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Synergistic effect of tocotrienol-rich fraction (TRF_{25}) of rice bran and lovastatin on lipid parameters in hypercholesterolemic humans

Asaf A. Qureshi*[,a], Saeed A. Sami[b], Winston A. Salser[c], Farooq A. Khan[b]

[a]*Advanced Medical Research, 8251 Raymond Road, Madison, WI 53719, USA*
[b]*Armed Forces Institute of Pathology, Rawalpindi, Pakistan*
[c]*Department of Molecular Cell and Developmental Biology, and the Molecular Biology Institute, University of California, Los Angeles, CA 90024, USA*

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Abstract

Tocotrienols exert hypocholesterolemic action in humans and animals. Lovastatin is widely used for that purpose. Both agents work by suppressing the activity of β-hydroxy-β-methylglutaryl coenzyme A reductase through different mechanisms, post-transcriptional vs competitive inhibition. A human study with 28 hypercholesterolemic subjects was carried out in 5 phases of 35 days each, to check the efficacy of tocotrienol-rich fraction (TRF_{25}) of rice bran alone and in combination with lovastatin. After placing subjects on the American Heart Association (AHA) Step-1 diet (phase II), the subjects were divided into two groups, A and B. The AHA Step-1 diet was continued in combination with other treatments during phases III to V. Group A subjects were given 10 mg lovastatin, 10 mg lovastatin plus 50 mg TRF_{25}, 10 mg lovastatin plus 50 mg α-tocopherol per day, in the third, fourth, and fifth phases, respectively. Group B subjects were treated exactly to the same protocol except that in the third phase, they were given 50 mg TRF_{25} instead of lovastatin.

The TRF_{25} or lovastatin plus AHA Step-1 diet effectively lower serum total cholesterol (14%, 13%) and LDL-cholesterol (18%, 15% $P < 0.001$), respectively, in hypercholesterolemic subjects. The combination of TRF_{25} and lovastatin plus AHA Step-1 diet significantly reduces of these lipid parameters of 20% and 25% ($P < 0.001$) in these subjects. Substitution of TRF_{25} with α-tocopherol produces insignificant changes when given with lovastatin. Especially significant is the increase in the HDL/LDL ratio to 46% in group (A) and 53% ($P < 0.002$) in group (B). These results are consistent with the synergistic effect of these two agents. None of the subjects reported any side-effects throughout the study of 25-weeks. In the present study, the increased effectiveness of low doses of tocotrienols (TRF_{25}) as hypocholesterolemic agents might be due to a minimum conversion to α-tocopherol. The report also describes *in vivo* the conversion of γ-[4-^{3}H]-, and [^{14}C]-desmethyl (*d*-P_{21}-T3) tocotrienols to α-tocopherol.

Keywords: Hypercholesterolemic human subjects; Lovastatin; Tocotrienols; Lipid parameters

1. Introduction

Hypercholesterolemia and inflammations of coronary artery are the major risk factors for the development of coronary heart disease (CHD) in humans [1–5]. The most effective approach for controlling hypercholesterolemia is by inhibiting cholesterol synthesis and degrading cholesterol into bile acids in the body [1–4]. The most important enzymes in this respect are liver β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase and cholesterol 7α-hydroxylase, respectively. These enzymes are generally considered to be the rate-limiting steps, respectively, for cholesterol and for bile acid synthesis in liver [6,7]. It has been demonstrated that 7α-hydroxycholesterol, bile acids and cholesterol are very effective feedback repressors of both of these enzymes [7]. Their activities are regulated coordinately but in opposite directions [7]. Long-term changes in the activities of these two enzymes have been attributed to changes in the rates of synthesis and degradation of the enzymes [7].

Moderate hypercholesterolemia can frequently be controlled with diet [8–10]. The use of American Heart Asso-

Abbreviations: AHA = American Heart Association; apo A1 = apolipoprotein A1; apo B = apolipoprotein B; HDL-cholesterol = high density lipoprotein cholesterol; HPLC = high performance liquid chromatography; HMG-CoA = β-hydroxy-β-methylglutaryl coenzyme A; LDL-cholesterol = low density lipoprotein cholesterol; TRF_{25} = Tocotrienol-rich fraction of stabilized and heated rice bran.

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* Corresponding author. Tel.: +1-608-845-5445; fax: +1-608-845-5425.

E-mail address: nqureshi@mhub.facstaff.wisc.edu (A.A. Qureshi).

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ciation Step-1 diet (restricted intake of cholesterol-300 mg/day and fat-30%/day) lowers serum total cholesterol less than 5% to 8% in humans [11,12]. Therefore, dietary therapy only is insufficient as the sole intervention when the serum cholesterol concentration is elevated substantially. The hypocholesterolemic therapeutic agent, lovastatin continue to be useful in the management of hypercholesterolemia despite unwanted side-effects [11]. At the same time tocotrienols have been demonstrated to possess cholesterol-lowering properties in humans and various experimental animals in the last few years [12–22]. Tocotrienols, a group of unsaturated analogs with antioxidant, vitamin E activity, and lovastatin both act by inhibiting the activity of HMG-CoA reductase enzyme through different mechanisms [11, 22]. Lovastatin competitively inhibits the synthesis of mevalonic acid, a rate-limiting substance for the synthesis of cholesterol [11]. Adoptive responses in rat hepatocytes incubated with 10–30 μM lovastatin incorporate three actions: an 8-fold increase in HMG-CoA reductase mRNA, a 5-fold increase in the rate of the translation of HMG-CoA reductase mRNA, and a 5-fold decrease in the degradation of HMG-CoA reductase—the multiplicative effect of which yields a 200-fold increase in HMG-CoA reductase mass [11]. While tocotrienols exert a hypocholesterolemic effect by the post-transcriptional mechanism [22].

Recently, it was demonstrated that tocotrienols and lovastatin may be blocking each other's actions at different doses [22]. When HepG2 cells incubated with 10 μM γ-tocotrienol at various intervals (5′, 10′, 15′, 20′), the activity of HMG-CoA reductase was 76, 57, 40, and 20%, respectively, of control values due to a decrease in the translation rate of HMG-CoA reductase mRNA and increased degradation of the enzyme [22]. These findings, relevant to the present work show that 10 μM γ-tocotrienol blocked the adaptive response to 5 μM lovastatin on the activity of HMG-CoA reductase [22]. On the other hand, α-tocopherol has an opposite (induce) effect on HMG-CoA reductase activity [23], and, as tocotrienols are converted to tocopherols in vivo [14–16], it is important not to exceed a restrictive dose of tocotrienols, as overdosing of tocotrienols would override their inhibitory effects on cholesterogenesis [14–20].

The present study, a double-blind, cross-over, controlled clinical trial, was accordingly carried out in hypercholesterolemic human subjects to test the effectiveness of a therapy consisting of a low dose (10 mg/day) of lovastatin alone or in combination with a minimum effective dose of TRF_{25} (50 mg/day) for the treatment of elevated serum total cholesterol and LDL-cholesterol levels. The last phase of the study comprised the addition of α-tocopherol (vitamin E) to lovastatin. The tocotrienol-rich fraction (TRF_{25}) was prepared from stabilized and heated rice bran as described recently [21]. The TRF_{25} is a mixture of tocols (tocopherols plus tocotrienols), consisting of α-tocopherol (8.7%), α-tocotrienols (15.5%), β-tocotrienol (1.6%), γ-tocotrienol (39.4%), δ-tocopherol (4.4%), δ-tocotrienol (5.2%), desmethyl (d-P_{21}-T3) and didesmethyl (d-P_{25}-T3) tocotrienols (20.9%), and unidentified tocotrienols (4.3%). The TRF_{25} (mixture of tocopherols and tocotrienols) is "generally regarded as safe" (GRAS) and has no known side-effects [24], whereas lovastatin has side-effects, such as headaches, nausea, fatigue, insomnia, skin rashes, stomach cramps, and pain in the stomach [25].

2. Materials and methods

2.1. Chemicals

Sources of chemicals, substrates, and diagnostic kits have been identified previously [14]. Chemicals and solvents were of analytical grade. The [^{3}H] sodium borohydride and [2-^{14}C] sodium acetate were purchased from ICN, Biochemical Research Products, 3300 Hyland Ave., Costa Mesa, CA. Aquasol scintillation solution was obtained from New England Nuclear, Boston, MA.

