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Similar cholesterol-lowering properties of rice bran oil, with varied γ-oryzanol, in mildly hypercholesterolemic men[1]

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Summary *Background* The cholesterol lowering properties of rice bran oil (RBO) containing differing amounts of non-saponifiable components have not been studied in humans, to our knowledge. *Aim of the study* To evaluate cholesterol lowering effects of RBO, with low and high amounts of γ-oryzanol (ferulated plant sterols) in mildly hypercholesterolemic men. *Methods* Mildly hypercholesterolemic men, 38–64 y, starting cholesterol 4.9–8.4 mmol/l ($n = 30$), consumed 50 g/d peanut oil (PNO) in vehicles for 2 wks during a run-in period, then, without wash-out, were randomly equilibrated (based on initial level of cholesterol) into two groups to consume 50 g/d RBO low (0.05 g/d) or high (0.8 g/d) γ-oryzanol for 4 wks, in a randomized, controlled, parallel design study. Subjects were free-living and consumed habitual diets with some restrictions. Plasma concentrations of total, LDL-, HDL-cholesterol and triacylglycerol were measured at base line and after 2, 4, and 6 wks. *Results* The two RBO types were not significantly different with respect to effects on various cholesterol parameters, at 2 and 4 wks, including total cholesterol, LDL-, HDL- and LDL/HDL cholesterol ratio. Low and high γ-oryzanol-containing RBO feeding for 4 wks lowered total plasma cholesterol (6.3 %), LDL-C (10.5 %) and the LDL-C/HDL-C ratio (18.9 %). *Conclusions* RBO supplementation at ca. 50 % total fat intake improved lipoprotein pattern in mildly hypercholesterolemic men. Methylated sterols in γ-oryzanol are thought to be largely ineffective at inhibiting dietary cholesterol absorption, but could enhance cholesterol-lowering ability of 4-desmethylsterols. Assuming all ferulated sterols become de-ferulated in the gut, low and high γ-oryzanol-containing RBOs provided intestinal loads of 453 and 740 mg/d free 4-desmethylsterols, respectively. This intestinal load of 453–740 mg/d of efficacious free plant sterol equivalents had identical effects on lipoproteins.

Key words cardiovascular risk – cholesterol – HDL – LDL – γ-oryzanol – peanut oil – rice bran oil

Abbreviations

ANOVA	analysis of variance
BMI	body mass index
C	cholesterol
D	day
HDL	high density lipoprotein
HPLC	high performance liquid chromatography

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LDL	low density lipoprotein
Lp(a)	Lipoprotein "little a"
n. d.	not detectable
P_1, P_{15}	denotes the peanut oil period, experimental days 1 or 15
PNO	peanut oil
R_{30}, R_{43}, R_{44}, R_{45}	denotes the rice bran oil period, experimental days 30, 43, 44, or 45
$R_{43–45}$	denotes the rice bran oil period, average values for days 43–45
RBO	rice bran oil
TAG	triacylglycerol
TC	total cholesterol

Introduction

Rice bran oil (RBO) is traditionally consumed in Asian rice producing countries, and there is now interest for Western markets [22, 26, 35, 43]. A potential advantage of RBO over oils with similar fatty acid composition may be its oxidative stability, imparted by high levels of tocopherols, tocotrienols, and γ-oryzanol [26]; and its cholesterol lowering potential [78]. Thus, there has been effort to develop RBO retaining its non-saponifiable components, while minimizing levels of problematic free fatty acids [14].

γ-Oryzanol is a mixture of 4,4'-dimethyl- and 4-desmethylsterols esterified to ferulic acid (4-hydroxy-3-methoxycinnamic acid). Specific examples of ferulated 4,4'-dimethyl- and ferulated 4-desmethylsterols in RBO are cycloartenol, 24-methylenecycloartanol, β-sitosterol, and campesterol [11]. RBO also contains non-ferulated 4-desmethylsterols.

γ-Oryzanol and RBO are generally purported to have cholesterol lowering properties. However, the exact cholesterol lowering compounds, mechanisms of actions of these compounds, and possible synergies amongst active components remain unidentified.

Older studies investigating cholesterol lowering properties of RBO were confounded in that investigators did not have placebo controls; used mixed clinical populations [81] and populations such as schizophrenics who concurrently consumed medications [53]; did not quantify non-saponifiable oil components [30]; extrapolated benefits achieved with de-fatted rice bran or γ-oryzanol itself, to benefits achieved with RBO [80, 81]; and used non-optimal animal models with high amounts of dietary cholesterol, completely shutting down LDL receptor activity.

There have been animal studies examining effects of different amounts of γ-oryzanol [60, 61] and non-saponifiable components [23]; however only one study evaluated non-saponifiable components in a RBO matrix [34]. The cholesterol lowering properties of RBO containing differing amounts of non-saponifiable components have not been studied in humans, to our knowledge. Thus, we conducted a clinical trial in hypercholesterolemic men to compare cholesterol lowering properties of RBOs with low and high quantities of γ-oryzanol.

Methods

Recruiting

Advertising was via E mail and TV screens at the Nestlé Research Center in Lausanne, and a nearby Product Technology Center and a Nestlé factory in Orbe, Switzerland, for a "vegetable oil cholesterol study". The study was approved by the Nestlé Research Centre Ethics Committee. Subjects were financially compensated Nestlé employees or direct family members. Nurses screened subjects 4 wks prior to study commencement on 5 different days. TC was measured at the beginning of the recruitment period.

