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ORIGINAL CONTRIBUTION

The α′ subunit of β-conglycinin and the A1–5 subunits of glycinin are not essential for many hypolipidemic actions of dietary soy proteins in rats

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Abstract
Purpose This study examined the effects of dietary soy protein (SP) lacking different storage protein subunits and isoflavones (ISF) on the abdominal fat, blood lipids, thyroid hormones, and enzymatic activities in rats.
Methods Weanling Sprague–Dawley rats (8 males and 8 females/group) were fed diets containing either 20 % casein without or with supplemental isoflavones or alcohol-washed SP isolate or SP concentrates (SPC) prepared from 6 different soy bean lines for 8 weeks.
Results Feeding of diets containing SPC regardless of their subunit compositions significantly lowered relative liver weights, blood total, free, and LDL cholesterol in both genders ($P < 0.05$) and also reduced serum free fatty acids (FFA) and abdominal fat in females ($P < 0.05$) compared to the casein or casein + ISF diets. Dietary SPC significantly elevated the plasma free triiodothyronine (T3) in both genders and total T3 in females compared to the casein diet ($P < 0.05$). The SPC lacking β-conglycinin α′ and either the glycinin A1–3 or A1–5 subunits increased total T3 in males and reduced plasma enzymatic activities of creatine kinase and lactate dehydrogenase compared to casein or casein + ISF diet ($P < 0.05$).
Conclusions Soy isoflavones were mainly responsible for the hypocholesterolemic effects and increased plasma free T3, whereas reduction in FFA, abdominal fat, liver weight and increased plasma total T3 were the effects of the soy proteins. Neither the α′ subunit of β-conglycinin nor the A1–5 subunits of glycinin are essential for the hypolipidemic properties of soy proteins.

Keywords Soy protein · Isoflavones · Lipid profiles · Thyroid hormones · Rats

Abbreviations

ALP	Alkaline phosphatase
CK	Creatine kinase
FFA	Free fatty acids
LDH	Lactate dehydrogenase
SP	Soy protein
SPI	Soy protein isolate
SPC	Soy protein concentrate
T3	Triiodothyronine
T4	Thyroxine

Introduction

Soy consumption is associated with many health benefits such as reduced risk for cardiovascular diseases, type 2 diabetes, atherosclerosis, and non-alcoholic fatty liver diseases [1–5]. The hypolipidemic functions of soybean components have been demonstrated in human clinical trials [1, 6] and various animal studies [7–9], as well as cultured cells [10, 11]. Soy proteins (SP) and associated

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isoflavones (ISF) are the most studied soy components which are believed to have lipid-lowering properties. Studies in monkeys [12] and mildly hypercholesterolemic human subjects [13] have shown that isoflavones play an important role in the reduction of blood cholesterols. However, an increasing body of evidence tends to support a major role of SP in promoting the lipid-lowering effects observed in both animals and humans [3, 14–17].

Soybean seeds contain approximately 36–38 % protein on a dry-weight basis. Glycinin, an 11S globulin, and β-conglycinin, a 7S globulin, are the two major storage proteins, which account for ~40 and ~20 % of the total proteins, respectively. β-conglycinin is composed of three subunits, α′, α, and β. Glycinin consists of six subunits, each made up of an acidic (A) and a basic (B) polypeptide component. Glycinin and β-conglycinin differ in their amino acid composition. For example, β-conglycinin contains a low level of methionine (0.61 %), whereas glycinin has relatively higher sulfur-containing amino acids (methionine, 2.23 %; cysteine, 1.83 %) [18, 19]. As a result, the nutritional quality of soybeans in terms of their amino acid scores can be improved by modulating the ratio of glycinin and β-conglycinin through conventional breeding or genetic engineering [20, 21]. Numerous soybean lines with depletions of storage protein subunits have been developed by conventional breeding, and although they are different in the content of glycinin and β-conglycinin subunits and amino acid profiles, they are similar in total seed protein content [20]. Furthermore, the content and ratio of glycinin and β-conglycinin may also vary depending on the environmental conditions of plant growth [22].

The hypolipidemic properties of SP, β-conglycinin or its subunits (α′, α, and β), and glycinin have been investigated in humans [3, 4, 23], animals [7, 9, 24], and cultured cells [10, 11]. It has been shown that the α′ subunit of the β-conglycinin may play a major role in the regulation of cholesterol homeostasis [25, 26]. However, this appears to be inconsistent with some other studies demonstrating that isoflavones are associated with hypocholesterolemic actions of soy [13], whereas soy proteins are shown to be the main contributors to the lowering of triglycerides [3, 14–16]. The use of various soybean lines with altered glycinin profiles to study the hypolipidemic effects of dietary consumption has not been reported before. The proteins or subunits in soy responsible for the lipid-lowering properties remain unknown. Since the protein compositions of different soybean cultivars can be quite variable, consumption of soy may not always have the same health benefits or physiological functions as expected due to the absence of or lower content of the bioactive components. Many studies on soy proteins and isoflavone consumption in both animals and humans have demonstrated marked variations in the hypolipidemic functions [27, 28], which might be caused by differences in the unknown bioactive component(s) within the seeds.

The identification of the bioactive component(s) responsible for different physiological functions such as lipid-lowering action of soy would assist government regulatory agencies in the evaluation of related health claims or post-market monitoring of the safety of soy foods such as soy-based infant formulas and soy milk. It is also important for soybean producers and processors to develop functional soybeans with increased health properties through conventional breeding or genetic engineering. The objectives of this study were, using rats as a model, (a) to examine the effects of different dietary proteins and soy isoflavones on growth, organ development, and blood biochemical parameters; and (b) to identify the bioactive component(s) responsible for the hypolipidemic actions of soy using proteins prepared from soybean lines with very different protein subunit composition.

Materials and methods

Chemicals and reagents

Vitamin-free casein was purchased from Harlan Teklad (Madison, WI, USA). Alcohol-washed SP isolate (SPI) (PRO-FAM 930) and Novasoy, isoflavone concentrate, were purchased from Archer Daniels Midland Company (Decatur, IL, USA). Lipid assay kits were from Wako Pure Industries, Ltd. (Osaka, Japan). ELISA kits of total triiodothyronine (T3) and thyroxine (T4) were from Alpha Diagnostic International (San Antonio, Texas). Free T3 and T4 ELISA kits were from Crystal Chem Inc (Downers Grove, IL, USA). Assay reagents for enzymatic activities of amylase, alkaline phosphatase (ALP), creatine kinase (CK), and lactate dehydrogenase (LDH) were from Horiba ABX Diagnostics (Irvine, CA, USA).

