Journal of Clinical & Experimental Cardiology

Qureshi et al., J Clin Exp Cardiolog 2015, 6:4
DOI: 10.4172/2155-9880.1000367

Research Article Open Access

Impact of δ-Tocotrienol on Inflammatory Biomarkers and Oxidative Stress in Hypercholesterolemic Subjects

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Received date: March 23, 2015; **Accepted date:** April 20, 2015; **Published date:** April 27, 2015

Abstract

Background: Tocotrienols have hypocholesterolemic, anti-inflammatory, and anti-cancer properties. Clinical studies using tocotrienol-rich fraction (TRF) from palm oil yielded inconsistent results with regards to its efficacy due to presence of tocopherols in TRF mixture.

Objectives: The impact of tocopherol-free δ-tocotrienol on inflammatory and oxidative stress biomarkers, plasma cytokines/proteins, their gene expression, and microRNAs was studied in hypercholesterolemic subjects.

Design: Hypercholesterolemic (n=31; serum cholesterol >5.2 mmol/L) subjects were enrolled in the study. All hypercholesterolemic subjects were given increasing doses of δ-tocotrienol (125, 250, 500, 750 mg/d) plus AHA Step-1 diet for 4 weeks each during a 30 week study period. Serum nitric oxide (NO), C-reactive protein (CRP), malondialdehyde (MDA), δ-glutamyl-transferase (δ-GT), total antioxidant status (TAS), cytokines/proteins, cDNA, and microRNAs were determined.

Results: All concentrations of δ-tocotrienol reduced serum levels of NO, CRP, MDA, δ-GT. The most effective dose (250 mg/d) decreased serum NO (40%), CRP (40%), MDA (34%), δ-GT (22%) significantly (P<0.001), while TAS levels increased 22% (P<0.001). The 500 mg/d and 750 mg/d doses were less effective in improving oxidative stress compared to the 250 mg/d dose. Inflammatory plasma cytokines (resistin, IL-1α , IL-12, IFN-γ) were reduced 15-17% (P<0.05-0.01), while cardiac angiogenic fibroblast growth factor-b (FGF-b) and platelet-derived growth factor (PDGF) were decreased by 11% and 14% (P<0.05-0.01), respectively, with 250 mg/d δ-tocotrienol treatment. Similar results were obtained for cytokine gene expression. Several plasma miRNAs (miRNA-16-1, miRNA-125a, miRNA-133, miRNA-155, miRNA-223, miRNA-372, miRNA-10b, miRNA-18a, miRNA-214) associated with cardiovascular disease and cancer were modulated by δ-tocotrienol treatment.

Conclusions: In a dose-dependent study of 125-750 mg/d, δ-tocotrienol maximally reduced inflammation and oxidative stress parameters with a 250 mg/d dose in hypercholesterolemic subjects, and may be a potential therapeutic alternative natural product for the maintenance of health during aging process.

Keywords: Tocotrienols; Inflammatory biomarkers; Serum NO; hsCRP, Malondialdehyde; γ-GT; Total antioxidant status; Plasma cytokines; Circulatory miRNAs

Abbreviations:

AHA Step-1 diet: American Heart Association Step-1 diet; CRP: C-reactive Protein; FGF-b: Fibroblast Growth Factor-b; IFN-γ: Interferon-γ; FGF-b: Fibroblast Growth Factor-b; mRNA: Messenger Ribonucleic Acid; miRNA: MicroRNA; NO: Nitric Oxide; PDGF: Platelet-derived Growth Factor; ROS: Reactive Oxygen Species; TAS: Total Antioxidant Status; TNF-α: Tumor Necrosis Factor-alpha

Introduction

It is well-established that low-grade inflammation causes progressive damage to organs in chronic diseases. Our recent studies have demonstrated that serum nitric oxide (NO) levels are significantly increased with aging, and administration of δ-tocotrienol along with other nutritional supplements (resveratrol, quercetin, and niacin) has beneficial effects in lowering NO, γ-glutamyl-transferase (γ-GT), C-reactive protein (CRP), and uric acid in normal-cholesterolemic seniors [1]. Moreover, intake of these nutritional supplements in hypercholesterolemic individuals along with AHA Step-1 diet has proved to considerably reduce the serum total cholesterol, LDL-cholesterol and triglyceride levels [2]. We have recently reported that reduction or induction in lipid parameters in hypercholesterolemic subjects by δ-tocotrienol is concentration-dependent [3]. The present investigation is an extension of above

study [3] to check the novel properties of δ-tocotrienol on inflammatory biomarkers (NO, CRP, MDA, γ-GT), cytokines/proteins, their gene expression, and circulatory miRNAs in cardiovascular risk.

It has long been postulated that supplementation with dietary antioxidants like vitamins A, C, and E can alleviate the redox imbalance and thereby protect against the deteriorating effects of oxidative stress and inflammation in aging [4]. Inflammation enhances NO production by inducible nitric oxide synthase (iNOS). It was also reported that nitrate levels in plasma are considerably higher in older people, as compared to young people [1,5]. The increased levels of NO reacts with reactive oxygen species (ROS; superoxide) to form pro-oxidant species, such as peroxy-nitrites, that can potentiate inflammatory injury to vascular cells [6]. Hypercholesterolemia is associated with increased level of ROS through activation of NADPH oxidase and Xanthine oxidase [7,8]. Moreover, oxidative stress increases in the aging process are mainly due to mitochondrial injury, and lead to excessive production of reactive oxygen species (ROS) from the electron transport chain [9]. This causes the activation of β-hydroxy-β-methylglutaryl coenzymeA (HMG-CoA) reductase (rate-limiting enzyme in the body's cholesterol biosynthesis) and leads to increased levels of cholesterol during the aging process [10]. ROS are also responsible for activation of nuclear factor-kappa B (NF-κB), a stimulator of inflammatory interleukins like tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) [11]. These inflammatory mediators lead to increased levels of CRP. CRP has both pro-inflammatory as well as pro-atherogenic potential [12-14]. It is proven to be an important risk factor for cardiovascular events in the elderly [15,16].

