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Dose-dependent suppression of serum cholesterol by tocotrienol-rich fraction (TRF_{25}) of rice bran in hypercholesterolemic humans

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Abstract

Tocotrienols are effective in lowering serum total and LDL-cholesterol levels by inhibiting the hepatic enzymic activity of β-hydroxy-β-methylglutaryl coenzymeA (HMG-CoA) reductase through the post-transcriptional mechanism. α-Tocopherol, however, has an opposite effect (induces) on this enzyme activity. Since tocotrienols are also converted to tocopherols in vivo, it is necessary not to exceed a certain dose, as this would be counter-productive. The present study demonstrates the effects of various doses of a tocotrienol-rich fraction (TRF_{25}) of stabilized and heated rice bran in hypercholesterolemic human subjects on serum lipid parameters. Ninety (18/group) hypercholesterolemic human subjects participated in this study, which comprised three phases of 35 days each. The subjects were initially placed on the American Heart Association (AHA) Step-1 diet and the effects noted. They were then administered 25, 50, 100, and 200 mg/day of TRF_{25} while on the restricted (AHA) diet. The results show that a dose of 100 mg/day of TRF_{25} produce maximum decreases of 20, 25, 14 ($P < 0.05$) and 12%, respectively, in serum total cholesterol, LDL-cholesterol, apolipoprotein B and triglycerides compared with the baseline values, suggesting that a dose of 100 mg/day TRF_{25} plus AHA Step-1 diet may be the optimal dose for controlling the risk of coronary heart disease in hypercholesterolemic human subjects.

Keywords: Hypercholesterolemic human subjects; Novel tocotrienols (TRF_{25}); Lipid parameters; Total cholesterol; LDL-cholesterol; HDL-cholesterol

1. Introduction

The cholesterol-lowering properties of tocotrienols have been demonstrated in chickens, swine, and humans by a number of investigators [1–9], and these natural substances can be used to manage mild hypercholesterolemia. Tocotrienols are characterized by a total lack of side-effects, and act by inhibiting the enzymic activity of β-hydroxy-β-methylglutaryl coenzymeA (HMG-CoA) reductase through the post-transcriptional mechanism [10]. On the other hand, α-tocopherol induces HMG-CoA reductase activity [11,12].

Tocotrienols are naturally occurring farnesylated unsaturated analogs of α-, β-, γ- and δ-tocopherols [1,2]. In contrast to corn, wheat, and soybean (contain mainly tocopherols), barley, oats, palm, and commercial rice brans contain $>70\%$ tocotrienols (known as tocotrienol-rich fraction: TRF), which consists of α-, γ-, β-and δ-tocotrienols [1,2]. The tocotrienols from these cereals have been demonstrated to lower cholesterol levels in animals and humans [1–10].

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 coenzymeA; LDL-cholesterol, low density lipoprotein cholesterol; TRF_{25}, tocotrienol-rich fraction of stabilized and heated rice bran.

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TRF_{25} from stabilized and heated rice brans not only consists of α-, γ-, δ-tocotrienols but also contains other novel compounds, desmethyl (D-P_{21}-T3) and didesmethyl (D-P_{25}-T3) tocotrienols [13]. Recently, we have demonstrated that the intake of TRF_{25} (200 mg/day) in hypercholesterolemic human subjects for four weeks in combination with AHA Step-1 diet lowered the serum total cholesterol, and low-density lipoprotein (LDL) cholesterol levels by 17 and 24%, respectively, as compared with their baseline values [3]. As mentioned above α-tocopherol opposes the effect of tocotrienols on the activity of HMG-CoA reductase [11,12]. Further, tocotrienols are also converted to tocopherols in vivo as reported recently [3–6,8,9]. Therefore, it is necessary not to exceed a certain dose of tocotrienols, as this conversion to tocopherol could override its effects on cholesterogenesis and may be counter-productive [3,4].

The present study was carried out to evaluate the hypocholesterolemic effects of different doses of TRF_{25} of stabilized and heated rice brans plus AHA Step-1 diet in hypercholesterolemic human subjects (serum total cholesterol levels >6.5 mmol/l). More specifically to determine if various doses of TRF_{25} 25, 50, 100, 200 mg/day in addition to a restricted diet regimen (AHA Step-1 diet) for five weeks results in dose-dependent effects on serum total cholesterol, LDL-cholesterol, apolipoprotein B, triglycerides, HDL-cholesterol and apolipoprotein A1 concentrations in hypercholesterolemic human subjects, and to determine the optimal dose of TRF_{25} in these subjects.

