Dose-dependentModulation of Lipid Parameters,Cytokines and RNA by δ-TocotrienolinHypercholesterolemic SubjectsRestricted to AHA Step-1 Diet

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ABSTRACT

Aims: Evaluate the consumption of δ-tocotrienol(free from tocopherols) on serum lipid parameters, and several cytokines (TNF-α, IL-4, IL-6, IL-8, IL-10), including gene expression and circulating microRNAs (miRNAs) in hypercholesterolemic subjects.

Study Design: The present preliminary dose-response study consisted of six phases. All hypercholesterolemic subjects took increasing doses of δ-tocotrienol (125, 250, 500,750 mg/d) plus AHA Step-1 diet for 4-weeks during 30-week study period.

Methodology: Hypercholesterolemic (*n* = 31; serum cholesterol > 5.2 mmol/L) subjects (males-26/females 5; age range 50-71 years) were enrolled in study from Wah Cantonment, Pakistan. Serum lipids parameters were measured by autoanalyzers. Various plasma cytokines, cDNA, and miRNAs were estimated by using Signosis kits.

Results: All participants (*n* = 31) completed all phases of study. The δ-tocotrienol plus AHA Step-1 diet caused reductions in lipid parameters in dose-dependent manner with maximum effects on serum total cholesterol (15%), LDL-cholesterol (18%), triglycerides (14%) with 250 mg/d dose (***P***< 0.001) and above 500 mg/d dose resulted ininduction in the levels of all lipid parameters, except HDL-cholesterol. The cytokines associated with cardiovascular disease (plasma TNF-α, IL-2, IL-4, IL-6, IL-8, IL-10) were all down-regulated 39%-64% byδ-tocotrienol treatment (***P***< 0.01). Similar results were obtained with gene expression of these cytokines using whole blood messenger-RNA. In contrast, circulating miRNA-7a, miRNA-15a, miRNA-20a (anti-angiogenic), miRNA-21, miRNA-29a, miRNA-92a, miRNA-200, miRNA-206(skeletal muscle regeneration) down-regulated in hypercholesterolemic subjects, were up-regulated by δ-tocotrienol treatment as compared to baseline (***P***< 0.01).

Conclusions: The present results confirm that consumption of δ-tocotrienol plus AHA Step-1 diet causes significant reduction in serum lipid parameters and several cytokines (TNF-α, IL-2, IL-4, IL-6, IL-8, IL-10) at low optimal dose of 250 mg/d. The capacity of δ-tocotrienol to modulate inflammation is partly attributable to dose-dependent properties of inhibition/activation, which may play a major role in future treatment of cardiovascular diseases.

***Keywords*:** DeltaGold-90% δ-tocotrienol + 10% γ-tocotrienol, lipid parameters, inflammatory biomarkers, cytokines, TNF-α, gene expression, circulatory miRNAs.

ABBREVIATIONS:

Palm oil TRF: Palm oil tocotrienol rich fraction (14.64% α-tocotrienol, 27.59% γ-tocotrienol, 6.33%

DeltaGold : 90% δ-tocotrienol+ 10% γ-tocotrienol

AHA Step-1 diet: American Heart Association Step-1 diet

HDL: High Density Lipoprotein

LDL: Low Density Lipoprotein

HMG-CoA reductase: β-hydroxy-β-methylglutaryl-coenzyme A reductase

TNF-α: Tumor necrosis factor-alpha

IL: Interleukin

mRNA: messenger ribonucleic acid

miRNAs: micro-Ribonucleic acids

1. INTRODUCTION

We have been studying lipid lowering effects of naturally-occurring compoundsfor several years,such as tocotrienols isolated from palm oil known as tocotrienol rich fraction (TRF), and its components, α-tocopherol, α-tocotrienol, γ-tocotrienol, and δ-tocotrienol in chickens and humans [1,2].The tocotrienol rich fraction (TRF) from palm oil, comprising of a mixture of tocopherols and tocotrienols, has shown both positive [3-12] and negative [13-17] hypocholesterolemic effects in a number of reported clinical studies [2-17]. Palm TRF (palmvitee capsule, 200 mg/day) or rice bran TRF_{25} preparation low in α-tocopherol concentration (< 10%) combined with AHA Step-1 diet have been effective in lowering serum total cholesterol, LDL-cholesterol, and triglycerides levels in hypercholesterolemic human subjects [2,8]. A major factor underlying the failure of other studies to exhibit beneficial effects is attributable to the presence of over 20% α-tocopherol in palm TRF. This probably inhibited TRF from lowering serum total cholesterol or LDL-cholesterol levels in four major studies [14-17]. Palm TRF also does not reduce serum total cholesterol level in free-living hypercholesterolemic patients [15-17], or healthy humans even if the TRF contained less than 15% α-tocopherol [13]. Furthermore, large doses of tocotrienolshave also proved ineffective (8,18-20) perhaps owing to bioconversion of tocotrienols to α-tocopherol, which antagonizes this beneficial effect [8]. The serum level of α-tocopherol was 2 to 4 fold higher, as compared to the placebo group in these studies [14-17].

We accordingly carried out a study with pure tocotrienols devoid of contamination with tocopherols instead of TRF from palm oil, which lacked good quality control. The availability of tocopherol-free DeltaGold from annatto seeds (consisting of 90% δ-tocotrienol and 10% γ-tocotrienol; Figure 1) made this

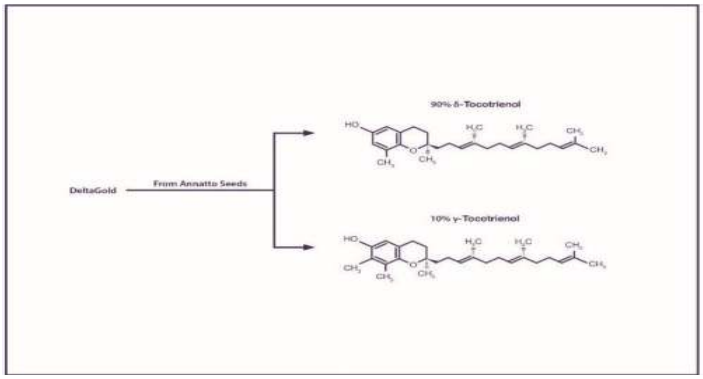

Figure 1: Chemical structures ofDeltaGold (90%δ-tocotrienol + 10% γ-tocotrienol).

humanstudy possible. We have previously demonstrated the underlying mechanism through which tocotrienols exert their effects by suppressing the activation of nuclear factor-κB (NF-κB) in various experimental models [21]. Moreover, the order of potency of various tocotrienols for acting as cholesterol-lowering,anti-oxidant, anti-inflammatory and as anticancer agents were as follows:δ-tocotrienol>γ-tocotrienol>α-tocotrienol>α-tocopherol [22,23]. Recently, a comprehensive review has compared the various biological properties of tocotrienols, including results of various clinical studies of palm TRF and pure tocotrienols [24].