Nutritional strategies are an important aspect of both prevention and treatment of chronic conditions. Of the vitamin E isomers, δ-tocotrienol was the most potent antioxidant and had the greatest lipophilic antioxidant capacity in human plasma (Figure 1) [17]. The present study examined NO and MDA as oxidative stress markers, total antioxidant status (TAS), and CRP. Moreover, γ-GT activity- a useful predictor of non-fatal myocardial infarction and fatal coronary heart disease- was examined [18]. Inflammatory cytokines implicated in aging and heart disease, including interleukin-1α (IL-1α), IL-12, and IFN-γ were also evaluated. In addition, resistin, which is involved in insulin resistance and induces inflammation, and two growth factors associated with cardiac angiogenesis, fibroblast growth factor-b (FGF-b) and platelet-derived growth factor (PDGF), were examined.

Moreover, microRNAs (miRNAs), small non-coding RNAs involved in many biological processes and important mediators of transcriptional and post-transcriptional gene expression, were also assessed [19,20]. The present study evaluated the effect of δ-tocotrienol on selected miRNAs associated with cardiovascular, cancer and aging diseases, including miRNA-16-1, miRNA-10b, miRNA-18a, miRNA-125a, miRNA-133a, miRNA-155, miRNA-214, miRNA-223 and miRNA-372.

The present study tested the effects of DeltaGold (90% δ-tocotrienol +10% δ-tocotrienol) at dosages of 125, 250, 500, or 750 mg/d plus AHA Step-1 diet in hypercholesterolemic subjects on inflammatory biomarkers, cytokines/proteins and their mRNA gene expression, and miRNAs associated with aging, cardiovascular, and other diseases.

Materials and Methods

Reagents

DeltaGold 125 mg softgels from annatto seeds (typical composition 90% δ-tocotrienol and 10% δ-tocotrienol) were supplied by American River Nutrition, Inc. (Hadley, MA. USA).

Study design

The study was a forced titration design, where all subjects took increasing doses of δ-tocotrienol (125, 250, 500, and 750 mg/d) plus AHA Step-1 diet after baseline (Phase I), and AHA Step-1 diet (Phase II). A sample size (n=31) of this study was based on data derived from senior citizens with alpha 0.05 and beta 0.8 to determine the effectiveness of δ-tocotrienol in various doses (Mammatech Inc., Coppell, Texas, USA). The same serum/plasma samples obtained in a previous study on the estimation of lipid parameters were used [3]. In short, the study subjects were screened for high cholesterol from the general community at Wah Cantonment, Pakistan. Clinical history was taken and physical examination carried out for each participant.

The inclusion criteria

Adults male/female, age >50 years with cholesterol level ≥ 5.2 mmol/L labelled as hypercholesterolemic were included [21].

The exclusion criteria

Any subject having weight >125% of Metropolitan Life relative weights and taking cholesterol lowering medication or anti-inflammatory drugs in the last 2 weeks were excluded. Subjects suffering from elevated serum transaminase activity, serum urea, glucose, thyroid stimulating hormone, liver, renal, diabetes, or thyroid diseases were excluded from the study. A total of (n=31) hypercholesterolemic subjects (26 males+5 females) were enrolled in the study [3].

All subjects signed informed-consent forms, and the study protocol was approved by the Institutional Review Board of the Armed Forces Institute of Pathology, Rawalpindi, Pakistan. Each participant was individually counselled to use AHA Step-1 diet (restricted intake of fat <30%/d, and cholesterol <300 mg/d) throughout the study period. Participants of the study were also advised to stop using cholesterol-lowering drugs or antioxidants and counselled individually to modify food intake to meet the goals of the AHA Step-1 diet. Subjects were asked to stop the intake of whole milk, butter, cheese, eggs, animal fat and ice cream. Experimental design of effects of δ-tocotrienol plus AHA Step-1 diet in hypercholesterolemic subjects was exactly the same as reported previously [3].

Experimental design

The present study was carried out in six phases; phase 1 consisted of 4 weeks of an alcohol-free, which was followed by 4 weeks phase II, in which all participants were restricted to American Heart Association AHA) Step-1 diet (intake of fat 30%, and cholesterol 300 mg/d), and all the participants continued AHA Step-1 diet during phase III, IV, V, VI. The subjects were administered δ-tocotrienol 125 mg/d (one capsule, 8 am after breakfast; phase III) for 4 weeks, followed by 250 mg/d (one capsules 8 am, and second capsule 8 pm after dinner; phase IV), four capsules (two at 8 am, and two capsules at 8 pm; phase V)

and six capsules of d-tocotrienol (two at 8 am, two after lunch 2 pm and two after dinner, 8 pm; phase VI). Each phase lasted for 4 weeks. There was a 2 weeks washout-period after each δ-tocotrienol treatment period (Figure 2). At the end of each phase, blood samples were collected after overnight fast of each subject to carry out estimations of cardiovascular risk factors and inflammatory biomarkers.