Inclusion criteria

The goal was to obtain 32 mildly hypercholesterolemic men with defined criteria. Of 56 men screened, 34 had TC in the preferred 5.7–8.0 mmol/l range, but due to factors emerging prior to the study (unexpected absences, dislike of fatty foods), the TC range was widened to 5.1–8.4 mmol/l to have a sufficient number of participants. The study started and finished with 30 mildly hypercholesterolemic males, from the Nestlé Research Center [n = 16], Orbe Product Technology Center/Orbe factory [n = 10], and external (4 subjects), who were 40–65 y old, with BMI < 28 kg/m^2, and baseline TC on day 1 of 4.9–8.4 mmol/l. Starting mean TC values (6.1 mmol/l) were lower than mean screening values (6.4 mmol/l), likely because subjects contemplated diet and cholesterol prior to study commencement, and made dietary changes.

Additional inclusion criteria were: no smoking > 20 cigarettes/d; no excessive exercise; no consumption of prohibited foods; ≤ 2 servings wine or beer/d and no consumption of liquor, aperitifs or alcoholic beverages with > 40 % alcohol; and no acute or chronic metabolic diseases (*e. g.*, hemophilia, inflammatory; liver, renal or hypertensive disease; elevated glucose and diabetes; elevated glutamate-pyruvate and glutamate-oxaloacetate transaminases). Limits on prohibited foods were: no fondue or raclette; < 20 g/d of cheese (necessitating limits on Swiss specialities such as Gâteau au fromage, croûte au fromage, etc.); no liver, kidney, and brain offals; ≤ 3–4 eggs/wk; no charcuterie, lard and rillette; no molluscs, fish eggs, and eel; and ≤ 1 serving/2 wks of shrimp, squid, or shellfish. Use of cholesterol-altering medication was not permitted; other drugs and medica-

tions were individually considered by the nurses. Finally, extensive vacations abroad during the study were not permitted.

Expulsion criteria

Subjects completed a daily log, a 3-d dietary recall once biweekly, and were randomly telephoned to monitor compliance. Expulsion criteria were: < 57 % of provided food items actually consumed/d; < 83 % of provided food items actually consumed/wk; < 85 % of provided food items consumed/mo; and missing one of the critical blood draw periods (days 1, 15, 30, 43–45). Blood was not drawn from non-fasted subjects. Sickness and medication, injuries, unusual stresses, family crises, and extreme physical exercise were evaluated on an individual basis. Other upper limits were: 21 acceptable alcohol beverages/wk; 2 prohibited alcoholic beverages/wk; and 1.5 X specified limits for cheese and egg consumption. The minimum compliance for the daily breakfast was 6 d/wk. No subjects were expelled from the study based on the above criteria.

Food diary and food recall

In the daily food diary, subjects initialized the time of consumption of vehicles, and recorded consumption of cigarettes, alcohol, cheese, meat, charcuterie, crustaceans, medicines, vitamins, intense physical efforts, and sickness. For the food recall, subjects recorded: all foods and beverages consumed; any reductions in habitual food intake; whether the amount of oil provided was too much in their judgement; perceptions of vehicle taste; whether vehicles were supplemented with the provided syrups; and diarrhea incidence. Compliance and feedback were also monitored via random phone calls.

Study design

After the recruitment period, subjects consumed a peanut oil (PNO) based diet for 2 wks (run-in period, time points P_1-$P_{15;}$ P, peanut oil) and were then randomly equilibrated based on P_1 TC to consume low or high γ-oryzanol RBO vehicles for 4 wks (RBO period, time points R_{16}-$R_{45;}$ R, rice bran oil) in a randomized, parallel experimental design. Blood (10 ml) was taken at the beginning of study (P_1), at the end of the run-in period (P_{15}), in the middle of the RBO period (R_{30}) and at the end of the RBO period on 3 consecutive days ($R_{43–45}$). After screening $R_{43–45}$ values for outliers with box plots, an average was calculated [52]. On R_{43}, to determine cholesterol synthesis rates, subjects received two oral bolus doses of 99.8 % concentrated deuterated water (Cambridge Isotope Laboratories, Andover, MA, USA) at 19:00 and 21:00–22:00, providing deuterium at 0.3 % of body water, with Wakat's body weight correction [75]. Participants then received 2 l deuterated drinking water (4.5 g D_2O/l) during $R_{43–45}$ to maintain blood D_2O concentration.

Preparation of test oils and food vehicles

PNO was from Oleificio Sabo (Manno, Switzerland); both γ-low and γ-high RBOs were produced from hexane-extracted crude RBO supplied by Rito Inc. (Stuttgart, AK, USA). Tests oils were analyzed chemically for fatty acids, regiospecific distribution of fatty acids, and non-saponifiable components (Tables 1 and 2). Vehicles consisted of: salad oil (10 g serving providing 10 g test oil and 377 kJ (90 kcal); spread (10 g serving providing 6 g test oil and 226 kJ (54 kcal); refilled UHT Nesquik-type milk shakes in Tetrapaks (215 g serving providing 11 g test oil and 477 kJ (114 kcal); chocolate and strawberry flavors; and refilled Greek-type (high fat) yogurt (125 g serving providing 16 g test oil and 753 kJ (180 kcal), excluding contribution from optional vanilla and chocolate flavoring provided). Nutrient values for each food vehicle were calculated from proximate analysis compositional measurements and/or from the USDA/ARS Nutrient Data Laboratory database (www.nal.usda.gov/fnic/cgi-bin/nut_search.pl).

