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β-Conglycinin (7S) and glycinin (11S) exert a hypocholesterolemic effect comparable to that of fenofibrate in rats fed a high-cholesterol diet

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ABSTRACT

The hypocholesterolemic effect of isolated soybean proteins and fenofibrate in rats was compared. Forty-five rats were divided into five groups: standard (STD; casein), high cholesterol (HC; STD plus 1% cholesterol/0.5% cholic acid), HC + β-conglycinin, HC + glycinin and HC + fenofibrate. The proteins and the drug were administered by gavage for 28 days. The proteins decreased total cholesterol (TC) and triacylglycerol (TG) in the plasma of the rats fed HC diet, to values very close to those fed on fenofibrate. High density lipoprotein cholesterol (HDL-C) levels in the plasma were increased by the β-conglycinin, glycinin and fenofibrate groups. The largest TC reduction in the liver was observed in the fenofibrate group; in contrast, the β-conglycinin and glycinin groups exhibited reduced the levels of hepatic TG and TC. Based on these data, it could be suggested that the oral daily administration of isolated soybean proteins, in the range of 2.75% of the protein ingested daily, can promote a reduction in TC and TG in the plasma of rats fed hypercholesterolemic diets.

1. Introduction

The growing importance of treating hyperlipidemia and hypercholesterolemia with a diet-drug combination is well recognized and increasingly diet therapy moves in this direction (Knopp, 1999; Poli et al., 2008). Thus, several studies have highlighted the diet as an important element among the possible forms of intervention aimed at reducing lipids (cholesterol, LDL-C, TG) in the plasma (Poli et al., 2008; Scarafoni, Magni, & Duranti, 2007). The inclusion of some foods as dietary supplements takes into account the context of hypercholesterolemia and/or tolerance to a pharmacological intervention. Leguminous seeds are among the richest food sources of protein for humans and animals nutrition. In addition to the nutritional properties of their proteins, the beneficial effects of dietary seeds on lipid metabolism have been demonstrated in a variety of animal models and humans (Scarafoni et al., 2007). In this respect, the soybean stands out, since it contains higher protein content (~35–40%) than most other legumes. Several studies have shown evidence that soybean proteins and not other compounds present in the grain are responsible for its beneficial effects

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Abbreviations: STD, standard diet; HC, hypercholesterolemic; TC, total cholesterol; TG, triacylglycerols; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; LDL/HDL, low density lipoprotein/high density lipoprotein ratio; β-Conglycinin, 7S fraction; Glycinin, 11S fraction; FF, fenofibrate; FDA, Food and Drug Administration; SPI, soy protein isolate; ANOVA, analysis of variance.

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on the lipid metabolism (Desroches, Mauger, Ausman, Lichtenstein, & Lamarche, 2004; Duranti, 2006; Rho, Park, Ahn, Shin, & Lee, 2007; Torres, Torre-Villalvazo, & Tovar, 2006; Wilson, Nicolosi, Kotyla, & Fleckinger, 2007) and scientific data in support of this central role of soy protein have led the American Food and Drug Administration (FDA) to authorize a specific health recommendation to include 25 g of soy protein a day in a diet low in saturated fat and cholesterol, on the basis of a number of previous clinical observations (FDA, 1999). Soybean protein, in the form of isolate, concentrate and isolated protein fractions, has shown activity on cholesterol metabolism in studies *in vitro* and *in vivo* (Scarafoni et al., 2007; Torres et al., 2006). A meta-analysis published recently reported that soy protein, when partially or fully substituted for dietary animal protein, induced a decrease in plasma total and LDL-cholesterol and it was observed that this reduction was greater in subjects with established hypercholesterolemia and practically negligible in those with baseline cholesterolemia (Reynolds, Chin, & Lees, 2006; Sirtori, Ederini, & Arnoldi, 2007).

Storage globulins comprise two main constituents of the protein in soybean seeds that can be differentiated by their sedimentation coefficients, the 7S protein or β-conglycinin and the 11S protein or glycinin. These constitute nearly 90% of all the protein in the seed. While the 11S fraction has not attracted much attention in the studies on lipid metabolism, the 7S protein has been extensively studied in several experimental designs, involving various animals, such as rats, normal and genetically obese mice and monkeys (Duranti et al., 2004; Fukui et al., 2004). All of these studies show the 7S fraction as the only protein source in the diet, but in a hypercholesterolemic validated model, Lovati et al. (1992) had already observed, and recently Duranti et al. (2004) confirmed that the administration of a daily dose of the 7S fraction significantly decreased plasma cholesterol and triacylglycerols in a dose-dependent fashion. The former authors showed that the 11S fraction had a much lower effect than the 7S, while the latter demonstrated the key role played by the α′ chain on the cholesterolemic and triacylglycerolidemic levels, owing to the isolation of relatively large amounts of this subunit from the 7S globulin. These authors suggested that peptides produced *in vivo* would be the active elements in the observed effects.

