Original Research

Soybean Protein Hydrolysate Improves Plasma and Liver Lipid Profiles in Rats Fed High-Cholesterol Diet

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Key words: soybean protein, hydrolysate, hypercholesterolemia, 7α-hydroxylase, lipid metabolism, rat

Objective: This investigation attempted to clarify the hypolipidemic effects of non-dialyzed soybean protein hydrolysate (NSPH), which is hydrolyzed by pepsin from soybean acid-precipitated protein (APP), in rats fed a cholesterol-rich diet.

Methods: Forty Sprague-Dawley rats were divided into four groups as the control group (19.7% casein), the APP group (14.7% casein + 5% APP), the NSPH group (14.7% casein + 5% NSPH), and the ISO group (19.7% casein + 0.0013% soy isoflavone).

Results: After 12-week experimental period, the APP and NSPH groups had a significant lower plasma total cholesterol, triglycerides, and LDL-cholesterol concentrations compared with the control group. Additionally, the atherosclerosis index in APP and NSPH group had also markedly decreased. Liver cholesterol and triglyceride contents of the APP and NSPH group were significantly lower than those of the control group. There were no different in plasma LDL-C, liver cholesterol and triglycerides between the ISO group and control group. Fecal excretion of neutral steroids and nitrogen compounds was significantly higher in the APP and NSPH groups than that in the control group. An *in vitro* study also showed that NSPH, compared with casein, obviously decreased cholesterol micellar solubility.

Conclusion: These results suggested that NSPH may decrease lipid accumulation in the liver and have a hypolipidemic effect by enhancing excretion and inhibiting absorption of lipids.

INTRODUCTION

Cardiovascular disease (CVD), which is often associated with hypercholesterolemia, has become the major cause of death in many countries [1]. Dietary lipids and cholesterol are important factors that affect lipid metabolism. Although the primary emphasis has traditionally been put on the quality and quantity of lipid intake, effects of other food components on lipid metabolism should not be ignored [2–4].

Soybean and soybean products have been widely used in the oriental countries as an important dietary proteins source for more then 1000 years [5]. According to epidemiological surveys, researchers suggested that the lower incidence of CVD in Asia countries than western countries might be related with the greater consumption of soybean foods [6]. In 1999, the US Food and Drug Administration also published a health claim in which indicated that a daily intake of 25 g of soybean protein could prevent CVD [7].

Soybean protein hydrolyzed by certain enzymes, such as pepsin or trypsin, can be separated into the digestible low-molecular-weight fraction (LMF) and the undigestible high-molecular-weight fraction (HMF). Sugano *et al.* [8] indicated that LMF increases plasma cholesterol concentrations, but HMF obviously lowers plasma cholesterol. Furthermore, HMF reduced plasma cholesterol to a greater extent than intact soybean protein, and promoted fecal steroid excretion [9]. Non-dialyzed soybean protein hydrolysate (NSPH), a product of peptic-digested soybean protein which contains mostly the high-molecular weight fraction, has been reported that replacing 5% casein in rat diet by NSPH effectively lowered plasma cholesterol concentration [10], but the mechanism still remained unknown.

Soy isoflavones, a kind of phytoestrogens, have similar

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structure to mammalian estrogen and was reported to have cholesterol-lowering effects [11]. However, some studies have also shown that soy isoflavone extracts diminish the lipid-lowering effects of soybean protein [12]. The aim of the study is to investigate the mechanism of the hypolipidemic effects of NSPH in rats fed a cholesterol-rich diet and to clarify the effects of isoflavones in the beneficial effects of soy protein.

MATERIALS AND METHODS

Preparation of Soybean Acid-Precipitated Protein

Soybean acid-precipitated protein (APP) was prepared from *Glycine max* using the method of Iwabuchi and Yamauchi [13]. Fifty grams of defatted soybean powder was dissolved in 500 mL Tris-HCl buffer (0.03 M, pH 8.0), and the solution was mixed well and centrifuged for 20 min (12,000 $\times g$, 30 °C). The precipitates were discarded and the pH value of the supernatants was adjusted to 4.6. After 60 min in a 4 °C ice-bath, the solution was centrifuged for 20 min (10,000 $\times g$, 4 °C) and then the precipitates were dissolved in 0.05 M PBS buffer (pH 7.6). After dialyzing against water for 48 h, the APP was lyophilized and stored at 4 °C until use.