Consumption of vehicles

For variety, subjects consumed food vehicles in 10 combinations, providing 42.4–63.8 g PNO or RBO/d and a total of 1833–2787 kJ/d (438–666 kcal/d, Table 3). Average daily amounts of oil provided over the 14 d PNO and 30 d RBO feeding period were 51.2 and 52.2 g oil/d, respectively. A target of 50 g oil/d was chosen, as 50 g RBO was fed in previous cholesterol lowering trials [7, 30, 37, 67, 68, 78, 79]. Assuming a 10460 kJ (2500 kcal) diet, with 40 en% fat, vehicles provided ca. 20 en% of to-

Table 1 Profile and regiospecific distribution of fatty acids in test oils (wt%)

	PNO			RBO high γ-oryzanol		
Fatty acids	Overall	sn-2	sn-1,3	Overall	sn-2	sn-1,3
16:0	15.2	2.0	21.8	15.3	2.8	21.5
18:0	1.7	0.4	2.4	1.7	0.8	2.2
18:1n-7/n-9	43.2	46.2	41.6	43.3	45.1	42.5
18:2n-6	36.4	49.9	29.7	36.3	49.9	29.6
18:3n-3	1.1	1.1	1.1	1.1	1.1	1.1
20:0	0.6	n. d.	0.9	0.6	n. d.	0.9
20:1n-9	0.6	0.1	0.8	0.5	0.1	0.8

Excluded are fatty acids < 0.4 wt%. Low and high γ-oryzanol RBOs had a very similar fatty acid profile; for simplicity only the latter is shown

Table 2 γ-Oryzanol, 4-desmethylsterols, tocopherols, and tocotrienols in test oils (mg/50 g test oil)

Components	PNO	RBO low γ-oryzanol	RBO high γ-oryzanol
γ-Oryzanol*	n. d.	50	800
Total 4-desmethylsterols	113	453	740
β-Sitosterol	64	211	323
Campesterol	17	80	166
Stigmasterol	10	59	96
$^{\Delta 7}$-Stigmastenol	1	17	23
$^{\Delta 5}$-Avenasterol	12	13	16
$^{\Delta 7}$-Avenasterol	2	15	16
$^{\Delta 7}$-Campesterol	1	12	12
Campestanol	1	6	20
Sitostanol	1	7	19
$^{\Delta 5,23}$-Stigmastadienol	1	9	12
$^{\Delta 5,24}$-Stigmastadienol	1	7	8
Clerosterol	1	4	7
Cholesterol	trace	3	4
Brassicasterol	trace	1	1
24-Methylenecholesterol	1	4	5
Other sterols	n. d.	5	12
Total tocopherols	15.0	16.0	17.0
α-Tocopherol	10.0	11.0	12.0
β-Tocopherol	n. d.	0.5	0.6
γ-Tocopherol	5.4	4.0	4.4
Total tocotrienols	n. d.	30.0	33.0
α-Tocotrienol	n. d.	8.1	9.0
γ-Tocotrienol	n. d.	21.0	23.0
δ-Tocotrienol	n. d.	0.7	0.9

*γ-oryzanol was shown to contain 4,4′-dimethylsterol ferulates (33 % cycloartenyl ferulate, 27 % 24-methylenecycloartanyl ferulate and 2 % cyclobranyl ferulate) as well as 4-desmethylsterol ferulates (24 % campesteryl ferulate, 11 % β-sitosteryl ferulate and 2 % stigmasteryl ferulate). Ferulated 4-desmethylsterols are de-ferulated during analysis and thereby included in total 4-desmethylsterols. *Trace* at the limit of detection and not quantifiable

tal energy intake, and replaced half of habitual fat intake.

Participants consumed continental-style breakfast and dinner at home (of their own choosings), and a choice of provided lunches at the Nestlé Research Center or Orbe Product Technology Center/Factory. Other than some specific restrictions (see Inclusion and Expulsion criteria), the dietary intake was not controlled. Breakfast was provided to subjects following blood draws at the above centers. Food vehicles were prepared several times throughout the course of the study as needed; *e.g.,* yogurts were prepared 3 times. Vehicles were coded.

Food vehicles were distributed weekly (with adjustments to accommodate short vacations), and subjects were weighed at the same time. Participants were instructed: not to heat or cook with the vehicles; to keep the yogurt, spread, and shake refrigerated; not to refrigerate the salad oil, to avoid any possible precipitation problems; and not to place the vegetable oil near bright light or sun light.

Analysis of test oils

Fatty acids in test oils were analyzed as their methyl esters by gas chromatography [4]. Fatty acid profiles at the *sn*–1,3 and *sn*–2 positions were determined with IUPAC standard method 2.210. Phytosterols were determined after Biedermann et al. [5], and validated [8].