2.2. Purification of TRF_{25} by flash chromatography

The purification of large quantities of TRF_{25} (free from γ-oryzanols and most of α-tocopherol) from stabilized and heated rice bran was carried out by flash chromatography, using silica gel as described recently [21]. The composition of various tocols was determined by high pressure liquid chromatography (HPLC [14]). TRF_{25} contains 8.7% α-tocopherol, 15.5% α-tocotrienol, 1.6% β-tocotrienol, 39.4% γ-tocotrienol, 4.4% δ-tocopherol, 5.2% δ-tocotrienol, 20.9% d-desmethyl-(d-P_{21}-T3), plus d-didesmethyl (d-P_{25}-T3) tocotrienols, and 4.3% unidentified tocotrienols. The molecular structures of desmethyl (d-P_{21}-T3) and didesmethyl (d-P_{25}-T3) tocotrienols have been established as 3,4-dihydro-2-methyl-2-(4,8,12-trimethyltrideca-3′(E),7′(E),11′-trienyl)-2H-1-benzopyran-6-ol, and 3,4-dihydro-2-(4,8,12-trimethyltrideca-3′,(E),7′(E),11′-trienyl)-2H-1-benzopyran-6-ol, respectively[21]. The compounds were identified according to the retention time and absorption profiles against standards of tocols described earlier [14]. Capsules containing TRF_{25} (25 mg) and 225 mg of rice bran oil stripped tocols (by extracting with methanol) were prepared.

2.3. Study population

Subjects were recruited from a hypercholesterolemic population (serum total cholesterol level $>$ 5.7 mmol/L) screened at the Armed Forces Institute of Pathology, Rawalpindi, Pakistan. Prospective participants were grouped according to cholesterol level ($<$median$>$) and subgrouped by sex. Volunteers were excluded on the basis of weight ($>$150% of Metropolitan Life ideal weight), use of cholesterol-altering medication, an elevated serum glutamate-pyruvate or glutamate oxaloacetate transaminase activity,

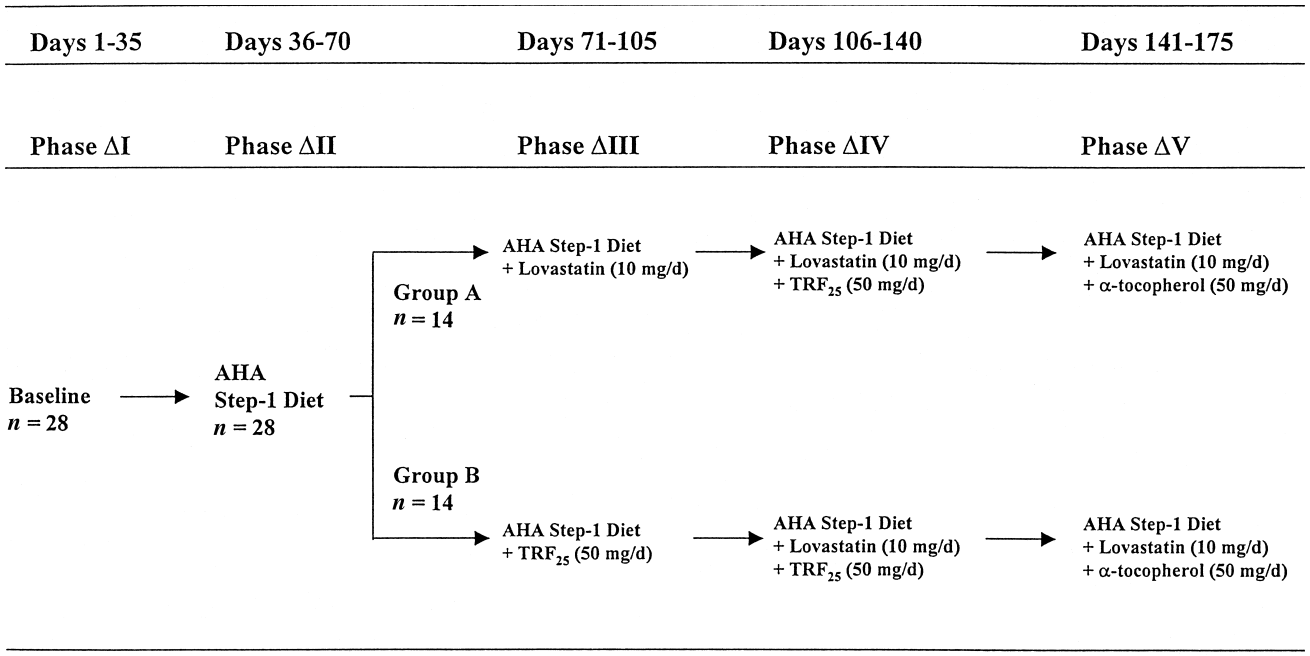

Fig. 1. Complete study design for groups A and B.

an elevated blood urea nitrogen or glucose level, diabetes, or a history of a liver, renal, or hypertensive diseases. Subject screening was accomplished during a five-week period. A fasting blood (5 ml) sample was collected for cholesterol determination at the initial session following the determination of eligibility. Following blocking by gender and stratification according to low and high cholesterol levels as determined at screening, the subjects (serum total cholesterol level 6.86 ± 0.48 mmol/L) were randomized into two groups (A and B). The average age (43.77 ± 7.5 years), height (166.78 cm), and body weight (72.16 ± 9.23 Kg) was for all the subjects (n = 36).

All subjects signed an informed-consent form which was approved by the Institutional Review Board of Armed Forces Institute of Pathology, Rawalpindi, Pakistan. This study was carried out under a FDA approved IND number 30906.

2.4. Experimental design

Out of 55 hypercholesterolemic subjects (serum total cholesterol levels >6.5 mmol/L), 36 subjects were enrolled in the study, and randomly assigned to two (A and B) groups (18 participants/group). The study was carried out in 5 phases (I–V), each of which lasted for 35 days. All the subjects were asked to take lovastatin (1 tablet, 10 mg/day) or TRF_{25} (2 capsules of 25 mg/day) alone or in combination with AHA Step-1 diet during phases III and IV after dinner. In the final phase (V) of the study comprised addition of α-tocopherol (vitamin E) plus AHA Step-1 diet and lovastatin. A complete study design is outlined in Fig. 1. The TRF_{25} consists of 8.7% α-tocopherol, 4.4% δ-tocopherol, and 86.9% tocotrienols and tocotrienol-like compounds. Each capsule contained 25 mg of TRF_{25} of rice bran [14].

2.4a. Phase I. Blood (5 ml) samples after an overnight fast were collected to establish the baseline lipid parameters at the start of the study during phase I, and the subjects met individually and in small group sessions with counselors. The individual sessions focused on 24-h recalls of food consumption, and the group sessions provided instruction for keeping 3-day records of food intake (2 weekdays, 1 weekend day). Subjects were encouraged to follow their typical dietary pattern and were instructed to keep food records for the terminal 3 days of this and the remaining phases. Subjects also received an unanticipated telephone call for a 24-h recall of food intake.

2.4b. Phase II. In the second phase, all the subjects were limited to an American Heart Association (AHA) Step-1 diet; limited to intake of 300 mg/day cholesterol and 30% energy from fat by stopping intake of whole milk, ice-cream, cheese, eggs, butter, and pure ghee, and using skim milk) for 35 days and a blood (5 ml) sample was drawn from each subject at the end of this phase. Subjects met in small groups for discussions of the relationship between diet and cardiovascular risk factors and for instruction on the AHA Step-1 diet. Each subject received a copy of the 1988 AHA Step-1 diet, Patient Manual, and the telephone number of a staff contact person. This counselling on adherence to the AHA step-1 diet continued for the study's duration. After 35 days a second blood (5 ml) sample was drawn in the fasting state to study the effects of AHA Step-1 diet, which constituted the second phase of the study. Following

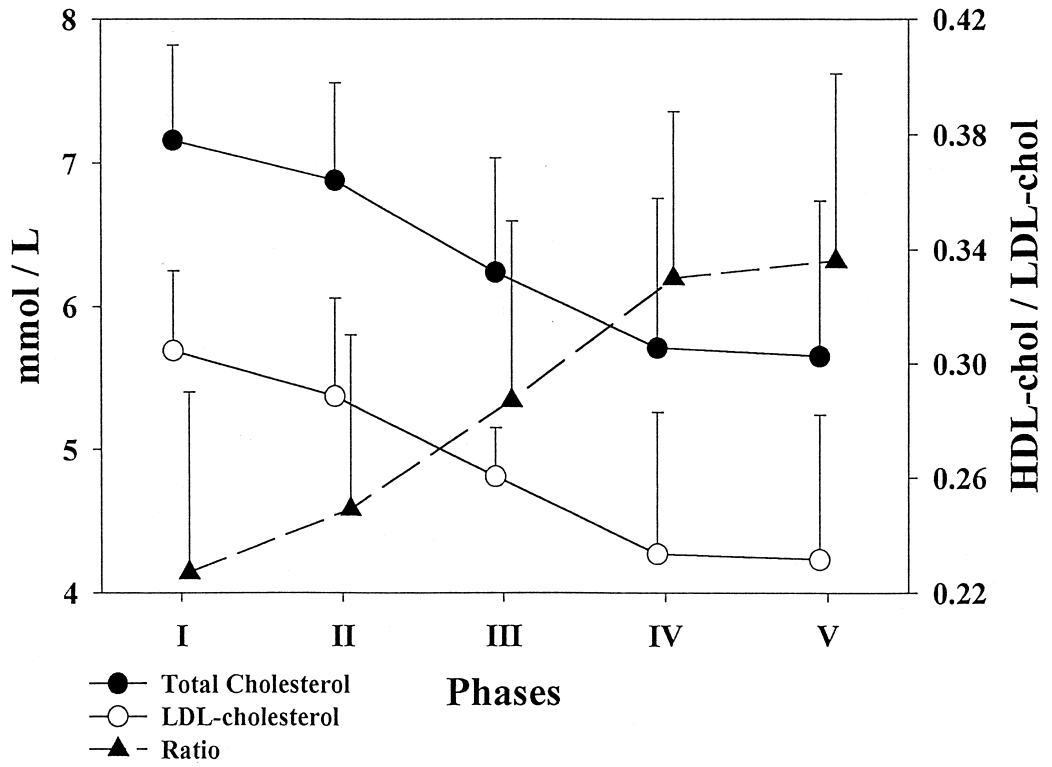

Fig. 2. The comparative effects of different treatments of various phases on serum total-cholesterol, LDL-cholesterol and HDL-cholesterol/LDL-cholesterol ratio of group A. Phase I = baseline, Phase II = AHA Step-1 diet, Phase III = AHA Step-1 diet + lovastatin, Phase IV = AHA Step-1 diet + lovastatin + TRF_{25}, Phase V = AHA Step-1 diet + lovastatin + α-tocopherol.

this, the subjects were divided randomly in two: group A and group B.

