Influence of a Protein Concentrate from *Amaranthus cruentus* Seeds on Lipid Metabolism

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It is widely known that elevated cholesterol and triglycerides levels favor the development of heart disease. In this paper we studied the effect of a protein concentrate from *Amaranthus cruentus* (Ac) on the lipid content in serum and liver tissue of male Wistar rats. The animals were separated into two groups, each group with 16 rats. The control diet had casein as protein source (CD), and the experimental one had Ac protein concentrate (PCAcD). The diets contained 1% cholesterol. Parameters of oxidative stress in liver with CD and PCAcD were also evaluated. No significant differences were observed in serum total cholesterol, whereas LDL decreased and HDL increased ($P < 0.001$), and the amount of triglycerides decreased in PCAcD as compared to CD. In liver, a decrease of total cholesterol and triglycerides ($P < 0.001$) was observed in the experimental group in relation to control. Fatty acid synthase (FAS) activity decreased significantly in the experimental group. The mRNA of HMG-CoA reductase did not change, and mRNA of FAS decreased in rat liver fed with PCAcD compared with CD. The excretion of total lipids in feces increased with PCAcD compared to CD ($P < 0.001$). The activity of reactive substances to thiobarbituric acid in liver showed no significant differences between the control and experimental diets. However, total glutathione and reduced glutathione increased in PCAcD compared to CD ($P < 0.001$). It can be concluded that PCAcD has a hypotriglyceridemic effect, affects the metabolism of liver lipids, and increases parameters of antioxidant protection in male Wistar rats. Exp Biol Med 231:50–59, 2006

Key words: *Amaranthus cruentus;* protein concentrate; hypolipidemic effect; oxidative stress; FAS mRNA; HMG-CoAR mRNA; LDL-cholesterol

Introduction

It is widely known that elevated cholesterol and triglycerides levels favor the development of cardiovascular disease. The World Health Organization (WHO) has established that one of the keys to increasing life expectancy in a population is the prevention of such disease. The effect of dietary protein on cholesterol levels has been the focus of many investigations. There are numerous reports about the hypocholesterolemic effect of plant proteins, particularly those obtained from soybean as well as wheat gluten and potato and oat proteins compared to casein as reference animal protein (1, 2).

Amaranth grains contain about 15% protein and 60% starch. Its high content of lysine makes amaranth an attractive source of protein because, if consumed along with other cereals, it can provide a "balanced" protein source (3, 4). There is therefore increasing interest in the use of amaranth flour in blends with wheat or maize (5). Amaranth species are also receiving a great deal of attention in developing countries as a means to fight protein malnutrition. Tocopherols in amaranth seeds include γ- and δ-tocotrienols, the unsaturated forms of vitamin E. All of them have antioxidant activity, and they are under scrutiny as hypocholesterolemic agents (6).

The amaranth grain has been chemically characterized, and its nutrient composition has been determined (7, 8). In addition, some researchers have drawn attention to the hypocholesterolemic effect of amaranth grains. Berger *et al.* have reported that diets containing 20% *Amaranthus cruentus* (Ac) grains and 5% crude amaranth oil have a decreasing effect on total cholesterol and low- or very low-density lipoprotein in hamsters (9). Plate and Arêas demonstrated that the consumption of extruded amaranth (*Amaranthus caudatus*) reduces LDL and total cholesterol in hypercholesterolemic rabbits (10). However, little is still known about the physiologic effects of amaranth, and even less information is available about the protein concentrate obtained from the amaranth seed flour (11, 12).

On the other hand, epidemiologic studies indicate that

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the ingestion of vegetables protects against oxidative stress. Oxidative cellular stress, an alteration of the balance between prooxidants and antioxidants, may arise from excessive production of reactive oxygen species (ROS) and/or from a deficiency in antioxidant mechanisms, leading to cell damage. Within the cellular context, the redox status depends on the relative amounts of oxidized and reduced major redox molecules, such as glutathione. The glutathione disulfide (GSSG)/glutathione (GSH) ratio reflects the redox status within the cell (13). Potter suggests that fruits and vegetables act as a "polypharmacy," providing antioxidant agents (14).

The purpose of this work was to compare the Ac protein concentrate with a casein protein source by studying lipid metabolism and antioxidant defense capacity in rats with induced hypercholesterolemia.

Materials and Methods

Chemicals. Lipid and protein standards were acquired from Sigma Chemical Company (St. Louis, MO). Serum parameters were evaluated by enzymatic methods using kits from Boehringer Mannheim Diagnostics (Indianapolis, IN). [^{14}C]Sodium acetate (specific activity 23 mCi/mmol) was purchased from DuPont, New England Company (Boston, MA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco BRL, Life Technologies Inc. (Rockville, MD). All other chemicals were of reagent grade and were purchased from Merck Laboratory.

Amaranthus Flour Preparation. The Ac seeds, obtained from the 1999 harvest of an experimental cultivation, were provided by the Agronomy Department of La Pampa National University, Argentina, and the flour was prepared in our laboratory. Raw seeds were dried in an air current oven at 45°C for 48 hrs. The dried product was ground in an electric coffee grinder (CG-8 Stylo, 220 V-50 Hz 90 W, China) and sieved through a 200-μm nylon sieve. A light brown and slightly yellowish flour was obtained.