Preparation of soy protein concentrate

Soy protein concentrates (SPC) were prepared from 6 soybean lines including 1 wild type (Harovinton) and 5 null lines with depletion of different subunit(s) of the two major storage proteins (Table 1) as described previously [20]. Briefly, dry soybeans were washed, drained, and soaked in cool tap water in a ratio of 1:5 (w/v) at room temperature for 16–18 h. The soaked soybeans were ground in a Soy-Cow "E" system which consists of a grinder, a pressure cooker/boiler, and a filtering unit (Malnutrition Matters, Ottawa, Ontario, Canada). The resulting slurry was heated to 105 °C with a pressure of approximately 25 psi and maintained for 2 min. Soy milk was recovered by filtering

Table 1 Contents of main nutrients and isoflavones in the proteins used in diets

Protein source	Diets	Protein (%)	Fat (%)	Carbohydrate (%)	Fiber (%)	Isoflavones (mg/g)
Vitamin-free casein	Diets 1, 2	90.00				
SPI (PROFAM 930)	Diet 3	90.00				0.23
SPC from Harovinton	Diet 4	66.22	28.29	1.73	2.44	2.43
SPC from A3 null line	Diet 5	60.24	31.00	1.97	2.16	2.15
SPC from A3, A4, A5 null line	Diet 6	63.02	28.69	2.00	3.41	1.81
SPC from A1, A2, A4, and A5 null line	Diet 7	60.28	29.45	1.73	1.77	2.15
SPC from α′, A1, A2, and A3 null line	Diet 8	63.43	29.02	1.66	2.74	1.54
SPC from α′, A1 to A5 null line	Diet 9	60.24	31.25	2.17	2.20	2.17

SPI alcohol-washed soy protein isolate, *SPC* soy protein concentrate

the cooked slurry. The acid coagulant glucono-delta-lactone (Sigma-Aldrich, St-Louis, MO, USA) was added to the hot soy milk to a final concentration of 0.3 % (w/v) to coagulate for at least 1 h. The curds were recovered from the whey and frozen at −20 °C for 24–48 h and then defrosted to allow the remaining whey to be pressed out before going to an industrial freeze-dryer (AAFC, St-Hyacinthe, Quebec).

Nutrient concentrations in SPC were determined by AACC International Approved Methods of Analysis [29]. Protein concentrations of SPC were determined by measuring their nitrogen contents (% N) using Kjeldahl (approved method AACCI 46-16). A factor of 6.25 was used to convert nitrogen into protein. Total fat concentrations were determined by accelerated solvent extraction (ASE) following DIONEX application note No. 314 (Determination of unbound fat in various food matrices using ASE). Carbohydrate contents were determined by solvent extraction followed by HPLC analysis. Total fiber contents were measured using the Megazyme (Ireland) kit following approved method AACCI 32-07. Isoflavone contents were determined by HPLC following approved method AACCI 20-20.

Characterization of soy protein compositions

Protein compositions of the SPC were confirmed by SDS-PAGE as described previously [20]. Briefly, 25 mg of SPC samples or SPI was resuspended in 1 mL 1× SDS loading dye and centrifuged at 16,000×*g* for 10 min. The supernatants were separated on a 12.5 % SDS-PAGE gel (Protean II xi cell, BioRad). The gel was stained with 0.25 % Commassie Brilliant Blue R250 for 1 h and then destained. The gel was then scanned on an Epson Perfection V300 Photo.

Animals and diets

Animal experimental protocol was approved by the Health Canada Animal Care Committee, and all animal handling and care followed the guidelines of Canadian Council for Animal Care. Weanling Sprague–Dawley rats (Charles River, St Constant, Quebec, Canada) were housed individually in wire bottom cages in an environmentally controlled room maintained at 22 °C and 60 % relative humidity with a 12-h day/12-h night cycle. Rats were randomly divided into 9 groups (8 males and 8 females/group). After acclimation, rats had free access to water and one of the 9 experimental diets. All diets (Table 2) were formulated according to AIN-93G recommendations for rodents [30] except that casein was replaced by the equal amounts of alcohol-washed SPI (PRO-FAM 930) in Diet 3 or different SPC in Diets 4–9. In addition, the fat contents were increased from 7 to 15.6 % in all diets by the addition of soybean oil and lard as needed. Novasoy was added to make the final isoflavone contents to about 50 mg/kg in Diet 2 to provide the same amount of isoflavones as contained in Diet 3, and 700 mg/kg in Diets 6 and 8. Diets were made into feed pellets and stored at 4 °C until used.

Body weight and food intake were recorded twice weekly. After 8 weeks on the test diets, 12-h food-deprived rats were euthanized via exsanguination from the abdominal aorta while under 3 % isoflurane anesthesia. Blood was collected, and plasma and serum were isolated. Liver, kidneys, heart, thyroid, spleen, thymus, brain, uterus, ovaries, testis, prostate, and abdominal fat (visceral, mesenteric, and retroperitoneal fat) were collected, weighted, and frozen immediately in liquid nitrogen and stored at −80 °C.

Plasma lipid profiles and thyroid hormones

Plasma concentrations of total, HDL, LDL, and free cholesterol, triglyceride, and FFA were analyzed using commercial kits according to the manufacturer's instructions. Plasma total and free T3 and T4 levels were measured using ELISA kits. The absorbance was read with the Infinite 200 PRO Microplate Reader (Tecan Asutria GmbH, Salzburg, Austria). The concentrations were calculated using standard curves.