2.4c. Phases III–V: Group A subjects (n = 14) were administered 10 mg lovastatin plus AHA Step-1 diet in the third phase (III) for 35 days, and at the end of the phase another fasting blood (5 ml) was taken. In the fourth phase (IV), the subjects were administered 50 mg TRF_{25} with 10 mg lovastatin plus the AHA Step-1 diet for 35 days. After taking another blood (5 ml) sample after an overnight fast, 50 mg α-tocopherol was substituted for TRF_{25} and given along with 10 mg lovastatin plus the AHA Step-1 diet during the fifth (V) and final phase of the study of 35 days. A final blood (5 ml) sample was taken after an overnight fast at the conclusion of this phase.

Group B subjects (n = 14) followed a very similar protocol as described above, except that in the third phase (III) they were administered each day 50 mg TRF_{25} instead of lovastatin, along with AHA Step-1 diet. In the fourth (IV) phase, 50 mg TRF_{25} and 10 mg lovastatin plus AHA Step-1 diet were given for 35 days, and in fifth (V) phase TRF_{25} was replaced with 50 mg/day α-tocopherol along with 10 mg/day lovastatin plus AHA Step-1 diet for 35 days. Blood (5 ml) was drawn from each subject after an overnight fast at the end of each phase of 35 days. The serum was harvested and kept at −70°C for later analyses of lipid parameters. The stored samples were analyzed for various lipid parameters after all the phases were completed in both groups.

Twenty-eight subjects completed the entire study in both group (14/group).

2.5. Analyses of serum cholesterol values and different lipid parameters

Initial measures included the subject's height, initial body weight, final body weight, blood pressure, history of significant diseases, and medications (no alcohol use was allowed throughout the study). Body weights were recorded weekly. Venous blood samples were drawn at 7:00–9:00 a.m. following an overnight fast at the baseline phase and at the end of the study. The analyses of the coded samples were performed at Advanced Medical Research (Madison, WI). The serum total cholesterol (total-chol), HDL-cholesterol (HDL-chol), and triglycerides concentrations were estimated with reagent kits from Sigma Chemical Co. (St. Louis, MO).

Serum LDL-cholesterol (LDL-chol) was precipitated from 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 × g for 19 min. The supernatant was decanted and analyzed for HDL-cholesterol. The precipitate was dissolved in 200 μl of 0.1 M sodium citrate and the

level of LDL-cholesterol was estimated, as described, for total cholesterol.

Serum apolipoprotein A1 (apo A1), and apolipoprotein B (apo B) levels were determined by radioimmunoassay using kits from Sigma Chemical Co. (St. Louis, MO) in all groups. All assays for each subject were carried out at the same time under similar conditions to minimize standard deviation. To determine if the subjects complied with the request to fast prior to blood drawing, aliquots were used at the site to detect the presence of chylomicrons.

Diet records and 24-h recalls were analyzed (Nutrition Co-ordinating Center, University of Minnesota, Minneapolis, MN); if required, subjects were individually counseled to modify food intake to meet the goals of the AHA Step-1 diet or to maintain weight.

2.6. Syntheses and conversion of γ-[4-^{3}H]-tocotrienol and [^{14}C]-desmethyl tocotrienol (d-P_{21}-T3) into various tocols including α-tocopherol

The pathway for the biosynthesis of tocols (tocopherols and tocotrienols) in plants is established by decarboxylation of homogentistic acid (derived from *p*-hydroxyphenylpyruvic acid), followed by attachment of geranylgeranyl group at *meta* to the methyl group and cyclization, which leads to the formation of δ-tocotrienol [13,26–28]. The δ-tocotrienol then gives rise to γ-, β-, and α-tocotrienols by successive methylation, and δ-, γ-, β-, and α-tocopherols by reduction of the double bonds of the geranylgeranyl side-chain of each tocotrienols [13]. The δ-tocotrienol is also converted directly to δ-tocopherol by reduction, which is then converted to γ-, β-, and α-tocopherols by successive methylation [13,26–28].

The biosynthesis of desmethyl tocotrienol (*d*-P_{21}-T3) takes on gentisic acid, which is derived from phenylalanine. This route involves decarboxylation of gentisic acid and attachment of geranylgeranyl group at the position *para* to the hydroxy group and cyclization. In order to demonstrate the conversion of tocotrienols into tocopherols and confirm these routes, labelled γ- and desmethyl (*d*-P_{21}-T3) tocotrienols were prepared and fed to animal and plant systems.

2.7. Synthesis of γ-[4-^{3}H]-tocotrienol

The following experiments were carried out in the "Hood" with special permission from the Radiation Safety Department (RSD) of University of Wisconsin. All the residues were disposed off by RSD of the University of Wisconsin.

2.8. Reduction of γ-4-oxytocotrienol to its alcohol

The γ-4-oxytocotrienol (4.0 g; supplied by Dr. B.C. Pearce of Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT) was dissolved in methanol (50 mL). Sodium borohydride [^{3}H, 500 mCi, sp.act. 5–15 Ci/mmol] plus cold (1.0 g) was added slowly in the above methanol solution, while stirring continuously at room temperature. The mixture was stirred overnight at 25–30°C. The reaction mixture was poured over ice cold (5°C) 1 N hydrochloric acid (100 mL). This aqueous mixture was extracted with ether (4 × 200 mL each time). The remaining aqueous mixture was saturated with sodium chloride, and again extracted with ether twice (200 mL each time). The combined ether extracts (six) were dried with anhydrous magnesium sulfate, filtered and concentrated to 20 mL under vacuum yielded γ-[4-^{3}H]-hydroxytocotrienol (3.4 g).

2.9. Reduction of γ-[4-^{3}H]-hydroxytocotrienol to γ-[4-^{3}H]-tocotrienol

Aluminum chloride (4.84 g; 20 mM) was added in dry ether (20 mL), then cooled to −5°C under nitrogen. Lithium aluminum hydride (0.38 g; 10 mM) was added to the above ether solution portionwise. The slurry was stirred for 5 min at −5°C. The γ-[4-^{3}H]-hydroxytocotrienol (1.70 g; 4 mM) in ether (15 mL) was added in aluminum chloride/lithium aluminum hydride mixture and stirred for 2 h at 0°C. The mixture was saturated with anhydrous sodium sulfate. The aluminum salts were removed by filtration and washed with methanol (4 × 100 mL each time) and then ether (4 × 100 mL each time). The combined methanol and ether washings were poured into 1 N hydrochloric acid and extracted with fresh ether (4 × 100 mL of each). The ether solutions were dried with anhydrous magnesium sulfate and concentrated under vacuum [29]. The crude γ-[4-^{3}H]-tocotrienol was purified by flash chromatography (gradient 45:1 to 30:1 hexane-ether) to give pure γ-[4-^{3}H]-tocotrienol [21,30].

2.10. Conversion of γ-[4-^{3}H]-tocotrienol to various tocols including α-tocopherol in chickens.

Six 2-week-old white Leghorn male chickens (Poultry Research Laboratory, University of Wisconsin, Madison), weighing 80–95 g each were fed a commercial corn-soybean meal diet supplemented with γ-[4 ^{3}H]-tocotrienol (2,031,821 dpm) for 4 weeks. The diet was isonitrogenous and isocaloric, and its composition was as reported earlier [21]. The labelled γ-tocotrienol was dissolved in ether (100 ml) and mixed with the diet in the Hood in the dark and stirred for 4 h to remove ether. The birds were housed in a single brooder with continuous light and free access to water and diet. At the end of 24 d feeding period, each bird was injected 200,000 dpm in 0.1% triethylamine emulsion (0.5 mL) at 9 am for 4 d. After 28 d the blood was drawn from the heart (10 mL) from each bird. The collected blood was processed for serum, which was held at −20°C for the analyses of various tocols by high pressure liquid chromatography (HPLC), as described in detail earlier [14,21]. The birds were killed by carbon dioxide overdose. The protocol was reviewed and approved by the University of Wisconsin-Madison College of Agriculture and Life Sciences Animal Care Committee.