Protein Concentrate Preparation. The preparation of protein concentrate was done in our laboratory as previously described (12). From the flour aqueous dispersion of Ac, the protein was solubilized at pH 11 with 5 *M* NaOH and then precipitated with 5 *M* HCl at pH 4.5. The precipitate was dried and ground, producing a light-colored protein concentrate.

Diets. The diets were prepared according to recommendations of the American Institute of Nutrition 1993 AIN-93 M (15), which requires 12% protein in the diet. Casein (Inmobal Nutrer, Buenos Aires, Argentina) was used as protein source in both the prestudy diet (PD) and the control diet (CD). In the experimental diet, the protein source was the Ac protein concentrate (PCAcD). The control and experimental diets contained 1% crystalline cholesterol. All the diets were isonitrogenous and isocaloric. Table 1 shows the ingredients of the diets used. The composition of the protein concentrate (12) and the casein source are also described in Table 1.

Experimental Design. Sixteen male Wistar rats (Romanelly, Buenos Aires, Argentina), starting weight 200 g, were kept in individual cages at 25°C and exposed to 12-hr light:dark cycles, with food and water *ad libitum*. The animals were subjected to a 10-day adaptation period with PD. On the 10th day, after a 12- to 14-hr fast period, the rats were weighed, and serum cholesterol was assessed. They were subsequently separated into two groups. For 28 days, one group was fed the CD and the other one the experimental diet. Food intake, water consumption, and body weight were recorded every 3 days. During the last week of the experiment, the feces were collected daily, weighed, placed into aluminum foil, and stored in a freezer. On Day 28, the rats were sacrificed 12 hrs after the last feeding. Blood was collected and processed for serum. The livers were extracted, weighed, and stored at −70°C for subsequent analyses (experimental Group 1). An additional group of 16 rats (experimental Group 2) was subjected to the same experimental protocol, except that they were not starved on Day 28, and the organs were stored at −70°C until they were analyzed. All determinations were performed in duplicate.

All studies involving experimental animals in this work were conducted in accordance with national and institutional guidelines for the protection of animal welfare. The study was approved by the Animal Care Committee of San Luis National University.

Blood Analytes. Total cholesterol (Total-C), HDL-cholesterol (HDL-C), triglycerides (TG), glucose, total proteins, and albumin concentrations were determined by enzymatic methods, using commercial kits. LDL-cholesterol (LDL-C) was calculated by using the Friedwald formula (16). Serum T_4 concentrations were determined by a commercial kit.

Hepatic Determinations. *Lipid Analyses.* Lipids were extracted from liver tissue (300 mg) in a hexane/isopropanol mixture (3:2, v:v) containing butylated hydroxytoluene as antioxidant (17). Total lipids were determined by dry weight (18). Lipids were resuspended in hexane, and aliquots were taken for determining phospholipids and measuring phosphorus (19). Total cholesterol (20) was determined using another aliquot, which was subjected to saponification with 2% KOH in 95% ethanol for 1 hr at 50°–60°C (21). After cooling, hexane and distilled water were added. The sample was stirred, and the up phase that contained the cholesterol was separated and then evaporated under N_2 at 37°C. The extract was resuspended in glacial acetic acid, and the cholesterol content was determined using the color reagent $FeCl_3$, 10% in glacial acetic acid:sulfuric acid concentrate (1:100, v:v). The sample was kept in the dark for 30 mins. The concentration of cholesterol was calculated from the absorbance at 560 nm. Other aliquots of the extracts were used for the separation of the different lipids on thin-layer chromatography (TLC)

Table 1. Diets Composition

Components	Prestudy diet casein (g/kg)	Control diet casein (g/kg)	Experimental diet *A. cruentus* protein concentrate (g/kg)
tert-Butylhydroquinone	0.008	0.008	0.008
Choline bitartrate	2.50	2.50	2.50
L-Cystine	1.80	1.80	1.80
Vitamin mix[a]	10.00	10.00	10.00
Mineral mix[a]	35.00	35.00	35.00
Fiber	50.00	50.00	50.00
Sucrose	100.00	100.00	100.00
Protein Sources[b]	156.87	156.87	230.00
Cornstarch	465.69	465.69	465.69
Dextrinized cornstarch	138.13	128.93	55.00
Soybean oil	40.00	40.00	40.00
Cholesterol	—	10.00	10.00
Protein sources			
Casein			
Protein (dw)[c]	758.57 ± 59.60		
Oils (dw)	52.27 ± 10.23		
Ash (dw)	51.30 ± 0.93		
A. cruentus protein concentrate			
Protein (dw)	525.63 ± 4.53		
Moisture	31.04 ± 0.44		
Ash (dw)	37.03 ± 0.52		
Soluble dietary fiber (dw)	129.01 ± 3.24		
Insoluble dietary fiber (dw)	206.92 ± 5.73		
Total lipids (dw)	59.01 ± 7.54		

[a] AIN-93 M [15].
[b] The amounts of casein and protein concentrate of *Amaranthus cruentus* added to the diets depend on the pure protein content in each diet (N protein content was determined by Kjeldhal).
[c] dw, dry-weight basis.

plates coated with silica gel G, using hexane/diethyl ether/acetic acid (80:20:1, by volume) as solvent. The lipids were detected by exposing the plates to iodine vapors. They were scraped off and were used for determination of triglycerides (22) and free and esterified cholesterol (20).