γ-Oryzanol composition was determined by HPLC with a LaChrom acquisition system and D-7000 HPLC system software (E. Merck, Darmstadt, Germany; Hitashi Ltd, Tokio, Japan). The HPLC pre-column was a 5 µm, 4 mm × 4 mm Licospher 100 RP-18 column (E. Merck). The HPLC column was a 5 µm, 250 mm × 4 mm TSK gel ODS-80Tm column (TosoHaas, Stuttgart, Germany). The mobile phase was acetonitrile:MeOH:2-propanol (50:45:5, v/v/v) run under reverse phase con-

Table 3 Food vehicle combinations used to deliver test oils during the 45 d trial

	Days									
Food Item	1	2	3	4	5	6	7	8	9	10
Spread	0	0	0	1	1	0	1	0	0	1
Shake (2 varieties)	3	2	2	1	3	2	2	3	1	3
Yogurt	0	1	2	1	1	1	1	1	2	1
Salad oil	2	1	0	1	1	2	1	0	1	0
PNO or RBO/d (g)	52.2	47.1	52.7	42.4	63.8	57.1	53.1	47.8	52.0	53.8
Energy from vehicles (kJ)	2185	2084	2460	1833	2787	2460	2310	2185	2360	2410

Shown are 10 combinations used during the first 10 study days. Values above the bold line represent number of servings of each food item consumed each day. The bottom values show daily oil consumption and energy from the food items. The vehicle combination on day 11 corresponded to that of day 1, etc. There were 5 patterns of daily vehicle consumption. Shakes were consumed at breakfast, and 1–2 before or after lunch and dinner; salads were consumed at lunch; yogurts were consumed at breakfast or dinner; and spreads were consumed at breakfast

ditions isocratically for 50 min at 1 ml/min, with 325 nm UV detection.

Peaks were identified by LC-MS with a Waters (Millford, MA, USA) HPLC system (757 autosampler, 600-MS pump, 486-MS UV detector). Column and chromatographic conditions were identical to that used for HPLC. The mass spectrometer was a Thermo Finnigan TSQ 700 triple quadrupole mass spectrometer (San Jose, CA, USA) equipped with APCI source. The vaporizer and transfer capillary were heated at 450 and 200 °C, respectively. Ion production was increased with a 5 µA corona discharge. Mass spectra were acquired in positive mode via scanning from 100–800 Da in 1 s. γ-Oryzanol composition was confirmed with GC/MS, following collection of the HPLC fraction containing ferulate derivatives, hydrolysis in ethanolic NaOH, hexane extraction, and trimethylsilyl derivatization [50]. An HP 5890 GC equipped with J&W Sci D-1701 column (30 m, 0.32 mm i. d., film thickness 0.25 µm) was connected to a Finnigan SSQ-7000 mass spectrometer. Temperature programming was 60 °C (1 min), then 5 °C/min to 280 °C (held 10 min). Mass spectra were acquired from 100–800 Da after 70 eV electron impact ionization. Tocopherols and tocotrienols were measured after Bertoli et al. [4].

Determination of total and lipoprotein cholesterol, lipoprotein subclasses and Lp(a)

Venous blood was collected and plasma separated by centrifugation (1500 g, 10 min, 4 °C). TC and triacylglycerol (TAG) were determined enzymatically on fresh plasma using a Cobas Mira Chemistry Analyzer (Hoffmann-La Roche, Basel, Switzerland), with Sigma kit 352 (Fluka Chemie AG, Buchs, Switzerland). Plasma for other analysis was stored at –80 °C.

Lipoproteins were separated with size-exclusion HPLC [25]. Briefly, 20 µl plasma, diluted 1:1 (v/v) with Dulbecco's phosphate buffered saline (pH 7.4, containing 0.02 % sodium azide; PBS), was injected onto a Superose 6HR FPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). Lipoproteins were eluted with 0.6 ml/min PBS. Cholesterol was determined with a post-column reactor, consisting of a mixing coil in a 40 °C water bath and HPLC pump to deliver cholesterol reagent (CHOD-PAP, Roche Diagnostics, Rotkreuz, Switzerland) at 0.1 ml/min. Absorbance was recorded at 500 nm (Waters absorbance detector 484) and cholesterol concentrations calculated from these ratios and TC concentration. TC, HDL-C, LDL-C, and TAG were also determined enzymatically with manual commercial kits for comparative purposes. The overall interpretation of the lipoprotein and TAG findings was the same with the Cobas, manual, or HPLC methods; for simplicity HPLC values are discussed herein.

Proton nuclear magnetic resonance spectroscopy (Lipomed Inc, Raleigh, NC, USA) was used to quantify 16 lipoprotein subclasses of VLDL, LDL, and HDL in 5 plasma samples on R_{30} and R_{45}. LDL particle concentration, average VLDL, LDL, and HDL particle size and cholesterol content were measured. Selected plasma samples had maximal differences in R_{45} LDL-C, following low and high γ-oryzanol RBO feeding.

Plasma Lp(a) was measured with a 1-step sandwich latex enhanced immunoassay using monospecific polyclonal anti-apo(a) antibodies (Immuno GmbH, Heidelberg, Germany). Turbidity changes were detected nephelometrically on a Cobas Mira Chemistry Analyzer, according to Immuno's instructions.

Measurement of cholesterol biosynthesis

Cholesterol biosynthesis was assessed using deuterium stable isotopes [16]. Results are reported as fractional synthesis rate (FSR), percent newly synthesized cholesterol in the cholesterol pool.

Statistical analysis

The statistical hypothesis was that high γ-oryzanol RBO would lower cholesterol relative to low γ-oryzanol. A statistically significant lowering was > 0.6 mmol/l. A 2-tailed, homoscedastic, unpaired *t*-test verified TC values were not statistically significantly different for subjects destined to consume low and high γ-oryzanol RBOs on P_1 (Table 4; $p > 0.05$). Subjects were not informed of their dietary oil treatment, but RBO taste was difficult to mask; thus the study was likely not fully blinded. There were no differences in taste between the two RBO types.

