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Hypocholesterolemic effects of soy protein isolates from soybeans differing in 7S and 11S globulin subunits vary in rats fed a high cholesterol diet

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ABSTRACT

The objective of this study was to determine if genotypes with differing globulin subunit compositions affect the hypocholesterolemic effect (HCE) of soy protein isolates (SPIs). Six SPIs (300 mg/kg/day), which differed in the 11S/7S globulin subunit composition, and fenofibrate (FF) (30 mg/kg/day), a hypocholesterolemic drug, were administered by gavage for 28 days to hypercholesterolemic model rats (HMRs). Blood samples were collected to measure serum lipid parameters. Hepatic histopathologic and lipid biochemical data were collected, and the expression of cholesterol metabolism-related key genes was analyzed. Our results demonstrated that soybeans differing in genotype generate SPIs with marked different HCEs, and the mechanism underlying the various HCEs of SPIs is associated with differential expression of hepatic cholesterol metabolism-related key genes in HMRs. 7S (α′+α)-Null genotype provided a greater effect on increasing HDL-C level, and daily ingestion of SPI-2 (null α′, α) might significantly contribute to increase HDL-C level in HMRs.

1. Introduction

Soybean protein is effective to prevent or treat hypercholesterolemia and cardiovascular disease (Anderson, & Bush, 2011; Lee et al., 2012). The more potent hypocholesterolemic effect (HCE) of soy proteins relative to animal proteins has been reported for>40 years (Sirtori et al., 1979). Many studies have shown that consumption of soy food products is effective to reduce the plasma total cholesterol (TC) concentration and has cardiovascular benefits for humans (Gardner, Messina, Kiazand, Morris, & Franke, 2007; Oldewage, & Egal, 2013; Blanco et al., 2019) and animals (Takahashi, & Konishi, 2011; Kobayashi, Hirahata, Egusa, & Fukuda, 2012). Recent research has also demonstrated that substantial soy protein consumption is similarly effective as clofibrate, an antilipidemic drug (Duranti et al., 2004), and fenofibrate (FF), a triacylglycerol (TG)-lowering drug (Knopp, 1999; Ferreira, Silva, Demonte, & Neves, 2010), at reducing TC and TG concentrations in

Abbreviations: DN47, Dongnong 47 strain; FF, fenofibrate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCD, high-cholesterol diet; HCE, hypocholesterolemic effect; HDL-C, high-density lipoprotein-cholesterol; HMR, hypercholesterolemic model rats; LDL-C, low-density lipoprotein-cholesterol; NAS, nonalcoholic fatty liver disease activity score; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPI, soybean protein isolate; STD, standard diet; TC, total cholesterol; TG, triacylglycerol; WSP, whole soybean powder.

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hypercholesterolemic rats. Therefore, it has been suggested that a diet containing soy proteins may be effective to reduce plasma lipid concentrations, particularly in patients who do not respond or are intolerant to drug therapy. Accordingly, the US Food and Drug Administration (2013) and Canadian Government (2015) have approved soy protein as a "food for a specified health use" because of its cholesterol-lowering effects.

Soybean seeds contain two principal classes of proteins, β-conglycinin (7S globulin) and glycinin (11S globulin), which constitute approximately 70 % of the total protein content in a typical soybean seed (Derbyshire, Wright, & Boulter, 1976). The HCEs of soy protein are associated with both 7S and 11S fractions, and suggest that biologically active peptides produced by gastrointestinal digestion of soy proteins may influence cholesterol and TG homeostasis (Ferreira, Silva, Demonte, & Neves, 2010; Nagaoka, 2019; Caponio, Wang, DiCiaula, De Angelis, & Portincasa, 2020; Kim, Yang, & Kim, 2021). Lovati et al. (1998) showed that β-conglycinin is much more effective than 11S soy glycinin at upregulating low-density lipoprotein (LDL) receptor expression and degradation of LDL in cultured HepG2 human hepatoma cells (Lovati et al., 1992, 1996). Additionally, β-conglycinin reduces plasma TG concentrations in humans (Kambara, Hirotsuka, Takamatsu, & Kito, 2002) and hyperlipidemic rats (Aoyama et al., 2001). Soy 7S globulin is a trimeric protein composed of three major subunits: α′ (71 kDa), α (67 kDa), and β (50 kDa), and the subunits of 7S have differential effects on cholesterol: the α′ subunit induces upregulation of LDL receptor, whereas the β subunit does not induce LDL receptor activation (Manzoni, Lovati, Gianazza, Morita & Sirtori, 1998). These findings suggest that inclusion of soy 7S globulin in a diet may help prevent hypertriglyceridemia, hyperinsulinemia, and hyperglycemia, which are risk factors for cardiovascular diseases.

Cultivation and gene modification strategies have been successfully employed to alter the protein composition of soybean seeds in a manner that facilitates their use in food processing and animal feed, and as functional dietary protein components (Ogawa, Tayama, Kitamura, & Kaizuma, 1989; Takahashi et al., 2003; Kita et al., 2010; Harada, Hayashi, & Tsubokura, 2013). We have recently developed a series of soybean cultivars that lack specific 7S and 11S protein subunits (Song et al., 2014, 2016; Song, Oehrle, Liu, & Krishnan, 2018; Wei et al., 2020), but the effects of consuming protein derived from these cultivars on hypercholesterolemia have not yet been studied.

In the present study, we measured serum TC, TG, LDL-C, and high-density lipoprotein-cholesterol (HDL-C) concentrations, and assessed hepatic and adipose histologies as well as the relative mRNA expression levels of major cholesterol and lipid metabolism-related genes in the livers of rats fed a high-cholesterol diet (HCD). We aimed to elucidate the different effect of raw soybean genotype (Table 1 and Fig. 1) on the HCE of SPI in hypercholesterolemic model rats (HMRs) and to compare the HCE of SPIs with those of FF, a hypolipidemic and hypocholesterolemic drug, in the same experimental model.

2. Materials and methods

2.1. Selected soybean genotypes

Soybean 'Dongnong47′ (DN47; contains all 7S and 11S subunits) and five soybean cultivars that each lacks specific 7S and 11S subunits were used in this study (Table 1). Subunit-null soybean genotypes were developed by crossing and backcrossing DN47 and HS99B cultivars. The latter lacks the α′ and α subunits of 7S and all 11S subunits. All plants were grown in a field in the Soybean Experimental Plot at the Chinese Agriculture Ministry′s Key Laboratory of Soybean Biology and Breeding/Genetics research farm. Four rows of each strain were planted, each of which was 3 m in length and spaced 70 cm apart, with interplant spacing of 14 cm using a randomized complete block design with three replicates. Dry DN47 seeds and the five subunit-null genotypes were harvested at maturity in 2019 and stored at room temperature.

The seed protein composition of DN47 and the five subunit-null genotypes was analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The proteins were extracted from whole soybean powder (WSP) and SPIs using SDS sample buffer (2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol, 5 M urea, and 62.5 mM Tris aminomethane) and then centrifuged at 15,000 × *g*. Ten microliters of each supernatant were separated on 4.5 % stacking and 12.5 % separating polyacrylamide gels and stained with Coomassie Brilliant Blue R 250. The gels were scanned using a Sharp scanner (JX-330, Amersham Biosciences, Amersham, UK).

