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Combined effect of *n*-3 fatty acids and phytosterol esters on alleviating hepatic steatosis in non-alcoholic fatty liver disease subjects: a double-blind placebo-controlled clinical trial

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Abstract

The aim of this study was to investigate the combined effect of *n*-3 fatty acids (EPA and DHA, at an EPA:DHA ratio of 150:500) and phytosterol esters (PS) on non-alcoholic fatty liver disease (NAFLD) patients. We conducted a randomised, double-blind, placebo-controlled trial. Ninety-six NAFLD subjects were randomly assigned to the following groups: the PS group (receiving 3·3 g/d PS); the FO group (receiving 450 mg EPA + 1500 mg DHA/d); the PS + FO combination group (receiving 3·3 g/d PS and 450 mg EPA + 1500 mg DHA/d) and the PO group (a placebo group). The baseline clinical characteristics of the four groups were similar. The primary outcome was liver:spleen attenuation ratio (L:S ratio). The percentage increase in liver–spleen attenuation (≤1) in the PS + FO group was 36 % ($P = 0{\cdot}083$), higher than those in the other three groups (PS group, 11 %, $P = 0{\cdot}519$; FO group, 18 %, $P = 0{\cdot}071$; PO group, 15 %, $P = 0{\cdot}436$). Compared with baseline, transforming growth factor-β (TGF-β) was significantly decreased in the three study groups at the end of the trial (PS, $P = 0{\cdot}000$; FO, $P = 0{\cdot}002$; PS + FO, $P = 0{\cdot}001$) and TNF-α was significantly decreased in the FO group ($P = 0{\cdot}036$), PS + FO group ($P = 0{\cdot}005$) and PO group ($P = 0{\cdot}032$) at the end of the intervention. Notably, TGF-β was reduced significantly more in the PS + FO group than in the PO group ($P = 0{\cdot}032$). The TAG and total cholesterol levels of the PS + FO group were reduced by 11·57 and 9·55 %, respectively. In conclusion, co-supplementation of PS and EPA + DHA could increase the effectiveness of treatment for hepatic steatosis.

Key words: **Phytosterol esters: *n*-3 Fatty acids: Non-alcoholic fatty liver disease: Clinical trials**

With the prevalence of obesity and its associated metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease and threatens human health worldwide[1]. The reported prevalence of NAFLD is 30 % in Western countries and 12–24 % in Asia[2]. NAFLD comprises a spectrum of liver damage that ranges from simple steatosis to non-alcoholic steatohepatitis and from fibrosis to cirrhosis, end-stage liver disease and occasionally hepatocellular carcinoma; therefore, NAFLD has been recognised as a major burden on public health.

Diet and physical exercise are considered the first line of treatment for patients with NAFLD, but there is contradictory evidence regarding the therapeutic efficacy of these measures, and compliance is poor[3]. Despite an abundance of clinical trials, NAFLD therapy remains a challenge for the scientific community, and there are no licenced therapies for NAFLD[4]. New pharmacological approaches are urgently needed.

n-3 Long-chain PUFA, especially EPA (C20 : 5*n*-3) and DHA (C22 : 6*n*-3), are safe dietary supplements that have shown efficacy in the prevention and therapy of NAFLD, effectively lowering abnormal levels of TAG by controlling key pathways involved in hepatic lipid metabolism through the regulation of transcription factors (i.e. PPARα, PPARγ, sterol regulatory element-binding protein 1 and carbohydrate-responsive element-binding protein)[5–7]. Despite available studies that provide encouraging data about the efficacy of *n*-3 PUFA as a treatment for NAFLD in humans,

Abbreviations: CT, computerised tomography; FO, fish oil capsules 450 mg EPA + 1500 mg DHA; NAFLD, non-alcoholic fatty liver disease; PO, placebo; PS, phytosterol esters; TC, total cholesterol; TGF-β, transforming growth factor-β.

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different trial designs, intervention durations, supplementation compositions and supplementation concentrations have yielded various results regarding the efficacy of PUFA in the treatment of NAFLD.

Plant sterols and plant stanols are natural dietary ingredients that are well known to interfere with intestinal cholesterol absorption. Daily intake of plant sterols or stanols is generally accepted to lower serum cholesterol concentrations by up to 10 %[8]. Our previous animal experimental study showed that intake of phytosterol esters (PS) could effectively decrease the levels of hepatic TAG, total cholesterol (TC) and NEFA; down-regulate the expression levels of transforming growth factor-β (TGF-β), TNF-α and uncoupling protein 2; and up-regulate the expression of liver X receptor-α. These variables are involved in the development of liver fibrosis, hepatic inflammation, lipid metabolism and energy homoeostasis. In addition, PS could also regulate the expression of PPARα and PPARγ, which are treatment targets for NAFLD therapy[9,10].

Therefore, in the present study, we designed a randomised, double-blind, placebo-controlled clinical trial to evaluate whether combined supplementation with PS (3·3 g/d, equal to 2·5 g phytosterol in the free form/d) and *n*-3 PUFA (450 mg EPA + 1500 mg DHA) could effectively ameliorate liver steatosis in NAFLD patients.

Materials and methods

Trial registration, ethical issues and informed consent process

This randomised, double-blind, placebo-controlled parallel trial was registered in the Chinese Clinical Trial Registry (http://www.chictr.org.cn; registration number: ChiCTR1800014419; date of registration: 12 January 2018; date of enrolment of the first participant to the trial: 21 January 2018). Additionally, the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of The First People's Hospital of Ningyang County, Tai'an City, Shandong Province (LL20170001). The investigators provided all related information at a level of complexity that was understandable to subjects. All participants provided written informed consent before participation. Each group was randomly assigned a predetermined number of participants, who then received the intended treatment. Randomisation was conducted using computer-generated random numbers by a trained staff member at the physical examination centre of The First People's Hospital of Ningyang County, Tai'an City, Shandong Province; all experimenters and subjects were blinded to the group allocations during the study.

Inclusion criteria for the clinical trial

This study recruited adult participants (aged 30–67 years) who had an average liver:spleen attenuation ratio of ≤1·2 or liver attenuation ≤52 Hounsfield units (HU), as determined by unenhanced computerised tomography (CT) in the present study[11], and were dyslipidaemic (TC ≥ 5·2 mmol/l, LDL-cholesterol ≥ 3·36 mmol/l or TAG ≥ 1·7 mmol/l) or overweight (BMI ≥ 24 kg/m^2).

