enhanced the T-lymphocytes response to PHA and Con A, as well as B-lymphocytes responses to LPS at all concentration used (0-300 µM). γ-Tocotrienol was observed to affect cell proliferation more than α-tocopherol. The uptake of γ-tocotrienol and α-tocopherol into the splenocytes was determined by high performance liquid chromatography (HPLC). γ-Tocotrienol was absorbed into the cells at markedly higher levels than α-tocopherol with the ratio of 4.8 : 1 ($p<0.01$, $n=4$) at 300 µM of treatment. This may be the reason of the higher proliferation affect of γ-tocotrienol as compared to α-tocopherol. In conclusion, we are of the opinion that palm oil γ-tocotrienol and α-tocopherol are able to

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synergistically influence splenocytes proliferation thus enhancing the cell-mediated immune system.

Keywords: *Gamma-tocotrienol, α-tocopherol, splenocytes, immune response*

INTRODUCTION

The immune system has the ability to identify, destroy and defend the body from viral, bacterial infections and growth of tumor cells [1]. The ability of T-lymphocytes in recognizing and eliminating tumor antigens and enhancement of antibodies produced by B-lymphocytes is crucial in the enhancement of immune response [2]. The incidence of viral-associated cancers especially in Human Herpes Virus 8-associated Kaposi's sarcoma, Epstein-Barr virus-associated Non-Hodgkin's lymphoma and HPV-associated squamous carcinoma are enhanced in immune-suppressed patients' due to the lack of protection against viral infections or reactivation in the absence of T cells [3, 4]. Other studies using Rag2-/- mice, which lack B and T-lymphocytes, have reported to develop a 35 % and 15 % of spontaneous malignancies in the intestine and lungs respectively [5].

Approximately, 30 % to 40 % of all cancer types can be averted with a proper diet and healthy lifestyle [6, 7]. Vitamin E, in combination with omega-3 polyunsaturated fatty acids, restores immunodeficiency and prolongs the survival rate of severely ill patients with generalized malignancy [8]. Daily supplementation of alpha-tocopherol caused one third reduction in the incidence of prostate cancer among Finnish men [9]. Other study demonstrated that gamma-tocopherol-rich mixture of tocopherols efficiently inhibits the tumorigenesis of colon, prostate, mammary and lung in animal models [10]. The possible mechanism involved in the inhibition of carcinogenesis may be through the inhibition of cell cycle and enhancement of immune system. Alpha-tocopheryl succinate has the ability to induce a prolonged S phase, thus contributes to sensitization of cancer cells to drugs destabilizing DNA during replication [11]. It also exhibits a cooperative antitumor effect in combination with immunological agents by inducing INF-gamma production in $CD4^+$ and $CD8^+$ T lymphocytes, resulting in a significant tumor growth inhibition or in complete tumor regression [11].

The present study demonstrated that palm oil vitamin E isomers *i.e.*, γ-tocotrienol and α-tocopherol could enhance the mitogenesis of normal splenic T- and B-lymphocytes extracted from male *Mus musculus*. The study thus indicated their role in inducing cell-mediated immune response.

MATERIALS AND METHODS

Cell Culture

Splenocytes, freshly extracted from normal male *Mus musculus* (Animal Unit, Institute of Medical Research, Malaysia), were cultured in RPMI. It was supplemented with 10% fetal bovine serum, 20 mM Hepes, 20 mM sodium bicarbonate, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 1% penicillin and streptomycin. Culture media and the above chemicals were purchased from FLOWLAB, Sydney, Australia.

Vitamin E Treatment

Palm oil γ-tocotrienol and α-tocopherol of 80 % concentration (single peak by high performance liquid chromatography) were obtained from the Malaysian Palm Oil Board. Stock solutions of both compounds were dissolved in absolute alcohol at 500 μM and then diluted. This was to the final concentration of alcohol in the culture media < 0.1%.