2.2. Defatted soybean powder preparation

Seeds from the six soybean types (DN47, DND360, DND358, BSH-3, DND359, and BSH-2; harvested in Harbin, China in 2019) (Table 1) were heated in a microwave at 90 °C for 2 min and then defat using a small expeller. The extruded defatted soybean flakes were then crushed into particles that were passed through a 60 mesh sieve. The extruded defatted soybean powder was stored at 4 °C in plastic bags until SPI preparation.

2.3. SPI preparation

Defatted soybean powder was prepared from the six soybean cultivars. The SPI was prepared from powder using alkaline extraction at pH 8.0 followed by precipitation at pH 4.5. The precipitate was redissolved in distilled water and then neutralized to pH 7.5 using 2 M NaOH. Subsequently, the protein solution was dialyzed against distilled water at 4 °C for 48 h and then lyophilized. The protein content in SPIs was determined using the Dumas method (N × 6.25, Friedman et al., 1979) (Rapid N Cube, Elementar Analytical, Villeurbanne, France).

Table 1
Subunit composition of 7S and 11S globulin, and 11S/7S and α'/(7S + 11S) ratios of SPIs prepared from Dongnong 47 (DN47; control) and five null soybean genotypes administered by oral gavage to hypercholesterolemic model rats for 4 weeks[1].

Soybean genotypes		Subunit absent		Protein (g/100 g dry weight)		11S/7S	α′/(7S + 11S)
Line	Designation	(7S)	(11S)	WSP	SPI		
DN47	SPI-1			39.35 ± 0.02^{f}	83.63 ± 0.02^{e}	1.57 ± 0.23^{c}	18.33 ± 3.14^{b}
DND360	SPI-2	α′, α		47.69 ± 0.01^{a}	87.33 ± 0.02^{a}	6.55 ± 0.41^{a}	0.00 ± 0.00^{c}
DND358[2]	SPI-3	α	G1, G2, G4	44.06 ± 0.01^{b}	84.87 ± 0.01^{b}	0.95 ± 0.09^{c}	28.58 ± 0.85^{a}
BSH-3[3]	SPI-4	α′	G1, G2, G4	41.73 ± 0.02^{d}	84.56 ± 0.01^{d}	1.24 ± 0.20^{c}	0.00 ± 0.00^{c}
DND359	SPI-5	α′, α	G1, G2, G4	41.51 ± 0.02^{e}	82.91 ± 0.02^{f}	3.01 ± 0.52^{b}	0.00 ± 0.00^{c}
BSH-2	SPI-6		G1, G2, G4	42.01 ± 0.02^{c}	84.79 ± 0.01^{c}	1.25 ± 0.24^{c}	18.87 ± 4.36^{b}

[1] Data are expressed as mean ± SD. Labeled means in a column without a common superscript letter differ, $P < 0.05$. SPI, soybean protein isolate; WSP, whole soybean powder. 11S/7S and α′/(7S + 11S) ratios were calculated using specific band intensities in the SDS-PAGE gel shown in Fig. 1.
[2] DND358, Song et al., 2022.
[3] BSH-3, Song et al., 2018.

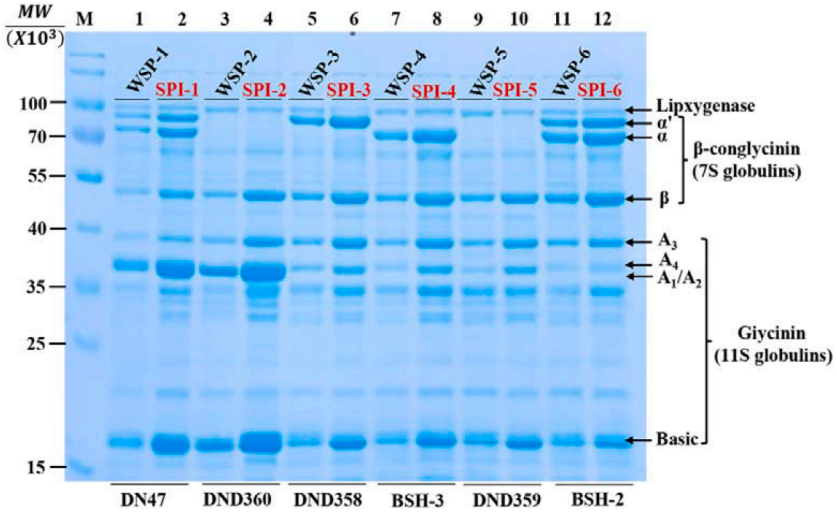

Fig. 1. SDS-PAGE profile of whole soybean powder (WSP) and soy protein isolate (SPI) fractions from DN47 and five null soybean genotypes differing in their subunit composition. Hypercholesterolemic model rats were administered the six SPIs daily by oral gavage for 4 weeks. The lanes are marked at the top as follows: Lanes 1 and 2: total proteins of WSP and SPI fractions from DN47 containing all 7S and 11S subunits; 3 and 4: DND360 (null α′ and α subunits of 7S); 5 and 6: DND358 (Song et al., 2022) (null α and G1, G2, and G4 of 11S); 7 and 8: BSH-3 (Song et al., 2018) (null α′ and G1, G2, and G4); 9 and 10: DND359 (null α′, α and G1, G2, and G4); 11 and 12: BSH-2 (null G1, G2, and G4). Lane marked M contains standard molecular weight markers.

2.4. Animals, diets, and experimental protocol

Animal experiments were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Laboratory Animal Ethics Committee.

Fifty-four male Sprague–Dawley rats (5 weeks old, 220–250 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., ltd. (Beijing, China). The rats were individually housed in wire-bottomed cages at 23 °C with a 12 h/12 h light/dark cycle. Rats had free access to food and water throughout the study and were allowed to acclimate to the new environment for 1 week before the experiments. The rats were then divided into two groups as follows: i) standard diet (STD) group (n = 6), in which the rats were fed a STD continued until the end of experiment; and ii) HCD group (n = 48), in which the rats were fed cholesterol-enriched feed (1.0 % cholesterol and 0.5 % sodium cholate, Table S1) for 4 weeks to induce the HMRs. Blood samples were taken from the caudal vein to determine serum TC, TG, LDL-C, and HDL-C levels at the end of the high-cholesterol diet inducing for 4 weeks, and all 48 rats met the following hypercholesterolemic criteria:i) the difference in TC, TG, LDL-C, and HDL-C levels was statistically significant in the HCD-fed group compared with that in the STD-fed group; and ii) no statistically significant differences in TC, TG, LDL-C, or HDL-C levels were found among HMRs (Table S2). After hypercholesterolemia confirmation, the 48 HMRs were then randomly allocated to eight experimental groups as follows: HCD; HCD + 30 mg·kg^{-1}·day^{-1} FF, and HCD + 300 mg·kg^{-1}·day^{-1} of one of the six SPIs that differed in their 7S and 11S seed protein composition (Table 1 and Fig. 1). During the experimental period, SPI and FF suspensions were prepared by suspending each substance in 5 mL of purified water at a final concentration of 40 mg/ml. FF and six SPIs were administered to the respective groups daily by gavage at 09:00. Rats in the STD group (used as normal reference) and the HCD group (hypercholesterolemia control) received water by gavage. The rats were also checked daily regarding their general health. Body weight and food intake for the rats were recorded weekly. The treatment was conducted for 28 days, and the rats were then sacrificed immediately. A flow diagram for the study is shown in Fig. S1.

