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RESEARCH PAPER

Conjugated and free sterols from black bean (*Phaseolus vulgaris* L.) seed coats as cholesterol micelle disruptors and their effect on lipid metabolism and cholesterol transport in rat primary hepatocytes

Rocio A. Chávez-Santoscoy · Armando R. Tovar · Sergio O. Serna-Saldivar · Nimbe Torres · Janet A. Gutiérrez-Uribe

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Abstract Phytosterols have been widely studied for their cholesterol-lowering effect. Conjugated phytosterol forms have been found more active than free moieties. There are no reports about the sterol profile of black bean seed coats neither its effects on cholesterol metabolism. The aim of this research was to identify and quantify phytosterols from black bean seed coats and to determine their effects on cholesterol micellar solubility and on mRNA and key protein levels involved in lipid/cholesterol metabolism and cholesterol transport in primary rat hepatocytes. Free phytosterols, acylated steryl glycosides, and steryl glycosides were extracted from black bean seed coats. They were identified through HPLC–MS–TOF and quantified through HPLC equipped with UV–visible and evaporative light-scattering detectors. Free and conjugated phytosterols from the coats significantly increased the inhibitory effect of cholesterol micelle formation compared with stigmasterol, which was used as control ($P < 0.05$). In addition, phytosterols of black bean seed coat decreased lipogenesis by the downregulation of lipogenic proteins such as sterol regulatory element-binding protein 1 and fatty acid synthesis (FAS) in primary rat hepatocytes. Regarding β-oxidation, phytosterols upregulated the expression of carnitine palmitoyltransferase I and promoted the β-oxidation of long-chain fatty acids. Phytosterols inhibited cholesterol micellar solubility and reduced the activation of the liver X receptor, decreasing hepatic FAS and promoting hepatic β-oxidation of long-chain fatty acids.

Keywords Black bean · Cholesterol-lowering · Phytosterol composition · Lipid metabolism · Lipogenesis · β-Oxidation

Abbreviations

ABCG	ATP-binding cassette subfamily G members
ASG	Acylated steryl glycosides
CPT1	Carnitine palmitoyltransferase I
ELSD	Evaperative light-scattering detector
FAS	Fatty acid synthesis
HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase
LXR	Liver X receptor
Ph	Phytosterols from black bean seed coat
SG	Steryl glycosides
SREBP	Sterol regulatory element-binding protein
T	T0901317

Introduction

Phytosterols are structurally similar to cholesterol and act in the intestine lumen to lower cholesterol absorption via the higher excretion of fecal cholesterol (Ostlund 2004). The Food and Drug Administration (FDA) (2000) has considered these plant sterols as generally recognized as safe (GRAS) and established that the dietary intake necessary to achieve significant cholesterol plasma reductions

R. A. Chávez-Santoscoy · S. O. Serna-Saldivar · J. A. Gutiérrez-Uribe (✉)
Departamento de Biotecnología e Ingeniería de Alimentos, Centro de Biotecnología FEMSA, Tecnológico de Monterrey-Campus Monterrey, Av. Eugenio Garza Sada 2501 Sur, C.P. 64849 Monterrey, NL, Mexico
e-mail: jagu@itesm.mx

A. R. Tovar · N. Torres
Departamento de Fisiología de la Nutrición, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, C.P. 14000 Mexico, DF, Mexico

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(5–15 %) is 2 g per day. Similarly, the National Cholesterol Education Program (NCEP) ( ) recommends for adults the daily intake of 2 g phytosterols to reduce plasma LDL and the risk of cardiovascular diseases. However, a conventional Western diet provides only an average of 250 mg/day of phytosterols.

Studies with the free forms of phytosterols related to their cholesterol-lowering effects have only been addressed toward the reduction in cholesterol absorption (Carr ; Guderian et al. ; Lee et al. ; Rasmussen et al. ; Trautwein and Duchateau). One of the proposed mechanisms for the interference of cholesterol intestinal absorption is the inhibition of micellar solubility (Jesch and Carr ; Trautwein and Duchateau). Remarkably, esterified phytosterols have been found more active in the cholesterol-lowering effect than free counterparts (Guderian et al. , Rasmussen et al.). Glycoside sterols (GS) and acylated steryl glycosides (ASG) are the major forms of phytosterols in many foods (Ostlund), and according to Lin et al. ( ), these chemical forms have a similar reduction effect of cholesterol absorption compared with sterol esters. Further studies have demonstrated that the bioactive components responsible for reducing cholesterol absorption are ASG and SG, but not the free phytosterols (Lin et al.). Lin et al. ( ) reported that ASG and SG present in the lumen and mucosa of mice reduced cholesterol absorption, and despite their low absorption, they significantly reduced both plasma and hepatic cholesterol. Other reports showed that ASG have also been used to enhance drug delivery because they are well recognized by some hepatic receptors, allowing the administrated drug to accumulate up to 80 % in the liver (Maitani et al.). Despite the limited absorption of ASG, there is not knowledge whether small amounts of these compounds may exert biological effects in liver cells.

There is limited information about the effect of specific free phytosterols (sitosterol and stigmasterol) on the expression of key genes of cholesterol metabolism. A significant decrease in the relative expression of hepatic sterol regulatory element-binding protein 2 (SREBP-2), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), and intestinal ATP-binding cassette subfamily G 5 (ABCG5) has been observed in hamsters fed with a high-cholesterol diet and pure sitosterol or stigmasterol (Liang et al.). SREBP is a family of transcription factors that regulate lipid metabolism and activate the expression of more than 30 genes dedicated to the synthesis and uptake of cholesterol, as well as fatty acid synthesis (Horton ; Sato). On the other hand, ABCG5 and 8 are transporters that belong to the family of reverse cholesterol transport and are also responsible to limit intestinal absorption and promote biliary excretion of hepatic sterols (Graf et al.).

Beans are an important source of several phytosterols, but these phytochemicals have been quantified after the hydrolysis of the seed (Iriti et al. ; Nyström et al. ; Ryan et al.). For instance, the most abundant phytosterol in kidney beans is sitosterol, containing 86.5 mg/100 g (Ryan et al.). In addition, other authors reported that sitosterol is the main phytosterol found in common beans with a concentration of 27.2 mg/100 g (Iriti et al.). For black beans, the most abundant reported phytosterol is also sitosterol, and the proportion of acylated steryl glycosides is greater than that of steryl glycoside sterols (Nyström et al.). To our knowledge, it has not been reported the sterol profile of black bean (*Phaseolus vulgaris* L.) seed coats nor their effects on cholesterol metabolism. It is important to point out that black bean is one of the most frequent legume seeds consumed in the Mexican diet and is extensively used to prepare an array of foods of the Mexican cuisine. However, the consumption of beans has decreased during the past decades, and interestingly during this time span, there has been an increase in the incidence of hypercholesterolemia and cardiovascular diseases (Díaz-Batalla et al.). Thus, black bean consumption could benefit individuals from high serum cholesterol concentration and thus reduction in the risk of cardiovascular diseases.

Thus, the aim of this study was to identify and quantify phytosterols, in their free and conjugated forms, in black bean (*Phaseolus vulgaris* L.) seed coats, and to determine their effects in cholesterol micellar solubility and in key protein levels and gene expression involved in lipid/cholesterol metabolism and cholesterol transport in primary rat hepatocytes.

Materials and methods

Seed coat samples

Phaseolus vulgaris L. var. San Luis was obtained from Sinaloa, Mexico, during the month of March of 2011. The seeds were stored at 4 °C and relative humidity of 85 %. Black beans were wiped with a flannel and then tempered in a plastic bag with distilled water in a 100:1 (w/v) ratio at room temperature for 24 h. The conditioned black beans were placed on trays for drying at 60 °C for 6 h in an oven (Electrolux, EOB31003X, Spain). Later, the seed coats were removed using a mechanical seed decorticator (Square D, SC-DGE 4364, India) for 90 s. The mixture of decorticated beans was separated by three sieves into cotyledons (2.81 mm); seed coats (1.00 mm); a mixture of

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fine particles of cotyledons, hilum, and seed coats (0.251 mm); and a bottom pan that collected the finest particles. Subsequently, the seed coats were milled with a coffee grinder (Krups GX4100, Mexico).

Extraction of sterols associated with black bean seed coats

Dihydrocholesterol (5α-cholestan-3β-ol, Sigma, St. Louis, MO, USA) was used as internal standard. The extractions were performed with hexane using a mass–solvent ratio of 1:10 w/v. The mixture was stirred for 3 h at 250 rpm at a temperature of 35 °C and left for one additional hour. The supernatant was recovered and vacuum filtered through a Whatman filter paper No. 1. The resulting extract was concentrated in a rotary evaporator to remove the organic solvent. The bath temperature was set at 40 °C, and pressure in the vacuum pump at a range of −70 to −90 kPa. Once hexane was removed, the extract rich in phytosterols was lyophilized and the resulting powder stored at −80 °C until analyses.

Hydrolysis of extract rich in phytosterols

Two grams of lyophilized extract was hydrolyzed with 10 mL of 6 M HCl for 60 min at 70 °C; every 10 min the samples were manually shaken. After cooling the samples for 20 min at room temperature, lipids were extracted twice with 7 mL of hexane/diethylether 1:1 (v:v). The organic layer was recovered and evaporated to dryness. Then for saponification, the dried organic layer was dissolved in 4 mL of ethanol, and 5 mL of KOH was added. The mixture was shaken in a vortex and hydrolyzed for 30 min at 70 °C. After that, the mixture was cooled at room temperature, and 4 mL of distilled water and 7 mL of hexane were added. The organic layer was recovered, and other 7 mL of hexane was added and recovered. The total organic layer was evaporated to dryness. The dried fraction was dissolved in 1 mL of hexane for HPLC analysis.

Sterols identification and quantification

The identification of phytosterols was performed in a HPLC–MS–TOF (Model G1969A Agilent 1100 Santa Clara, CA, USA) with the same chromatographic conditions described above for the HPLC–DAD–ELSD analysis. The free forms were confirmed by retention time of each standard in the HPLC column. For the identification of conjugated forms, ionization tests were performed to fragment the molecular ion and obtain the corresponding fragmentation pattern. Mass spectra were collected using electrospray source in positive mode (ESI+) under the following conditions: *m/z* range, 100–1,400; nitrogen gas; gas temperature, 250 °C; drying gas flow rate, 13 L/min; nebulizer pressure, 50 psig; capillary voltage, 4,000 V; and fragment voltage, 80 V. Extracted ion chromatograms were obtained considering accurate mass of phytosterols or their adducts, with an error range of 0.01 units using the Analyst QS 1.1 software (Applied Biosystems, Carlsbad, CA, USA).

Phytosterol quantifications were performed in HPLC equipped with UV–Visible detector and an Evaporative Light Scattering Detector, HPLC–UV–VIS–ELSD (1200 Series, Agilent Technologies, Santa Clara, CA, USA). The HPLC was equipped with a Luna C8 column (250 × 4.6 mm i.d; Phenomenex, Torrance, CA, USA); injection volume was 20 μL, and the mobile phase consisted of (A) acetonitrile and (B) 55 % methanol and 45 % water (acidified with 1 % formic acid). The elution gradient for B was as follows: 0–7 min, 0 % (flow rate of 0.6 mL/min); 7–15 min, 0–15 % (0.6–1.2 mL/min); 15–20 min, 15–80 % (1.2–1.5 mL/min); and 20–50 min, 80–100 % (1.5 mL/min). The column temperature was maintained at 40 °C. ELSD was adjusted to 50 °C with a nitrogen pressure of 1.8 bar. ELSD signal was used to quantify free and conjugated forms of stigmasterol and sitosterol. Campesterol, free or conjugated, and cholesterol were measured at 205 nm. Quantifications were performed using calibration curves of standards of stigmasterol, sitosterol, and campesterol (Sigma, St. Louis, MO, USA), and conjugated forms were quantified as equivalents of the corresponding phytosterol in its free form. In the case of Δ^5-avenasterol forms, cholesterol was used for quantification.

Inhibition of in vitro cholesterol micellar solubility

The in vitro micellar solubility of cholesterol was measured according to the method described before (Zhong et al. 2007). Micellar solutions (7 mL) containing 10 mM sodium taurocholate (Sigma, St. Louis, MO, USA), 2 mM cholesterol (Sigma, St. Louis, MO, USA), 5 mM oleic acid (Sigma, St. Louis, MO, USA), 132 mM NaCl, 15 mM sodium phosphate (pH 7.4), and 5 mg/mL of the non-hydrolyzed phytosterols extract or 5 mg/mL of sitosterol or 3 mg/mL of sitosterol or 5 mg/mL of stigmasterol were prepared by sonication (Ultrasonic Homogenizer VP-5, Taitec Co. Ltd., Japan). The sitosterol concentration of 3 mg/mL was selected to assay the effect of the same proportion of sitosterol contained in the phytosterol extract, and 5 mg/mL of sitosterol and stigmasterol was evaluated to compare the effect of free forms of phytosterol with the effect of the non-hydrolyzed phytosterol extract. The mixture was incubated at 37 °C for 24 h and centrifuged at 100,000*g* for 60 min at 37 °C. The supernatant portion was collected for the determination of cholesterol concentration

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using the method previously described (Chávez-Santoscoy et al. 2013). Data were expressed as inhibition of cholesterol micellar solubility (%) obtained as [Cs/(Ci − Co)] × 100, where Cs is cholesterol concentration in supernatants with the tested extracts or control, Co is cholesterol concentration in supernatant without disruptor, and Ci was the initial cholesterol concentration, which in this particular case was 2 μg/mL.

Culture of primary rat hepatocytes

Wistar rat hepatocytes were isolated by collagenase perfusion and separated from non-parenchymal liver cells by centrifugation at 325*g* as previously described (Berry and Friend 1969). Primary hepatocytes were plated in a six-well plate (9.6 cm^2/well) (CorningCellBIND, Tewksbury, MA). At 4 h after plating hepatocytes, phytosterol extracts at different dosages up to 76.1 μg/L and/or 10 μmol/L of T0901317 (Sigma, St. Louis, MO, USA) were added. The hepatocytes were stimulated for 24 h by each treatment. Total RNA from the hepatocytes was obtained using Trizol reagent (Life Technologies, Carlsbad, CA, USA) with the protocol recommended for the provider, quantified in a NanoDrop spectrophotometer (NanoDrop Technologies, Houston, TX, USA), and reverse-transcribed. RT-PCR amplification was performed in a Roche Lightcycler 480 II (Switzerland) using TaqMan assays. Relative expression of SREBP1, fatty acid synthase (FAS), carnitine palmitoyltransferase I (CPT1), ABCG5, and actin as housekeeping gene was calculated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Protein extraction and Western blotting

Protein extraction and Western blotting were performed as previously reported (González-Granillo et al. 2012). Briefly, primary rat hepatocytes were homogenized in lysis protein RIPA buffer and complete protease inhibitor cocktail tablets (Roche Applied Science, Germany). Total protein (30 μg) was loaded on 8 % polyacrylamide gels, separated by SDS–PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane. Blots were blocked with nonfat dry milk (Bio-Rad, Hercules, CA, USA) and incubated overnight at 4 °C with the following primary antibodies: anti-SREBP1, anti-FAS, anti-CPT1, anti-HMGCR, anti-tubulin, and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then, blots were incubated with the secondary antibody and revealed in ChemiDoc™ XRS + System with Image Lab™ Software (Bio-Rad, Hercules, CA, USA). The bands were analyzed using the ImageJ 1.42p digital imaging processing software (http://rsbweb.nih.gov/ij/download/2012/).

Statistical analysis

Results were expressed as mean ± standard error. Data were analyzed with Graphpad Prism 6.0 and MINITAB 16 software. The statistical analysis was performed by one-way ANOVA followed by Tukey's test to identify significant differences between groups. Differences between means were compared at a level of significance of $P < 0.05$. Every experiment was performed in triplicate.

Results

Identification and quantification of phytosterols

It was possible to identify specific free and conjugated sterols (Table 1) by comparing the mass spectra with the compounds that have been previously identified in the Fabaceae family, according to the fragmentation pattern and molecular ions previously reported (Kaloustian et al. 2008; Leisso et al. 2013; Ongoka et al. 2008), as well as the coincidence in the retention time of the respective standards. Stigmasterol was only found in its free form, whereas campesterol was found as free, acylated steryl glycoside and steryl glycoside; sitosterol was found in free and acylated steryl glycoside forms. Finally, Δ^5-avenasterol was found as free and steryl glycoside forms (Table 1).

The 44.2, 30.1, and 25.4 % of the identified phytosterols were acylated steryl glycosides, free forms, and steryl glycosides, respectively (Table 2). The most abundant free phytosterol found in the extract before and after acid hydrolysis was sitosterol with a concentration of 29.4 and 51.2 mg/100 g, respectively (Table 2), and the most abundant conjugated phytosterols found in black bean seed coats were β-sitosteryl (6′-*O*-linoleoyl)-β-D-glucoside (41.2 ± 0.1 mg/100 g) and campesteryl-β-D-glucoside (32.9 ± 1.2 mg/100 g) (Table 2).