2.11. Synthesis of [^{14}C]-desmethyl tocotrienol ([^{14}C] d-P_{21}-T3)

Rice (20 g; Texamati/Jasmine, 1998 crop; supplied by Dr. A.M. McClung, USDA, Agriculture Research Service, Beaumont, TX) and barley (20 g; Morex, 1999 crop; Dr. D.M. Peterson, USDA, Cereal Crops Research Unit, Madison, WI) kernels were grown separately in petridishes at 37 and 24°C, respectively for 7 d. After 2 d of germination, [2-^{14}C] sodium acetate (1.0 mL of 1.0 mCi in 2 mL sterilized water; 55.7 mCi/mmol) was added in each petridish and were grown for 5 more d. The 7 d-old seedlings were ground in the coffee mill. The ground seedlings were heated (150°C) under vacuum (1550 mmHg) in a Precision vacuum oven connected with a water aspirator. The dried material was extracted with methanol (4 × 100 mL each time). The solvent of the combined methanol fractions was removed under vacuum at 50°C. The [^{14}C]-*d*-desmethyl tocotrienol ([^{14}C] *d*-P_{21}-T3) was purified by a combination of silica gel (Merk, 230–400 mesh, 60 Å) chromatography, and normal phase and chiral HPLC as described earlier [21].

2.12. Conversion of [^{14}C]-d-desmethyl tocotrienol ([^{14}C] d-P_{21}-T3) into various tocols including α-tocopherol in rice and barley seedlings

The rice (10 g; Jasmine variety) and barley (10 g; Morex variety) kernels were grown separately in petridishes for 7 d at 37 and 24°C, respectively. The purified [^{14}C]-*d*-P_{21}-T3 (1,321,205 dpm) was dispersed in 0.1% triethylamine aqueous solution (2 mL), and 1 mL of this solution was added in each petridish at the start of the germination. Sterilized water (1 mL) was added every day. At the end of 7 d, the seedlings were combined and ground in a coffee mill and worked up as described above. The barley seedlings were included to make sure of the high concentrations of α-tocopherol and α-tocotrienol in the mixture because barley kernels contain high levels of α-tocopherol and α-tocotrienol and rice kernels contain γ-tocotrienol as a major tocol [13]. The individual tocols from this mixture were separated by HPLC, as reported recently in cereals and serum [14,21]. The purified individual tocols were counted in aquasol scintillation solution.

2.13. Statistical analysis

The data were analyzed using the GLM procedure of SAS (Statistical Analysis System) for personal computers to test the study hypothesis. Duncans multiple-range test was used to determine whether the treated groups differed from the placebo for serum lipid parameters. Repeated-measures, two-way ANOVA, was used to test whether changes in serum lipid parameters occur in the course of supplementation, and whether there were between- and -within-subjects differences. Because all observations were required, available degrees of freedom were reduced by this statistical approach. The treatment effects on cholesterol were also evaluated using the paired, two-tailed *t*-test (StatView, Abacus Concepts, 1992, Berkeley, CA, USA). Data are reported as mean ± SD in the text. The statistical significance level was set at 5%. The data was also analyzed using changes from baseline and from the treatment's values of phase I, phase II, and phase III, using a multiclinic extension of the SAS or Duncan's matched pairs signed-rank *t*-test.

3. Results

3.1. Group A: Study on lowering lipid parameters by lovastatin, TRF_{25} and α-tocopherol

The subjects ($n = 14$) in this group (age-40.14 ± 8.98 years; height-166.79 ± 4.72 cm) showed no significant change or gain in body weights (initial weight 72.57 ± 8.25 and final weight 71.93 ± 8.56 Kg) throughout the study. All changes in serum lipid parameters with various treatments (AHA Step-1 diet, lovastatin, TRF_{25} or α-tocopherol) are compared with the baseline (pre-AHA Step-1 diet) values throughout the study.

The serum total cholesterol, LDL-cholesterol, apo B, and triglycerides levels decreased by 4%, 6%, 5%, and 5%, respectively, from the baseline values during the AHA step-1 diet (Tables 1 and 2). During the third phase (III), administration of 10 mg/day lovastatin plus AHA Step-1 diet for 35 days resulted in pronounced decreases of 13%, 15%, 13%, and 15%, respectively, in the above parameters as compared to baseline values (Tables 1 and 2). These decreases below the baseline are significant at $P < 0.002$, except that of triglycerides (Tables 1 and 2). However, the decreases below the baseline values achieved with the AHA Step-1 diet alone are not significant for these four lipid parameters (Tables 1 and 2).

Significant decreases below the AHA Step-1 diet values were achieved by addition of TRF_{25} to lovastatin plus AHA Step-1 diet in the next phase (IV) of 35 days, which showed significant decreases of 20%, 25%, 16%, ($P < 0.001$) and 18%, respectively, in serum total-chol, LDL-chol, apo B, and triglycerides levels as compared to their baseline values (Tables 1 and 2). In the fifth and final phase (V), the substitution of α-tocopherol with TRF_{25} gave total decreases of 19%, 26%, 17%, ($P < 0.001$) and 19%, respectively, in serum total-chol, LDL-chol, apo B, and triglycerides for the entire study, as compared to baseline values (very little changed from the previous phase: Tables 1 and 2). Therefore, the maximum decreases occurred with combination of TRF_{25} plus lovastatin in these parameters, and the substitution of TRF_{25} with α-tocopherol did not cause further reduction in these four parameters (Tables 1A and 2A). Although, the levels of triglycerides were decreased by 5%, 15%, and 19% with various treatments, but statistically it is not significant due to the large standard deviation (Table 2A). The HDL-cholesterol (10%) and apolipoprotein

Table 1
Effects of combined therapy of TRF_{25} plus lovastatin on serum total-, HDL-, and LDL-cholesterol levels in hypercholesterolemic human subjects*

Treatments	Total cholesterol (mmol/L)	HDL-cholesterol (mmol/L)	LDL-cholesterol (mmol/L)	Ratio	
				HDL-chol/total-chol	HDL-chol/LDL-chol
Baseline	7.16 ± 0.66[a]** (100)***	1.29 ± 0.27[a] (100)	5.69 ± 0.56[a] (100)	0.180 ± 0.032[c] (100)	0.227 ± 0.063[c] (100)
AHA Step-1 Diet	6.88 ± 0.68[a,b] (96)	1.34 ± 0.29[a] (104)	5.37 ± 0.69[a,b] (94)	0.194 ± 0.034[b,c] (108)	0.249 ± 0.061[b,c] (110)
AHA Step-1 Diet + Lovastatin (10 mg)	6.24 ± 0.80[b,c] (87)	1.38 ± 0.26[a] (107)	4.81 ± 0.34[b,c] (85)	0.221 ± 0.035[a,b] (123)	0.287 ± 0.063[a,b] (126)
AHA Step-1 Diet + Lovastatin (10 mg) + TRF_{25} (50 mg)	5.71 ± 1.05[c] (80)	1.41 ± 0.26[a] (109)	4.27 ± 0.99[c] (75)	0.247 ± 0.034[a] (137)	0.330 ± 0.058[a] (145)
AHA Step-1 Diet + Lovastatin (10 mg) + Vitamin E (50 mg)	5.65 ± 1.09[c] (79)	1.42 ± 0.26[a] (110)	4.23 ± 1.01[c] (74)	0.248 ± 0.035[a] (138)	0.336 ± 0.065[a] (148)
ANOVA (*P*-values)	0.0001	NS (0.6876)	0.0001	0.0001	0.0002

* Time of drawing blood was 0800 hr. The subjects ($n = 14$) were fasted for 12 hr. before blood samples were taken.
** X ± SD (mean ± standard deviation).
*** Percentage with respect to baseline values are in parentheses.
[a,b,c] Values in columns with a different superscript letter are significantly different at $P < 0.05$. NS = not significant.

A1 (15%; $P < 0.001$) levels were increased significantly with lovastatin plus TRF_{25}, as compared to the baseline values (Tables 1A and 2A). Similarly, the maximum increases also occurred in HDL-cholesterol and apolipoprotein A1 levels with the combination of TRF_{25} plus lovastatin.

The apolipoprotein A1 levels were not increased significantly over the AHA Step-1 diet levels with lovastatin but were increased significantly when TRF_{25} was added in phase four: 10% over AHA Step-1 diet and 15% over baseline levels (Table 2A). The HDL-chol/total chol ratio was increased 8%, 23%, 37%, and 38% ($P < 0.001$), respectively, as compared to the baseline value with the AHA Step-1 diet and in the combination of the AHA Step-1 diet with lovastatin, lovastatin plus TRF_{25}, lovastatin plus α-tocopherol (Table 1A). Similarly, the HDL-chol/LDL-chol ratio was increased by 10% over the baseline by the AHA Step-1 diet alone, climbed to 26% over the baseline, with lovastatin and climbed more to 45% ($P < 0.001$) over baseline with the combination of TRF_{25} and lovastatin (Table 1A). The comparative effects of various treatments on total-chol, LDL-chol and HDL-chol/LDL-chol ratio of group A study are summarized in Figure 2. No side-effects were reported by any subjects with combined therapy of TRF_{25} plus lovastatin.