2.5. Blood collection and analysis

At the end of weeks 4 and 8 (Fig. S1), rats were fasted overnight, and 0.5-mLblood samples were collected from the caudal vein into tubes and immediately centrifuged at 1,900 × *g* for 15 min to obtain the serum. The serum samples were stored at − 80 °C until the TC, TG, LDL-C, and HDL-C concentrations were measured using the respective enzymatic assay commercial kits (AU5800, Beckman Coulter, California, USA).

2.6. Liver and adipose tissue analyses

Following blood sampling at the end of week 8, the rats were euthanized and their livers as well as their perirenal and epididymal white adipose tissue were excised, rinsed with cold saline, weighed, frozen, and stored at − 80 °C for < 1 month before analysis.

Hepatic TC and TG concentrations were analyzed using commercial assay kits (AU5800, Beckman Coulter, California, USA).

The liver and adipose tissue were embedded in paraffin and cut into 5-μm-thick sections. Samples were stained with hematoxylin and eosin and examined under a light microscope (×200 magnification) (BX53, Olympus, Tokyo, Japan).

All sections were evaluated under a light microscope by one expert pathologist who was blinded to the treatments. Nonalcoholic fatty liver disease activity scores (NASs), which were proposed by Kleiner et al. (2005), were used to assess liver histology. Lobular inflammation was scored from 0 to 3 (0, no foci;1, < 2 foci; 2, 2–4 foci; and 3, > 4 foci), steatosis was scored from 0 to 3 (0, < 5 %; 1, 5–33 %; 2, 34–66 %; and 3, > 66 %), and ballooning degeneration was scored from 0 to 2 (0, none; 1, few; and 2, many). One section from each sample was analyzed, and five views were selected in each section (200 × magnification).

The adipocyte area was measured by randomly selecting five complete closed-loop fat cells from each sample in each group at 200 × magnification.

2.7. Real-time quantitative polymerase chain reaction analysis

RNA was extracted from rat liver using TRIzol Reagent (TaKaRa, Kusatsu, Shiga, Japan) in accordance with the manufacturer's instructions. RNA concentrations were measured using a microspectrophotometer (K5600, Kaiao, Beijing, China). Reverse transcription was performed using a Prime Script RT reagent Kit with gDNA Eraser (TaKaRa). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using TB Green Premix Ex Taq II (TaKaRa) on a CFX96 RT-PCR Detection System (Bio-Rad, Hercules, CA, USA). Target mRNA expression was quantified in triplicate relative to expression of the reference gene *Gapdh* using the $2^{-\Delta\Delta Ct}$method. Primer sequences are listed in Table S3.

2.8. Statistical analysis

Test for equal variances for all data was conducted using a Levene method and data with unequal variances were subjected to Box-Cox transformation to follow assumption of equal variance. Student's *t* test was used to compare differences between the STD (normal reference)

and HCD groups in order to confirm the establishment of hypercholesterolemic rat model in the first 4 weeks. The protein content and subunit ratio, serum and liver lipid, and the expression of key genes related to cholesterol and lipid metabolism were analyzed by one-way ANOVA, and multiple comparisons among the groups were performed using Tukey-HSD test. Data are presented as mean ± standard deviation (SD). P values < 0.05 was considered to indicate statistical significance. All analyses were conducted using SPSS version 22.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Characterization of raw soybeans and its corresponding SPIs

SPIs were prepared from soybean cultivars that lacked specific subunits of 7S and 11S globulin proteins. Distinct electrophoretic patterns of total soybean protein of the six cultivars and their corresponding SPIs were observed (Fig. 1). SPI-1 prepared from DN47 contained all 7S and 11S subunits (Fig. 1, Lanes 1 and 2); SPI-2 prepared from DND360 lacked the α′ and α subunits of 7S (Fig. 1, Lanes 3 and 4); SPI-3 prepared from DND358 (Song et al., 2022) lacked the α subunit of 7S and the G1, G2, and G4 subunits of 11S (Fig. 1, Lanes 5 and 6); SPI-4 prepared from BSH-3 (Song et al., 2018) lacked the α′ subunit of 7S and the G1, G2, and G4 subunits of 11S (Fig. 1, Lanes 7 and 8); SPI-5 prepared from DND359 lacked the α′ and α subunits of 7S and the G1, G2, and G4 subunits of 11S (Fig. 1, Lanes 9 and 10); SPI-6 prepared from BSH-2 lacked the G1, G2, and G4 subunits of 11S (Fig. 1, Lanes 11 and 12). The protein expression profiles of each WSP preparation and the corresponding SPI were similar.

Table 1 summarizes the protein subunit composition, protein content, 11S:7S ratio, and α′:(7S + 11S) ratio of the six soybean cultivars. The protein contents of WSPs and SPIs ranged from 39.4 % to 47.7 % and 82.9 % to 87.3 %, respectively. The 11S:7S ratio ranged from 0.95 to 6.55. SPI-3 made from DND358 (null 7S α & 11S G1, G2, G4) had a higher α′:(7S + 11S) ratio compared with that of the other five SPIs. The SPIs prepared from DN47 and the other five soybeans that lacked specific subunits of 7S and 11S globulins were used in subsequent experiments.

3.2. Effects of SPI administration on serum and liver lipid parameters

Table 2 summarizes the effects of the six SPIs and FF on serum and liver lipid parameters. The HCD group had significantly higher TC, TG, and LDL-C levels, and a significantly lower HDL-C level in serum than the STD group ($p < 0.05$) (Table S2).

Oral administration of the six SPI experimental diets and FF to HMRs significantly reduced TC, TG, and LDL-C concentrations, and elevated the HDL-C level (except for SPI-4) compared with the HCD after 28 days of treatment (Table 2).

SPIs prepared from different genotypes of soybean differed in their HCE effects (Table 2). After 4 weeks of supplementation, relative to the HCD group, SPI groups exhibited 12.4 %–32.5 % lower serum TC, 17.3 %–27.4 % lower serum TG, and 27.4 %–44.3 % lower serum LDL-C. Furthermore, compared with that in the HCD group, the SPI groups showed a reduction in hepatic TC and TG by 13.1 %–43.4 % and 8.3 %–30.9 %, respectively (Table 2).

After 4 weeks of supplementation, rats in the six HCD + SPI groups showed a 20.0 %–63.3 % increase in HDL-C levels compared with the HCD group. It is important to note that the HCD + SPI-2 group showed higher HDL-C levels than the other five HCD + SPI and the HCD + FF group (Table 2). Administration of SPI-2 and FF increased HDL-C by 63.3 % and 51.7 %, respectively, relative to the HCD group. Consequently, marked improvements were evident in TC/HDL-C and TG/HDL-C ratios in SPI-2 groups (Table 2).

3.3. Effects of SPI administration on body weight, liver and adipose tissue weight and histology

Table 3 summarizes the effects of daily administration of SPIs and FF for 28 days on the body weight, liver weight, visceral fat weight, hepatosomatic index, and visceral somatic fat index of rats. No differences in initial body weight and final body wight were observed among the eight HCD-fed groups, whereas the HCD group (hypercholesterolemic control) had the lowest daily food intake value. Except for the liver weight and hepatosomatic index in the SPI-5 group in which no significant differences were identified, all values were significantly lower in the SPI and FF treatment groups compared with those in the HCD group, and the minimum liver weight and visceral fat weight were achieved in the SPI-3 group (Table 3). Moreover, a similar hepatosomatic index and visceral somatic fat index were observed in SPI-3 group compared with those in

Table 2
Serum and liver lipid profiles of HCD-fed HMRs untreated or administered FF or 1 of 6 SPIs for 4 weeks[1].