Inhibition of in vitro cholesterol micellar solubility

The inhibition of cholesterol micellar solubility due to phytosterols of black bean seed coats was significantly higher than the effect achieved by any of the two phytosterol standards used as control (Fig. 1). Particularly, sitosterol at a concentration of 3 mg/mL inhibited cholesterol micellar solubility by 3.39 ± 0.46 % when commercial standard was used and 14.24 ± 1.55 % when it was

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Table 1 Free phytosterols, steryl glycosides and acylated steryl glycosides found in black bean seed coat based on accurate mass and comparison with literature

Compound	Molecular mass	m/z	Positive MS assignment	References
Free sterols				
Stigmasterol	412	395.36	$[M + H - H_2O]^+$	Ongaka et al. (2011), Rozenberg (2004)
Campesterol	400	383.35	$[M + H - H_2O]^+$	Rozenberg (2004)
		411.35	$[M + H - 2H]^-$	Rozenberg (2004)
		621.69	$[M + M - H]^-$	
β-Sitosterol	414	397.37	$[M + H - H_2O]^+$	Kishore et al. (2011), Rozenberg (2004)
Δ^7-Avenasterol	412	395.35	$[M + H - H_2O]^+$	Rozenberg (2004)
		413.26	$[M - H]^-$	
Steryl glycosides				
Δ^7 Avenasterol-β-D-glucoside	574	395.27	$[Aglycone - 3H - H_2O]^+$	Rozenberg (2004)
		413.26	$[Aglycone + H]^+$	
		592.48	$[M + H_2O]$	
Campesteryl β-D glucoside	562	383.35	$[Aglycone + H - H_2O]^+$	
		419.25	$[Aglycone + H_2O + H]^+$	Dweck (2009), Rozenberg (2004)
		579.28	$[M + H_2O - H]^-$	
		595.25	$[M + H_2O + 3H]^+$	
Acylated steryl glycosides				
Campesteryl (6′-*O*-linoleoyl)-β-D-glucoside	824	383.36	$[Aglycone - H - H_2O]^+$	Lassa et al. (2011)
		821.69	$[M + H - 4H]^-$	Rozenberg (2004)
β-Sitosteryl(6′-*O*-linoleoyl)-β-D-glucoside	838	837.56	$[M + H - 2H]^-$	Rozenberg (2004)
		397.37	$[Aglycone + H - H_2O]^+$	Rozenberg (2004)

Table 2 Concentrations of phytosterols in extracts of black bean (mg/100 g of seed coats) before and after acid hydrolysis

Extract	Stigmasterol mg/100 g seed coats[a]	Campesterol	β-Sitosterol	Δ^7-Avenasterol	Total	ASG/SG ratio
Non hyd						
Free	3.21 ± 0.13	10.20 ± 1.54	29.42 ± 0.51	1.00 ± 0.41	43.92 ± 2.50	
ASG	n.d	23.60 ± 0.85	41.23 ± 0.11	n.d	64.81 ± 1.01	1.74
SG	n.d.	32.91 ± 1.21	n.d.	4.41 ± 0.08	37.32 ± 1.20	
Total	3.21 ± 0.13	66.71 ± 1.99	70.65 ± 1.51	5.50 ± 0.51	146.07 ± 3.54	
Hyd						
Free	9.02 ± 1.12	21.17 ± 1.10	51.21 ± 1.18	2.95 ± 0.94	84.35 ± 1.62	

ASG acylated steryl glycosides, *SG* steryl glycosides, *Hyd* hydrolyzed extract, *n.d.* not detectable

[a] Mean ± SE of at least three replicates, the conjugated forms are quantified as equivalent of the free forms

used along with the rest of the components in the phytosterol extract from black bean seed coats.

Relative expression and Western blotting

Phytosterols contained in black bean seed coat at a concentration of 76.1 μg/L were able to significantly reduce the expression of SREBP1 and FAS in cultured rat hepatocytes. Moreover, they suppressed the effect of the liver X receptor (LXR) inductor, T0901317, reducing the relative mRNA abundance and protein expression of two of the main proteins involved in liver lipogenesis (FAS and SREBP) to the levels presented in non-stimulated primary hepatocytes (Fig. _). ABCG5 was also downregulated by the phytosterol-rich extract, before and after the induction of LXR (Fig. _) at mRNA and protein levels.

Interestingly, it was observed that the phytosterol extract was able to upregulate the expression of CPT1 at mRNA

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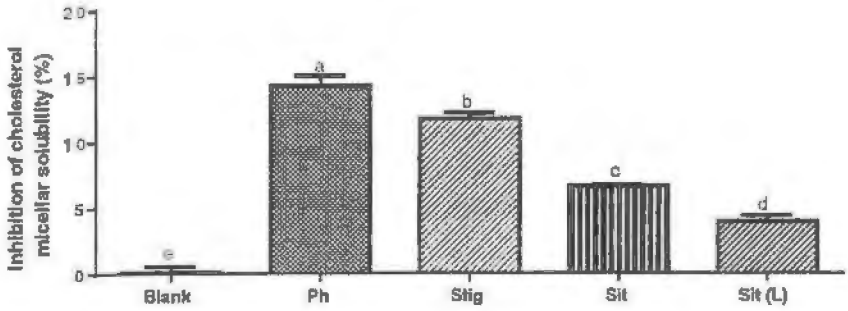

Fig. 1 Inhibition of cholesterol micellar solubility. The tested treatments were blank, the micellar mixture without any disruptor; Ph, the extract rich in phytosterols (free and conjugated) of black bean seed coats (5 mg/mL); Stig, stigmasterol (5 mg/mL); Sit, sitosterol (5 mg/mL); and Sit (L), sitosterol at a dose contained in phytosterols extract (3 mg/mL). Every experiment was performed in triplicate ($n = 3$) Data are presented in mean ± SE. *Bars with different letter* indicate statistically significant difference. $P < 0.05$

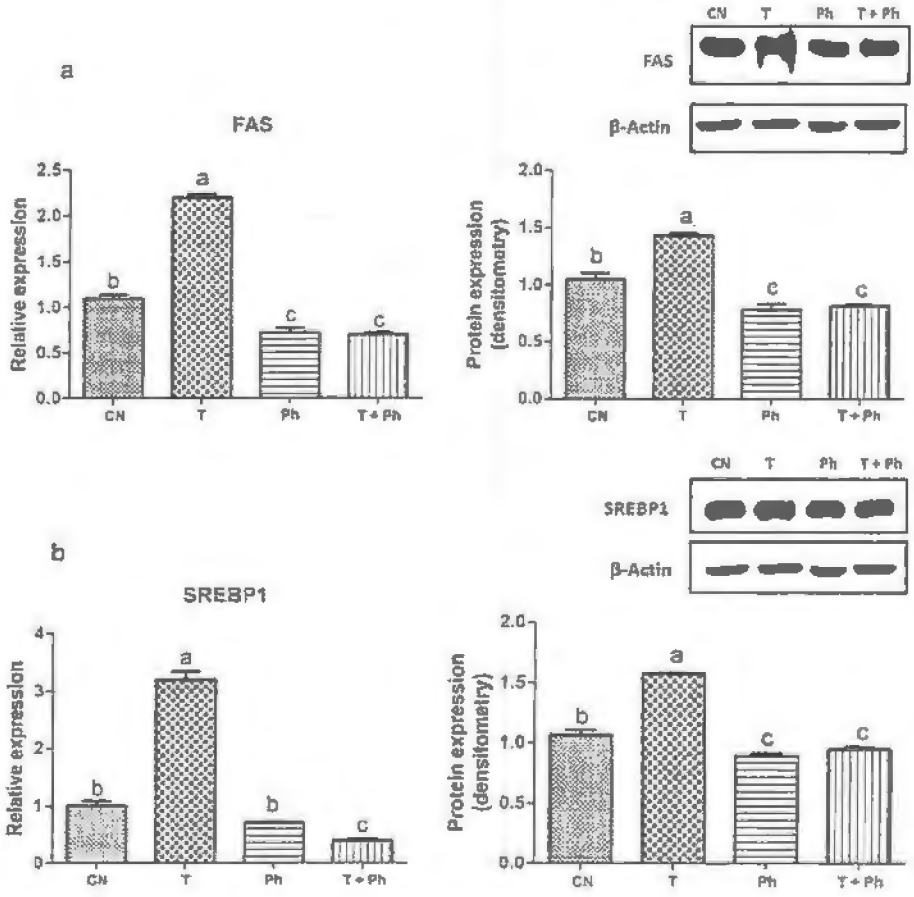

Fig. 2 Effect of phytosterols of black bean seed coats on the expression of lipogenic genes of the liver. It is presented at the *left* the relative gene expression, and at the *right* the protein expression of **a** FAS and **b** SREBP1 of primary hepatocytes treated; CN = no stimulus, T = the synthetic LXR agonist T0901317, Ph = phytosterols from black bean seed coat extract at 76.13 μg/L, T + Ph = T0901317 and the extract rich in phytosterols associated with black bean seed coat at the same dose (76.13 μg/L). Every experiment was performed in triplicate ($n = 3$). Data are presented in mean ± SE. *Bars with different letter* indicate statistically significant difference, $P < 0.05$

and protein level (Fig.). The LXR induction by T0901317 reduced the mRNA abundance and protein expression of the key enzyme of β-oxidation CPT1 (Fig.) but the addition of phytosterols extract to stimulate hepatocytes increased CPT1 expression to normal levels (Fig.).

Discussion

This research is the first to report the profile of phytosterols associated with bean seed coats. Sitosterol was the major phytosterol, and the concentration determined herein is similar to that reported for other related seeds of the

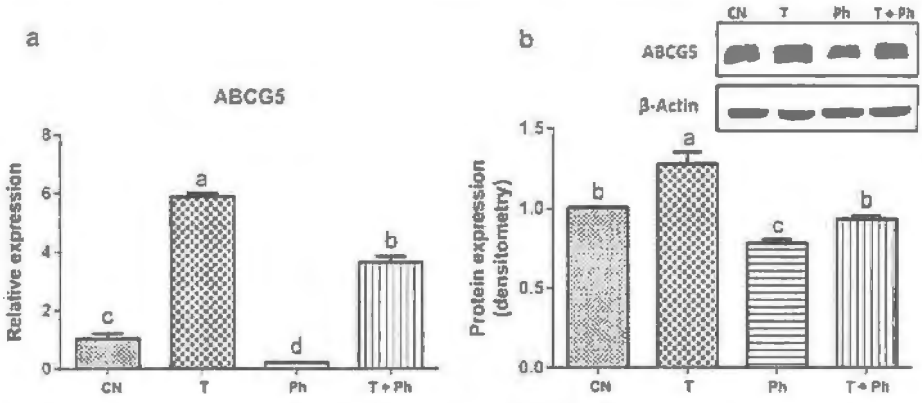

Fig. 3 Effect of phytosterols of black bean seed coats on the expression of ABCG5 (transport protein responsible for biliary excretion of sterols in the liver). It is presented the a relative expression and b protein expression of ABCG5 in primary hepatocytes treated with control or CN = no stimulus, T = the synthetic LXR agonist T0901317, Ph = extract rich in phytosterols associated with black bean seed coat at a dose of 76.13 μg/L. T + Ph = T0901317 and the extract rich in phytosterols associated with black bean seed coat at the same dose (76.13 μg/L). Every experiment was performed in triplicate ($n = 3$). Data are presented in mean ± SE. *Bars with different letter* indicate statistically significant difference, $P < 0.05$

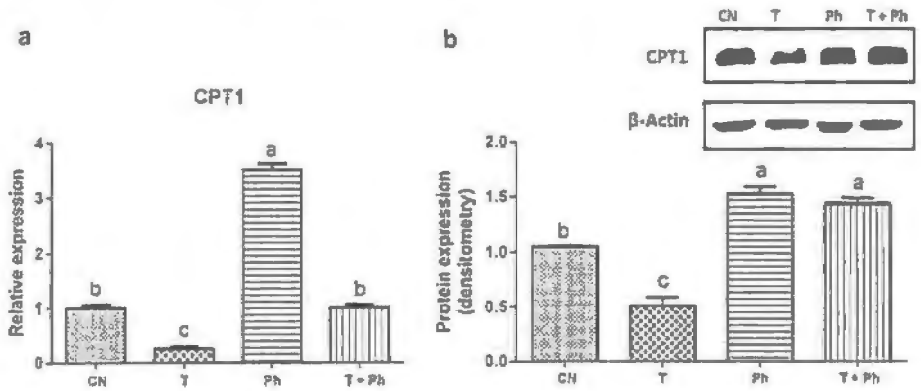

Fig. 4 Effect of phytosterols of black bean seed coats in the expression of CPT1 (rate-limiting enzyme for β-oxidation of long chain fatty acids in the liver). It is presented the a relative expression and b protein expression of CPT1, which mediates the transport of long-chain fatty acids across the mitochondrial membrane by binding them to carnitine for β-oxidation, in primary hepatocytes treated with CN = no stimulus, T = the synthetic LXR agonist T0901317, Ph = extract rich in phytosterols associated with black bean seed coat at a dose of 76.13 μg/L. T + Ph = T0901317 and the extract rich in phytosterols associated with black bean seed coat at the same dose (76.13 μg/L). Every experiment was performed in triplicate ($n = 3$). Data are presented in mean ± SE. *Bars with different letter* indicate statistically significant difference, $P < 0.05$

Fabaceae family such as kidney and common beans that contained 86.5 and 27.2 mg/100 g of sitosterol, respectively (Iriti et al. [illegible]; Ryan et al. [illegible]). Sitosterol, campesterol, and stigmasterol contents found after hydrolysis were comparable to the concentrations previously reported in linseed, spelt, and rye (Ryan et al. [illegible]). As expected, from previous reports in common beans (Nyström et al. [illegible]), the amount of acylated steryl glycosides was higher than that of steryl glycosides (Table [illegible]). Furthermore, the ASG/SG ratio obtained was 1.74, which is similar to the ratio 1.79 previously reported for black bean (Nyström et al. [illegible]). This ratio is important in estimating the bioactivity of the phytosterols associated with seed coats. It has been reported that ASG are more bioactive than the GS in lowering cholesterol absorption in mice (Lin et al. [illegible]), and therefore, the phytosterol profile of black bean seed coats contains more bioactive conjugated sterols.

Results of the in vitro studies suggest that the enhancement of the inhibition of cholesterol micellar solubility was due to the synergistic effect of free and conjugated phytosterols contained in the extract. There is evidence that dietary phytosterols reduced serum and hepatic cholesterol levels by interfering with the intestinal absorption, through the inhibition of its micellar solubility (Carr et al. [illegible]; Trautwein and Duchateau [illegible]). Additionally, it has been demonstrated that ASG reduced

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cholesterol absorption by 45 ± 6 % in mice in contrast to the null effect of the free forms (Lin et al. 2013). Other compounds contained in black bean seed coats have been studied for their effect on the inhibition of cholesterol micellar solubility, such as saponins, which strongly inhibited cholesterol micellization with values higher than 50 % (Chávez-Santoscoy et al. 2013).

The downregulatory effect on lipogenic genes (SREBP1 and FAS) of the phytosterol extract was consistent with previous reports that demonstrated that sitosterol and stigmasterol downregulated the expression of other relevant proteins involved in cholesterol biosynthesis, such as SREBP-2 and HMGCR (Liang et al. 2011). Moreover, it has been demonstrated hepatic downregulation of SREBP1 in C57BL/6 mice fed with a mixture of phytosterols in their free forms (Rideout et al. 2010). It is well established that the expression of the transcription factor SREBP-1, essential for the transcriptional control of lipogenic genes, is strongly upregulated by the transcription factor LXR. Particularly, the isoform LXR-α is the mainly responsible for upregulating the expression of SREBP-1 when stimulated with either natural oxysterols or synthetic LXR ligands such as T0901317. Interestingly, phytosterols were able to almost abolish the stimulating effect of T0901317 on SREBP1 and FAS expression to the levels observed in non-stimulated primary hepatocytes.

Furthermore, phytosterols of black bean seed coats decreased the relative expression of ABCG5, another target of the transcription factor LXR, in primary hepatocytes incubated with T0901317. Nonetheless, as expected, ABCG5 was overexpressed by the LXR inductor T0901317 (Fig. 3) (Calpe-Berdiel et al. 2008; Pawar et al. 2003), in primary hepatocytes non-incubated with phytosterols.

The downregulation of SREBP1, FAS, and ABCG5 by phytosterols of black bean seed coats in hepatocytes incubated with T0901317 suggests that the sterols inhibited the LXR activity. There is evidence that other bioactive compounds such as isoflavones have similar effects on LXR activity (González-Granillo et al. 2012). It has been suggested that these compounds could regulate the activity of the enzyme adenosine monophosphate kinase (AMPK), and this in turn could modulate the phosphorylation state of LXR modifying its biological activity (González-Granillo et al. 2012). However, more studies are needed to understand whether the phytosterols are able to alter the LXR phosphorylation state. Nonetheless, it is known that systemic LXR activation increases hepatic fatty acid synthesis and steatosis (De Smet et al. 2012), and therefore, phytosterols of black bean seed coats can potentially be used to reduce or prevent these abnormalities.

Interestingly, it was observed that the synthetic ligand T0901317 reduced the mRNA and protein expression of the key enzyme of β-oxidation CPT1. In contrast, phytosterols associated with black bean seed coats upregulated the expression of CPT1, promoting β-oxidation of long-chain fatty acids. Moreover, they were able to suppress the downregulating effect of T0901317 over the expression of CPT1, causing the enzyme to be expressed at similar levels observed in non-stimulated primary hepatocytes (Fig. 4a). Remarkably, at the protein level, the expression of CPT1 of primary hepatocytes treated with T0901317 with phytosterols of black bean seed coat (T + Ph) was higher than that observed in non-stimulated primary hepatocytes (Fig. 4b).