2. Materials and methods

2.1. Chemicals

Sources of chemicals, substrates, and diagnostic kits have been identified previously [3,13]. Chemicals and solvents were of analytical grade.

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 [13]. The composition of various tocols was determined by high pressure liquid chromatography (HPLC; 3) in TRF_{25} was 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, 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 [13]. The compounds were identified according to the retention time and absorption profiles against standards of tocols described earlier by HPLC [3]. Capsules containing TRF_{25} (25 mg) and 225 mg of rice bran oil stripped tocols (by extracting with methanol) were prepared. The TRF_{25} consists of 8.7% α-tocopherol, 4.4% δ-tocopherol, and 86.9% tocotrienols and tocotrienol-like compounds. The tocols stripped rice bran oil (250 mg) was used to prepare capsules for the placebo group.

2.3. Study population

Subjects were recruited from a hypercholesterolemic population (serum cholesterol levels >5.7 mmol/l) screened at the Armed Forces Institute of Pathology, Rawalpindi, Pakistan. The cholesterol level of 5.7 mmol/l was the cut-off level for selecting the hypercholesterolemic human subjects for the present study. Prospective participants were grouped according to the cholesterol level (the population was divided into high- and low-cholesterol levels groups and randomized into five groups to achieve 'median' cholesterol level in each and every group), and sub-grouped by sex. Volunteers were excluded (35 out of 125) on the basis of weight (>150% of metropolitan life ideal weight), use of cholesterol-altering medication, an elevated serum glutamate–pyruvate or glutamate–oxalo-acetate transaminase activity, an elevated blood urea nitrogen or glucose level, diabetes, or a history of a liver, renal, or hypertensive disease. Subject screening was accomplished during a five-week period. A fasting blood 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 during screening, the subjects (serum cholesterol level >6.5 mmol/l) in each of the five groups were randomized into four experimental groups and a placebo group, age (males <50 year old and females <40 year old), and body weight (between 60 and 80 kg). Each group consisted of 15 males and 3 females.

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 125 hypercholesterolemic subjects, 90 subjects (serum total cholesterol levels >6.5 mmol/l), were di-

vided into five groups (18 participants/group). The study was carried out in three phases, each of which lasted for 35 days.

2.4.1. Phase-I

Blood samples after an overnight fast was collected to establish the baseline lipid parameters at the start of the study during phase I of the first 35 days, and the subjects met individually and in small group sessions with counselors. The individual sessions focused on the 24 h recalls of food consumption, and the group sessions provided instructions for keeping three-day records of food intake (two weekdays and one weekend day). Subjects were encouraged to follow their typical dietary pattern and were instructed to keep food records for the terminal three days of this and the remaining phases. Subjects also received an unanticipated telephone call for a 24 h recall of food intake.

2.4.2. Phase II

In the second phase, all the subjects were limited to an AHA Step-1 diet (limited to intake of 300 mg (7.76 mmol/l)/day cholesterol and 30% energy from fat by stopping the intake of whole milk, ice-cream, cheese, eggs, butter, and pure ghee, and using skim milk) for 35 days and a sample of blood (5 ml) 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. The dietary restriction of AHA Step-1 diet was continued through out the study.

2.4.3. Phase III

In the third phase, capsule of 25, 50, 100, and 200 mg/day of TRF_{25} were given, respectively, plus AHA Step-1 diet to subjects of the four experimental groups.. The subjects of 25 mg/day dose group were asked to take one capsule of TRF_{25} after dinner (20:00 h). The subjects of 50 or 100 mg/day groups were asked to take the capsule of TRF_{25} after breakfast (one or two in the morning; 08:00 h) and one or two after dinner (20:00 h), respectively. However, for the intake of eight capsules (200 mg/day), subjects were requested to take two capsules every 4 h (08:00, 12:00, 16:00 and 20:00 h). Subjects in the placebo group received eight capsules containing tocols stripped rice bran oil (250 mg/capsule) after every 4 h (as described above for 200 mg/day) plus AHA Step-1 diet. At the end of the phase of 35 days, blood (5 ml) after an overnight fast was drawn from each subject; the serum was harvested and kept at -70 °C for later analyses of lipid parameters.