Proteins were determined by the method of Biuret (23) with bovine serum albumin as a standard.

Incorporation of [^{14}C]Acetate into Fatty Acids. The synthesis rate of fatty acids in liver slices was determined by the rate of [^{14}C]sodium acetate incorporation in the saponifiable fraction. The samples were resected from the same lobe of each liver. Liver slices (100 mg) of 1 mm thickness from CD and PCAcD were rapidly cut with blade scissors and incubated by triplicate in individual metabolic flasks containing 2 ml of phenol red-free DMEM supplemented with 10% FCS, 50 μg/ml gentamicin, 50 μg/ml penicillin, 6 m*M* glucose, and 4% delipidated bovine serum albumin, pH 7.4. The flasks were continuously flushed with 95% O_2/5% CO_2. After the slices were preincubated for 20 mins at 37°C, 0.1 μCi/ml [^{14}C]acetic acid sodium salt was added to each flask. After 3 hrs of incubation, the incubation medium was removed by aspiration. Tissues were washed three times with ice-cold 0.9% NaCl until no more radioactivity was detected in the wash water. The tissue was submitted to saponification by treatment with 1 ml of 10% (w:v) KOH in ethanol plus 1 ml of methanol to facilitate the subsequent extraction step. The fatty acids were recovered from the saponifiable fraction after acidification with HCl and extracted with petroleum ether (bp 30–40). This fraction was dried down in a stream of nitrogen and then dissolved in hexane. The incorporation of radioactivity was measured in a Beckman LS100C liquid scintillation counter and results were expressed as cpm/g of tissue (24).

Thiobarbituric Acid Reactive Substances (TBARS) Determination. Samples of liver were homogenized in 10 volumes of 50 m*M* HEPES buffer, pH 7.4, 125 m*M* KCl, containing protease inhibitor cocktail, at 4°C. The suspension was centrifuged at 800 *g* for 10 mins at 4°C to remove nuclei and cell debris. The pellet was discarded, and the supernatant was used as homogenate.

Liver homogenates were used for thiobarbituric acid (TBA) assay (25) and the levels of lipid peroxidation products were determined spectrophotometrically as TBARS.

Antioxidant Parameters. Total glutathione, reduced glutathione (26), and total nonprotein thiols (27) were measured. Total glutathione (GSH + GSSG) was determined on perchloric acid extracts. Livers were thawed and immediately homogenized in perchloric acid. After centrifugation at 10,000 *g* for 5 mins, the supernatant fraction (neutralized extracts) was used in the determination. The

concentration of total GSH in the supernatant was measured using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and glutathione reductase. Glutathione disulfide was determined on perchloric acid extracts. After centrifugation, the acid extracts were neutralized with 3 *M* K_3PO_4. The oxidized glutathione in the supernatant was measured using glutathione reductase. Reduced glutathione was obtained as a difference between total and oxidized glutathione.

For the determination of total nonprotein thiols (NPT), tissue was suspended in 0.5 *M* $HClO_4$. The suspension was centrifuged 5000 *g* for 5 mins. The supernatant was added with a 0.5 *M* solution of KH_2PO_4/K_2HPO_4, pH 8, containing 1 n*M* EDTA. The NPT content was spectrophotometrically measured at 412 nm by the addition of 6 m*M* DTNB in potassium phosphate buffer, pH 7.4.

Glycogen Analysis. Glycogen concentration (28) was analyzed as follows. The extraction was performed with 6% $HClO_4$ and centrifuged, and the supernatant was precipitated with absolute ethanol. After washing and reprecipitation, the precipitate was dried overnight at 37°C. Hydrolysis was performed with 0.6 *M* HCl for 2 hrs in a boiling bath. Subsequently, the hydrolysis product was neutralized with 0.5 *M* NaOH, pH 7. Glucose was determined using commercial kits.

Fecal Matter Determinations. Total lipids and total cholesterol were determined using the same method described for liver.

Experimental Group 2: Hepatic Determinations. *Fatty Acid Synthase Activity.* Livers were homogenized in an Ultra Turrax T25 machine (Ramm Doman, Buenos Aires, Argentina) with a 0.5 *M* potassium phosphate buffer (pH 7) containing 0.1 m*M* EDTA and 10 m*M* D,L-dithiothreitol (DTT). The homogenate was centrifuged at 100,000 *g* for 1 hr to yield the cytosolic fraction by a Beckman model L2 65B ultracentrifuge with a Ty rotor. Fatty acid synthase (FAS) activity was determined spectrophotometrically by a modified version of the method of Roncari (29). The oxidation of NADPH at 30°C was monitored at 340 nm.