Table 2 Composition of experimental diets (g/kg diet)

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9
Casein (vitamin free)	222.20[a]	222.20							
Soy protein isolate (SPI)			222.20[b]						
Soy protein concentrate (SPC)				302.00[c]	332.03[c]	317.35[c]	331.77[c]	315.31[c]	331.99[c]
Corn starch	339.29	339.12	339.29	357.61	346.10	351.88	339.06	348.09	347.69
Dextrinized cornstarch	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00	82.00
Soybean oil	105.00	105.00	105.00	19.50	2.10	14.00	7.30	13.50	1.30
Lard	51.00	51.00	51.00	51.00	51.00	51.00	51.00	51.00	51.00
Sucrose	100.00	100.00	100.00	94.78	93.46	93.65	94.26	94.77	92.80
Cellulose-type fiber	50.00	50.00	50.00	42.60	42.80	39.20	44.10	44.10	42.70
L-Cystine	3.00	3.00							
L-Methionine			3.00	3.00	3.00	3.00	3.00	3.00	3.00
Novasoy[d]		0.167				0.416		0.720	
Miscellaneous[e]	47.514	47.514	47.514	47.514	47.514	47.514	47.514	47.514	47.514
Total isoflavones		0.05	0.05	0.70	0.70	0.70	0.70	0.70	0.70

Total protein (200 g/kg) = amounts of Casein, SPI or SPC × protein (%) in Table 1; Total fat (156 g/kg) = 51 g (lard) + soybean oil added + amounts of each SPC × respective fat (%) in Table 1

[a] Casein from Harlan Teklad (Madison, WI, USA) contains 90 % crude protein

[b] Alcohol-washed SPI (Archer Daniels Midland) contains 90 % crude protein

[c] Subunit composition, contents of protein, fat, carbohydrate, fiber, and isoflavones of SPC are listed in Table 1

[d] Novasoy isoflavone concentrate contains 30 % total isoflavones

[e] AIN-93G mineral mix (35 g/kg), AIN-93-V vitamin mix (10 g/kg), choline bitartrate (2.5 g/kg), and *tert*-butylhydroquinone (0.014 g/kg)

Biochemical parameter in serum

Serum enzymatic activities of amylase, ALP, CK, and LDH were measured with the ABX Pentra 400 Clinical Chemistry System (Horiba ABX Diagnostics, Irvine, CA, USA) with appropriate reagents according to the manufacturer's instructions.

Statistical analysis

Data are presented as the mean ± SEM. All data were evaluated for equality of variance prior to statistical analysis. Variables with skewed distribution were logarithmically transformed. One- or two-way analyses of variance followed by Fisher's Least Significant Difference post hoc test were used to determine the dietary and gender effects and to compare differences among group means. Differences were considered significant when $P < 0.05$. Statistical analyses were performed using Statistica 8.0 software (StatSoft, Tulsa, OK, USA).

Additionally, the contrasts presented in Table 5 were constructed in SAS to compare the effects of casein versus SP diets (i.e., Diets 1–2 vs. all other diets), wild-type SPC diet versus the SPC diet with most depleted seed storage protein subunits (Diet 4 vs. Diet 9), and the presence or absence of 4 groups of seed storage protein subunits (A1 and A2, A3, A4, and A5 glycinin, and α′ β-conglycinin). The analysis was carried out with PROC GLM of SAS (SAS Institute, Cary, NC). Significance of contrasts was indicated at the 0.05, 0.01, or 0.001 level or was considered not significant when $P > 0.05$.

Results

SPC compositions

The protein contents of the SPC prepared from 6 soybean lines varied from 60.24 to 66.22 %, fat from 28.29 to 31.25 %, carbohydrate from 1.66 to 2.17 %, fiber from 1.77 to 3.41 %, and total isoflavones from 1.54 to 2.43 mg/g (Table 1). The depletion of storage protein subunit(s) in each SPC was confirmed by SDS-PAGE (Fig. 1).

Food consumption and body weight gain

The initial and final body weights were not different among dietary groups in both genders ($P > 0.05$, Table 3). Body weight gains and food intake did not differ among dietary groups in males ($P > 0.05$, Table 3). The female rats fed Diets 4 and 6 had lower body weight gains even though their food intakes were similar (Diet 6) or even higher (Diet 4) than the rats fed casein or SPI diets ($P < 0.05$). Additionally, the food intakes of the female rats fed Diet 5 or 7

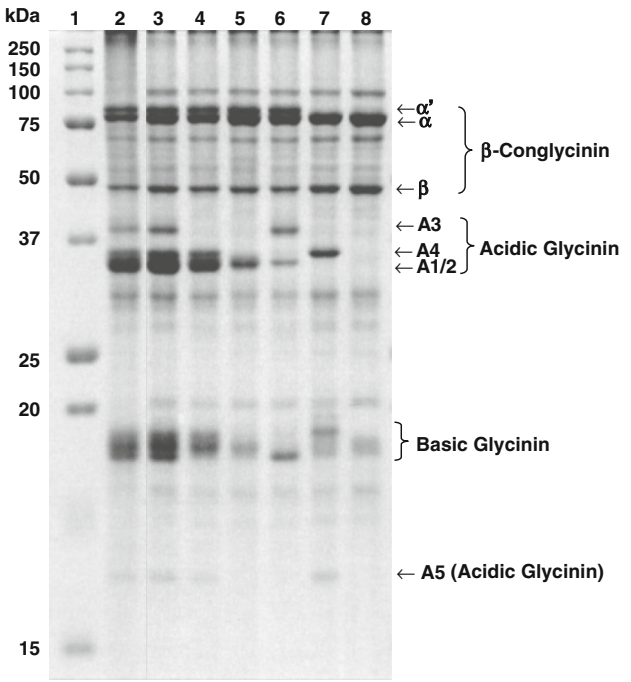

Fig. 1 One-dimensional SDS-PAGE image of soy proteins tested in the study. *Lane 1* is the molecular mass marker, *lanes 2–8* are commercial alcohol-washed soy protein isolate, soy protein concentrates prepared from soybean lines of Harovinton (wild type), A3 null, A3–5 null, A1–2, A4–5 null, α′ A1–3 null, and α′ A1–5 null, respectively

were higher than those of the rats fed casein diets (Diets 1 and 2, $P < 0.05$), but their body weight gains did not differ ($P > 0.05$, Table 3).

Tissue and organ weights

The relative liver weights (% body weight) of the female rats fed all SPC diets except Diet 7 were remarkably lower than those of the rats fed casein + ISF diet (Diet 2, $P < 0.05$, Table 3). In addition, the female rats fed Diet 6 had the lowest relative liver weight. All the male rats fed diets containing SPC had significantly lower relative liver weights compared to those fed casein + ISF (Diet 2) or casein (Diet 1) diet except Diet 5 ($P < 0.05$). Interestingly, the relative liver weights were significantly reduced in males but increased in females by feeding SPI (Diet 3) compared to casein (Diet 1, $P < 0.05$).