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 07:00–09:00 h 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 assays for each lipid parameter for all the subjects in each group were carried out at the same time under similar conditions to minimize standard deviation. When the frozen samples were thawed, most of them (11–14 out of 18) have precipitates in each group due to high levels of triglycerides (> 3.5 mmol/l) found in these subjects. The number of samples have precipitates in groups one [12], two [14], three [11], four [14] and, five [13], respectively, out of the 18 samples in each group. Therefore, samples were vortexed just prior to removing the aliquot samples for manual analyses of each lipid parameter. The serum total cholesterol, HDL-cholesterol, and triglycerides concentrations were estimated with reagent kits from Sigma Chemical Co. (St. Louis, MO).

Serum LDL-cholesterol 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 $12\,000 \times g$ 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 concentration of LDL-cholesterol was estimated as described for the total cholesterol.

Serum apolipoprotein A1 (apo A1), and apolipoprotein B (apo B) concentrations were determined by radioimmunoassay using kits from Sigma Chemical Co. (St. Louis, MO) in the placebo and treatment groups. To determine if subjects have 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 the food intake to meet the goals of the AHA Step-1 diet or to maintain weight.

2.6. Statistical analyses

The data were analyzed by using the GLM procedure of SAS (Statistical Analysis System) for personal computers to test the study hypothesis. Duncans multiple-range test was used to test whether the treated groups differed from the baseline values and placebo group 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, and available degrees of freedom were reduced by this statistical approach. The treatment effects on gain in body weight, total cholesterol, and LDL-cholesterol were also evaluated using the paired, two-tailed t-test (StatView, Abacus Concepts 1992, Berkeley, CA. USA). Data are reported as mean $\pm$ SD in the text. The statistical significance level was set at 5%. The data was also analyzed by using changes from baseline and from the treatment's values of phases I–III using a multiclinic extension of the SAS or Duncan's matched pairs signed-rank t-test ($P < 0.05$).

3. Results

The main characteristics (number of subjects, age, body weights, heights, body mass index and initial cholesterol levels) of the study population are outlined in Table 1. There was a significant ($P < 0.001$) change in body weights in subjects on AHA Step-1 diet and four treatment groups of TRF_{25} as compared with the baseline values (Table 1). The analyses of all the subjects during the last two phases (5–10 and 10–15 weeks) of AHA Step-1 diet and various doses of TRF_{25} capsule plus AHA Step-1 diet revealed that all the subjects reduce their intakes of energy (30%; $P < 0.2$–0.001), fat (20%; $P < 0.01$), carbohydrate (18%; $P < 0.05$), and cholesterol (35%; $P < 0.001$) significantly as compared with the baseline values (Table 2).

The dietary restriction (AHA Step-1 diet, second phase of 35 days) alone lowered the serum total cholesterol, LDL-cholesterol, apo B and triglycerides concentrations by 4, 5, 5, and 4%, respectively, from the baseline values (Table 3), without affecting the levels of HDL-cholesterol and apo A1.

During the third phase (35 days), TRF_{25} (25 mg/day) was administered, which showed further decreases of 6% in serum total and LDL-cholesterol concentrations as compared with the baseline values. The serum total cholesterol, LDL-cholesterol, apo B, and triglycerides lowered significantly ($P < 0.05$) by 17, 21, 11, and 12%, respectively, in the third phase with TRF_{25} (50 mg/day) in these subjects (Table 3). The serum HDL-cholesterol also increased by 15% while apo A1 increased by 11% ($P < 0.05$) during this phase, as compared with the baseline values (Table 3).