Preparation of NSPH

Fifty grams of APP was dissolved in 1 L of deionized water. The pH of the solution was adjusted to 2.0 with 2 N HCl, and 0.15 g of pepsin (from porcine gastric mucosa, EC.3.4.23.1, Merck, Darmstadt, Germany) was added. After 24 h of digestion at 37°C, the hydrolysates were neutralized against water for 48 h. The NSPH fraction was lyophilized and stored at 4 °C until use. The isoflavone concentration was determined using high-performance liquid chromatography with UV detection by the method of Klump *et al.* [14]. APP and NSPH used in this study contained 262 μg/g and 102 μg/g isoflavone, respectively. The *in vitro* micellar solubility of cholesterol with various proteins was measured by the method of Ikeda *et al.* [15].

Animals, Diets, and Experimental Design

Forty male Sprague-Dawley rats were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Rats were individually housed in a room maintained at 23 ± 2 °C with 55% ± 10% humidity and a 12-h light/dark cycle. Investigators followed Taipei Medical University Laboratory Animal Center as described in the guide for the care and use of laboratory animals. Animals were fed a standard rat chow diet (Rodent Laboratory Chow 5001, Purina Mills, Inc., St. Louis, MO) for 1 week, and then randomly divided into four groups (n = 10). Rats were then given control or experimental diets with 0.5% cholesterol for 12 weeks. Rats were fed a casein control diet (19.7% casein; the control group), a 5% APP-replaced diet (14.5% casein + 5% APP; the APP group), a 5% NSPH-replaced diet (15% casein + 5% NSPH; the NSPH group), or an isoflavone-treated diet (19.7% casein + 0.0013% soy isoflavone; the ISO group) which contained the same amount of isoflavone as the APP group; other components were mixed in appropriate proportions based on an AIN-93M diet [16] (Table 1). Energy contents of all diets were 17.85 kJ/g. Food intake and body weight were recorded daily during the experimental period.

Table 1. Composition of the Experimental Diets (%)

Ingredients[1]	Group			
	Control	APP	NSPH	ISO
Maize starch	51	51	51	51
Casein	19.7	14.7	14.7	19.7
APP	-	5	-	-
NSPH	-	-	5	-
Sucrose	10.9	10.9	10.9	10.9
Soybean oil	10.5	10.5	10.5	10.5
Mineral	4	4	4	4
Cellulose	2	2	2	2
Vitamin	1	1	1	1
Cholesterol	0.5	0.5	0.5	0.5
Methionine	0.3	0.3	0.3	0.3
Choline	0.05	0.05	0.05	0.05
Cholic acid	0.05	0.05	0.05	0.05
Isoflavone[2]	-	-	-	0.0013

[1] Casein (high nitrogen), sucrose (food grade), soybean oil, mineral mixture (AIN-93M mineral mixture), cellulose (non-nutritive bulk), and vitamin mixture (AIN-93M vitamin mixture) were obtained from ICN Biochemicals (Aurora, OH). Maize starch was purchased from Samyang Genex Corp. (Seoul, Korea). Cholesterol, choline bitartrate, and cholic acid were obtained from Sigma (St. Louis, MO). APP, soybean acid precipitated protein. NSPH, non-dialyzed soybean protein hydrolysate.

[2] Contains 58% genistin, 30% diazin, 6% ginistein, and 6% diazein, and was purchased from Sigma (St. Louis, MO). The isoflavones profile is similar to soybean acid precipitated protein.