Aside from measuring lipid parameters (total cholesterol and LDL-cholesterol) as classic indicators of cardiovascular disease risk, the present study also examinedinflammatory cytokines implicated in heart disease and their gene expression. These included tumor necrosis factor-alpha (TNF-α), a cytokine which is an important contributor to atherosclerotic lesion development [25], interleukin-2 (IL-2) level of which is significantly elevated in patients with stable angina [26], interleukin-4 (IL-4), an activator of collagen synthesis that may be involved in cardiac fibrosis [27], interleukin-6 (IL-6) continuous production of which promotes production myocardial injury and can cause cardiac hypertrophy [28], and interleukin-8 (IL-8), a cytokine found in vascular injury sites that plays a role in various stages of atherosclerosis [29].

The expression ofcirculating microRNAs (miRNA)which are small non-coding RNAs, that are likely involved in many biological processes were also analyzed [30,31]. The present study evaluated theδ-tocotrienol's effect on selected miRNAs associated with cardiovascular disease such as miRNA-7a, miRNA-15a, miRNA-20a, miRNA-21, miRNA-29a, miRNA-92a, miRNA-200, and miRNA-206. Particularly, miRNA-29a was examined, a family that accounts for ~4% of all miRNAs in the murine heart [36]. MicroRNA-29a is down regulated after myocardial infarction (MI), and targets genes involved in fibrosisand are known as a fibrotic inhibitor. Other miRNAs examined in the present study included anti-angiogenic miRNA-20a, and miRNA-206, which mainly promotes skeletal muscle regeneration, but may also play a pivotal role in the heartmuscle [31].The present study of dose-response (125, 250, 500,750 mg/d) offeeding DeltaGold (90% δ-tocotrienol + 10% γ-tocotrienol) plus AHA Step-1 diet tohypercholesterolemic subjectswas carried out on serum lipid parameters, various plasma cytokine levels, and their gene expression and plasma circulating miRNA levelsassociated with cardiovascular disease.

2. MATERIALS AND METHODS

The study was carried out in the Department of Chemical Pathology & Endocrinology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan in collaboration with the Department of Basic Medical Sciences, University of Missouri-Kansas City, MO, USA The study protocol was registered and approved by Institutional Review Board of AFIP, Rawalpindi, Pakistan.The study was carried out under a FDA approved IND number 36906**.**

2.1 Materials

DeltaGold 125 mg softgels from annatto seeds (composition 90% δ-tocotrienol +10%γ-tocotrienol)were supplied by American River Nutrition, Inc. (Boston, MA. USA). Serum total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides levels were estimated by using reagent kits from Sigma Chemical Co., St. Louis.Pure total RNA was obtained from the EDTA treated fresh whole blood by using"total RNA purification kit # 17200 (NORGEN Bioteck Corporation, Thorold, ON, Canada). The various plasma cytokines, cDNA, and miRNA were estimated by using Signosis, Inc. (1700 Wyatt Drive Suite 10-12. Santa Clara, CA, 95054) Human Cytokine Elisa Plate Array I (chemiluminescence), Catalog number EA-4001, Customized Human cDNA Plate Array (Catalog Number AP-UM000416) from messenger ribonucleic acid (mRNA). The mRNA was extracted from each sample and converted to cDNA and plated on a cytokine cDNA array plate (Signosis, Inc.). Estimation of circulating microRNAs (miRNAs) was carried out using customized MiRNA Direct Hybridization Plate Array(chemiluminescence; Catalog Number Inv-00465)according to the manufacturer's instructions(Signosis, Inc.).

2.2 Study design:

The present study was a forced titration design, where all subjects took increasing doses (125, 250, 500, 750 mg/d) of δ-tocotrienol plus AHA Step-1 diet after baseline (phase I) and AHA Step-1diet (phase II). A sample size of this study (*n* = 31) was based on data derived from senior citizens with alpha 0.05 and beta 0.8 to assess the effectiveness of the tocotrienols in different doses (Mammatech Inc., Coppell, Texas, USA).The study subjects were screened for high cholesterol from general community at Wah Cantonment, Pakistan. Clinical history was taken and physical examination was carried out for each participant. The initial measures included the participant's height, weight, systolic and diastolic blood pressure at rest, history of significant diseases, medications (including statins, nitrates, calcium antagonists, angiotensin-converting enzyme [ACE] inhibitors, and/or diuretics) and tobacco smoking. The height and weight were measured in light clothing and without shoes. Body mass index (BMI, kg/m^2) was calculated for each subject.**The inclusion criteria:**Adults male /female, age >50 years with cholesterol level ≥ 5.2mmol/L labeled as hypercholesterolemic were included in the study (32). **The exclusion criteria:**Any subject having weight (> 125% of Metropolitan Life relative weights), taking cholesterol

lowering medication or anti-inflammatory drugs in the last 2 weeks were excluded. The subjects with elevated serum transaminase activity, serum urea, glucose,thyroid stimulating hormone (TSH), liver, renal, diabetes, and thyroid diseases were excluded from the study.A total of (*n* = 31)hypercholesterolemic subjects (26 males + 5 females) were enrolled in thisstudy.