3.2. Group B: Study on lowering of lipid parameters by TRF_{25}, lovastatin and α-tocopherol

The subjects ($n = 14$) in this group (age-42.57 ± 6.36 years; height-166.11 ± 5.88 cm) showed no significant change or gain in body weights (initial weight 72.01 ± 9.46 and final weight 70.71 ± 9.48 Kg) throughout the study.

The subjects in this group showed reductions of 6%, 7%, 1%, and 2% in the levels of serum total cholesterol, LDL-cholesterol, apo B, and triglycerides, respectively, at the end of the second phase with the AHA Step-1 diet alone as compared to baseline values. On administering 50 mg/day TRF_{25} plus the AHA Step-1 diet for the next 35 days during the third phase (III), significant decreases of 14%, 18%, 4%, and 10% ($P < 0.05$), respectively, occurred in these parameters as compared to baseline values (Tables 3 and 4).

Table 2
Effects of combined therapy of TRF_{25} plus lovastatin on serum apolipoprotein A1, apolipoprotein B, and triglycerides levels in hypercholesterolemic human subjects*

Treatments	Apolipoprotein A1 (g/L)	Apolipoprotein B (g/L)	Triglycerides (mmol/L)
Baseline	1.23 ± 0.10[a,]** (100)***	2.38 ± 0.27[a] (100)	2.42 ± 0.60[a] (100)
AHA Step-1 Diet	1.25 ± 0.10[a] (102)	2.25 ± 0.31[a,b] (95)	2.29 ± 0.64[a] (95)
AHA Step-1 Diet + Lovastatin (10 mg)	1.33 ± 0.10[b] (108)	2.08 ± 0.28[b,c] (87)	2.05 ± 0.57[a] (85)
AHA Step-1 Diet + Lovastatin (10 mg) + TRF_{25} (50 mg)	1.41 ± 0.11[c] (115)	2.00 ± 0.27[c] (84)	1.99 ± 0.56[a] (82)
AHA Step-1 Diet + Lovastatin (10 mg) + Vitamin E (50 mg)	1.43 ± 0.11[c] (116)	1.97 ± 0.27[c] (83)	1.96 ± 0.55[a] (81)
ANOVA (*P*- values)	0.0001	0.0009	NS (0.2427)

* Time of drawing blood was 0800 hr. The subjects ($n = 14$) were fasted for 12 hr. before blood samples were taken.
** X ± SD (mean ± standard deviation).
*** Percentage with respect to baseline values are in parentheses.
[a,b,c] Values in columns with a different superscript letter are significantly different at $P < 0.05$. NS = not significant.

Table 3
Effects of combined therapy of TRF_{25} plus lovastatin on serum total-, HDL-, and LDL-cholesterol levels in hypercholesterolemic human subjects*

Treatments	Total cholesterol (mmol/L)	HDL-cholesterol (mmol/L)	LDL-cholesterol (mmol/L)	Ratio	
				HDL-chol/total-chol	HDL-chol/LDL-chol
Baseline	$7.44 \pm 0.85^{a,**}$ (100)***	0.97 ± 0.30^{a} (100)	6.34 ± 0.93^{a} (100)	0.139 ± 0.038^{c} (100)	0.153 ± 0.063^{b} (100)
AHA Step-1 Diet	$6.96 \pm 0.83^{a,b}$ (94)	0.99 ± 0.28^{a} (102)	$5.89 \pm 0.91^{a,b}$ (93)	$0.142 \pm 0.049^{b,c}$ (109)	0.168 ± 0.056^{b} (110)
AHA Step-1 Diet + TRF_{25} (50 mg)	$6.38 \pm 0.81^{b,c}$ (86)	1.09 ± 0.29^{a} (112)	$5.21 \pm 0.89^{b,c}$ (82)	$0.171 \pm 0.042^{a,b}$ (132)	$0.209 \pm 0.062^{a,b}$ (137)
AHA Step-1 Diet + TRF_{25} (50 mg) + Lovastatin (10 mg)	6.00 ± 0.81^{c} (81)	1.13 ± 0.26^{a} (116)	4.80 ± 0.88^{c} (76)	0.188 ± 0.043^{a} (145)	0.235 ± 0.059^{a} (154)
AHA Step-1 Diet + Vitamin E (50 mg) + Lovastatin (10 mg)	5.88 ± 0.86^{c} (79)	1.14 ± 0.30^{a} (118)	4.77 ± 0.96^{c} (75)	0.194 ± 0.042^{a} (149)	0.239 ± 0.057^{a} (156)
ANOVA (*P*-values)	0.0001	NS (0.4490)	0.0001	0.0039	0.0047

* Time of drawing blood was 0800 hr. The subjects ($n = 14$) were fasted for 12 hr. before blood samples were taken.
** X ± SD (mean ± standard deviation).
*** Percentages with respect to baseline values are in parentheses.
[a,b,c] Values in columns with a different superscript letter are significantly different at $P < 0.05$. NS = not significant.

The addition of 10 mg/day lovastatin and TRF_{25} plus AHA Step-1 diet in the fourth phase (IV) resulted in pronounced decreases in the levels of serum total cholesterol (19%), LDL-cholesterol (24%), apolipoprotein B (5%), and triglycerides (13%, $P < 0.05$) as compared to baseline values (Tables 3 and 4). As was the case in group A, insignificant decreases were seen in the final phase (V) with the administration of lovastatin and α-tocopherol (Tables 3 and 4). The effects of TRF_{25} on these subjects would, perhaps, have been more visible at a slightly higher dose (75 mg/d).

The serum HDL-cholesterol and apolipoprotein A1 showed remarkable increases of 12% and 8% with TRF_{25} and further increases to 16% and 11% over the baseline when lovastatin was added (the combined therapy of TRF_{25} and lovastatin: Table 4). The HDL-chol/total chol ratio increased with AHA Step-1 diet (9%), AHA Step-1 diet + TRF_{25} (32%), AHA Step-1 diet + TRF_{25} + lovastatin (45%), and AHA Step-1 diet + lovastatin + α-tocopherol (49%), as compared to baseline values (Table 3). The HDL-chol/LDL-chol ratio rose 10% above the baseline with the AHA Step-1 diet, increased to 37% when TRF_{25} was added, and further increased to 54% ($P < 0.005$) over baseline with both TRF_{25} and lovastatin in combination with the AHA Step-1 diet (Table 3). The comparative effects of various treatments on serum total-chol, LDL-chol and HDL-chol/LDL-chol ratio of group B study are summarized in Fig. 3. No side-effects were reported in either of the study.

4. Discussion

The results of the present study clearly demonstrate that with AHA Step-1 diet, the minimum effective dose of TRF_{25} (50 mg/day) of rice bran or a low dose of lovastatin (10 mg/day) individually or in combination are effective in lowering serum total- (14% and 13%) and LDL-cholesterol levels (18% and 15%), respectively, in hypercholesterolemic human subjects. The combination of both TRF_{25} and lovastatin plus AHA Step-1 diet significantly reduces these lipid parameters to 20% and 25% over the baseline values in hypercholesterolemic human subjects (Figs. 2 and 3).

In the present study 10 mg/day lovastatin was used, although the lowest dose usually recommended for control-

Table 4
Effects of combined therapy of TRF_{25} + lovastatin on serum apolipoprotein A1, apolipoprotein B, and triglycerides levels in hypercholesterolemic human subjects*

Treatments	Apolipoprotein A1 (g/L)	Apolipoprotein B (g/L)	Triglycerides (mmol/L)
Baseline	$1.19 \pm 0.09^{a,**}$ (100)***	2.22 ± 0.14^{a} (100)	2.99 ± 0.39^{a} (100)
AHA Step-1 Diet	$1.23 \pm 0.10^{a,b}$ (103)	2.19 ± 0.14^{a} (99)	2.93 ± 0.38^{a} (98)
AHA Step-1 Diet + TRF_{25} (50 mg)	$1.29 \pm 0.10^{b,c}$ (108)	$2.13 \pm 0.14^{a,b}$ (96)	$2.69 \pm 0.39^{a,b}$ (90)
AHA Step-1 Diet + TRF_{25} (50 mg) + Lovastatin (10 mg)	1.32 ± 0.10^{c} (111)	2.11 ± 0.14^{b} (95)	$2.59 \pm 0.39^{a,b}$ (87)
AHA Step-1 Diet + Vitamin E (50 mg) + Lovastatin (10 mg)	1.35 ± 0.11^{c} (113)	2.06 ± 0.14^{b} (93)	$2.58 \pm 0.39^{a,b}$ (86)
ANOVA (*P*-values)	0.0007	0.0466	0.017

* Time of drawing blood was 0800 hr. The subjects ($n = 14$) were fasted for 12 hr. before blood samples were taken.
** X ± SD (mean ± standard deviation).
*** Percentage with respect to baseline values are in parentheses.
[a,b,c] Values in columns with a different superscript letter are significantly different at $P < 0.05$.