	Treatment								
	STD	HCD	HCD + FF	HCD + SPI-1	HCD + SPI-2	HCD + SPI-3	HCD + SPI-4	HCD + SPI-5	HCD + SPI-6
Serum lipid (mmol/L)									
TC	1.69 ± 0.18	2.74 ± 0.44^{a}	1.80 ± 0.21^{c}	1.86 ± 0.20^{c}	2.07 ± 0.35^{bc}	1.85 ± 0.18^{c}	2.19 ± 0.20^{bc}	2.40 ± 0.20^{ab}	2.28 ± 0.28^{abc}
TG	0.81 ± 0.24	1.68 ± 0.24^{a}	1.01 ± 0.26^{c}	1.35 ± 0.22^{abc}	1.22 ± 0.12^{bc}	1.22 ± 0.11^{bc}	1.39 ± 0.18^{ab}	1.25 ± 0.17^{bc}	1.37 ± 0.14^{ab}
LDL-C	0.31 ± 0.07	1.06 ± 0.16^{a}	0.58 ± 0.03^{c}	0.71 ± 0.05^{b}	0.71 ± 0.04^{b}	0.59 ± 0.02^{c}	0.70 ± 0.03^{b}	0.71 ± 0.08^{b}	0.77 ± 0.12^{b}
HDL-C	1.05 ± 0.13	0.60 ± 0.10^{d}	0.91 ± 0.07^{ab}	0.86 ± 0.07^{abc}	0.98 ± 0.07^{a}	0.83 ± 0.07^{bc}	0.72 ± 0.08 ^{cd}	0.74 ± 0.05^{c}	0.84 ± 0.05^{bc}
TC:HDL-C	1.63 ± 0.32	4.64 ± 0.67^{a}	1.99 ± 0.34^{c}	2.20 ± 0.33^{c}	2.09 ± 0.27^{c}	2.25 ± 0.36^{c}	3.05 ± 0.45^{b}	3.28 ± 0.46^{b}	2.72 ± 0.31^{bc}
TG:HDL-C	0.78 ± 0.27	2.94 ± 1.04^{a}	1.11 ± 0.25^{d}	1.60 ± 0.35^{bc}	1.25 ± 0.17 ^{cd}	1.48 ± 0.14^{bcd}	1.95 ± 0.39^{ab}	1.72 ± 0.34^{bc}	1.64 ± 0.25^{bc}
LDL-C:HDL-C	0.30 ± 0.08	1.81 ± 0.39^{a}	0.64 ± 0.06^{d}	0.83 ± 0.07^{bc}	0.72 ± 0.04 ^{cd}	0.72 ± 0.06 ^{cd}	0.98 ± 0.12^{b}	0.97 ± 0.14^{b}	0.92 ± 0.14^{b}
Liver lipid (nmol/g)									
TC	12.74 ± 2.74	25.51 ± 3.68^{a}	14.26 ± 2.86^{d}	15.55 ± 2.32 ^{cd}	19.85 ± 3.32^{abcd}	14.44 ± 3.39^{d}	18.64 ± 3.40^{bcd}	21.20 ± 4.23^{abc}	22.18 ± 4.68^{ab}
TG	16.39 ± 3.54	29.15 ± 2.59^{a}	19.22 ± 1.19^{d}	21.58 ± 1.48 ^{cd}	23.23 ± 1.81^{bcd}	20.13 ± 2.50^{d}	25.59 ± 3.82^{abc}	26.41 ± 2.68^{ab}	26.72 ± 1.55^{ab}

[1] Data are presented as the mean ± SD (n = 6/group). Labeled means in a row without a common superscript letter differ, $P < 0.05$. FF, fenofibrate; HCD, high-cholesterol diet; HDL-C, high-density lipoprotein-cholesterol; HMRs, hypercholesterolemic model rats; LDL-C, low-density lipoprotein-cholesterol; SPI, soy protein isolate; STD, standard diet used as normal reference; TC, total cholesterol; TG, triacylglycerol.

Table 3
Change in food consumption, body weight, liver and adipose tissues weights and relative organ weights in STD-fed rats and HCD-fed HMRs untreated or administered FF or 1 of 6 SPIs for 4 weeks[1].

	Dietary groups								
	STD	HCD	HCD + FF	HCD + SPI-1	HCD + SPI-2	HCD + SPI-3	HCD + SPI-4	HCD + SPI-5	HCD + SPI-6
Daily food intake(g/rat/day)	27.61 ± 1.65	22.85 ± 0.73^{b}	26.85 ± 1.13^{a}	26.00 ± 0.54^{a}	25.93 ± 0.88^{a}	26.43 ± 2.07^{a}	27.08 ± 1.97^{a}	26.60 ± 2.66^{a}	25.91 ± 1.23^{a}
Body weight (g)									
Initial body weight(g)	424.83 ± 29.84	433.50 ± 15.25	435.00 ± 15.59	432.00 ± 13.43	429.67 ± 17.20	431.50 ± 18.05	437.17 ± 24.40	429.67 ± 32.36	431.50 ± 26.10
Final body weight(g)	513.17 ± 38.06	535.00 ± 24.11	518.67 ± 28.37	513.67 ± 25.11	515.67 ± 33.67	515.83 ± 32.87	518.83 ± 37.34	513.50 ± 50.34	511.67 ± 33.90
Live and Adipose tissues weight (g)									
Liver weight (g)	17.22 ± 2.31	31.93 ± 3.05^{a}	15.64 ± 1.27^{d}	15.83 ± 1.69^{d}	20.61 ± 1.02^{c}	15.47 ± 1.60^{d}	23.00 ± 3.81^{bc}	30.97 ± 2.14^{a}	25.62 ± 0.85^{b}
Visceral fat weight (g)	13.49 ± 2.83	24.44 ± 0.94^{a}	15.00 ± 3.83^{bc}	15.06 ± 3.16^{bc}	14.66 ± 1.48^{bc}	10.58 ± 1.28^{c}	16.30 ± 3.23^{bc}	17.87 ± 3.89^{b}	17.42 ± 4.77^{b}
Relative organ weight (% body weight)									
Hepatosomatic index[2]	3.37 ± 0.49	5.96 ± 0.40^{a}	3.01 ± 0.10^{d}	3.08 ± 0.27^{d}	4.00 ± 0.20^{c}	3.00 ± 0.32^{d}	4.48 ± 0.93^{bc}	6.05 ± 0.39^{a}	5.02 ± 0.26^{b}
Visceral somatic fat index[3]	2.62 ± 0.46	4.58 ± 0.26^{a}	2.87 ± 0.63^{bc}	2.92 ± 0.49^{bc}	2.84 ± 0.13^{bc}	2.06 ± 0.30^{c}	3.13 ± 0.47^{b}	3.47 ± 0.65^{b}	3.37 ± 0.72^{b}

[1] Values are given as mean ± SD for six animals in each group (n = 6). Labeled means in a row without a common superscript letter differ, $P < 0.05$. FF, fenofibrate; HCD, high-cholesterol diet; HMRs, hypercholesterolemic model rats; SPI, soy protein isolate; STD, standard diet used as normal reference.
[2] Hepatosomatic index: liver weight (g)/body weight(g) 100.
[3] Visceral somatic fat index: visceral fat weight (g) /body weight (g) × 100.

the FF treatment group.