Exclusion criteria for the clinical trial

The following exclusion criteria for the trial, partially derived from a previous study[12], were applied in the present study: pregnancy, CVD, cancer, disability, diabetes mellitus, excessive alcohol consumption (≥30 g/d for men and ≥20 g/d for women), hepatitis B or C or other liver diseases, use of hypoglycaemic or lipid-regulating drugs (statins, fibrates) or other drugs that may impact glucose and lipid metabolism and intolerable adverse events from soyamilk products. The other exclusion factors were diseases that impact the participant's metabolism or ability to participate in this study, for example, hyperthyroidism, mental disorder, or diseases associated with serious dysfunction of the heart, liver or kidney. All subjects underwent comprehensive physical examinations and routine biochemical analyses of blood.

Study design

A total of 103 subjects of Han Chinese origin who were diagnosed with fatty liver by ultrasound at the Physical Examination Center of The First People's Hospital of Ningyang County were recruited for this study. The diagnosis of participants was verified with unenhanced CT before stratified randomisation, and the primary outcome was the liver:spleen attenuation ratio (L:S). Seven participants failed to meet the inclusion criteria. Ninety-six subjects were enrolled and randomly assigned to three study groups and a control group, with stratified randomisation according to the average L:S. Each day the participants in the PS alone group received PS-enriched soyamilk powder containing 3·3 g of PS (Vegapure 67 WDP, equivalent to 2·5 g phytosterol in the free form) and placebo capsules (vegetable oil blend: 30 % palmitic acid, 40 % oleic acid and 20 % linoleic acid obtained by blending rapeseed, sunflower and palm oils); the participants in the FO alone group received fish oil capsules containing highly concentrated EPA and DHA (PronovaPure 150:500TG, 450 mg EPA + 1500 mg DHA) and placebo soyamilk powder; the PS + FO group received PS-enriched soyamilk powder containing 3·3 g of PS plus fish oil capsules containing highly concentrated EPA and DHA (450 mg EPA + 1500 mg DHA); the PO group received the placebo soyamilk and placebo capsules. The intervention lasted for 12 weeks.

According to a previous study, the increasing extent of the L:S in the placebo group was 0·06 from baseline to the end of treatment[13]. We expected the L:S of the combined treatment group to be 0·25 with a standard deviation of 0·20. A sample size of nineteen per group was calculated as sufficient to achieve 80 % power to reject the null hypothesis. With an assumed drop-out rate of 20 %, the sample size for each group was twenty-four (as shown in Fig. 1).

By the end of the intervention, three patients in the control group, three in the FO group, eight in the PS group and seven in the PS + FO group withdrew from the study because of business trips and/or poor compliance. Therefore, seventy-five people participated in the entire trial, and the numbers of participants in the PO, FO, PS and PS + FO group were 21, 21, 16 and 17, respectively (as shown in Fig. 1).

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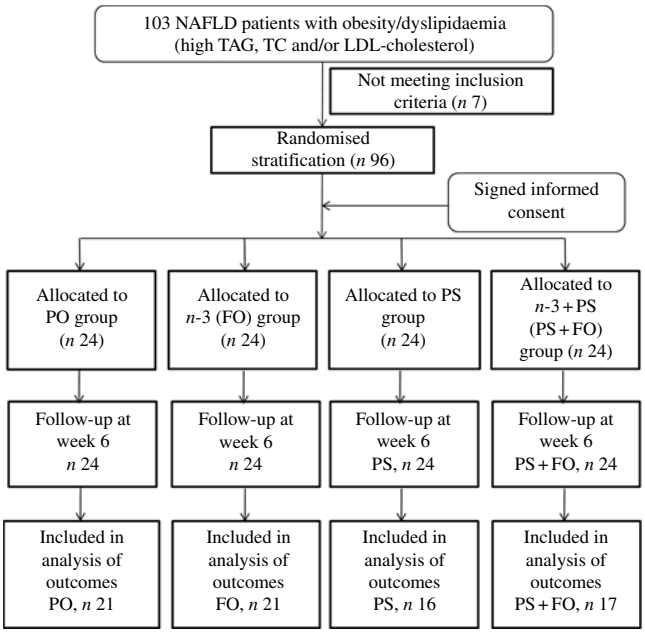


Fig. 1. CONSORT flow diagram. NAFLD, non-alcoholic fatty liver disease; TC, total cholesterol; PO, placebo; PS, phytosterol esters; FO, fish oil capsules 450 mg EPA + 1500 mg DHA.

All containers of soyamilk powder were similar in shape and size, and the same was true of the capsules. The fish oil capsules, PS-fortified soyamilk powder and the corresponding placebo samples were supplied by Yanlin Natural Hygigen Sdn. Bhd.

Bottles were given to the participants at the beginning and in the middle of the intervention period. Compliance was assessed by having the participants return the used containers at every visit and by measuring serum concentrations of EPA + DHA at the end of the trial. During the 12 weeks of intervention in this trial, no dietary intervention management was performed in the interest of achieving high compliance and isolating the effect of the supplements we used in the trial. The participants were not encouraged to specifically modify their lifestyles (including dietary habits) but were instructed to refrain from consuming supplements or other products claimed to reduce blood cholesterol. All participants, care providers and outcome assessors were blinded to the treatment allocations.

Anthropometric and biochemical measurements

Anthropometric, biochemical and CT scanning data were collected at baseline (the initiation of the trial) and after 12 weeks (the end of the trial).

BMI was calculated as body weight in kg divided by the square of height in m. Waist circumference was measured at the midpoint between the inferior costal margin and the superior border of the iliac crest on the mid-axillary line. Hip circumference was measured at the fullest part of the buttocks. Blood samples were drawn after an overnight (8–10 h) fast and immediately centrifuged. Serum TC, TAG, HDL-cholesterol, LDL-cholesterol, glucose, liver function markers (alkaline phosphatase, alanine aminotransferase, γ-glutamyl transferase, aspartate aminotransferase) and kidney function markers (uric acid, creatinine) were determined using a Hitachi 7600 analyser (Hitachi, Ltd).

Computerised tomography examination

Unenhanced CT scanning was performed at the beginning and end of the trial by the same doctor. The liver:spleen attenuation ratio was measured with non-enhanced scans (SOMATOM Definition AS, Siemens). Considering the regional characteristics of hepatic steatosis, the person performing the CT scan selected three points on the right lobe of the liver, two points on the left and two points on caudate lobes of the liver.

Cytokine determination

The serum levels of TGF-β (cat. no.: ELH-TGFb1; Ray Biotech; 1:20 dilution) and TNF-α (Procartaplex™ Multiplex Immunoassay; Thermo Fisher) in all patients from the three study groups and the control group were retrospectively measured at the beginning and end of the trial.