Blood sample collection

Venous blood samples (12 hours fast, 9:00 pm-9:00 am) were drawn after screening at the termination of base line phase and at end of each phase. Blood (5 ml) was collected into plain tubes; the remaining (5 ml) was collected into EDTA plasma tubes. The samples were then centrifuged at 3000 × g for ten minutes. Processed samples were stored in Eppendoff tubes at -70°C until further analysis.

Biochemical analyses

The analyses of the coded samples were performed at the Department of Chemical Pathology and Endocrinology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan. The estimation of nitric oxide was done by colorimetric assay based on Griess reagent at 540 nm on an ELISA Reader. Serum hS-CRP was analysed by two-site sequential chemi-luminescence immunometric assay kit (Seimen, LA, CA, USA). Serum MDA was assayed by using serum MDA Catalog No. 10009055 kit (Cayman Chemical Company, USA) on an ELISA Microplate Reader. The estimation of serum γ-GT activity was carried out by kinetic colorimetric assay based on a standard kit procedure (Randox, UK). Serum TAS was estimated by kinetic colorimetric assay kit (Randox, Crumlin, UK). All of these analytes were measured on the automated clinical chemistry analyzer, Selectra E (Vita Lab, Netherland). The analyses of serum/plasma samples of all subjects of each group were carried out simultaneously to avoid large standard deviation.

Purification of pure total RNA from plasma

The plasma total messenger RNAs (mRNAs) were extracted from EDTA treated fresh whole blood of δ-tocotrienol, 250 mg/d plus AHA Step-1 diet treated subjects for 4 weeks by using total RNA purification kit 17200 (NORGEN Bioteck Corporation, Thorold, ON, Canada). The circulating microRNAs (miRNAs) were purified by purification mini kit (Slurry Format) product 51000 (NORGEN Bioteck Corporation, Thorold, ON, Canada).

Estimation of plasma cytokines/proteins, cDNA, and miRNAs

The plasma cytokines/proteins, cDNA, and miRNAs were estimated by Human Cytokines/proteins Elisa Plate Array I (chemiluminescence)-EA-4001, Customized Human cDNA Plate Array-AP-UM000416 from messenger RNAs (Signosis Inc., Santa Clara, CA) according to their protocols as described in detail in our recent publication [3].

Statistical analysis

Data was analysed using SPSS 16 version (SPSS Inc, Chicago). Descriptive statistics, comprising means and SD or SE were calculated. Parametric variables were summarized as Means SD (standard deviation). Percent difference was calculated from baseline value of each analyte. Analysis of one-way and two-way variance was used (GraphPad Prism 5) to test whether changes in serum levels of NO, CRP, MDA and γ-GT activity occur in the course of supplementation, and whether there were between- and within-subject differences; because all observations were required, available degree of freedom was reduced by this statistical approach [22]. Data are reported as mean ± SD (standard deviation). The statistical significance level was set at $P<0.05$.

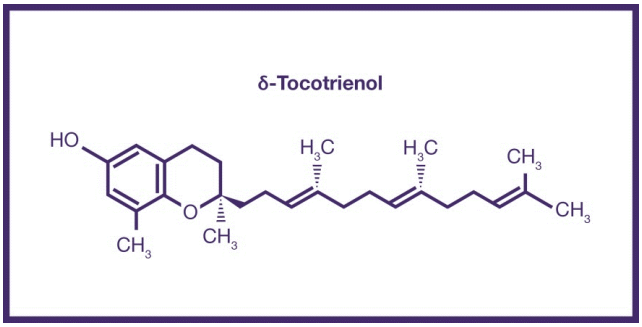

Figure 1: Chemical structure of δ-tocotrienol.

#	Parameters	Means ± SD
1	**Age**	57.84 ± 8.07
2	**Males/Females (n)**	26/5
3	**Height (meter)**	1.74 ± 0.07
4	**Weight (kg)**	69.0 ± 7.0
5	**BMI (kg/m^2)**	25.30 ± 1.86
6	**Systolic BP (mmHg)**	140.16 ± 6.26
7	**Diastolic BP (mmHg)**	90.32 ± 5.31
8	**Serum creatinine (mmol/L)**	93.39 ± 10.12
9	**Serum ALT (U/L)**	36.68 ± 7.97
10	**Serum Total Cholesterol (mmol/L)**	5.44 ± 1.06
11	**Serum LDL-cholesterol (mmol/L)**	3.44 ± 0.78
12	**Serum Triglyceride (mmo/L)**	1.81 ± 0.54
13	**Serum Apo B mg/L)**	91.65 ± 6.75
14	**Serum Apo A-1 mg/L)**	118.97 ± 4.91
15	**Serum glucose (mmol/L)**	4.22 ± 0.98
16	**Serum NO (μmol/L)**	11.79 ± 1.22
17	**Serum CRP (mg/L)**	4.39 ± 0.64
18	**Serum MDA (μmol/L)**	6.26 ± 1.13
19	**Serum γ-GT (U/L)**	33.61 ± 3.61
20	**Serum TAS (mmol/L)**	3.00 ± 1.31

Table 1: Baseline characteristics of hypercholesterolemic subjects.