In the STD group (Normal reference), liver histology showed that hepatocytes were arranged in order and clearly, dense, and uniform, and nuclei were round, while no liver histological abnormalities were observed (Fig. 2A), whereas hepatocytes in the HCD group contained large fat vacuoles, nuclei were pushed to one side (Fig. 2B), and NAS scores were increased significantly (Fig. 2J). These results indicated that the rats had developed steatosis. Histological changes were also present after 28 days of SPI or FF administration. The degree of steatosis and ballooning degeneration in rats were reduced significantly and NAS scores were decreased significantly (Fig. 2J), but SPI groups differed in the severity of hepatocyte steatosis (Fig. 2D–I). These histological observations indicated that intake of SPIs prepared from different genotypes relieved fatty liver symptoms or hepatic lipid accumulation to various degrees in the livers of HMRs. A particularly marked amelioration of steatosis was present in the SPI-3 treatment group (Fig. 2F) in which liver histology was similar to that of the FF group (Fig. 2C and J).

Photomicrographs of white adipose tissue from each group are shown in Fig. 3. Adipose tissue cells in the STD group were arranged normally (Fig. 3A). The HCD increased the volume of adipose cells in HMRs (Fig. 3B). The adipose cell area in the HCD group (Fig. 3B) was significantly larger than that in the STD group (Fig. 3A). Compared with the HCD group, adipocytes were reduced in size to varying extents by the SPI treatments (Fig. 3D–I), which was particularly marked in the SPI-3 group (Fig. 3F). The area score of adipose cells in the SPI-3 group was significantly lower than that of the HCD group and very close to that of the FF group (Fig. 3J).

3.4. Effects of SPI administration on hepatic gene expression

We next evaluated the effects of SPIs on the expression of major cholesterol and lipid metabolism-related genes in the livers of HMRs by quantitative real-time PCR (Fig. 4). The mRNA expression of all genes was affected by FF to varying degrees. FF significantly reduced the expression of *Hmgcr*, *Srebp1*, and *Srebp2* ($p < 0.05$) (Fig. 4A–C), and significantly increased expression of *Cyp7a1* and *Ldlr* ($p < 0.05$) (Fig. 4D, and E) compared with the HCD group.

Daily administration of the six SPIs for 28 days resulted in similar trends in the expression changes of these genes in the FF group, although overall differences did not all achieve statistical significance. *Hmgcr* mRNA expression was significant lower in SPI-1, SPI-3, and SPI-6 groups compared with HCD groups ($p < 0.05$), and the most significant reduction in *Hmgcr* mRNA expression occurred in the SPI-6 group (Fig. 4A). Additionally, SPI-6 made from the BSH-2 (null 11S G1, G2, G4) genotype showed lower *Hmgcr* mRNA expression compared with that of the FF group (Fig. 4A). Compared with the HCD group, SPI-3 administration caused most significant downregulation in both *Srebp1* ($p < 0.05$) and *Srebp2* expression ($p < 0.05$) among six detected SPIs (Fig. 4B, C). In contrast to the decreases in *Hmgcr*, *Srebp1*, and *Srebp2* mRNA levels, *Cyp7a1* and *Ldlr* mRNA levels were generally upregulated in the six SPI groups compared with the HCD group. In particular, compared with the HCD group, SPI-3 administration caused a more obvious upregulation in *Cyp7a1* and *Ldlr* mRNA expression by 886 %, and 368 %, respectively (Fig. 4D, E). Compared with the FF treatment group, *Cyp7a1* and *Ldlr* mRNA levels were upregulated by 53.8 % and 59.8 %, respectively, in the SPI-3 group (Fig. 4D, E).

4. Discussion

SPI affects the plasma cholesterol level and processed foods prepared from SPI are also effective to reduce human plasma cholesterol levels (Anderson, Johnstone, & Cook-Newell, 1995; Komatsu, Komatsu, Matsuo, Nagata, & Yamagishi, 1990; Kito, Moriyama, Kimura, & Kambara, 1993). However, limited information is available on the effect of raw soybean genotypes on the HCE of SPI. The SPIs used in the present study were obtained by direct processing of six WSPs. Protein analysis by SDS-PAGE revealed that the protein bands were similar between each WSP and its SPI, indicating that all SPIs investigated were not significantly altered from the major constituents of the corresponding raw soybean (Fig. 1). The relative abundance of each band was enhanced in the corresponding SPI, indicating that each subunit content of raw soybean was enriched in its SPI (Fig. 1).

The hypocholesterolemic activity of soy components (mainly proteins and associated peptides) has been well-known for decades (Anderson, & Bush, 2011; Lee et al., 2012; Sirtori et al., 1979; Gardner, Messina, Kiazand, Morris, & Franke 2007; Oldewage-Theron, & Egal, 2013; Blanco et al., 2019; Takahashi, & Konishi, 2011; Kobayashi, Hirahata, Egusa, & Fukuda, 2012; Fukui et al., 2004; Kohno, Hirotsuka, Kito, & Matsuzawa, 2006). The observed effects are likely not due to intact proteins, but instead to peptides derived from the proteins by gastrointestinal digestion, which after absorption and transport to the liver, modulate cholesterol homeostasis (Nagaoka, 2019; Caponio,