In summary, our results suggest that phytosterols of black bean seed coats can potentially reduce cholesterol levels by inhibiting its absorption in the intestinal lumen through the inhibition of cholesterol micellar solubility. Furthermore, the downregulation of LXR target genes (SREBP1, FAS, and ABCG5) suggested that these phytosterols were able to suppress the activity of LXR, reducing the expression of lipogenic genes that are known to reduce hepatic lipogenesis. In addition, phytosterols of black bean seed coats upregulated the expression of CPT1, which promoted the β-oxidation of long-chain fatty acids. Further experiments are needed to determine the in vivo effects of these phytochemicals in humans.

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Conflict of interest None.

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Flavonoids and saponins extracted from black bean (*Phaseolus vulgaris* L.) seed coats modulate lipid metabolism and biliary cholesterol secretion in C57BL/6 mice

Rocio A. Chavez-Santoscoy[1], Janet A. Gutierrez-Uribe[1], Omar Granados[2], Ivan Torre-Villalvazo[2], Sergio O. Serna-Saldivar[1], Nimbe Torres[2], Berenice Palacios-González[2] and Armando R. Tovar[2]*

[1]*Centro de Biotecnología FEMSA, Escuela de Biotecnología y Alimentos, Tecnológico de Monterrey, Campus Monterrey, Avenida Eugenio Garza Sada 2501 Sur, C.P. 64849 Monterrey, NL, Mexico*
[2]*Departamento de Fisiología de la Nutrición, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, C.P. 14000 Mexico, DF, Mexico*

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Abstract

Black bean (*Phaseolus vulgaris* L.) seed coats are a rich source of natural compounds with potential beneficial effects on human health. Beans exert hypolipidaemic activity; however, this effect has not been attributed to any particular component, and the underlying mechanisms of action and protein targets remain unknown. The aim of the present study was to identify and quantify primary saponins and flavonoids extracted from black bean seed coats, and to study their effects on lipid metabolism in primary rat hepatocytes and C57BL/6 mice. The methanol extract of black bean seed coats, characterised by a HPLC system with a UV–visible detector and an evaporative light-scattering detector and HPLC–time-of-flight/MS, contained quercetin 3-*O*-glucoside and soyasaponin Af as the primary flavonoid and saponin, respectively. The extract significantly reduced the expression of *SREBP1c*, *FAS* and *HMGCR*, and stimulated the expression of the reverse cholesterol transporters *ABCG5*/*ABCG8* and *CYP7A1* in the liver. In addition, there was an increase in the expression of hepatic *PPAR-α*. Consequently, there was a decrease in hepatic lipid depots and a significant increase in bile acid secretion. Furthermore, the ingestion of this extract modulated the proportion of lipids that was used as a substrate for energy generation. Thus, the results suggest that the extract of black bean seed coats may decrease hepatic lipogenesis and stimulate cholesterol excretion, in part, via bile acid synthesis.

Key words: Black beans: Lipid metabolism: Saponins: Flavonoids

Dysfunctional lipid homeostasis plays an important role in the initiation and progression of atherosclerotic lesions[1]. Statins are clinically used to reduce the levels of cholesterol in serum and thus reduce the risk of heart disease[2]. However, it has been stated by the National Cholesterol Education Program Expert Panel (ATP III) that dietary strategies must be used before introducing medications for the treatment of hypercholesterolaemia[3,4]. A number of dietary modifications have been proposed to reduce the levels of cholesterol in serum, including the consumption of soya protein, PUFA and dietary phytosterols, among other bioactive compounds[5–10]. Interestingly, natural phytochemicals can have synergistic effects, and lower doses of each bioactive compound can attenuate hypercholesterolaemia[11–15].

Several studies have suggested that bean consumption has a hypocholesterolaemic effect[14,15]. However, in these studies, bean components that exerted the hypocholesterolaemic effect were not identified. Black bean (*Phaseolus vulgaris* L.) seed coats are a rich source of natural compounds, such as flavonoids and saponins, that have beneficial effects on human health[16]. Flavonoids are polyphenols that have long been recognised as having many beneficial activities, such as antioxidant, anti-inflammatory and hepatoprotective activities[17]. Saponins are secondary plant metabolites with multiple biological activities being attributed, including hypocholesterolaemic effects[14,18].

It has not been clearly established whether dietary bioactive compounds from black bean could modulate cholesterol

Abbreviations: ABCG, ATP binding cassette, subfamily G; AMPK, AMP-activated protein kinase; CPT1, carnitine palmitoyltransferase 1; CYP7A1, cholesterol 7α-hydroxylase; FSE, extract rich in flavonoids and saponins from black bean seed coats; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; INSIG, insulin-induced gene; LXR, liver X receptor; SREBP, sterol regulatory element-binding protein.

* **Corresponding author:** Dr A. R. Tovar, fax +52 55 36553038, email tovar.ar@gmail.com

Table 1. Diet composition of the six tested experimental diets offered to C57BL/6 mice*

Ingredients (g/kg)	CN	CN+FSE (L)	Chol	Chol+FSE (H)	Chol+FSE (L)	Chol+Sim
Casein	200	200	200	200	200	200
Dextrin	132	132	127	127	127	127
Soyabean oil	70	70	70	70	70	70
Cellulose	50	50	50	50	50	50
Choline	2·5	2·5	2·5	2·5	2·5	2·5
Saccharose	100	100	100	100	100	100
Starch	397·486	397·486	397·486	397·486	397·486	397·486
Vitamins	10	10	10	10	10	10
Minerals	35	35	35	35	35	35
Cystein	3	3	3	3	3	3
tert-Butylhydroquinone	0·014	0·014	0·014	0·014	0·014	0·014
FSE	0	2·5	0	5	2·5	0
Cholesterol	0	0	5	5	5	5
Simvastatin	0	0	0	0	0	0·3

CN, control diet; CN+FSE (L), control diet with the flavonoid- and saponin-rich extract (0·25 %); Chol, control diet with cholesterol (0·5 %); Chol+FSE (H), control diet with cholesterol (0·5 %) and the extract (0·5 %); Chol+FSE (L), control diet with cholesterol (0·5 %) and the extract (0·25 %); Chol+Sim, control diet with cholesterol (0·5 %) and simvastatin (0·03 %).
* Diets were made according to the standards of the AIN-93G diet (control).

homeostasis via the transcription factors sterol regulatory element-binding proteins (SREBP) and liver X receptor (LXR). Lipid homeostasis in vertebrate cells is regulated by a family of membrane-bound transcription factors such as SREBP. On the one hand, SREBP directly activate the expression of more than thirty genes involved in the synthesis and uptake of cholesterol and fatty acids[20,21]. On the other hand, LXR can modulate the expression of the transporters ATP-binding cassette subfamily G members (*ABCG5/ABCG8*). These reverse cholesterol transporters are glycoproteins synthesised in the endoplasmic reticulum[22,23]. They are expressed in a tissue-specific manner in the liver, colon and intestine[24], and are responsible for limiting intestinal absorption of cholesterol and promoting biliary excretion of sterols[25].

Therefore, the aim of the present study was to identify and quantify the main saponins and flavonoids extracted from black bean seed coats, and to study their effects on the expression of lipogenic genes, specifically those involved in reverse cholesterol transport and β-oxidation in primary rat hepatocytes. The study also assessed the intestinal and hepatic effects of these compounds on genes involved in the same aforementioned pathways in primary rat hepatocytes and in bile acid synthesis in C57BL/6 mice.

Experimental methods

Samples of seed coats

P. vulgaris L. var. San Luis was obtained from Sinaloa, Mexico and stored at 4°C and 85 % relative humidity. The beans were soaked in a plastic bag with distilled water at a ratio of 100:1 (w/v) for 24 h at room temperature. The conditioned black beans were dried for 6 h at 60°C. Later, seed coats were removed using a mechanical seed decorticator (SC-DGE 4364; Square D). The decortication time was 90 s. The resulting milled material was separated by three nested sieves into cotyledons (2·81 mm), seed coats (1·00 mm), and a mixture of fine cotyledon, hilum and seed coat debris (0·251 mm).

Extraction of flavonoids and saponins from black bean seed coats

The extraction of flavonoids and saponins was performed at 35°C for 3 h at 250 rpm with 80 % methanol in water (v/v) using a mass:solvent ratio of 1:10 (w/v). The supernatant was recovered and vacuum filtered through a Whatman filter paper no. 1. The resulting extract was concentrated in a rotary evaporator to remove methanol. The bath temperature was set at 60°C, and the pressure in the vacuum pump set at a range of −70 to −90 kPa. After the removal of methanol, the concentrated extract was lyophilised, and the resulting freeze-dried powder was stored at −80°C.

Fractionation of the methanol extract

The fractionation of the extract was performed by solid phase extraction using a Strata C18 cartridge (55 µm, 20 g/60 ml; Phenomenex). First, the black bean seed coat extract (FSE), rich in flavonoids and saponins, was resuspended in methanol (0·1 g/ml). The column was conditioned first with methanol and then with distilled water (−50 kPa). Subsequently, the soluble extract was passed through the cartridge, followed by 60 % (v/v) methanol acidified with 0·1 % (v/v) HCl and then by 60 % (v/v) methanol without acidification. This first elution yielded the flavonoid-rich fraction. Lastly, methanol was passed through the cartridge to obtain the saponin-rich fraction. Both fractions were concentrated and lyophilised as described previously.

Quantification and identification of flavonoids and saponins

The quantification of flavonoids and saponins was performed using a HPLC system with a UV–visible detector and an evaporative light-scattering detector (1200 Series; Agilent Technologies). The HPLC system was equipped with a Zorbax SB-Aq (3 × 150 mm, 3·5 µm) column, and data were collected using Agilent ChemStation. Separation conditions used were as reported previously[10]. The concentrations of saponins

Table 2. Sequences of real-time PCR primers* designed for expression studies in *Mus musculus*

Genes		Primer (5′–3′)	T_m (°C)	Amplified fragment size (bp)
ABCG5	Forward	cagaggagatgcttggcttc	60·01	240
	Reverse	tgaaaggaaccgtgggtaag	59·96	
ABCG8	Forward	cctctcaggtgccttggttt	59·89	175
	Reverse	acgtcgagtagtgaggctct	60·04	
AMPK	Forward	acctgagaacgtcctgcttg	59·97	155
	Reverse	ggcctgcgtacaatcttcct	60·11	
CYP7A1	Forward	tgggctgtgctctgaagttc	60·25	211
	Reverse	ctgtgtccaaatgccttcgc	60·11	
FAS	Forward	caaaggaccaagcattgccc	55·00	238
	Reverse	tacaacagcctcagagcgac	55·00	
FXR	Forward	ggcactcccatttacaggct	60·03	260
	Reverse	accagtcttccggttgttgg	60·18	
HMGCR	Forward	gggtattgctggcctcttca	59·74	215
	Reverse	ggattgccattccacgagct	60·75	
INSIG1	Forward	tagccaccatcttctcctcc	58·21	300
	Reverse	gccaacgaacacggcaatac	60·45	
INSIG2	Forward	ggtttggccttggtgttgg	60·18	153
	Reverse	tcccatcgttatgcctccag	59·24	
LXR-α	Forward	aagatgcaggagaccaggga	60·25	207
	Reverse	gctcgttccccagcatttttg	60·11	
LXR-β	Forward	ttcgggcttccactacaacg	60·32	326
	Reverse	tgcttgcttccgaagtgc	58·55	
SREBP1c	Forward	agacaaactgcccatccacc	60·25	216
	Reverse	aagcggatgtagtcgatggc	60·25	
SREBP2	Forward	atgatcaccccgacgttca	59·83	120
	Reverse	gtcacgaggcttgcacttg	60·04	

ABCG5/G8, ATP-binding cassette subfamily G5/G8; *AMPK*, AMP-activated protein kinase; *CYP7A1*, cholesterol 7α-hydroxylase; *FAS*, fatty acid synthase; *FXR*, farnesoid X receptor; *HMGCR*, 3-hydroxy-3-methylglutaryl-CoA reductase; *INSIG1/2*, insulin-induced gene 1/2; *LXR-α/β*, liver X receptor-α/β; *SREBP1/2*, sterol regulatory element-binding protein 1/2.

*Primers were designed using the program Primer3 based on the NCBI reference sequence of each protein.

and flavonoids were calculated as soyasaponin I and aglycone equivalents, respectively, using standards (Sigma).

The identities of flavonoids and saponins were confirmed by HPLC–time-of-flight/MS (Model G1969A Agilent 1100; Agilent Technologies) with the same chromatographic conditions as described previously. Mass spectral data were collected using electrospray ionisation in a positive mode under the following conditions: m/z range, 100–1400; N_2 gas; gas temperature, 250°C; flow rate of the dry gas, 13 litres/min; nebuliser pressure, 344738 Pa; capillary voltage, 4000 V; fragment voltage, 70 V. Extracted ion chromatograms were obtained based on the accurate masses obtained for the compounds or their adducts with Na or K with an error range of 0·01 units, using Analyst QS 1.1 software (Applied Biosystems).

Culture of primary rat hepatocytes

Rat hepatocytes were isolated by collagenase perfusion and separated from non-parenchymal liver cells by centrifugation at 325 *g*, as reported previously[26]. Primary hepatocytes were plated in a six-well plate (9·6 cm²/well) (Corning CellBIND). At 4 h after plating the hepatocytes, FSE, the flavonoid- or saponin-rich fraction and/or T0901317, a high-affinity LXR agonist (10 μM), was added. The concentrations used were based on the most abundant compounds, such as quercetin 3-O-glucoside (100 μM) and soyasaponin Af1 (1 μM). The hepatocytes were stimulated for 24 h for each treatment. Each treatment was performed in triplicate.

Animals and dietary groups

Male C57BL/6 mice were bred and raised at the animal facility of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán in Mexico City. A total of forty-eight mice (21 d old, initial weight 12–15 g) were randomly assigned to six experimental groups; the size of the sample was determined by the well-known method called power analysis. Each group was fed *ad libitum* for 35 d with one of the experimental diets detailed in Table 1. The diets were carefully prepared according to the standards of the AIN-93 diet for laboratory rodents[27]. All animals were maintained under standard environmental conditions (20°C, diurnal cycle 12 h light–12 h dark).

Food consumption and body weight were recorded every 3–5 d. The effects of the diets on energy metabolism were determined during the 4th week (detailed in the next section). During the last week, faeces were collected daily. At the end of the study, mice were killed in a CO_2 chamber after 12 h of food deprivation. Blood was collected via the portal vein, and serum was obtained by centrifugation at 1000 *g* for 10 min and stored at −80°C until further analysis. The liver and ileum were rapidly excised, frozen in liquid N_2 and stored at −80°C. The animal protocol was approved by the Institutional Animal Care and Research Advisory Committee of the Instituto Nacional de Ciencias Médicas y Nutrición in México City.

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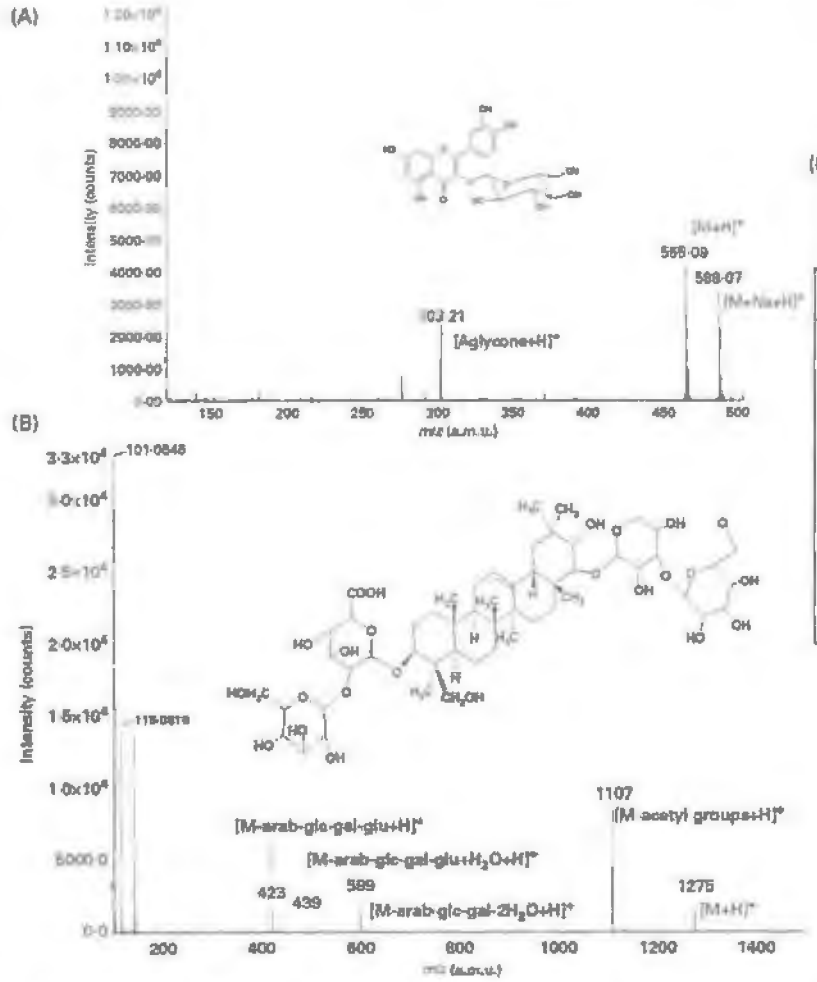

(C)

Identification and concentration of compounds from the black bean seed coat extract

	mg/g extract*	
	Mean	SE
Identified flavonoids		
Myricetin 3-*O*-glucoside	14·83	1·18
Quercetin 3-*O*-glucoside	118·68	4·03
Kaempferol 3-*O*-glucoside	0·75	0·02
Identified saponins		
Phaseoside I	0·17	0·03
Soyasaponin Af	1·42	0·13
Deacetyl soyasaponin Af	0·26	0·08
Soyasaponin Ba	0·34	0·29
Soyasaponin αg	1·26	0·02
Soyasaponin βg	0·50	0·01
Soyasaponin γg	0·20	0·01

Fig. 1. Extraction and identification of compounds from black bean seed coats. Mass spectra of the major (A) flavonoid (quercetin 3-*O*-glucoside) and (B) saponin (soyasaponin Af) extracted from black bean seed coats. (C) Amounts of other compounds identified in the black bean seed coat extract. a.m.u., Atomic mass unit; arab, arabinose; glc, glucoside; gal, galactose; glu, glucose.