Table 2. Changes in Body Weight, Daily Food Intake, Feeding Efficiency, and Liver Weight of SD Rats Fed Different Diets[1]

	Diet group			
	Control	APP	NSPH	ISO
Initial body weight (g)	228.9 ± 11.9	231.4 ± 16.1	231.1 ± 13.3	230.8 ± 12.9
Final body weight (g)	613.3 ± 40.3^{ab}	602.8 ± 44.8^{ab}	618.8 ± 21.5^{a}	581.9 ± 40.6^{b}
Daily weight gain (g)	4.5 ± 0.5^{ab}	4.4 ± 0.5^{ab}	4.6 ± 0.4^{a}	4.1 ± 0.5^{b}
Daily food intake (g)	24.0 ± 1.2	23.8 ± 1.1	24.2 ± 0.6	23.8 ± 0.8
Feeding efficiency[2] (%)	18.8 ± 1.8	18.3 ± 1.4	18.8 ± 1.2	17.4 ± 2.3
Liver weight (g)	26.6 ± 4.4	23.0 ± 4.1	24.6 ± 2.7	23.2 ± 5.0
Hepatosomatic index[3] (%)	4.3 ± 0.6	3.8 ± 0.4	4.0 ± 0.4	4.0 ± 0.7

[1] Data are expressed as the mean ± SD ($n = 10$); values in a row with different superscripts significantly differ ($p < 0.05$) as analyzed by Duncan's multiple range test.
[2] Feeding efficiency: (daily weight gain/daily food intake) × 100%.
[3] Relative liver weight: (liver weight/body weight) × 100%.

Sample Collection and Analysis

Blood was collected from the tail vein of rats every 2 weeks for lipid analysis. At the end of the experiment, rats were fasted for 12 h and then sacrificed. Blood was collected from an abdominal vein. Plasma total cholesterol (TC) and triglyceride (TG) concentrations were assayed enzymatically using the method described by Richmond [17] and Mcgowan *et al.* [18], respectively. The plasma from animals was collected in EDTA tubes for lipoprotein separation according to the method described by Rayssiguier *et al.* [19]. Samples were supplemented with different densities of NaBr ($d = 1.006$, 1.055 and 1.210) and ultracentrifugation was performed at 4 °C and 120,000 × *g* for 6 h. Lipoproteins were then stored at −20 °C until analysis. Liver was perfused with cold normal saline before excision. One part of the liver was excised and soaked in 10% formaldehyde, and the rest part was stored at −70 °C for lipids analysis. Liver samples fixed with 10% formaldehyde were sliced and then stained with haematoxylin and eosin stain. Degrees of fatty liver was measured according to the method of Teramoto *et al.* [23] by a pathologist. Liver lipids were extracted by the method of Folch *et al.* [20]. Cholesterol and triglyceride concentrations in the liver were determined with diagnostic kits (Randox Laboratories, Antrim, UK) with cholesterol and glycerol as standards, respectively. The activity of 7α-hydroxylase was determined by the method of Rudel *et al.* [21]. Briefly, microsomes were separated from the livers and then quick-frozen in liquid nitrogen and stored at −70 °C until the assay. An RP-HPLC method was used for the 7α-hydroxylase assay based on Ogishima & Okuda [22]. Feces samples were collected for 2 days every 4 weeks during the experimental period, dried for 48 h and stored at −70 °C till analysis. Fecal neutral steroids and bile acids concentrations were analyzed according to the method of Suckling *et al.* [24] and Chezem & Story [25], respectively. Fecal nitrogen compounds were determined using the Kjeldahl method.

Statistical Analysis

Results were analyzed by SAS (Version 8.0, Clay, NC). One-way ANOVA and Duncan's multiple range test were used to determine the treatment effect among three groups and the differences between two groups, respectively. Differences were analyzed at each time point. All values were shown as mean ± SD. A difference of $p < 0.05$ was considered significant.

RESULTS

Food Intake, Body Weight, and Feeding Efficiency

As shown in Table 2, none of the experimental diets significantly affected food intake, feeding efficiency, or liver weight ($p > 0.05$). However, the final body weight and the daily weight gain of the ISO group were significantly lower than those of the NSPH group ($p < 0.05$).

Micellar Solubility

APP and NSPH both significantly decreased the micellar solubility of cholesterol compared to the control group ($p < 0.05$), and that of the NSPH group was obviously lower than the APP group ($p < 0.05$, Table 3). However, there were no significant differences in the micellar solubility of bile acids for all proteins ($p > 0.05$).