All subjects signed an informed-consent form, which was approved by Institutional Board of AFIP, Rawalpindi, Pakistan. Each participant was individually counseled to restricted intake of fat (< 30%), and (< cholesterol 300 mg/d; AHA Step-1 diet) throughout the study period. Participants of the study were also advised to stop using cholesterol-lowering drugs or antioxidants and counseled 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. In order to ascertain full adherence to dietary recommendations and intake of nutritional supplements, participants were contacted by telephone during each phase.

2.3 Experimental design:

Effect of δ-tocotrienol plus AHA Step-1 diet in hypercholesterolemic subjects

The experiment consisted of six phases; the first (**phase I**), an alcohol-free choice diet phase (baseline) was followed by a 4-week second phase (**phase II**), during which all participants were counseled to follow the American Heart Association Step-1 diet (AHA Step-1diet). All participants were continued on the AHA Step-1 diet during phases III, IV, V and VI. During **phase III**, all participants were administered 1 capsule (125 mg/d) of δ-tocotrienol (8 pm after food) for 4-weeks. During **phase IV**, participants were administered 2 capsules of 125 mg (250 mg/d; one at 8 am and one at 8 pm after breakfast and dinner) for 4-weeks, followed by 4 capsules of 125 mg (500 mg/d; two at 8 am and two at 8 pm) in **phase V** and during last **phase VI,** 6 capsules of 125 mg (750 mg/d; two at 8 am, two at 2 pm and two at 8 pm after food) was administered for 4-weeks as outlined in Figure 2. There was a 2-week washout period after the

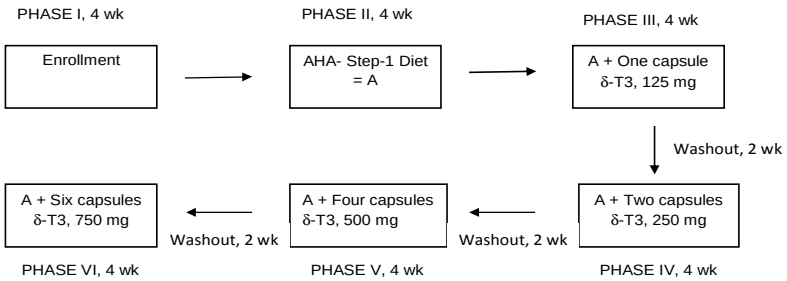

Figure 2: Annatto-based δ-tocotrienol treatment study protocol corresponds to six phases, and each phase lasted for 4 weeks: Enrollment (baseline) = I; AHA Step-1 diet = II; δ-tocotrienol 125 mg/d + AHA Step-1 diet = III; δ-tocotrienol 250 mg/d + AHA Step-1 diet = IV; δ-tocotrienol-500 mg/d + AHA Step-1 diet = V; δ-tocotrienol 750 mg/d = VI, fed to hypercholesterolemic subjects.

treatment of first dose of 125 mg/d, however, all subjects were continued on AHA Step-1 diet for the rest of the treatment period. At the end of the each phase, blood samples were collected after overnight fast of each participant to carry out estimation of lipid parameters and several inflammatory biomarkers.Serum/plasma samples from all the subjects of each group were studied simultaneously to avoid large standard variation/deviation.

2.4 Blood collection:

Venous blood samples (12 h fast, 9:00 pm – 9:00 am) were drawn at screening. At screening, the participants were counseled to follow their normal dietary intake. Screening was accomplished during three to four weeks (baseline). Venous blood samples were drawn at the termination of baseline phase, and at week four of the treatment. The processed samples were coded and held at -72ºC until analyses were carried out, following the completion of treatment phases.

2.5 Analyses:

The analyses of the coded samples were performed at the department of Chemical Pathology & Endocrinology, Armed Forces Institute of Pathology, in Rawalpindi, Pakistan. The analyses of samples of all the phases of each parameter were carried outat the same time to avoid large variation.Serum total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides levels were measured in each sample for every subject. Automated clinical laboratory procedures were used for determining lipid parameters at the end of phase I (4 weeks); II (8 weeks); III (12 weeks), IV (18 weeks), V (24 weeks), and VI (30 weeks). Serum LDL-cholesterol levels were estimated by precipitating 200 µL of serum with 25 µL of a mixture of 9.7 mMphosphotungstic acid and 0.4 M $MgCl_2$. The preparation was mixed for 10 min at room temperature and then centrifuged at 12,000 x g for 10 min. The supernatant fraction was decanted and analyzed for HDL-cholesterol level. The precipitate was dissolved in 200 µL of 0.1 M sodium citrate and LDL-cholesterol level was determined [33]. Serum total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides levels were estimated by using reagent kits from Sigma Chemical Co., St. Louis.

2.6Analyses of total RNA from EDTA treated whole blood after feeding δ-tocotrienol plus AHA step-1 diet for 4- weeks to hypercholesterolemic subjects.

The pure total RNA was extracted from EDTA treated fresh whole blood of subjects treated with most effective dose of δ-tocotrienol (250 mg/d) plus AHA Step-1 diet fed for four weeks, by using total RNA purification kit # 17200 (NORGEN Bioteck Corporation, Thorold, ON, Canada). The purity of total RNA was carried out by measuring the absorption at several wavelengths using a Thermo Scientific NanoDrop 1000 Spectrophotometer. The purity of total RNA was determined by the ratio of 260/280 (2.02 - 2.08). The plasma miRNAs (dose of 250 mg/d of δ-tocotrienol plus AHA Step-1diet fed for four weeks) were also purified by using Plasma/Serum Circulating miRNA Purification Mini Kit (Slurry Format) Product # 51000 (NORGEN Bioteck Corporation, Thorold, ON, Canada).