The TRF_{25} (100 mg/day) showed maximum decreases in serum levels of total cholesterol, LDL-cholesterol, apo B, and trglycerides by 20, 25, 14 ($P < 0.05$), and 12%, respectively, from the baseline values, while HDL-cholesterol and apolipoprotein A1 levels increased by 19% and 14% ($P < 0.02$), respectively, as compared with baseline values (Table 3). The administration of TRF_{25} (200 mg/day) in the third phase did not bring about any further reduction in the lipid parameters of these subjects. These results indicate that the decreases

Table 1
Characteristics of the study population of hypercholesterolemic human subjects

Characteristics	Baseline	AHA Step-1 diet	AHA Step-1 diet + TRF_{25} (25 mg/day)	AHA Step-1 diet + TRF_{25} (50 mg/day)	AHA Step-1 diet + TRF_{25} (100 mg/day)	AHA Step-1 diet + TRF_{25} (200 mg/day)
Subjects						
Males (15)	(83%)	(83%)	(83%)	(83%)	(83%)	(83%)
Females (3)	(17%)	(17%)	(17%)	(17%)	(17%)	(17%)
Age (years)						
Males	47.8 ± 6.8 [a]	48.4 ± 5.8	47.3 ± 5.2	49.2 ± 7.8	48.3 ± 6.9	47.9 ± 6.3
Females	32.7 ± 4.7	34.6 ± 5.1	33.9 ± 5.8	34.1 ± 5.7	33.8 ± 4.5	34.6 ± 4.6
Initial body weight (kg)	78.4 ± 8.7[a]	74.5 ± 6.6[a]	72.2 ± 8.5[a]	79.7 ± 7.1[a]	73.2 ± 9.9[a]	75.7 ± 7.9[a]
Final body weight (kg)	78.6 ± 7.8[a]	71.8 ± 6.7[b]	67.8 ± 6.5[b]	74.7 ± 7.8[b]	68.6 ± 7.4[b]	72.6 ± 7.8[b]
t #	−0.21	9.9	3.7	3.4	3.9	13.7
Height (cm)	165.7 ± 8.7	164.6 ± 7.4	166.9 ± 7.8	162.2 ± 8.3	169.2 ± 8.6	161.3 ± 7.1
Body mass index (kg/m^2)	28.6 ± 4.5	27.5 ± 3.7	25.9 ± 4.1	30.3 ± 3.3	25.6 ± 3.6	29.1 ± 3.2
Initial cholesterol (mmol/l)	6.6 ± 1.12[a]	6.9 ± 0.89[a]	7.1 ± 0.81[a]	6.9 ± 0.71[a]	6.8 ± 0.81[a]	6.7 ± 0.77[a]

Superscripted letters a and b: values in a row not sharing a common superscript letter are significantly different at $P < 0.001$.
[a] Data expressed as mean ± SD (standard deviation), $n = 18$ subjects/group (15 males and 3 females); t # = paired, two-tailed t-test.

Table 2
Impact of AHA Step-1 diet plus TRF_{25} regimen on dietary intake during 5–15 weeks of hypercholesterolemic human subjects

Dietary intake	Baseline	AHA Step-1 diet	AHA Step-1 diet + TRF_{25} (25 mg/day)	AHA Step-1 diet + TRF_{25} (50 mg/day)	AHA Step-1 diet + TRF_{25} (100 mg/day)	AHA Step-1 diet + TRF_{25} (200 mg/day)
Energy (kJ/day) [a]	7304 ± 2684^{a} (100) [b]	$5866 \pm 2521^{a,b}$ (80)	4960 ± 1671^{b} (68)	5368 ± 1986^{b} (73)	4803 ± 1627^{b} (66)	4848 ± 1789^{b} (66)
$t\#$ [P-values]	–	6.3 [0.01]	4.5 [0.01]	5.2 [0.05]	1.9 [0.1]	1.8 [0.2]
Protein (g/day)	67.6 ± 23.2^{a} (100)	62.5 ± 22.6^{a} (92)	64.3 ± 24.2^{a} (95)	61.8 ± 21.5^{a} (91)	63.7 ± 19.8^{a} (94)	64.9 ± 22.8^{a} (96)
Fat (g/day) [c]	65.9 ± 20.7^{a} (100)	52.5 ± 19.5^{b} (80)	52.5 ± 18.7^{b} (80)	51.3 ± 18.2^{b} (77)	50.7 ± 18.8^{b} (77)	50.9 ± 18.9^{b} (77)
$t\#$ (P-values)	–	4.7 [0.01]	4.5 [0.01]	4.8 [0.01]	4.7 [0.01]	4.7 [0.01]
Carbohydrate (g/day)	231.8 ± 61.5^{a} (100)	190.3 ± 66.9^{b} (82)	186.9 ± 63.8^{b} (81)	191.4 ± 66.1^{b} (83)	194.5 ± 62.8^{b} (84)	188.5 ± 65.7^{b} (81)
$t\#$ [P-values]	–	9.5 [0.05]	2.8 [0.05]	2.7 [0.05]	2.6 [0.05]	2.8 [0.05]
Fiber (g/day)	16.9 ± 6.3^{a} (100)	16.3 ± 6.9^{a} (96)	15.7 ± 7.3^{a} (93)	16.7 ± 6.9^{a} (99)	17.4 ± 6.4^{a} (103)	16.6 ± 6.3^{a} (98)
Cholesterol (g/day)	255.7 ± 38.8^{a}	165.6 ± 32.8^{b}	162.3 ± 36.9^{b}	157.8 ± 31.1^{b}	161.6 ± 29.2^{b}	158.7 ± 32.4^{b}
Cholesterol (mmol/l/day)	6.61 ± 1.00^{a} (100)	4.28 ± 0.85^{b} (65)	4.19 ± 0.95^{b} (63)	4.08 ± 0.80^{b} (62)	4.18 ± 0.76^{b} (63)	4.10 ± 0.84^{b} (62)
$t\#$ [P-values]	–	10.8 [0.001]	10.5 [0.001]	10.6 [0.001]	10.1 [0.001]	10.5 [0.001]
Alcohol	–	–	–	–	–	–