Feeding diets containing SPC except Diets 5 and 6 markedly reduced the relative abdominal fat in female rats ($P < 0.05$, Table 3) compared to casein diets (Diets 1 and 2) but no effects were observed in male rats ($P > 0.05$). Additionally, the female rats fed Diet 6 had lower relative kidney weights than those fed casein diets, while the male rats fed Diet 7 had higher relative thyroid weights than the rats fed the other diets ($P < 0.05$, data not shown). All other tissue and organ weights measured were not different among the diets ($P > 0.05$, data not shown).

Blood lipid profiles

The male rats fed diets containing SP (Diets 3–9) or casein + ISF (Diet 2) had remarkably lower blood total and free cholesterol compared to feeding a casein diet (Diet 1, $P < 0.05$, Fig. 2b, d). Moreover, the SPC diets (Diets 4–9) further lowered the total cholesterol in males compared to casein + ISF (Diet 2, $P < 0.05$, Fig. 2b). The female rats fed SPC diets except Diets 7 and 8 had significantly lower blood total cholesterol than the rats fed casein or SPI diets (Diets 1–3, $P < 0.05$, Fig. 2a). Free cholesterol levels in the female rats fed SPC diets (Diets 4–9) were markedly lower than those in the rats fed casein + ISF or SPI (Diets 2–3, $P < 0.05$, Fig. 2c). Interestingly, the diets containing casein + ISF (Diet 2) or SPI (Diet 3) reduced both total and free cholesterol in male rats compared to casein diet (Diet 1, $P < 0.05$, Fig. 2b, d), but not in female rats ($P > 0.05$, Fig. 2a, c). Blood FFA levels were significantly lower in female rats fed all soy diets (Diets 3–9) compared to a casein diet (Diet 1, $P < 0.05$, Fig. 2e), however, were unchanged in male rats ($P > 0.05$, Fig. 2f). Blood triglycerides were not different among diets in both male and female rats ($P > 0.05$, data not shown).

The male rats fed all SPC diets (Diets 4–9) had markedly lower LDL and HDL cholesterol levels compared to the rats fed the casein diet (Diet 1, $P < 0.05$, Fig. 3b, d). Particularly, the rats fed Diet 9 had the lowest LDL (Fig. 3b) and the highest HDL cholesterol levels following the casein diet (Diet 1, Fig. 3d). The female rats fed all SPC diets except for Diet 7 had significantly lower LDL cholesterol than those fed casein + ISF (Diet 2, $P < 0.05$, Fig. 3d). HDL cholesterol levels in the females fed SPC diets (Diets 4–8) except for Diet 9 were reduced compared to feeding the casein diet (Diet 1, $P < 0.05$, Fig. 3c). Feeding the SPI diet (Diet 3) lowered HDL cholesterol ($P < 0.01$, Fig. 3c, d), but had no effect on LDL cholesterol ($P > 0.05$, Fig. 3a, b), compared to a casein diet (Diet 1) in both male and female rats.

Thyroid hormone levels

Plasma total and free T4 levels were not different among the dietary groups in both genders ($P > 0.05$, data not shown). However, the plasma free T3 (active form) in both genders fed SPC diets (Diets 4–9) was significantly higher than in those fed casein diet (Diet 1, $P < 0.05$, Fig. 4c, d). Moreover, feeding soy diets (Diets 3–9) remarkably increased plasma total T3 in female rats compared to casein diets (Diets 1 and 2, $P < 0.05$, Fig. 4a), while total T3 levels were higher in the male rats fed Diets 8 and 9 compared to the casein diets (Diets 1 and 2, $P < 0.01$, Fig. 4b).

Table 3 Food consumption, body weight gain, and tissue weights of the rats fed experimental diets for 8 weeks

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	*P* value
Food intake (g)										
Female	837* ± 20^{a}	835 ± 27^{a}	864 ± 22^{ab}	978 ± 45^{c}	957 ± 52^{bc}	853 ± 44^{ab}	943 ± 36^{bc}	840 ± 32^{a}	890 ± 48^{abc}	0.04
Male	1,014 ± 34	1,075 ± 34	1,097 ± 50	1,135 ± 25	1,168 ± 44	1,170 ± 35	1,087 ± 44	1,054 ± 41	1,043 ± 45	0.06
Initial BW (g)										
Female	121 ± 4	120 ± 6	121 ± 4	123 ± 1.4	125 ± 4	124 ± 5	124 ± 2	124 ± 6	125 ± 4	0.99
Male	126 ± 3	127 ± 2	126 ± 5	126 ± 3	127 ± 6	131 ± 3	127 ± 4	130 ± 5	127 ± 3	0.98
Final BW (g)										
Female	245 ± 5	242 ± 8	248 ± 8	227 ± 5	240 ± 6	229 ± 7	238 ± 5	241 ± 8	233 ± 9	0.40
Male	376 ± 7	391 ± 10	385 ± 20	395 ± 10	385 ± 19	398 ± 13	382 ± 16	382 ± 20	371 ± 10	0.95
BW gain (g)										
Female	124 ± 4^{ab}	122 ± 7^{ab}	127 ± 7^{a}	103 ± 6^{c}	115 ± 6^{abc}	105 ± 6^{c}	114 ± 4^{abc}	117 ± 5^{abc}	109 ± 6^{bc}	0.05
Male	251 ± 5	264 ± 8	259 ± 16	269 ± 9	270 ± 8	267 ± 11	255 ± 13	252 ± 16	244 ± 8	0.71
Liver/BW (%)										
Female	2.39 ± 0.06^{ab}	2.51 ± 0.09^{ac}	2.56 ± 0.04^{c}	2.32 ± 0.04^{b}	2.35 ± 0.07^{b}	2.12 ± 0.04^{d}	2.38 ± 0.05^{ab}	2.34 ± 0.07^{b}	2.25 ± 0.05^{bd}	0
Male	2.88 ± 0.14^{ab}	2.94 ± 0.09^{a}	2.59 ± 0.06^{c}	2.44 ± 0.05^{c}	2.66 ± 0.12^{bc}	2.44 ± 0.07^{c}	2.55 ± 0.08^{c}	2.44 ± 0.05^{c}	2.48 ± 0.08^{c}	0
Abd fat/BW (%)										
Female	3.91 ± 0.46^{ab}	4.20 ± 0.20^{a}	3.27 ± 0.24^{bc}	3.07 ± 0.17^{c}	3.52 ± 0.31^{abc}	3.83 ± 0.20^{abc}	3.03 ± 0.24^{c}	2.94 ± 0.27^{c}	3.15 ± 0.44^{c}	0.05
Male	2.93 ± 0.32	3.17 ± 0.14	3.16 ± 0.31	3.26 ± 0.18	3.27 ± 0.35	3.86 ± 0.51	3.19 ± 0.35	3.10 ± 0.30	2.66 ± 0.31	0.45