2.7 Estimation of human plasma cytokines, cDNA, and miRNA:

The various plasma cytokines, cDNA, and microRNAs (miRNAs) were estimated by usingHuman Cytokine Elisa Plate Array I (chemiluminescence), Catalog number EA-4001, Customized Human cDNA Plate Array (Catalog Number AP-UM000416) frommessenger-RNA (mRNA)(Signosis, Inc., Santa Clara, CA, 95054), The mRNA was extracted from each sample was converted to cDNA and plated on a cytokine cDNA array plate (Signosis, Inc.). Assays for estimating the plasma cytokines (protein) and gene expression of messenger RNAs were carried out according to the protocols provided by Signosis, Inc. The incubation of each assay mixture at various temperatures was carried out by usingEnviro-Genie Shaker/incubator (Enviro-Genie Industries, Bohemia, NY). The intensity of chemiluminescence was detected using a MicroplateLuminometer (GloMaxPromega, Madison, WI) at 500 nm, and luminescence was monitored over 20 min period. Estimation of circulating miRNAs was carried out using “Customized miRNA Direct Hybridization Plate Array”, chemiluminescence; Catalog Number Inv-00465 (Signosis, Inc).

2.8 Statistical analyses:

The data were analyzed by using the GLM procedure of SAS (Statistical Analysis System) for personal computers to test the study hypothesis.Analysis of two-way variance was used to test whether changes in

serum lipid parameters occur in the course of supplementation, and whether there were between- and within-subject differences; because all observations were required, available degree of freedom were reduced by this statistical approach [34]. Data are reported as mean ± SD (Standard Deviation). The statistical significance level was set at $P < 0.05$.

3. RESULTS

3.1 Inhibitory effects of δ-tocotrienol plus AHA Step-1 diet on lipid parameters in hypercholesterolemic subjects

The commercial availability of DeltaGold (90% δ-tocotrienol + 10% γ-tocotrienol) from annatto seeds enabled us to carry out dose-response study of 125 mg, 250 mg, 500 mg and 750 mg/d with restricted intake of fat (< 30%), and (< cholesterol 300 mg/d; AHA Step-1 diet) in hypercholesterolemic subjects. All participants (*n* = 31) completed all phases of study, and there was no change in the body weight, and other physical characteristics of the participants during the treatment period (Table 1). Therewere

Table 1. Baseline characteristics of study population

Parameters	Means ± SD
Age (years)	57.84 ± 8.07
Male/Female(n)	26/5
Height (meter)	1.74 ± 0.07
Weight (Kg)	69 ± 7
BMI (Kg/m2)	25.30 ± 1.86
Systolic BP (mmHg)	140.16 ± 6.26
Diastolic BP (mmHg)	90.32 ± 5.31
Blood glucose (mmol/L)	4.22 ± 0.43
Serum Creatinine (µmol/L)	93.39 ± 10.12
Serum ALT (U/L)	36.68 ± 7.97
Serum Cholesterol (mmol/L)	5.44 ± 1.06
Serum triglyceride (mmol/L)	1.81 ± 0.54

insignificant reductions of 2%, 3%, 3% in serum levels of total cholesterol, LDL-cholesterol and triglycerides, respectively, due to dietary restriction (AHA Step-1 diet) after 4-weeks, as compared to

baseline values (Figures 3–5). However, consumption of δ-tocotrienol plus AHA Step-1 diet lowered serum totalcholesterol, LDL-cholesterol and triglycerides levels in a dose-dependent manner below 500 mg/d, in contrast, higher dose of 750 mg/d increased levels of these lipid parameters (Figures 3-5).

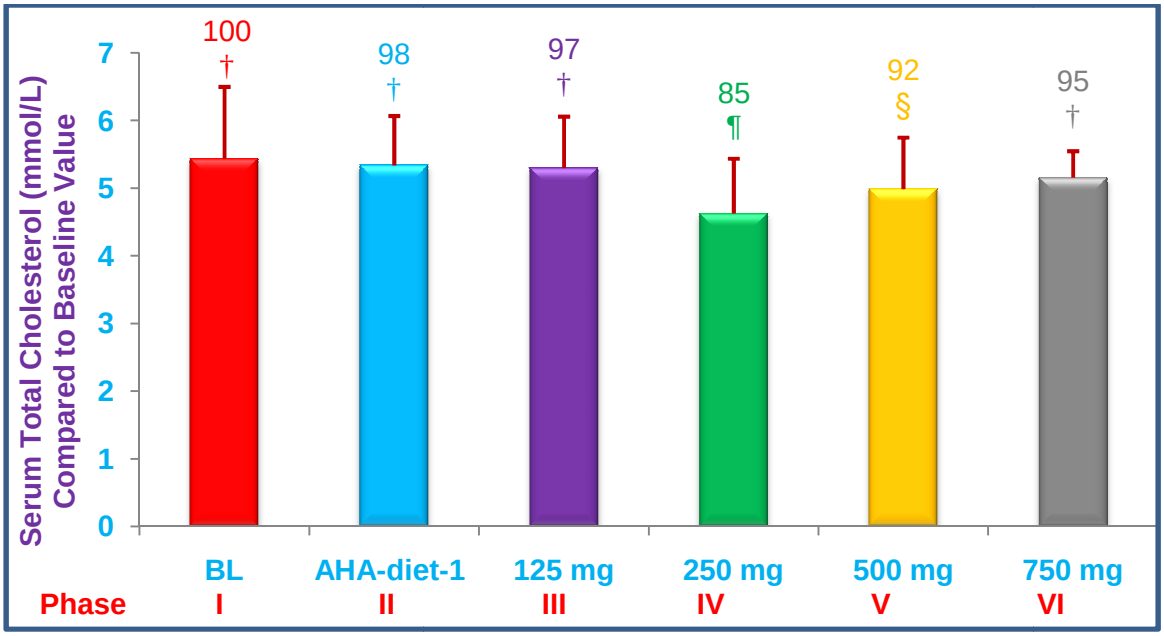

Figure 3: Inhibitory effects of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of total cholesterol in hypercholesterolemic subjects: The treatments 1- 6 correspond to six phases. Data are means ± SD (Standard Deviation). Values in a column not sharing a common symbol are significantly different at ¶ = ***P***< 0.001; § = ***P***< 0.05.