To determine sample size, we assumed starting TC = 5.9 mmol/l, minimal cholesterol reduction = 10 % ($\Delta = 0.6$; TC = 5.3 at 4 wks), COV = 8 % of mean (thus SD = 0.45) [70], $\beta = 0.1$ (1-sided), and $\alpha = 0.05$ (2-sided). Minimum sample size was 28 for a paired, parallel design, 14 per group; 32 starting subjects was considered ideal to allow for outliers, drop-outs, and disqualifiers.

Additional specific statistical tests employed were paired and unpaired, homoscedastic 2-tailed *t*-tests to evaluate group and time effects. Correlation matrices

Table 4 Characteristics of subjects on the first day of the study (mean ± SD)

Characteristic	All (n = 30)	RBO low γ-oryzanol (n = 15)	RBO high γ-oryzanol (n = 15)
Age (y)	47.8 ± 6.5	47.3 ± 6.0	48.4 ± 7.2
Body weight (kg)	78.9 ± 9.7	80.2 ± 10.1	77.7 ± 9.5
Height (m)	1.75 ± 0.08	1.75 ± 0.08	1.74 ± 0.09
BMI (kg/m^2)	25.9 ± 2.6	26.2 ± 2.6	25.6 ± 2.7
Total cholesterol (mmol/l)	6.13 ± 0.73	6.17 ± 0.69	6.09 ± 0.79

Based on P_1 TC, subjects were split on P_{15} into low and high γ-oryzanol groups

evaluated links between lipoprotein parameters, and compared Cobas and HPLC methods for quantifying lipoproteins (data not shown). Box plots evaluated outliers (data not shown). F-tests evaluated differences in variance for the two RBO treatment groups. The above analyses were performed with Statview® software (Albacus Concepts, Berkeley, CA, USA). All results refer to a *P* value of 0.05.

Results

Chemical composition of test oils

Fatty acids in test oils were very similar, with $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$ being the major fatty acids (Table 1). Regiospecific distribution of fatty acids was also very similar for the three test oils. These oils differed in their unsaponifiable composition (Table 2). Free and esterified 4-desmethylsterols increased in the order PNO < low γ-oryzanol RBO < high γ-oryzanol RBO. The chemical composition of γ-oryzanol is shown in Table 2.

Baseline characteristics of subjects

On P_1, mean age, body weight, height, BMI and TC did not statistically differ between low and high γ-oryzanol RBO groups (Table 4). Weight and BMI remained constant during the study.

Dietary compliance and food vehicle acceptance

There were no dropouts and expulsions during the 6 wk experiment. Compliance was excellent with very few missed vehicle servings. In the daily logs, no serious adverse effects were reported. PNO or RBO containing food vehicles were generally well accepted, with no objections to smell, taste or aftertaste. With respect to smoking, during the 45 d experiment, there were 5 people who smoked 20–896 cigarettes (20, 86, 133, 393, and 896 cigarettes); the rest were non-smokers.

Plasma lipid profiles and cholesterol synthesis

Total-, VLDL-, LDL- and HDL-C and TAG were assayed on P_1, P_{15}, R_{30} and $R_{43–45}$. For these parameters there were no statistically significant differences ($P > 0.10$, two tailed unpaired *t*-tests; $n = 15$ for all measurements, except $n = 12$ for Lp(a) measurements) between the two RBO types at any time points (Table 5).

These two groups were thus statistically combined (Table 6). TC decreased 4.3 % during the screening period prior to P_1, 4.8 % after 2 wks PNO feeding (P_1 *vs.* P_{15}), and 9 % after 2 wks RBO feeding (P_{15} *vs.* R_{30}). These decreases in TC were however not statistically different for PNO and RBO (statistical evaluation of P_{15}-P_1 *vs.* R_{30}-P_{15}). TC increased slightly (2.9 %) during the final 2 wks feeding RBO, relative to R_{30} baseline.

LDL-C did not change during PNO supplementation, but decreased 11.9 % after 2 wks RBO feeding (P_{15} *vs.* R_{30}), and was not further reduced in the remaining 2 wks RBO feeding. HDL-C decreased slightly during the first 2 wks PNO and RBO feeding, but the changes were not statistically significant. HDL levels were restored to P_1 values by $R_{43–45}$. The LDL-C/HDL-C ratio was unchanged during PNO feeding, but decreased significantly during both RBO feeding periods, reaching 18.9 % below P_{15} baseline on $R_{43–45}$. Specifically, the ratio decreased 10.5 % relative to P_{15} baseline, on R_{30}; and by 9.4 % relative to R_{30} baseline, on $R_{43–45}$. VLDL-C was a minor lipoprotein particle that decreased most noticeably during the final 2 wks RBO feeding.

Lp(a) was analyzed on P_1, P_{15} and R_{45}. Only subjects displaying "normal" Lp(a) values less than 1.0 mmol/l were analyzed at these time points (6 subjects in P_1, and 3 subjects each in the low and high γ-oryzanol RBO groups were excluded. Plasma Lp(a) concentrations increased with PNO but not RBO feeding.

There were no significant differences in 16 lipoprotein subclasses of VLDL, LDL, and HDL, LDL particle concentration, average VLDL, LDL, and HDL particle size and cholesterol content in selected low and high γ-oryzanol RBO samples after 2 and 4 wks. Although the sample size of 5 per group was not adequate, it is unlikely γ-oryzanol had/has striking effects on the measured parameters. PNO and RBO periods were not compared.