BW body weight, *Abd fat* abdominal fat

* Values are presented as mean ± SEM, $n = 8$. Means in a row with different letters differ ($P < 0.05$)

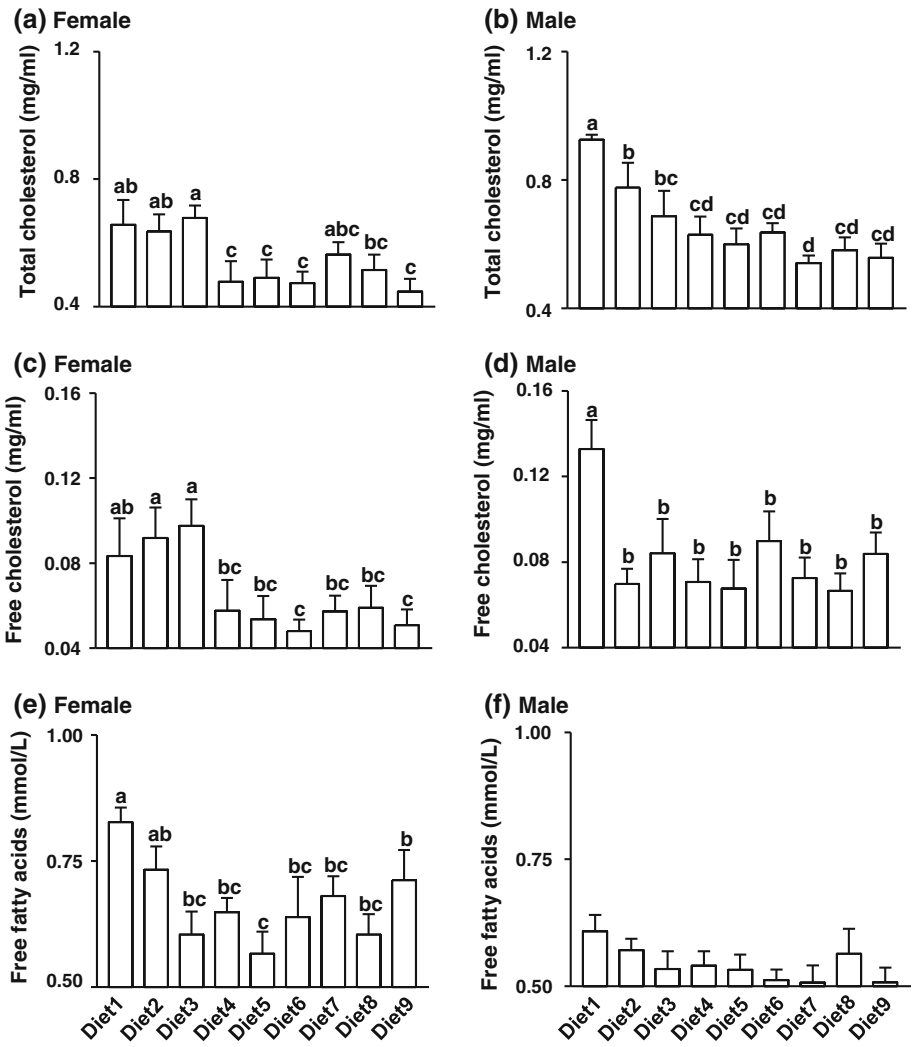

Fig. 2 Plasma total (**a**, **b**), free (**c**, **d**) cholesterol and free fatty acid (**e**, **f**) levels in the rats fed diets containing 20 % protein from either casein in the absence (Diet 1) or presence (Diet 2) of 50 mg/kg diet of isoflavones, or alcohol-washed SPI (Diet 3), or SPC prepared from soybean lines of Harovinton (wild type, Diet 4), A3 null (Diet 5), A3–5 null (Diet 6), A1–2 and A4–5 null (Diet 7), α′ and A1–3 null (Diet 8) or α′ and A1–5 null (Diet 9) for 8 weeks. Values are mean ± SEM ($n = 8$). Means with *different letters* differ, $P < 0.05$

Blood biochemical parameters

Serum ALP activities were markedly lowered by all soy diets (Diets 3–9) and casein + ISF diet (Diet 2) compared to the casein diet (Diet 1) in the female rats ($P < 0.05$), but were not different in the male rats ($P > 0.05$, Table 4). Interestingly, serum amylase activities in the female rats fed SPC diets except Diet 5 were significantly lower than in the rats fed casein diets ($P < 0.05$, Table 4). Additionally, serum enzymatic activities of CK and LDH in the male rats fed Diets 7, 8, and 9 were significantly lower than in the rats fed casein and SPI diets ($P < 0.05$, Table 4). The female rats fed Diet 9 had significantly lower serum CK and LDH activities than in the rats fed all other diets except Diet 8 ($P < 0.05$).

Effects of different soy protein subunits

The food intake, body weight gain, relative liver weight, relative abdominal fat content, blood FFA, total, free, and LDL cholesterol, free T3, amylase, and ALP were not significantly different between the rats fed diets containing SPC lacking storage protein subunits (α′ of β-conglycinin and A1–A5 of glycinin, Diet 9) and those fed the wild-type SPC (Diet 4, $P > 0.05$, Table 5). Presence of the α′ subunit of β-conglycinin was associated with lower blood HDL cholesterol, higher blood CK and LDH activities ($P < 0.05$) in both genders and lower total T3 in males. Presence of the A1 and A2 subunits of glycinin were related to lower blood HDL cholesterol and higher LDH activities ($P < 0.05$) in both genders, while the A1, A2, A4, and A5 subunits were associated with higher blood CK activities ($P < 0.05$).