Determination of the effects of the diets on energy metabolism

O_2 consumption, CO_2 production and food intake were measured using a laboratory animal monitoring system (Columbus Instruments). From each experimental group, three mice were housed individually in Plexiglas cages for 3 d and three nights, and 0·6 litres of air per min were passed through each cage. Detectors that were previously calibrated with primary gas standards of high purity were used to measure the O_2 and CO_2 levels sequentially in each chamber for 45 s. The RER was calculated as the average volume of CO_2 produced (VCO_2, ml/kg per h) by the three mice over the 3 d and three nights divided by the volume of O_2 consumed (VO_2, ml/kg per h).

Quantification of serum biochemical parameters and lipids in the liver

The levels of total cholesterol, TAG, HDL- and LDL-cholesterol were measured in serum obtained from food-deprived mice using a COBAS C111 analyser (Roche). The concentration of insulin in serum was measured by ELISA according to the manufacturer's protocol (Merck KGaA). Total lipids were extracted twice from homogenised hepatic tissue with chloroform–methanol (2:1) solution. The organic layer was dried under N_2, solubilised in isopropanol/Triton X-100 (10 %) and then assayed to determine the concentrations of total cholesterol and TAG using enzymatic kits (DiaSys Diagnostic Systems GmbH).

Histological analysis

Liver samples were dissected and immediately fixed with ice-cold 4 % (w/v) paraformaldehyde in PBS and embedded in paraffin. For each block, two 4 μm sections were then stained with haematoxylin and eosin. To visualise neutral lipids, frozen liver sections (8 μm) were stained with Oil Red O (Sigma). Stained slides were imaged with a Sony CCS-IRIS digital camera coupled to a Leica microscope at 20× magnification.

RNA extraction and real-time RT-PCR

Total RNA from the hepatocytes and tissue was obtained using TRIzol reagent, quantified using a NanoDrop spectrophotometer

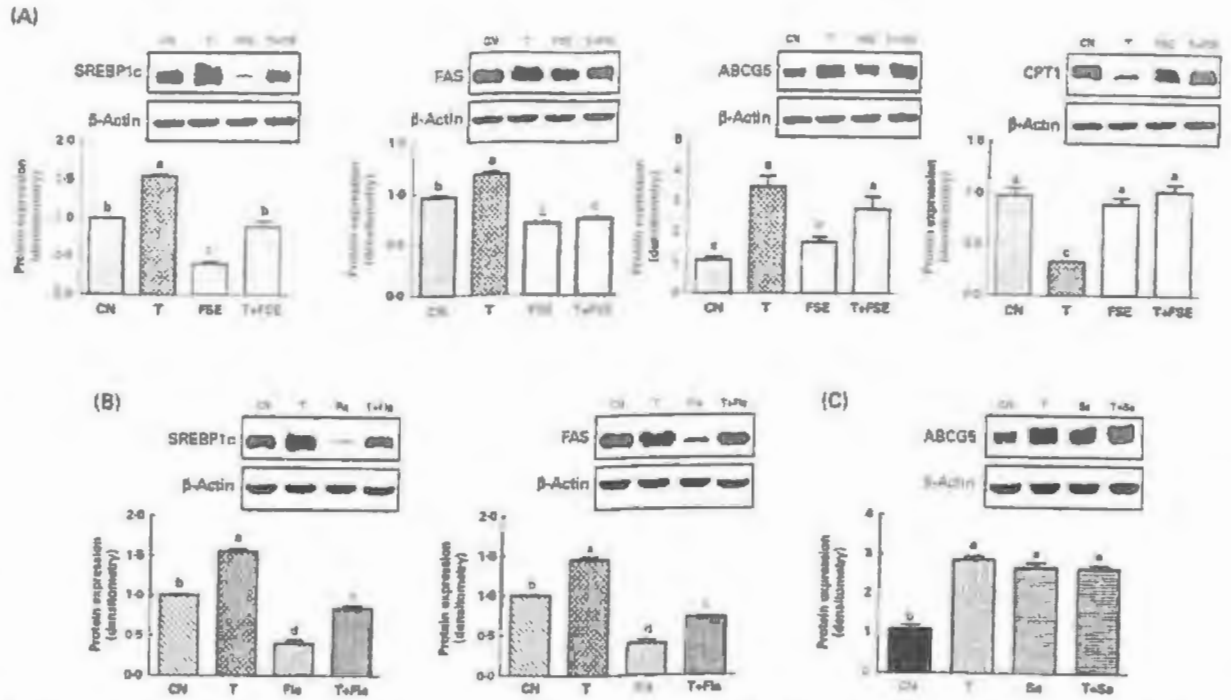

Fig. 2. Effects of the black bean seed coat extract on the expression of key proteins involved in lipid metabolism. (A) Relative expression and protein levels of sterol regulatory element-binding protein 1c (SREBP1c) and fatty acid synthase (FAS) (key lipogenic proteins), ATP-binding cassette, subfamily G5 (ABCG5, a key protein in reverse cholesterol transport) and carnitine palmitoyltransferase 1 (CPT1, a key protein in β-oxidation) in primary rat hepatocytes treated with no stimulus (control diet; CN), the synthetic liver X receptor (LXR) agonist T0901317 (T; 10 μM), the flavonoid- and saponin-rich extract (FSE) at a concentration of 100 μM based on the major compound (quercetin 3-*O*-glucoside), or T0901317 and the extract at the same dose (T+FSE). (B) Relative expression and protein levels of SREBP1c and FAS in primary rat hepatocytes treated with no stimulus (CN), the synthetic LXR agonist T0901317 (T; 10 μM), the flavonoid-rich fraction (Fla) at a dose of 100 μM based on the major compound (quercetin 3-*O*-glucoside), or T0901317 and the flavonoid-rich fraction at the same dose (T+Fla). (C) Relative expression and protein levels of ABCG5 in primary rat hepatocytes treated with no stimulus (CN), the synthetic LXR agonist T0901317 (T; 10 μM), the saponin-rich fraction (Sa) at a dose of 1 μM based on the major compound (soyasaponin Af), or T0901317 and the saponin-rich fraction at the same dose (T+Sa). Values are means, with their standard errors represented by vertical bars. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P<0.05$).

(NanoDrop Technologies), and reverse transcribed. PCR amplification was performed in a Roche LightCycler 480 II using the TaqMan or SYBR Green System. Primers for PCR amplification were designed using the program Primer3 (Howard Hughes Medical Institute) to obtain an amplicon size of 100–350 bp (Table 2). Relative expression levels were calculated by the $2^{-\Delta\Delta C_T}$ method[28]. Assays for each gene were performed in triplicate in ninety-six-well optical plates.

Protein extraction and Western blotting

Protein extraction and Western blotting were performed as reported previously[28]. Briefly, for each experimental group, equal quantities of protein from each mouse were pooled for Western blotting analysis of tissues. Total protein (30 or 40 μg) was loaded onto 8% polyacrylamide gels, separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with non-fat dry milk (Bio-Rad) and incubated overnight at 4°C with the following primary antibodies: anti-SREBP1c; anti-SREBP2 (Millipore); anti-ABCG5 and ABCG8; anti-fatty acid synthase (FAS); anti-carnitine palmitoyltransferase 1 (CPT1); anti-3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR); anti-insulin-induced gene 1 and 2 (INSIG1 and INSIG2); anti-total and phosphorylated AMP-activated protein kinase (AMPK and p-AMPK); anti-peroxisome proliferator-activated α receptors (PPAR-α) (Santa Cruz Biotechnology). Then, the blots were incubated with a secondary antibody and visualised using a ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad). Bands were analysed using the ImageJ 1.42p digital imaging processing program (http://rsb.info.nih.gov/ij/; March 27, 2012).

Faecal bile acid analysis by GC

Dried faecal samples from each experimental group (100 mg) were diluted in saline solution, and norcholic acid was added as an internal standard (100 mg/l). Bile acids from the faeces were extracted and quantified as described previously[30].

Statistical analysis

Results are expressed as means with their standard errors. Data were analysed with GraphPad 6.0 (Prism) and MINITAB 16 (Minitab Inc.) software. To identify significant differences among the groups, statistical analysis was performed by one-way ANOVA followed by Tukey's test. The distribution type of the variables was examined using the Kolmogorov–Smirnov 2 test. Logarithmic transformation was performed before

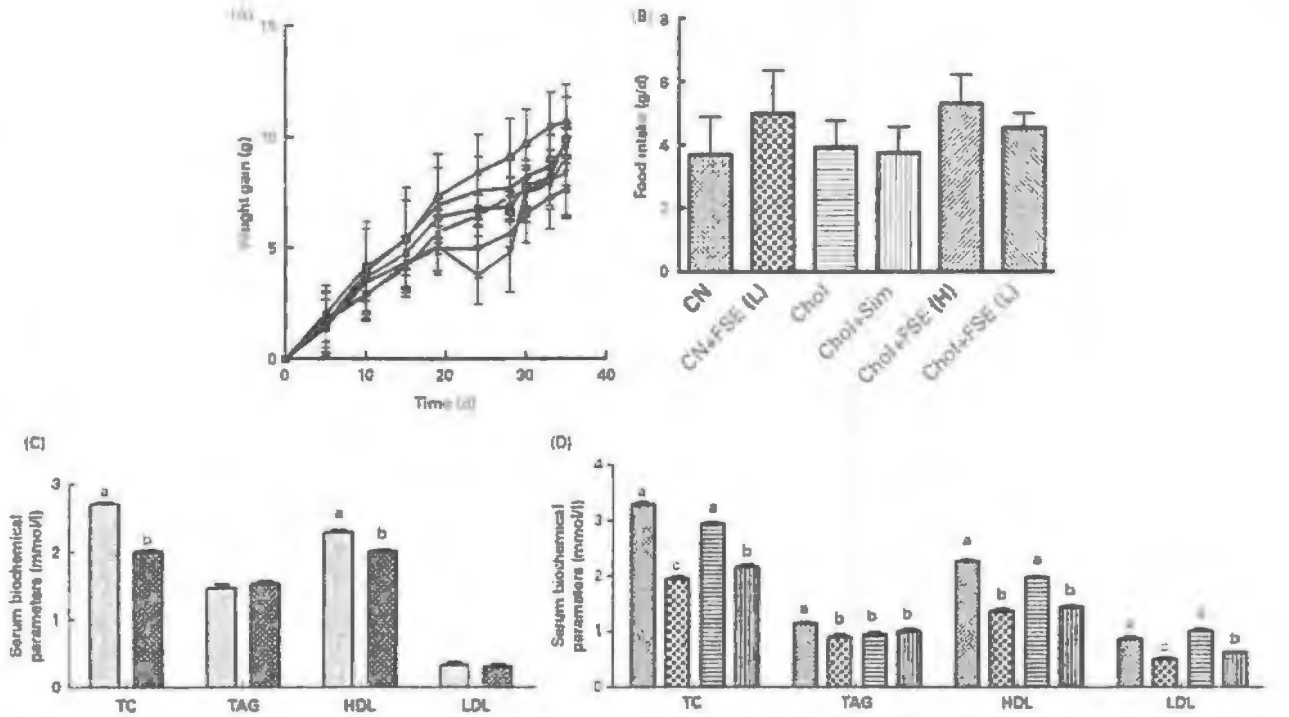

Fig. 3. Effects of the black bean seed coat extract on (A) body-weight gain, (B) food intake and (C, D) serum biochemical parameters in C57BL/6 mice. A total of forty-eight mice were randomised into six groups that received the following experimental diets for 5 weeks: CN, control diet (*n* 7; (A) –●– and (C) ■); CN+FSE (L), control diet with 0·25 % (low-dose) black bean seed coat extract (*n* 8; (A) –●– and (C) ■); Chol, control diet with 0·5 % cholesterol (*n* 8; (A) –▲– and (D) ■); Chol+Sim, control diet with 0·5 % cholesterol and 0·03 % simvastatin (statin) (*n* 8; (A) –■– and (D) ■); Chol+FSE (H), control diet with 0·5 % cholesterol and 0·5 % (high-dose) black bean seed coat extract (*n* 9; (A) –◆– and (D) ■); Chol+FSE (L), control diet with 0·5 % cholesterol and 0·25 % (low-dose) black bean seed coat extract (*n* 8; (A) –●– and (D) ■). Body weight (A) and food intake (B) were measured every 3 to 5 d. (C, D) At the end of the study, plasma was obtained from C57BL/6 mice that were fasted for 12 h to determine the serum biochemical parameters. Values are means, with their standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P < 0·05$). TC, total cholesterol.

analysis if the variables did not exhibit a normal distribution. Differences were considered to be significant at $P<0·05$.

Results

Characterisation and composition of the black bean seed coat extract

Quercetin 3-*O*-glucoside was the primary flavonoid identified in the extract of black bean seed coats (Fig. 1(A)), and the most abundant saponin was soyasaponin Af (Fig. 1(B)). In addition, other flavonoids and saponins were identified and quantified (Fig. 1(C)).

Expression of lipogenic proteins and ATP-binding cassette subfamily G, member 5

The extract rich in flavonoids and saponins reduced the expression of both *SREBP1c* and *FAS* in primary rat hepatocytes. The synthetic ligand of LXR, T0901317, increased the expression of lipogenic proteins compared with the levels in the control group, as expected. Interestingly, the addition of the FSE partially repressed the stimulatory effect of T0901317 on the expression of the lipogenic proteins *SREBP1c* and *FAS*. In contrast, the expression of *ABCG5* was not repressed by the FSE, and this extract did not reduce the stimulatory effect of T0901317 on the expression of *ABCG5*. The potential effects of the FSE on genes involved in the β-oxidation of fatty acids, especially the rate-limiting enzyme *CPT1*, were also analysed in primary rat hepatocytes. The FSE had no effect on the expression of *CPT1*, however, it inhibited the suppressive effect of T0901317 on genes in primary hepatocyte cultures (Fig. 2(A)).

The analysis of the FSE fractions revealed that both families of compounds, flavonoids and saponins, had effects similar to those observed with the FSE. The flavonoid-rich fraction had a stronger effect on the down-regulation of the expression of lipogenic proteins (Fig. 2(B)), whereas the saponin-rich fraction had a greater effect on the up-regulation of the expression of the transporter ABCG5 similar to the synthetic ligand of LXR, T0901317 (Fig. 2(C)).