In the present study, the effects of a daily dose of the 7S and 11S soybean protein fractions on total cholesterol (TC), lipoproteins (LDL-C, HDL-C) and triacylglycerol (TG) levels in the plasma were assayed, as well as TC and TG in the liver of rats fed a hypercholesterolemic (HC) diet for 28 days. In addition, the effects of fenofibrate, a hypolipidemic and hypocholesterolemic drug, were also studied in the same model.

2. Methods and materials

2.1. Isolation and characterization of β-conglycinin (7S) and glycinin (11S)

Soybean flour (60 mesh) was defatted with hexane (1:8, w/v) flour to solvent ratio) by rocking at room temperature for 12 h followed by reextraction in the same solvent (1:6, w/v); evaporated to dryness at room temperature and used for protein extraction. Soybean fractions β-conglycinin and glycinin were isolated following the method of Nagano, Hirotsuka, Mori, Kohyama, and Nishinari (1992) with some modifications. Defatted soybean flour was extracted with water (1:15 w/v flour to water ratio) adjusted to pH 7.5. The soluble extract was centrifuged at 2000 *g* for 30 min. The supernatant was treated with anhydrous sodium bisulphite (0.98 g/L) and the pH adjusted to 6.4. The solution was stirred and stored on an ice bath overnight, followed by centrifugation at 6500 *g* for 20 min. The insoluble fraction, containing 11S globulin, was washed, centrifuged again under the same conditions, suspended in distilled water, dialyzed overnight against distilled water in appropriate dialysis sacs (pore size of about 10 kDa) and lyophilized. The soluble fraction was treated with 0.25 mol/L NaCl and adjusted to pH 5.0 and, after 1 h, centrifuged at 9000 *g* for 30 min. The precipitate (7S or β-conglycinin) was solubilized in 0.25 mol/L sodium phosphate buffer (pH 7.0) and stored at −14 °C. All steps were realized at 4 °C. All chemicals were of analytical purity.

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (BSA) as a standard. Electrophoresis of soybean total protein, 7S-globulin and 11S-globulin was performed on 10 g/100 g polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS), as described by Laemmli (1970), in a Mini Protean II cell (Bio-Rad, Hercules, CA,USA) (SDS–PAGE). About 10 μg of sample was loaded on each gel. The gels were stained in Coomassie Brilliant Blue (R-250) and destained by diffusion in methanol–acetic acid–water (1:1:8, v/v/v). Marker proteins of known molecular weight (MW) were rabbit muscle phosphorylase b, bovine serum albumin, hen egg white albumin, bovine carbonic anhydrase, soybean trypsin inhibitor and hen egg white lysozyme. Gel images were digitalized and analyzed by densitometry with an alpha Imager 6.0 scanner (Alpha Innotech®, San Leandro, CA, USA), to quantify the bands and level of purity (data not shown). The content and homogeneity of the isolated β-conglycinin and glycinin, determined by SDS–PAGE and densitometric gel analysis, showed that these were the main fractions, containing 92.2% and 91% protein, respectively. The gels showed the characteristic bands of conglycinin (7S), due to the subunits α, α′, β, and glycinin (11S), with six subunit bands made up of acidic and basic polypeptides. The procedure used to isolate 7S and 11S globulins did not cause changes in the subunits; this step was essential to obtain a purified protein suitable for the *in vivo* studies (data not shown).

2.2. Animals, diets and experimental protocol

Forty-five male weanling Wistar rats, weighing 148 ± 12.3 g, were obtained from the Central Laboratory for animals of the São Paulo State University (UNESP) at Botucatu. All procedures were performed in accordance with the principles in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1985) and animal maintenance followed the guidelines of the Committee On Animal Experiments of the University; these experiments were approved by the Research Ethics Committee of the School of Pharmaceutical Sciences, São Paulo State University (UNESP), under

resolution 19/2007. The animals were acclimatized for a week and fed standard laboratory rat chow (Purina®). After this period, they were randomly divided into five groups ($n = 9$): a standard group (STD) that received a normal casein-based diet, following the recommendations of The American Institute of Nutrition (AIN-93) for maintenance (Reeves, Nielsen, & Fahey, 1993), and four hypercholesterolemic groups (HC): (1) control HC, (2) 7S globulin, 300 mg/kg/day (HC + 7S), (3) 11S globulin, 300 mg/kg/day (HC + 11S) and (4) fenofibrate, 30 mg/kg/day, (HC + FF). All HC groups were fed with a AIN-93 diet, modified by adding of 1 g/100 g cholesterol, and 0.5 g/100 g cholic acid (Duranti et al., 2004; Nath, Wiener, Harper, & Elvehjem, 1959), as described in Table 1.

