Original Research Article

Dose-dependent Modulation of Lipid Parameters and Inflammatory Biomarkers by δ-Tocotrienol in Hypercholesterolemic Subjects

ABSTRACT

Aims: Evidence suggests that while tocotrienols lower serum total cholesterol and LDL-cholesterol levels in hypercholesterolemic subjects, the tocotrienol rich fraction (TRF) of palm oil has been inconsistent in achieving such effects. This is perhaps attributable to the presence of substantial amounts (> 20%) of α-tocopherol in TRF, which inhibits the benefits achieved with tocotrienols.

Study Design: The present dose-response study examined the effects of δ-tocotrienol free from tocopherols on serum lipid parameters, and several inflammatory biomarkers (TNF-α, IL-4, IL-6, IL-8) including circulating miRNAs expression in hypercholesterolemic subjects for 30-weeks.

Results: The δ-tocotrienol (125, 250, 500,750 mg/d) plus AHA Step-1 diet fed to hypercholesterolemic subjects (***n*** = 31) for 4-weeks, effected reductions in total cholesterol (12-14%; ***P*** < 0.05), LDL-cholesterol (16-19%; ***P*** < 0.03), triglycerides (11-14%; ***P*** < 0.05) in a dose-dependent manner below 500 mg/d, and 750 mg/d dose resulted induction in the levels of these lipid parameters (9%, 8%, 11%; ***P*** < 0.05), without affecting HDL-cholesterol. The inflammatory cytokines/proteins associated with cardiovascular disease (plasma TNF-α, IL-2, IL-4, IL-6, IL-8) were all down-regulated by 30-60%, while angiogenic growth factors important for regeneration of ischemic myocardium (FGF-b, PDGF) were up-regulated (3-6 fold) by δ-tocotrienol treatment. The gene expression of cytokines/proteins using mRNA followed the similar pattern. Circulating miRNA-20a (anti-angiogenic), miRNA29a (skeletal muscle regeneration) were down-regulated in hypercholesterolemic subjects, and were up-regulated by δ-tocotrienol treatment compared to baseline.

Conclusions: The present results confirm that consumption of δ-tocotrienol plus AHA Step-1 diet causes significant reduction in serum lipid parameters and down-regulation of several inflammatory biomarkers (TNF-α, IL-2, IL-4, IL-6, IL-8) at a low dose of 250 mg/d, while higher doses up-regulate these biomarkers. The capacity of tocotrienol to modulate inflammation is partly attributable to their dose-dependent properties of inhibition and activation, with major implications for the future management of cardiovascular diseases and cancers.

***Keywords*:** DeltaGold-90% δ-tocotrienol, lipid parameters, inflammatory biomarkers, cytokines, gene expression, TNF-α, FGF-b, PDGF, Circulatory miRNAs.

ABBREVIATIONS:

Palm oil TRF: palm oil tocotrienol rich fraction (14.64% α-tocotrienol, 27.59% γ-tocotrienol, 6.33% δ-tocotrienol, 33.28% α-tocopherol, 7.31% phytosterol, 10.85% terpenes

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-2: interleukin-2

FGF-b: fibroblast growth factor-b

PDGF: platelet derived growth factor

miRNAs: micro-ribonucleic acids.

1. INTRODUCTION

The tocotrienol rich fraction (TRF) from palm oil, comprising of a mixture of tocopherols and tocotrienols, has shown both positive [1-11] and negative [12-16] hypocholesterolemic effects in a number of reported clinical studies [1-16]. 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 [6,8]. A major factor underlying their failure 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 [13-16]. Palm TRF also does not reduce serum total cholesterol level in free-living hypercholesterolemic patients [14-16], or healthy humans even if the TRF contained less than 15% α-tocopherol [12]. Furthermore, large doses of tocotrienols have also proved ineffective (6, 17-19) perhaps owing to bioconversion of tocotrienols to α-tocopherol, which antagonizes this beneficial effect [6]. The serum level of α-tocopherol was 2 to 4 fold higher, as compared to the placebo group in these studies [13-16].

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

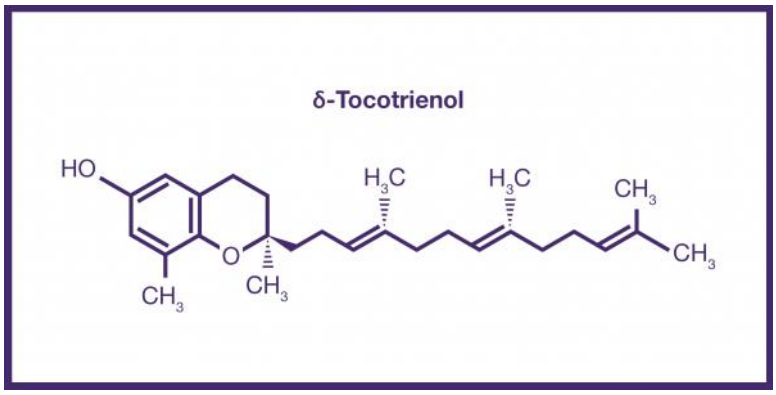


Figure 1: Chemical structure of δ-tocotrienol.

this human study possible. We have previously demonstrated the mechanism through which tocotrienols exert their effects by suppressing the activation of nuclear factor-κB (NF-κB) in various experimental models [20]. Moreover, the order of potency of various tocotrienols for acting as cholesterol-lowering,

Anti-oxidant, anti-inflammatory and anticancer agents were as δ-tocotrienol > γ-tocotrienol > α-tocotrienol > α-tocopherol [21,22]. Recently, a comprehensive review has reported the various biological properties of tocotrienols, including results of various clinical studies of palm TRF and pure tocotrienols [23].