RNA Isolation and RT-PCR Analysis. Total RNA was isolated by using TRIzol (Invitrogen, Buenos Aires, Argentina). All RNA isolations were performed as directed by the manufacturers. Gel electrophoresis and ethidium bromide staining confirmed the purity and integrity of the samples. Quantification of RNA was based on spectrophotometric analysis at 260/280 nm. Ten micrograms of total RNA was reverse transcribed at 42°C using random hexamer primers and Moloney murine leukemia virus RT (Invitrogen/Life Technologies, Buenos Aires, Argentina) in a 20-μl reaction mixture. The primer sequences used in the PCR amplification of cDNAs were L19 primers of oligonucleotides (sense 5′,3′-CGCCAATGCCAACTCTCGTCA, antisense 5′,3′-TTCCGTCGGGCCAAAGGTGTTC) Genbank Accession X82202. Size of amplification product 120 pb; FAS primers of oligonucleotides (sense 5′,3′-GTTTGATGGCTCACACACCT, antisense 5′,3′-TACACTCACTCGAGGCTCAG) Genbank Accession M84761; size of amplification product 515 pb; hydroxymethyl glutaryl coenzyme A reductase (HMG-CoAR) primers of oligonucleotides (sense 5′,3′-GTGATTACCCTGAGCTTAGC, antisense 5′,3′-TGGGATGTGCTTAGCATTGA) Genbank Accession NM013134; size of amplification product 462 pb.

Aliquots of the reverse transcription reaction mix cDNA corresponding to different quantities of cDNA for each reaction, such as to give optimal signals (FAS, 50 ng; HMG-CoAR, 100 ng; and L 19, 50 ng) were amplified with primers specific for rat FAS (30), HMG-CoAR (31), and L19 (32) used as control. All reactions were carried out for 25 cycles for L 19 and 30 cycles for all the rest, with the following cyclic parameters for all the reactions: 95°C for 1 min, 57°C 1 min, and 72 °C 1 min, and then terminated with a 5-min extension at 72°C. The conditions and quantities of cDNA added were such that the amplification of the products was in the exponential phase and the assay was linear with respect to the amount of input RNA. RNA samples were assayed for DNA contamination by PCR without prior reverse transcription. The PCR products were analyzed on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and photographed with a Polaroid camera. Band intensities of RT-PCR products were quantified using NIH Image software. Relative levels of mRNA were expressed as the ratio of signal intensity for the target genes relative to that for L 19 cDNA.

Statistical Analysis. Results are expressed as mean ± standard deviations. Statistical differences were tested by Student's *t*-test. A probability of 0.05 or less indicated significant difference (33).

Results

Body weight gain, total food intake, and percentage of food efficiency (FE) (weight gain/food intake × 100) did not show significant differences between the control and experimental groups (Table 2). The growth was linear throughout the experiment for rats fed with CD and PCAcD.

In rats fed with PCAcD, serum Total-C was not modified, but HDL-C levels increased, which provoked a significant increase of the HDL-C/Total-C ratio ($P < 0.001$; Table 3). This group showed a significant decrease of LDL-C ($P < 0.001$). A decrease of triglycerides was observed in rats fed with PCAcD when compared to those fed with CD ($P < 0.001$). The values of glucose, total protein, and albumin were not affected by the experimental diet. Likewise, tetraiodothyronine (thyroxine) did not show significant differences between the control and experimental groups.

Protein and glycogen concentration in liver did not show significant differences between rats fed the experimental and control diets (Table 4). The rats fed with PCAcD showed a significant decrease of total cholesterol and triglycerides ($P < 0.001$). The decrease of total

Table 2. Effect of Diets on Weight Gain, Food Intake, and Food Efficiency (FE) in Rats[a]

Parameters	Casein	*A. cruentus* protein concentrate
Body weight gain (g)	86.56 ± 13.83	83.11 ± 14.20
Food intake (g)	559 ± 24	523 ± 35
FE (%)[b]	15.48 ± 2.35	15.89 ± 3.85

[a] Values represent means ± SD, $n = 8$, Student *t*-test.
[b] FE (%) = weight gain/food intake × 100.

cholesterol led to a decrease in esterified cholesterol ($P < 0.001$) with no changes in free cholesterol. Animals fed both diets did not show significant differences in phospholipid levels. Regarding enzymatic activity of FAS, a significant decrease was observed in rats fed with the experimental diet when compared to CD ($P < 0.01$). *In vitro* lipogenesis determination showed a decrease of [^{14}C]acetate incorporation in the saponifiable fraction in PCAcD as compared to CD ($P < 0.01$).

To determine whether the decrease observed in lipid content was caused by changes in the expression of lipogenic enzymes, FAS mRNA was measured. To study the effect of the decrease on hepatic cholesterol levels with PCAcD diets, the rate-limiting enzyme for cholesterol biosynthesis was evaluated by the expression of HMG-CoAR. The relative amount of the different mRNAs was expressed relative to L 19. Liver FAS mRNA abundance was significantly diminished ($P < 0.01$) in the rats fed with the experimental diet (Fig. 1), in accordance with the changes in enzymatic activity observed in this group. The HMG-CoAR mRNA was not modified (Fig. 2) when both diets were compared.