Superscripted letters a and b: values in a row not sharing a common superscript letter are significantly different at $P < 0.2$–0.05.
[a] Data expressed as mean ± SD (standard deviation); $n = 18$ subjects/group (15 males and 3 females); $t\#$ = paired, two-tailed t-test.
[b] Percentage with respect to baseline values are in parentheses.
[c] Fat = Prior to study, the subjects were consuming butter (desi ghee), vanaspati ghee (partially hydrogenated soybeen seeds oil), palm oil, or Dalda (hydrogenated cotton seeds oil). At the sart of the study subjects were restricted to corn or soybean oils throughout the study.

in the serum total cholesterol, LDL-cholesterol, apo B and triglycerides concentrations are maximum with the dose of 100 mg/day of TRF_{25} of rice bran. The dose-dependent decreases in serum total cholesterol, and LDL-cholesterol levels during the second and third phases (35 days of each) are summarized in Fig. 1 as compared with the baseline values. Moreover, significant increases in HDL-cholesterol (19%) and apo A1 (14%) concentrations were also witnessed at this dose (Table 3).

The subjects of the placebo group were given eight capsule of tocols stripped rice bran oil (200 mg/day) plus AHA Step-1 diet, and as reported earlier that intake of 200 mg/day corn oil capsule plus AHA Step-1 diet had insignificant affects on the lipid parameters of hypercholesterolemic human sujects [3]. Most of the subjects in each group had very high levels of triglycerides (> 3.5 mmol/l), and when the frozen samples were thawed, some of them (at least 11–14 samples out of 18 in each and every group) had precipitated. Therefore, each sample was vortexed just prior to taking the aliquot sample for the manual analyses of each of the lipid parameters. The results obtained for each lipid parameter are based on manual analysis and not auto-analyzer because of the presence of precipitates caused by the high levels of triglycrides (> 3.5 mmol/l) found in these subjects.

4. Discussion

The results of the present study clearly demonstrate that doses of 50–100 mg/dayof TRF_{25} are most effective in lowering the serum total cholesterol and LDL-cholesterol concentrations, suggesting that a dose of 100 mg TRF_{25} plus AHA Step-1 diet may be the optimal dose for controlling the risk factors of heart disease in these hypercholesterolemic subjects. TRF_{25} (mixture of tocopherols and tocotrienols) is placed in the generally recognized as safe (GRAS) category by the FDA and are remakably free of adverse effects [14], also observed in the present study. The dose of 100 mg of TRF_{25} produced the maximal decreases of 20, 25, 14 ($P < 0.05$), and 12% in serum total cholesterol, LDL-cholesterol, apolipoprotein B and triglycerides levels, respectively, as compared with the baseline values, only when all the subjects are following a strict AHA Step-1 diet during the ten-week study period. The concentrations of HDL-cholesterol (19%) and apolipoprotein A1 (14%) are significantly ($P < 0.05$) increased during this period.