The present study also examined the inflammatory 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 [24], interleukin-2 (IL-2) level is elevated in patients with stable angina [25], interleukin-4 (IL-4), an activator of collagen synthesis that may be involved in cardiac fibrosis [26], interleukin-6 (IL-6) production promotes myocardial injury [27], and interleukin-8 (IL-8), a cytokine found in vascular injury sites [28]. In addition, two growth factors associated with cardiac angiogenesis, fibroblast growth factor-b (FGF-b) and platelet-derived growth factor (PDGF), were also estimated.

The expression of circulating microRNAs (miRNA) that are small non-coding RNAs, likely involved in many biological processes were also analyzed [29,30]. The present study will also evaluate δ-tocotrienol's effect on selected miRNAs associated with cardiovascular disease. Particularly, miRNA-7a, miRNA-15a, miRNA-20a, miRNA-21, miRNA-29a, miRNA-92a, miRNA-200, and miRNA-206 were examined. The present study of effects of dose-response (125, 250, 500,750 mg/d) of DeltaGold (90% δ-tocotrienol + 10% γ-tocotrienol) plus AHA Step-1 diet in hypercholesterolemic subjects was carried out on serum lipid parameters, various cytokine levels, and their gene expression and circulating miRNA levels associated with cardiovascular disease.

2. MATERIALS AND METHODS

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

2.1 Study Design

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

Hypercholesterolemic subjects (*n* = 31; 26 males + 5 females; age > 50 years; serum total cholesterol level > 5.50 mmol/L were enrolled in study at Wah Cantonment, Pakistan. The study protocol was registered at a governmental agency (University of Health Science, Lahore, Pakistan), and study protocol was approved by Institutional Review Board of Armed Forces Institute of Pathology, Rawalpindi, Pakistan and also University of Health Science, Lahore, Pakistan. All subjects signed an informed-consent form, which was approved by Institutional Board of Armed Forces Institute of Pathology, Rawalpindi, Pakistan.

2.2 Study Subjects

Exclusion criteria included weight (> 125% of Metropolitan Life relative weights), use of cholesterol altering medication, elevated serum glutamate-pyruvate or glutamate-oxaloacetate transaminase activity, an elevated blood urea nitrogen or glucose value, diabetes, or a liver, renal, or hypertensive disease. Clinical history was taken and physical examination carried out for each participant. The initial measures included the participant's height, weight, and 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 used as a measure of relative body weight. Weights were recorded daily. 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. All relevant investigations were carried out in the Department of Chemical Pathology & Endocrinology, Armed Forces Institute of Pathology, 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. **The study was carried out under a FDA approved IND number 36906.**

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 the participants were continued on the AHA Step-1 diet during phases III, IV, V and VI. During **phase III**, all the participants were administered 1 capsule (125 mg/d) of δ-tocotrienol (8 pm after food) for 4-weeks. During **phase IV**, the participants were administered 2 capsules of 125 mg (250 mg/d; 1 at 8 am and 1 at 8 pm after breakfast and dinner) for 4-weeks, followed by 4 capsules of 125 mg (500 mg/d; 2 at 8 am and 2 at 8 pm) in **phase V** and during last **phase VI,** 6 capsules of 125 mg (750 mg/d; 2 at 8 am, 2 at 2 pm and 2 at 8 pm after food) was administered for 4-weeks as outlined in Figure 2. There was a 2-week washout period (continue on AHA Step-1 diet) after the treatment of first dose of 125 mg/d for the rest of the treatments, including washout 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.