Serum DHA and EPA concentration analysis

Sample preparation. The sample preparation for the detection of NEFA in serum followed the method of Browse *et al.* with some modifications[14]. Briefly, 100 µl of serum, 10 µl of C19 : 0 methyl ester internal standard solution (1 mg/ml) and 1 ml of 5 % sulphuric acid–methanol were mixed by vortexing. The N_2 was purged for 10 s, and the vessel was sealed and reacted at 80°C for 90 min and then cooled down for 10 min at 4°C. Water (2 ml) and n-hexane (2 ml) were sequentially added and mixed for 3 min, stood for 30 min and centrifuged (2600 rpm) for 10 min to obtain the fatty acid methyl esters. The organic phases were evaporated to dryness under N_2 gas and dissolved into n-hexane (100 µl), and 2 µl for GC analysis was used.

GC analysis condition. GC analysis was performed using a GC-2010 gas chromatograph (Shimadzu). Separation was performed on a DB-5 ms capillary column (30 m × 0·25 mm × 0·25 µm). N_2 was used as the carrier gas at a flow rate of 1 ml/min. The temperature of the injector was 270°C and the split ratio was 1:1. The following temperature programme was employed: (1) 70°C for 5 min; (2) increase to 200°C at 25°C/min and (3) increase to 240°C at 2°C/min. A flame ionisation detector was used at a temperature of 320 °C.

Statistical analysis

All parameters are expressed as mean values and standard deviations. Analysis was performed with the statistical software package SPSS (SPSS, 20.0). All parameter changes within each group during the study were evaluated using paired *t* tests. Among-group comparisons of the rates of the changes in all parameters from baseline to 12 weeks were statistically analysed by one-way ANOVA, followed by least significant difference multiple comparison tests. Patients with abnormal TAG, TC and/or LDL-cholesterol were defined as a subgroup and analysed in the same way. Patients whose liver:spleen attenuation ratio was ≤1·0

and the mean value of minimum ratio of each group were also defined as subgroups and analysed in the same way. The criterion for significance was $P < 0{\cdot}05$.

Results

Baseline characteristics

In this trial, the average ages of patients in the PO, FO, PS and PS + FO groups ranged from 44 to 47 years. The groups were similar with respect to their sex ratios and baseline biochemical characteristics (including BMI, waist:hip ratio, blood pressure, blood glucose, blood lipids, liver function, kidney function and degree of hepatic steatosis; Table 1), and the percentages of subjects with abnormal characteristics at baseline were also similar among all groups (online Supplementary Table S1). After 12 weeks of intervention, BMI did not exhibit a significant change within any group compared with the beginning of the trial and minor reduction after the trial was similar among the four groups (Table 2).

Blood lipids

The TAG levels of the FO and PS + FO groups showed downward trends over the course of the trial (Table 2), while the TAG levels in the PO and PS groups showed upward trends over the same period. The TAG level of the PS + FO group was reduced by 11·57 %, which was significantly higher than those of the PO ($P < 0{\cdot}05$) and PS groups ($P < 0{\cdot}01$) and was the largest reduction among the groups (Fig. 2(a)). The TC levels of the FO, PO and PS + FO groups showed downward trends over the course of the intervention (Table 2). The TC level of the PS + FO group was reduced by 9·55 %, which was significantly higher than that of the phytosterol intervention alone group ($P < 0{\cdot}05$) and was the largest reduction among the groups (Fig. 2(b)). In addition, compared with baseline, the LDL-cholesterol levels of

Table 1. Comparison of baseline characteristics, biochemical results and lipid profiles of the groups (*n* 75)
(Mean values and standard deviations; numbers and percentages; minimum, median and maximum values)

	Control group: PO (*n* 21)		Study groups: FO (*n* 21)		PS (*n* 16)		PS + FO (*n* 17)	
Baseline characteristics	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Sex								
Female								
n	3		2		2		1	
%	14		10		12		6	
Male								
n	18		19		14		16	
%	86		90		88		94	
Age (years)	47	6	46	5	46	8	44	7
BMI (kg/m^2)	27·92	2·39	29·43	3·42	29·20	4·45	27·80	2·58
WHR	0·92	004	0·92	0·04	0·93	0·04	0·92	0·06
SBP (mmHg)	146·90	17·97	134·65	11·22	149·60	18·10	146·00	18·92
DBP (mmHg)	91·28	11·18	91·52	9·52	95·18	15·42	95·12	14·34
FPG (mmol/l)	6·16	1·83	5·87	1·16	6·06	1·93	6·41	2·38
TC (mmol/l)	5·50	0·83	5·46	1·20	5·32	1·21	6·28	2·21
TAG (mmol/l)	2·11	1·42	4·43	5·84	1·74	0·87	3·86	6·21
HDL-cholesterol (mmol/l)	1·45	0·30	1·47	0·38	1·38	0·32	1·88	1·06
LDL-cholesterol (mmol/l)	3·63	0·79	3·21	0·88	3·54	0·96	3·64	0·89
ApoA	0·90	0·20	0·83	0·20	0·91	0·18	0·96	0·21
ApoB	0·97	0·18	0·82	0·25	0·97	0·20	0·90	0·29
ALP (U/l)	76·37	14·69	80·11	17·54	80·48	21·10	80·64	15·50
ALT (U/l)	34·99	11·33	41·35	24·01	42·91	23·35	39·80	20·12
GGT (U/l)	50·53	25·94	54·60	32·29	59·88	28·64	62·34	48·70
AST (U/l)	25·26	4·90	30·96	13·60	27·24	6·97	29·37	13·19
UA (μmol/l)	358·84	77·83	402·53	92·20	398·31	78·81	390·72	57·20
Cr (μmol/l)	101·08	11·29	80·11	17·53	100·33	9·48	99·47	10·60
Concentration of DHA (μg/ml)	431·53	136·31	482·44	311·63	384·76	114·14	506·63	416·63
Concentration of EPA (μg/ml)	124·21	69·19	154·47	198·36	116·21	32·17	151·65	91·60
Liver attenuation (HU)	48·63	3·56	45·30	4·45	47·23	4·68	45·80	4·30
Minimum L:S (mean of all subjects)	0·96	0·17	0·92	0·19	0·97	0·20	0·88	0·25
L:S (mean of all subjects)	1·08	0·15	1·05	0·18	1·08	0·21	1·01	0·27
Minimum	0·5		0·5		0·7		0·5	
Median	1·0		0·9		0·9		0·9	
Maximum	1·2		1·1		1·1		1·2	
L:S (mean of the subjects in the ≤1 subgroup)	0·85	0·15	0·87	0·13	0·92	0·11	0·74*†	0·19
Number of subjects with mean L:S ≤ 1								
n	8		9		5		7	
%	38		43		31		41	

PO, placebo; FO, fish oil capsules 450 mg EPA + 1500 mg DHA; PS, phytosterol esters; WHR, waist:hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; TC, total cholesterol; ALP, alkaline phosphatase; ALT, alanine aminotransferase; GGT, γ-glutamyl transferase; AST, aspartate aminotransferase; UA, uric acid; Cr, creatinine; HU, Hounsfield units; L:S, liver:spleen attenuation ratio.
* Mean value was significantly different from that of the FO group ($P<0{\cdot}05$).
† Mean value was significantly different from that of the PS group ($P<0{\cdot}05$).