In our first human 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 four weeks caused decreases of 15, and 8%, respectively, in

Table 3
Effects of AHA Step-1 diet and different doses of TRF_{25} on serum lipid parameters in hypercholesterolemic human subjects [a]

Treatments	Total cholesterol (mmol/l)	LDL-cholesterol (mmol/l)	Apolipoprotein B (g/l)	Triglycerides (mmol/l)	HDL-cholesterol (mmol/l)	Apolipoprotein A1 (g/l)
Baseline	$6.79 \pm 0.45^{a,b}$ (100.00) [c]	5.95 ± 0.48^{a} (100.00)	0.85 ± 0.11^{a} (100.00)	2.85 ± 0.86^{a} (100.00)	0.79 ± 0.16^{a} (100.00)	1.10 ± 0.16^{a} (100.00)
AHA Step-1 diet	6.50 ± 0.32^{a} (95.71)	5.66 ± 0.42^{a} (95.04)	0.81 ± 0.10^{a} (95.33)	2.73 ± 0.83^{a} (95.72)	$0.82 \pm 0.16^{a,b}$ (103.89)	$1.12 \pm 0.16^{a,b}$ (102.19)
AHA Step-1 diet + TRF_{25}, 25 mg	6.12 ± 0.39^{b} (90.12)	5.28 ± 0.44^{b} (88.72)	$0.79 \pm 0.01^{a,b}$ (94.31)	2.71 ± 0.83^{a} (95.01)	$0.84 + 0.15^{a,b,c}$ (107.16)	$1.12 \pm 0.17^{a,b}$ (102.32)
AHA Step-1 diet + TRF_{25}, 50 mg	5.66 ± 0.46^{c} (83.44)	4.72 ± 0.48^{c} (79.33)	$0.76 \pm 0.02^{a,b}$ (89.79)	2.52 ± 0.81^{a} (88.30)	$0.90 \pm 0.14^{b,c}$ (115.12)	$1.22 \pm 0.17^{a,b}$ (111.11)
AHA Step-1 diet + TRF_{25}, 100 mg	5.46 ± 0.51^{c} (80.42)	4.49 ± 0.54^{c} (75.39)	0.73 ± 0.06^{b} (86.27)	2.51 ± 0.81^{a} (88.20)	$0.93 \pm 0.12^{b,c}$ (118.76)	$1.25 \pm 0.18^{b,c}$ (113.78)
AHA Step-1 diet + TRF_{25}, 200 mg	5.52 ± 0.47^{c} (81.25)	4.55 ± 0.50^{c} (76.50)	0.74 ± 0.04^{b} (87.49)	2.53 ± 0.82^{a} (88.83)	$0.91 \pm 0.12^{b,c}$ (115.52)	$1.25 \pm 0.18^{b,c}$ (113.43)
ANOVA (P-values)	0.0001	0.0001	0.0422	NS (0.7581)	0.0152	0.0133

Superscripted letters a–c: values in a row not sharing a common superscript letter are significantly different at $P < 0.05$.
[a] Time of drawing blood was 08:00 h. The subjects were fasted for 12 h before samples were taken.
[b] Data expressed as means $\pm$ SD (standard deviation); $n = 18$ per group.
[c] Percentage with respect to baseline values are in parentheses.

the concentrations of serum total and LDL-cholesterol [4]. 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 were not statistically significant for the entire goup [4]. Estimation of serum tocols (tocopherols and tocotrienols) by HPLC in these subjects given Palmvitee capsules for four weeks showed a higher concentration of α-tocopherol than tocotrienols [4].

Atroshi et al. have also examined the effect of the same Palmvitee capsules (four capsules/day; 50 mg/capsule contains α-tocopherol 16%, plus tocotrienols 84%) on lipid parameters in Finnish healthy adults for six weeks. They also observed an insignificant reduction in the concentrations of serum total cholesterol, LDL-cholesterol and confirmed our results of an increase in the serum α-tocopherol concentration in these subjects [15]. Although, it is not clear why they were trying to lower the serum total cholesterol and LDL-cholesterol concentrations in the healthy subjects with Palmvitee capsules.