Blood Lipids

As shown in Fig. 1, plasma total cholesterol concentration significantly increased ($p < 0.05$) after animals were fed a

Table 3. Levels of Cholesterol and Bile Acid Micellar Solubility of Different Proteins in Feces[1]

Micellar solubility (mmol/L)	Protein		
	Casein	APP	NSPH
Cholesterol	0.48 ± 0.03^{a}	0.40 ± 0.02^{b}	0.20 ± 0.04^{c}
Bile acid	5.62 ± 0.20	5.52 ± 0.28	5.57 ± 0.19

[1] Data are expressed as the mean ± SD ($n = 3$); values in a row with different superscripts significantly differ ($p < 0.05$) as analyzed by Duncan's multiple range test.

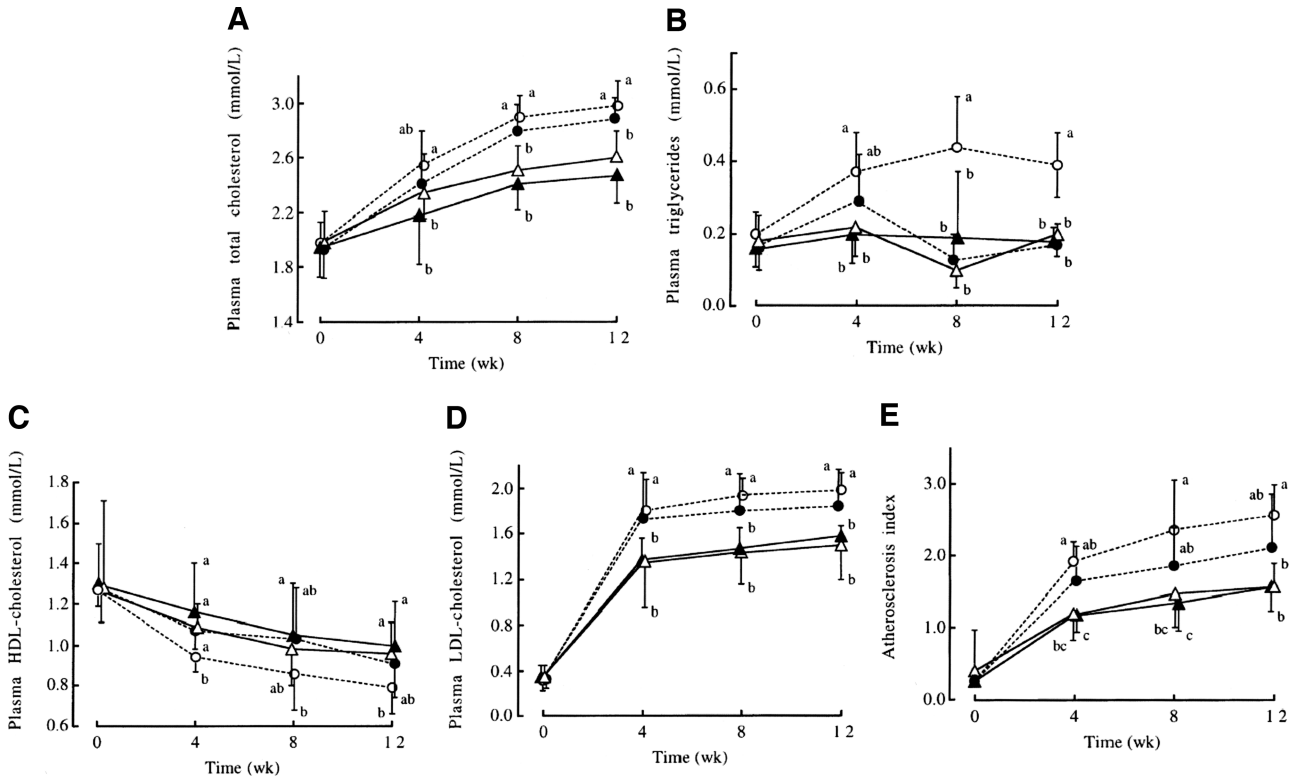

Fig. 1. Effects of different diets on plasma total cholesterol (a), triglyceride (b), HDL-cholesterol (c), and LDL-cholesterol (d) concentrations (mmol/L), and the atherosclerosis index (e) in SD rats. Data are expressed as the mean ± SD ($n = 10$); values in a row with different superscripts significantly differ ($p < 0.05$) as analyzed by Duncan's multiple range test.