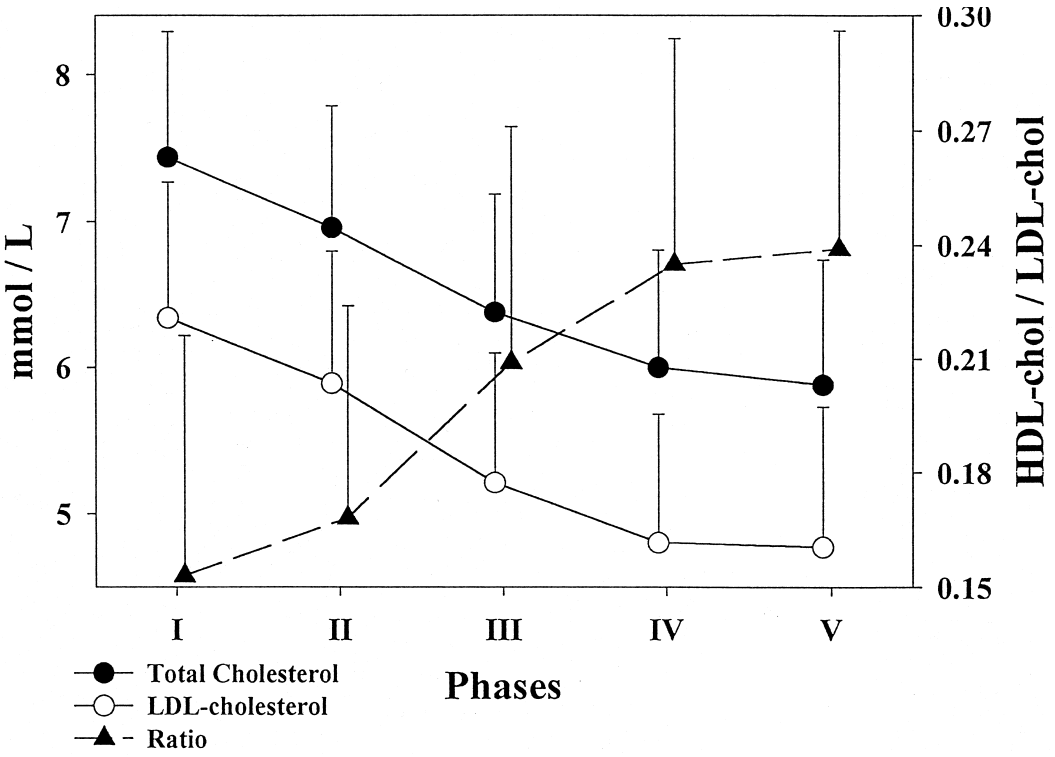

Fig. 3. The comparative effects of different treatments of various phases on serum total-cholesterol, LDL-cholesterol and HDL-cholesterol/LDL-cholesterol ratio of group B. Phase I = baseline, Phase II = AHA Step-1 diet, Phase III = AHA Step-1 diet + TRF_{25}, Phase IV = AHA Step-1 diet + TRF_{25} + lovastatin, Phase V = AHA Step-1 diet + lovastatin + α-tocopherol.

ling hypercholesterolemia is 40 mg/day. However, even this low dose of lovastatin (10 mg/day) when combined with 50 mg of TRF_{25} (the optimal dose for TRF_{25} is 100 mg/day-unpublished results) lowers serum total cholesterol and LDL-cholesterol significantly in hypercholesterolemic human subjects. The most remarkable improvement occurs in the ratio of HDL-chol/LDL-chol to 46% over baseline value in one group (A) and 53% over baseline in the other experimental group (B). These results are consistent with the synergistic effect of these two agents. None of the subjects reported any side-effects throughout the study of 25-weeks.

It is well established that coronary heart disease or stroke is caused by impaired circulation due to atherosclerosis. The major risk factors for atherosclerosis are an elevated level of serum total cholesterol, LDL-cholesterol, and inflammation of coronary arteries caused by increased levels of C-reactive protein [1–5]. Dietary regulation often proves insufficient in the management of moderate or severe hypercholesterolemia, and antilipemic drugs need to be used [31–34]. The commonly used antilipemic drugs include bile acid sequestrants, fibric acid derivatives, niacin, D-thyroxine, and the statins. The statins, particularly lovastatin, are most effective in controlling hypercholesterolemia as they competitively inhibit the synthesis of mevalonic acid, a rate-limiting substance for the synthesis of cholesterol in the liver by the enzyme, HMG-CoA reductase [25]. Lovastatin, a potent competitive inhibitor of HMG-CoA reductase, suppresses hepatic cholesterol synthesis and upregulates the number of hepatic LDL receptors [25]. The increase in the receptor-mediated removal of LDL-cholesterol from serum accounts for the large reduction in LDL-cholesterol with lovastatin [25]. The daily intake of 40 to 80 mg of lovastatin reduced serum total-cholesterol and LDL-cholesterol levels by 25% to 38% in heterozygous hypercholesterolemic human subjects [25]. The hypercholesterolemic effects of lovastatin are potentiated when it is combined with bile acid sequestrant (cholestyramine or colestipol or nicotinic acid). The combination resulted in more than 50% reduction in serum total- and LDL-cholesterol levels in familial hypercholesterolemic human subjects [31–34].

There was a 200-fold increase in HMG-CoA reductase mass in rat hepatocytes incubated with lovastatin [11]. On the other hand, tocotrienols caused 20% decrease in its mass, 76% in the level of HMG-CoA reductase mRNA, 57% in the rate of translation of HMG-CoA reductase mRNA, 235% in the degradation of HMG-CoA reductase activity of the control value. Further, its half life decreases from 3.7 to 1.6 h [22].

Recently, it was demonstrated that exogenous or endogenously produced 24 (S), 25-oxidolanosterol (OLAN) can also regulate HMG-CoA reductase activity [35]. Moreover, the metabolic conversion of OLAN in cell culture to both 24 (S), 25-epoxycholesterol or 25-hydroxycholesterol results in the transcriptional regulation of HMG-CoA reductase activity[35]. The results reported by Parker et al. [22] clearly demonstrate that the primary block effected by γ-to-

cotrienol lies between acetate and mevalonate, and a direct correlation exits between inhibition of cholesterol synthesis and suppression of HMG-CoA reductase activity by tocotrienols. In conclusion, similar to 24 (S), 25-epoxylanosterol, tocotrienols also act as post-transcriptional suppressors of HMG-CoA reductase activity [11,22].

As discussed above, the inhibition mechanism of HMG-CoA reductase by lovastatin and tocotrienols is quite different (competitive inhibition vs post-transcriptional inhibition, respectively). Therefore, the simultaneous administration of these two agents, even in low doses of each in combination with the AHA Step-1 diet to hypercholesterolemic human subjects causes significant decreases in serum total cholesterol, LDL-cholesterol, apo B and triglycerides levels more efficiently than either agent alone and without any side-effects. In the present study 10 mg/day lovastatin was used, although the lowest dose usually recommended for controlling hypercholesterolemia is 20 mg twice a day (40 mg/day), which resulted in some side-effects, such as constipation, headaches, nausea, insomnia, fatigue and skin rashes in several cases [25]. The present study of combined therapy of these two agents in low doses eliminates all such lovastatin side-effects.

The hypocholesterolemic effects of TRF, TRF_{25}, and individual tocotrienols have been confirmed by a number of investigators [12–22], however, the tocotrienol-rich fraction (TRF) mixture of palm oil containing high α-tocopherol ($>$20%) concentration (Palmvitee capsule) had no impact on serum total cholesterol or LDL-cholesterol in hypercholesterolemic human subjects as reported by Wahlqvist [36] and Mensink et al. [37] A review of the literature shows that TRF preparations (Palmvitee) low in α-tocopherol concentration ($<$20%), plus AHA Step-1 diet, were consistently effective in both lowering serum total cholesterol, LDL-cholesterol, and inhibiting HMG-CoA reductase activity [14,16,41].

Two major differences exist between the protocols and tocol mixture used in the present and other studies. The tocol mixture (TRF, Palmvitee capsule) of palm oil used in the studies by Wahlqvist [36] and Mensink et al. [37] contains higher levels of α-tocopherol (30% and 36%, respectively), whereas present mixture (TRF_{25}) contains 13% α-tocopherol + δ-tocopherol and 87% tocotrienols, and is obtained from stabilized and heated rice bran. The TRF_{25} not only contains known (α-, β-, γ-, and δ-) tocotrienols but also contains two new more potent ones, desmethyl (d-P_{21}-T3) and didesmethyl (d-P_{25}-T3) tocotrienols [12,13,21]. We recently demonstrated that the ratio of the tocopherols and tocotrienols play an important role in determining the hypocholesterolemic properties of tocotrienols [38]. The presence of more than 20% α-tocopherol in tocotrienol-rich fraction (TRF) from palm oil results in an attenuation of the hypocholesterolemic effect of tocotrienols [38]. These findings have been confirmed by Khor and Ng [39]. Therefore, it is desirable to use TRF preparations from any natural source with minimal ($<$15%) concentrations of tocopherols in the TRF mixture.

