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Basic nutritional investigation

A diet rich in dietary fiber from cocoa improves lipid profile and reduces malondialdehyde in hypercholesterolemic rats

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Abstract

Objective: The potential hypolipidemic effect of a new cocoa product rich in dietary fiber (DF) naturally containing antioxidant polyphenols (cocoa fiber [CF]) was studied in a rat model of dietary-induced hypercholesterolemia.

Methods: For 3 wk animals were fed normal, cholesterol-free diets or diets supplemented with cholesterol to evoke hypercholesterolemia. Control diets contained 10% cellulose as DF, and test diets were supplemented with 165 g of CF per kilogram (providing 10% DF). Lipid profile, total antioxidant capacity, and malondialdehyde were measured in serum in addition to the activity of the antioxidant enzymes catalase, glutathione reductase, glutathione peroxidase, and superoxide dismutase and concentrations of glutathione and malondialdehyde in the liver.

Results: Hypercholesterolemia and hypertriglyceridemia were established as a consequence of the cholesterol-rich diets. CF showed an important hypolipidemic action, returning triacylglycerol levels in hypercholesterolemic animals to normal values. The hypocholesterolemic effect was also patent, reducing total and low-density lipoprotein cholesterol, yet basal values were not attained. Decreased lipid peroxidation in serum and liver as a consequence of CF intake was patent not only in hypercholesterolemic but also in normocholesterolemic animals. No apparent effects on serum total antioxidant capacity or on the activity of antioxidant enzymes and hepatic levels of glutathione were observed. These effects might be attributed to the high DF content of CF and to the natural presence of antioxidant polyphenols.

Conclusion: The consumption of CF with a hypercholesterolemic diet improved the lipidemic profile and reduced lipid peroxidation, suggesting that CF might contribute to a reduction of cardiovascular risk.

Keywords: Cocoa fiber; Hypolipidemic effect; Lipid peroxidation; Antioxidant status

Introduction

Much attention has been paid in recent years to cocoa and cocoa products due to their potential implication in cardiovascular health. Animal and human intervention studies have shown that cocoa products (cocoa powder, chocolate, cocoa drinks) inhibit platelet activation and function [1,2], favorably alter eicosanoid synthesis [3,4], suppress the production of proinflammatory cytokines and lipoxygenase activity [5,6], stimulate nitric oxide production [7,8], and improve endothelial function [7–9]. In addition, cocoa was found to positively affect serum lipid and lipoprotein profiles and to decrease levels of markers of lipid peroxidation such as F_2-isoprostanes, malondialdehyde (MDA), or low-density lipoprotein (LDL) oxidizability [10–13]. All these data are indicative of a putative cardioprotective action of cocoa.

Most of these effects are attributed to the polyphenolic fraction of cocoa, especially to the flavonoid group of poly-

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phenols. Flavonoids in cocoa are mainly flavan-3-ols, either monomeric (catechin and epicatechin) or oligomeric procyanidins (ranging from dimers to decamers), with appreciable amounts of anthocyanins (especially cyanidin glycosides) and flavonols (quercetin glycosides) [14,15]. Cocoa polyphenols have been shown to have antioxidant and antimutagenic activities in vitro [14,16–18] and in vivo, increasing the total antioxidant capacity of serum [4,19–22], which implies the bioavailability of cocoa polyphenols. Monomeric epicatechin and dimeric procyanidins have been shown to be absorbed in humans [19,20,23–25].

Edible cocoa products are obtained from the beans of the plant *Theobroma cacao* (L) after industrial manufacture with several processing steps including fermentation, roasting, alkalinization, drying, etc. After these treatments, beans skins are removed, constituting a sizeable byproduct of the important cocoa industry. These cocoa husks are a good source of dietary fiber (DF), mostly insoluble fiber [26], which retains important amounts of polyphenolic compounds with antioxidant activity [27]. Such a product might be of interest for the food industry, with potential use as a functional ingredient similarly to winery byproducts, should its putative nutritional and functional properties be proved. However, despite the composition of this cocoa fiber (CF) source and the enormous production of cocoa byproducts worldwide, little attention has been paid to this material.