Plasma lipid profile in mice

The addition of the FSE to the diets did not significantly change the body-weight gain among the groups during the 5-week study (Fig. 3(A)). We measured the average daily food intake of mice in all experimental groups, and no significant difference was found during the 5-week study (Fig. 3(B)). Interestingly, the FSE significantly reduced the levels of total cholesterol and HDL-cholesterol in the serum of mice fed the control diet. These effects of the FSE were significantly enhanced in mice fed a high-cholesterol diet by decreasing the levels of

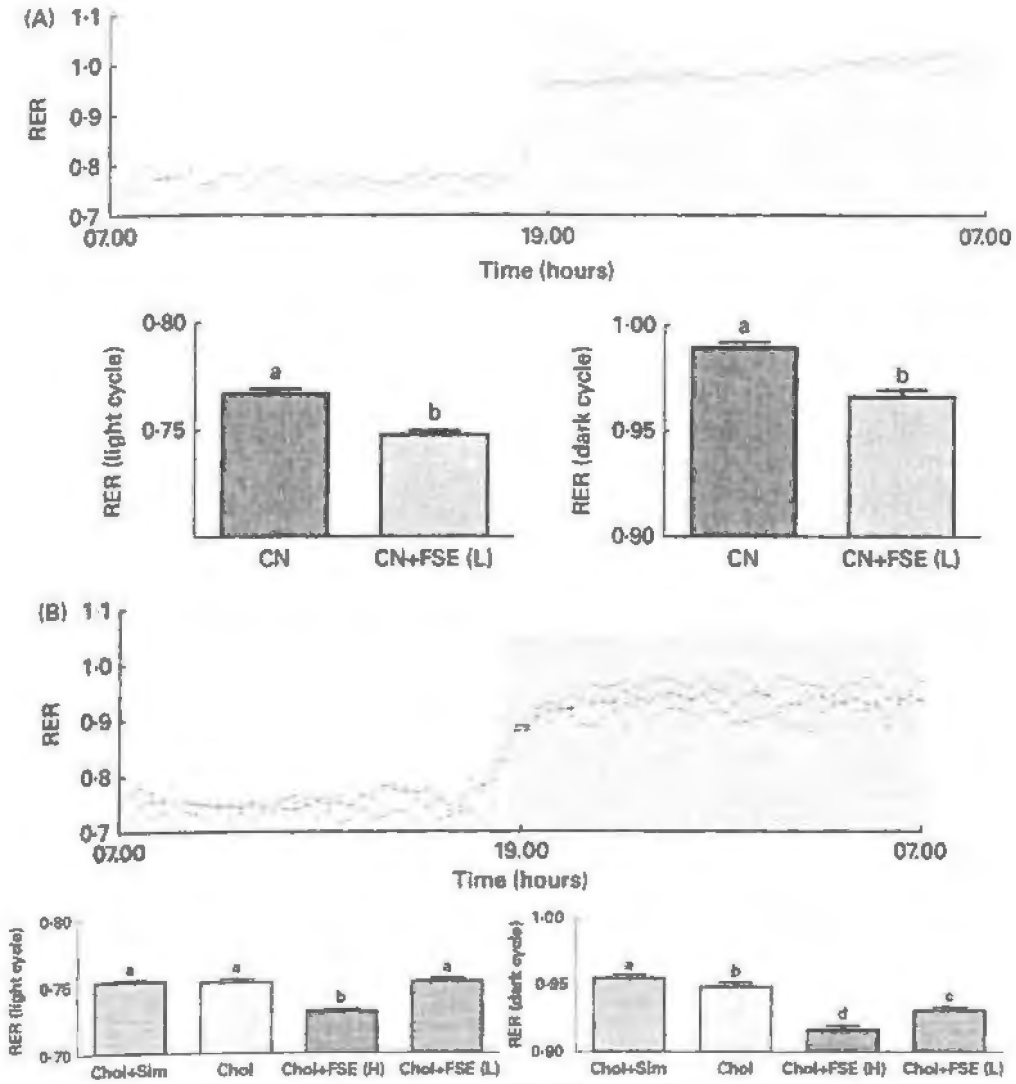

Fig. 4. Effects of the experimental diets on the average RER of C57BL/6 mice. C57BL/6 mice (n 3 per group) were placed in metabolism cages and analysed for 3 d. (A) Average RER of mice fed the control diet (CN; —•—) and the control diet with the black bean seed coat extract at a low dose (CN+FSE (L); —○—). RER (light cycle): CN – 0·76 (SE 0·002), lipids = 90·80 %, carbohydrates = 19·20 %; CN–FSE (L) – 0·74 (SE 0·001), lipids = 88 %, carbohydrates = 12 %. RER (dark cycle): CN – 0·98 (SE 0·002), L = 6·37 %, carbohydrates = 93·7 %; CN–FSE (L) – 0·96 (SE 0·003), lipids = 12·8 %, carbohydrates = 87·2 %. (B) Average RER of mice fed the control diet with cholesterol (0·5 %, Chol;), the control diet with cholesterol (0·5 %) and the extract (0·5 %, Chol+FSE (H); —○—), the control diet with cholesterol (0·5 %) and the extract (0·25 %, Chol+FSE (L); —○—) and the control diet with cholesterol (0·5 %) and simvastatin (0·03 %, Chol+Sim; —•—). RER (light cycle): Chol+Sim – 0·75 (SE 0·001), lipids = 84·4 %, carbohydrates = 15·6 %; Chol – 0·75 (SE 0·002), lipids = 84·4 %, carbohydrates = 15·6 %; Chol+FSE (H) – 0·73 (SE 0·001), lipids = 91·6 %, carbohydrates = 8·4 %; Chol+FSE (L) – 0·75 (SE 0·002), lipids = 84·4 %, carbohydrates = 15·6 %. RER (dark cycle): Chol+Sim – 0·96 (SE 0·002), lipids = 12·8 %, carbohydrates = 87·2 %; Chol – 0·95 (SE 0·003), lipids = 16·0 %, carbohydrates = 84·0 %; Chol+FSE (H) – 0·92 (SE 0·003), lipids = 25·9 %, carbohydrates = 74·1 %; Chol+FSE (L) – 0·93 (SE 0·002), lipids = 22·6 %, carbohydrates = 77·4 %. The RER was calculated from O_2 consumption and CO_2 production at week 4. The black bean seed coat extract decreased energy expenditure and induced lipid oxidation. Values are means, with their standard errors represented by vertical bars. ^{a,b,c,d}Mean values with unlike letters were significantly different ($P < 0·05$). A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn

LDL-cholesterol and TAG in addition to the levels of total cholesterol and HDL-cholesterol (Fig. 3(C) and (D)). No significant differences were observed in the levels of serum insulin and glucose among the groups (data not shown).

Effects of the black bean seed coat extract on the RER in mice

The present results showed that the RER was significantly lower in the group fed with the FSE than in the control group, with a dose-dependent effect being observed. Although the RER differed between night and day, the trend remained the same. The effect of the FSE on the RER was more evident in mice fed the cholesterol-containing diets (Fig. 4).

Effects of the black bean seed coat extract on lipogenesis in the liver of C57BL/6 mice

The *in vitro* assays revealed that the FSE significantly decreased the expression of lipogenic proteins in the liver of

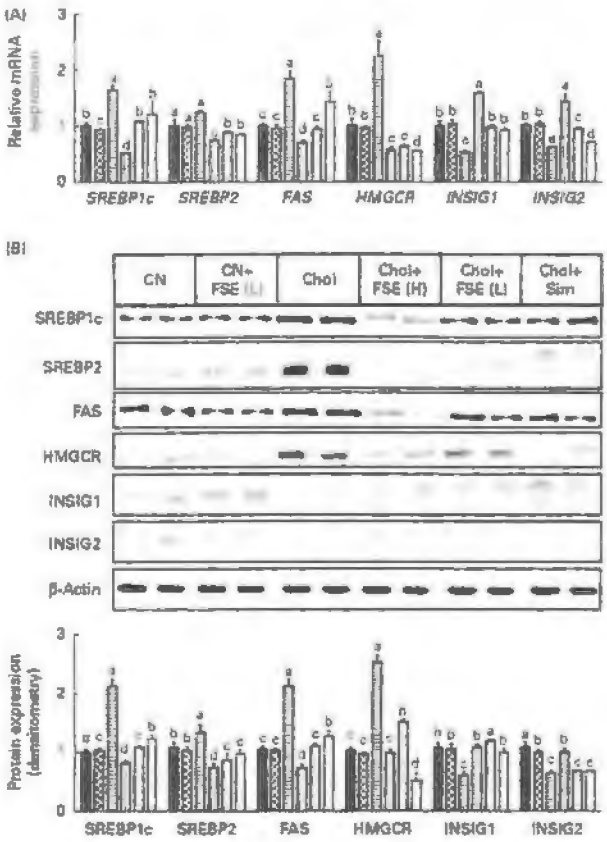

Fig. 5. Effects of the experimental diets on liver lipogenesis in C57BL/6 mice. (A) Relative mRNA expression levels of the lipogenic proteins sterol regulatory element-binding protein 1c (*SREBP1c*), *SREBP2*, fatty acid synthase (*FAS*) and 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*) and of insulin-induced gene 1 (*INSIG1*) and *INSIG2*, key proteins involved in the degradation of SREBP proteins. (B) Protein expression levels of the same proteins determined by Western blotting. The experimental diets were as follows: CN, control diet (■); CN+FSE (L), control diet with the flavonoid- and saponin-rich extract (0·25 %) (▩); Chol, control diet with cholesterol (0·5 %) (▩); Chol+FSE (H), control diet with cholesterol (0·5 %) and the extract (0·5 %) (▩); Chol+FSE (L), control diet with cholesterol (0·5 %) and the extract (0·25 %) (▩); Chol+Sim, control diet with cholesterol (0·5 %) and simvastatin (0·03 %) (▩). The black bean seed coat extract significantly decreased the expression levels of lipogenic proteins in C57BL/6 mice. Values are means, with their standard errors represented by vertical bars. ^{a,b,c,d,e} Mean values with unlike letters were significantly different ($P < 0·05$).

C57BL/6 mice. This decrease in the expression of lipogenic proteins was observed at both the mRNA (Fig. 5(A)) and protein (Fig. 5(B)) levels. Moreover, a dose-dependent effect was observed among the experimental groups fed with the FSE. Interestingly, we observed that the FSE induced the over expression of *INSIG1*/*INSIG2* (Fig. 5).

Histological analysis

Histological analysis of liver sections stained with haematoxylin and eosin (Fig. 6(A)) or Oil Red O (Fig. 6(B)) showed that mice fed the cholesterol diets had significantly greater hepatic fat accumulation than the control mice, and these results were confirmed by the quantification of hepatic lipid levels (Fig. 6(C)). Mice fed a diet containing cholesterol and FSE at a low dose had a significant decrease in hepatic lipid depots; however, this reduction was only modest when compared with that in the group of mice fed a cholesterol-containing diet with simvastatin. However, the addition of the FSE at a high dose to the cholesterol-containing diet had effects on the reduction of hepatic lipid levels similar to those observed in mice fed a cholesterol-containing diet with simvastatin. There was no difference in the hepatic lipid levels between the control groups fed the diets with and without the FSE (Fig. 6(C)).

Effects of the black bean seed coat extract on bile acid synthesis

Bile acid formation depends on the synthesis of bile acids as well as on the hepatic transport of cholesterol to the bile. These processes are mainly regulated by the enzyme cholesterol 7α-hydroxylase (CYP7A1) and by the reverse cholesterol transporters, respectively. The present results showed that the expression of ABCG5/ABCG8 in the liver (Fig. 7(A)) and ileum (Fig. 7(B)) was significantly increased by the addition of the FSE to the diet. However, the cholesterol-containing diet with simvastatin down-regulated the expression of ABCG5/ABCG8 in the ileum, promoting the absorption of cholesterol (Fig. 7(B)). Intriguingly, the FSE did not further stimulate LXR-α or PPAR-α expression in the ileum, suggesting that the FSE could modify LXR activity without an increase in the levels of LXR (data not shown). Additionally, the FSE stimulated bile acid synthesis via the up-regulation of CYP7A1 (Fig. 7(C)). As a result, we observed that the inclusion of the FSE in the diet increased the amount of bile acid in the stools of mice (Fig. 7(D)).

Effects of black bean seed coat extract on liver X receptor activity

To determine how the FSE increased the activity of LXR, we first found that feeding the FSE resulted in a significant increase in the relative expression level of LXR as well as the protein level of LXR-α (Fig. 8(A) and (B), respectively) without any significant changes in the level of LXR-β or farnesoid X receptor. We then found that LXR activity could be modulated by its phosphorylation. Particularly, phosphorylation of LXR-α by the enzyme AMPK reduced its capacity to stimulate the lipogenic genes. The present results suggest that the FSE could activate this mechanism, since the intake of the FSE increased the phosphorylation of AMPK which is the active form of this enzyme (Fig. 8(C)).

Effects of the black bean seed coat extract on the expression of carnitine palmitoyltransferase I and PPAR-α

Surprisingly, cholesterol intake by C57BL/6 mice decreased the expression levels of hepatic PPAR-α and CPT1. In contrast, mice fed the high-cholesterol diet with the FSE did not exhibit decreases in the expression levels of these proteins (Fig. 9). These results are in agreement with the *in vitro* data as

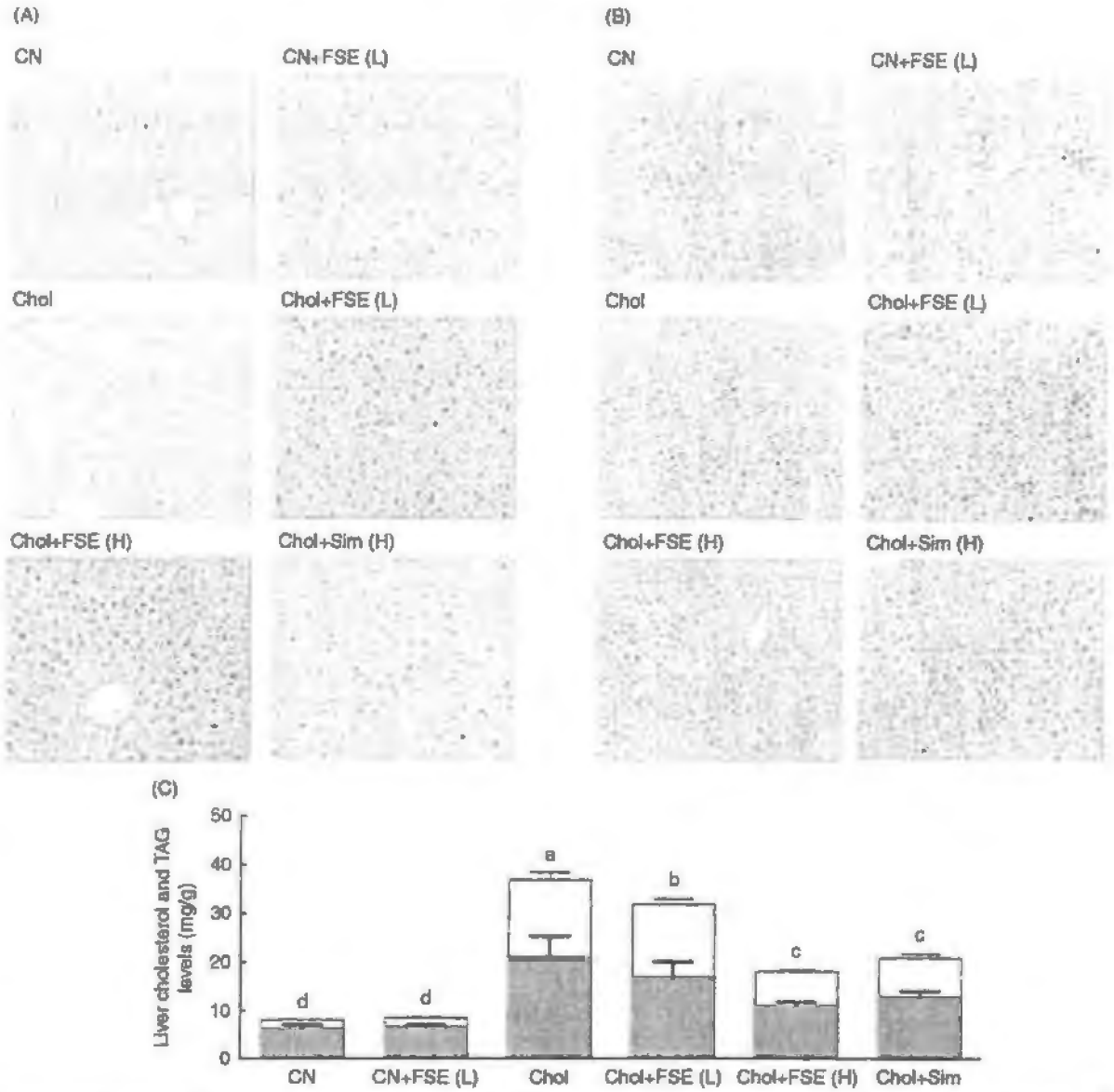

Fig. 6. Effects of the experimental diets on lipid accumulation in the liver of C57BL/6 mice. Hepatic tissue stained with (A) haematoxylin and eosin and (B) Oil Red O, showing differences in lipid accumulation between the experimental groups. (C) Lipid accumulation in the liver was confirmed by the measurement of the cholesterol (□) and TAG (▨) levels. The experimental diets were as follows: CN, control diet; CN–FSE (L), control diet with the flavonoid- and saponin-rich extract (0·25 %); Chol, control diet with cholesterol (0·5 %); Chol+FSE(L), control diet with cholesterol (0·5 %) and the extract (0·25 %); Chol+FSE (H), control diet with cholesterol (0·5 %) and the extract (0·5 %); Chol+Sim, control diet with cholesterol (0·5 %) and simvastatin (0·03 %). Values are means, with their standard errors represented by vertical bars. [a–d] Mean values with unlike letters were significantly different ($P<0·05$). A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn

well as the RER, suggesting that the FSE could stimulate the β-oxidation of fatty acids.