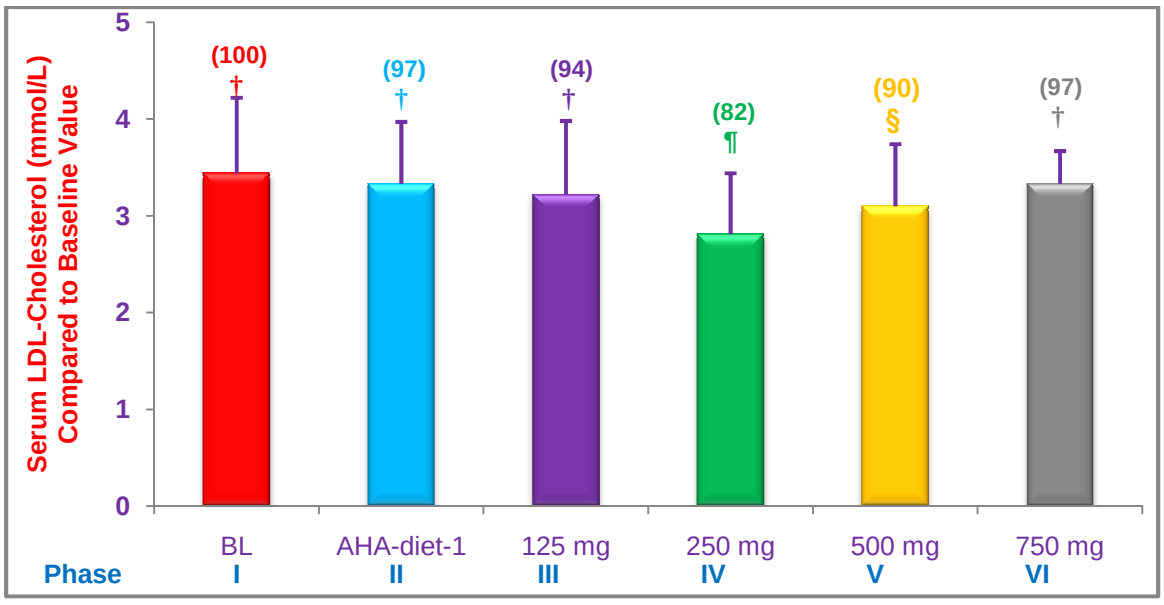

Figure 4: Inhibitory effects of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of LDL-cholesterol in hypercholesterolemic subjects: The treatments 1- 6 correspond to six phases. Data are means ± SD (Standard Deviation). Values in a column not sharing a common symbol are significantly different at ¶ = *P*< 0.001; § = ***P***< 0.03.

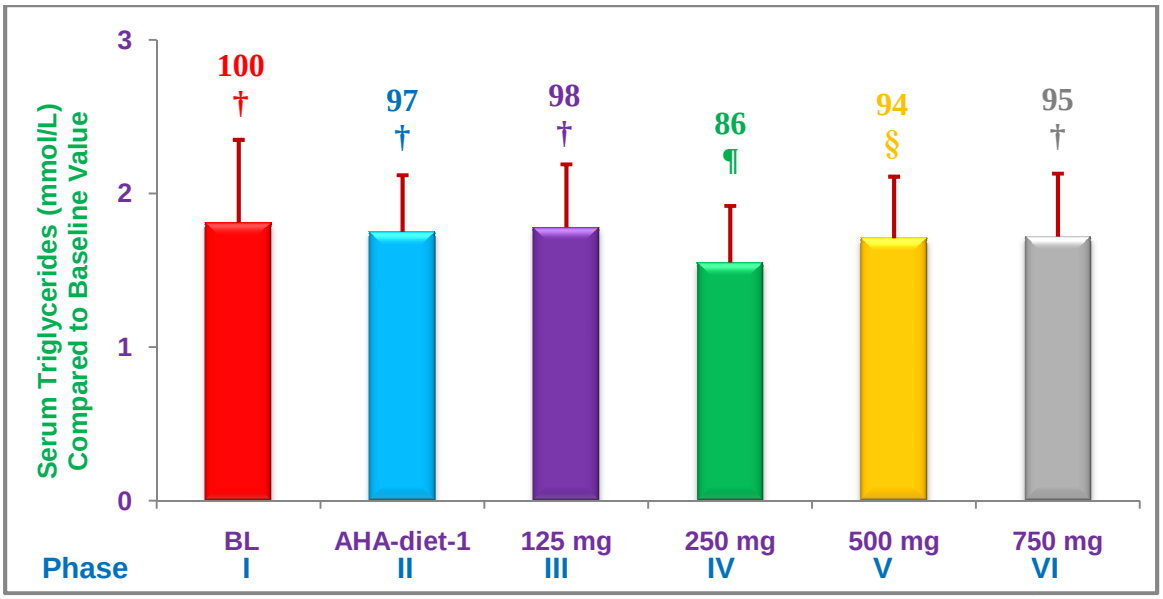

Figure 5: Inhibitory effects of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of triglycerides in hypercholesterolemic subjects: The treatments 1- 6 correspond to six phases. Data are means ± SD (Standard Deviation). Values in a column not sharing a common symbol are significantly different at ¶ = ***P***< 0.001; § = ***P***< 0.05.

The optimal dose was found to be 250 mg/d of δ-tocotrienol, plus AHA Step-1 diet after 4-weeks caused significant reduction of serum total cholesterol (15%; ***P***< 0.001), LDL-cholesterol (18%; ***P***< 0.001) and triglycerides (14%; ***P***< 0.001) levels, compared to baseline (Figures 3, 4, 5). The administration of minimum dose of 125 mg/d of δ-tocotrienol plus AHA Step-1 diet did not cause any remarkable reductions in serum levels of total cholesterol, LDL-cholesterol and triglycerides (3%, 6%, 2%), respectively, compared to baseline (Figures 3-5). This slight reduction might be due to AHA Step-1 diet restriction. Administration of highest dose (750 mg/d) of δ-tocotrienol plus AHA Step-1 diet after 4-weeks resulted in moderate increases of 5%, 3%, 5% in serum levels of total cholesterol, LDL-cholesterol, and triglycerides respectively, compared to baseline, which might be due to novel properties of δ-tocotrienol(Figures 3, 4, 5). Serum HDL-cholesterol level was not affected compared to baseline under these conditions (data not shown). Similar trends of increases or decreases in levels of total cholesterol, LDL-cholesterol, triglycerides, and HDL-cholesterol with various doses also impact the ratios of total cholesterol/HDL-cholesterol and LDL-cholesterol/HDL-cholesterolas compared to baseline (data not shown). The efficacy and safety assessment was done at the end of each phase of increasing doses of tocotrienol treatment. The efficacy was analyzed based on the changes in the lipid parameters as compared withbaseline levels. The safety and tolerability of different doses of tocotrienolsdid not report any adverse events