Plasma TAG was significantly decreased 28.2 % with PNO feeding, whereas RBO feeding increased TAG 16.5 % after 2 wks, relative to P_{15} baseline. After 4 wks RBO feeding, TAG remained below P_1 values, and was not statistically significantly different from P_{15} values.

The statistical variance was greater for several cholesterol parameters with high as compared to low γ-oryzanol RBO (Table 7).

FSR on $R_{43–45}$ was not statistically significantly different for the low and high γ-oryzanol RBO groups, averaging 6.01 ± 0.41 ($n = 12$) and 6.26 ± 0.68 ($n = 11$), respectively. Several samples in each group of 15 were however lost due to a technical problem.

Discussion

Decrease in total and LDL-cholesterol

PNO is generally considered a healthy oil due to its monounsaturated and polyunsaturated fatty acid content [17]. The 4.8 % decrease in TC observed with 2 wks PNO

Table 5 Plasma cholesterol parameters, low and high γ-oryzanol RBOs separate (mean mmol/l ± SD)

Cholesterol parameter	TC	LDL-C	HDL-C	LDL-C/HDL-C	VLDL-C	Lp(a)	TAG
R_{30} Low γ-oryzanol RBO	5.24 ± 0.48	3.46 ± 0.48	1.60 ± 0.31	2.25 ± 0.56	0.15 ± 0.09	0.19 ± 0.14	1.35 ± 0.54
R_{30} High γ-oryzanol RBO	5.38 ± 0.82	3.59 ± 0.97	1.58 ± 0.40	2.49 ± 1.01	0.20 ± 0.08	0.18 ± 0.13	1.73 ± 0.80
$R_{43–45}$ Low γ-oryzanol RBO	5.42 ± 0.56	3.54 ± 0.55	1.75 ± 0.29	2.09 ± 0.50	0.07 ± 0.05	0.23 ± 0.15	1.24 ± 0.50
$R_{43–45}$ High γ-oryzanol RBO	5.52 ± 0.77	3.61 ± 0.98	1.79 ± 0.48	2.21 ± 0.89	0.09 ± 0.08	0.25 ± 0.18	1.59 ± 0.76
$P_{15}–R_{30}$ Low γ-oryzanol RBO	0.64 ± 0.50	0.57 ± 0.42	0.10 ± 0.50	0.28 ± 0.50	0.00 ± 0.10	0.04 ± 0.05	−0.25 ± 0.34
$P_{15}–R_{30}$ High γ-oryzanol RBO	0.41 ± 0.54	0.39 ± 0.47	0.02 ± 0.38	0.28 ± 0.84	0.00 ± 0.17	0.07 ± 0.06	−0.19 ± 0.39
$R_{30}–R_{43–45}$ Low γ-oryzanol RBO	−0.17 ± 0.35	−0.08 ± 0.39	−0.15 ± 0.13	0.17 ± 0.32	0.07 ± 0.07	−0.03 ± 0.03	0.11 ± 0.24
$R_{30}–R_{43–45}$ High γ-oryzanol RBO	−0.13 ± 0.28	−0.02 ± 0.26	−0.22 ± 0.15	0.28 ± 0.36	0.11 ± 0.08	−0.07 ± 0.07	0.14 ± 0.46
$P_{15}–R_{43–45}$ Low γ-oryzanol RBO	0.47 ± 0.46	0.48 ± 0.33	−0.05 ± 0.51	0.45 ± 0.47	0.07 ± 0.05	0.01 ± 0.03	−0.14 ± 0.33
$P_{15}–R_{43–45}$ High γ-oryzanol RBO	0.27 ± 0.46	0.36 ± 0.46	−0.20 ± 0.39	0.56 ± 0.83	0.11 ± 0.15	0.01 ± 0.06	−0.05 ± 0.45

P_{15} experimental day 15, PNO feeding, and the first day of RBO feeding; *R_{30}* day 30, and RBO feeding; *$R_{43–45}$* the average value for days 43–45. There were no significant differences between subjects consuming low and high-γ-oryzanol RBO at each time point, nor for the difference of any two time points ($P > 0.05$, unpaired, homoscedastic 2-tailed t-tests)

Table 6 Plasma cholesterol parameters, low and high γ-oryzanol RBOs combined (mean mmol/l ± SD)

Day	TC	LDL-C	HDL-C	LDL-C/HDL-C	VLDL-C	Lp(a)	TAG
P_1	6.13 ± 0.73^{a}	4.15 ± 0.75^{a}	1.75 ± 0.44^{ab}	2.54 ± 0.79^{a}	0.22 ± 0.19^{a}	0.18 ± 0.11^{ac}	1.84 ± 1.12^{a}
P_{15}	5.84 ± 0.70^{b}	4.00 ± 0.70^{a}	1.64 ± 0.49^{ab}	2.65 ± 0.89^{a}	0.18 ± 0.13^{ab}	0.24 ± 0.16^{b}	1.32 ± 0.61^{b}
R_{30}	5.31 ± 0.66^{c}	3.52 ± 0.76^{bc}	1.59 ± 0.35^{a}	2.37 ± 0.81^{b}	0.18 ± 0.10^{b}	0.18 ± 0.13^{a}	1.54 ± 0.70^{c}
$R_{43–45}$	5.47 ± 0.66^{d}	3.58 ± 0.78^{c}	1.77 ± 0.39^{b}	2.15 ± 0.71^{c}	0.08 ± 0.06^{c}	0.24 ± 0.16^{bc}	1.42 ± 0.66^{bc}