Animals were individually housed in stainless steel cages, with free access to food and water. The room temperature was maintained at a constant 23 ± 2 °C and relative humidity of 60 ± 5%, with a 12–12 h light–dark cycle, lights on at 6:00 a.m. The proteins (7S and 11S globulins) and fenofibrate were administered daily by gavage at 9:00 and 14:00 h, in proportion to the body weight of the animals, to the groups HC + 7S, HC + 11S and HC + FF; they were dissolved in saline and given at the rates of 300 and 30 mg/kg/day, respectively. Vehicle alone was given to the HC group. Body weight, food intake, fecal excretion and feeding efficiency were measured every other day for comparative analysis between groups during the 28 days of the experiment. The feeding efficiency coefficient was calculated from the ratio of weight gain/daily intake × 100. On the last day, the animals were deprived of food for 12 h and euthanized by guillotine. Blood was then collected in tubes containing gel separator SST II (Vacutainer BD D®, Franklin Lakes, NJ, USA) and centrifuged at 1900 *g* for 15 min. The serum was separated, stored at −24 °C and used for biochemical analysis. Retroperitoneal fat, liver and heart were removed, washed immediately in cold saline, weighed; frozen and stored at −40 °C for time less than a month for subsequent comparative analysis.

2.3. Biochemical analysis in plasma and liver

To find out the effects of diets and treatment on the rat groups, plasma total cholesterol (TC) was measured by the liquid cholesterol CHOD–PAP method described by Stockbridge, Hardy, and Glueck (1989); serum high density lipoprotein (HDL-C) was measured by HDL-C precipitation as described by Assmann (1979); triacylglycerols (TG) were measured by the liquid triglyceride GPO–PAP method, as described by Annoni, Botasso, Ciaci, Donato, and Tripodi (1982). All analysis were carried out by enzymatic colourimetric methods, using commercially available reagent kits (Laborlab® Co., Ribeirão Preto, Brazil). The non-HDL-cholesterol fraction (non-HDL-C or LDL-C + VLDL-C) was calculated as the difference between the TC and HDL-C. The atherogenic index (TC − HDL-C/HDL-C) was calculated as proposed by Liu et al. (2006) and compared between groups. Hepatic-somatic and visceral fat indexes were respectively calculated as follows: liver weight/body weight × 100, and fat visceral weight/body weight × 100, as described by Chen, Chiou, Suetsuna, Yang, and Yang (2003). Liver lipids were extracted by the method of Haug and Hostmark (1987). The liver TC and TG concentrations were measured as described earlier for plasma analysis.

2.4. Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA) and Student–Newman–Keuls multiple range tests, employing SigmaStat® 3.5 software (Dundas software, Germany, 1999). All values are presented as mean ± standard error for nine values per group. A difference was considered statistically significant when $P < 0.05$.

Table 1 – Composition (percent) of the diets and treatments of the experimental groups.[a]

	STD	HC	HC + 7S	HC + 11S	HC + FF
Ingredients					
Casein[b]	15	15	15	15	15
Starch	61.07	59.57	59.57	59.57	59.57
Sucrose	10	10	10	10	10
Soybean oil	4	4	4	4	4
Cellulose powder	5	5	5	5	5
Mineral mixture[c]	3.5	3.5	3.5	3.5	3.5
Vitamin mixture[c]	1	1	1	1	1
L-Cystine	0.18	0.18	0.18	0.18	0.18
Choline bitartrate	0.25	0.25	0.25	0.25	0.25
Cholesterol[d]	0	1	1	1	1
Cholic acid[b]	0	0.5	0.5	0.5	0.5
Treatment					
β-Conglycinin (mg/kg/day)	–	–	300.0	300.0	–
Fenofibrate			–	–	30

[a] STD = standard diet, AIN-93 composition; HC = hypercholesterolemic diet: AIN-93 plus 1 g/100 g cholesterol and 0.5 g/100 g cholic acid; HC + 7S = HC diet + β-conglycinin (300 mg/kg/day); HC + 11S = HC diet + glycinin (300 mg/kg/day) and HC + FF = HC diet + fenofibrate (30 mg/kg/day).
[b] Sigma–Aldrich, Co., USA.
[c] PragSoluções®, Co., Brazil.
[d] Reagen Co., USA.

3. Results

3.1. Parameters of the in vivo experiment

In this study, 7S and 11S isolated soybean globulins and fenofibrate were given in single daily doses in an experimental model described in methods. Table 2 shows the effects of daily administration of β-conglycinin, glycinin and fenofibrate on the food consumption, weight gain, feeding efficiency ratio and fecal excretion in rats fed the diets for 28 days. There were no statistical differences ($P > 0.05$) in body weight gain between groups during the experimental period (Table 2) and also no significant ($P > 0.05$) alterations in the feeding efficiency ratio or food intake between groups (Table 2).

The HC group showed an increase of 24.2% and 21.1% in the relative liver weight of rats and in the hepatosomatic index, respectively, relative to the casein group ($P < 0.01$) (Table 3). However, the rats treated with fenofibrate showed increases of 55.8% and 63.6% in these values, respectively, relative to the hypercholesterolemic group ($P < 0.001$). Although the weight of the liver in the animals of the glycinin group were lower than in the fenofibrate group, there was no statistical difference; indeed, the hepatosomatic index for this group was still 28.8% higher than for HC ($P < 0.001$). Meanwhile, no significant difference was found between these values in the β-conglycinin group and the HC group ($P > 0.05$) (Table 3). The ratios of fat to body weight and heart to body weight did not vary significantly between the groups. However, animals in the glycinin group showed a decrease of 34% and 32.5% in visceral somatic fat and epididymal adipose tissue, respectively, relative to other groups.