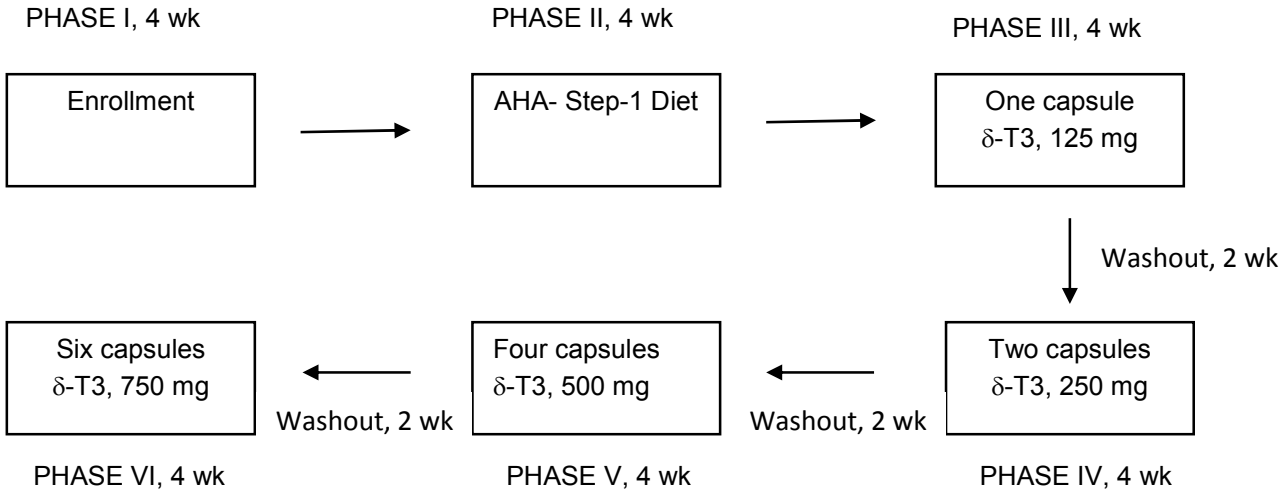


Figure 2: Annatto-based δ-tocotrienol treatment study protocol corresponds to six phases, and each phase lasted for 4 weeks: Enrollment (baseline) = I.; AHA Step-1 diet = II; δ-tocotrienol 125 mg/d + AHA Step-1 diet = III; δ-tocotrienol 250 mg/d + AHA Step-1 diet = IV; δ-tocotrienol-500 mg/d + AHA Step-1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

2.4 Analyses:

The analyses of the coded samples were performed at the department of Chemical Pathology & Endocrinology, Armed Forces Institute of Pathology, in Rawalpindi, Pakistan. Serum total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides levels were measured of 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 was estimated by precipitating 200 µL of serum with 25 µL of a mixture of 9.7 mM phosphotungstic 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 [31-33]. The LDL-cholesterol was estimated as described above, and also calculated by Friedwald's formula by subtracting the total cholesterol from (HDL-cholesterol + triglycerides/5) [34]. Serum total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides levels were estimated by using reagent kits from Sigma Chemical Co., St. Louis.

2.5 Microarray analyses 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 obtained from the EDTA treated fresh whole blood of most effective dose of δ-tocotrienol (250 mg/d) plus AHA Step-1 diet fed for four weeks, by using NORGEN Bioteck Corporation kit (Thorold, ON, Canada) of total RNA purification kit # 17200. 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 NORGEN Bioteck Corporation kit (Thorold, ON, Canada of Plasma/Serum Circulating miRNA Purification Mini Kit (Slurry Format) Product # 51000.

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

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 mRNA. The mRNA was extracted of each sample and converted to cDNA and plated on a cytokine cDNA array plate (Signosis, Inc.). The assays for estimating the plasma cytokines (protein) and gene expression of mRNA were carried out according to the protocols provided by Signosis, Inc. The incubations of each assay mixtures at various temperatures were carried out by Enviro-Genie Shaker/incubator (Enviro-Genie Industries, Bohemia, NY). The intensity of chemiluminescence was detected using a Microplate

Luminometer (GloMax Promega, Madison, Wisconsin) at 500 nm, and emission was monitored over period of 20 min period. Similarly, estimation of miRNAs (Circulating RNAs) was also carried out using Signosis, Inc. (1700 Wyatt Drive Suite 10-12. Santa Clara, CA, 95054) Customized MiRNA Direct Hybridization Plate Array (chemiluminescence; Catalog Number Inv-00465).

2.7 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 [35]. Data are reported as mean ± SE (Standard Error). 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). There were

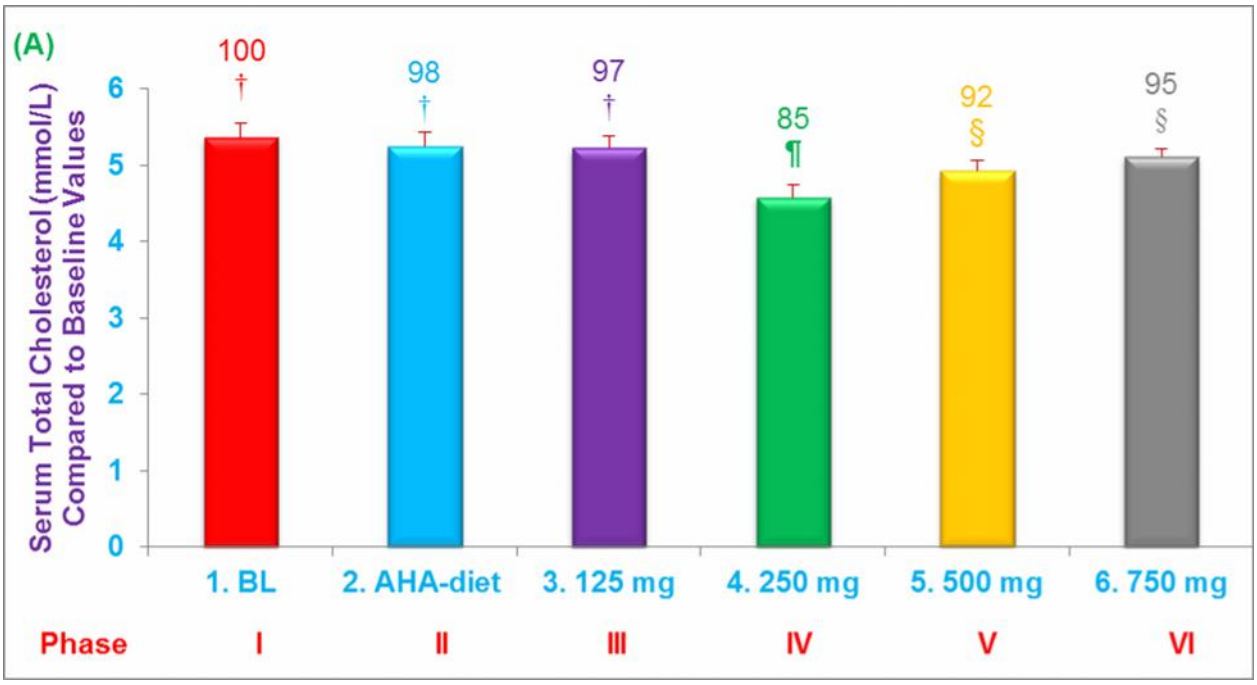
Table 1: Impact of δ-tocotrienol + AHA Step-1 diet on physical characteristics of the study population of hypercholesterolemic subjects[1].