○= Control group, △ = APP group, ▲= NSPH group, ● = ISO group.

cholesterol-rich diet. Plasma total cholesterol concentrations of the APP and NSPH groups were significantly lower than that of the control group since week 4 ($p < 0.05$, Fig. 1a). Plasma triglyceride concentrations of the APP and NSPH groups were significantly lower than that of the control group from week 4 to the end of the experiment ($p < 0.05$, Fig. 1b). However, the plasma triglyceride concentration of the ISO group was significantly lower than that of the control group since week 8 ($p < 0.05$). There were significant differences in HDL-cholesterol concentrations between the NSPH and control groups ($p < 0.05$, Fig. 1c). Additionally, NSPH obviously decreased LDL-cholesterol concentrations and the atherosclerosis index (AI, as the LDL/HDL-cholesterol ratio; Potter, 1995) from weeks 4 to 12 when compared with the control group ($p < 0.05$, Fig. 1d, e).

Liver

Different diets had no significant effect on the liver weight or the relative liver weight of any group ($p > 0.05$, Table 2). Liver total cholesterol and triglyceride concentrations of the APP and NSPH groups were both significantly lower than those of the control group ($p < 0.05$, Table 4). Nevertheless, there were no significant differences in 7α-hydroxylase activity among the groups ($p > 0.05$, data not shown). The extents of

Table 4. Changes in Liver Total Cholesterol and Triglyceride Concentrations of SD Rats Fed Different Diets[1]

	Diet group			
	Control	APP	NSPH	ISO
Total cholesterol				
μmol/g liver	30.5 ± 4.2^{a}	23.7 ± 5.3^{b}	24.0 ± 3.3^{b}	29.6 ± 6.0^{a}
μmol/liver	726.6 ± 176.8^{a}	562.7 ± 121.6^{b}	527.5 ± 133.4^{b}	752.1 ± 164.2^{a}
Triglyceride				
μmol/g liver	44.9 ± 11.3^{a}	35.3 ± 6.4^{b}	35.2 ± 5.6^{b}	38.4 ± 5.9^{ab}
μmol/liver	1090.2 ± 228.0^{a}	867.6 ± 169.9^{bc}	808.4 ± 173.4^{c}	1025.6 ± 281.2^{ab}

[1] Data are expressed as the mean ± SD ($n = 10$); values in a row with different superscripts significantly differ ($p < 0.05$) as analyzed by Duncan's multiple range test.

fatty liver of the APP and NSPH groups were both grade 2, while those of the ISO and control groups were both grade 4. Additionally, there were significant differences between the APP/NSPH and the ISO/control groups ($p < 0.05$, Fig. 2).

Feces

Fecal wet weights of the APP and NSPH groups were significantly higher than that of the control group ($p < 0.05$, Table 5). Results for fecal dry weight were similar to wet weight, but that of the NSPH group was significantly higher than the control group at week 12. Moreover, APP and NSPH replacement to the diet significantly increased fecal neutral steroid excretion compared to the control group in weeks 8 and 12 ($p < 0.05$, Fig. 3a). However, fecal bile acid excretion showed no significant differences among all groups ($p > 0.05$, Fig. 3b). APP and NSPH also significantly enhanced the excretion of fecal nitrogen compounds compared to the control and ISO groups since week 4 ($p < 0.05$, Fig. 3c).