Discussion

In the present study, we examined the effect of flavonoids and saponins extracted from black bean seed coats on the expression of key proteins involved in the regulation of lipid and cholesterol metabolism. We first examined the effect of the FSE on proteins regulated by the transcription factor LXR in primary rat hepatocytes. The results showed that the extract reduced the expression of lipogenic proteins, but induced the expression of reverse cholesterol transport such as ABCG5. Furthermore, the addition of the FSE to the diet repressed the stimulatory effect of synthetic LXR ligands on lipogenic proteins, but, again, this extract did not reduce the stimulatory effect of T0901317 on the expression of ABCG5. Interestingly, despite the FSE having no effect on the expression of CPT1, it inhibited the suppressive effect of T0901317 on this rate-limiting enzyme of fatty acid β-oxidation. These results

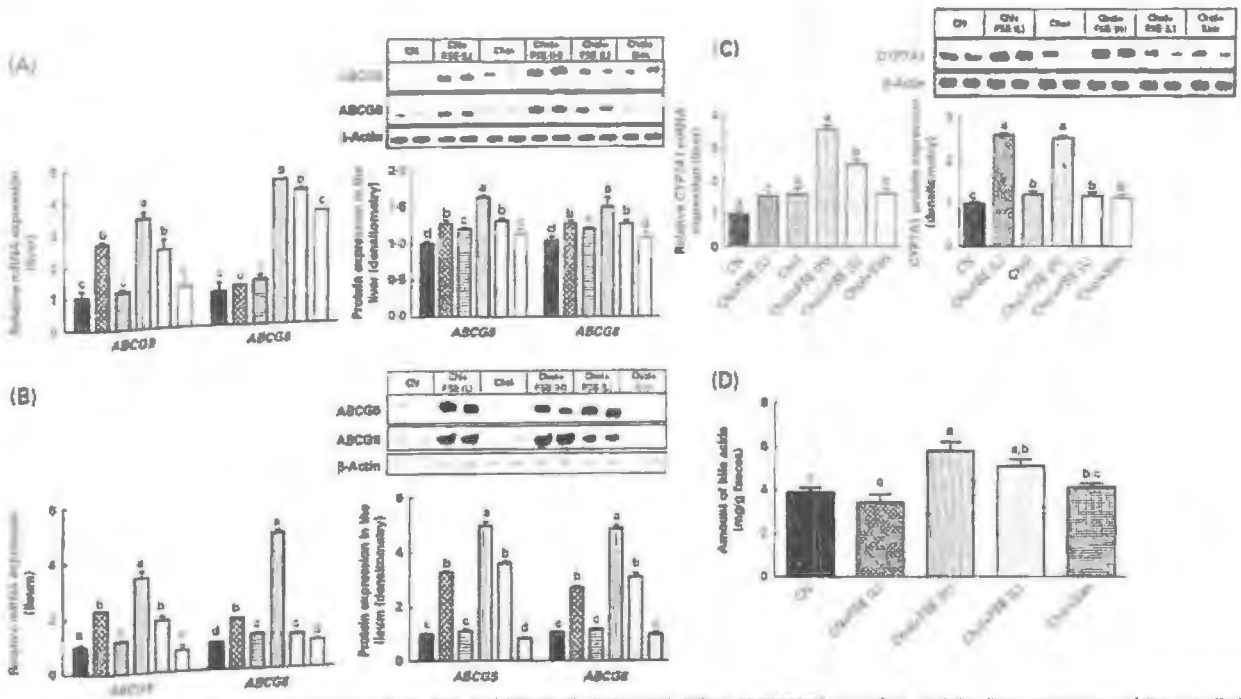

Fig. 7. Effects of the black bean seed coat extract on liver and intestinal cholesterol, biliary cholesterol secretion, and the liver expression of the rate-limiting enzyme in the synthesis of bile acids. (A) In the liver, the black bean seed coat extract significantly increased the relative protein expression levels of ATP-binding cassette, subfamily G5 (ABCG5)/ABCG8. These proteins mediated the excretion of free cholesterol into the bile. (B) In the ileum, ABCG5/ABCG8 suppressed the absorption of sterols. This suppressive effect was increased in the experimental groups fed the extract. (C) The level of cholesterol 7α-hydroxylase (CYP7A1), which catalysed the rate-limiting step in the synthesis of bile acids, was up-regulated in the liver by the extract. (D) The amount of bile acid was increased in the stools of mice fed the extract. The experimental diets were as follows: CN, control diet (■); CN+FSE (L), control diet with the flavonoid- and saponin-rich extract (0·25 %) (■); Chol, control diet with cholesterol (0·5 %) (■); Chol+FSE (H), control diet with cholesterol (0·5 %) and the extract (0·5 %) (■); Chol+FSE (L), control diet with cholesterol (0·5 %) and the extract (0·25 %) (■); Chol+Sim, control diet with cholesterol (0·5 %) and simvastatin (0·03 %) (■). Values are means, with their standard errors represented by vertical bars. [a,b,c,d] Mean values with unlike letters were significantly different ($P<0·05$)

suggested that flavonoids and saponins extracted from black bean seed coats were capable of regulating the activity of LXR. Moreover, studies with flavonoid- and saponin-rich fractions have suggested that there is a synergistic effect exerted by flavonoids and saponins contained in the extract to control lipogenesis as well as reverse cholesterol transport.

To understand the potential physiological relevance of *in vitro* studies on cholesterol and fatty acid metabolism, we then studied the effects of the FSE in C57BL/6 mice fed diets with or without cholesterol. Metabolic changes were not associated with differences in food intake among the groups, and as a result, at the end of the study, there was no significant difference in weight gain among the groups. The present data showed that the plasma lipid profile was indeed improved by feeding the extract in mice. Additionally, analysing the RER, we observed that the FSE was able to modulate the type of energy substrate for energy generation used by mice. The present data indicated that the proportion of lipids used as a substrate for energy generation was greater in mice fed the FSE than in mice fed the control diets or diets with simvastatin. The control diet containing cholesterol increased the expression of *SREBP1c* possibly by activating LXR and hence the lipogenesis programme[31].

The beneficial effect of the FSE on decreasing the levels of serum cholesterol in C57BL/6 mice was associated with a reduction in hepatic lipogenesis and cholesterol biosynthesis through the down-regulation of *SREBP1c*, *FAS* and *HMGCR* and the up-regulation of *INSIG1*/*INSIG2*. It has been demonstrated that overexpression of *INSIG1* traps the SCAP (SREBP cleavage-activating protein)/SREBP complex in the endoplasmic reticulum, promoting SREBP degradation and reducing the ability of SREBP to activate the transcription of genes that encode enzymes involved in cholesterol and fatty acid biosynthesis[32]. A similar down-regulatory effect of lipogenic proteins by the consumption of wild blueberry-enriched diet[33] and coumarin has been reported, the latter was related to an attenuation of hepatic steatosis[34].

We then studied cholesterol metabolism, which showed an increment in the mRNA abundance of *SREBP2* and *HMGCR* in mice fed the control diet with cholesterol. In agreement with previous studies, we showed that simvastatin decreased the levels of *HMGCR* more than those of the other lipogenic proteins. Interestingly, the addition of cholesterol to the diet did not significantly change the abundance of *SREBP2* mRNA; however, the level of the mature form of *SREBP2* was significantly increased, indicating an increase in the processing of the precursor form of *SREBP2* in the Golgi[35]. The increase in the concentration of this transcription factor was markedly reduced when mice were fed the FSE, reducing the cholesterol biosynthetic programme.

To reduce the concentration of hepatic cholesterol, the FSE increased the expression of genes involved in bile acid synthesis,

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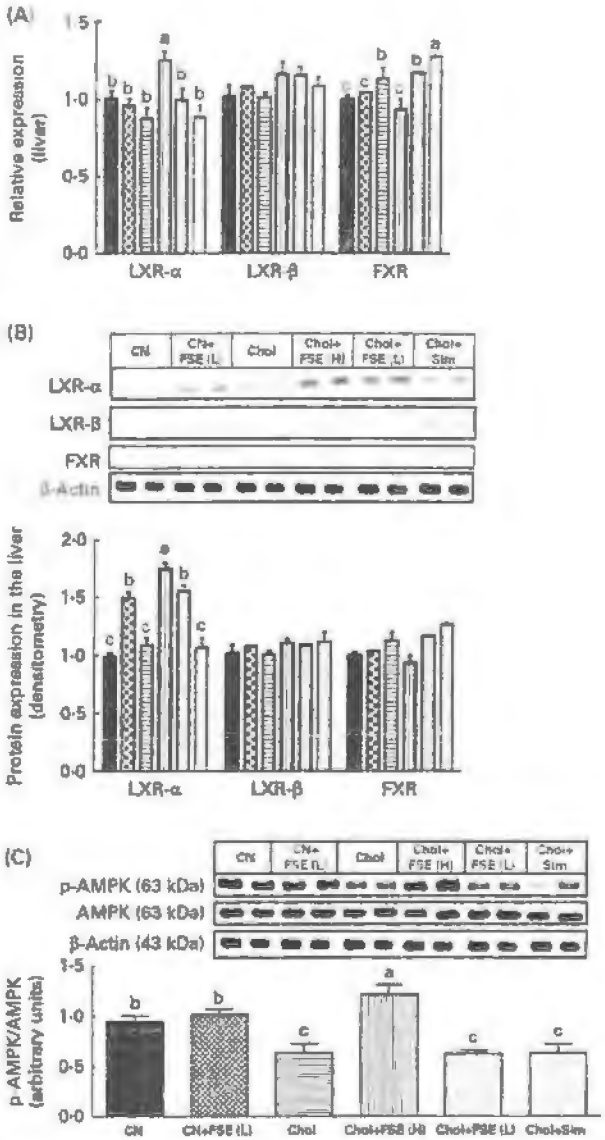

Fig. 8. Effects of the black bean seed coat extract on liver X receptor (LXR), farnesoid X receptor (FXR) and the phosphorylation of AMP-activated protein kinase (p-AMPK). The extract significantly increased (A) the relative expression levels of hepatic FXR and LXR-α, (B) the protein abundance of LXR-α and (C) the phosphorylation of AMPK in the liver, which might activate LXR. The experimental diets were as follows: CN, control diet (■); CN+FSE (L), control diet with the flavonoid- and saponin-rich extract (0·25 %) (■); Chol, control diet with cholesterol (0·5 %) (■); Chol+FSE (H), control diet with cholesterol (0·5 %) and the extract (0·5 %) (■); Chol+FSE (L), control diet with cholesterol (0·5 %) and the extract (0·25 %) (■); Chol+Sim, control diet with cholesterol (0·5 %) and simvastatin (0·03 %) (■). Values are means, with their standard errors represented by vertical bars. [a,b,c,d]Mean values with unlike letters were significantly different ($P<0·05$).

particularly *CYP7A1*, as well as in reverse cholesterol transport, such as *ABCG5/ABCG8*; in addition, the FSE stimulated the expression of *ABCG5/ABCG8* in the intestine. In the liver, *ABCG5/ABCG8* drives the elimination of hepatic cholesterol into the bile, whereas in the intestine, it suppresses the absorption of biliary and dietary sterols[36,37]. The effective suppression of the accumulation of dietary cholesterol appears to require the overexpression of both intestinal and hepatic *ABCG5/ABCG8*[37]. This effect has been shown to be related to protection against atherosclerosis[38]. Additionally, CYP7A1 is the rate-limiting step in the synthesis of bile acid from cholesterol[39]. Hence, the results indicated that the FSE promoted the secretion of biliary cholesterol and reduced the absorption of dietary cholesterol. This result showing the stimulation of the expression of these genes by the FSE was associated with an increase in faecal bile acids. Interestingly, we observed a down-regulation of *ABCG5* in the intestine due to simvastatin, an effect that has been reported previously[40], suggesting that statins potentially can increase the absorption of intestinal cholesterol. Therefore, the FSE was represented as a prospectively more effective treatment to decrease cholesterol absorption by the up-regulation of *ABCG5/ABCG8*.

To elucidate the possible mechanism by which the FSE regulated the expression of genes involved in lipogenesis and reverse cholesterol transport (Fig. 10), it is important to point out that these genes are primarily controlled by the transcription factor LXR. The activity of these transcription factors is regulated by the binding of their natural or synthetic ligands, and also by its phosphorylation state. Recent evidence shows that isoflavones, particularly genistein, can modulate LXR activity by controlling the phosphorylation state of this nuclear receptor[20]. Phosphorylation of LXR-α and LXR-β can be triggered by the enzyme AMPK, resulting in the modulation of their biological activity in an opposite manner. In the present study, we demonstrated that the FSE stimulated the phosphorylation state of AMPK. Therefore, it is possible to suggest that the FSE up-regulates *ABCG5/ABCG8* and *CYP7A1*, suggesting tentatively the promotion of the excretion and

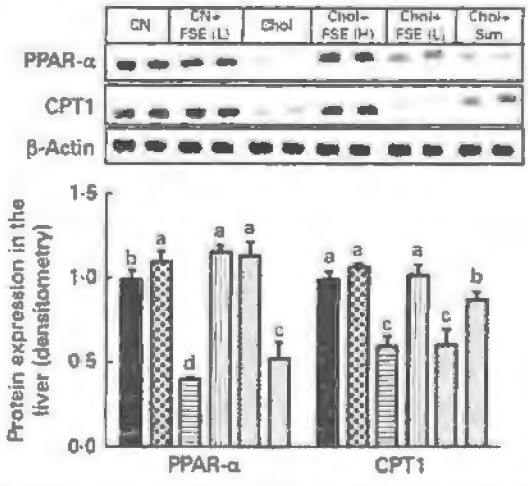

Fig. 9. Effects of the experimental diets on β-oxidation in the liver of C57BL/6 mice. The black bean seed coat extract at a high concentration significantly suppressed the effect of cholesterol on the protein expression levels of PPAR-α and carnitine palmitoyltransferase 1 (CPT1). The experimental diets were as follows: CN, control diet (■); CN+FSE (L), control diet with the flavonoid- and saponin-rich extract (0·25 %) (■); Chol, control diet with cholesterol (0·5 %) (■); Chol+FSE (H), control diet with cholesterol (0·5 %) and the extract (0·5 %) (■); Chol+FSE (L), control diet with cholesterol (0·5 %) and the extract (0·25 %) (■); Chol+Sim, control diet with cholesterol (0·5 %) and simvastatin (0·03 %) (■). Values are means, with their standard errors represented by vertical bars. [a,b,c,d]Mean values with unlike letters were significantly different ($P<0·05$).

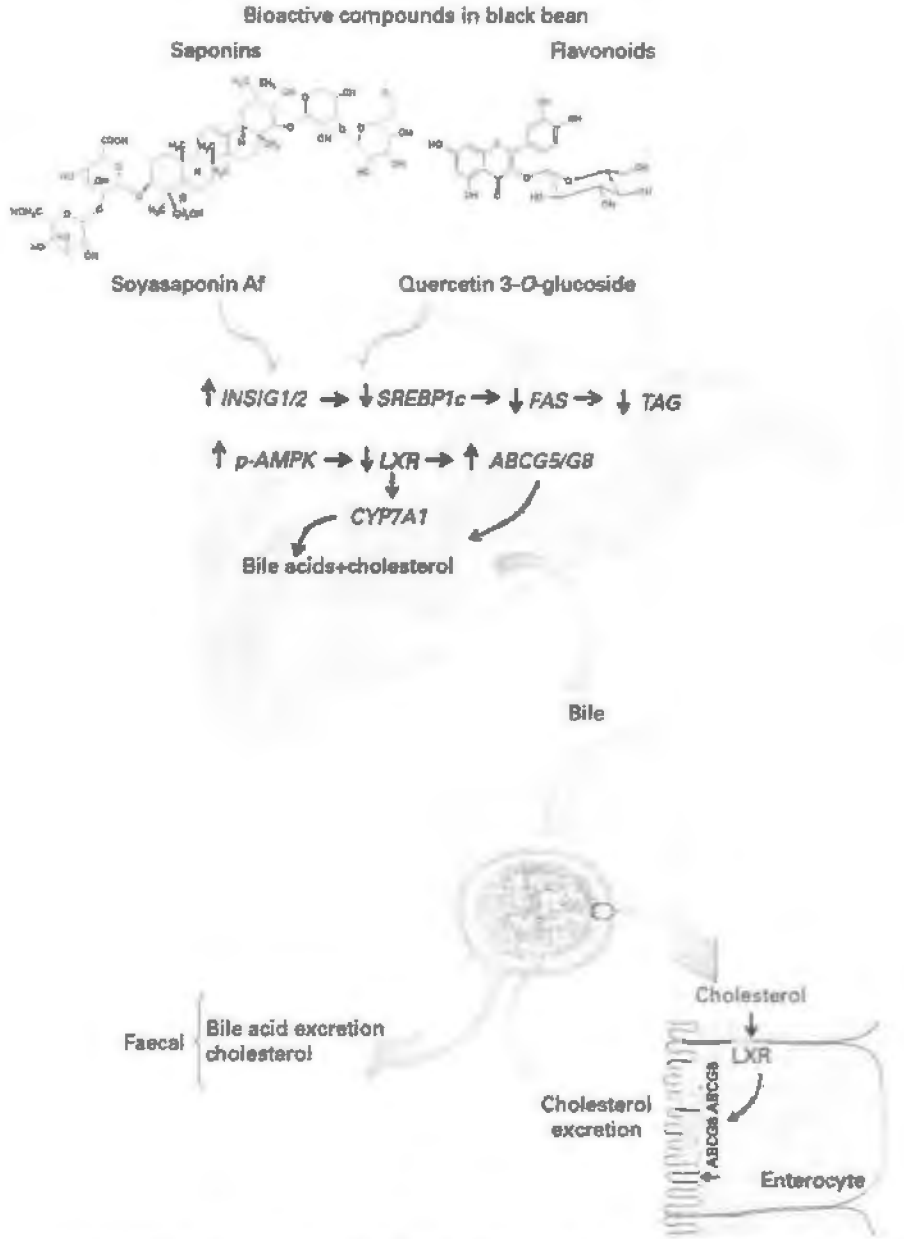

Fig. 10. Proposed model for the mechanism by which flavonoids and saponins extracted from black bean (*Phaseolus vulgaris* L.) seed coats modulate lipid metabolism and biliary cholesterol secretion. *INSIG1/2*, insulin-induced gene 1/2; *SREBP1c*, sterol regulatory element-binding protein 1; *FAS*, fatty acid synthase; *p-AMPK*, phosphorylation of AMP-activated protein kinase; *LXR*, liver X receptor; *ABCG5/G8*, ATP-binding cassette, subfamily G5/G8; *CYP7A1*, cholesterol 7α-hydroxylase. A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn

catabolism of cholesterol[41], but suppresses the expression of lipogenic proteins via LXR. In contrast, it has been suggested that LXR activation inhibits fatty acid oxidation by interfering with the binding of PPAR-α to its target sites[42]. We observed that cholesterol decreased the expression of *PPAR-α* and *CPT1*. Surprisingly, the FSE prevented the decrease in the expression of these genes in the liver, possibly stimulating fatty acid oxidation despite the presence of cholesterol in the diet.

In summary, the present results suggest that flavonoids and saponins extracted from black bean seed coats could potentially prevent hepatic lipid accumulation by attenuating lipogenesis and stimulating fatty acid oxidation and biliary cholesterol excretion through the differential regulation of LXR probably mediated by the phosphorylation of AMPK.