3.2. Plasma parameters

Oral administration of glycinin, β-conglycinin and fenofibrate to hypercholesterolemic rats significantly reduced the levels of TC and TG in the plasma of the animals relative to HC, by the end of the 28-day treatment. In rats fed on the hypercholesterolemic diet (HC), the plasma and hepatic lipid levels were significantly higher than in those given the standard diet (STD) ($P < 0.001$) (Figs. 1–3). However, the daily administration of β-conglycinin and glycinin to animals promoted an attenuation of the effects of the HC diet, as also observed with fenofibrate. The animals that ingested β-conglycinin showed reductions of 22.95% and 34.8% in the levels of TC and TG in the plasma, respectively, relative to the HC group ($P < 0.01$), while those that ingested glycinin showed reductions of 16% and 16.3%, respectively ($P < 0.05$)(Fig. 1). On the other hand,

Table 2 – Body weight change, food intake and feeding efficiency ratio (FER) of rats after 28 days of feeding experimental diets.

	Dietary groups[1]				
	STD	HC	HC + 7S	HC + 11S	HC + FF
Initial body weight (g/rat)	195.23 ± 5.03	196.55 ± 4.63	195.26 ± 3.64	194.55 ± 3.93	193.26 ± 3.64
Final body weight (g/rat)	287.77 ± 7.07	295.01 ± 5.43	300.64 ± 7.71	298.11 ± 5.30	281.14 ± 4.25
Weight gain (g/rat/day)	3.31 ± 0.24	3.51 ± 0.32	3,76 ± 0.29	3.71 ± 0.23	3.86 ± 0.29
Food intake (g/rat/day)	15.47 ± 0.27	16.74 ± 0.33	16.25 ± 0.22	16.78 ± 0.33	15.35 ± 0.22
Fecal excretion (g/rat/day)	2.61^{c} ± 0.08	3.59^{a} ± 0.12	2.94^{b} ± 0.09	3.38^{a} ± 0.12	2.94^{b} ± 0.09
Feeding efficiency (%)	21.36 ± 1.67	21.01 ± 1.44	23.05 ± 1.93	21.41 ± 1.07	23.21 ± 1.93

[1] Values with different superscript letters in the same row differ significantly ($P < .05$) by the Student–Newman–Keuls multiple range tests. Each value is the mean ± SE ($n = 9$). STD = standard diet, AIN-93 composition; HC = hypercholesterolemic diet: AIN-93 plus 1 g/100 g cholesterol and 0.5 g/100 g cholic acid; HC + 7S = HC diet + β-conglycinin (300 mg/kg/day); HC + 11S = HC diet + glycinin (300 mg/kg/day) and HC + FF = HC diet + fenofibrate (30 mg/kg/day).

Table 3 – Tissue weights and ratios of rats after 28 days of feeding experimental diets.

	Dietary groups[1]				
	STD	HC	HC + 7S	HC + 11S	HC + FF
Liver weight (g/rat)	10.90^{c} ± 0.45	13.54^{b} ± 0.36	13.55^{b} ± 0.87	17.63^{a} ± 0.28	21.10^{a} ± 0.69
Epididymal adipose tissue (g/rat)	5.05^{a} ± 0.36	5.54^{a} ± 0.23	5.39^{a} ± 0.46	3.74^{b} ± 0.30	4.97^{a} ± 0.24
Heart (g/rat)	1.08 ± 0.04	1.03 ± 0.04	0.94 ± 0.03	1.06 ± 0.02	0.95 ± 0.03
Heart weight/body weight (g/100 g)	0.37 ± 0.01	0.34 ± 0.01	0.30 ± 0.02	0.35 ± 0.01	0.34 ± 0.01
Hepatosomatic index[2]	3.79 ± 0.12	4.59^{c} ± 0.14	4.41^{c} ± 0.20	5.91^{b} ± 0.12	7.51^{a} ± 0.27
Visceral somatic fat[3]	1.75^{a} ± 0.11	1.89^{a} ± 0.10	1.79^{a} ± 0.13	1.25 ± 0.10	1.76^{a} ± 0.09
Atherogenic index[4]	1.02 ± 0.03	8.09^{a} ± 0.07	3.52^{c} ± 0.11	5.04^{b} ± 0.13	1.96^{d} ± 0.12

[1] Values with different superscript letters in the same row differ significantly ($P < .05$) by the Student–Newman–Keuls multiple range tests. Each value is the mean ± SE ($n = 9$). STD = standard diet, AIN-93 composition; HC = hypercholesterolemic diet: AIN-93 plus 1 g/100 g cholesterol and 0.5 g/100 g cholic acid; HC + 7S = HC diet + β-conglycinin (300 mg/kg/day); HC + 11S = HC diet + glycinin (300 mg/kg/day) and HC + FF = HC diet + fenofibrate (30 mg/kg/day).

[2] Defined as liver weight/body weight × 100.

[3] Defined as fat visceral weight/body weight × 100.