DISCUSSION

In the present study, APP and NSPH both significantly reduced plasma total cholesterol and LDL-cholesterol concentration ($p < 0.05$). Previous studies had reported that people who consumed more soybean products had lower plasma total cholesterol concentrations and they suggested that the lipid-lowering effect were caused by their protein contents [26]. Sugano *et al.* [27] also indicated that the high-molecular-weight fraction derived from soybean protein isolate effectively lowered plasma lipids even when there was no cholesterol contained in diet. Moreover, we found that neither the soybean protein nor the soy isoflavones had effect on the growth of experimental animals. The results showed that the hypocholesterolemic effects of soybean protein might be mainly from the undigestible portion of soybean protein for because NSPH was the non-dialyzed high-molecular-weight fraction prepared from pepsin-digested APP and the hypolipidemic effects of soybean protein was not caused by affecting normal growth. Besides,

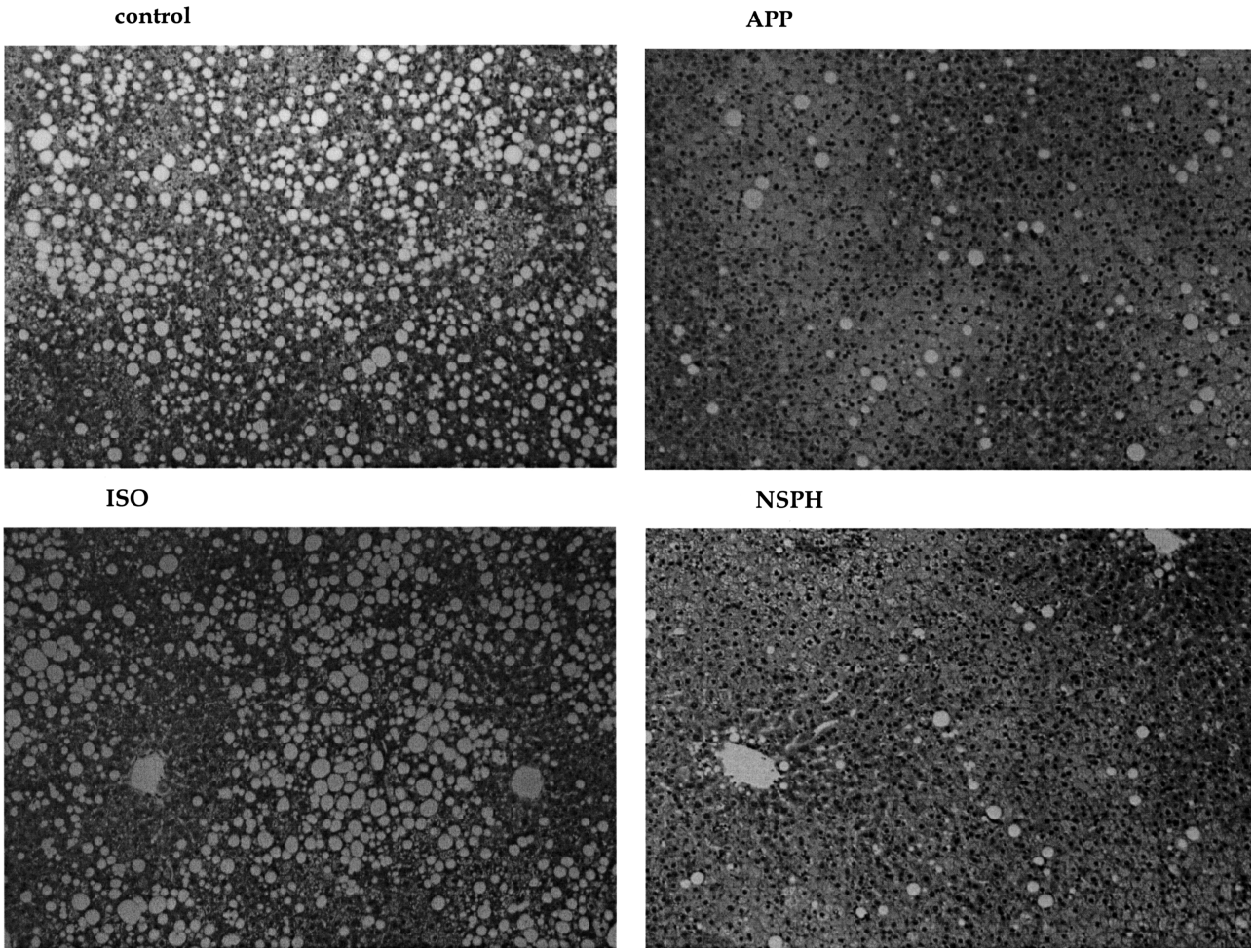

Fig. 2. Representative micrographs (×200) of liver stained by haematoxylin and eosin were samples per group. CC, control group; APP, APP group; NSPH, NSPH group; ISO, ISO group.