Mitogenesis of Splenocytes

A total of 2×10^4 cells were cultured in 100 μl medium and plated into each well of a 96-well plate. The cells were treated separately with different final concentrations of γ-tocotrienol and α-tocopherol (0, 10 μM, 50 μM, 100 μM, 150 μM, 200 μM and 300 μM) for 48 hours. Mitogens such as PHA (0.25 μg/mL), LPS (1.0 μg/mL) and Con A (1.0 μg/mL) were added separately into each well and the cells were incubated in 5 % CO_2 at 37 °C for 24 hours. The effect of both compounds on cell mitogenesis was determined using a 5-Bromo-2'-deoxy-uridine (BrdU) labeling and detection method (Roche Diagnostics Corporation, USA). The assays were repeated 4 times and in triplicates. Mitogens used were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Vitamin E Uptake

Cells were plated at 10×10^6 into 6 microtiter well plates and treated with γ-tocotrienol and α-tocopherol at different final concentrations of 0-300 μM for 24 hours. The cells were collected and washed thrice with ice-cold phosphate buffer saline (10 mM NaF, 0.9 % NaCl, pH7.2). A 50 μL of 10 mg/mL β-hydroxytoluene was added to prevent oxidation of vitamin E's isomers. Cells pellets were collected, washed, dissolved with 100 μL of 95% ethanol and β-hydroxytoluene and sonicated for 40 seconds. The samples were then dissolved with 100 μL of high performance liquid chromatography grade-hexane containing β-hydroxytoluene, centrifuged and the top phase was air dried. The samples were finally dissolved in 100 μL of hexane, filtered with 0.4 μm nitrocellulose membrane and injected into a 150×4.6 mm normal silica column with a mobile phase of hexane:methanol (99.75 : 0.25) at a rate of 1.50 mL/min. Vitamin E isomers were quantified with a fluorescence detector at 294 nm and emission detection at 330 nm. Assays were repeated four times and in triplicates. All the above chemicals were purchased from Sigma Chemical Company.

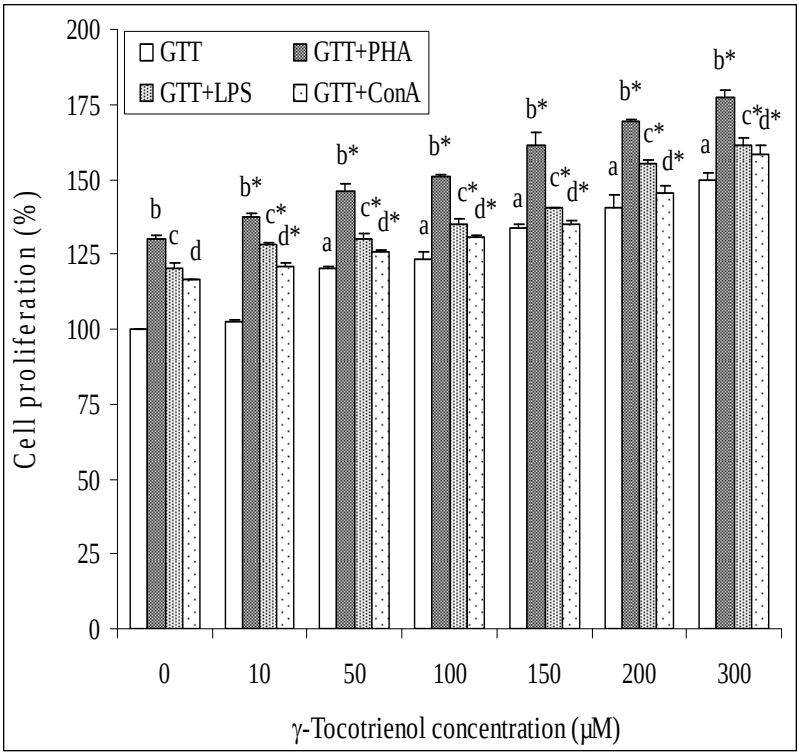
Statistical Analysis

ANOVA test was used to compare between the control and the treated cells at different concentrations of treatment on the measured parameters. Each treatment was conducted in triplicates (Technical, $n = 3$) and repeated four times (Biological, $n = 4$). The sample size was 12 with the significance set at $p < 0.05$.

RESULTS

Mitogenic Responses of Splenocytes

The effect of γ-tocotrienol and α-tocopherol on the mitogenesis of splenic T-lymphocytes (PHA and Con A) and B-lymphocytes (LPS) were investigated by determining the BrdU incorporation to freshly synthesized cellular DNA at S phase after 24 hours of treatment.