Discussion

Soy consumption has been linked to many health benefits such as decreased risk of coronary heart disease [3], type 2 diabetes [31], and atherosclerosis [32, 33]. Coronary heart

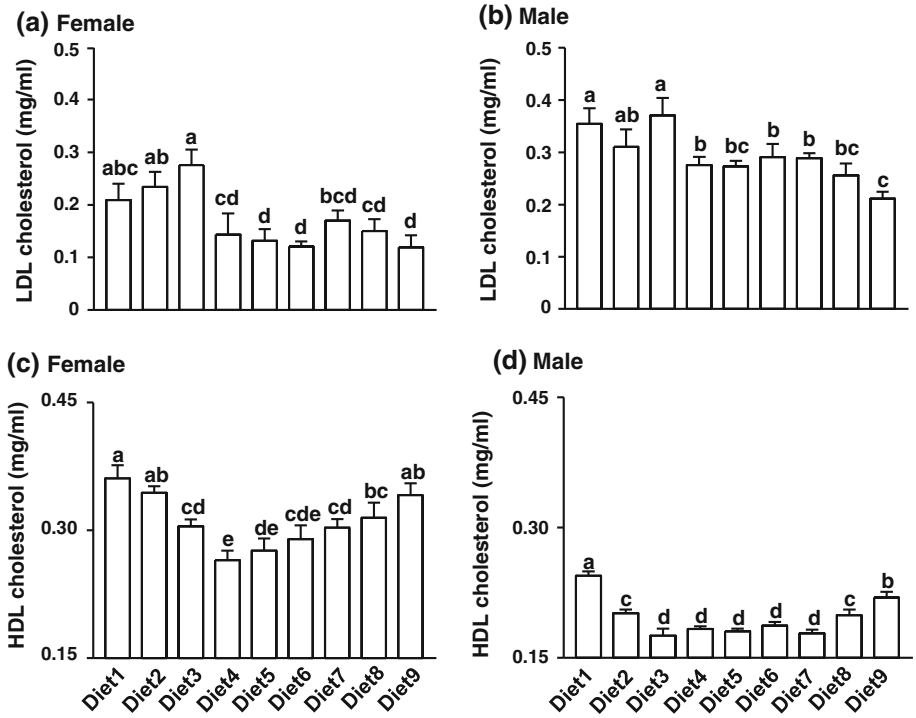

Fig. 3 Plasma LDL (**a**, **b**) and HDL (**c**, **d**) cholesterol levels in the rats fed diets containing 20 % protein from either casein in the absence (Diet 1) or presence (Diet 2) of 50 mg/kg diet of isoflavones, or alcohol-washed SPI (Diet 3), or SPC prepared from soybean lines of Harovinton (wild type, Diet 4), A3 null (Diet 5), A3–5 null (Diet 6), A1–2 and A4–5 null (Diet 7), α′ and A1–3 null (Diet 8) or α′ and A1–5 null (Diet 9) for 8 weeks. Values are mean ± SEM ($n = 8$). Means with *different letters* differ, $P < 0.05$

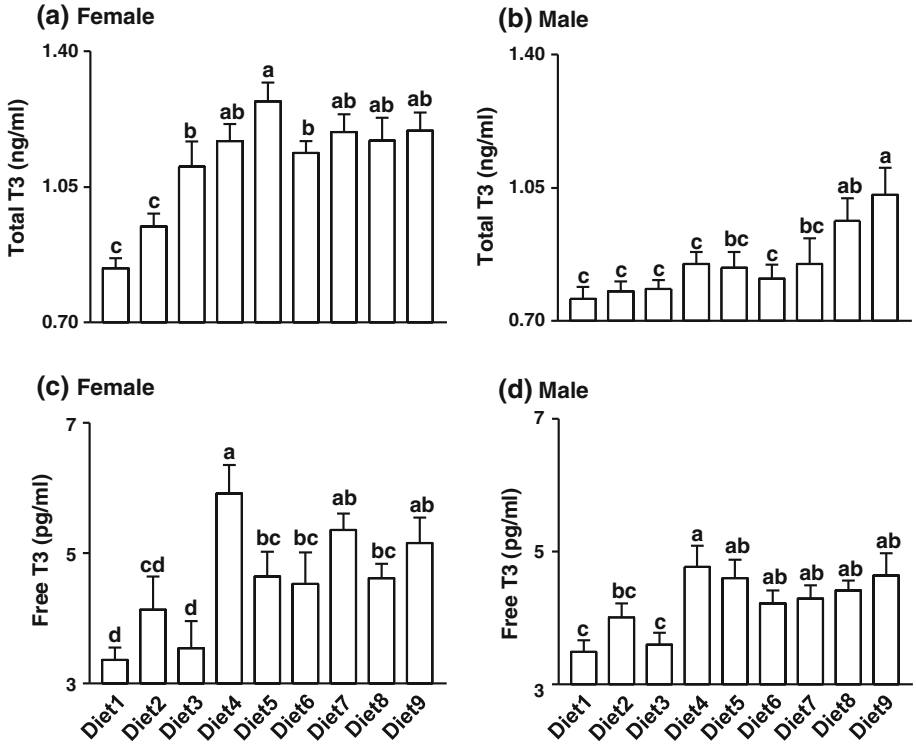

Fig. 4 Plasma total (**a**, **b**) and free (**c**, **d**) triiodothyronine (T3) levels in the rats fed diets containing 20 % protein from either casein in the absence (Diet 1) or presence (Diet 2) of 50 mg/kg diet of isoflavones, or alcohol-washed SPI (Diet 3), or SPC prepared from soybean lines of Harovinton (wild type, Diet 4), A3 null (Diet 5), A3–5 null (Diet 6), A1–2 and A4–5 null (Diet 7), α′ and A1–3 null (Diet 8) or α′ and A1–5 null (Diet 9) for 8 weeks. Values are mean ± SEM ($n = 8$). Means with *different letters* differ, $P < 0.05$

disease is a leading cause of mortality and morbidity [34]. Increased blood LDL-cholesterol concentration is a risk factor of coronary heart disease [35]. The present study showed that feeding SPC diets prepared from wild type and certain null lines reduced total, LDL, and free cholesterol compared to casein or casein + ISF diets. These results support the notion that consumption of SP may reduce the risk of coronary heart disease and hypercholesterolemia-associated disorders.