Results

Baseline physical characteristics of hypercholesterolemic subjects participating in study

Serum or plasma samples (n=31) were used to estimate cardiovascular and oxidative risk factors (NO, CRP, MDA, γ-GT activity, and total antioxidant status (TAS), as well as various cytokines/proteins, their gene expression and miRNAs. The physical characteristics of pre-treatment values (Physical Exam) of these subjects are reported in Table 1. There were no changes in the body weight, height, and body mass index, systolic and diastolic blood pressure at the end of each phase (Data not shown).

Effects of δ-tocotrienol + AHA Step-1 diet on aging and inflammation biomarkers in hypercholesterolemic subjects

The AHA Step-1 diet treatment resulted in a modest 3% to 6% decrease (non-significant) in aging and inflammation biomarkers in hypercholesterolemic subjects (Figures 3-7). The most important aging and inflammatory biomarkers, such as NO and CRP were 2 to 4 times higher compared to normal values in these hypercholesterolemic subjects [2]. There were significant (P<0.001) reductions in the serum levels of NO (40%), CRP (40%), MDA (34%), and γ-GT activity (22%; Figures 3-6), while an induction in TAS (22%; P<0.001; Figure 7) occurred with a 250 mg/d dose treatment of δ-tocotrienol plus AHA Step-1 diet.

There were slight increases in levels of NO, CRP, MDA, and γ-GT activity with doses of 500 mg/d and 750 mg/d of δ-tocotrienol plus AHA Step-1 diet (2-5%, P<0.001; 9-12%, P<0.001; 4-7%, P<0.05; 2-6%) as compared to their respective dose of 250 mg/d plus AHA Step-1 diet, while the 125 mg/d dose showed a less significant change (P<0.01 vs P<0.001) in these parameters compared to the 250 mg/d dose (Figures 3-6).

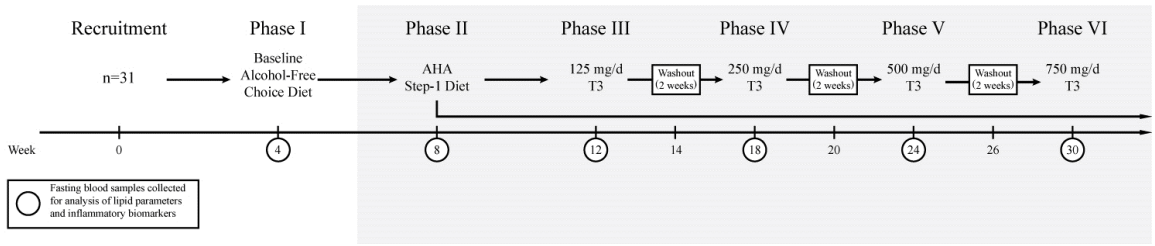

Figure 2: Study protocol of δ-tocotrienol corresponds to six phases, and each phase lasted for 4 weeks.

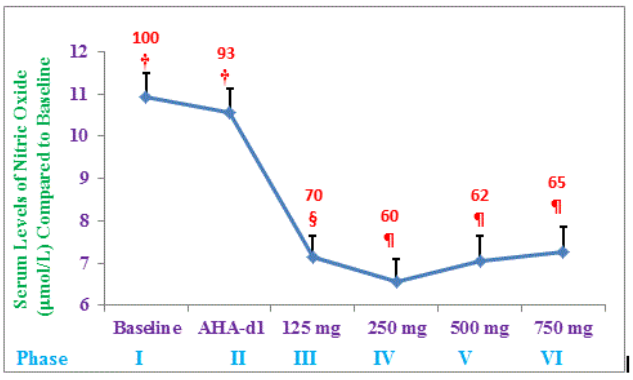

Figure 3: Role of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of nitric oxide (NO) in hypercholesterolemic subjects. The treatments I-VI correspond to six phases and each phase lasted for 4 weeks- I: baseline (n=31); II: AHA Step-1 diet; III: δ-tocotrienol 125 mg/d+AHA Step-1 diet; IV: δ-tocotrienol 250 mg/d+AHA Step-1 diet; V: δ-tocotrienol 500 mg/d+AHA Step-1 diet; VI: δ-tocotrienol 750 mg/d+AHA Step-1 diet. Data are means ± SD (standard deviation). Percentages of each treatment compared to baseline values are above the column. Point on a line not sharing a common symbol are significantly different at §P<0.01; ¶P<0.001.

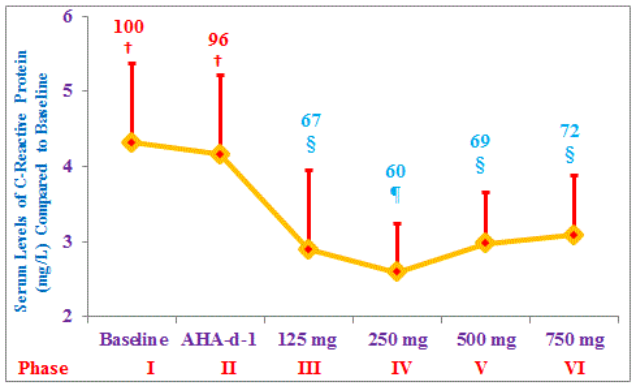

Figure 4: Role of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of C-reactive protein (CRP) in hypercholesterolemic subjects. The treatments I-VI corresponds to six phases and each phase lasted for 4 weeks- I: baseline (n=31); II: AHA Step-1 diet; III: δ-tocotrienol 125 mg/d+AHA Step-1 diet; IV: δ-tocotrienol 250 mg/d+AHA Step-1 diet; V: δ-tocotrienol 500 mg/d+AHA Step-1 diet; VI: δ-tocotrienol 750 mg/d+AHA Step-1 diet. Data are means ± SD (standard deviation). Percentages of each treatment compared to baseline values are above the column. Points on a line not sharing a common symbol are significantly different at §P<0.01; ¶P<0.001.