Table 2. Changes in characteristics and biochemical parameters between baseline and the end of the 12-week intervention (*n* 75) (Mean values and standard deviations)

	Study groups											
	Control group: PO (*n* 21)			FO (*n* 21)			PS (*n* 16)			PS + FO (*n* 17)		
Characteristics or parameters	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*
ΔBMI (kg/m^2)	−0·35	1·60	0·527	−0·35	0·83	0·719	−0·42	0·85	0·790	−0·28	0·81	0·763
ΔWHR	0·007	0·041	0·527	0·001	0·039	0·851	−0·0121	0·035	0·417	0·004	0·039	0·803
ΔSBP (mmHg)	1·75	12·92	0·825	2·81	11·47	0·412	−1·23	11·37	0·804	2·23	17·74	0·746
ΔDBP (mmHg)	0·50	11·55	0·834	−2·09	7·04	0·469	−0·76	8·09	0·928	−1·00	10·62	0·833
ΔTAG (mmol/l)	0·38	1·74	0·397	−1·49	4·69	0·283	1·18	2·84	0·171	−1·88	5·89	0·248
ΔTC (mmol/l)	−0·23	0·78	0·414	−0·15	0·62	0·643	0·11	0·92	0·772	−0·79	1·59	0·212
ΔLDL-cholesterol (mmol/l)	−0·23	0·54	0·370	0·06	0·54	0·787	−0·14	0·56	0·659	−0·18	0·79	0·553
ΔHDL-cholesterol (mmol/l)	−0·02	0·23	0·794	−0·02	0·18	0·842	0·16	0·30	0·155	−0·23	0·77	0·416
ΔALT (U/l)	−2·89	15·16	0·536	−3·06	17·06	0·650	−5·63	16·98	0·487	−0·53	12·76	0·939
ΔGGT (U/l)	−0·23	21·63	0·981	1·75	13·73	0·864	−2·74	19·87	0·705	−6·78	8·15	0·677
ΔAST (U/l)	0·25	7·19	0·884	−3·22	7·54	0·356	−0·18	6·74	0·942	−1·12	11·65	0·775
ΔUA (μmol/l)	−19·03	44·05	0·418	−15·85	46·87	0·571	−8·49	54·74	0·761	−8·11	50·94	0·674
ΔLiver attenuation (HU)	0·87	3·50	0·756	1·31	6·81	0·590	−2·57	6·50	0·279	1·75	9·93	0·633
ΔMinimum L:S (mean of all subjects)	0·10	0·13	0·121	0·08	0·17	0·163	0·04	0·19	0·449	0·16	0·19	0·090
ΔL:S (mean of the all subjects)	0·09	0·16	0·093	0·07	0·19	0·188	0·04	0·19	0·546	0·14	0·25	0·155
ΔL:S (mean of the subjects in the ≤1 subgroup)	0·11	0·17	0·436	0·14	0·20	0·071	0·07	0·17	0·519	0·21	0·31	0·083
ΔTGF-β (ng/ml)	−5·94	11·89	0·068	−10·00*	12·16	0·002	−14·52*	12·34	0·000	−13·11*	11·66	0·001
ΔTNF-α (pg/ml)	−0·48*	0·33	0·032	−0·43*	0·49	0·036	−0·30	0·42	0·102	−0·58*	0·36	0·005
ΔConcentration of DHA (μg/ml)	83·10	171·39	0·101	435·11*	286·31	0·000	57·67	84·67	0·234	594·02*	620·47	0·001
ΔConcentration of EPA (μg/ml)	16·71	80·27	0·456	199·85*	219·01	0·002	18·75	48·88	0·270	257·65*	225·89	0·000

PO, placebo; FO, fish oil capsules 450 mg EPA + 1500 mg DHA; PS, phytosterol esters; Δ, the index value after intervention – the index value at baseline; WHR, waist:hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; ALT, alanine aminotransferase; GGT, γ-glutamyl transferase; AST, aspartate aminotransferase; UA, uric acid; HU, Hounsfield units; L:S, liver:spleen attenuation ratio; TGF-β, transforming growth factor-β.
* $P < 0{\cdot}05$ *v.* baseline within group.