These results are similar to those reported by Wahlqvist et al. and Mensink et al. that the inability of tocotrienol-rich fraction of palm oil (TRF as Palmvitee capsules) to lower serum total and LDL-cholesterol concentrations in free-living hypercholesterolemic human subjects [16,17]. These results are at odds with the present study and the results of the recently reported human studies [3,18]. In our recent human studies, hypercholesterolemic subjects were first conditioned for at least four weeks to a restricted dietary regimen using AHA Step-1 diet [3,18]. These subjects on an AHA Step-1 diet for four weeks resulted in decreases in serum total and LDL-cholesterol levels by 5 and 8%, respectively. The subjects that received TRF_{25} plus AHA Step-1 diet for 12 weeks showed decreases of 12 and 16% in serum total and LDL-cholesterol levels [3].

The hypocholesterolemic effects of tocotrienols have been confirmed by a number of investigators [1–10]. However, the TRF mixture of palm oil containing high α-tocopherol (> 20%) concentration had no impact on the serum total and LDL-cholesterol levels in hypercholesterolemic human subjects as reported by Wahlqvist et al. [16] and Mensink et al. [17]. There are two major differences between the protocols and tocol mixtures used in their studies, and other studies [4,15–17]. The first main difference in our first trial [4], and those of Atroshi [15], Wahlqvist [16], and Mensink [17] was not using the restricted intake of cholesterol (< 300 mg (7.76 mmol/l)/day), and energy (< 30% fat/day)

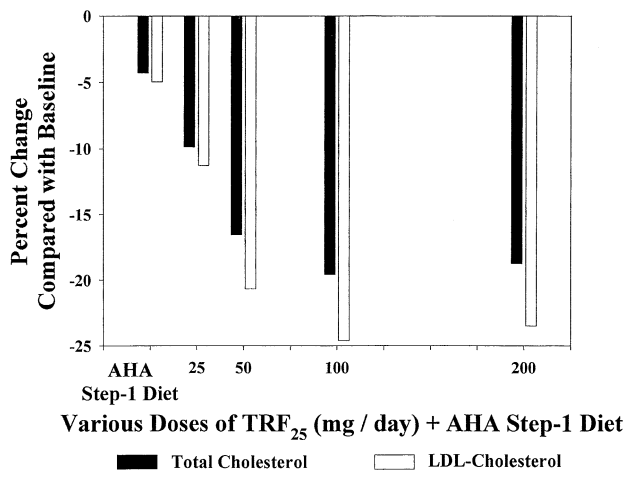

Fig. 1. The dose dependant decreases of TRF_{25} plus AHA Step-1 diet on the concetrations of serum total cholesterol and LDL-cholesterol as compared to their respective baseline values.

as recommended by the AHA Step-1 diet by the human subjects in all these studies. [4,15–17]

The second difference is the mixture of tocol (Palmvitee capsules) used in the studies by Wahlqvist and Mensink, contained higher concentrations of α-tocopherol 30 and 36%, respectively [16,17], whereas the present mixture (TRF_{25}) contained tocopherols 13, and 87% tocotrienols, obtained from stabilized and heated rice bran [3,13,18]. The TRF_{25} not only contains α-, β-, γ-, and δ-tocotrienols but two new more potent ones, desmethyl (D-P_{21}-T3), and didesmethyl (D-P_{25}-T3) tocotrienols also [3,13,18].

We have recently reported that the ratio of the tocopherols and tocotrienols play an important role in determining the hypocholesterolemic properties of tocotrienols [12]. The presence of more than 20% α-tocopherol in TRF from palm oil results in an attenuation of the hypocholesterolemic effect of tocotrienols [12]. These findings have been confirmed by Khor and Ng [19]. Therefore, it is desirable to prepare TRF from any natural source with minimal concentrations of tocopherols in the mixture.

As mentioned above, the estimation of various tocols (tocopherols + tocotrienols) of the serum showed the higher concentrations of α-tocopherol compared with tocotrienols after consuming Palmvitee, TRF_{25}, or γ-tocotrienol capsules by the subjects in all these studies [3–9,15–20]. Large quantities of TRF, TRF_{25}, and γ-tocotrienol (200 mg/day or more) were used in all human studies [3,4,15–18]. Recently, Khor et al. and Watkins et al. have reported that lower doses (5 or 50 mg/kg) of γ-tocotrienol are much more effective in inhibiting the activity of HMG-CoA reductase and lowering the serum total cholesterol, and LDL-cholesterol levels than their respective higher doses (50 or 100 mg/kg) in guinea pigs and rats [6,8,19]. The reason for low 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 increase three to fourfold in guinea pigs treated with 50 mg/kg γ-tocotrienol [6,19], and an increased level of α-tocopherol induces the activity of HMG-CoA reductase [11,12].