Characteristics	[a]Pre-treatment values	[b]Post-treatment values	[c]Post-treatment values
	Baseline	(250 mg/d)	(750 mg/d)
Subjects- Males	26	26	26
Subjects-Females	5	5	5
Total	**31**	**31**	**31**
Age (years)	57.84 ± 8.07	58.34 ± 7.48	58.44 ± 7.52

Body weight (kg)	69 ± 7	68 ± 7	66 ± 8
Height (cm)	174.2 ± 7.7	174.1 ± 7.7	174.1 ± 7.8
Body mass index (kg/m^2)	25.3 ± 1.86	24.4 ± 2.3	24.4 ± 2.3

[1]Baseline physical profiles of subjects (n = 31) enrolled at the start of the study.
[a]Physical profiles of subjects (n = 31) completing all the phases (I, II, III, IV, V, VI) of the study.
[b]Values represent at the end of (250 mg/d) phase IV for 4 weeks.
[c]Values represent at the end of (750 mg/d) all phases for 4 weeks.

218 insignificant reductions of 3%, 4%, 6% in serum levels of total cholesterol, LDL-choleserol and
219 triglycerides, respectively, due to dietary restriction (AHA Step-1 diet) after 4-weeks, as compared to
220 baseline values (Figures 3A – 5C). However, consumption of δ-tocotrienol plus AHA Step-1 diet lowered
221 serum total cholesterol, LDL-cholesterol and triglycerides levels in a dose-dependent manner below 500
222 mg/d, in contrast, at higher dose of 750 mg/d increased levels of these lipid parameters (Figures 3A-5C).