[4] Defined as (TC − HDL-C)/HDL-C ratio.

the administration of fenofibrate decreased the same variables by 35.8% and 45.7%, respectively ($P < 0.001$). These latter data indicate that the proteins β-conglycinin and glycinin produced a considerable reduction of plasma TC and TG, compared to fenofibrate (Fig. 1A/B) and there was no statistical difference between the effects of the two proteins on TC levels. In the case of TG, the 7S protein group showed a remarkable fall reaching the same level obtained with the drug.

The animals of the HC group showed a decrease of 43.6% in HDL-C levels in the plasma, compared to the STD group ($P < 0.001$), while those of rats fed β-conglycinin, glycinin and fenofibrate were 55.5%, 20.0% and 95.5% higher, respectively, than the levels of the HC group; however, in the glycinin group, these values were not significant at $P < 0.05$ (Fig. 2A). Fig. 2B shows the effect of the diets on the non-HDL-C fraction. It can be observed that this fraction was four times higher in the HC group than in STD rats ($P < 0.001$). In contrast, the administration of glycinin, β-conglycinin and fenofibrate decreased this fraction by 20.7%, 33.2% and 53.1%, relative to the HC group, respectively.

In the present study, the atherogenic index, a possible indicator of a predisposition to heart diseases, was seven times greater in the animals of the HC group than in STD ($P < 0.001$); however, a decrease was observed for rats that

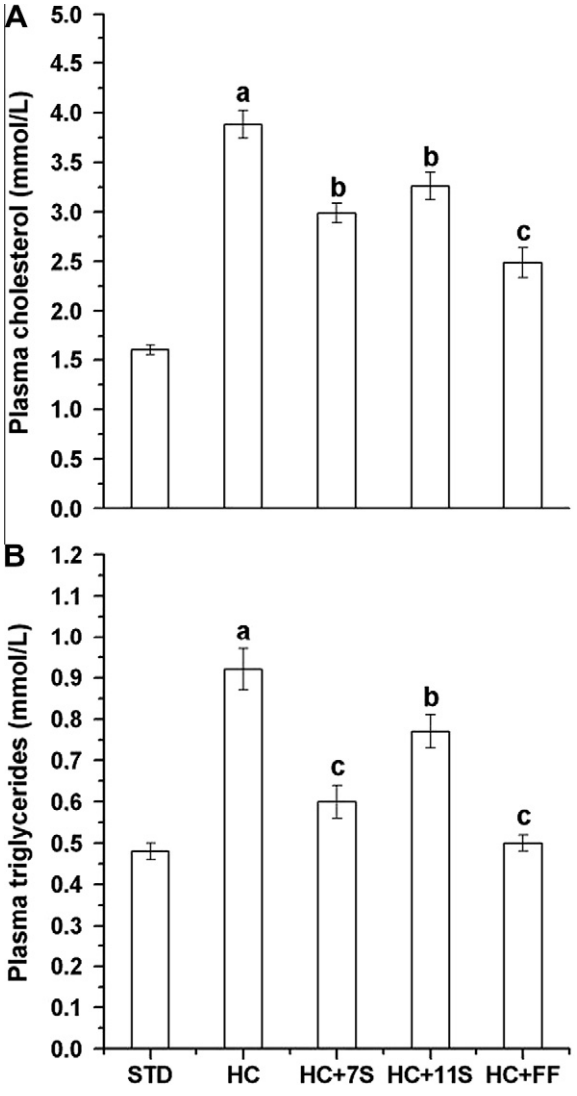

Fig. 1 – Plasma total cholesterol (A) and triglyceride (B) levels of rats fed HC diet without and with oral daily doses of β-conglycinin, glycinin or fenofibrate for 28 days. Values are means ± SE for nine rats. Different letters indicate differences between means. STD = standard diet: AIN-93 Composition; HC = hypercholesterolemic diet: AIN-93 plus 1 g/100 g cholesterol and 0.5 g/100 g cholic acid; HC+7S = HC diet + β-conglycinin (300 mg/kg/day); HC+11S = HC diet + glycinin (300 mg/kg/day) and HC+FF = HC diet + fenofibrate (30 mg/kg/day).