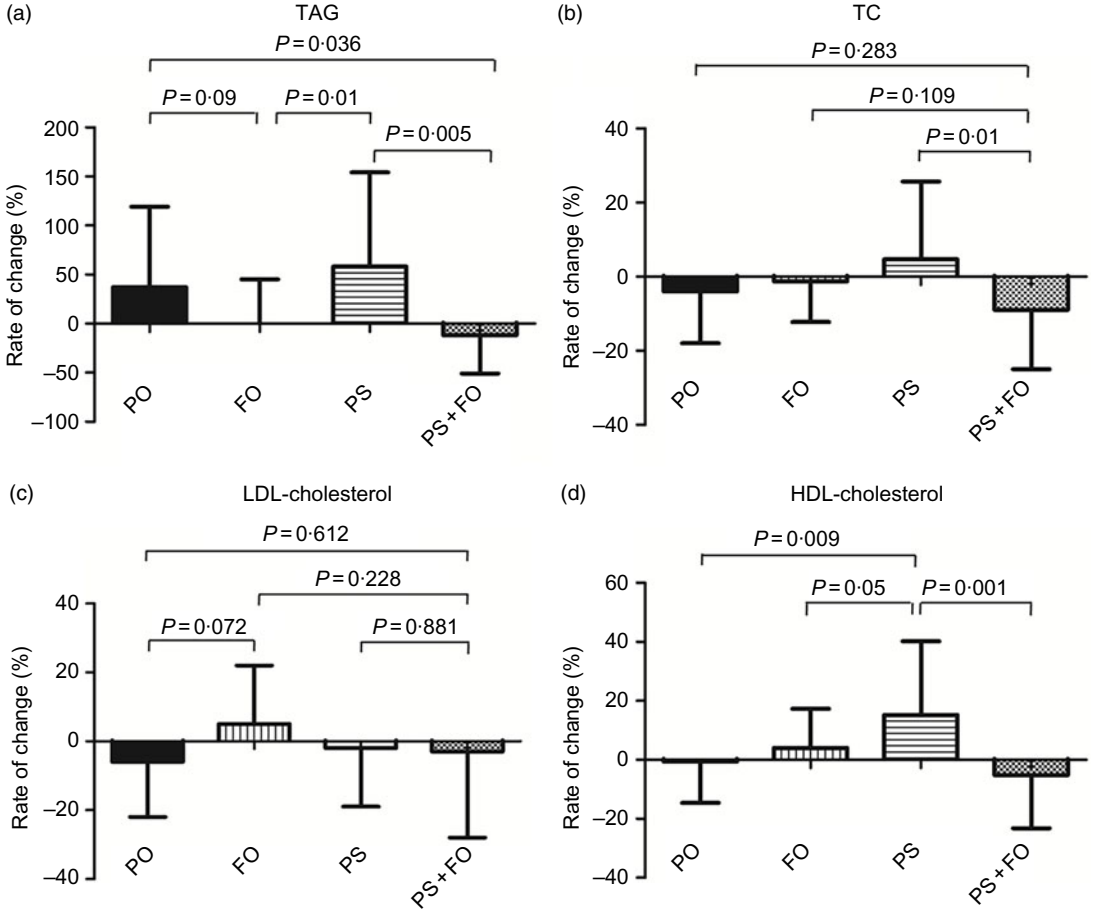


Fig. 2. Percentage changes in the TAG, total cholesterol (TC), LDL-cholesterol and HDL-cholesterol levels of each group after 12 weeks of intervention. (a)–(d) Lipid profiles of all participants in each group. Values are means, with standard deviations represented by vertical bars. PO, placebo; PS, phytosterol esters; FO, fish oil capsules 450 mg EPA + 1500 mg DHA.

the PS, PO and PS + FO groups showed downward trends over the trial (Table 2). However, the LDL-cholesterol level of the PO group decreased more than that of the other groups (Fig. 2(c)). The percentage increase in HDL-cholesterol in the PS group was significantly higher than that in the PO ($P < 0·01$), FO ($P = 0·05$) or PS + FO ($P < 0·01$) groups (Fig. 2(d)).

Moreover, we analysed the changes in the blood lipid profiles of the subgroups in which all the subjects exhibited hyperlipidaemia, and the trend in the reduction rates of lipid profiles was similar to the average trend for all subjects in each group (online Supplementary Fig. S1A–D).

Cytokines

Serum levels of TNF-α and TGF-β were retrospectively measured in all patients at the beginning and end of the trial. Compared with the beginning of the trial, the TGF-β levels of the PS, FO and PS + FO groups were significantly decreased ($P < 0·01$); the TNF-α levels of the PO, FO and PS + FO groups were significantly decreased ($P < 0·01$) at the end of the intervention (Table 2).

Notably, the percentage decrease in TGF-β in the PS + FO group was significantly higher than that in the PO group ($P < 0·05$) (Fig. 3(a)). TNF-α was reduced by slightly more in the PS + FO group than in the other three groups ($P = 0·072$ *v.* PS group, $P = 0·318$ *v.* FO group, $P = 0·511$ *v.* PO group) (Fig. 3(b)).

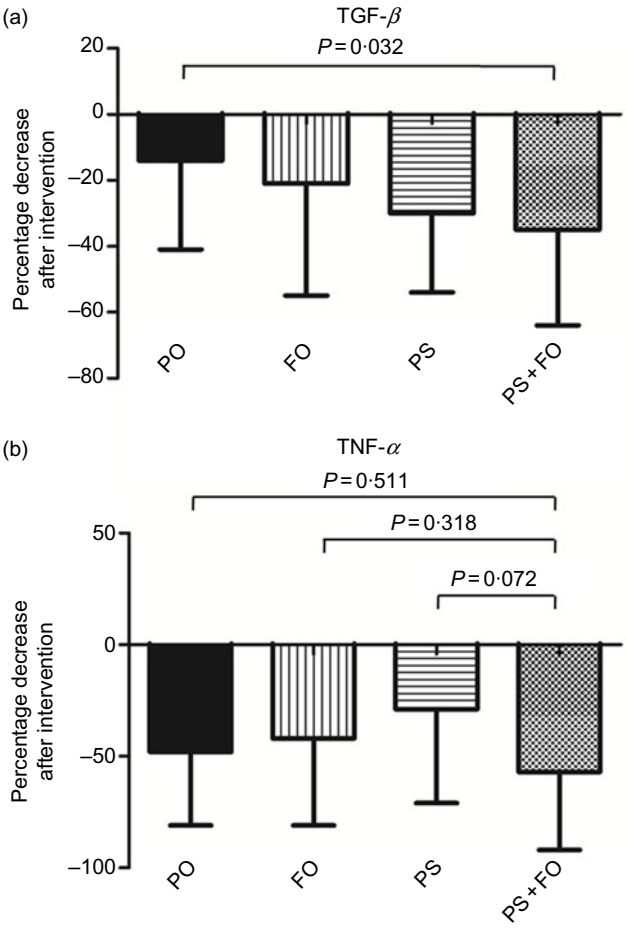


Fig. 3. Reduction rates of the transforming growth factor β (TGF-β) (a) and TNF-α (b) levels of the study and control groups. Values are means, with standard deviations represented by vertical bars. PO, placebo; PS, phytosterol esters; FO, fish oil capsules 450 mg EPA + 1500 mg DHA.

Hepatic steatosis measured by unenhanced computerised tomography

As shown in Table 1, the mean liver attenuation ranged from 45 to 48 Hounsfield units, the average L:S ranged from 1·01 to 1·08 and the mean of minimum L:S of all subjects ranged from 0·88 to 0·97 in each group at the beginning of the trial.

After 12 weeks of intervention, patients in the PS + FO group showed more obvious improvement compared with baseline: the Δmean minimum L:S, the Δmean L:S across all CT scanning sites, the Δmean L:S in the subgroup with a baseline value of less than 1 and the Δliver attenuation value were 0·16 (SD 0·19), 0·14 (SD 0·25), 0·21 (SD 0·31) and 1·75 (SD 9·93), respectively, and these changes were larger than those in the PO, FO and PS groups (Table 2). The typical characteristics and changes in the CT scan results of a patient from the PS + FO group are shown in Fig. 4(e) and (f). The average L:S was increased from 0·57 (SD 0·11) at baseline to 1·11 (SD 0·14) after 12 weeks of intervention.

The percentage increases in the average L:S, minimum L:S across all CT scanning sites, the L:S in the subgroup with a baseline value of less than 1 and the liver attenuation (Hounsfield units) in the PS + FO group were 19, 24, 36 and 9·6 %, respectively (Fig. 4(a)–(d)); the above indexes in the PS + FO group were higher than those in other two study groups and the PO group, which were as follows: PS group, 4 %; FO group, 9 %; PO group, 10 % (Fig. 4(a)); PS group, 6 %; FO group, 11 %; PO group, 12 % (Fig. 4(b)); PS group, 8 %; FO group, 18 %; PO group, 16 % (Fig. 4(c)); PS group, −4·6 %; FO group, 4·8 %; PO group, 2·9 % (Fig. 4(d)). However, the difference among the groups was not significant, probably because of the high SD and small sample size. The above-mentioned data imply that the combination of PS and EPA + DHA may effectively improve hepatic steatosis in NAFLD, especially for those patients whose L:S is less than 1.