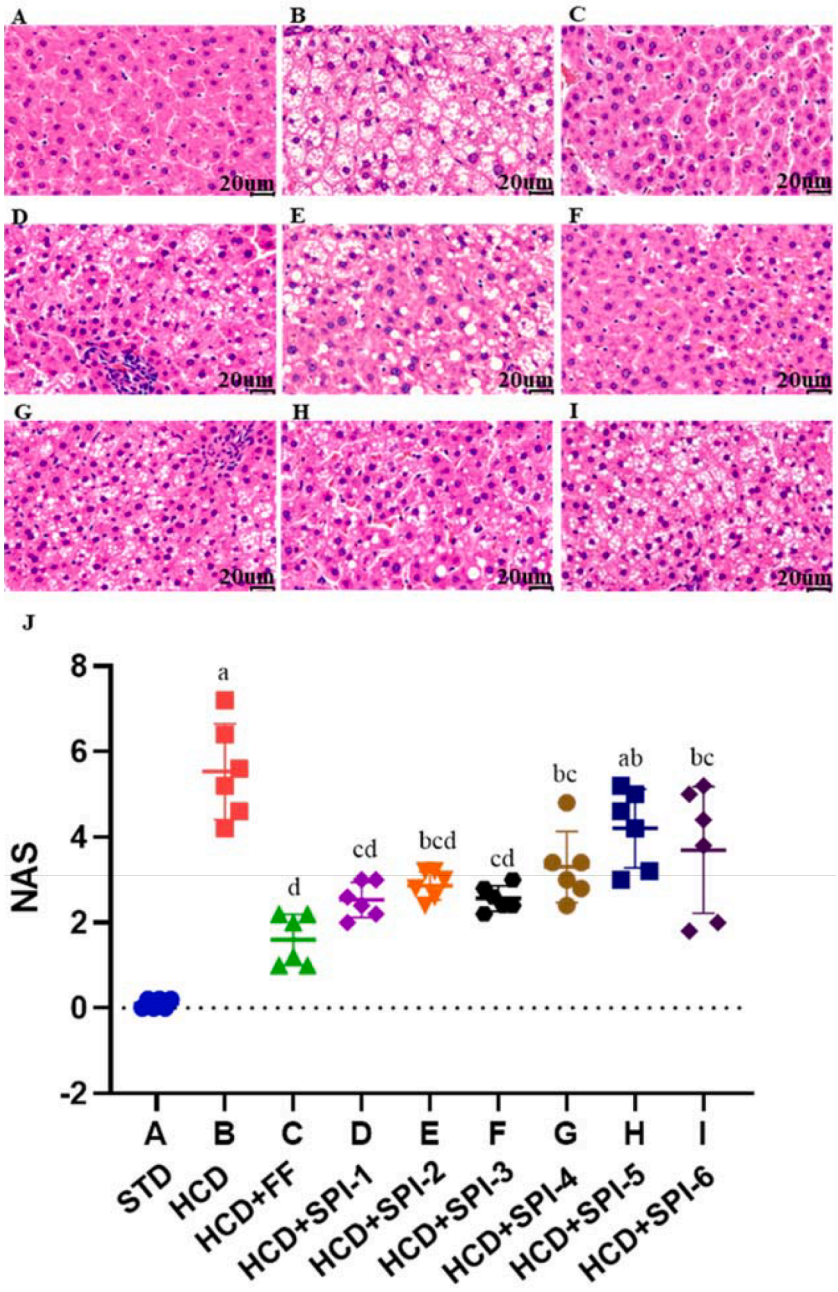

Fig. 2. Hematoxylin and eosin staining of liver tissue from STD-fed rats (normal cholesterol reference) and HCD-fed HMRs untreated or administered FF or SPI-1 to 6 for 4 weeks (200 × magnification). (A) STD group, (B) HCD group (hypercholesterolemia control), (C) HCD + FF group, (D) HCD + SPI-1 group, (E) HCD + SPI-2, (F) HCD + SPI-3, (G) HCD + SPI-4, (H) HCD + SPI-5, (I) HCD + SPI-6. (J) Nonalcoholic fatty liver disease activity score (NAS) in the liver tissue of the nine groups. FF, fenofibrate; HCD, high-cholesterol diet; HMR, hypercholesterolemic model rat; SPI, soy protein isolate; STD, standard diet.

Wang, DiCiaula, & De Angelis, 2020; Kim, Yang, & Kim, 2021; Yamamoto, Fukuda, Zhang, & Sakai, 1996; Kwon et al., 2002; Pak, Koo, Kasymova, & Kwon, 2005; Mochizuki et al., 2009; Sugano et al.,1990; Lovati et al., 2000). Two peptides from soybean 7S globulin, i.e., YVVNPDNDEN (peptide 2) and YVVNPDNNEN (peptide 3), are absorbed by human enterocytes. The former were shown to increase LDL uptake and degradation in hepatocytes, and both peptides act as competitive inhibitors of HMGCoAR with a statin-like behavior (Lammi, Zanoni, Arnoldi, & Vistoli, 2015). FVVNATSN is another 7S peptide that increases *Ldlr* transcription (Cho, Juillerat, & Lee, 2008). IAVPGEVA, IAVPTGVA, and LPYP, are three peptides from soy glycinin hydrolysis, and they are able to interfere with the catalytic activity of HMGCoAR and to modulate cholesterol metabolism through the activation of the LDLR-SREBP2 pathway (Lammi, Zanoni, & Arnoldi, 2015).

The present study showed that SPI-1, SPI-2, and SPI-3 reduced serum TC levels, SPI-2 and SPI-3 reduced TG levels, and SPI-3 reduced LDL-C levels to a similar extent as FF treatment (Table 2). In addition, the histological observations indicated that intake of SPIs prepared from different soybean genotypes relieved fatty liver symptoms and suppressed the expansion of white adipose tissue to various degrees in the liver and adipose tissue of HMRs (Fig. 2 and Fig. 3). All of the above effects may be caused by different specific peptides derived from the different SPI subunit compositions through gastrointestinal digestion. The extent to which the SPI protein or its peptides exert cholesterol-lowering effects requires further investigation.

Intestinal cholesterol absorption is an important determinant of circulating cholesterol levels and thus has an impact on cardiovascular health. The intestinal bile acids (BAs) are taken up into the enterocytes by the apical sodium-dependent BAs transporter and then secreted via the basolateral site into the portal circulation. Terpstra et al. (1982) suggested that the hypocholesterolemic action of soy protein might be due to increased fecal steroids. It has been reported that soybean peptides contain a high-molecular-weight (undigested) fraction (HMF), which is responsible for the simulation of fecal steroid excretion, HMF