Acknowledgements

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R. A. C.-S. was responsible for the experimental design, analysed the data and wrote the manuscript; J. A. G.-U., D. G. and I. T.-V. analysed data; S. O. S.-S., and N. T. were responsible for the experimental design and data analysis; B. P.-G. analysed the data; A. R. T. contributed to the experimental design and discussion and wrote the manuscript.

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INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

"2017. Año del Centenario de la Promulgación de la Constitución Política de los Estados Unidos Mexicanos"

Ciudad de México abril 27 de 2017

Dra. Norma Bobadilla Sandoval
Coordinadora de la Comisión de
Investigación en animales
Presente

Por medio de la presente informo que se concluyó satisfactoriamente el proyecto titulado **"Efecto de compuestos bioactivos asociadoas a la testa de frijol negro (phaseolus vulgaris L.)en el metabolismo de colesterol en ratones C57BL/6"** con referencia **CINVA-781**, del cual se obtuvieron dos publicaciones.

1. Conjugated and free sterols from black bean (Phaseoulus vulgaris L.) seed coats as cholesterol micelle disruptors and their effect on lipid metabolism and cholesterol transport in rat primary hepatocytes. *Genes Nutr.* 9:367, 2014.

2. Flavonoids and saponins extracted from black bean (Phaseolus vulgaris L.) seed coats modulate lipid metabolism and biliary cholesterol secretion in C57BL/6 mice. *Brit J Nutr.* 112: 886-899, 2014.

Atentamente

Dr. Armando Tovar Palacio
Responsable del proyecto

Avenida Vasco de
Quiroga No. 15
Colonia Belisario
Dominguez Sección XVI
Delegación Tlalpan
Código Postal 14080
México, Distrito Federal
Tel. (52)54870900
www.incmnsz.mx

INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

México, D.F. a 10 de Febrero del 2016

Dra. Norma Bobadilla Sandoval
Coordinadora de la CINVA
Presente

Estimada Dra. Bobadilla:

Por este conducto me permito solicitar el cierre del protocolo: " Efecto de compuestos bioactivos asociados a la testa de frijol negro (phaseolus vulgaris L) en el metabolismo de colesterol en ratones C57BL/6 " con registro CINVA: 781, debido a que el protocolo ha concluido

Sin otro particular por el momento quedo de usted

Atentamente

Dr. Armando Tovar Palacio

Nombre y Firma del (a) Investigador (a)

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN SALVADOR ZUBIRAN

México D F a 10 de Febrero de 2016

Dr. Armando R. Tovar Palacios
Depto. de Fisiología de la Nutrición
Presente

Estimado Dr. Tovar:

Por este conducto le informo que su proyecto: **"EFECTO DE COMPUESTOS BIOACTIVOS ASOCIADOS A LA TESTA DE FRIJOL NEGRO (PHASEOLUS VULGARIS L.) EN EL METABOLISMO DE COLESTEROL EN RATONES C57BL/6."**, con registro **CINVA: 781-13/13-1** finalizó en mayo del 2015. Por lo que le solicito de la manera mas atenta me haga saber si el proyecto requerirá una prórroga. En caso afirmativo, favor de enviar a la **CINVA** el periodo de extension que solicita y de requerir un mayor número de animales especificar y justificar como se utilizarán y los procedimientos experimentales que se llevarán a cabo con los mismos. En caso de no requerir una prórroga favor de llenar el formato de cierre del protocolo que se anexa a la presente. De no recibir respuesta de su parte en el plazo de 30 días, el protocolo se dará por cerrado.

Sin otro particular por el momento, quedo de usted.

Atentamente

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la CINVA

INS. NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN - SALVADOR ZUBIRÁN - 10 FEB 2016 DIRECCIÓN DE INVESTIGACIÓN

INCMNSZ DIRECCIÓN DE NUTRICIÓN
DEPARTAMENTO DE FISIOLOGÍA DE LA NUTRICIÓN

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN 10 FEB 2016 INVESTIGACIÓN EXPERIMENTAL

c.c.p. Dr. Gerardo Gamba Ayala, Director de Investigación
MVZ Mariela Contreras Escamilla, Jefa del DIEB

NAB/mlm

Acuse

INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

"2015, Año del Generalísimo José María Morelos y Pavón"

México, D.F. a 23 de Junio del 2015

INCMNSZ
DIRECCIÓN DE NUTRICIÓN
23 JUN
DEPARTAMENTO DE
FISIOLOGÍA DE LA NUTRICIÓN

Dr. Armando Roberto Tovar Tovar
Depto. de Fisiología de la Nutrición
Presente

Estimado Dr. Tovar

Por este conducto le informo que su proyecto: **"EFECTO DE COMPUESTOS BIOACTIVOS ASOCIADOS A LA TESTA DE FRIJOL NEGRO (PHASEOLUS VULGARIS L.) EN EL METABOLISMO DE COLESTEROL EN RATONES C57BL/6."**, con registro **CINVA: FNU-781-13/13-1** finalizó en mayo de este año. Por lo que, le solicito de la manera más atenta me haga saber si el proyecto requerirá una prórroga. En caso afirmativo, favor de enviar a la **CINVA** el periodo de extensión que solicita y de requerir un mayor número de animales especificar y justificar como se utilizarán y los procedimientos experimentales que se llevarán a cabo con los mismos. En caso de no requerir una prórroga favor de llenar el formato de cierre del protocolo que se anexa a la presente.

Sin otro particular por el momento, quedo de usted

Atentamente,

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la CINVA

INS. NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN
26 JUN 2015
DIRECCIÓN DE INVESTIGACIÓN

928

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN SALVADOR ZUBIRAN
INCMNSZ
26 JUN 2015
DEPARTAMENTO DE INVESTIGACIÓN EXPERIMENTAL Y BIOTERIO

c.c.p. Dr. Gerardo Gamba Ayala, Director de Investigación.
MVZ Mariela Contreras Escamilla, Jefa del Bioterio.

Avenida Vasco de Quiroga No. 15
Colonia Belisario Domínguez Sección XVI
Delegación Tlalpan
Código Postal 14080
México, Distrito Federal
Tel. (52)54870900
www.incmnsz.mx

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

Mayo 30, 2013

Dr. Armando RobertoTovar Palacio
Jefe del Departamento de Fisiología de la Nutrición
P r e s e n t e

Con referencia al proyecto de investigación: "Efecto de compuestos bioactivos asociados a la testa de frijol negro (Phaseolus vulgaris L.) en el metabolismo de colesterol en ratones C57BL 6".

Registro CINVA: **781**

Clave: FNU-781 13/13-1

La Comision de Investigación en Animales (CINVA) revisó su respuesta a las observaciones emitidas por la CINVA, y se decidió **APROBARLO** para su desarrollo.

A t e n t a m e n t e

Dr. Rafael Hernández González
Coordinador de la Comisión de Investigación en Animales

ccp. Dr. Rubén Lisker Y.- Director de Investigación
MVZ. Griselda Salmerón Estrada. Secretaria CINVA
Dra. Nimbe Torres y Torres.- CINVA
MVZ.M.C. Ma. de la Luz Streber J. CINVA
Dr. Gonzalo M. Torres Villalobos. CINVA
Dr. Emiliano Tesoro Cruz CINVA

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DIRECCIÓN DE NUTRICIÓN
4 JUN 2013
DEPARTAMENTO DE FISIOLOGÍA DE LA NUTRICIÓN

Investigacion
Tradicion Servicio
Asistencia Docencia

• Vasco de Quiroga 15.
• Delegación Tlalpan
• C. P. 14000 México, D. F.
• Tel. 54-87-09-00

Folio del registro: FNU-781-13/13-1

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Formato Único de Registro

(0) Comentarios

Título del proyecto: Efecto de compuestos bioactivos asociados a la testa de frijol negro (Phaseolus vulgaris L.) en el metabolismo de colesterol en ratones C57BL/6

Tipo de proyecto: Investigación Experimental

Antecedentes:

Las enfermedades cardiovasculares como la aterosclerosis, accidentes cerebrovasculares y ataques cardíacos representan un problema de salud pública a nivel mundial (Erdmann et al., 2008). Algunos estudios han encontrado una fuerte correlación entre el desarrollo de enfermedades cardiovasculares y el desbalance en las concentraciones de lípidos en plasma (Chen et al., 2008), así como concentraciones elevadas de colesterol total y lipoproteínas de baja densidad (LDL) (Erdmann et al., 2008).

Uno de los productos más utilizados para disminuir las concentraciones de colesterol son los inhibidores de la enzima Hidroxi-3-metilglutaril-coenzima A (HMG-CoA) reductasa, como lo son las estatinas. Sin embargo, el uso prolongado de estos compuestos están relacionados con efectos adversos como lo son falla hepática (Calpe-Berdiel et al., 2009).

Productos bioactivos extraídos de plantas han demostrado ser útiles en la prevención y tratamiento de concentraciones elevadas de colesterol. Por ejemplo, se ha demostrado que los fitoesteroles reducen las concentraciones de colesterol en plasma (Gupta et al., 2011; Hayes et al., 2002; Moreau et al., 2002; Ostlund, 2004). La FDA ha establecido una dosis de ingesta diaria de 2g para reducir los niveles de colesterol en plasma de 5 a 15%(Food and Drug Administration, 2010).

Por otro lado, se ha comprobado que es posible alcanzar un efecto sinérgico de varios fitoquímicos para combatir la hipercolesterolemia, reduciendo las dosis utilizadas y así mejorar la seguridad de los suplementos. Por ejemplo, se ha reportado la reducción de colesterol y triglicéridos in humanos con suplementación de fitoesteroles y aceite de pescado, ricos en ácidos grasos polinsaturados(Micallef & Garg, 2008). De manera similar, los estanoles y la berberina han mostrado un efecto sinérgico en la reducción de colesterol a través de la inhibición de absorción de colesterol en roedores (Wang et al., 2010).

Definición del problema:

La obesidad es un padecimiento que afecta a millones de personas en nuestro país y en el mundo. Esta es causante de las altas concentraciones de colesterol en plasma, que a su vez, ha sido relacionado con numerosas enfermedades crónicas. Es necesario identificar compuestos que tengan un efecto significativo en la disminución de colesterol y triglicéridos, y que sean capaces de equilibrar el metabolismo de lípidos. Además es necesario elucidar sus posibles mecanismos de acción sobre el metabolismo de lípidos.

Justificacion:

El frijol es una fuente muy importante de proteína, fibra dietaria, hierro, carbohidratos complejos, minerales y vitaminas de millones de personas en México, y en otros países en desarrollo(Messina, 1999). Algunos reportes sugieren el efecto hipocolesterolemico obtenidos por el consumo de frijol, atribuyendo dicho efecto al catabolismo de LDL (Marzetti, Amigo & Nervi, 1993). Otros estudios proponen que el frijol negro promueve la excreción de colesterol en heces(Rosa et al., 1998). Además, se encontró que el colesterol en plasma se veía reducido de manera significativa si se incluía frijol cocido en la dieta (Shuler et al., 1989). Se realizo también un estudio con extracto de vaina de frijol donde se observó un efecto hipocolesterolemico e hipoglicémico en ratas diabéticas, sin encontrar efecto alguno en ratas normales (Pari & Venkateswaran, 2004).Sin embargo, no se encontró qué molécula o familia de compuestos ni el mecanismo por el cual causaron este efecto positivo en las ratas diabéticas.

Una vez que el proyecto se concrete se espera generar información científica sobre los compuestos o familia de compuestos presentes en la testa de frijol negro que tienen un efecto hipocolesterolemico.

Hipótesis:

La Hipótesis central del proyecto es que los flavonoides, saponinas y fitoesteroles asociados a la testa de frijol negro, son los componentes principales que tienen un efecto sinérgico en la disminución de colesterol y por tanto pueden ser potencialmente utilizados en los tratamientos de hipercolesterolemia.

Fecha estimada de inicio: 14/01/2013

Fecha estimada de término: 04/03/2013

Comisión a la que somete

¿Incluye documentos anexos?: No

Investigadores participantes

(0) Comentarios

Investigador	Participacion	Orden de participación	Investigador responsable
Tovar Palacio, Armando Roberto	Investigador responsable	1	Si

Población vulnerable

(0) Comentarios

Población vulnerable vinculado al protocolo Ninguna de las anteriores

Otra población:: ratones

Objetivos

(0) Comentarios

Objetivo:

Determinar si los flavonoides, saponinas y fitoesteroles contenidos en la testa de frijol negro tienen un efecto significativo en la disminución de colesterol y triglicéridos en plasma y en tejidos de ratones C57BL/6

Tipo de objetivo: General

Objetivo:

1. Caracterización del extracto de testa de frijol negro para determinar las concentraciones y el perfil de flavonoides, saponinas y fitoesteroles contenidas en el mismo.
2. Determinación del efecto del extracto en:
    1. Niveles de colesterol, triglicéridos (en tejido y en plasma).
    2. Niveles de glucosa y leptina en plasma
    3. Concentración de sales biliares
3. Determinación del cambio en la expresión genética en tejidos que propicien el efecto observado en los niveles de colesterol, triglicéridos, glucosa y leptina.
    1. Medición de expresión de proteínas en tejidos hepático y muscular (ABCG5, ABCG8, SREBP-1, SREBP-2,FAS, CYP7, CPT-1, LxR)
    2. Medición de AMPK fosforilado en la proteína del tejido hepático

Tipo de objetivo: Específico (s)

Metodología: Diseño general

(0) Comentarios

Metodología gral:

A) El diseño del estudio es aleatorio, las unidades experimentales serán bloqueadas por tratamiento de manera aleatoria. Se realizarán 5 tratamientos
    a. Dieta control (estandar)
    b. Dieta control + extracto de testa de frijol negro
    c. Dieta hipercolesterolemica
    d. Dieta hipercolesterolemica + estatinas (tratamiento comun)

e. Dieta hipercolesterolemica + extracto (concentracion alta)
f. Dieta hipercolesterolemica + extracto (concentracion baja)

A continuación se describe el contenido de cada una de las dietas a utilizar:

	g/kg					
	Control	Control + extracto	Hipercolesterolemica	Hipercolesterolemica + Extracto BAJA	Hipercolesterolemica + Extracto concentración ALTA	Hipercolesterolemica + estatina
Caseína	200	200	200	200	200	200
Dextrina	132	132	127	127	127	127
Aceite de soya	70	70	70	70	[illegible]	70
Fibra	50	50	50	50	50	50
Colina	2.5	2.5	2.5	2.5	2.5	2.5
Sacarosa	100	100	100	100	100	100
Almidon	397.486	397.486	397.486	397.486	397.486	397.486
Vitaminas	10	10	10	10	10	10
Minerales	35	35	35	35	35	35
Cisteína	3	3	3	3	3	3
Extracto	NA	7.5	NA	2.5	5	NA
Colesterol	NA	NA	5	5	5	5
Estatina	NA	NA	NA	NA	NA	0.3

B) Para estimar el efecto del consumo de extracto de testa de frijol negro sobre la obesidad se utilizarán ratones de la cepa C57BL/6. Los animales se mantendrán en microaislamiento con un ciclo de luz/oscuridad de 12 horas con libre acceso a la dieta correspondiente a su grupo experimental y al agua.

Los grupos anteriores serán alimentados por un periodo de 56 días con la dieta respectiva. Con la finalidad de determinar el efecto del consumo de del extracto probado sobre la obesidad, se registrara diariamente el peso, el consumo de alimento y el consumo de energía (calorías totales y contenidas en la dieta) durante los 56 días de estudio.

Después de cumplir con el respectivo tiempo de estudio los ratones de la cepa C57BL/6 con ayuno de 12 horas, se procederá a realizar la eutanasia a través de inhalación de CO_2 y decapitación. Posteriormente se recolectara la sangre por gravedad en tubos con gel separador y activador de coagulación para la determinación de parámetros bioquímicos (glucosa, triglicéridos, colesterol, insulina y leptina). De estos mismos animales se extraerá el músculo esquelético (cuádriceps), hígado, intestino y sales biliares para la medición de niveles de [illegible] y colesterol en cada uno de los tejidos, además de la extracción del RNA total para su posterior análisis. El tejido inmediatamente se depositara en nitrógeno líquido y se guardará a -70°C hasta el momento de realizar las determinaciones correspondientes.

C) El tamaño de muestra será de 8 ratones de la cepa C57BL/6 por grupo.

D) Los ratones son machos de 4 semanas de edad, de la cepa C57BL6.

E) La asignación del tratamiento se realizará de forma aleatorizada.

F) Se tendrán 6 grupos de tratamientos
    a. Dieta control (estándar)
    b. Dieta control + extracto de testa de frijol negro
    c. Dieta hipercolesterolemica
    d. Dieta hipercolesterolemica + estatinas (tratamiento control)
    e. Dieta hipercolesterolemica + extracto de testa de frijol negro (concentración baja)
    f. Dieta hipercolesterolemica + extracto de testa de frijol negro (concentración alta)

G) La duración del estudio será de 36 días

C) El tamaño de muestra será de 8 ratones de la cepa C56BL6 por grupo

D) Los ratones son machos de 4 semanas de edad, de la cepa C56BL6

E) La asignación del tratamiento se realizará de forma aleatorizada

F) Se tendrán 6 grupos de tratamientos:

a. Dieta control (estándar)
b. Dieta control + extracto de testa de frijol negro
c. Dieta hipercolesterolemica
d. Dieta hipercolesterolemica + estatinas (tratamiento común)
e. Dieta hipercolesterolemica + extracto de testa de frijol negro (concentración baja)
f. Dieta hipercolesterolemica + extracto de testa de frijol negro (concentración alta)

G) La duración del estudio será de 36 días

Metodología: Criterios de selección

(0) Comentarios

Criterios de selección del protocolo:

a) Ser ratones machos de la cepa C56BL6 de cuatro semanas de edad de peso entre 15-17 g

b) Ratones hembra de la cepa C56BL6

c) Se eliminarán del estudio los ratones C57BL/6, que no aumenten de peso, que presenten baja en la ganancia de peso

Beneficio (s) del estudio

(0) Comentarios

Beneficio:

1) Encontrar una disminución significativa (semejante a los normales) en los niveles de lípidos en plasma y tejido

2) Encontrar un efecto mayor al proporcionado por tratamientos utilizados actualmente como el uso de estatinas y fitoesteroles comerciales

3) Encontrar que dicho efecto mayor se debe al efecto sinérgico de los componentes del extracto de testa de frijol negro proporcionado (flavonoides, saponinas, fitoesteroles)

Tipo de beneficio: Beneficios directos esperados

Beneficio:

Generar información científica valiosa sobre el efecto que tendrían los flavonoides, saponinas y fitoesteroles contenidos en la testa de frijol negro en la disminución de colesterol en otros modelos y posteriormente en seres humanos.