A panel of six key plasma cytokines/proteins (IFN-γ, resistin, IL-1α, FGF-b, PDGF) was selected to investigate the effect of δ-tocotrienol (250 mg/d) taken orally by hypercholesterolemic subjects (Table 2). The quantitative value of plasma levels of each cytokine/protein was estimated against their respective standard as shown in Table 2. The AHA Step-1 diet alone did not have any significant effect on the levels of plasma cytokines/proteins (Table 2). However, the treatment with δ-tocotrienol plus AHA Step-I diet (250 mg/d) down-regulated levels of resistin (16%), IL-1α (17%), IL-12 (15%), and interferon-γ (17%) significantly ($P<0.01$) as compared to baseline values (Table 2). The plasma growth factors (FGF-b and PDGF) were modestly down-regulated (11%, and 14%, $P<0.01$, respectively) by δ-tocotrienol treatment (Table 2). These cytokine/protein and growth factor data correlated directly with gene expression of messenger RNAs (mRNAs) purified from fresh EDTA treated whole blood obtained from subjects on the same treatments (250 mg/d; Figure 8).

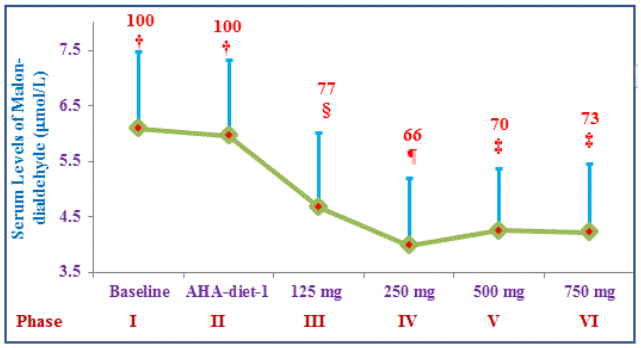

Figure 5: Role of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of Malondialdehyde (MDA) in hypercholesterolemic subjects. The treatments I-VI correspond to six phases and each phase lasted for 4 weeks- I: baseline (n=31); II: AHA Step-1 diet; III: δ-tocotrienol 125 mg/d+AHA Step-1 diet; IV: δ-tocotrienol 250 mg/d+AHA Step-1 diet; V: δ-tocotrienol 500 mg/d+AHA Step-1 diet; VI: δ-tocotrienol 750 mg/d+AHA Step-1 diet. Data are means ± SD (standard deviation). Percentages of each treatment compared to baseline values are above the column. Points on a line not sharing a common symbol are significantly different at $^{§}P<0.05$; $^{‡}P<0.01$; $^{¶}P<0.001$.

Many miRNAs appear to be dysregulated during cellular senescence, aging, cancer, and during various diseases. However, only few miRNAs have been linked to age-related changes in cellular and organ functions. δ-tocotrienol upregulated miR-16-1, miR-125a, miR-133, miR-155, miR-223, and miR-372 compared to pre-treatment baseline (Figure 9). On the other hand, miR-10b, miR018a, and miR-214 were down-regulated significantly ($P<0.05$-0.001) with δ-tocotrienol treatment in the present study (Figure 9). These results indicate that δ-tocotrienol treatment modulates miRNAs in hypercholesterolemic subjects, thus potentially lowering risk of aging, cardiovascular, and other diseases.

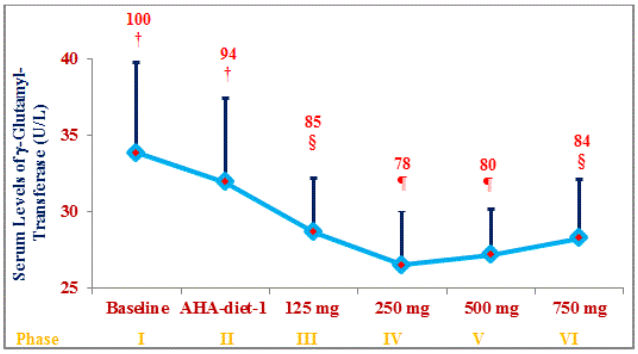

Figure 6: Role of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of γ-glutamyl-transferase (γ-GT) in hypercholesterolemic subjects: The treatments I-VI corresponds to six phases, and each phase lasted for 4 weeks- I: baseline (n=31); II: AHA Step-1 diet; III: δ-tocotrienol 125 mg/d+AHA Step-1 diet; IV. δ-tocotrienol 250 mg/d+AHA Step-1 diet; V: δ-tocotrienol 500 mg/d+AHA Step-1 diet; VI: δ-tocotrienol 750 mg/d+AHA Step-1 diet. Data are means ± SD (standard deviation). Percentages of each treatment compared to baseline values are above the column. Points on a line not sharing a common symbol are significantly different at $^{§}P<0.01$; $^{¶}P<0.001$.