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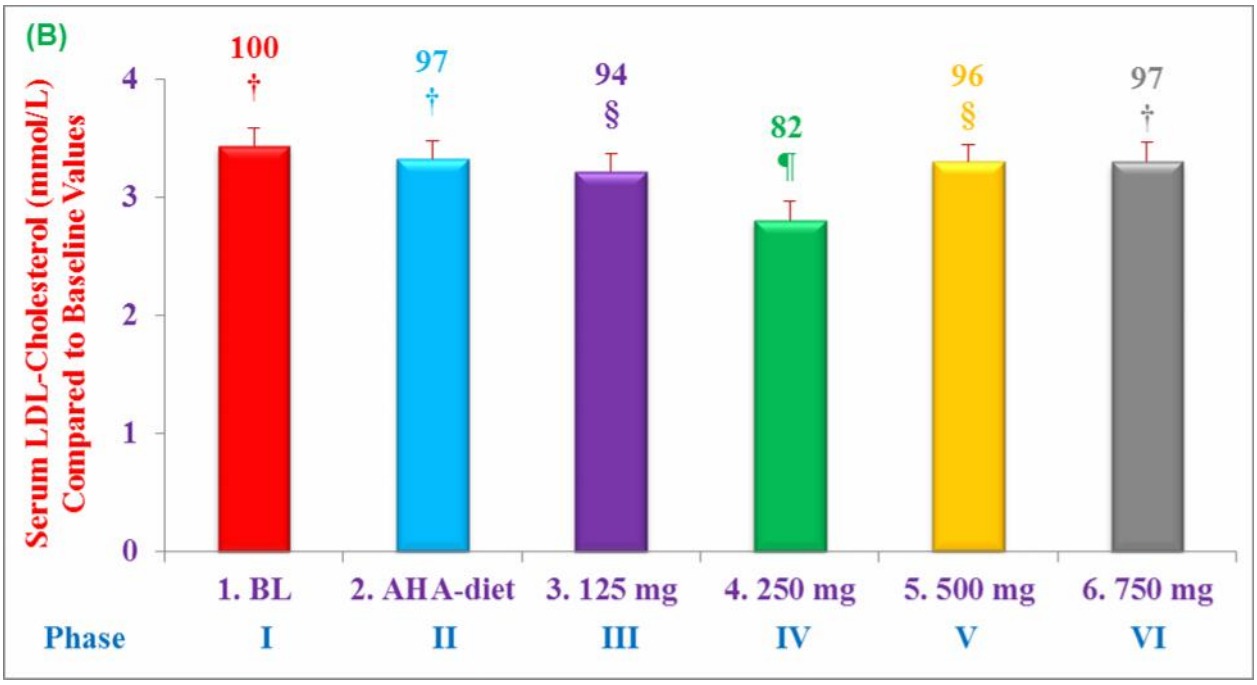
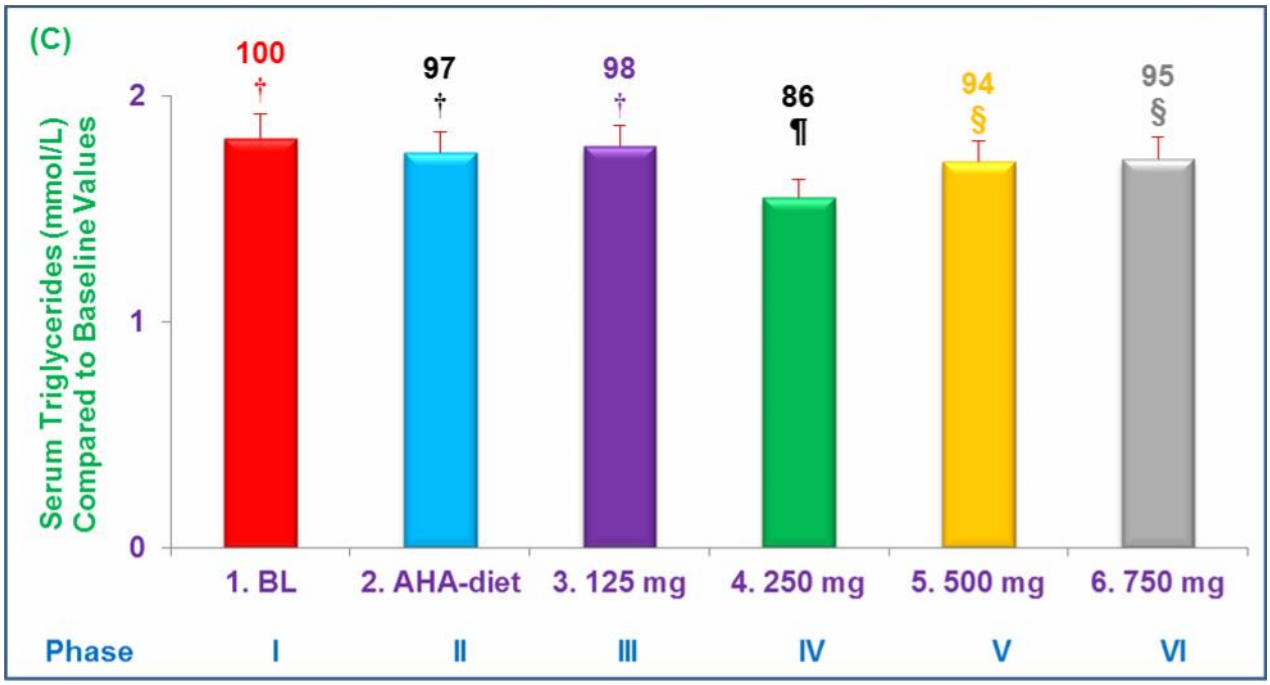
224 **Figure 3A: Inhibitory effects of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of total-**
225 **cholesterol (A) in hypercholesterolemic subjects:** The treatments 1- 8 correspond to six phases. Data are means ±
226 SE (standard error). Values in a column not sharing a common symbol are significantly different at ***P*** < 0.05.

227 The optimal dose was found to be 250 mg/d of δ-tocotrienol, plus AHA Step-1 diet after 4-weeks caused
228 significant reduction of serum total cholesterol (14%; ***P*** < 0.05), LDL-cholesterol (19%; ***P*** < 0.03) and
229 triglycerides (14%; ***P*** < 0.05) levels, compared to baseline (Figures 3A, 4B, 5C). The administration of
230 minimum dose of 125 mg/d of δ-tocotrienol plus AHA Step-1 diet did not cause any remarkable reductions