In a previous study we reported the composition and some physicochemical properties (glucose retardation index, hydration properties) of a CF product obtained from cocoa shells [27]. The objective of the present work was to assess in vivo some nutritional properties derived from regular consumption of this CF, especially its potential effect on cardiovascular disease in an animal model of dietary-induced hyperlipidemia. Animals were fed normal, cholesterol-free diets or diets supplemented with cholesterol to evoke hypercholesterolemia. The lipid profile and total antioxidant capacity were measured in rat serum in addition to levels of MDA as a biomarker of lipid peroxidation. Also, the activity of the antioxidant enzymes catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), and superoxide dismutase (SOD) and concentrations of glutathione and MDA were determined in the liver to further evaluate the effect of CF consumption on markers of oxidative status in vivo.

Materials and methods

Chemicals

The commercial kit Bioxytech SOD-525 was obtained from Oxis Health Products Inc. (Portland, Oregon, USA). The Bradford reagent was from BioRad Laboratories S.A. (reference no. 500-0006, Madrid, Spain), and the Folin-Ciocalteau reagent was from Panreac S.A. (Barcelona, Spain). All other chemicals, including GR, reduced glutathione (GSH), oxidized glutathione, hydrogen peroxide, reduced nicotinamide adenine dinucleotide phosphate, *o*-phtaldehyde, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), tripyridyltriazine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid (Trolox), 1,1,3,3-tetraethoxypropane, and dinitrophenylhydrazine were purchased from Sigma-Aldrich Quimica S.A. (Madrid, Spain). Other reagents were of analytical or chromatographic quality.

Cocoa fiber

Cocoa fiber was supplied by Nutrexpa S.A. (Barcelona, Spain) as a fine powder. It contained 600 g/kg (dry matter) of total DF, >80% of which was insoluble DF (503 g/kg). Total polyphenols amounted to 58 g/kg, mostly condensed tannins and procyanidins. A detailed description of this fiber is given elsewhere [27].

Animal experiment

Thirty-two male Wistar rats (8 wk old) were obtained from the School of Medicine, Universidad Autonoma (Madrid, Spain). Animals were individually housed in wire-bottomed metabolic cages and kept in a room with controlled conditions (19–23°C, 60% humidity, 12-h light/dark cycles) at the animal facility of the School of Pharmacy, Universidad Complutense (Madrid, Spain), where the experiment was conducted. Rats were weighed and randomly assigned to the different experimental groups (eight animals per group), with free access to food and water. All diets were prepared from a fiber-free AIN-93M Purified Rodent diet (Panlab S.L., Barcelona, Spain), which provides the macro- and micronutrients required by adult rats according to guidelines from the National Research Council [28]. Two sets of diets were prepared: normal, cholesterol-free diets and cholesterol-supplemented diets, with control and test diets (containing cellulose or the CF product as a source of DF, respectively) in each set. The composition of the four diets is presented in Table 1. Cellulose (10%) was added to the control diets (normo- and hypercholesterolemic diets) as a DF source. Because CF contained approximately 60% of total DF, 16.5% of the cocoa powder was added to the experimental diets to provide a similar amount of DF. CF was added to the basal, fiber-free diet at the expense of starch. The hypercholesterolemic diets (control and CF diets) were supplemented with cholesterol and cholic acid (10 and 2 g/kg of diet, respectively), also at the expense of starch.

Animals were adapted to the diets and metabolic cages for 4 d before the 3-wk experimental period. During this time, body weight and food intake were monitored daily. Feces were collected daily, weighed, freeze-dried, and weighed again before milling for analysis. At the end of the experimental period, fasting rats were sacrificed by decapitation, and blood and livers were collected. Livers were immediately frozen in liquid nitrogen and kept at −80°C until analysis. Blood was centrifuged (1500 rpm, 10 min, 4°C), and serum was separated and stored at −80°C. All

Table 1
Composition of experimental diets (g/kg dry weight)