#	Cytokines	Baseline		AHA-1=A		A+δ-T3		Description	Functions
		ng/ml	%	ng/ml	%	ng/ml	%		
1	**Resistin**	1.34 ± 0.11	100	1.27 ± 0.92	95	1.13 ± 0.12**	84	Plasmenogen Activator Inhibitor-1.	A multitask cytokine involved in various types of inflammation.
2	**IL-1α**	2.7 ± 0.12	100	2.64 ± 0.15	98	2.24 ± 0.11**	83	Interleukin-1α	Important agonist mediating inflammatory and Immune-modulatory effects.
3	**IL-12**	1.36 ± 0.11	100	1.31 ± 0.11	96	1.13 ± 0.11*	85	Interleukin-12	It plays a key role in the activities of natural killer cells and T lymphocytes.
4	**IFN-γ**	3.38 ± 0.10	100	3.31 ± 0.12	98	1.73 ± 0.11**	83	Interferon-γ	Potent mediators of host defense system and homoeostasis.

5	**FGF-b**	1.77 ± 0.13	100	1.726 ± 0.11	98	1.57 ± 0.11**	89	Fibroblast Growth Factor-b	A stimulator of angiogenesis *in-vivo*.
6	**PDGF**	1.46 ± 0.11	100	1.41 ± 0.77	97	1.26 ± 0.11*	86	Platelet Derived Growth Factor	Important to embryonic development, cell proliferation and in blood formation (angiogenesis)

Table 2: Effects of δ-tocotrienol (250 mg/d)+AHA Step-1 diet on various plasma cytokines/proteins in hypercholesterolemic subjects. *-**Number of asterisk/s in a row indicate significantly different from the respective control values, *P<0.05; **P<0.01.

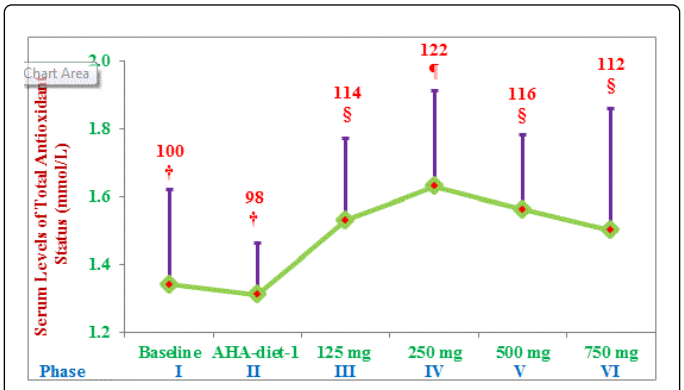

Figure 7: Role of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of total antioxidant status (TAS) in hypercholesterolemic subjects. The treatments I- VI correspond to six phases and each phase lasted for 4 weeks- I: baseline (n=31); II: AHA Step-1 diet; III: δ-tocotrienol 125 mg/d+AHA Step-1 diet; IV: δ-tocotrienol 250 mg/d+AHA Step-1 diet; V: δ-tocotrienol 500 mg/d+AHA Step-1 diet; VI: δ-tocotrienol 750 mg/d+AHA Step-1 diet. Data are means ± SD (standard deviation). Percentages of each treatment compared to baseline values are above the column. Points on a line not sharing a common symbol are significantly different at §P<0.01; ¶P<0.001.

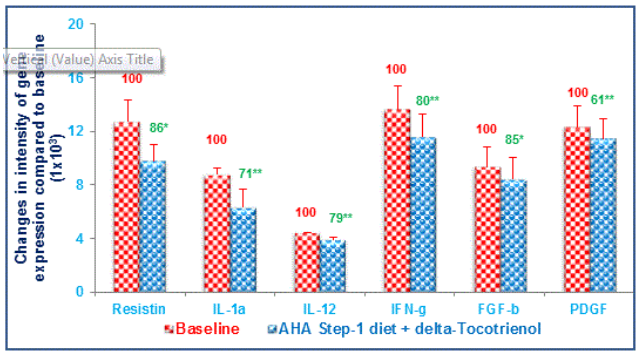

Figure 8: Effects of δ-tocotrienol (250 mg/d)+AHA Step-1 diet on various plasma gene expression in hypercholesterolemic subjects. *-***Number of asterisks indicate differences from respective control values, *P<0.05; **P<0.01.

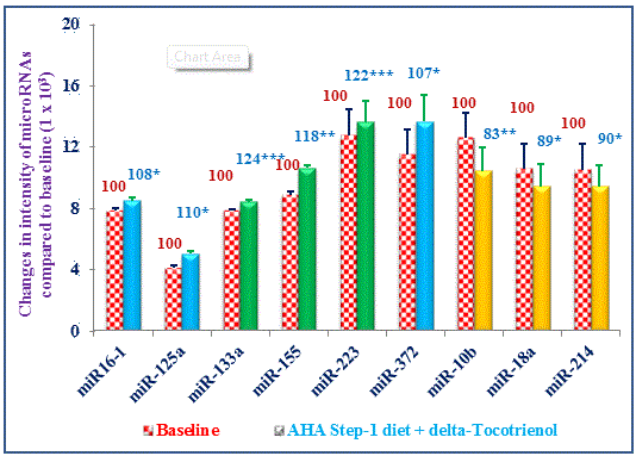

Figure 9: Effects of δ-tocotrienol (250 mg/d)+AHA Step-1 diet on various microRNA in hypercholesterolemic subjects. *-***represent statistical significance from respective control value, *P<0.05; **P<0.01; ***P<0.001.