We have recently demonstrated the conversion of labeled γ-[4-^{3}H]-tocotrienol and [^{14}C]-D-desmethyl (D-P_{21}-T3) tocotrienol into α-tocopherol in chickens and rice/barley seedlings [21]. Therefore, it is important to know the effective dose of tocotrienols, and not to exceed a restrictive dose of tocotrienols, as overdosing of tocotrienols would overide their hypocholesterolemic effects, and may be counter-productive. The earlier trials were carried out without knowing the effective dose of tocotrienols [3,4,18].

It is well established that lipoprotein disorders are amongst the most common metabolic disorders encountered in clinical practice, and often cause coronary heart disease, peripheral vascular disease, dermatological manifestations, pancreatitis and sometimes, neurological or ocular abnormalities [22,23]. The raised serum total cholesterol and LDL-cholesterol levels are important risk factors for the development of coronary artery disease. Serum total cholesterol and lipoprotein analyses frequently provide clinicians the basic essential data with which to devise further screening, diagnosis, and dietary and therapeutic interventions in the management of primary hyperlipoproteinaemia [22–24]. While mild to moderate hypercholesterolemia may frequently be controlled by the dietary intake of fibers, the latter prove insufficient when the serum cholesterol level is elevated substantially [24]. Therefore, antilipemic drugs such as statins need to be used as adjuncts to diet to control LDL-cholesterol levels [25]. Lovastatin (one of the statins drugs) is a competitive inhibitor of cholesterol biosynthesis and lowers serum total cholesterol and LDL-cholesterol levels in animals and humans [26]. Lovastatin is very effective in lowering total, and LDL-cholesterol levels when used in combination of AHA Step-1 diet in hypercholesterolemic human subjects [25–27].

Tocotrienols inhibit cholesterol synthesis by suppressing HMG-CoA reductase activity through a post-transcriptional mechanism [10]. HMG-CoA reductase is the rate-limiting enzyme in the mevalonate pathway of endogenous cholesterol synthesis in the liver. Tocotrienols act in a similar manner to oxysterols [10]. A number of oxysterols have been shown to regulate cholesterol biosynthesis by a transcriptional down-regulation of the reductase gene [28]. It has been postulated that endogenously produced oxysterols are natural regulators of cholesterol biosynthesis. In particular, 24(S), 25-epoxycholesterol (OLAN) and 25-hydroxycholesterol have been found in human liver, in vivo, in concentrations high enough for cholesterol regulation [29]. Tocotrienols, natural regulators of cholesterol biosynthesis have a similar function and it appears, they are very effective in reducing cholesterol levels in hypercholesterolemic subjects [1–5].

It is well known that a diet rich in soluble fibers of plant origin can retard the development of coronary heart disease [30,31]. The rice, oat, and barley brans can lower total and LDL-cholesterol levels in humans and animals due to their contents of β-glucan, and a number of lipid soluble components, including tocotrienols [32–34]. The consumption of 100 gm/day of rice bran or oat bran lowers serum total cholesterol level about 7% in humans [32] Whereas, TRF_{25}, 100 mg/day of rice bran plus AHA Step-1 diet lowers the serum total and LDL-cholesterol levels by 20 and 25% ($P < 0.001$), respectively, in hypercholesterolemic humans (Fig. 1), and has no known side-effects. This reduction is similar to lovastatin, a drug extensively

used in the management of hypercholesterolemia, despite some unwanted side-effects [25]. The synergistic effect of low dose of lovastatin (10 mg/day), and minimum effective dose of TRF_{25} (50 mg/day) plus AHA Step-1 diet has been reported to lower total cholesterol, and LDL-cholesterol levels by 20 and 25%, respectively, over the baseline values in hypercholesterolemic human subjects, and also eliminated the side-effects of lovastatin [21].

The results of the present study demonstrate that a dose of TRF_{25} of rice bran 100 mg/day (tocotrienols mixture with less than 15% α-tocopherol) not only is an effective cholesterol lowering natural product but also a very useful agent for combined therapy with other cholesterol lowering drugs because of its role in eliminating side-effects of these drugs in humans.

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