Our results also showed that feeding SP diets reduced the HDL cholesterol in both genders compared to a casein diet (except Diet 9 in females). This is consistent with

Table 4 Blood biochemical parameters in the rats fed experimental diets for 8 weeks

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	*P* value
ALP (U/L)										
Female	94.54* ± 7.36^{a}	73.41 ± 6.04^{b}	67.76 ± 6.47^{b}	69.83 ± 5.15^{b}	67.13 ± 4.49^{b}	64.53 ± 2.88^{b}	64.53 ± 4.38^{b}	59.23 ± 3.24^{b}	73.65 ± 6.10^{b}	0.00
Male	99.51 ± 7.39	96.29 ± 6.77	95.11 ± 8.43	86.30 ± 7.69	106.9 ± 8.96	89.25 ± 7.38	86.39 ± 4.43	94.86 ± 6.68	87.31 ± 3.78	0.48
Amy (U/L)										
Female	1,394 ± 89^{ab}	1,452 ± 80^{a}	1,331 ± 44^{abc}	1,171 ± 78^{cd}	1,257 ± 81^{bc}	1,019 ± 65^{d}	1,209 ± 35^{cd}	1,142 ± 48^{cd}	1,140 ± 71^{cd}	0.00
Male	2,468 ± 136	2,658 ± 129	2,152 ± 107	2,308 ± 90.3	2,371 ± 134	2,326 ± 81.2	2,269 ± 105	2,377 ± 149	2,121 ± 62.2	0.06
CK (U/L)										
Female	314.9 ± 55.2^{ab}	348.0 ± 52.7^{a}	357.7 ± 70.5^{a}	305.8 ± 35.8^{ab}	301.5 ± 70.1^{ab}	320.8 ± 56.2^{ab}	264.5 ± 32.9^{abc}	188.9 ± 44.3^{bc}	120.0 ± 17.4^{c}	0.04
Male	396.1 ± 70.2^{a}	388.5 ± 57.8^{a}	304.0 ± 42.4^{ab}	340.9 ± 65.6^{ab}	280.6 ± 44.3^{abc}	196.4 ± 21.9^{bc}	183.9 ± 19.8^{cd}	192.8 ± 32.5^{cd}	128.6 ± 27.8^{d}	0.00[1]
LDH (U/L)										
Female	472.8 ± 98.16^{ab}	523.1 ± 96.6^{a}	539.6 ± 110.3^{a}	470.2 ± 44.9^{ab}	461.3 ± 135.1^{ab}	504.6 ± 90.3^{a}	397.0 ± 53.0^{ab}	252.8 ± 75.6^{bc}	140.7 ± 27.3^{c}	0.04
Male	482.8 ± 73.0^{ab}	575.8 ± 94.8^{a}	452.7 ± 68.3^{ab}	384.6 ± 81.0^{abc}	406.0 ± 79.1^{abc}	318.6 ± 71.2^{bc}	231.3 ± 37.5^{cd}	239.5 ± 58.0^{cd}	112.9 ± 44.6^{d}	0.00

Amy amylase, *ALP* alkaline phosphatase, *CK* creatine kinase, *LDH* lactate dehydrogenase

* Values are presented as mean ± SEM, $n = 8$. Means in a row with different letters differ ($P < 0.05$)

[1] Logarithmic transformation prior to one-way ANOVA

another study conducted in hamsters [9]. However, the rats fed either β-conglycinin [7] or glycinin [36] fractions had higher HDL levels than those fed casein. Interestingly, the highest levels of HDL cholesterol among the animals fed SP diets in our study were observed in those fed the most knocked-out diets (Diets 8 and 9). Furthermore, Diet 9 was associated with the lowest LDL cholesterol level. These two diets are devoid of the α′ subunit of β-conglycinin which was shown to be related to lower HDL cholesterol. Since these lines still maintain a high seed protein content, the α and β subunits are likely enriched in these lines and could potentially be associated with the higher HDL levels.

Consumption of alcohol-washed SPI (Diet 3) in this study demonstrated gender-specific effects on total and free cholesterol levels as well as FFA content in the plasma. Feeding of SPI reduced both total and free cholesterol levels in males, but had no significant effect in the female rats. This hypocholesterolemic action of SPI in males could be attributed to the low levels of isoflavones associated with the proteins because addition of a similar amount of isoflavones (50 mg/kg diet) to the casein diet (Diet 2) showed the same effects. It appears that the male rats are more sensitive than the female rats to soy isoflavone stimulation in the regulation of cholesterol metabolism. This might be due to the lower endogenous estrogen levels in male rats which may lead to the binding of more soy isoflavones to the hepatic estrogen receptors (ER) that mediate the cholesterol metabolism.

The major soy isoflavones, genistein and daidzein, are structurally similar to that of mammalian estrogens and can bind to both ERα and β. However, their binding affinities to ERα are ∼500–850 times weaker than to ERβ and ∼500–10,000 times weaker than that of estradiol to ERα [37]. This suggests that the presence of high levels of endogenous estrogens in female rats can diminish the effects of soy isoflavones through competitive binding to the ERs. This has been shown in ovariectomized hamsters fed diets supplemented with soy isoflavones (9.5–38 mg/kg diet) [33]. The effect of isoflavones was dose-dependent in reducing blood total cholesterol and preventing the formation of atherosclerotic lesions and was also independent of proteins.