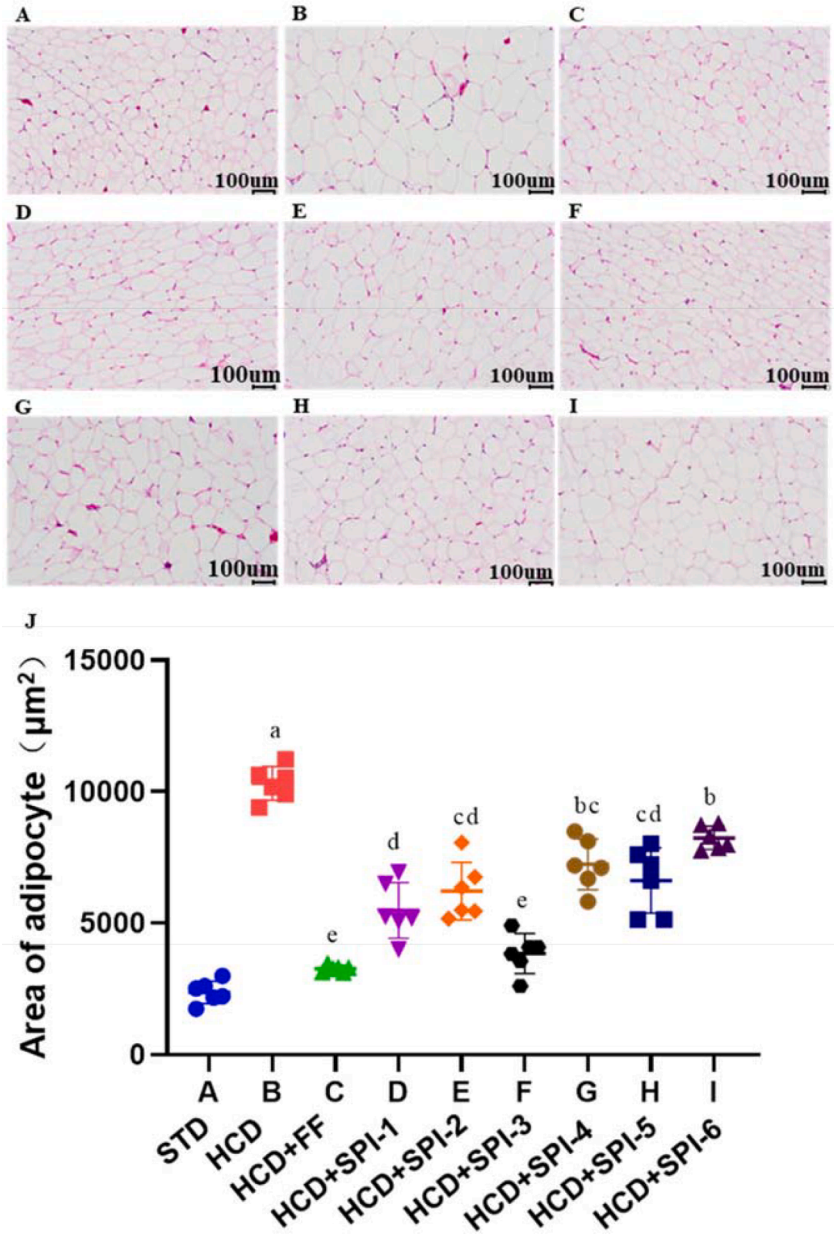

Fig. 3. Hematoxylin and eosin staining of adipose tissue from STD-fed rats (normal cholesterol reference) and HCD-fed HMRs untreated or administered FF or SPI-1 to 6 for 4 weeks (200 × magnification). (A) STD group (normal reference), (B) HCD group (hypercholesterolemia control), (C) HCD + FF group, (D) HCD + SPI-1 group, (E) HCD + SPI-2, (F) HCD + SPI-3, (G) HCD + SPI-4, (H) HCD + SPI-5, and (I) HCD + SPI-6. (J) Adipocyte area scores in the nine groups. FF, fenofibrate; HCD, high-cholesterol diet; HMRs, hypercholesterolemic model rats; SPI, soy protein isolate; STD, standard diet.

appears to be of substantial benefit for the treatment of hypercholesterolemia since it disrupts intestinal absorption not only of acidic but also neutral steroids without largely influencing intestinal function (Sugano et al., 1988; Sugano, 1990; Sugano, & Goto, 1990; Sugano et al., 1990). Furthermore, another study showed that the absorption of cholesterol was significantly lower from diets containing soy protein, but bile acid excretion was not significantly affected, supporting the hypothesis that the hypocholesterolemic effect of soy protein is mediated in part by decreased cholesterol absorption (Greaves, Wilson, Rudel, Williams, &, Wagner, 2000).

Additionally, 7S and 11S globulins have a sequestering action on bile acids, and they reduce intestinal cholesterol absorption and promote a plasma cholesterol-lowering effect (Pak, Koo, Kasymova, & Kwon, 2005; Lammi, Zanoni, Arnoldi, & Vistoli, 2015; Cho, Juillerat, & Lee, 2008; Lammi, Zanoni, & Arnoldi, 2015; Nagaoka, Nakamura, Shibata, & Kanamaru, 2010). Also, recently, Liu et al. (2017) demonstrated that the 7S protein was an effective cholesterol-lowering ingredient in soybean as it was able to inhibit the cholesterol absorption, increase the bile acid synthesis and decrease the cholesterol synthesis. Moreover, the contributions of individual 7S and 11S subunits to cholesterol-lowering properties were different, and the subunit involved includes α′ from 7S (Lammi, Zanoni, Arnoldi, & Vistoli, 2015) and A1aB1b and A2B1a from 11S (Choi, Adachi, & Utsumi, 2002, 2004; Takenaka, Utsumi, & Yoshikawa, 2000). The α′ subunit plays a major role in the LDL-C-lowering properties of the 7S globulin (Duranti et al., 2004; Anderson, Johnstone, & Cook-Newell, 1995; Manzoni et al., 2003; Consonni et al., 2010, 2011; Cabanos et al., 2014). Oral administration of the α′ subunit at a dose of 10 mg·kg body weight-1·day-1 to a model hypercholesterolemic rat for 28 days significantly decreased serum LDL-C levels compared with that of control rats (Duranti et al., 2004). Moreover, the α′ subunit increases hepatic LDL uptake, thereby improving hypercholesterolemic conditions, which is accompanied by a decrease in serum triglycerides (Duranti et al., 2004). Using the 7S α′ subunit, Cabanos et al. developed transgenic rice that exhibits hypocholesterolemic activity in rats (Cabanos et al., 2014).

The choice of raw soybean material determines its SPI product characteristics, which is of great importance with respect to functional properties and applications. In the present study, SPI-3 was prepared

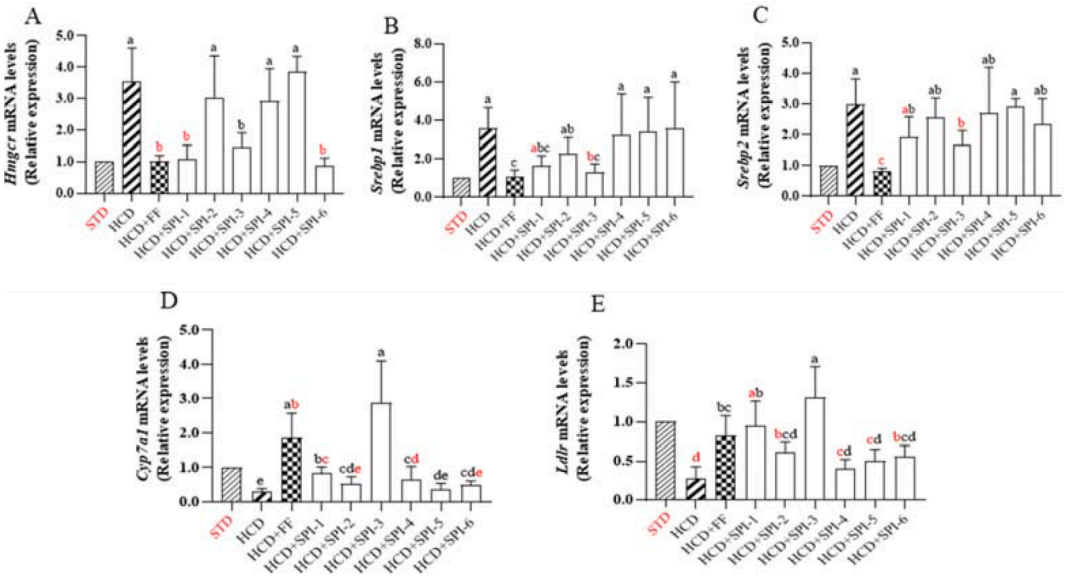

Fig. 4. Effect of ingestion of SPIs with different genotypes on the expression of crucial cholesterol and lipid metabolism-related hepatic genes in HCD-fed HMRs untreated or administered FF or SPI-1 to 6 for 4 weeks. (A) *Hmgcr*, (B) *Srebp1*, (C) *Srebp2*, (D) *Cyp7a1*, and (E) *Ldlr*. Values were normalized to the reference gene *Gapdh*. Each bar represents the mean ± SD (n = 6 per group). Labeled means without a common superscript letter differ, $P < 0.05$. *Cyp7a1*, Cholesterol 7α-hydroxylase; FF, fenofibrate; *Gapdh*, Glyceraldehyde3-phosphate dehydrogenase; HCD, high-cholesterol diet; *Hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase; HMRs, hypercholesterolemic model rats; *Ldlr*, low-density lipoprotein receptor; SPI, soy protein isolate; STD, standard diet used as normal reference. *Srebp1*, Sterol regulatory element-binding transcription factor 1; and *Srebp2*, Sterol regulatory element-binding transcription factor 2.

from DND358 that lacks the α subunit of 7S and the G1, G2, and G4 subunits of 11S (Table 1, Fig. 1). Loss of these subunits results in a compensatory increase in the proportion of the α′ subunit in DND358. Consequently, DND358 had the higher α′-subunit content (Table 1) and the enriched α′-subunit content of dietary SPI-3 might contribute to its pronounced HCE similar to that of FF. The null α, G1, G2, and G4 genotype of the soybean cultivar DND358 used to prepare SPI-3 is a soybean protein source suitable to prepare SPI products for cholesterol-lowering dietary therapy (Song et al., 2022). Our results suggest that, in combination with a high yield, an enriched α′ subunit of 7S can be used in hypocholesterolemic soybean development (Song et al., 2022).