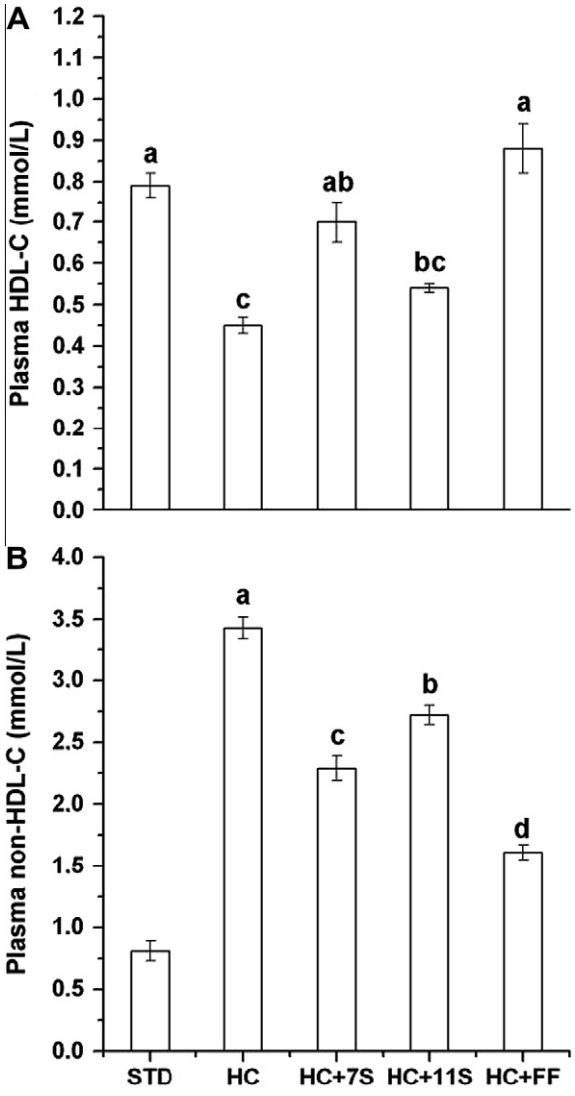

Fig. 2 – Plasma HDL-C (A) and non-HDL-C (B) levels of rats fed HC diet without and with oral daily doses of β-conglycinin, glycinin or fenofibrate for 28 days. Values are means ± SE for nine rats. Different letters indicate differences between means. STD = standard diet: AIN-93 Composition; HC = hypercholesterolemic diet: AIN-93 plus 1 g/100 g cholesterol and 0.5 g/100 g cholic acid; HC+7S = HC diet + β-conglycinin (300 mg/kg/day); HC+11S = HC diet + glycinin (300 mg/kg/day) and HC+FF = HC diet + fenofibrate (30 mg/kg/day).

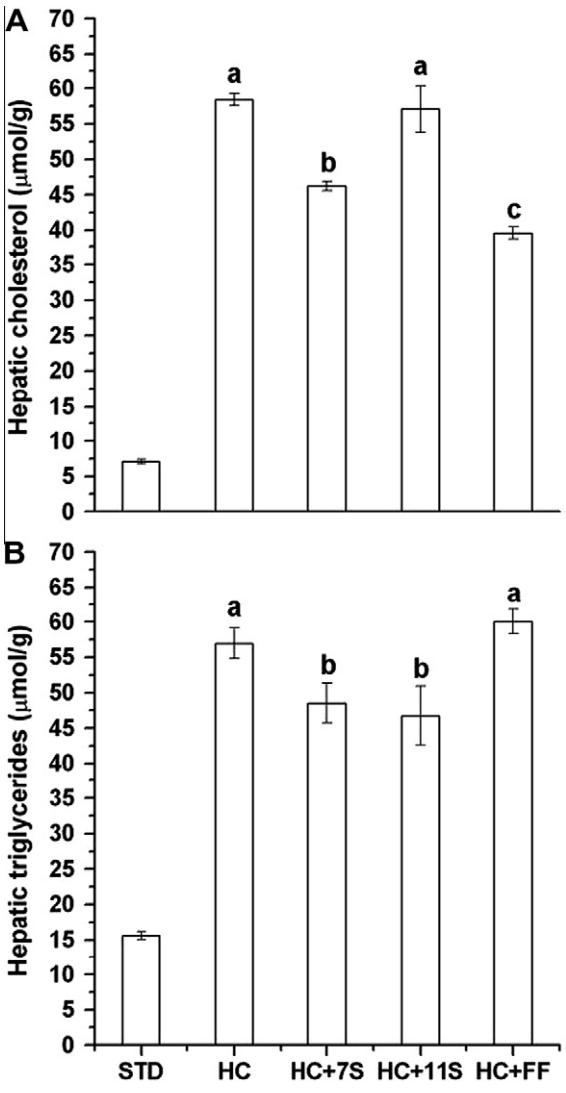

Fig. 3 – Hepatic cholesterol (A) and triglycerides (B) concentration of rats fed a HC diet without and whit oral daily doses of β-conglycinin, glycinin or fenofibrate for 28 days. Values are means ± SE for nine rats. Different letters indicate differences between means. STD = standard diet: AIN-93 Composition; HC = hypercholesterolemic diet: AIN-93 plus 1 g/100 g cholesterol and 0.5 g/100 g cholic acid; HC+7S = HC diet + β-conglycinin (300 mg/kg/day); HC+11S = HC diet + glycinin (300 mg/kg/day) and HC+FF = HC diet + fenofibrate (30 mg/kg/day).

ingested glycinin, β-conglycinin and fenofibrate, relative to the HC group (Table 3). Thus, β-conglycinin achieved 58%, 62.6% and 74.5%, and glycinin 21.7%, 39.0% and 49.7%, of the performance of fenofibrate with respect to HDL-C, non-HDL-C and atherogenic index in the animals, respectively.