	Normal cholesterol-free diets		Cholesterol-rich diets	
	Control	Cocoa fiber	Control	Cocoa fiber
Casein	140	140	140	140
Dextrose	155	155	155	155
Sucrose	100	100	100	100
Fat	40	40	40	40
t-BHQ	0.008	0.008	0.008	0.008
Mineral mixture	35	35	35	35
Vitamin mixture	10	10	10	10
L-cysteine	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5
Cholesterol	—	—	10	10
Sodium cholate	—	—	2	2
Cellulose	100	—	100	—
Starch	415.692	350.692	403.692	338.692
Cocoa fiber	—	165	—	165

t-BHQ, *tert*-butyl hydroquinone

animal procedures were carried out in accordance to National Institutes of Health guidelines for animal care [28].

Analysis of samples

Serum antioxidant activity was analyzed by two different methods. The ferric reducing/antioxidant power (FRAP) assay [29] was used to estimate the reducing power of samples and measured the increase in absorbance at 595 nm of the complex tripyridyltriazine/Fe(II) in the presence of serum reducing agents. The capacity of samples to scavenge the stable radical $ABTS^+$ was determined by the Trolox equivalent antioxidant capacity (TEAC) decoloration assay of Re et al. [30] by measuring the absorbance decrease at 730 nm of the radical cation $ABTS^+$. The area under the absorbance curve taken between 0 and 6 min was used for calculations. In both cases, Trolox was used as a standard and results were expressed as milli- or micromoles of Trolox equivalents per liter.

The lipid profile was determined in serum samples immediately after being obtained. Free fatty acids (FAs) were analyzed by the procedure of Nagele et al. [31]. Determination of total cholesterol, high-density lipoprotein (HDL) cholesterol, and triacylglycerols (TGs) has been described elsewhere [32]. LDL cholesterol was calculated as the difference between total and HDL cholesterol.

Malondialdehyde was determined as its hydrazone by high-performance liquid chromatography using dinitrophenylhydrazine for derivatization [33]. Livers (0.5 g) were homogenized in ice-cold 0.25 M Trizma base buffer, pH 7.4 (containing 0.2 M sucrose and 5 mM dithiothreitol) using a Teflon glass homogenizer. After centrifugation (10 000 *g*, 30 min, 4°C), supernatants were collected for MDA quantification. Serum samples were analyzed directly. Standard MDA was prepared by acidic hydrolysis of 1,1,3,3-tetraethoxypropane in 1% sulfuric acid. Concentrations were expressed as nanomoles of MDA per milligram of protein in liver tissue and per milliliter in serum samples. Protein content in liver homogenates was estimated by the Bradford method [34] using a Bio-Rad protein assay kit.

The activity of antioxidant enzymes and glutathione concentration were determined in liver homogenates. For the GR, GPx, and SOD assays, livers were homogenized (1:5 w/v) in 0.25 M Tris, 0.2 M sucrose, and 5 mM dithiothreitol buffer, pH 7.4; for determination of CAT activity and GSH levels, livers were homogenized (1:5 w/v) in 50 mM phosphate buffer, pH 7.0. GSH concentrations and enzyme activities of GR, GPx, and CAT were determined spectrophotometrically according to methodologies previously described [35], whereas SOD activity was measured by the Oxis commercial kit Bioxytech SOD-525. Results are expressed as milligrams of GSH per gram of liver and specific enzyme activities per milligram of protein in liver homogenates, which was determined by the Bradford assay.

Feces were analyzed for their protein, fat, and polyphenolic content. Protein was determined by thermal conductivity (Dumas method) using an automated nitrogen analyzer (LECO FP-2000, St. Joseph, Michigan, USA). Protein was calculated as nitrogen multiplied by 6.25. Fat was quantified after extraction with light petroleum in a Soxtec System HT (Tecator, Högannas, Sweden). Polyphenols were measured spectrophotometrically by the Folin-Ciocalteau method [36] in the solutions obtained after sequentially extracting dry feces with acidic 50% aqueous methanol and 70% aqueous acetone [37].