To assess the degree of lipoperoxidation in liver tissue, the levels of substances that react to thiobarbituric acid were measured, and no significant differences were found between the control and experimental diets (Table 4).

To determine if the antioxidant defense system is modified by the protein concentrate of Ac in the diet, total glutathione, reduced glutathione, and total nonprotein thiols were assessed. The results of these determinations were expressed in relation to the data obtained with CD as 100%. In animals fed with PCAcD, total, reduced glutathione ($P < 0.001$), and total nonprotein thiols ($P < 0.05$), were increased when compared to CD (Fig. 3).

The effect of the diet on feces is shown in Table 5. No significant differences in feces weight were observed between the means of the control group and those of the experimental group. Total lipids and cholesterol in feces were higher in rats fed with PCAcD when compared to CD ($P < 0.001$ and $P < 0.05$, respectively).

Discussion

In this study, we examined the effects of a protein concentrate from *Amaranthus cruentus* on serum and hepatic lipids, and we demonstrated a marked improvement of these parameters in hypercholesterolemic rats fed with this protein with respect to rats fed with casein, evidenced by a decrease of serum triglycerides, hepatic triglycerides, total cholesterol, FAS activity, and [^{14}C]acetate incorporation and increase of lipids and total cholesterol in feces. Furthermore, the antioxidant defense system (total and reduced glutathione and nonprotein thiols) increased in liver.

The average value of serum cholesterol determined after the intake of CD was 23% higher than that obtained after the administration of PD (1.86 ± 0.13 m*M*), which demonstrates the hypercholesterolemic action of the addition of 1% cholesterol. PCDAcD did not decrease Total-C, but HDL-C was increased, and LDL-C was decreased in relation with CD. Similar variations in HDL-C and LDL-C values were reported by Chaturvedi *et al.* (34). Czerwin'ski *et al.* observed that the rise in the plasma lipids including Total-C was significantly hindered, and there were no significant changes in the concentration of HDL-C (35). In

Table 3. Effect of Diets on Blood Serum in Rats[a]

	Diet	
Parameters	Casein	*A. cruentus* protein concentrate
Total-C[b] (m*M*)	2.35 ± 0.23	2.38 ± 0.21
HDL-C (m*M*)	0.67 ± 0.10	1.24 ± 0.08*
LDL-C (m*M*)[c]	1.37 ± 0.18	0.91 ± 0.10*
HDL -C/ Total-C	0.28 ± 0.03	0.52 ± 0.04*
Triglycerides (m*M*)	0.68 ± 0.03	0.53 ± 0.04*
Glucose (m*M*)	3.22 ± 1.17	3.44 ± 1.50
Total proteins (g/L)	66.40 ± 4.70	66.80 ± 9.10
Albumin (g/L)	35.40 ± 0.30	35.50 ± 1.50
T_4 (n*M*)	58.00 ± 8.00	55.00 ± 3.00

[a] Values represent means ± SD, $n = 8$.
[b] C, Cholesterol.
[c] LDL-C = C-Total – HDL-C – (0.45 × triglycerides).
* $P < 0.001$ compared with control diet.

Table 4. Effect of Diets on Weight and Liver Parameters in Rats[a]

Parameters	Diet	
	Casein	*A. cruentus* protein concentrate
Liver weight (g)	10.36 ± 0.85 (3.50)[b]	11.47 ± 1.09 (3.94)
Total proteins (g/g liver)	0.61± 0.09	0.65 ± 0.03
Glycogen (μ*M* of glucose/g liver)	189.36 ± 26.64	235.14 ± 52.7
Total cholesterol (μ*M*/g liver)	11.01 ± 0.91	6.17 ± 1.13*
Esterified cholesterol (μ*M*/g liver)	9.64 ± 1.22	4.44 ± 1.08*
Free cholesterol (μ*M*/g liver)	1.37 ± 0.63	1.73 ± 0.35
Triglycerides (μ*M*/g liver)	3.36 ± 0.80	0.89 ± 0.41*
Phospholipids (μ*M* P/g liver)	13.17 ± 3.45	11.61 ± 0.84
Fatty acid synthetase (FAS) (μM NADPH transf./min/mg of protein)	0.0332 ± 0.011	0.0089 ± 0.001**
Incorporation of [^{14}C] sodium acetate in the saponifiable fraction (cpm/g liver)	1823.74 ± 307.70	331.80 ± 189.10**
TBARS (n*M*/mg prot)	$1.56 \times 10^{-6} \pm 4.82 \times 10^{-7}$	$1.79 \times 10^{-6} \pm 3.86 \times 10^{-7}$

[a] Values represent means ± SD, $n = 8$.
[b] Values in parentheses correspond to the percentage of liver weight in relation to body weight.
* $P < 0.001$ compared with control diet; ** $P < 0.01$ compared with control diet.