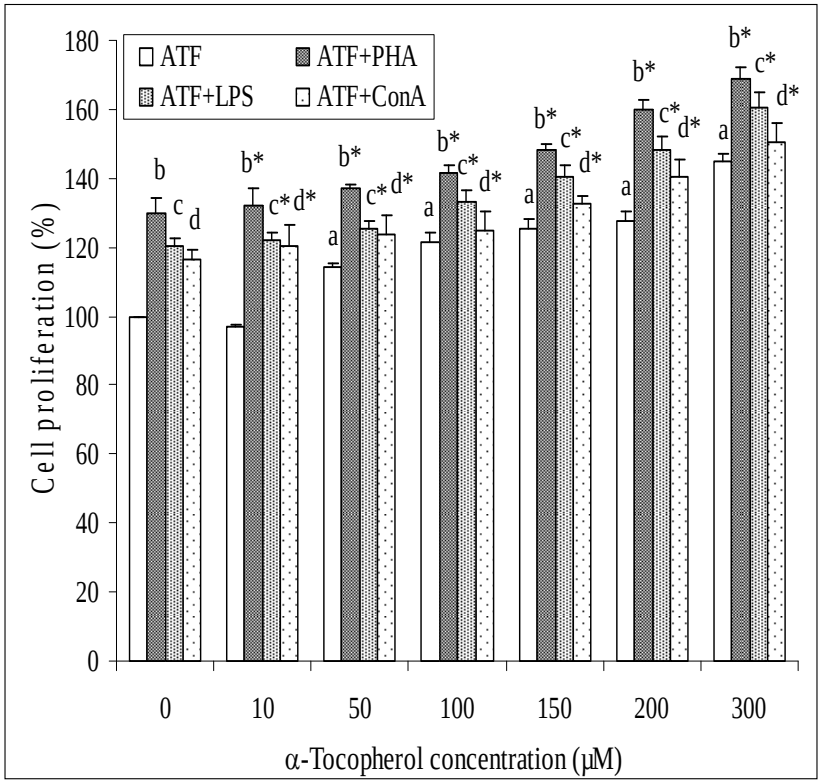
a significant as compared to control without γ-tocotrienol and mitogen at p<0.05.
b,c,d significant as compared to control without γ-tocotrienol and mitogen at p<0.01.
* significant as compared to control without γ-tocotrienol but in the presence of mitogen at p<0.01.

Figure 1a: Effect of γ-tocotrienol (GTT) on the Mitogenic Responses of Splenic T- and B-lymphocytes via BrdU Detection

Gamma-tocotrienol enhanced the T-lymphocytes response to PHA, LPS and Con A by 37.6 % to 89.4 % ($p < 0.01$, $n = 4$), 28.5 % to 76.2 % ($p < 0.01$, $n = 4$) and 20.7 % to 70.8 % ($p < 0.01$, $n = 4$), respectively at all concentrations used.

The proliferation of splenocytes was unaffected by the treatment with 10 μM of γ-tocotrienol. Interestingly, γ-tocotrienol enhanced the cell proliferation by 20.4 % to 58.4 % ($p < 0.05$, $n = 4$) beginning with a dose of 50 μM and above after 24 hours. The addition of both PHA and γ-tocotrienol

increased the proliferation response by 37.6 % to 89.4 % ($p < 0.01$, $n = 4$) as compared to 30.0 % ($p < 0.01$, $n = 4$) increment with treatment of PHA alone. While the addition of both LPS and γ-tocotrienol had enhanced the proliferation response by 28.5 % to 76.2 % ($p < 0.01$, $n = 4$) as compared to 20.4 % ($p < 0.01$, $n = 4$) increment with the treatment of LPS alone. Con A and γ-tocotrienol caused a 20.7 % to 70.8 % ($p < 0.01$, $n = 4$) increment in the proliferation response as compared to 16.5 % ($p < 0.01$, $n = 4$) with treatment of Con A alone. PHA was the most potent inducer to T-lymphocytes as compared to Con A ($p < 0.01$, $n = 4$) (Figure 1a).



a significant as compared to control without α-tocopherol and mitogen at $p<0.05$.
b,c,d significant as compared to control without α-tocopherol and mitogen at $p<0.01$.
* significant as compared to control without α-tocopherol but in the presence of mitogen at $p<0.01$.