3.3. *Liver parameters*

All the rat groups that received the hypercholesterolemic diet showed significantly higher TC and TG levels in the liver than those on the STD diet ($P < 0.001$), as shown in Fig. 3. However, relative to the HC rats, the β-conglycinin group exhibited a reduction of 20.9% and 14.8% for hepatic TC and TG, respectively; while the glycinin group showed no change in TC levels and a decrease of 17.9% in hepatic TG ($P < 0.001$). A greater reduction in TC level in the liver was observed in the rats that had received fenofibrate: 32.1% lower than the HC group ($P < 0.001$); however, the levels of triacylglycerols were 5.4% higher than in the HC group ($P < 0.05$).

4. Discussion

It has been demonstrated that fenofibrate can reduce food intake and body weight gain when administered at doses of 100 mg/day or during prolonged periods (Ferreira, Pereira, Green, & Botion, 2006; Mancini et al., 2001), however, in our experiment the dose of 30 mg/kg/day did not affect these variables. In addition, similar results have been described by other authors, who used these soybean proteins as the only protein source and observed no effect on the feeding efficiency ratio and food intake after 28 days of experiment (Fukui et al.,2002, 2004). In a study on humans, Kohono, Hirotsuka, Kito, and Matsuzawa (2006) observed a significant reduction of the adipose tissue weight and visceral fat weight in 65 people of both sexes after 20 days of administration of 650 mg/day β-conglycinin in the form of candy, compared to a placebo. The effects of β-conglycinin on TC and TG in the plasma of rats confirm the observations made by other authors (Duranti et al., 2004) using this experimental model. However, our study represents the *in vivo* confirmation that the administration of a daily dose of isolated glycinin is sufficient to reduce plasma TC and TG in hypercholesterolemic rats, as already described *in vitro* (Kwon et al., 2002; Pak, Koo, Kasimova, & Kwon, 2005). Lovati et al. (1992) did not observe alterations in TG but only in TC levels in the plasma of rats given the same two proteins in a similar experimental model.

Investigations have shown that the relative low risk of coronary heart disease (CHD) in Asian people is related to the use of soy protein as a food source. The use of soy protein products at least four times per week decreases the risk of heart disease by 22% (Herbert, Graziano, Chan, & Hennekens, 1997). The ratio of LDL-C to HDL-C is commonly used in order to assess the risk of CHD, on the basis of evidence that an elevated LDL-C concentration is atherogenic, whereas a greater level of HDL-C is cardioprotective (Castelli et al., 1986). Sawashita et al. (2006) reported that the antithrombotic effect observed with a whole soy powder was due to inhibition of the atherosclerotic process rather than to platelet inhibition. Adams et al. (2004) found that β-conglycinin showed effects on the inhibition of atherosclerosis in mice however; these were not associated with the role of LDL receptors, and minimally related to the concentrations of plasma lipoproteins. Rho et al. (2007) observed significant and progressive reductions of the atherogenic index in obese rats fed a diet containing black soy peptides at 2%, 6% and 10% of their energy expenditure for 20 days, compared to a casein diet. In addition, Aoyama et al. (2001) found a 16% reduction for atherogenic index in young rats and an increase of 7% in adult rats that ingested β-conglycinin as a single protein source, rel-

ative to casein. By contrast, in the present study, the atherogenic index, exhibited a decrease of 37.7%, 56.5% and 75.6% for rats that ingested glycinin, β-conglycinin and fenofibrate, respectively, relative to the hypercholesterolemic group (Table 3). These data indicate that both fenofibrate and the soybean protein fractions diminish the cardiovascular risks in hypercholesterolemic rats.

There is a lot of evidence to suggest that the concentrations of triacylglycerols in the liver and plasma depend mainly on the rate of hepatic lipogenesis (Brandsch, Shukla, Hirche, Stangl, & Eder, 2006; Moriyama et al., 2004; Pak et al., 2005). Raised TG levels in the liver and liver weight in rats that ingested fenofibrate have also been reported by other authors. Yamamoto, Fukuda, Zhang, and Sakai (1996) found a reduced secretion of hepatic TG but not cholesterol in the liver of rats that had ingested fenofibrate. Ferreira et al. (2006) showed that fenofibrate treatment prevents hypertriglyceridemia, hypercholesterolemia and weight gain in rats by reducing lipoprotein lipase activity in various tissues.