Statistical analysis

Data are presented as mean ± standard deviation. Variance homogeneity was checked by Cochran's test before application of one-way analysis of variance, followed by Duncan's multiple comparison test. To discriminate among means, the Fisher's least significant difference test was used. No transformation of the data was required. The level of statistical significance was $P < 0.05$. Statgraphic Plus 5.1 (Statistical Graphics Corp.) was used.

Results

Food intake, weight gain, and fecal output

The addition of CF to the test diets affected the mean food intake and body weight gain of animals in different ways depending on the experimental groups. In animals fed the standard, cholesterol-free diets (denoted as normocholesterolemic groups), CF led to a slight yet statistically significant decrease in total food intake as compared with the controls; body weight, however, was similar in control and CF-fed animals (Table 2). As to the groups consuming

Table 2
Food intake, body weight gain, and fecal excretion of rats in control and cocoa fiber groups fed the cholesterol-free (normocholesterolemic) and cholesterol-rich (hypercholesterolemic) diets*

	Normocholesterolemic groups		Hypercholesterolemic groups	
	Control	Cocoa fiber	Control	Cocoa fiber
Food intake (g/21 d)	398.56 ± 15.70[a]	372.01 ± 16.24[b]	394.67 ± 28.89[ab]	461.54 ± 38.08[c]
Body weight gain (g/21 d)	80.43 ± 9.29[ab]	71.29 ± 11.38[a]	85.00 ± 14.67[b]	74.83 ± 13.23[ab]
Food efficiency†	0.20 ± 0.02[a]	0.20 ± 0.02[a]	0.21 ± 0.03[a]	0.16 ± 0.03[b]
Feces (g/21 d)				
Dry weight	31.95 ± 3.27[a]	51.96 ± 5.46[b]	47.90 ± 6.99[b]	66.39 ± 4.92[c]
Fresh weight	54.42 ± 7.40[a]	105.05 ± 18.72[b]	100.24 ± 18.77[b]	140.12 ± 10.74[c]
Water in feces (%)	42.76 ± 6.54[a]	50.82 ± 3.76[b]	50.85 ± 7.68[b]	45.75 ± 9.59[ab]

* Data are presented as mean ± SD ($n = 8$). Data in a row with different superscript letters are statistically different ($P \leq 0.05$).
† Food efficiency = body weight gain × (food intake)$^{-1}$.

the cholesterol-rich diets (hypercholesterolemic groups), food intake was significantly higher in rats fed CF. Nevertheless, pondered growth of these animals was not increased in parallel to the increased food intake, and body weight gain was comparable to that of the normocholesterolemic groups, resulting in a lower food efficiency index (Table 2).

As expected, CF had a remarkable fecal bulking effect, with the fresh and dry weights of feces significantly higher than in the respective cellulose controls (Table 2). It is worth noting that the fecal output was higher in the hypercholesterolemic than in the normocholesterolemic group. This difference might be due in part to a higher fat excretion. Fecal excretion of fat was much higher in animals fed the cholesterol-rich diets, with 12.6 and 9.2 g of fat excreted in the control and CF hypercholesterolemic groups, respectively, in comparison with <1 g of fat eliminated by normocholesterolemic animals (Table 3).

Cocoa fiber contained 6.5% fat [27], contributing with approximately 10 g of fat per kilogram of diet; therefore, fat intake was higher in the groups fed the CF diets (Table 3). A slightly higher fat excretion was observed in the CF normocholesterolemic group in comparison with its control, yet the apparent digestibility of fat in both normocholesterolemic groups was similar. Conversely, regardless of the higher fat intake by the CF hypercholesterolemic animals, excretion of fat in feces was lower than in the corresponding hypercholesterolemic control animals, resulting in a low apparent fat digestibility in these control animals (Table 3).

Protein intake was also significantly higher in the CF fed groups due to the contribution of the cocoa powder to the total protein content of diets with an extra 3% protein. Fecal excretion of protein was higher in both CF-fed groups, resulting in lower apparent digestibility and protein efficiency ratio indexes (Table 3). This higher protein excretion might be attributed in part to the effect of DF decreasing protein digestibility but also to a similar effect of the polyphenols in CF.