Transaminases

The patients we recruited in the present study exhibited no significant differences in the levels of alanine aminotransferase, γ-glutamyl transferase or aspartate aminotransferase at baseline or after 12 weeks of intervention within/among groups, and the same was true in the subgroup with liver–spleen attenuation of less than 1 (Tables 1 and 2).

Compliance and adverse effects

In order to confirm subjects' compliance during the trial, this study determined the serum levels of DHA and EPA before and after intervention. Before the intervention, there was no significant intergroup difference in serum DHA or EPA concentrations ($P > 0·05$; shown in Table 1). After 12 weeks of intervention, the concentrations of DHA and EPA in the serum of the FO group and PS + FO group were significantly higher than that

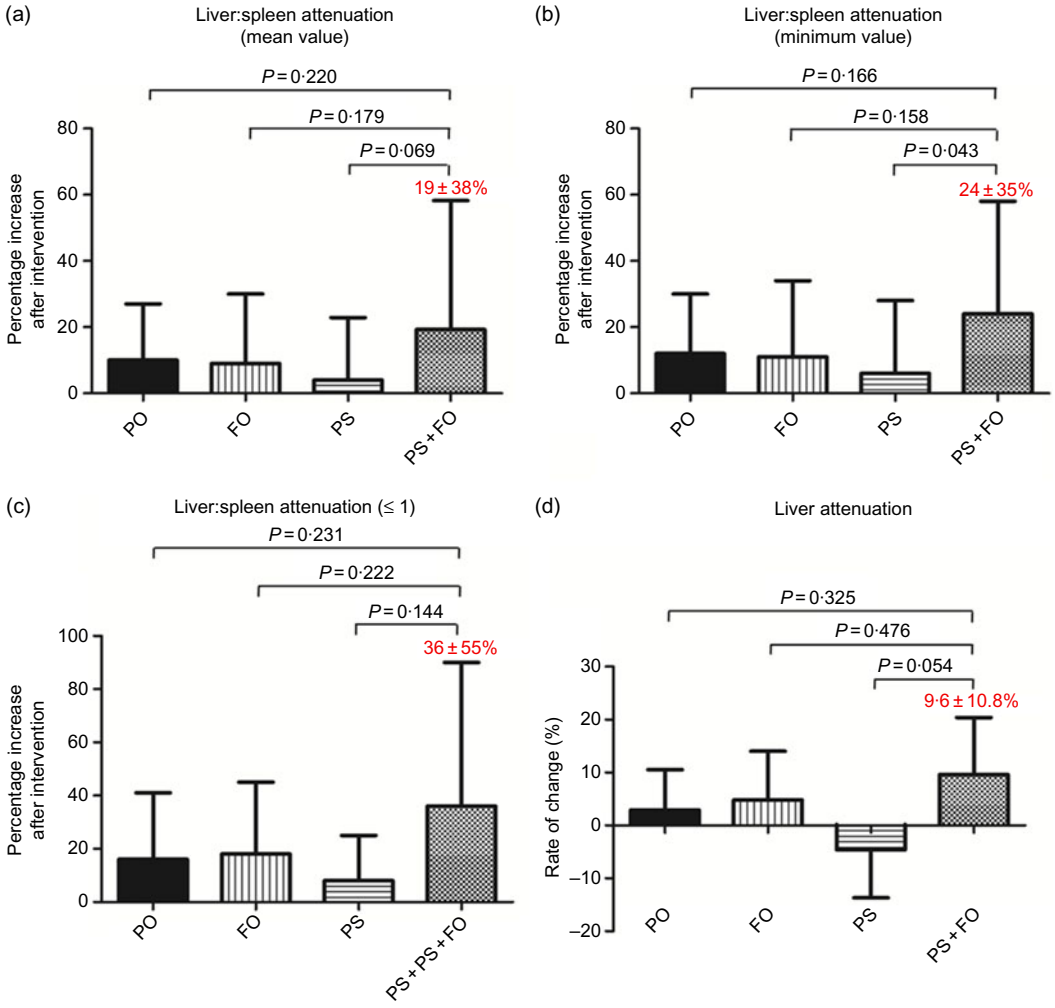


Fig. 4. Increase rates of the liver:spleen attenuation ratio and liver attenuation of the study and control groups. (a) Groupwise comparison of the percentage increases in the ratio of mean liver:spleen attenuation. (b) Groupwise comparison of the percentage increases in the ratio of minimum liver:spleen attenuation. (c) Groupwise comparison of the percentage increase in the ratio of liver:spleen attenuation within the subgroup whose baseline was less than 1. (d) Groupwise comparison of the mean liver attenuation value. (e) and (f) Representative unenhanced computerised tomography scans from participants in the PS + FO group before and after intervention. Values are means, with standard deviations represented by vertical bars. PO, placebo; PS, phytosterol esters; FO, fish oil capsules 450 mg EPA + 1500 mg DHA.