HDL-C levels are a strong, independent inverse predictor of cardiovascular events (Nakanishi et al., 2009; Horie et al., 2014). In the Framingham Heart Study, the HDL-C level was a stronger risk factor for coronary heart disease than the LDL-C level (Castelli et al., 1977). Data analysis of four large studies concluded that each increase of 1 mg/dL (0.3 mmol per liter) in HDL-C is associated with a decrease of 2 %–3% in the risk of future heart disease (Gordon et al., 1989). Recently, increasing evidence indicates that lipoprotein ratios, which have atherogenic components (TC, TG, and LDL-C) in the numerator and anti-atherogenic components (HDL-C) in the denominator, may be better predictors to evaluate the severity of coronary heart disease than individual lipid parameters (Ballantyne, & Hoogeveen, 2003). In the present study, after 28 days of treatment, the HDL-C level in the SPI-2 group was higher than that in the FF group (Table 2).The above result indicates that consumption of SPI-2 may induce a higher serum HDL-C level than that induced by the FF treatment (Table 2). The marked increase of the HDL-C level in the SPI-2 group consequently resulted in a significant decrease in HDL-C-related ratios (Table 2). The ratios of TC/HDL-C and TG/HDL-C in the SPI-2 group were closer to the outcome of FF treatment. Furthermore, SPI-2 and SPI-3 groups had the similar LDL-C/HDL-C ratios (Table 2). Although the values are very similar, it is clear that the mechanism is quite different. Different from the lower LDL-C level induced by SPI-3, the remarkable increase in the HDL-C level was a crucial factor for the pronounced LDL-C/HDL-C ratio in the SPI-2 group. Further study is needed to elucidate the molecular mechanism of the significant increase in the HDL-C level due to SPI-2 consumption by HMRs.

To gain a better understanding of the SPI effect on cholesterol metabolism in HMRs, we measured the expression of genes involved in cholesterol homeostasis. SPIs had differential effects on expression of *Hmgcr*, *Srebp1*, *Srebp2*, *Cyp7a1*, and *Ldlr* genes encoding six major proteins that regulate cholesterol and fatty acid metabolisms, and the overall trends were similar to those of fenofibrate (Fig. 4).

HMGCR is the rate-controlling enzyme in the endogenous cholesterol biosynthesis pathway. At the transcriptional level, HMGCR expression is regulated by sterol regulatory element-binding proteins (SREBPs) and it is well established that SREBPs are adipogenic transcription factors that control the metabolism of cellular cholesterol and fatty acids in hepatocytes (Brown, & Goldstein, 1997; Yang et al., 2019). In the present study, compared with the HCD group, *Hmgcr* mRNA levels were significantly reduced in the SPI-6 group (75.4 %) (Fig. 4A). Furthermore, *Srebp1* mRNA expression was significantly reduced in the SPI-3 group (63.4 %) (Fig. 4B). These results suggested that the cholesterol-lowering mechanism of SPI-3 and SPI-6 may be related to inhibition of the cholesterol synthesis pathway by downregulation of HMGCR/SREBP signaling pathways.

Cholesterol and BAs have been classically connected in a simple model in which cholesterol is the biosynthetic precursor of BAs and BAs are participants in intestinal cholesterol absorption. CYP7a1 is considered to be the rate-limiting enzyme for cholesterol catabolism into BAs. CYP7a1 induction stimulates the conversion of cholesterol to BAs, resulting in a relative deprivation of hepatic microsomal cholesterol content, followed by upregulation of LDL receptor expression and activity, which consequently reduces plasma LDL cholesterol levels (Cohen, 1999). Indeed, increased CYP7a1 has been demonstrated to increase the rate of cholesterol conversion to bile acids in hepatocytes (Férézou et al., 1997; García-Mediavilla, Villares, Culebras, Báyon, & González-Gallego, 2003). In the present study, hepatic mRNA expression levels of *Cyp7a1* were significantly elevated in rats treated with SPI-3 by 8.86-fold, compared with the HCD group ($p < 0.05$) (Fig. 4D). These data indicate that SPI-3 effectively induces expression of the Cyp7a1 gene. To clarify cholesterol absorption mechanism of different soybean genotype, further in-depth studies on effect of dietary SPI-3 on hepatic

bile acid metabolism is necessary.

The LDLR is a single transmembrane glycoprotein that is ubiquitously expressed on the cell surface and mediates LDL entry into cells via clathrin-dependent vesicular endocytosis (Brown, & Goldstein, 1986; Goldstein, & Brown, 2009). It internalizes LDL to lower plasma LDL and cholesterol concentrations. Previous studies have demonstrated that the soybean 7S globulin, particularly the α′-subunit, is responsible for directly upregulating LDLR activation (Lovati, Manzoni, Gianazza, & Sirtori, 1998; Lovati et al., 1992; Manzoni, Lovati, Gianazza, Morita, & Sirtori, 1998; Sirtori et al., 1984; Lovati, et al., 1987, 1991). In the present study, SPI-3 treatment showed a significant effect on *Ldlr* gene expression. Hepatic mRNA expression levels of *Ldlr* were increased 3.68-fold and 0.6-fold in the HCD + SPI-3 group compared with the HCD and HCD + FF groups, respectively (Fig. 4F). These data suggest that the cholesterol-lowering activity of SPI-3 is associated with upregulation of LDLR-mediated cholesterol uptake and may be due to the enriched α′-subunit in the SPI-3 genotype (Table 1).

Taken together, these findings indicate that soybeans that differ in their globulin subunit composition generate SPIs with marked different HCEs, which provide different supplemental therapy for hypercholesterolemia in HMRs and represent different molecular mechanisms. Hepatic gene expression analysis (Fig. 4) indicated that the cholesterol-lowering effect of SPIs in HMRs is caused at least in part by regulation of cholesterol synthesis (e.g., SPI-3, SPI-6, downregulation of HMGCR/SREBP signaling pathways), cholesterol catabolism (e.g., SPI-3, upregulation of *Cyp7a1*), and upregulation of LDLR-mediated cholesterol uptake (e.g., SPI-3, upregulation of *Ldlr* gene expression). To summarize, hepatic gene differential expression generated to date suggest that the HCE of a certain genotype would be expected to be derived from the interactions of several cholesterol metabolic pathways, further studies are required to determine precisely and confirm the mechanisms involved in the various effects of SPIs with different genotypes.

Ethics statement

The animal experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Laboratory Animal Ethics Committee.

CRediT authorship contribution statement

Shanshan Liu: Conceptualization, Writing – original draft. **Tingting Luo:** Project administration. **Yanru Song:** Data curation. **Hongbo Ren:** Investigation. **Zhendong Qiu:** Data curation. **Chongxuan Ma:** Formal analysis. **Yusu Tian:** Formal analysis. **Qi Wu:** Formal analysis. **Fu Wang:** Data curation. **Hari B. Krishnan:** Writing – review & editing. **Wenhua Yu:** Data curation. **Jiliang Yang:** Writing – review & editing, Writing – review & editing. **Pengfei Xu:** Data curation. **Shuzhen Zhang:** Funding acquisition. **Bo Song:** Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105347.

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