Tipo de beneficio:

Beneficios indirectos esperados

Metodología: Desenlace y variables

(0) Comentarios

Metodología de desenlace y variables:

La ganancia de peso y consumo de alimentos

Las concentraciones en ayuno de glucosa, insulina, leptina, ácidos graso libres y triglicéridos

- La expresión génica de ABCG5, ABCG8, SREBP-1, SREBP-2,FAS, CYP7, CPT-1, LxR en músculo esquelético, tejido hepático e intestinos

- La concentración de lípidos en el interior del músculo esquelético, hígado y plasma

- La concentración de sales biliares en la bilis

-Medición de AMPK fosforilado en la proteína del tejido hepático

Los resultados se presentarán como la media ± el error estándar de la media (EEM). Los datos se evaluarán por análisis de varianza (ANOVA) de una vía, con la dieta utilizada como variable independiente. El grado de significancia de las diferencias entre los grupos control y experimentales se determinará por medio de la prueba protegida de la diferencia de los cuadrados mínimos de Fisher (Fisher PLSD), usando el programa Minitab 16.0. Las diferencias se considerarán significativas con una $p<0.05$.

Manejo de confidencialidad

(0) Comentarios

Acciones, estrategias y precauciones que serán tomadas para proteger la confidencialidad de la información de los pacientes.:

El presente estudio no se llevará a cabo en pacientes, únicamente en animales de investigación

Ponderación general de riesgos contra beneficios del estudio propuesto

(0) Comentarios

Ponderación general de riesgos contra beneficios del estudio propuesto:	No existe riesgo de no encontrar diferencias significativas entre los tratamientos. Debido a que los fitoesteroles, como se mencionó en los antecedentes, están ampliamente estudiados por su efecto en el metabolismo del colesterol y la FDA ha aprobado su uso a dósis recomendada de 2g por día. Además las estatinas son el medicamento tradicional recetado por medicos para disminuir los niveles de colesterol en pacientes. También se ha estudiado el efecto sinérgico de productos naturales semejantes a los extraídos de testa de frijol negro que tiene un efecto significativo en los niveles de colesterol en plasma y tejido.

Riesgo (s) del estudio

(0) Comentarios

Molestias generadas por el estudio:	ninguna
Complicaciones del procedimiento:	ninguna
Efectos adversos reportados de medicamentos o sustancias utilizadas:	ninguna
Métodos de seguridad para el diagnóstico oportuno y prevención de los riesgos:	Observación diaria de los modelos animales para determinar si existe alguna enfermedad además de control de peso y consumo de alimento
Procedimientos a seguir para resolver los riesgos en caso de que se presenten:	
Otro tipo de riesgo:	

Consentimiento informado

(0) Comentarios

Hoja de informe al paciente:	NO APLICA.docx
Carta de consentimiento informado:	NO APLICA.docx

Declaración de los investigadores

(0) Comentarios

Archivo CEI 04 Declaración de investigadores:	CIBIS 04 - [illegible]

Instituto Nacional de Ciencias Médicas y Nutrición
Salvador Zubirán

INSTITUTO NACIONAL DE CIENCIAS MEDICAS Y NUTRICION

SALVADOR ZUBIRAN

Dirección de Investigación

FORMA ÚNICA PARA REGISTRO DE PROYECTOS

FECHA DE RECEPCIÓN: 11 1 20 2 CLAVE: INU-781-13 14-

TÍTULO: Efecto de compuestos bioactivos asociados a la testa de frijol negro (Phaseolus vulgaris L.) en el metabolismo de colesterol en ratones C57BL/6

INVESTIGADOR RESPONSABLE: Tovar Palacio Armando Roberto

DEPARTAMENTO O SERVICIO: DEPARTAMENTO DE FISIOLOGÍA DE LA NUTRICIÓN

TIPO DE INVESTIGACIÓN: Investigación Experimental

PATROCINADORES:

Patrocinador	Cantidad
Instituto Tecnológico y de Estudios Superiores de Monterrey	$ 400,000.00

VIGENCIA DEL PROYECTO: Del 01/10/2012 al 01/10/2014

Trimestre 1 Trimestre 2 Trimestre 3 Trimestre 4

COSTO TOTALES DE LA INVESTIGACIÓN	
Personal	$ 120,000.00
(sueldos y sobresueldos al personal)	
Equipos	$ 90,000.00
(de laboratorio, computo, transporte, etc.)	
Materiales	$ 100,000.00
(reactivos, consumibles, especificar, etc.)	
Animales	$ 50,000.00
(adquisición, cuidado, procedimientos, etc.)	
Estudios	$ 0.00
(de laboratorio, gabinete especiales, etc.)	
Viáticos	$ 0.00
(reuniones científicas y trabajo de campo)	
Publicaciones	$ 0.00

INSTITUCIONES PARTICIPANTES

FIRMAS

Investigador responsable

Jefe de Departamento

Comité de Investigación en Humanos

Comité de Investigación en Animales

Director de Investigación

Director General

Fecha de recepción

24-JUN-2013

(costo directos de publicacion, sobregiro)	
Suscripciones	$ 0.00
(libros, revistas, software, periodicos, etc	
Varios	$ 0.00
(teléfono, fax, fotocopias, mensajeria, etc	
Fondo de apoyo	$ 60,000.00
(5% de la cantidad total de proyecto)	
Admon. Gastos pacientes	$ 0.00
Total	$ 400,000.00

Titulo de proyecto:

Efecto de compuestos bioactivos asociados a la testa de frijol negro (*Phaseolus vulgaris L.*) en el metabolismo de colesterol en ratón C57BL/6

Tipo de proyecto: Investigación Experimental

Fecha estimada de inicio: 14/01/2013 **Fecha de Término:** 04/03/2013

Comisión a la que somete: Comisión de investigación en animales

Antecedentes:

Las enfermedades cardiovasculares como la aterosclerosis accidentes cerebrovasculares y ataques cardiacos representan un problema de salud publica a nivel mundial.(Erdmann et al. 2008) Algunos estudios han encontrado una fuerte correlación entre el desarrollo de enfermedades cardiovasculares y el desbalance en los niveles de lipidos en plasma (Chen et al., 2008) así como niveles altos de colesterol total y lipoproteínas de baja densidad (LDL) (Erdmann et al. 2008)

Uno de los productos más utilizados para disminuir los niveles de colestero son los inhibidores de Hidroxi-3-metilglutaril-coenzima A (HMG-CoA, reductasa, como lo son las estatinas. Sin embargo el uso prolongado de estos compuestos están relacionados con efectos adversos como lo son falla hepática(Calpe-Berdiel et al., 2009)

Productos bioactivos extraídos de plantas han demostrado ser útiles en la prevención y tratamiento de niveles altos de colesterol. Por ejemplo se ha demostrado que los fitoesteroles reducen los niveles de colesterol en plasma(Gupta et al. 2011 Hayes et al. 2002 Moreau et al. 2002 Ostlund 2004). La FDA ha establecido una dosis de ingesta diaria de 2g para reducir los niveles de colesterol en plasma de 5 a 15%(Food and Drug Administration. 2010).

Por otro lado se ha comprobado que es posible alcanzar un efecto sinérgico de varios fitoquímicos para combatir la hipercolesterolemia, reduciendo las dosis utilizadas y así mejorar la seguridad de los suplementos. Por ejemplo se ha reportado la reducción de colesterol y triglicéridos in humanos con suplementación de fitoesteroles y aceite de pescado ricos en ácidos grasos polinsaturados(Micallef & Garg 2008). De manera similar los etanoles y berberina han mostrado un efecto sinérgico en la reducción de colesterol a través de la inhibición de absorción de colesterol en roedores (Wang et al., 2010)

Definición del problema:

La obesidad es un padecimiento que afecta a millones de personas en nuestro pais y en el mundo. Ésta es causante de los altos niveles de colesterol en plasma, que a su vez, ha sido relacionado con numerosas enfermedades crónicas. Es necesario identificar compuestos que tengan un efecto significativo en la disminución de colesterol y lípidos, y que sean capaces de equilibrar el metabolismo de lípidos. Además es necesario elucidar sus posibles mecanismos de acción sobre el metabolismo de lípidos.

Justificación:

El frijol es una fuente muy importante de proteína, fibra dietaria, hierro, carbohidratos complejos, minerales y vitaminas de millones de personas en México, y en otros países en desarrollo(Messina, 1999). Algunos reportes sugieren el efecto hipocolesterolemico obtenidos por el consumo de frijol, atribuyendo dicho efecto al catabolismo de LDL(Marzolo, Amigo, & Nervi, 1993). Otros estudios proponen que el frijol negro promueve la excreción de colesterol en heces(Rosa et al., 1998). Además, se encontro que el colesterol en plasma se veia reducido de manera significativa, si se incluia frijol cocido en la dieta (Shutler et al., 1989). Se realizo también un estudio con extracto de vaina de frijol donde se observó un efecto hipocolesterolemico e hipoglicémico en ratas diabéticas, sin encontrar efecto alguno en ratas normales (Pari & Venkateswaran, 2004). Sin embargo, no se encontró qué molécula o familia de compuestos ni el mecanismo por el cual causaron este efecto positivo, en las ratas diabéticas.

Una vez que el proyecto se concrete se espera generar información científica sobre los compuestos o familia de compuestos presentes en la testa de frijol negro que tiene un efecto hipocolesterolemico.

Hipotesis:

La Hipotesis central del proyecto es que los flavonoides, saponinas y fitoesteroles asociados a la testa de frijol negro, son los componentes principales, que tienen un efecto sinérgico en la disminución de colesterol y por tanto pueden ser potencialmente utilizados en los tratamientos de hipercolesterolemia.

Investigadores participantes

Tovar Palacio, Armando Roberto

Torres, Nimbe

Metodología: Diseño general

A) El diseño del estudio es aleatorio, las unidades experimentales serán bloqueadas por tratamiento de manera aleatoria. Se realizarán 5 tratamientos
   a. Dieta control (estándar)
   b Dieta control + extracto de testa de frijol negro
   c. Dieta hipercolesterolemica
   d. Dieta hipercolesterolemica + estatinas (tratamiento común)
   e. Dieta hipercolesterolemica + extracto

A continuación se describe el contenido de cada una de las dietas a utilizar:

	g/Kg				
	Control	Control + extracto	Hipercolesterolemica	Hipercolesterolemica + Extracto	Hipercolesterolemica + estatina
Caseína	200	200	200	200	200
Dextrina	132	132	127	127	127
Aceite de soya	70	70	70	70	70
Fibra	50	50	50	50	50
Colina	2.5	2.5	2.5	2.5	2.5
Sacarosa	100	100	100	100	100
Almidón	397.486	397.486	397.486	397.486	397.486
Vitaminas	10	10	10	10	10
Minerales	35	35	35	35	35
Cisteína	3	3	3	3	3
Extracto	NA	2.5	NA	2.5	NA
Colesterol	NA	NA	5	5	5
Estatina	NA	NA	NA	NA	0.3

B) Para estudiar el efecto del consumo de extracto de testa de frijol negro sobre la obesidad se utilizaran ratones de la cepa C56BL6. Los animales se mantendrán en microaisladores con un ciclo de luz/oscuridad de 12 horas con libre acceso a la dieta correspondiente a su grupo experimental y al agua.

Los grupos anteriores serán alimentados por un periodo de 36 días con la dieta respectiva. Con la finalidad de determinar el efecto del consumo de del extracto probado sobre la obesidad, se registrará diariamente el peso, el consumo de alimento y el consumo de energía conforme a las kcal/g contenidas en la dieta durante los 36 días de estudio.

Después de cumplir con el respectivo tiempo de estudio los ratones de la cepa C57BL/6 con ayuno de 12 horas, se procederá a realizar la eutanasia a través de inhalación de CO_2 y decapitación. Posteriormente se recolectará la sangre

por gravedad en tubos con gel separador y activador de coagulación para la determinación de parámetros bioquímicos (glucosa, triglicéridos colesterol insulina y leptina) De estos mismos animales se extraerá el músculo esquelético (*vastus lateralis*) hígado intestinos y sales biliares, para la medición de niveles de lipidos y colesterol en cada uno de los tejidos además de la extracción del RNA total para su posterior análisis El tejido inmediatamente se depositará en nitrógeno liquido y se guardará a -70°C hasta el momento de realizar las determinaciones correspondientes

C) El tamaño de muestra será de 8 ratones de la cepa C57BL/6 por grupo
D) Los ratones son machos de 4 semanas de edad de la cepa C56BL6
E) La asignación del tratamiento se realizará de forma aleatorizada
F) Se tendrán 6 grupos de tratamientos
    a. Dieta control (estándar)
    b. Dieta control + extracto de testa de frijol negro
    c Dieta hipercolesterolemica
    d Dieta hipercolesterolemica + estatinas (tratamiento común)
    e Dieta hipercolesterolemica + extractc de testa de frijol negro
G) La duración del estudio será de 36 dias

Metodologia: Criterios de selección *

a) Ser ratones machos de la cepa C56BL6 de cuatro semanas de edad de peso entre 15-17 g

b) Ratones hembra de la cepa C56BL6

c) Se elimarán del estudio los ratones C57BL/6. que no aumenten de peso que presenten baja en la ganancia de peso

Beneficio (s) del estudio

1) Encontrar una disminución significativa (semejante a los normales) en los niveles de lipidos en plasma y tejido.
2) Encontrar un efecto mayor al proporcionado por tratamientos utilizados actualmente como el uso de estatinas y fitoesteroles comerciales
3) Encontrar que dicho efecto mayor se debe al efecto sinérgico de los componentes del extracto de testa de frijol negro proporcionado (flavonoides saponinas. fitoesteroles)

Metodología: Desenlace y variables

La ganancia de peso y consumo de alimentos

Las concentraciones en suero de glucosa, insulina leptina, ácidos graso libres y triglicéridos

- La expresión génica de ABCG5 ABCG8, SRBP-1 SRBP-2,FAS CYP7 CPT-1 LxR en músculo esquelético tejido hepático e intestinos.

- La concentración de lípidos en el interior del músculo esquelético hígado y plasma

- La concentración de sales biliares en la bilis

-Medición de AMPK fosforilado en la proteína del tejido hepático

Los resultados se presentarán como la media + el error estándar de la media (EEM). Los datos se evaluaran por análisis de varianza (ANOVA) de una vía con la dieta utilizada como variable independiente. El grado de significancia de las diferencias entre los grupos control y experimentales se determinará por medio de la prueba protegida de la diferencia de los cuadrados mínimos de Fisher (Fisher PLSD), usando el programa Minitab 16.0. Las diferencias se considerarán significativas con una $p<0.05$

Manejo de confidencialidad

El presente estudio no se llevara a cabo en pacientes, únicamente en animales de investigación

Citas bibliográficas

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Programa de trabajo

	Inicio	Término
Alimentación de ratones	07/01/2013	07/03/2013
Obtención de Tejido y suero	08/03/2013	08/03/2013
Expresión genética	09/03/2012	23/04/2013

DECLARACIÓN DE LOS INVESTIGADORES

TÍTULO DEL PROYECTO: *Efecto de compuestos bioactivos asociados a la testa de frijol negro (Phaseolus vulgaris L.) en el metabolismo de colesterol en ratones C57BL/6*

Número de Registro CIIBH: *SNE-781-13/13-1*

Los investigadores que participamos en el proyecto arriba mencionado sometemos voluntariamente a evaluación dicho proyecto ante el Comité Institucional de Investigación Biomédica en Humanos y libremente declaramos:

- ***Que conocemos todos los aspectos del estudio y contamos con la capacidad de llevarlo a buen término.***
- ***Que la revisión minuciosa de los antecedentes científicos del proyecto justifican su realización y nos comprometemos a mantener un estándar científico elevado que permita obtener información útil para la sociedad.***
- ***Que conocemos los riegos potenciales a los que exponemos a los pacientes invitados a participar los cuales hemos discutido ampliamente con ellos.***
- ***Que pondremos el bienestar y la seguridad de los pacientes sujetos de investigación por encima de cualquier otro objetivo.***
- ***Que nos conduciremos de acuerdo con los estándares de comportamiento ético y científico aceptados nacional e internacionalmente según lo establecido por la Ley General de Salud y el Reglamento en Materia de Investigación para la Salud de México, las Pautas Éticas Internacionales para la Investigación y Experimentación Biomédica en Seres Humanos de la Organización Mundial de la Salud así como la Declaración de Helsinky.***

Nombre del investigador	*Firma*
TOVAR PALACIO ARMANDO ROBERTO	