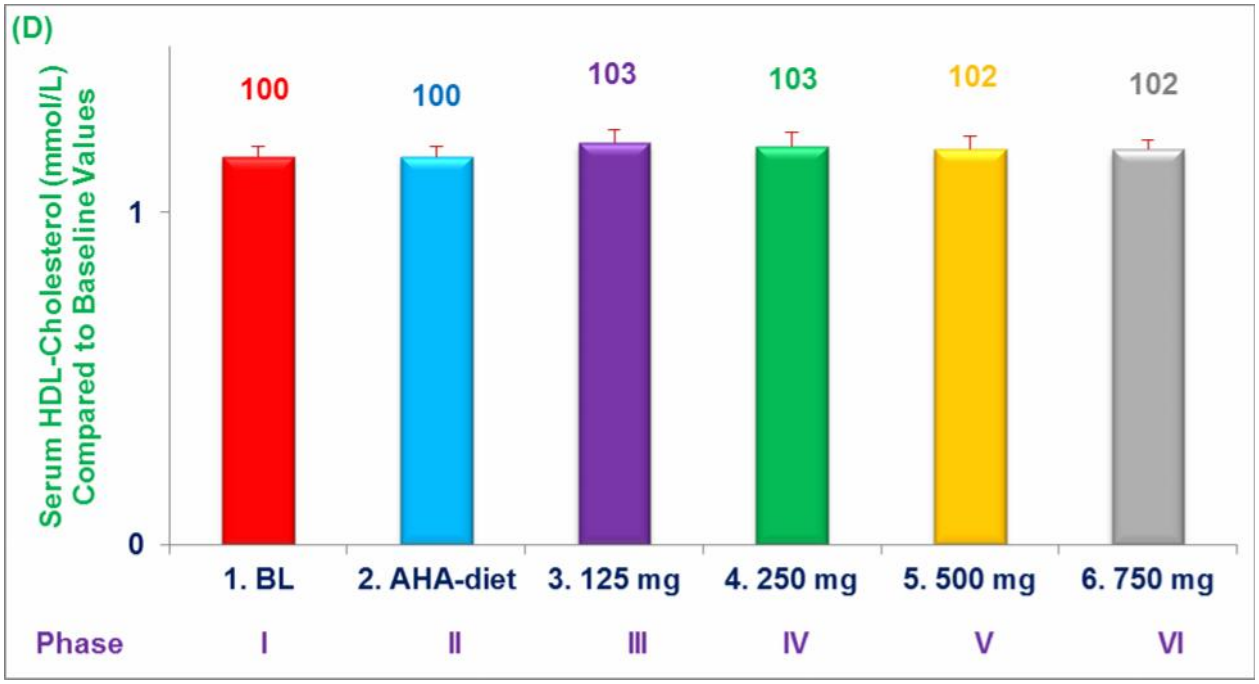
in serum levels of total cholesterol, LDL-cholesterol and triglycerides (4%, 6%, 9%), respectively, compared to baseline (Figures 3A - 5C). This slight reduction might be due to AHA Step-1 diet restriction. Administration of highest dose (750 mg/d) of δ-tocotrienol plus AHA Step-1 diet after 4-weeks resulted in significant (***P*** < 0.05) increases of 9%, 8%, 11% in serum levels of total cholesterol, LDL-cholesterol, and





Figures 4B, 5C: Inhibitory effects of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of LDL-cholesterol (B), triglycerides (C) in hypercholesterolemic subjects: The treatments 1- 8 correspond to six phases. Data are means ± SE (standard error). Values in a column not sharing a common symbol are significantly different at ***P*** < 0.03, and 0.05, respectively.

triglycerides respectively, which might be due to novel properties of δ-tocotrienol, as also reported for proteasome inhibitors (MG-132 and lactacystin ([36-38]; Figures 3A, 4B, 5C). Serum HDL-cholesterol level was not affected compared to baseline under these conditions (Figure 6D). Similar trends of increases or



Figures 6D: Inhibitory effects of various doses of δ-tocotrienol plus AHA Step-1 diet on serum levels of HDL-cholesterol (D) in hypercholesterolemic subjects: The treatments 1- 8 correspond to six phases. Data are means ± SE (standard error). Values in a column not sharing a common symbol are significantly different at ***P*** < 0.05.

decreases of total cholesterol, LDL-cholesterol, triglycerides, and HDL-cholesterol cholesterol of various doses

Table 2: Impact of δ-tocotrienol + AHA Step-1 diet on ratios of total cholesterol/HDL-cholesterol and LDL-cholesterol/HDL-cholesterol of hypercholesterolemic human subjects.

Treatments		Total cholesterol/HDL-cholesterol	LDL-cholesterol/HDL-cholesterol
1. Baseline[1]		4.55 (100)[2]	2.89 (100)[2]
2. AHA step-1 diet = A		4.46 (98)	2.81 (97)
3. A + δ-Tocotrienol	125 mg/d	4.38 (96)	2.76 (96)
4. A + δ-Tocotrienol	250 mg/d	3.91 (86)	2.36 (82)
5. A + δ-Tocotrienol	500 mg/d	4.50 (99)	2.98 (103)
6. A + δ-Tocotrienol	750 mg/d	4.95 (109)	3.12 (108)

[1]Baseline of subjects (*n* = 31) enrolled at the start of the study.

[2] Percentages as compared to baseline values.

249 impact were observed in the ratios of total cholesterol/HDL-cholesterol and LDL-cholesterol/HDL-
250 cholesterol compared to baseline (Table 2). There was no adverse effect or reaction of use of higher dose
251 of 750 mg/d or minimum dose of 125 mg/d of δ-tocotrienol reported by any participants throughout the
252 treatment periods of 4 weeks each. Therefore, administration of 125 - 750 mg/d of DeltaGold (90% δ-
253 tocotrienol + 10% γ-tocotrienol) was tolerable and safe for human consumption.