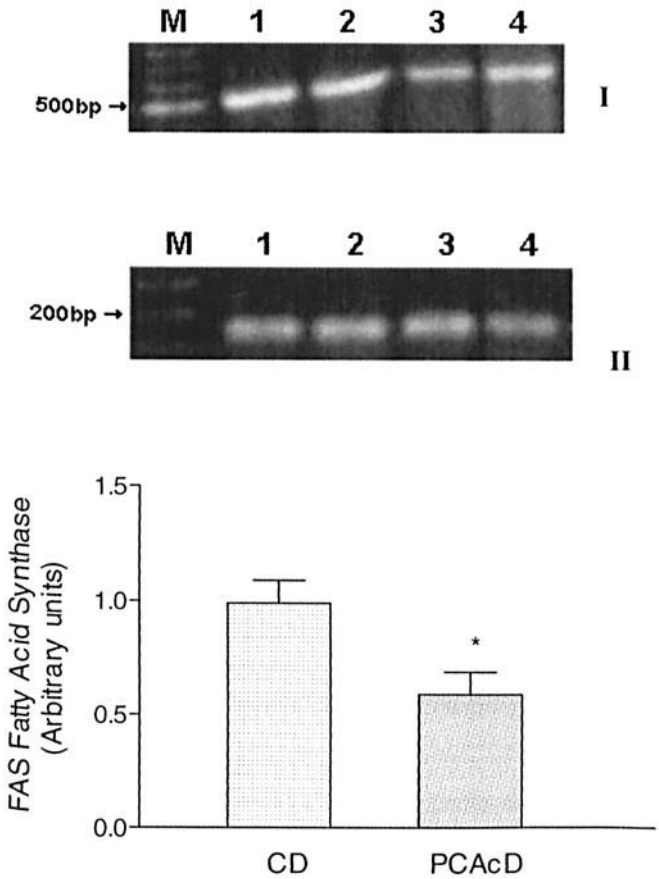

Figure 1. RT-PCR. (I) Ethidium bromide-stained agarose gel of FAS PCR products. (II) Ethidium bromide-stained agarose gel of L-19 PCR products, used as an internal control. M, molecular weight marker; Lanes 1–2, control diet; Lanes 3–4, experimental diet. Below, quantification of the intensity of the fragment bands in relation to the intensity of the internal control bands. Results are expressed as the mean ± SD. *$P < 0.001$ using Student's *t* test. CD, control diet with casein as protein source; PCAcD, experimental diet with protein concentrate of Ac as protein source.

the present experimental model no significant variations were observed in the levels of serum total cholesterol using 11.9% of protein content in the diet. It should be noted, however, that in previous studies carried out in our laboratory using a diet with 20% protein provided by an Ac protein concentrate, with addition of 1% cholesterol, the levels of serum total cholesterol were reduced when compared to 20% casein diet (unpublished results). This may suggest that higher contents of protein in the diet lead to a decrease of serum total cholesterol. Danz and Lupton (36) have suggested that Ac acts as a soluble fiber because of its hypocholesterolemic effect and as an insoluble fiber through its action in the colon, thus combining two beneficial effects. The effect of Ac and oat bran on lipids was studied in rats by Grajeta (37), who observed that amaranth led to a reduction of total cholesterol in serum and liver, this effect being higher when the diet was supplemented with sunflower oil.

It is known that serum TG concentration is the net result of several metabolic processes, including absorption of dietary fat (38). This is in agreement with our results regarding the hypotriglyceridemic effect of the PCAcD in relation to CD. This effect may be accounted for by the fact that the soluble fiber content in PCAcD decreases the absorption of dietary fat (38), in addition to decreased synthesis of fatty acid observed in our laboratory.

Liver tissue exhibited changes in the lipid metabolism of rats fed with the experimental diet. The percentage of esterified cholesterol in livers of rats fed with CD was 87.5% and 71.9% in livers of rats fed with PCAcD ($P < 0.001$). It has been reported that the percentage of esterified cholesterol in liver is increased by about 90% in rats fed with diets with 1% of cholesterol content (39). Rigotti *et al.* detected inhibition of cholesterol esterification in liver of rats fed with a bean diet and concluded that this diet could