during the study by any hypercholesterolemic subject, and there wasno adverse effect or reaction alter use of higher dose of 750 mg/d or minimum dose of 125 mg/d of δ-tocotrienol reported by any participant throughout the treatment period of 4-weeks each. Therefore, administration of 125 – 750 mg/d of DeltaGold (90% δ-tocotrienol + 10% γ-tocotrienol) was tolerable and safe for human consumption as tocotrienols has been granted "GRAS" statusby FDA recently [GRAS (Generally Regarded As Safe) Notice No:GRN 000471].

3.2 Evaluation of feeding δ-tocotrienol plus AHA Step-1 dieton levels ofcytokines, gene expression and miRNA in hypercholesterolemic subjects

A panel of six key plasma cytokines) associated with cardiovascular disease (TNF-α, IL-2, IL-4, IL-6, IL-8, IL-10) was selected to investigate the anti-inflammatory and cardio-protective effect of δ-tocotrienol taken orally. The functions of each of these cytokines are reported in Table 2. The AHA Step-1 diet alone did not have any significant effect on the levels of plasma cytokines except on IL-8 (Table 2). However, the

Table 2: Effects of δ-tocotrienol (250 mg/d) + AHA Step-1 diet on various plasma cytokines inhypercholesterolemic subjects.

#	Cytokines Down-Re	Baseline Percentages	AHA Step-1 diet =A Percentages	A = δ-T3 Percentages	Description	Functions
1	TNF-α	100	91.0 ± 1.41[a]	48.5 ± 0.70**	Tumor Necrosis Factor-α	Produced during inflammation.
2	IL-2	100	94.0 ± 1.41	55.5 ± 0.71**	Interleukin-2	for growth proliferation & differentiation of T cells to become "Effector T cells".
3	IL-4	100	93.0 ± 1.41	49.0 ± 1.41**	Interleukin-4	Activation of B-cells& T cells proliferation.
4	IL-6	100	98.0 ± 1.41	38.5 ± 2.21**	Interleukin-6	Regulates immune response& hematopoiesis.
4	IL-8	100	85.5 ± 2.12*	43.5 ± 0.71**	Interleukin-8	Potent anti-angiogenesis factor.
6	IL-10	100	92.5 ± 2.02	63.5 ± 2.12**	Interleukin-10	Immuno-regulation & inflammation.

[a]X ±SD (mean ±Standard Deviation); δ-T3 = δ-tocotrienol;
*-**Values in a row sharing a common symbol are significantly different at *P< 0.05; **P< 0.01.

treatment with δ-tocotrienol plus AHA Step-I diet showed down-regulated levels of TNF-α, IL-2, IL-4, IL-6, IL-8, and IL-10 (39% - 64%)as compared to baseline values. The maximum reduction was observed in IL-6 cytokine, which acts as both a pro-inflammatory and anti-inflammatory cytokine, and secreted by T-cells and macrophages to modulate immune response compared to baseline values (Table-2).The present results show down-regulation of IL-6 and IL-8 levels by δ-tocotrienol, confirming the anti-

angiogenicproperties of δ-tocotrienol. These cytokine data can be very well correlated to gene expression of messenger-RNA (mRNA) purified from fresh EDTA treated whole blood obtained from subjects on the same treatment (250 mg/d; Table 3).

Table 3: Effects of δ-tocotrienol (250 mg/d) + AHA Step-1 diet on various messenger-RNAgene expression in hypercholesterolemic subjects.

	Gene Expr	Baseline	AHA Step-1	AHA Step-1	Description	Functions
	Cytokines		diet	diet + δ-T3		
#		Percentages	Percentages	Percentages		
1	TNF-α	100	96.3 ±2.76[a]	84.5 ± 0.71[a,**]	Tumor Necrosis Factor-α	Inflammation
2	IL-2	100	98.8± 0.95	91.5 ± 3.54*	Interleukin-2	Cytokine involved in proliferation, & differentiation.
3	IL-4	100	96.2 ± 0.40	77.5 ± 2.12**	Interleukin-4	Activation of B-cells & T-cells proliferation
4	IL-6	100	95.0± 0.86	73.5 ± 0.71**	Interleukin-6	NF-κB and IL-6 signaling.
4	IL-8	100	97.0± 1.17	92.0 ± 2.83*	Interleukin-8	Chemokine (involved in angiogenesis).
6	IL-10	100	97.3± 0.65	89.0 ± 1.41*	Interleukin-10	Immuno-regulation and inflammation.

[a]X± SD (mean ±Standard Deviation); δ-T3 = δ-tocotrienol.

*-**Values in a row sharing a common symbol are significantly different at *P< 0.05; **P< 0.01.

The cluster of eight microRNAs(miRNA-7a, miRNA-15a, miRNA-20a, miRNA-21, miRNA-29a, miRNA-200, miRNA)were typicallydown-regulated in hypercholesterolemic subjects (baseline values) as shown in Table 4.

Table 4: The effect of δ-tocotrienol (250 mg/d) + AHA Step-1 diet on plasma circulating miRNAs of cardiovascular disease in hypercholesterolemic subjects.