Pronounced gender differences caused by endogenous estrogens have been previously shown in cholesterol metabolism. Endogenous estrogens lower plasma LDL cholesterol [38, 39] and induce the expression of hepatic LDL receptors in women [39] and rats [40]. Hepatic LDL receptors mediate the catabolism of ∼70 % of LDL cholesterol from circulation. Another possible mechanism explaining the hypocholesterolemic actions of soy isoflavones in male rats may be through induction of estrogen production. It has been shown that pre- and postnatal supplementation of soy isoflavones considerably increased

Table 5 Contrasts comparing the effects of dietary casein versus soy, wild-type versus most knockout soy proteins, and presence or absence of seed storage protein subunits on the body and blood biochemical parameters in rats

Contrast	Casein versus soy		Wild-type versus knockout		A1, A2 effect		A3 effect		A4, A5 effect		α′ effect	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
Food intake	*	*	ns	ns								
BW gain	*	ns	ns	ns								
Liver/BW	*	***	ns	ns								
Abd fat/BW	**	ns	ns	ns								
TC	**	***	ns	ns								
FC	**	*	ns	ns								
FFA	***	*	ns	ns								
LDL-C	**	**	ns	ns								
HDL-C	***	***	***	***	***	*	ns	***	*	ns	***	***
Total T3	***	**	ns	**		ns		ns		ns		***
Free T3	***	**	ns	ns								
ALP	***	ns	ns	ns								
Amylase	***	**	ns	ns								
CK	ns	***	*	**	ns	**	ns	ns	ns	*	**	*
LDH	ns	***	*	**	*	*	ns	ns	ns	*	***	**

BW body weight, *Abd fat* abdominal fat, *TC* total cholesterol, *FC* free cholesterol, *FFA* free fatty acids, *LDL-C* LDL cholesterol, *HDL-C HDL cholesterol*, *ALP* alkaline phosphatase, *CK* creatine kinase, *LDH* lactate dehydrogenase

*, **, ***, ns Contrast significant at the 0.05, 0.01, or 0.001 probability levels, respectively, or not significant ($P > 0.05$)

the blood estrogen concentrations in male rats [41]. The present study also showed that the total, free, and LDL cholesterol levels were higher in male rats compared to female rats. Likewise, the hypocholesterolemic effects of feeding SPC diets to female rats may be attributed to the high levels of isoflavones (700 mg/kg of diets) contained in the SPC diets. These results suggest that the amounts of soy isoflavones needed to achieve hypocholesterolemic actions in individuals with low endogenous estrogens such as males or postmenopausal females can be much lower than what is required for fertile females.

All SP diets significantly lowered plasma FFA compared to a casein diet, and the diets containing SPI and four SPC prepared from null lines reduced abdominal fat compared to casein + ISF in female rats in the present study. The addition of isoflavones into the casein diet did not lead to a significant reduction in both FFA and abdominal fat, suggesting that these were caused by the soy proteins. Interestingly, the SPC from an A3 null line (D5) resulted in the greatest reduction in FFA, whereas the presence of glycinin subunit A3 (D4 and D7) or the absence of the α′ subunit of β-conglycinin (D8 and D9) were associated with the lowest abdominal fat in female rats. Soy consumption has been shown to prevent non-alcoholic fatty liver diseases [2, 42, 43]. The formation and accumulation of lipid droplets in the liver is one of the main indicators of the disease and is usually associated with increased liver weight. The present study showed that feeding soy diets significantly reduced the relative liver weights compared to casein diets in male rats. This effect was independent of isoflavones. However, the female rats fed all SPC diets had significantly lower relative liver weights than those fed SPI and casein + ISF diets, which might be an effect of the higher level of isoflavones contained in the diets. Moreover, the SPC from the soybean line lacking glycinin subunits A3–5 (Diet 6) further reduced the liver weight.

Soy protein has been previously shown to increase blood T3 and/or T4 in rats [44] and pigs [45]. These observations led to the hypothesis that hypolipidemic actions of SP may be modulated by increased thyroid hormones [45]. Compared to a casein or a SPI diet, all SPC diets which contain higher levels of soy isoflavones in the present study increased free T3 in both genders, even though their protein compositions were different. This suggests an effect of isoflavones. However, the absence of different protein subunits attenuated the hyperthyroidism effects of isoflavones when compared to the wild-type SP, particularly in females. The total T3 levels in the female rats fed all SP diets are markedly higher than those fed casein diets. Since the SPI diet had very little isoflavone content (50 mg/kg diet) compared to SPC diets (700 mg/kg diet), the high T3 levels were independent of isoflavone quantity and might therefore be caused by the remaining component(s) other than the α′ subunit of β-conglycinin and A1–5 subunits of glycinin which were absent in Diet 9. Likewise, in male

rats, the highest total T3 levels were found in the two most knock-out diets (Diets 8 and 9). Both plasma free cholesterol and free T3 levels in the female rats of this study seem to be modulated by soy isoflavones. Whether the increased free T3 was responsible for the decreased free cholesterol by soy isoflavones remains to be elucidated.

The present study showed that the SPC lacking the α′ subunit of β-conglycinin and A1–5 subunits of glycinin (Diet 9) significantly lowered plasma CK and LDH activities compared to casein, wild-type, and the less knock-out (Diets 5 and 6) SPC diets in both females and males. Reduced serum activities of CK have been observed in a variety of clinical conditions such as alcoholic liver disease [46]. However, the mechanism(s) responsible for the reduction of these enzymes is unclear. It has also been shown that serum CK and LDH activities were related to thyroid status and that decreases in serum CK and LDH activities were associated with hyperthyroidism [47]. Whether the reduced plasma CK and LDH activities observed in this study is due to the increase in T3 levels remains to be investigated. Furthermore, the present study has demonstrated that female rats fed diets containing SP or casein + ISF had significantly lower plasma ALP activities compared to the casein diet, suggesting a role of isoflavones. Since increased blood ALP activity is a marker of greater bone turnover [48], this indicates that intake of soy isoflavones, even at a low concentration of 50 mg/kg diet, may reduce bone turnover, which would lead to increased bone strength and better structure [49, 50]. This is supported by the greater femoral bone density in the ovariectomized rats [48], reduced loss of bone quantity and increased proliferation of osteoblasts treated with soy isoflavones [51]. In addition, all SPC diets except Diet 5 resulted in lower amylase activity compared to the casein diets in females, which might be an effect of high levels of soy isoflavones. Soy isoflavones were shown to suppress α-amylase activity in the diabetic rats [52]. This is considered as an anti-diabetic feature of soy isoflavones because inhibition of α-amylase can slow down carbohydrate digestion thereby reducing postprandial serum glucose levels [53].

Overall, our results suggest that isoflavones in soy are mainly associated with the hypocholesterolemic actions and led to the increased plasma-free T3, whereas soy proteins were responsible for reductions in FFA, abdominal fat, liver weight, and increased plasma total T3. The SPC prepared from soybean lines devoid of β-conglycinin α′ and the glycinin A1–5 subunits (Diet 9) appears to have the best hypolipidemic functions.

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Conflict of interest The authors declare that they have no conflict of interest.

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