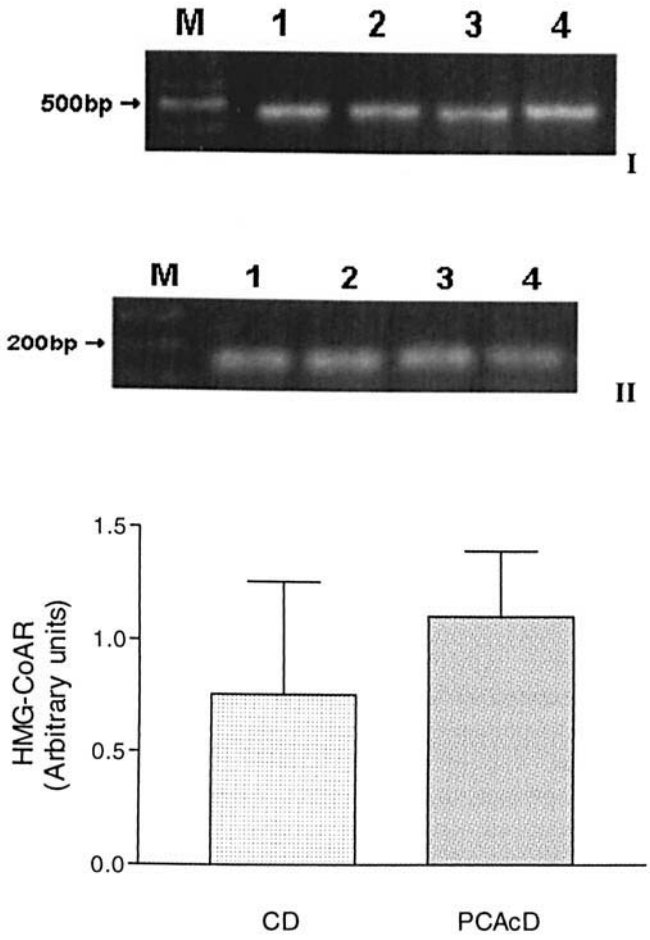

Figure 2. RT-PCR. (I) Ethidium bromide-stained agarose gel of HMG-CoAR PCR products. (II) Ethidium bromide-stained agarose gel of L-19 PCR products, used as an internal control. M, molecular weight marker; Lanes 1–2, control diet; Lanes 3–4, experimental diet. Below, quantification of the intensity of the fragment bands in relation to the intensity of the internal control bands. Results are expressed as the mean ± SD. CD, control diet with casein as protein source; PCAcD, experimental diet with protein concentrate of Ac as protein source.

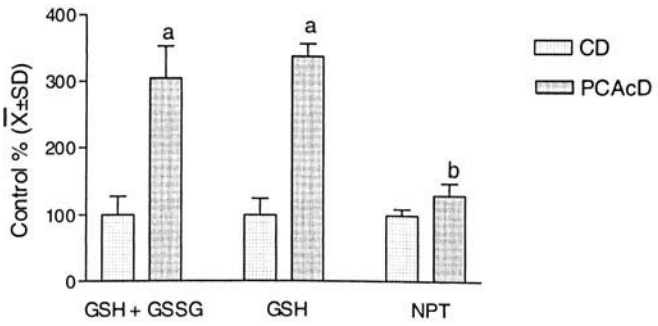

Figure 3. Influence of diet on antioxidant parameters in liver. Values are presented as mean ± SD for eight rats in each dietary group, analyzed in duplicate. The result of this determination was expressed in relation to CD as 100%. CD, control diet with casein as protein source; PCAcD, experimental diet with protein concentrate of Ac as protein source; GSH + GSSG, total glutathione; GSH, reduced glutathione; NPT, total nonprotein thiols. $^{a}P < 0.001$ compared with control diet. $^{b}P < 0.05$ compared with control diet.

have some component(s) with the capacity to inhibit hepatic cholesterol esterification and VLDL production (40). On the other hand, Berger *et al.*, using diets with amaranth oil (5%), found that the cholesterol synthesis rate increased, and hepatic cholesterol ester decreased, indicating reduced cholesterol ester availability for VLDL secretion and consistency with reduced VLDL cholesterol (9). Similar results were obtained in this work.

Shin *et al.* observed that amaranth grain and oil present in a diet with 1% cholesterol decrease cholesterol and tryglicerides in serum and liver. Similar results were observed when rats were injected with amaranth squalene. These authors also noted a decrease of the activity of HMG-CoAR in liver and simultaneous increase in cholesterol and biliary acids in feces (41). Other authors found that HMG-CoAR is not modified except when they used Ac popped in the diet, which decreased by about 7% the enzyme activity associated with diminution in the LDL-C. These authors suggest that the fiber and saponins present in the Amaranthus might produce the effect observed on cholesterol metabolism (42). In relation to the expression of HMG-CoAR, our results could be related to the effects of different Ac protein concentrate components, previously determined in our laboratory (12). These effects may be provoked by two factors. (i) Factors that increase HMG-CoAR activity, including the amount of soluble fiber (12.90%) in the protein concentrate, may decrease the absorption of cholesterol in intestine, leading to an increase of HMG-CoAR activity. On the other hand, the dietary soluble fiber can bond to biliary acids, which may increase cholesterol catabolism by transforming it into biliary acids in liver and consequently increase the HMG-CoAR activity. (ii) Factors that decrease this enzyme activity, including the squalene present in oil of the protein concentrate (9.53% Table 1) inhibits the activity of HMG-CoAR in liver. The soluble fiber fermentation in the colon produces short-chain fatty acids that may contribute to the decrease of cholesterol synthesis in liver. In our experimental model the interactions between these factors may have led to the decrease in hepatic cholesterol without changes in the enzyme expression.

The protein concentrate contains other components such as saponins (12), which could reduce blood cholesterol by increasing biliary excretion. Phytic acid is present in a similar concentration to that of soybean (12, 43). This component interferes with Zn absorption, modulating the Zn/Cu ratio in blood, and probably plays an important role in cholesterol regulation because high Zn/Cu ratios are associated with high cholesterol concentrations in humans (44). Trypsin inhibitors are present in considerable quantities in the concentrate (12). These probably led to gastrointestinal secretion including cholescystokinin, which causes muscle contraction in the biliary vesicle and sending of biliary secretion to the intestinal tract (44).