Discussion

The present results demonstrate that δ-tocotrienol given to elderly hypercholesterolemic humans had a positive effect on inflammatory biomarkers and oxidative stress by lowering NO, CRP, MDA, γ-GT activity, while TAS, cytokines/proteins, gene expression and miRNAs improved compared to baseline levels. The optimal dose was 250 mg/d, as reported previously [3]. Previous studies showed that δ-tocotrienol was more effective than other tocotrienol isomers in inhibiting reactive oxygen species formation induced by hydroperoxide as well as by hydrogen peroxide (H_2O_2) [23]. The possible reason for this increased antioxidant effect of δ-tocotrienol is thought to be due to its greater cellular uptake. It was reported that there was an increased cellular accumulation of δ-tocotrienol compared to α-tocotrienol and γ-tocotrienol [23]. Furthermore, it has also been reported that a decrease in the level of chromane ring methylation of tocotrienols-meaning delta- and gamma-isomers- results in a corresponding decrease in the partition coefficient of these compounds, which is responsible for a greater cellular uptake [24].

These results are consistent with the reported dose-dependent antioxidant effect of tocotrienol-rich fraction of various doses supplemented in rats [25]. The maximum decrease in MDA was observed at a concentration of 128 ppm of tocotrienol-rich fraction, which also exhibited 96% improvement in free radical scavenging

activity and reducing NO levels [25]. Our present results show that δ-tocotrienol decreased inflammation by reducing the levels of NO, MDA, and CRP at both 125 and 250 mg/d doses compared to baseline after 4 weeks, while slight increases were observed at doses of 500 mg/d and 750 mg/d compared to the 250 mg/d dose. For the 125 mg/d dose, a longer treatment period (>4 weeks) may have decreased inflammatory markers further. This dose-dependent trend, particularly above the 500 mg/d of δ-tocotrienol treatment matches our previous results observed with lipid parameters in hypercholesterolemic subjects [3]. The conclusions and justification of these results have been described in detail in our recent publication [3]. The reduction in serum level of NO by δ-tocotrienol is also supported by a recently published report [26].

As far as a mechanism by which δ-tocotrienol works is concerned, our earlier studies have revealed that δ-tocotrienol affects several different signalling pathways and transcriptional factors involved in inflammation and induction of TNF-α, IL-6, IL-1α, and iNOS, which are important inflammatory markers [27]. It has been reported that δ-tocotrienol might regulate the production of NO and inhibit the pro-inflammatory cytokines involved in normal and aging processes and atherosclerosis by two possible pathways involving NF-κB and toll-like receptors [27]. We have reported that the down-regulation of IL-6, IL-8 and IL-10 may affect NF-κB directly, hence suggesting a plausible mechanism for δ-tocotrienol's anti-inflammatory properties [3].

The traditional view that cardiovascular disease is directly related with elevated cholesterol is challenged by the fact that 50% of patients who die from heart attacks have normal cholesterol levels [28]. Furthermore, an extensive and growing base of evidence shows that inflammation participates centrally in all stages of atherosclerosis [8]. The present study demonstrates that δ-tocotrienol effectively down-regulates inflammatory cytokines and gene expression of resistin, IL-1α, IL-12 and IFN-γ.

Resistin has been linked to obesity, insulin resistance, and diabetes, while also playing a role in cardiovascular disease [29]. As such, it is an inflammatory mediator and biomarker particularly for atherosclerosis and heart failure [30]. Similarly, IL-1α may be an essential mediator in the pathogenesis of heart failure [31], and was shown to promote atheromatous plaque instability [32]. Further, IL-1α activates TNF-α, which in turn induces NF-κB and initiates the inflammatory process [33]. IL-12, a cytokine that stimulates growth and function of T-cells, is elevated in type 2 diabetes and upregulates cardiovascular disease in the presence of CRP [34]. While it has been found to accelerate atherosclerosis in mice [35], blocking IL-12 attenuates atherosclerosis [36]. IFN-γ, a cytokine critical for innate immunity against infection, is also a central player in atherogenesis and the development and progression of cardiovascular disease [37]. It is highly expressed in atherosclerotic lesions and emerged as significant factor in atherogenesis [38]. In the present study, δ-tocotrienol reduced all four of these cytokines implicated in atherosclerosis, suggesting that the supplement may reduce the risk of cardiovascular disease through its anti-inflammatory action.

δ-Tocotrienols in the present study down-regulated FGF-b and PDGF. Neo-angiogenesis plays an essential role in the process of cardiac repair after ischemic injury [39], and both FGF-b and PDGF are effective in inducing an angiogenic response. FGF-b can induce angiogenesis in animal models of myocardial ischemia, and has led to higher vessel counts and reduced infarct size [40]. Conversely, while FGF-b's neo-angiogenesis effect improves cardiac function in coronary artery disease, this angiogenic stimulation could also cause negative effects, such as atherosclerosis [41]. Similarly, PDGF pathways in the aging heart are cardioprotective, enhancing cardiac angiogenesis and protecting from myocardial infarction, but have also been found to have pro-atherosclerotic actions [42]. Both FGF-b and PDGF, due to their angiogenic activity, may also stimulate tumor growth [43]. It is possible that δ-tocotrienol's down-regulation of FGF-b and PDGF acts as an anti-angiogenic mechanism against both atherosclerosis (without the presence of a cardiovascular event) and malignant tumor growth. Tocotrienols anti-angiogenic properties have been confirmed by various prior studies [44,45].