In a similar experiment, Lovati et al. (1992) reported a significant fall in total TG concentration in the liver of the animals supplemented daily with 7S and 11S soybean proteins for 14 days, compared to casein–cholesterol fed rats; however, contrary to our results, the levels of cholesterol in the liver were unaffected by the soybean proteins. This difference could be related to the different concentrations of the proteins used since those authors offered only 30 mg/day/animal by gavage, or maybe the time of the experiment. Various experimental studies in which β-conglycinin and glycinin were used as the only protein source in the diet have demonstrated their effects on lipid metabolism in several animal models and *in vitro*. Thus, Aoyama et al. (2001) showed that rats fed a diet containing β-conglycinin without phytate and as the only source of protein presented a decrease of 34% in TC, 49% in TG and an increase of 19% in HDL-C, compared to rats fed a casein diet. Moriyama et al. (2004) found that β-conglycinin reduced the plasma TG in normal and obese rats, while glycinin did not alter its level. On the other hand, Adams et al. (2004) observed that β-conglycinin and glycinin in the diet had an effect on plasma TC levels that depended on sex and type of transgenic mice; while both proteins raised the TC levels in LDL-receptor-null male mice, they did not affect the Apo-E-null animals. By contrast, glycinin and β-conglycinin reduced the TC levels in the plasma of LDL-receptor-null females by 37.0% and 40.6%, while they increased the TC in Apo-E-null female mice by 43.6% and 11.2%, respectively. Recently, Adams, Anthony, Chen, and Clarkson (2008) observed that in monkeys fed on a diet with soy protein isolate, 7S and 11S soybean globulins, the TC levels in the plasma showed a modest reduction of 5% for soy isolate and the 11S fraction, while β-conglycinin increased TC by 6.6%, relative to animals fed a casein/lactalbumin diet. However, while these authors have used these fractions as the only source of protein in the diet, in our experiment these proteins represented only about 2.75% of the ingested total protein/day/animal and yet caused increases of 56.8% and 21% in the plasma HDL-C for the respective proteins, while the fenofibrate caused an increase of 97.8% in this lipoprotein. Accordingly, our experiment confirms, in fact, the observations of Duranti et al. (2004) in rats on a hypocholesterolemic diet, subjected to daily doses of 7S fraction and its α′ chain; however, in addition, we have recorded an increase of HDL-C in the plasma and a fall in TC and TG levels in the liver of the animals. The fibrates are effective triacylglycerol-lowering drugs (Knopp, 1999) and our results show that the administration of the isolated fractions had a significant effect on TG and TC levels in hypercholesterolemic rats, comparable to fenofibrate.

Although the mechanisms of the cholesterol-lowering effects of soybean proteins are not yet clear, it is important to raise the hypothesize that due to their large molecular size, it is likely that the effects observed are not due to the intact proteins, but certainly to peptides derived from them by gastrointestinal digestion, which, after absorption and transport to the liver, could modulate the homeostasis of cholesterol, as already discussed by other authors for soybean globulins (Kwon et al., 2002; Mochizuki et al., 2009; Pak et al., 2005; Yamamoto et al., 1996). Other studies suggest that the reduction of plasma cholesterol might be a consequence of an interaction between these peptides and cholesterol, or its metabolites, neutral sterols and bile salts, which promotes its excretion (Iwami, Sakakibara, & Ibuki, 1986; Kohono et al., 2006; Pak et al., 2005).

Lovati et al. (1996, 2000) have described a significant increase in the expression of LDL-receptors *in vitro* after exposure of HepG2 cells to the 7S fraction and the α' subunit. This increase in LDL-receptor expression may contribute to the observed decrease in ApoB-100 secretion, since LDL-receptor causes a post-translational degradation of ApoB-100 and thereby reduces VLDL-particle secretion, as observed by Mochizuki et al. (2009). Actually, these authors showed that peptides from highly purified β-conglycinin decreased the synthesis of TG and secretion of lipoproteins containing ApoB-100 in HepG-2 cells. Moreover, using the same experimental model, we found that the glycinin fraction had an effect similar to that of β-conglycinin on TC, TG and HDL-C in the plasma of the animals, though with less intensity. The present finding that the 11S fraction also has lipid-lowering effects are in accord with previous observations in studies *in vitro* and *in vivo* with diverse animals (Adams et al., 2008; Kwon et al., 2002; Lovati et al., 1992; Moriyama et al., 2004; Pak et al., 2005; Yoshikawa, Yamamoto, & Takenaka, 1999). Pak et al. (2005) showed that peptides from the pepsin hydrolysate of glycinin exhibited *in vitro* hypocholesterolemic activity. On the other hand, Kwon et al. (2002) isolated three different peptides from glycinin hydrolyzed by trypsin and also found a hypocholesterolemic effect *in vitro*. The authors also observed a 30% decrease in TC in the serum of mice fed the glycinin peptides, while cholestyramine (bile acid binding activity) and lovastatin (HMG CoA reductase inhibitor) caused reductions of 29% and 32%, respectively.

Despite many studies in animals and *in vitro* models, as already cited, the mechanisms of the reduction in cholesterol levels by ingestion of soy protein are still far from clear and, although advances have been made, controversy remains (Adams et al., 2008; Scarafoni et al., 2007; Torres et al., 2006). The data of the present study indicate that the hypocholesterolemic effects of the soybean protein are related to both the β-conglycinin and glycinin fractions, and suggest that biologically active peptides produced from by

gastrointestinal digestion of the proteins are capable of modulating cholesterol and triacylglycerol homeostasis.

In conclusion, our results show that oral ingestion of β-conglycinin and glycinin at around 2.75% of the daily total protein ingested, promoted the reduction of TC, LDL-C and TG in the plasma and an increase in HDL-C; besides this, they induced a small but significant decrease in TC and TG levels in the liver, as well as in the atherogenic index, in rats fed a hypercholesterolemic diet. Interestingly, the data show that the hypocholesterolemic and hypolipidemic effects of the proteins are comparable to those observed with the fenofibrate in the same experimental model. Studies are in progress to identify the effects of these proteins alone and/or combined with other hypocholesterolemic drugs, such as the statins.

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