The protein in the Ac concentrate is another component that might have influenced the effect on the cholesterol

Table 5. Effect of Diets on Weight and Total Lipids in Feces[a]

Parameters	Diet	
	Casein	*A. cruentus* protein concentrate
Feces weight per day (g)	2.31 ± 0.22	2.53 ± 0.31
Total lipids (mg LT/g fecal matter)	93.83 ± 12.25	138.58 ± 10.37*
Cholesterol (μ*M*/g fecal matter)	102.82 ± 9.31	117.63 ± 7.22**

[a] Values represent means ± SD, $n = 8$.
* $P < 0.001$ compared with control diet; ** $P < 0.05$ compared with control diet.

levels of rats. Plant proteins have hypocholesterolemic effects while animal proteins have the opposite effect. This is a result of the quality of amino acids rather than the amount of protein present in the diet (45). The hypocholesterolemic effect of diets containing soybean protein has been associated with arginine and glycine (46). According to data reported by Friedman (47), soybean protein has 73 mg/g of arginine and 40 mg/g of glycine. Determinations carried out in our laboratory indicate that the Ac protein concentrate contains 85 mg/g of arginine and 75 mg/g of glycine. Sánchez *et al.* studied this effect on rabbit using three different diets, in which the lysine/arginine ratio was 2.2 (casein), 0.9 (soybean), and 0.3 (almond), and concluded that the diminution of this ratio might be associated with a hypocholesterolemic effect (48). The Ac protein concentrate studied here presented a ratio of 0.85, similar to that in soybean.

On the other hand, the decrease of FAS activity in animals fed with PCAcD might be associated with a lower TG synthesis in liver. The activity and mRNA concentration of lipogenic enzymes such as FAS was lower in the livers of rats fed with the experimental diet than in the control animals. This is consistent with the decrease of TG synthesis in this tissue, probably as a result of a lower synthesis of fatty acids in liver, confirmed by [^{14}C]acetate incorporation in saponifiable fraction. The reduced biosynthesis of fatty acids probably reduces the production of VLDL particles, thereby limiting the formation of LDL particles and resulting in hypotriglyceridemia (49).

The excretion of total lipids in feces of animals fed with experimental diets was increased in relation to the control diet. This is in agreement with the amount of soluble fiber found in the Ac protein concentrate. Furthermore, greater cholesterol elimination in PCAcD-fed rats may indicate that cholesterol absorption in intestine decreased. Other causes leading to diminution in the absorption of lipids might be associated with the presence of saponin in the protein concentrate of Ac. In relation to that, Han *et al.* have reported that chikusetsusaponins isolated from *Panax japonicus* rhizomes in mice fed a high-fat diet may be partly mediated through delayed intestinal absorption of dietary fat from inhibition of pancreatic lipase activity (50).

The experimental model used in this work shows that the effect of DCPAc on the liver lipid metabolism could be extrapolated to humans. However, no hypocholesterolemic action was found when its effect was evaluated on lipid serum. This is in agreement with known metabolic differences among different animal species, particularly in rats, which are known to have an HDL pattern and atherogenic resistance (51). Besides, as mentioned above, hypocholesterolemic effects were observed when 20% of source protein was used in the diets. A hypocholesterolemic effect as reported in previous works (9, 10) might probably be obtained using rabbit or hamster instead of rat in our experimental conditions.

No significant differences were observed in TBARS between the experimental and control diets. These results are in agreement with those reported by Mahfouz and Kummerow, who found that diets rich in cholesterol did not affect lipid peroxidation in rats but led to a significant increase of catalase and glutathione peroxidase activity in liver. This may indicate that rats protect their liver from oxidation by avoiding the possible increase of lipoperoxidation and oxysterols, which can be secreted in lipoproteins from the liver to blood (39).

Our results are also in agreement with reports showing that plants have antioxidant activity and are potentially beneficial for health by inhibiting lipid peroxidation (52, 53). Our results also demonstrate a better response of the antioxidant system in organisms fed with a vegetable diet (*Amaranthus cruentus)* when compared to an animal diet.

In summary, the results show that the addition of protein concentrate from Ac to a rat diet caused (i) a significant increase in the concentration of HDL-C with decreases of LDL-C and TG in serum, (ii) a decrease of triglycerides and cholesterol in liver, (iii) increased excretion of lipids and cholesterol in feces, and (iv) increased antioxidant parameters. The hypotriglyceridemic action of *Amaranthus cruentus* in the diet is important. The association between TG levels and cardiovascular health is an issue for future investigation. Elevated plasma triglyceride concentration is becoming increasingly established as an independent risk factor for premature coronary artery disease (CAD; Ref. 54). The results obtained here suggest that the protein concentrate from *Amaranthus cruentus* as supplement in the diet contributes to the prevention of cardiovascular disease.

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