254 ***3.2 Effect of feeding δ-tocotrienol plus or minus AHA Step-1 diet on serum/plasma***
255 ***cytokines, gene expression and miRNA in hypercholesterolemic subjects***

256 A panel of eight key plasma proteins (cytokines) associated with cardiovascular disease (TNF-α, IL-2, IL-
257 4, IL-6, IL-8, FGF-b, PDGF) was selected to investigate the anti-inflammatory and cardioprotective effect
258 of δ-tocotrienol taken orally. The functions of each of these cytokines are reported in Table 3.The AHA

Table 3: Modulation of AHA Step-1 diet and δ-tocotrienol (δ-T3) + AHA Step-1 diet on the levels of protein expression in hypercholesterolemic subjects.

#	Cytokines	Baseline RLU*	%	AHA-Step-1 RLU*	%	AHA-Step-1+ δ-T3, RLU*	%	Description	Functions
1	TNF-α	185490	100	1704332	92	89605	48	Tumor necrosis factor-α	inflammation
2	IL-2	1762258	100	1715963	97	1057608	60	Interleukin -2	Proliferation & differentiation
3	IL-4	42548	100	40562	95	21310	50	Interleukin-4	Activation of B cells and T cells
4	IL-6	13426	100	129876	96	109127	40	Interleukin-6	NF-κB and IL-6 signaling
5	IL-8	149888	100	125487	84	65705	44	Interleukin-8	Chemokine
6	IL-10	310758	100	286528	92	207080	67	Interleukin-10	Immunoregulation
7	FGF-b	28295	100	32629	115	86339	305	Fibroblast growth factor-b	Angiogenesis
8	PDGF	37204	100	45982	124	250755	674	Platelet derived growth factor	Anti-inflammatory

*RLU= relative luminescence units.

Step-1 diet alone did not have any significant effect on the levels of plasma cytokines (Table 3). However, the treatment with δ-tocotrienol plus AHA Step-I diet showed down-regulated levels of TNF-α, IL-2, IL-4, IL-6, and IL-8 (40% - 60%), 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. The angiogenic growth factors, FGF-b, and PDGF were up-regulated 3- and 6-fold, respectively, by treatment of δ-tocotrienol plus AHA Step-1 diet (Table 3) compared to baseline values. The FGF-b is very effective in inducing angiogenic response *in vivo* and survival of many cell types *in vitro,* such as smooth muscle and endothelial cells. The PDGF protein also act as angiogenesis growth factor and is a dimeric glycoprotein composed of two A (-AA) chains or a combination of two (-AB). It has been linked to several diseases including atherosclerosis, fibrosis and

Table 4: Modulation of AHA Step-1 diet and δ-tocotrienol (δ-T3) + AHA Step-1 diet on the levels of gene expression in hypercholesterolemic subjects.

#	Cytokines	Baseline RLU*	%	AHA-Step-1 RLU*	%	AHA-Step-1+ δ-T3, RLU*	%	Description	Functions
1	TNF-α	45895	100	44986	98	38785	85	Tumor necrosis factor-α	inflammation
2	IL-2	39967	100	38234	96	36233	91	Interleukin -2	Proliferation & differentiation
3	IL-4	43678	100	43178	99	34896	79	Interleukin-4	Activation of B cells and T cells
4	IL-6	55806	100	54985	99	40804	73	Interleukin-6	NF-κB and IL-6 signaling
5	IL-8	36588	100	35882	98	32587	89	Interleukin-8	Chemokine
6	IL-10	42494	100	42185	99	37426	88	Interleukin-10	Immunoregulation
7	FGF-b	35872	100	36986	103	37923	106	Fibroblast growth factor-b	Angiogenesis
8	PDGF	38805	100	39234	101	42127	114	Platelet derived growth factor	Anti-inflammatory

*RLU= relative luminescence units.

malignant diseases. The present results show down-regulation of IL-6 and IL-8 levels by δ-tocotrienol, confirming the anti-angiogenic properties of δ-tocotrienol. These cytokine/growth factor data can be very well correlated to gene expression of mRNA purified from fresh EDTA treated whole blood obtained from subjects on the same treatments (Table 4), which indicates the down-regulation of cytokine IL-6 expressed by NF-κB, as reported earlier [20].

Recently, levels of miRNA have been shown as important regulators of gene expression that modify cellular responses and function [39-41]. 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 [41]. The miRNA-7a, miR-15a, miR-20a, miR-21, miR-29a, miR-200, and miR-206 were down-regulated in hypercholesterolemic human subjects (baseline values) as shown in Table 5 [41]. The δ-tocotrienol plus AHA Step-1 diet treatment in hypercholesterolemic subjects,

the cluster of above eight miRNA were up-regulated, as compared to baseline values (Table 5).The AHA Step-1 diet treatment resulted only in slight up-regulation in these miRNA's. These results indicated that

Table 5: The effect of δ-tocotrienol on plasma circulating microRNA (miRNA) of cardiovascular disease in hypercholesterolemic subjects.

	miRNA	Baseline		AHA Step-1 diet		AHA Step-1 diet + δ-Tocotrienol	
		Down-		**UP-**			
		regulation		**regulation**			
#		RLU	%	RLU	%	RLU	%
1	miRNA-7a	3074	100	3272	106	5552	181
2	**miRNA-15a**	**2122**	**100**	**2263**	**107**	**4036**	**190**
3	miRNA-20a	2546	100	2687	106	4337	170
4	**miRNA-21**	**874**	**100**	**948**	**108**	**1249**	**143**
5	miRNA-29a	3365	100	3478	103	4930	147
6	miRNA-92a	12607	100	13941	111	19971	153
7	miRNA-200	3927	100	4065	104	5720	146
8	**miRNA-206**	**1186**	**100**	**1243**	**105**	**1754**	**148**

RLU = Relative Luminescence Unit

δ-tocotrienol treatment up-regulated miRNA levels in plasmas of hypercholesterolemic subjects.