The second and the main difference in other studies is the absence of the restricted AHA Step-1 diet [36,37]. Both the Wahlqvist and Mensink groups have not only used large doses of Palmvitee capsules, but have not restricted the intake of cholesterol ($<$300 mg/day) and energy ($<$30% fat/day) in their hypercholesterolemic human subjects [36, 37], as recommended by the AHA Step-1 diet. In our first trial, administration of four capsules of TRF (Palmvitee; 50 mg/capsule contains 42 mg tocotrienols [84%] and 8 mg α-tocopherol [16%]) of palm oil to hypercholesterolemic subjects for 4 weeks causes decreases of 15%, and 8%, respectively, in the concentrations of serum total- and LDL-cholesterol [15]. All the subjects in this trial were allowed to consume their normal diets (free-living), and due to large number of nonrespondants (20%), these effects are not statistically significant for the entire group [15].

Atroshi *et al*. [40] have examined the effect of the same Palmvitee capsules (4 capsules/day; 50 mg/capsule contains α-tocopherol 16%, plus tocotrienols 84%) on lipid parameters in Finnish healthy adults for 6 weeks and observed an insignificant reduction in the concentrations of serum total cholesterol, LDL-cholesterol and, concomitantly, an increase in the serum α-tocopherol concentration [40]. It is not clear why they were trying to lower serum total- and LDL-cholesterol levels in the healthy subjects with Palmvitee capsules. Palmvitee's inability to lower serum total- and LDL-cholesterol concentrations in free-living healthy or hypercholesterolemic human subjects is similar to those reported by us, Wahlqvist, Mensink and Atroshi et al. [15, 36,37,40]. The failure of large doses of tocotrienols as effective hypocholesterolemic agents in all these studies is due to their conversion to α-tocopherol in vivo, because the level of α-tocopherol in serum of tocotrienols (TRF or TRF_{25}) treated subjects is substantially higher (2- to 4-fold) as compared to the placebo group [15,36,37,40].

As mentioned above, in our earlier human studies, various serum tocols show higher concentration of α-tocopherol as compared to tocotrienols after consuming Palmvitee (TRF) of palm oil, TRF_{25} of rice bran, or pure γ-tocotrienol capsules (200 mg/d) for 4 weeks [14,15]. This result is confirmed by others feeding Palmvitee (TRF) or pure α-, γ- or δ-tocotrienols to humans and animal models [17,19,36, 37,40]. Large quantities of TRF, TRF_{25}, and γ-tocotrienol (200 mg/d or more) are used in all these human studies [14,15,17,19,36,37,40].

Recently, Khor et al. reported a dose-dependent inhibition (17–50%) of HMG-CoA reductase activity after administration of γ-tocotrienol to guinea pigs for 6 d [17,39]. They reported that γ-tocotrienol in low doses (5 to 8 mg) but not higher doses (10, 15, and 50 mg) are much more effective in inhibiting the HMG-CoA reductase activity [17,39]. This conclusion is further supported by Watkins et al. findings, who find that γ-tocotrienol given at 50 mg/kg level significantly reduces total- and LDL-cholesterol lev-

Table 5
Conversion of γ-[4-^{3}H]-tocotrienol and [^{14}C]-desmethyl tocotrienol (*d*-P_{21}-T3) into various tocols in chickens and rice/barley seedlings

Nutritional state	α-tocopherol	α-tocotrienol	β-tocopherol	γ-tocopherol	β-tocotrienol	γ-tocotrienol	δ-tocopherol	δ-tocotrienol	*d*-P_{21}-T3
					dpm				
Chickens									
γ-[4-^{3}H]-tocotrienol*	3265 ± 58	5815 ± 76		4650 ± 96	256 ± 42	12,926 ± 465			
Rice/barley seedlings									
[^{14}C]-desmethyl tocotrienol** ([^{14}C] *d*-P_{21}-T3)	1448	2698	450	634	738	3498	246	478	8865

* γ-[4-^{3}H]-tocotrienol (2,031,821 dpm) was mixed with feed and fed for 4 weeks to 2-week-old male chickens ($n = 6$). Blood was collected 08.00 h and processed for the isolation of various tocols.

** [^{14}C] labeled *d*-P_{21}-tocotrienol (675,545 dpm in each) were added in Rice and barley kernels (10 g each) and were grown at 37°C and 24°C, respectively for 7 d. The seedlings were worked up as described in Methods.

els, when compared to the control, but γ-tocotrienol given at the 100 mg/kg level does not further decrease the total- and LDL-cholesterol levels in rats fed atherogenic diets [19]. The reason for this lesser inhibitory effect on the activity of HMG-CoA reductase by γ-tocotrienol at higher doses may be due to the bioconversion of γ-tocotrienol to α-tocopherol in the body. The levels of α-tocopherol in the serum and liver are increased 3- to 4-fold in guinea pigs that are treated with 50 mg/kg γ-tocotrienol [17,39], and increased levels of α-tocopherol induces HMG-CoA reductase activity [23,38].

In order to confirm this conversion of γ-tocotrienols to α-tocopherol, we fed radioactive synthetic γ-[4-^{3}H]-tocotrienol to chickens for 4 weeks, and the serum was subjected to HPLC analysis to separate individual tocopherols and tocotrienols [14]. Radioactivity was found in α-tocopherol, α-tocotrienol, δ-tocopherol, β-tocotrienol, and γ-tocotrienol but not in δ-tocopherol and δ-tocotrienol (Table 5). The enzymes of the gut bacteria in the chickens may be responsible for these conversions. To confirm this result, [^{14}C]-*d*-desmethyl (*d*-P_{21}-T3) tocotrienol was prepared and administered to rice and barley seedlings for 7 d, and methanol extract of the combined seedlings was subjected to HPLC analysis to separate individual tocols [14,21]. The radioactivity was found in α-tocopherol, α-tocotrienol, β-tocopherol, γ-tocopherol, β-tocotrienol, γ-tocotrienol, δ-tocopherol, δ-tocotrienol, and *d*-desmethyl tocotrienol (Table 5), indicating a similar metabolic biosynthetic pathway for tocotrienols in animals and plants, except that the first compound synthesized might be *d*-desmethyl tocotrienol (*d*-P_{21}-T3; no methyl group on benzene ring) instead of δ-tocotrienol in plants [13].

Later, most of our human trials were designed to avoid factors which underlie the failure of ours [15] and others [36,37,40] to demonstrate the cholesterol-suppressive action of the tocotrienols [14,41]. Hypercholesterolemic subjects were first conditioned for at least 4 weeks to a dietary regimen patterned after the AHA Step-1 diet [14,41]. These subjects on AHA Step-1 diet for 4 weeks resulted in decreases in serum total- and LDL-cholesterol by 5% and 8%, respectively. The maximal decreases in serum total- and LDL-cholesterol was 7% and 10% on AHA Step-1 diet for 8 weeks [14,41]. The subjects receiving TRF_{25} showed further decreases of 12% and 16% in serum total- and LDL-cholesterol concentrations [14].

5. Conclusions

The present study demonstrates that the even low dose of TRF_{25} (50 mg/day) not only is an effective cholesterol-lowering natural product but also a very useful agent for combined therapy with other cholesterol lowering drugs (lovastatin, or lipotor) because of its role in eliminating side-effects of these drugs in humans. An unexpected result of the present study is that improvement in the lipid parameters (achieved by the end of phase four) changes very little when TRF_{25} is replaced by α-tocopherol (during the final phase five). As expected, the addition of α-tocopherol might attenuate the action of tocotrienols (TRF_{25}), resulting in rapid worsening of the lipid parameters [38]. This is not the case in the present study because the use of minimum effective dose (50 mg/d instead of 200 mg/d) of tocotrienols [17,39], and also because the TRF_{25} appears to have a long-lasting effect as reported earlier in humans [14]. The levels of tocotrienols in serum increased 16-fold after a 4-week treatment with TRF_{25}; therefore, the reserves in serum and elsewhere in the body are adequate to confer this long-lasting effect of tocotrienols on lipid parameters.

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References

[1] Lipid Research Clinics Program: The lipid research clinics coronary primary prevention trial results. Reduction in incidence of coronary heart disease. J. Am. Med. Assoc. 251 (1984) 351–354.

[2] J.P. Strong, Natural history and risk factors for early human atherogenesis, Clin. Chem. 41 (1995) 134–138.

[3] M.S. Grundy, Cholesterol and coronary heart disease: a new era, J. Am. Med. Assoc. 256 (1986) 2849–2656.

[4] Nutrition Committee, American Heart Association, Dietary Guideline Healthy American Adults, Circulation, 77 (1988) 721–724A.