Recently, levels of miRNAs have been shown to be important regulators of gene expression that modify cellular responses and function [46-48]. The dysregulation of miRNA plays a crucial role in the development of cardiovascular disease, aging, diabetes and cancer. Several studies have provided evidence showing that miRNA participate in regulating cell cycle progression, proliferation, stem cell gene expression, and stress-induced responses [46]. Aging is the predominant risk factor for developing cardiovascular disease [49]. The present study has demonstrated that δ-tocotrienol modulated miRNAs associated with cardiovascular disease, including miRNA-133a, miRNA-155, miRNA-223, and miRNA-214. MiroRNA-133a is enriched in the cardiac muscle [46], and is down-regulated in cardiac hypertrophy and heart failure [50]. In mice where miRNA-133a was removed, animals experienced cardiac hypertrophy [19]. Hence, up-regulation of this miRNA with δ-tocotrienol, may prevent cardiac hypertrophy.

MicroRNA-155 may play a protective role in the development of endothelial inflammation and is reduced during aging [47]. A deficit of miRNA-155 could be implicated in hypertension and cardiovascular disease [51], and therefore up-regulation of this miRNA by δ-tocotrienol could be cardioprotective.

Although linked to various cancers, miRNA-223 plays not only an anti-inflammatory, but also cardioprotective role [52]. It was found to both coordinate cholesterol homeostasis [53] and protect the brain from neuronal cell death following transient global ischemia [54], and hence its up-regulation by δ-tocotrienol could indicate cardiovascular protection through diverse pathways.

Whereas cardiovascular risk markers were the focus of the present study, several of the miRNAs tested, including miRNA-16-1, miRNA-372, miRNA-10b, and miRNA-18a, have been linked to other aging diseases, particularly cancer. MicroRNA-16-1 reduces blood vessel formation and regulates angiogenesis, a crucial initiator of tumor growth. This miRNA is thought of as a tumor suppressor, and regulates vascular endothelial growth factor (VEGF) [55], which δ-tocotrienol was shown to reduce more potently than other vitamin E isomers in independent studies [44,45]. MicroRNA-372 is an anti-cancer miRNA that was shown to down-regulate hepatocellular carcinoma proliferation and metastasis [56]. Coincidentally, tocotrienols high in δ-tocotrienol were shown to reduce hepatocellular carcinoma when used in combination with epirubicin, and reduced the cardiotoxicity typically associated with this chemotherapy drug [57].

As opposed to miRNA-372, which appears to be protective against hepatocellular carcinoma and which was increased by δ-tocotrienol treatment, miRNA-10b is overexpressed in liver cancer [58], and in turn was down-regulated by δ-tocotrienol treatment in the present study. Down-regulation of miRNA-10b could also indicate prevention of epithelial-mesenchymal transition associated with breast cancer

[59]. MicroRNA-18a is a potential target for the treatment of glioblastoma, and its inhibition was shown to suppress this type of cancer [60]. Increased levels of miRNA-18a have also been associated with breast malignancy [61], indicating that down-regulation such as occurred with δ-tocotrienol treatment would be beneficial.

MicroRNA-125a is involved in the inflammatory chemokine pathway in systemic lupus erythematosus (SLE), and is reduced in those with the disease. Up-regulation of miRNA-125a with δ-tocotrienol treatment, could serve as a therapeutic target for the treatment of SLE via the regulation of inflammatory chemokines [62]. Separately, miRNA-125a was shown to increase hematopoietic stem cells [63]. Interestingly, several studies support δ-tocotrienol's effect in hematopoietic stem cell recovery following radiation injury [64,65].

Conclusion

The key findings of present study show that serum NO, CRP, MDA, γ-GT levels were significantly decreased, and TAS level was increased, suggesting greater protection against oxidative stress after consumption of δ-tocotrienol by hypercholesterolemic senior subjects. Decreased levels of these oxidative stress markers are of clinical importance with regards to host defense mechanisms and treatment of inflammatory diseases. While the 250 mg/d dose of δ-tocotrienol was most effective in modulating oxidative stress parameters. The results also indicate that a low dose of 250 mg/d of δ-tocotrienol administered for 4 weeks is effective in lowering several cardiovascular risk factors and down-regulating inflammatory biomarkers (resistin, IL-1α, IL-12, IFN-γ, FGF-b, and PDGF) associated with cardiovascular diseases. Further, δ-tocotrienol modulated miRNAs (miRNA-16-1, miRNA-125a, miRNA-155, miRNA-133a, miRNA-223, miRNA-214, miRNA-372, miRNA-10b, and miRNA-18a) which may play an important role in both cardiovascular, cancer and other inflammatory diseases. Taken together, these results suggest that δ-tocotrienol is a potential candidate for therapeutic applications in the maintenance of health and protection from aging diseases.

Acknowledgment

We thank American River Nutrition, Inc. for donating the DeltaGold softgels (American River Nutrition, Inc. Hadley, MA. USA). The study was supported by Advanced Medical Research (AMR), Madison, Wisconsin, USA. The study was carried out under an FDA-approved IND # 36906.

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