Values having a common superscript are not statistically significant ($P > 0.05$, ANOVA repeated measures and two tailed paired t-tests; $n = 30$ for all measurements, except $n = 24$ for Lp(a) measurements) . *P_1* and *P_{15}* experimental days 1 and 15, and PNO feeding; *R_{30}* day 30, and RBO feeding; and *$R_{43–45}$* the average value for days 43–45

Table 7 Variances for selected parameters

Group	TC R_{30}	TC R_{45}	LDL-C R_{30}	LDL-C R_{45}	HDL-C R_{30}	HDL-C R_{45}	LDL-C/HDL-C R_{30}	LDL-C/HDL-C R_{45}
Low γ-oryzanol RBO	0.231^{a}	0.315^{a}	0.229^{a}	0.301^{a}	0.098^{a}	0.081^{a}	2.252^{a}	2.084^{a}
High γ-oryzanol RBO	0.666^{b}	0.586^{a}	0.942^{b}	0.953^{b}	0.161^{a}	0.231^{b}	2.486^{b}	2.206^{b}

Shown are selected parameters that showed large differences in variance, evaluated using Bartlett's homogeneity of variances test. Groups having a common superscript are not statistically significant from one another ($P < 0.05$). *R_{30}* and *R_{45}* refer to day 30 and 45, respectively, and RBO feeding

feeding was likely due to replacement of saturated cholesterol-raising fats in the habitual diet at P_1, with the mono- and di-unsaturated fatty acids in PNO [17]. Other contributing factors were likely restrictions on specific fatty foods (see inclusion and expulsion criteria sections for details), and healthy dietary modifications made prior to P_1 during the recruitment period. These latter factors may also have contributed to TAG lowering with PNO feeding.

Although the PNO period was a run-in period, some interesting comparisons between 2 wks feeding PNO and RBO are notable. Both oils lowered TC statistically equivalently (4.8–9 %). RBO was likely more effective at lowering LDL-C than PNO. PNO lowered LDL-C only 3.7 % (which was not statistically significant), whereas RBO lowered LDL-C 11.9 % after 2 wks. Furthermore, the LDL-C/HDL-C ratio was unchanged during PNO feeding, but significantly decreased during both RBO feeding periods, reaching 18.9 % below P_{15} baseline on $R_{43–45}$. The inability of RBO to lower LDL-C further between R_{30} and $R_{43–45}$ may indicate compensatory mechanisms (such as increased cholesterol synthesis with both RBO types) preventing RBO from further lowering LDL-C.

In other human clinical trials, 50–65 g RBO/d, also lowered LDL-C [37, 47, 54, 67, 68], but γ-oryzanol quantity and oil characterization were not disclosed. Schwab et al. [54] found 20 en% RBO lowered LDL-C relative to beef tallow, but was not more effective than canola, corn, and olive oils. This is explainable: canola oil has lower levels of saturated cholesterol-raising fatty acids; and corn oil has higher levels of cholesterol-lowering

polyunsaturated fatty acids, than RBO. In primates, 20en% RBO lowered LDL-C equivalently to canola and corn oils [78]. Administering human subjects 12 en% RBO for 8 wks, providing 0.6 g γ-oryzanol/d, decreased LDL-C relative to an oil mixture with similar fatty acid profile as RBO [79]. Full fat rice bran lowered LDL-C more effectively than rice starch suggesting RBO components had cholesterol lowering properties [13]. Overall, there is LDL-C lowering potential for RBO, but γ-oryzanol's influence is not clear.

Similar to our results, Lichtenstein et al. [30] found LDL-C lowering potential for RBO low in γ-oryzanol, but high in non-ferulated plant sterols. Hypercholesterolemic subjects received canola, corn and olive oils for 32 d. Each 60 g daily RBO provided 30 mg γ-oryzanol, 1 g plant sterols and 30 mg tocotrienols. RBO lowered LDL-C equivalently to corn and canola oils (which typically have a more favorable fatty acid profile for lowering LDL-C), but more than olive oil, demonstrating the cholesterol lowering potential for RBO low in γ-oryzanol, but containing moderate amounts of plant sterols.

Changes in HDL-cholesterol

There was not strong evidence that RBO could raise HDL-C in the present study with only a hint of HDL-C raising potential during the final 2 wks RBO feeding. In rodents, RBO has been reported to raise HDL-C [44, 55, 56, 59, 66], but this can be artifactual. Rodents carry most cholesterol in HDL unless large amounts of bile acid and dietary cholesterol are provided [49].

Changes in LDL-cholesterol/HDL-cholesterol ratio

LDL-C/HDL-C ratio is a reliable marker for coronary heart disease, a low ratio being desirable [83]. RBO feeding significantly lowered the LDL-C/HDL-C ratio by 19% over 4 wks, being equally effective after each 2 wk period. This is a clear indication that RBO could be beneficial for maintaining a healthy plasma cholesterol distribution.

Lipoprotein (a)

Lp(a) is an apolipoprotein (a)-LDL complex, and a drug/nutrient-responsive cardiovascular disease risk factor [1]. Surprisingly, saturated fat lowers plasma Lp(a) [15]. Replacement of saturated fats in the basal diet with PNO (P_1 vs P_{14}) could have the opposite, observed effect of increasing Lp(a). During PNO and RBO feeding, equivalent amounts of saturated fats were likely provided, without affecting Lp(a). Effects of fatty acids, and non-saponifiable components in vegetable oils, on Lp(a) levels is poorly understood and merits further investigation.