Animals consuming the CF diets had a polyphenolic

Table 3
Intake and excretion of protein, fat, and polyphenols in control and cocoa fiber groups fed the basal (cholesterol-free) and cholesterol-rich diets*

	Normocholesterolemic groups		Hypercholesterolemic groups	
	Control	Cocoa fiber	Control	Cocoa fiber
Protein				
Intake (g/21 d)	55.80 ± 2.20[a]	62.91 ± 2.75[b]	55.25 ± 4.04[a]	78.05 ± 6.44[c]
Fecal excretion (g/21 d)	6.61 ± 0.68[a]	17.50 ± 1.84[b]	7.06 ± 1.03[a]	18.58 ± 1.38[b]
Apparent digestibility†	88.14 ± 1.22[a]	71.93 ± 2.89[b]	87.24 ± 1.34[a]	75.91 ± 2.07[c]
PER‡	1.38 ± 0.21[a]	1.05 ± 0.28[b]	1.53 ± 0.19[a]	0.96 ± 0.16[b]
Fat				
Intake (g/21 d)	15.94 ± 0.63[a]	19.90 ± 0.87[b]	23.68 ± 1.73[c]	33.92 ± 2.80[d]
Fecal excretion (g/21 d)	0.56 ± 0.06[a]	0.77 ± 0.08[b]	12.60 ± 1.84[c]	9.21 ± 0.68[d]
Apparent digestibility	96.49 ± 0.36[a]	96.13 ± 0.40[a]	46.87 ± 5.57[b]	72.75 ± 2.34[c]
Polyphenols				
Intake (mg/21 d)	—	632.42 ± 27.60[a]	—	784.61 ± 64.73[b]
Fecal excretion (mg/21 d)	61.66 ± 6.31[a]	343.94 ± 36.17[b]	43.99 ± 5.00[c]	444.84 ± 32.98[d]
Apparent digestibility	—	45.57 ± 5.60[a]	—	43.09 ± 4.89[a]

PER, protein efficiency ratio
* Data are presented as mean ± SD ($n = 8$). Data in a row with different superscript letters are statistically different ($P \leq 0.05$).
† Apparent digestibility = ([intake − fecal excretion] × intake^{-1}) × 100.
‡ PER = body weight gain × (protein intake)$^{-1}$.

Table 4
Serum lipids in control and cocoa fiber groups fed the basal (cholesterol-free) and cholesterol-rich diets*

	Normocholesterolemic groups		Hypercholesterolemic groups	
	Control	Cocoa Fiber	Control	Cocoa fiber
Total cholesterol (mg/dL)	80.0 ± 10.9[a]	80.5 ± 4.8[a]	299.3 ± 39.5[c]	218.5 ± 25.3[b]
LDL cholesterol (mg/dL)	28.0 ± 6.1[a]	29.4 ± 8.8[a]	229.8 ± 77.9[c]	140.6 ± 66.7[b]
HDL cholesterol (mg/dL)	50.0 ± 5.3[b]	53.2 ± 3.0[b]	35.0 ± 1.7[a]	35.5 ± 1.9[a]
Triacylglycerols (mg/dL)	41.8 ± 2.8[a]	37.8 ± 4.6[a]	68.0 ± 5.3[b]	40.2 ± 7.8[a]
Free fatty acids (mg/dL)	0.73 ± 0.06[a]	0.78 ± 0.04[a]	1.01 ± 0.13[b]	0.94 ± 0.18[b]
Atherogenic index[†]	0.60 ± 0.08[a]	0.51 ± 0.08[a]	9.32 ± 4.77[c]	5.00 ± 0.57[b]

HDL, high-density lipoprotein; LDL, low-density lipoprotein
* Data are presented as mean ± SD ($n = 8$). Data in a row with different superscript letters are statistically different ($P \leq 0.05$).
[†] Atherogenic index = (total cholesterol − HDL cholesterol) × (HDL cholesterol)$^{-1}$.

intake <0.8 g during the experimental period. Approximately 50% of these polyphenols were excreted in feces (Table 3). Although no polyphenols were added to the control diets, minor amounts of phenolic compounds were detected in the feces of these control animals. Some of the ingredients used in the formulation of the diets might have contributed trace amounts of phenols (e.g., soybean oil), yet overestimation of polyphenols in feces cannot be ruled out due to the non-specificity of the analytical method used.