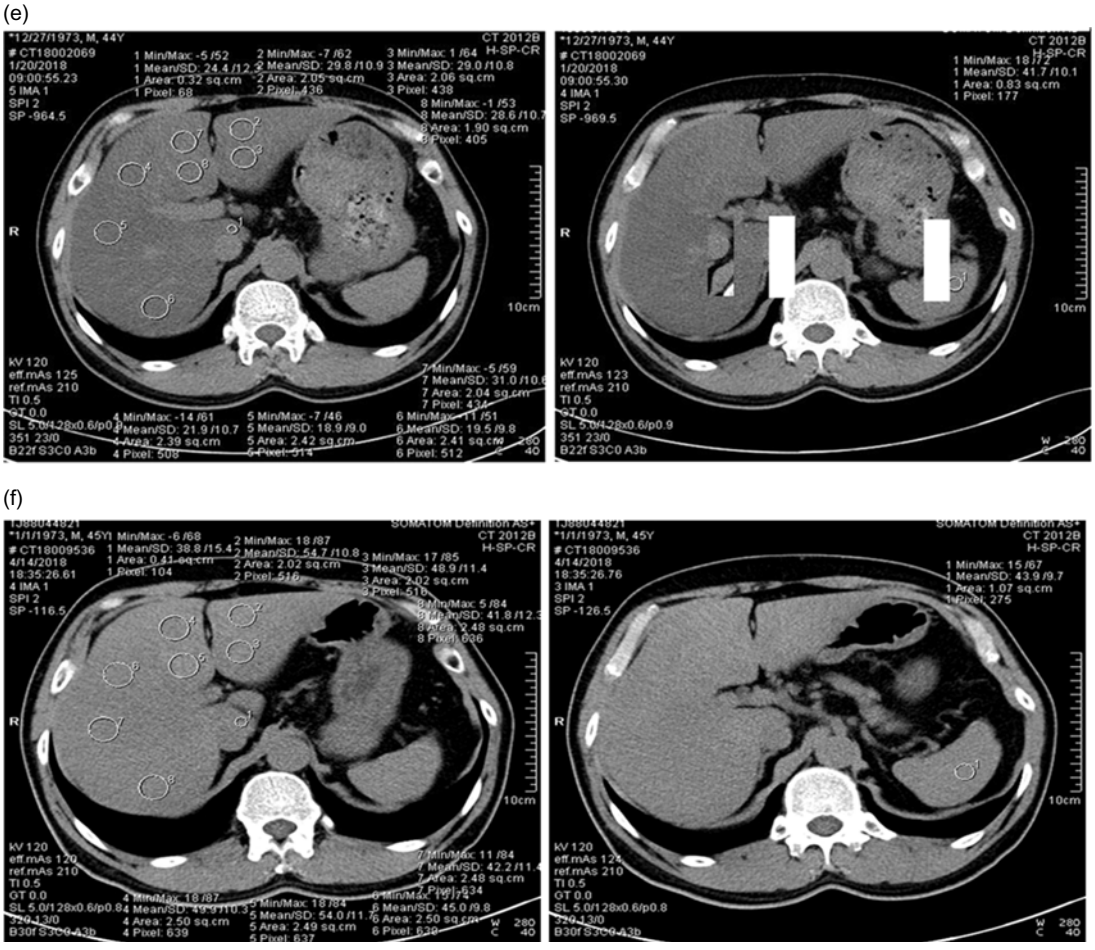
before the intervention ($P < 0{\cdot}001$) (Table 2). Compared with baseline data, the serum DHA and EPA concentrations in the FO group increased 1·14- and 2·22-fold, respectively, and the serum DHA and EPA concentrations in the PS + FO group increased 1·65- and 2·21-fold, respectively (Tables 1 and 2). The concentrations of DHA and EPA in the FO and PS + FO groups were significantly higher than those in the groups that did not consume EPA + DHA (the PS and PO groups) after 12 weeks of intervention ($P < 0{\cdot}001$) (Fig. 5(a)–(d)). These results suggest good compliance in this trial.

No other adverse clinical or laboratory events associated with the use of PS and/or *n*-3 fatty acids were observed or reported.

Discussion

NAFLD is often associated with other metabolic conditions, such as dyslipidaemia and visceral obesity, but it is highly non-specific. The subjects enrolled in this study exhibited a relatively low liver:spleen attenuation ratio (≤1·2) or liver attenuation ≤52 Hounsfield units; more than 85 % of the subjects had a BMI ≥ 24, and most of these subjects (71–94 %) also exhibited hyperlipidaemia. Some subjects exhibited hypertension or abnormal liver function. In the present study, a randomised, double-blind, placebo-controlled trial was conducted. It was found that co-supplementation of PS (3·3 g) and EPA (450 mg) + DHA (1500 mg) each day could effectively increase the liver:spleen attenuation ratio, ameliorate hepatic steatosis and decrease the serum level of TAG and TGF-β.

Weight reduction appears to be an effective therapy for NAFLD. In fact, a 5–10 % reduction in weight from baseline has been shown to significantly reduce hepatic steatosis in different studies[15,16]. A previous study showed that weight reduction was associated with improved hypertransaminasaemia and insulin resistance (IR) in NAFLD patients[17]. In the present study, the body weight of participants in all groups decreased by an average of only 1·1 % after 12 weeks of intervention. No significant changes were observed between or



	Spleen	SD	Right lobe of liver1	SD	Right lobe of liver2	SD	Right lobe of liver3	SD	Left lobe of liver1	SD	Left lobe of liver2	SD	Caudate lobe	SD
T0	41·7	10·1	21·9	10·7	18·9	9	19·5	9·8	29·8	10·9	29	10·8	24·4	12·3
T12	43·9	9·7	49·9	10·3	54	11·7	45	9·8	54·7	10·8	48·9	11·4	38·8	15·4

Fig. 4. Continued

within groups. Therefore, the apparent efficacy of the supplements in the present clinical trial is 'probably genuine'.

After 12 weeks' intervention, EPA + DHA supplementation could effectively decrease the level of TAG compared with baseline and with the PO and PS treatment groups. In particular, the decrease in TAG levels was strengthened following co-supplementation of *n*-3 PUFA and PS; co-supplementation of EPA + DHA and PS also led to the largest decrease in TC. These findings were consistent with a previous study that reported a regulatory effect of *n*-3 fatty acids on lipid metabolism[18]. The change in the lipid profile after treatment with EPA + DHA and PS was consistent with the CT results, in which the PS + FO intervention seemed to increase the liver: spleen attenuation ratio and liver attenuation value more effectively than the PS, FO and PO treatments. This improvement associated with PS and EPA + DHA co-supplementation was especially obvious in participants whose L:S was less than 1. It is well known that lipid overload plays a central role in the progression of hepatic steatosis. Liver TAG accumulation and hypercholesterolaemia are the main causes of NAFLD, and the evidence suggests a crucial role for cholesterol in hepatic inflammation[4,5]. The analysis of participant characteristics in the present study showed that hyperlipidaemic subjects accounted for 94 % of all participants in the PS + FO group, which made hyperlipidaemia the most common co-morbidity in NAFLD patients. This probably contribute to more beneficial effects we observed in the PS + FO group since co-supplementation could more effectively regulate lipid metabolism for hyperlipidaemia state. The synergetic regulatory effect and related mechanisms of phytosterol and *n*-3 fatty acids on lipid metabolism still need further exploration.