Cholesterol lowering properties of γ-oryzanol and 4-desmethylsterols in RBO

Isolated γ-oryzanol lowers LDL-C in animals [58] and humans [53, 81]; however, these results cannot be directly extrapolated to RBO containing γ-oryzanol as one of several possible interacting bioactive components.

Intact γ-oryzanol isolated from RBO (containing predominately 4,4'-dimethylsterols esterified to ferulic acid) is likely less effective than 4-desmethylsterols (isolated from various sources) at reducing LDL-C, in animal [21] and clinical trials [73, 77]. 4,4'-Dimethylsterols from non-RBO sources have also been found to be largely ineffective at lowering cholesterol [19, 21, 29, 62, 71, 73, 77]. Corn husk oil contains half of total sterols as ferulated 4-desmethylsterols. Since it is an effective cholesterol lowering agent, this suggests de-ferulated 4-desmethylsterols in RBO will also inhibit cholesterol absorption [48, 78].

Assuming all ferulated sterols are deferulated in the gut, low γ-oryzanol RBO provided 453 mg/d 4-desmethylsterols, a dose below that known to consistently lower LDL-C, but established to inhibit cholesterol absorption [38–40, 63]. The dose of 740 mg/d 4-desmethylsterols provided by the high γ-oryzanol RBO diet is similar to that previously established to lower LDL-C [3, 19, 32, 33, 42, 62, 72, 74].

The greater statistical variance in LDL-C, HDL-C and other cholesterol parameters observed with high- compared to low γ-oryzanol RBO feeding is curious (Table 7). Greater variance with high γ-oryzanol RBO was not due to some subjects trending to more LDL-C lowering with high- vs. low γ-oryzanol RBO.

Taken together, the 4-desmethylsterol RBO components are likely responsible for the observed cholesterol lowering, although other bioactive components in RBO and synergistic interactions with 4,4'-dimethylsterols could play roles. γ-Oryzanol may interact with 4-desmethylsterols to enhance cholesterol lowering [28], and with specific triacylglycerol molecular species [65].

Why is γ-oryzanol less effective at lowering LDL-C than 4-desmethylsterols?

For sterols to effectively displace cholesterol from micelles and inhibit cholesterol absorption, free sterols must be released via intestinal cholesterol esterase [9, 12, 69]. The extent of hydrolysis of γ-oryzanol is not clear [12, 20]; nor is it known whether all ferulated 4-desmethylsterols hydrolyze identically [51]. Low side

chain substitution in γ-oryzanol's 4,4'-dimethylsterols could make these sterols more absorbable [21, 71], and less able to inhibit cholesterol absorption relative to 4-desmethylsterols [18, 73]. The two extra methyl groups at C_4, a methyl group at C_{14}, and an extra cyclopropyl ring at C_{9-10} in 4,4'-dimethylsterols make these molecules less structurally similar to cholesterol than 4-desmethylsterols, and perhaps less able to compete with cholesterol for mixed micelle incorporation during digestion [73].

Bioactive components in RBO other than γ-oryzanol

Unsaturated fatty acids in PNO and RBO can lower LDL-C independently of plant sterols, but in our view, it is unlikely that fatty acids alone were responsible for the observed LDL-C lowering with RBO. First, following hydrolysis, free 4-desmethylsterols in RBO and other sources are established LDL-C lowering components, particularly at levels provided in the high γ-oryzanol RBO. Second, PNO, providing similar fatty acids as RBO, did not lower LDL-C significantly, whereas RBO did. Since there was no washout between PNO and RBO periods, one could still argue that fatty acids acted in a sustained manner to lower LDL-C. Applying equations of Yu et al. [82] and assuming our supplement replaced 4500 fat calories typical of a high fat diet [10], LDL-C would fall about 6 %. This is half of the ca. 12 % LDL-C reduction observed with RBO, arguing sterols did contribute to LDL-C lowering.

Tocotrienols and modified tocotrienols are reported to have cholesterol lowering potential [46] via effects on HMGCoA reductase [41]. While PNO did not contain tocotrienols, both our RBOs contained substantial quantities of γ- and α-tocotrienols (21–23, and 8.1–9.0 mg/50 g RBO, respectively). γ-Tocotrienol may have potent cholesterol lowering properties [45], but α-tocotrienol may attenuate its actions. Thus, the cholesterol lowering and synergistic [31] potential of tocotrienols in our RBOs is uncertain.

Ferulic acid may independently lower cholesterol [55, 57, 64]. As free ferulic acid can be absorbed by humans [6], ferulic acid from γ-oryzanol hydrolysis could lower cholesterol, and have antioxidant properties [76]. Supporting this hypothesis is the fact that the related molecule diferuloylmethane (curcumin, the major yellow pigment in turmeric) has reported to have cholesterol lowering and antioxidant properties [2, 36, 55].

In summary, RBO lowered LDL-C and LDL-C/HDL-C even when it provided only 50 and 453 mg/d γ-oryzanol and intestinally hydrolyzed (free) 4-desmethylsterols, respectively. Increasing daily dosage of γ-oryzanol and intestinally hydrolyzed (free) 4-desmethylsterols to 800 and 740 mg/d, respectively did not enhance the LDL-C and LDL-C/HDL-C ratio lowering potential. The presence of γ-oryzanol in RBO may still be important for preventing product oxidative damage and preserving vitamin E and tocotrienol levels before consumption and during heating and frying operations [26, 35], and possibly for preventing *in vivo* oxidation through its ferulate moiety [24, 27].

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