	MicroRNA =	Baseline	AHA Step-1 diet	AHA Step-1 diet + δ-T3
	miRNA	Percentages	Percentages	Percentages
1	miRNA-7a	100	103.5 ± 2.12[a]	168.0 ± 1.41**
2	miRNA-15a	100	107.6 ± 0.71*	179.0 ± 1.41**
3	miRNA-20a	100	102.5 ± 0.71	168.0 ± 2.24**
4	miRNA-21	100	108.0 ± 2.83*	143.0 ± 2.83**
5	miRNA-29a	100	102.5 ± 0.71	142.0 ± 2.83**
6	miRNA-92a	100	106.5 ± 2.12*	153.5 ± 2.12**
7	miRNA-200	100	104.0 ± 1.41	146.0 ± 1.41**
8	miRNA-206	100	109 .0 ± 2.83*	150.0 ± 2.83**

[a]X± SD (mean ±Standard Deviation); δ-T3 = δ-tocotrienol.

*-**Values in a row sharing a common symbol are significantly different at*P< 0.05; **P< 0.01.

The δ-tocotrienol plus AHA Step-1 diet treatment up-regulated miRNAs as compared to baseline values (Table 4). The AHA Step-1 diet treatment resulted only in slight up-regulation in these miRNAs. These results indicated that δ-tocotrienol treatment up-regulated a cluster of selected plasma miRNAs levels of hypercholesterolemic subjects.

5. DISCUSSION:

The maximum decreases of 2% to 3% resulted due to AHA Step-1 diet dietary modification in the present study confirming our earlier findings [7-9].The present results of dose-response study demonstrate that δ-tocotrienol specifically lowered the levels of serum total cholesterol, LDL-cholesterol, and triglycerides in a dose-dependent manner below 500 mg/d, and at higher dose of 750 mg/d increased the levels of these three lipid parameters (Figures 3-5). These results are consistent with our recent findings of dose-dependent inhibition of chymotrypsin-like activity of 20S rabbit muscle proteasomes between 5 μM and 40 μM for mevinolin and δ-tocotrienol, the inhibitory effects of mevinolin and δ-tocotrienol were reversed at higher concentrations between 80 μM and 320 μM [21]. This clearly demonstrates that δ-tocotrienol and mevinolin modestly inhibit or activate the proteasomal activity depending on its concentrations [21,35-37]. Thus, δ-tocotrienol is the first naturally-occurring compound, which blocks the proteasomal activity with low doses, and is able to halt and reduce the inflammatory response. This property of δ-tocotrienol may be useful for the control of cardiovascular disease, and at higher doses may cause apoptotic cell death in various types of cancers [38]. Similar dose-dependent activities (inhibition versus induction) and properties have been reported for synthetic proteasomal inhibitors, MG132 and lactacystin [35-37]. The aforementioned are very potent proteasome inhibitors in the range of 5 μM to 20 μM, but very toxic as well, barring their use in humans. Conversely, tocotrienols have been found safe even at doses of 1600-3200 mg/d in the treatment of pancreatic cancer [38].

Moreover, a dose of 250 mg/d caused significant reductions in all three lipid risk factors (total cholesterol, LDL-cholesterol, and triglycerides) after 4 weeks of treatment. The lower dose of 125 mg/d may have shown additional lipid lowering benefits provided the treatment period had been extended to 8 weeks or more. As reported earlier, the hepatic HMG-CoA reductase activity is inhibited by low doses of γ- andδ-

tocotrienols, whereas tocopherols, α-tocopherol in particular, induces the activity of HMG-CoA reductase (a rate-limiting enzyme in the biosynthesis of cholesterol) and consequently raises cholesterol [24,39]. This disadvantage of high dose of tocotrienols does not apply to their other functions, such as cancer chemoprevention and treatment, where large doses are used in current clinical trials, probably by activating the immune response [38].

It is also interesting to note that synthetic α-tocopherol at 400 IU/day was shown to increase the risk of prostate cancer by 17% in a large scale "Selenium and Vitamin E Cancer Prevention Trial (SELECT)" [40]. It is well documented that high cholesterol is associated with increased risk of prostate cancer [41-44], and prostate cancer cells accumulate cholesterol to spur their growth [45]. Thus it is plausible that the elevated prostate cancer risk of the above study is due to α-tocopherol's stimulation of the cholesterol synthesis pathway [40], while tocotrienols were indicated as potential therapeutic agents for prostate cancer owing to their ability to lower and degrade a major transcription factor in the cholesterol synthesis pathway [45]. Our present study reported no adverse events with large tocotrienol doses, suggesting that δ-tocotrienol at doses as high as 750 mg/day is safe for human consumption. Pure δ-tocotrienol may be safe for human consumption even at doses of 3,200 mg/d, as was shown in a recent Phase I Clinical Trial in patients with pancreatic cancer [38].

Recently, inflammation has been associated with several diseases including cardiovascular disorders [26]. The present study demonstrates that δ-tocotrienol effectively down-regulated inflammatory cytokines and gene expression of TNF-α, IL-2, IL-4, IL-6, and IL-8. The maximum down-regulation occurred with IL-6, which is both a pro-inflammatory cytokine (in the case of chronic inflammation and oncogenesis) and anti-inflammatory cytokine (in the case of immune regulation and support of hematopoiesis) [46]. While various studies have confirmed tocotrienol's anti-inflammatory functions, particularly for TNF-α and on a proteasomal level [21,23,47], they are also known to support the immune system [48]. Hence they do not appear to adversely affect the anti-inflammatory properties of IL-6. In the present study, results showing down-regulation of IL-6 and IL-8 levels by δ-tocotrienol confirm the anti-angiogenic properties of δ-tocotrienol in pathological conditions. The down-regulation of IL-6 also indicates an effect on NF-κB, by which this cytokine is expressed. Tocotrienol's effect on NF-κB and cytokine expression has been shown

earlier [21]. Interleukin-10 (IL-10) is capable of inhibiting several pro-inflammatory cytokines such as TNF-α, IL-2, IFN-γ, and granulocyte macrophage-colony stimulating factor produced by macrophages, regulatory T-cells (Th2), mast cells, stimulate B cell maturation and antibody production. IL-10 was modestly increased in premature coronary disease [49].