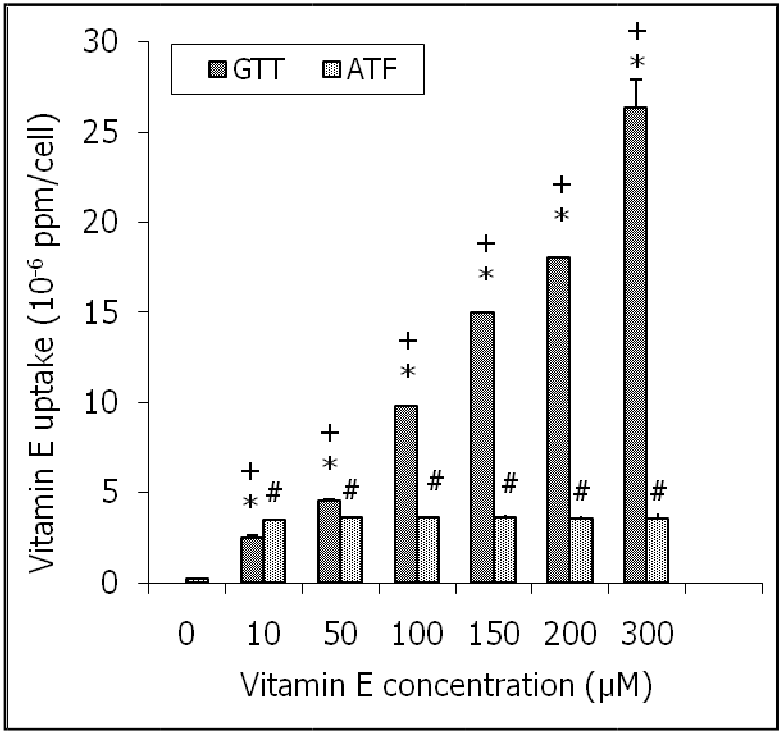
Figure 1b: Effect of α-tocopherol (ATF) on the Mitogenic Responses of Splenic T- and B-lymphocytes via BrdU Detection

The Alpha-tocopherol enhanced the T-lymphocytes response to PHA, LPS and Con A by 32.1 % to 75.4 % ($p < 0.01$, $n = 4$), 22.0 % to 65.1 % ($p < 0.01$, $n = 4$) and 20.1 % to 55.3 % ($p < 0.01$, $n = 4$), respectively at all concentrations used.

Similarly to γ-tocotrienol, treatment with α-tocopherol at the lowest dose of 10 μM also had no effect in the proliferation of splenocytes. But at the higher concentration of 50 μM to 300 μM, the α-tocopherol enhanced the cell proliferation by 14.2 % to 51.9 % ($p < 0.05$, $n = 4$) after 24 hours of treatment. The addition of both PHA and α-tocopherol had increased the proliferation response by 32.1 % to 75.4 % ($p < 0.01$, $n = 4$) as compared to 30.0 % ($p < 0.01$, $n = 4$) increment with treatment of PHA alone. While addi both LPS and α-tocopherol had increased the proliferation response by 22.0% to 65.1% ($p < 0.01$, $n = 4$) as compared to 20.4 % ($p < 0.01$, $n = 4$) increment with a treatment of just LPS. Con A and α-tocopherol caused a 20.1 % to 55.3 % ($p < 0.01$, $n = 4$) increment in the proliferation as compared to 16.5% ($p < 0.01$, $n = 4$) with treatment of only Con A (Figure 1b).

Vitamin E Uptake by Splenocytes

To ensure that both compounds were taken up by splenocytes, each concentration of treatment was analysed using a high performance liquid chromatography.



* significant as compared to control without γ-tocotrienol at p<0.01.
\# significant as compared to control without α-tocopherol at p<0.01.
\+ significant as compared between γ-tocotrienol and α-tocopherol at each concentration of treatment at p<0.01.

Figure 2: Evaluation of γ-tocotrienol (GTT) and α-tocopherol (ATF) Uptake by Splenocytes via High Performance Liquid Chromatography Detection

Gamma-tocotrienol was taken up most efficiently in a dose-dependent manner by splenocytes as compared to α-tocopherol with the ratio of 4.8 : 1 at 300 μM ($p < 0.01$, $n = 4$) of treatment.