Table 5. Changes in Fecal Weight of SD Rats Fed Different Diets[1]

g/d	Diet group			
	Control	APP	NSPH	ISO
Week 0				
Wet weight	5.18 ± 0.78	5.22 ± 0.82	5.19 ± 0.53	5.28 ± 0.58
Dry weight	3.29 ± 0.43	3.21 ± 0.39	3.35 ± 0.49	3.37 ± 0.53
Week 4				
Wet weight	2.42 ± 0.34	2.45 ± 2.62	2.41 ± 0.77	2.28 ± 1.76
Dry weight	2.09 ± 0.30	2.11 ± 1.42	2.17 ± 0.59	1.91 ± 1.42
Week 8				
Wet weight	2.54 ± 0.83^{a}	3.40 ± 0.77^{b}	3.38 ± 0.26^{b}	2.70 ± 0.96^{ab}
Dry weight	2.03 ± 0.83^{a}	2.85 ± 0.69^{b}	2.73 ± 0.52^{bc}	2.11 ± 0.85^{ac}
Week 12				
Wet weight	2.65 ± 0.83^{a}	3.51 ± 0.74^{b}	3.39 ± 0.33^{b}	2.82 ± 0.96^{ab}
Dry weight	2.16 ± 0.83^{a}	2.96 ± 0.71^{b}	2.90 ± 0.39^{b}	2.23 ± 0.83^{a}

[1] Data are expressed as the mean ± SD ($n = 10$); values in a row with different superscripts significantly differ ($p < 0.05$) as analyzed by Duncan's multiple range test.

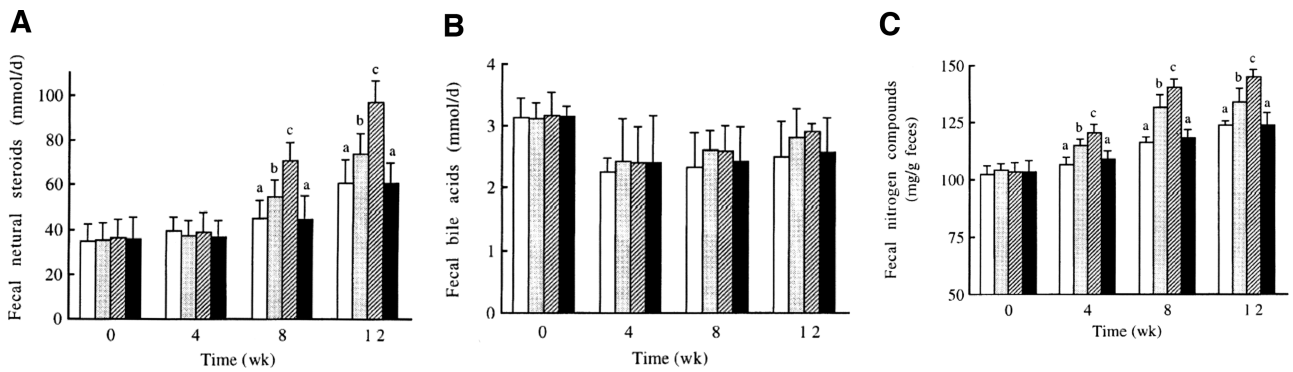

Fig. 3. Effects of different diets on concentrations of fecal neutral steroids (a), bile acids (b), and nitrogen compounds (c) in SD rats. Data are expressed as the mean ± SD ($n = 10$); values in a row with different superscripts significantly differ ($p < 0.05$) as analyzed by Duncan's multiple range test. **□= Control group, ▨= APP group, ▨= NSPH group, ■= ISO group.**

we found that the ISO group had no effect on total cholesterol and LDL-C. Anderson *et al.* [11] reported a dose-dependant response in hypocholesterolemic effects of soy isoflavones, Nestel *et al.* [28] indicated that highly purified soy isoflavones had no hypolipidemic effects. Our study was also in coincidence with the recent study that adding isoflavone supplement in diet had no improvement in cholesterol metabolism [29].