5. DISCUSSION

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 3A, 4B, 5C). 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 [20]. This clearly demonstrates that δ-tocotrienol and mevinolin modestly inhibit or activate the proteasomal activity depending on its concentrations [20,36-38]. 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 diseases, and at higher doses may cause apoptotic cell death in various types of cancers [42]. Similar dose-dependent activities (inhibition versus induction) and properties have been reported for synthetic proteasomal inhibitors, MG132 and lactacystin [36-38]. The aforementioned are very potent proteasome inhibitors in 5 μM to 20 μM, but very toxic as well, barring their used in humans.

Conversely, tocotrienols have been found safe even at doses of 1600-3200 mg/d in the treatment of pancreatic cancer [42].

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 were extended to 8 weeks or more. As reported earlier, the hepatic HMG-CoA reductase activity is inhibited by γ- and δ-tocotrienols, whereas tocopherols, α-tocopherol in particular, induce the activity of HMG-CoA reductase (a rate-limiting enzyme in the biosynthesis of cholesterol) and consequently raise cholesterol [23,31,43]. This disadvantage of high doses of tocotrienols does not apply to their other functions, such as cancer chemoprevention and treatment, where large doses are used in current clinical trials [42] probably by activating the immune response.

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)" [44]. It is well documented that high cholesterol is associated with increased risk of prostate cancer [45-48], and prostate cancer cells accumulate cholesterol to spur their growth [49]. 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 [44], 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 [49]. 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 [42].

Recently, inflammation has been associated with several diseases including cardiovascular disorders [25]. 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) [50]. While various studies have confirmed tocotrienol's anti-inflammatory functions, particularly for TNF-α and on a proteasomal level [20, 22, 51], they are also known to support the immune system [52]. Hence they do not appear to adversely affect the anti-inflammatory properties of IL-6.

As opposed to down-regulation of inflammatory cytokines, tocotrienols in the present study up-regulated FGF-b and PDGF. Neo-angiogenesis plays an essential role in the process of cardiac repair after ischemic injury [29], 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 infarction size [53]. Conversely, while FGF-b's neo-angiogenesis effect improves cardiac

function in coronary artery disease, this angiogenic stimulation could also result in adverse effects such as atherosclerosis [54]. Similarly, PDGF pathways in the ageing heart enhance cardiac angiogenesis and protect from myocardial infarction (MI), have also been found to have pro-atherosclerotic actions [55]. Both FGF-b and PDGF, due to their angiogenic activity, may also stimulate tumor growth [56]. 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 [20].

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-29a, miRNA-20a, and miRNA-206 in hypercholesterolemic humans. MiRNAs play multiple roles in various biological processes. For a single miRNA, these include normal physiological functions, but conversely may also display pathological activity. Since levels of miRNAs in the present study were down-regulated in the hypercholesterolemic population, up-regulation by δ-tocotrienol points to a beneficial effect. MiRNA-29a is enriched in fibroblasts and encodes proteins involved in fibrosis, including collagen, fibrillins, and elastin [57]. In myocardial infarction and associated cardiac hypertrophy, miRNA-29a is decreased, allowing for expression and deposition of collagen components in the fibrotic scar [29]. 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 [58]. It also prevents myocardial hypertrophy and angiogenesis during stress [59]. 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 [60], while suppressing gastric cancer cell growth and metastasis [61]. While there may be important implications for δ-tocotrienol in these applications [62], 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 [63], and hence miRNA-206 may play a pivotal role in the heart muscle [30] 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 [64].

6. CONCLUSION:

The present results indicate that low doses below 500 mg/d of δ-tocotrienol (250 mg/d) administered for 4 weeks is effective in lowering lipid parameters, down-regulate several inflammatory biomarkers (TNF-α, IL-4, IL-6, IL-8, and IL-10) and higher dose above 500 mg/d of δ-tocotrienol (750 mg/d) up-regulate these

biomarkers. Therefore, the capacity of tocotrienols to modulate inflammation may be attributable, in part, to their dose-dependent properties of inhibition in cardiovascular and activation of immune responses in cancer. δ-Tocotrienol was also found to be a potent naturally-occurring compound, which could remove 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 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 and cancers.

ETHICAL APPROVAL

The study protocol was registered at a governmental agency (University of Health Science, Lahore, Pakistan), and study protocol was approved by Institutional Review Board of Armed Forces Institute of Pathology, Rawalpindi, Pakistan and also University of Health Science, Lahore, Pakistan. All subjects signed an informed-consent form, which was approved by the Institutional Review Board of Armed Forces Institute of Pathology, Rawalpindi, Pakistan.

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