Gamma-tocotrienol and α-tocopherol were taken-up by splenocytes in a dose-dependent manner at all concentrations used (Figure 2). However, the extent of the cellular uptake varied between both compounds. The increment uptake of γ-tocotrienol by cells correlated with all the concentrations used (p

< 0.01, n = 4), while α-tocopherol showed plateau increment. γ-tocotrienol was taken up most efficiently by splenocytes as compared to α-tocopherol with the ratio of 4.8:1 ($p < 0.01$, $n = 4$) at 300 μM of treatment.

DISCUSSIONS

Our laboratory experiments demonstrated that both palm oil γ-tocotrienol and α-tocopherol, at increasing concentrations of 50 μM to 300 μM, efficiently enhanced the proliferation of splenocytes by 20.4 % to 58.4 % ($p < 0.05$, $n = 4$) and 14.2 % to 51.9 % ($p < 0.05$, $n = 4$) respectively (Figure 1a and b, respectively). Meanwhile, the treatment with γ-tocotrienol and α-tocopherol at increasing concentrations of 50 μM to 300 μM, were observed also to had enhanced the proliferation of T-lymphocytes in addition to the enhancement seen with mitogens, PHA and Con A. In the test, both compounds did enhance the proliferation of B-lymphocytes in addition to the enhancement seen with mitogen, LPS.

It is important to note that recent studies' reports stated that a diet supplemented with 20 % (wt/wt) of biscuits enriched with antioxidants (vitamin C, vitamin E, beta-carotene, zinc and selenium) for 5 weeks had enhanced the chemotaxis and phagocytosis as well as the intracellular free radical levels in macrophages, which are depressed in prematurely aging mice as compared to non-prematurely aging mice control. An increase also occured in lymphocyte chemotaxis, proliferation response to the mitogen concanavalin A, and interleukin-2 release, as well as in natural killer cell activity [12]. Another study using 30 clinically healthy dogs showed that the best immune response against *Taenia hydatigena* was observed in the vitamin E. The selenium-supplemented groups experienced increased production of antibody titer and IgG concentration in comparison with either vaccinated but without supplements of vitamin E and selenium or control groups (without both vaccination and supplements). These Vitamin E and selenium proved to be immuno-potentiating to dogs vaccinated with subunit and somatic antigens and increased the possibility for the protection against *Taenia hydatigena infection* [13].

Studies by Von Herbay *et al.* [14] reported that the levels of serum vitamin E in individuals with impaired immune responses associated with

viral infection were significantly lower as compared to healthy individuals. The serum vitamin E levels were significantly lower in patients with severe viral hepatitis than in controls and those levels returned to control levels when the hepatitis subsided. Their findings suggested that hepatitis involves oxidative reactions that consume vitamin E and as a consequence might decrease potential immune responses to the disease [14, 15]. Another study showed that the cellular immunity functions (quantity of T-, B-lymphocytes and natural killer cells) of 94 cervical cancer patients, aged 35 to 49, at II and III stage were suppressed in comparison with 69 healthy women taken as a control. The activity of the antioxidant system in patients with cervical cancer was impaired where the concentration of lipid peroxidation products was increased, the level of the endogenous antioxidant vitamin E and the activity of the antioxidant enzyme superoxide dismutase decreased in comparison with the control group. These data suggested the possibility of interaction between immune systems and antioxidant systems [16].

Data in our experiment showed that the γ-tocotrienol was more potent in inducing mitogenesis in splenocytes as compared to the α-tocopherol at all concentrations used ($p > 0.01$, $n = 4$). This may be due to its shorter unsaturated isoprenoid tail that allows easier mobility, more uniform distribution in cell membranes and greater recycling activity as compared to α-tocopherol [17]. This is in accordance with our findings of cellular uptake of both γ-tocotrienol and α-tocopherol by splenocytes using high performance liquid chromatography method. The γ-tocotrienol was taken up almost five times more efficiently by splenocytes as compared to α-tocopherol with the ratio of 4.8 :1 ($p < 0.01$, $n = 4$) at 300 μM of treatment (Figure 2).

CONCLUSIONS

In summary, it was demonstrated that the palm oil vitamin E, especially γ-tocotrienol, significantly enhanced the cellular immunity function in splenocytes by efficiently stimulating the proliferation of T- and B-lymphocytes. A better understanding of the cellular and molecular mechanism in the induction of mitogenesis by γ-tocotrienol may provide basic rational for clinical therapies in the future.

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