[5] L.E.P. Rohde, C.H. Hennekens, P.M. Ridker, Survey of C-reactive protein and cardiovascular risk factors in apparently healthy men, The Am. J. Cardiol. 84 (1999) 1018–1022.

[6] D.A. Kleinsek, A.M. Jabalquinto, J.W. Porter, In vivo and in vitro mechanisms regulating rat liver β-hydroxy-β-methylglutaryl coenzymeA reductase, J. Biol. Chem. 255 (1980) 3918–3923.

[7] S. Schefer, F.W. Cheng, S. Hauser, A.K. Batta, G. Salen, Regulation of bile acid synthesis. Measurement of cholesterol 7α-hydroxylase activity in rat liver microsomal preparations in the absence of endogenous cholesterol, J. Lipid Res. 22 (1981) 532–536.

[8] Rationale of the Diet-Heart Statement of the American Heart Association, Report of the AHA Nutrition Committee (Review), Arteriosclerosis, 2 (1982) 171–191.

[9] National Research Council Committee on Diet and Health, Diet and Health: implication of reducing chronic disease risk, Washington DC: National Academy of Sciences Press, 1989, USA.

[10] Report of National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, The Expert Panel. Arch. Intern. Med. 148 (1988) 36–69.

[11] J.L. Goldstein, M.S. Brown, Regulation of the mevalonate pathway, Nature 343 (1990) 425–430.

[12] A.A. Qureshi, R.H. Lane, A.W. Salser, Tocotrienols and tocotrienol-like compounds and method for their use, US Patent 5,919,818, (July 6, 1999).

[13] N. Qureshi, A.A. Qureshi, Novel hypercholesterolemic agents with antioxidant properties, In: Vitamin E in Health and Disease (L. Packer, J. Fuchs eds.) Mercel Dekker, Inc., New York, 1993, NY 247–267.

[14] A.A. Qureshi, B.A. Bradlow, W.A. Salser, L.D. Brace, Novel tocotrienols of rice bran modulate cardiovascular disease risk parameters of hypercholesterolemic humans, J. Nutr. Biochem. 8 (1997) 290–298.

[15] A.A. Qureshi, N. Qureshi, J.J.K. Wright, Z. Shen, G. Kramer, A. Gapor, Y.H. Chong, G. DeWitt, A.S.H. Ong, D.M. Peterson, B.A. Bradlow, Lowering of serum cholesterol in hypercholesterolemic humans by tocotrienols (Palmvitee), Am. J. Clin. Nutr. 53 (1991) 1021S–1026S.

[16] A.A. Qureshi, N. Qureshi, J.O. Hasler-Rapascz, F.E. Weber, V. Chaudhary, T.D. Crenshaw, A. Gapor, A.S.H. Ong, Y.H. Chong, D.M. Peterson, Y. Rapascz, Dietary tocotrienols reduce concentrations of plasma cholesterol, apoliprotein B, thromboxan B2, and platelet factor 4 in pigs with inherited hyperlipidemias, Am. J. Clin. Nutr. 53 (1991) 1042S–1046S.

[17] H.T. Khor, D.Y. Chieng, K.K. Ong, Tocotrienols inhibit HMG-CoA reductase activity in the guinea pig, Nutr. Res. 15 (1995) 537–544.

[18] R.L. Hood, G.S. Sidhu, Effects of guar gum and tocotrienols on cholesterol metabolism on the Japanese quail, Nutr. Res. 12 (1992) S117–S127.

[19] T. Watkins, P. Lenz, A. Gapor, M. Struck, A. Tomeo, M. Bierenbaum, γ-Tocotrienol as a hypocholesterolemic and antioxidant agent in rats fed atherogenic diets, Lipids 28 (1993) 1113–1118.

[20] R.L. Hood, Tocotrienols and cholesterol metabolism, In: Nutrition, Lipids, Health, and Diseases, (A.S.H. Ong, E. Niki, L. Packer) AOCS Press, Champaign, Illinois, (1995) 96–103.

[21] A.A. Qureshi, H. Mo, L. Packer, D.M. Peterson, Isolation and identification of novel tocotrienols from rice bran with hypocholesterolemic, antioxidant, and antitumor properties, J. Agr. Food Chem. 48 (2000) 3130–3140.

[22] R.A. Parker, B.C. Pearce, R.W. Clark, D.A. Gordon, J.J.K. Wright, Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl coenzymeA reductase, J. Biol. Chem. 268 (1993) 11298–11238.

[23] A.A. Qureshi, D.M. Peterson, C.E. Elson, A.R. Mangels, Z.Z. Din, Stimulation of avian cholesterol metabolism by α-tocopherol, Nutr. Rep. Int. 40 (1989) 993–1001.

[24] United States of America, Food and Drug Administration, Tocopherols and derivatives, Proposed affirmation of GRAS status for certain tocopherols and removal of certain others from GRAS status as direct human food ingredients, Federal-Register; 43 (209, October 27) (1978) 50193–50198.

[25] Mevacor (lovastatin, MSD), West Point, PA: Merck Sharp and Dohme; US package circular (July 1990).

[26] W. Janiszowska, J.F. Pennock, The biochemistry of vitamin E in plants, Vitam. Horm. 34 (1976) 77–105.

[27] D.R. Threlfall, The biosynthesis of vitamin E and K and related compounds, Vitam. Horm. 29 (1971) 153–200.

[28] J.F. Pennock, F.W. Hemming, J.D. Kerr, A reassessment of tocopherol chemistry, Biochem. Biophys. Res. Commun. 17 (1964) 542–548.

[29] B.C. Pearce, R.A. Parker, M.E. Deason, D.D. Dischino, E. Gillespie, A.A. Qureshi, K. Volk, J.J.K. Wright, Inhibitors of cholesterol biosynthesis, 2, Hypocholesterolemic and antioxidant activities of benzopyran and tetrahydronaphthalene analogues of the tocotrienols, J. Med. Chem. 37 (1994) 526–541.

[30] B.C. Pearce, R.A. Parker, M.E. Deason, A.A. Qureshi, J.J.K. Wright, Hypocholesterolemic activity of synthetic and natural tocotrienols, J. Med. Chem. 35 (1992) 3595–3606.

[31] D.R. Illingworth, Drug therapy of hypercholesterolemia, Clin. Chem. 34 (1988) B123–B132.

[32] D.R. Illingworth, Lipid-lowering drugs: an overview of indications and optimum therapeutic use, Drugs 33 (1987) 259–279.

[33] S.M. Grundy, G.L. Vega, D.W. Bilheimer, Influence of combined therapy with mevinolin and interruption of bile acid reabsorption on low density lipoproteins in heterozygous familial hypercholesterolemia, Annals of Internal Medicine 103 (1985) 339–343.

[34] D.R. Illingworth, Mevinolin plus colestipol in therapy for severe heterozygous familial hypercholesterolemia, Annals of Internal Medicine 101 (1984) 598–604.

[35] S.R. Panini, R.C. Sexton, H. Rudney, Regulation of 3-hydroxy-3-methylglutaryl coenzymeA reductase by oxysterol, by-products of cholesterol biosynthesis, J. Biol. Chem. 259 (1984) 7767–7771.

[36] M.L. Wahlqvist, Z. Krivokuca-Bogetic, C.H. Lo, B. Hage, R. Smith, W. Lukito, Differential serum responses to tocopherols and tocotrienols during vitamin E supplementation in hypercholesterolemic individuals without change in coronary risk factors, Nutr. Res. 12 (1992) S181–S201.

[37] R.P. Mensink, A.C. Van Houwelingen, D. Kromhout, G. Honstra, A vitamin E concentrate rich in tocotrienols had no effect on serum, lipoproteins, or platelet function in men with mildly elevated serum lipid concentrations, Am. J. Clin. Nutr. 69 (1999) 213–219.

[38] A.A. Qureshi, B.C. Pearce, R.M. Nor, A. Gapor, D.M. Peterson, C.E. Elson, Dietary α-tocopherol attenuates the impact of γ-tocotrienol on hepatic 3-hydroxy-3-methylglutaryl coenzymeA reductase activity in chickens, J. Nutr. 126 (1996) 389–394.

[39] H.T. Khor, T. T. Ng, Effects of administration of α-tocopherol and tocotrienols on serum lipids and liver HMG-CoA reductase activity, PORIM International Palm Oil Congress (PIPOC), Nutrition Conference, 1–6 February, 1999, pages 177–186.

[40] F. Atroshi, E. Antila, S. Sankari, J. Treuthardt, A. Gapor, Palm oil vitamin E effects in hypercholesterolemia, Lipid-Soluble Antioxidant: Biochemistry and Clinical Applications, A.S.H. Ong, L. Packer (eds). Birkhauser Verlag, Basel/Switzerland, 1995, pages 575–581.

[41] A.A. Qureshi, B.A. Bradlow, L. Brace, J. Manganello, D.M. Peterson, B.C. Pearce, J.J.K. Wright, A. Gapor, C.E. Elson, Response of hypercholesterolemic subjects to administration of tocotrienols, Lipids 30 (1995) 1171–1177.