Dewell *et al.* [30] showed that soybean protein isolate did not affect plasma HDL-cholesterol levels of moderately hypercholesterolemic patients after an 8-week experiment. On contrary, Greaves *et al.,* [31] stated that after consuming soybean protein for 20 weeks, HDL-cholesterol concentrations of female cynomolgus monkeys increased. The inconsistency of theses study may be caused by animal species, the experimental period, or the quantity of protein used in animal diet. In our study, we found that the NSPH group but the ISO group reduced plasma LDL/HDL cholesterol ratio and the atherosclerosis index (AI), which is related to CVD risk [32]. Thus, we suggested that NSPH might be useful in the prevention of CVDs. As for isoflavones, as Clarkson [33] stated, there is still no definite experimental evidence existing currently to establish that the cardiovascular benefits of soy protein are accounted for by its isoflavones.

High fat intake may result in the accumulation of lipids in the liver and we found that both APP and NSPH reduced cholesterol and triglyceride contents in the liver. Besides, the pathological analysis found that APP and NSPH retarded fatty vesicle accumulation in the liver. Iritani *et al.* [34] reported that soybean protein lowered fatty acid synthesis and lipogenic enzyme activities in the liver of Wistar rats. Additionally, soybean protein possibly enhanced plasma lipoprotein lipase activity when compared with casein [35]. Liver cholesterol metabolism was related to the excretion of fecal neutral steroids and bile acids. Previous studies showed that soybean protein reduced blood cholesterol by inhibiting cholesterol absorption [2] or increasing fecal bile acid excretion [27], thus affecting the enterohepatic circulation and accelerating cholesterol metabolism [36]. In consistency, we found that APP and NSPH, especially NSPH, increased the excretion of fecal neutral steroids. NSPH is the hydrophobic high-molecular-weight

fraction of soybean protein peptic hydrolysate. In the present study, we determined fecal nitrogen compounds and found that both APP and NSPH increased their excretion and this suggested that NSPH might play a role similar to dietary fiber in the gut.

Furthermore, we also measured the micellar solubility of cholesterol and bile acids *in vitro* and found that APP and NSPH significantly reduced the micellar solubility of cholesterol. The absorption of increate cholesterol suppressed the synthesis of cholesterol in the liver, and inhibited LDL-receptor activity [37] and exogenous cholesterol must be emulsified before it can be absorbed. Nagaoka *et al.* [38] indicated that soybean protein peptic hydrolysate lowers the micellar solubility of cholesterol to a greater extent than casein tryptic hydrolysate. Additionally, soybean protein peptic hydrolysate inhibited the absorption of micellar cholesterol in Caco-2 cells. However, APP and NSPH had no effects on the micellar solubility of bile acids and no significant differences in the activities of 7α-hydroxylase, the rate-limiting enzyme of bile acid synthesis, among the groups were found in this study. This suggested that APP and NSPH did not affect the reabsorption of bile acids in the enterohepatic circulation. Although Madani *et al.* [39] stated that dietary bile acids inhibit 7α-hydroxylase activity and our experimental diets contained 0.05% bile acids, the effect of bile acid might be neutralized by the exogenous cholesterol. Thus, there were no obvious effects on 7α-hydroxylase. Additionally, NSPH is the non-dialyzed high-molecular-weight fraction of soybean protein peptic hydrolysate and is not easily absorbed into the blood circulation to reach the liver where it had influence on enzyme activities. These results suggested that the hypocholesterolemic effects of NSPH might caused by binding to cholesterol or bile acid in the gut, and increasing their excretion, thus lowering plasma cholesterol concentrations.

CONCLUSION

We postulate that soy protein, based on the NSPH study group, binds cholesterol in the small intestine thereby inhibiting its absorption and also enhanced the emulsification of cholesterol secreted in the entrohepatic circulation, both of which reduce the liver content of cholesterol. Similar effects, however, were not found in the isoflavones group, which further suggests that protein in soy foods is the critical ingredient for lowering serum cholesterol and total body load.

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