In addition, Parker *et al.*[19] found that *n*-3 PUFA (total daily dose: 1728 mg marine TAG, including 588 mg EPA and 412 mg DHA) did not appear to be an effective agent for reducing liver fat in overweight men. Our results may provide useful diet intervention strategy for the practical application of *n*-3 PUFA and phytosterol in overweight NAFLD subjects. However, further investigation is needed to determine the mechanisms of

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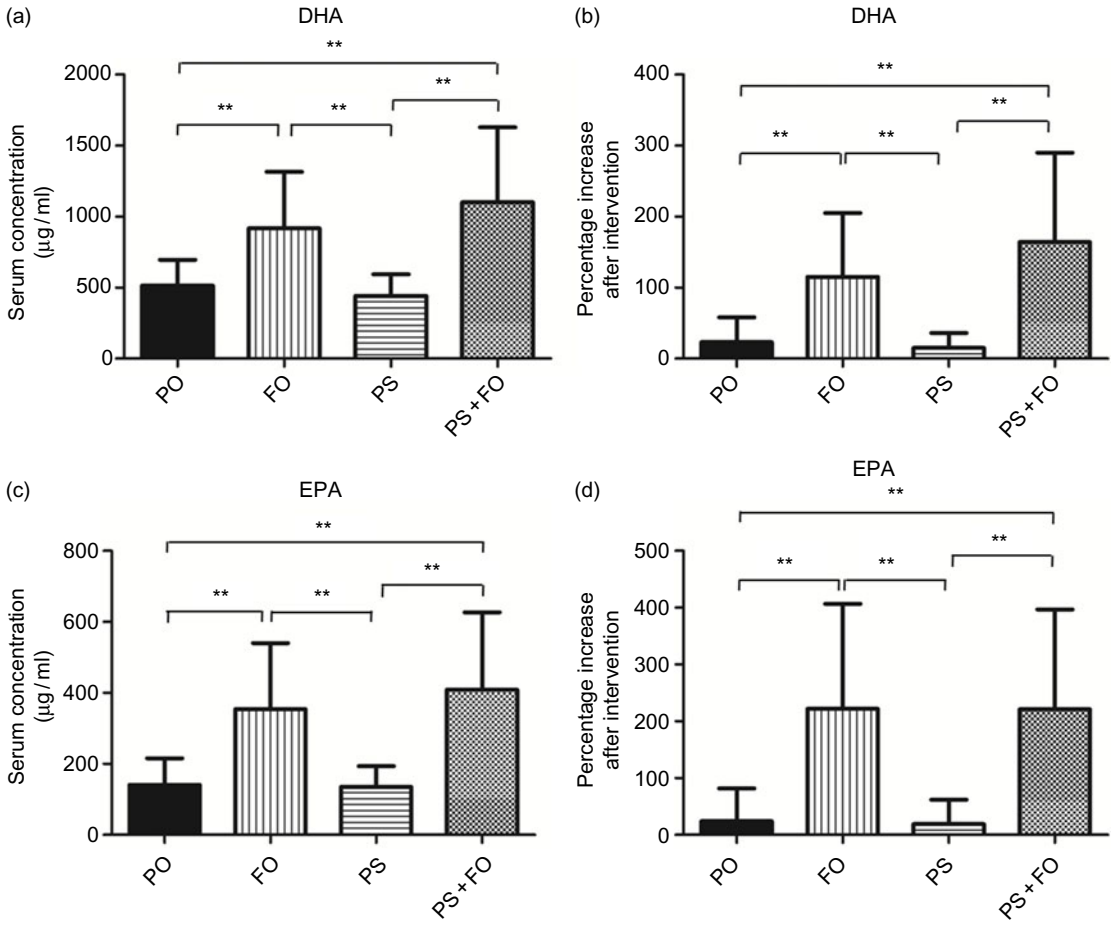

Fig. 5. Values of and increases in the serum DHA and EPA concentrations of the study and control groups. Values are means, with standard deviations represented by vertical bars. ** $P < 0{\cdot}01$. PO, placebo; PS, phytosterol esters; FO, fish oil capsules 450 mg EPA + 1500 mg DHA.

synergistic effects between phytosterol and *n*-3 PUFA on the alleviation of hepatic steatosis.

Furthermore, the abnormal serum cytokine levels of NAFLD patients may contribute to the progression of NAFLD to non-alcoholic steatohepatitis[20]. Among these abnormally expressed cytokines, TGF-β is a necessary cytokine for the induction of the fibrotic response and for the activation of the cancer stroma. Yang *et al.*[21] found that TGF-β signalling in hepatocytes contributes to hepatocyte death and lipid accumulation through Smad signalling and reactive oxygen species production, all of which promotes the development of non-alcoholic steatohepatitis. In addition to TGF-β, the pro-inflammatory cytokine TNF-α was found to promote IR directly or by inhibiting adiponectin[22]. In the present trial, we found that the serum levels of TGF-β in the three study groups (PS, FO and PS + FO) were significantly lower after 12 weeks of intervention than at the start of the trial. In particular, TGF-β was reduced significantly more in the PS + FO group than in the PO group (Fig. 3(a)). We also found that TNF-α levels were reduced slightly more in the PS + FO group than those in the other three groups (Fig. 3(b)). The above data implied that combination of phytosterol and EPA + DHA exhibited stronger anti-inflamatory activity.

In addition, we found that the serum concentrations of DHA and EPA in the PS + FO group were higher than those in the other three groups (Fig. 5(a) and (c)). Studies have shown that patients affected by NAFLD have high intakes of SFA and cholesterol[23,24]. The excessive ingestion of cholesterol has always been regarded as one of the risk factors for NAFLD[25,26]. Conversely, the consumption of PUFA from fish and flaxseed oils has beneficial effects on insulin sensitivity and promotes anti-inflammatory PG metabolism[27]. Other studies also showed that consumption of phytosterol-supplemented milk (1·6 g of plant sterols/250 ml of milk) induced a reduction in inflammatory molecules, and consumption of *n*-3-supplemented milk (131·25 mg EPA + 243·75 mg DHA/250 ml of milk) improved the lipid metabolic pathways in overweight healthy volunteers[28]. Another study showed that EPA could attenuate the progression of hepatic fibrosis in established steatohepatitis by inhibiting reactive oxygen species production[29]. Therefore, the excellent suppressive effect of PS + FO on TGF-β and TNF-α is probably attributable to the synergistic action of phytosterol and *n*-3 fatty acids and their comprehensive bioactivity profiles, which include the regulation of cholesterol and TAG metabolism as well as anti-inflammatory effects.

Unaccountably, some of the indexes (such as the levels of TC and LDL-cholesterol) were decreased in the PO group, while the liver:spleen attenuation ratio increased in the same group;

however, no significant differences in these metrics were observed between the PO group and any study group. Similar effects of placebo on some biochemical indexes of NAFLD were found in a previous study[30]. Therefore, a large population trial on the effect of co-supplementation with EPA, DHA and PS on NAFLD subjects is still needed.

In summary, the present trial showed that PS supplementation combined with EPA + DHA administration is more effective at reducing the levels of serum TAG, TC and LDL-cholesterol; decreasing the levels of inflammatory cytokines and reducing the severity of hepatic steatosis than treatment with PS alone, FO alone or placebo. The results of the present study require further verification in a trial with a large sample size, and the synergistic mechanisms of phytosterol and EPA + DHA still need further investigation.

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X. G. Z. generated the random allocation sequence, F. P. enrolled participants, and P. L. O. and Q. G. assigned participants to interventions and finished the detection of DHA and EPA analysis.

The authors declare no conflicts of interest.

Supplementary material

For supplementary materials referred to in this article, please visit https://doi.org/10.1017/S0007114520000495

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