The levels of miRNA have been shown to be important regulators of gene expression that modify cellular responses and function [50-52]. The dysregulation of miRNA plays a crucial role in the development of cardiovascular disease, diabetes and cancer. In the present study, we focused on miRNA involved only in cardiovascular disease [52]. The effect of δ-tocotrienol's on miRNAs may have important implications in the management of chronic diseases. The present study found that δ-tocotrienol up-regulated miRNA-7a, miRNA-15a, miRNA-20a, miRNA-21, miRNA-29a, miRNA-92a, miRNA-200 and miRNA-206 in hypercholesterolemic humans. MicroRNAs play multiple roles in various biological processes as well as normal physiological functions, and may also display pathological activity. Since levels of eight miRNAs tested in the present study were down-regulated in the hypercholesterolemic population as compared to normal cholesterolemicsubjects according to published report [52], up-regulation by δ-tocotrienol of these miRNAspoints to a beneficial effect of tocotrienols. MicroRNA-29a is enriched in fibroblasts and encodes proteins involved in fibrosis, including collagen, fibrillins, and elastin [53]. In myocardial infarction and associated cardiac hypertrophy, miRNA-29a is decreased, allowing for expression and deposition of collagen components in the fibrotic scar [30]. Up-regulation of miRNA-29a such as with δ-tocotrienol may provide a significant therapeutic option for myocardial infarction (MI), reducing scar formation in post-myocardial infarction remodeling.

MiRNA-20a is anti-angiogenic and known to inhibit the proliferation and metastasis of pancreatic cancer [54]. It also prevents myocardial hypertrophy and angiogenesis during stress [55]. By up-regulating miRNA-20a, δ-tocotrienol may decrease angiogenesis during stress situations to prevent abnormal increase of heart size. Similarly, miRNA-206 that is essential in promoting skeletal muscle regeneration delays the progression of amyotrophic lateral sclerosis [56], while suppressing gastric cancer cell growth and metastasis [57]. While there may be important implications for δ-tocotrienol in these applications [58], the present study focused on the supplement's relevance in cardiovascular diseases. Skeletal muscle

degeneration, ameliorated by miRNA-206, was found to contribute to cardiac dysfunction [59], and hence miRNA-206 may play a pivotal role in the heart muscle [31].δ-Tocotrienol's up-regulation of miRNA-206 may contribute to myocardial and vascular regeneration, as demonstrated by a previous study in murine chronically failing hearts [60]. The positive impact of γ-tocotrienol of remaining miRNAs has been described in detail in recent publication[61].

Itshould be pointed out that the present preliminary study that started as a double-blind study, but during phase V, most of the participants figured out that they were involved in a dose-response study, however,most effective dose of 250 mg/d of δ-tocotrienol wasfound to be responsible for lipid-lowering in hypercholesterolemic subjects. In order to validate the results of present study on the efficacy of δ-tocotrienol as hypocholesterolemic and anti-inflammatory agent, a larger, more comprehensive double-blind long-term (12-months) study should be carried out by enrolling equal number of male and female hypercholesterolemic subjects (50 of each), a placebo group (corn oil or olive oil stripped tocols after extraction with hexane) should be added, and subjects of one more group should be kept on AHA Step-1 diet for at least 4-months (administered placebo capsule) to establish the long-term impact of dietary restriction in hypercholesterolemic subjects. The lipid profile of all the subjects should be monitored every 2-weeks by using Cholestech LDX unit, which requires only 10 μL blood sample obtained by pricking finger, and gives accurate values of total cholesterol, LDL-cholesterol, triglycerides, and glucose within 5 minutes. Furthermore, in our earlier double-blind studies, the impact of AHA Step-1 diet, and changes in lipid profile occurred mostly between 7 to 10-days, and after first 4-weeks on restricted diet, the decrease in serum total cholesterol was 5%, and feeding for an additional 4-weeks along with restricted diet resulted in only 2% more decrease compared to baseline values [7-9]. The 2-week washout period was used due to these observations in present and previous studies [62]. During washout period, all the subjects were provided placebo capsules. The estimation of various cytokine/protein, and their gene expression levels could be carried out using microarray analyses, coupled with RT-PCR, instead of customized array plates.The present studylacked the discussion about bioavailability and absorption of tocotrienols, because serum/plasma levels of tocotrienols were not determined in the present study, and will be studied in a future study.

6. CONCLUSION:

The present results indicate that doses below 500 mg/d of δ-tocotrienol (250 mg/d) administered for 4-weeks is effective in lowering lipid parameters, down-regulating several inflammatory biomarkers (TNF-α, IL-4, IL-6, IL-8, and IL-10) and doses above 500 mg/d of δ-tocotrienol (750 mg/d) up-regulate these biomarkers and possibly kill cancer cells. Therefore, the capacity of tocotrienols to modulate inflammation may be attributable, in part, to their dose-dependent properties of inhibitionof gene expression in cardiovasculardiseaseand for activation of immune responses to killcancer cells. δ-Tocotrienol was also found to be a potent naturally-occurring compound, which could alter the dysregulation of a number of miRNAs (miR-7a, miR-15a, miR-20a, miR-20, miR29a, miR-92a, miR-200, and miR-206) levels in hypercholesterolemic subjects. Future investigations may explore the combined therapy of δ-tocotrienol and other naturally-occurring compounds(resveratrol,quercetin, curcumin) having complementary mechanisms of action as more effective agents for patients with dyslipidemia, and hypercholesterolemia, and may play a major and significant role in the future management of cardiovascular disease.

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COMPETING INTEREST

The authors declare that they have no competing interests.

Ethical Approval

All authors hereby declare that the trial has been examined and approved by the Independent Ethics Committee of Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Author's Contributions

The present study was carried out in collaboration between all authors. AAQ was responsible for research planning, analysis of cytokines/miRNA, interpretation and writing the manuscript for publication. DAKcarried out the study/performed lipids analysis, compilation of study results, the statistical analyses, and revision of manuscript. WM did screening/clinical examination of the hypercholestrolemic subjects, data collection and follow up of the subjects. NQcritically reviewed protocol and manuscript for publication.

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CONSENT

All authors declare that they have read the manuscript, and written informed consent was obtained from each subject (or any other approved parties) for publication of this study.

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