Serum lipids and atherogenic index

The groups fed the cholesterol-rich diets had altered serum lipid concentrations, causing a marked hyperlipidemia. Serum lipid profile showed increased levels of total and LDL cholesterol, TG, and FA and decreased concentrations of HDL cholesterol (Table 4). This resulted in very high atherogenic indices of control and CF hypercholesterolemic groups in comparison with normocholesterolemic ones. Although addition of CF to the diet partly limited these deleterious effects of the cholesterol-rich diet, with lower levels of total and LDL cholesterol in comparison with the hypercholesterolemic control group, it failed to totally overcome such effects. Only TG levels were decreased to values similar to the normocholesterolemic groups. Therefore, CF supplementation of a fatty diet such as the one tested in this study had a slight hypocholesterolemic effect and a marked hypotriglyceridemic action. Conversely, when CF was incorporated into a normal diet, with no added cholesterol, no effect on the serum lipid profile was observed (Table 4).

Serum antioxidant capacity, liver glutathione, and MDA levels

Polyphenols present in the CF diets did not significantly modify the antioxidant capacity of serum. Neither the ferric reducing ability of serum nor its capacity to scavenge the free radical ABTS were increased in rats fed the CF diets in comparison with control animals fed polyphenol-free diets (Table 5). In contrast, the free radical scavenging capacity of serum in the normocholesterolemic CF group was significantly lower than that in the control. Groups consuming the hypercholesterolemic diets had a lower serum antioxidant capacity than did the normocholesterolemic ones, which might be indicative of a poorer antioxidant status as a result of the intake of fatty diets.

As a biomarker of lipid peroxidation, MDA levels were measured in serum and liver homogenates (Table 5). Serum MDA was significantly increased in the hypercholesterolemic control in comparison with the normocholesterolemic one, sustaining the impaired antioxidant status of these animals mentioned above. Although CF failed to increase the total antioxidant capacity of serum, it did dimin-

Table 5
Serum antioxidant capacity (FRAP and TEAC values), MDA concentration in serum and liver, and glutathione levels in livers of animals fed the control and cocoa fiber basal (cholesterol-free) and cholesterol-rich diets*

	Normocholesterolemic groups		Hypercholesterolemic groups	
	Control	Cocoa fiber	Control	Cocoa fiber
FRAP (μmol eqivalent Trolox/L)	341.42 ± 7.20[b]	383.38 ± 25.89[b]	317.41 ± 26.29[ab]	299.57 ± 26.02[a]
TEAC (mmol equivalent Trolox/L)	4.09 ± 0.42[c]	2.80 ± 0.31[a]	3.24 ± 0.56[b]	3.39 ± 0.29[b]
Serum MDA (nmol/mL)	2.2 ± 0.1[b]	1.8 ± 0.1[a]	2.8 ± 0.1[c]	2.0 ± 0.1[ab]
Liver MDA (nmol/mg protein)	0.9 ± 0.1[a]	0.8 ± 0.1[a]	1.2 ± 0.1[b]	0.8 ± 0.1[a]
Glutathione (mg/g liver)	4.82 ± 0.67[ab]	4.41 ± 0.41[a]	5.79 ± 0.86[b]	5.78 ± 0.71[ab]

FRAP, ferric reducing/antioxidant power; MDA, malondialdehyde; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid
* Data are presented as mean ± SD ($n = 8$). Data in a row with different superscript letters are statistically different ($P \leq 0.05$).

Table 6
Activity of liver antioxidant enzymes in control and cocoa fiber groups fed the basal (cholesterol-free) and cholesterol-rich diets*

	Normocholesterolemic groups		Hypercholesterolemic groups	
	Control	Cocoa fiber	Control	Cocoa fiber
SOD (U/mg protein)	62.31 ± 25.21[a]	96.43 ± 33.57[a]	92.75 ± 13.05[a]	44.76 ± 3.60[a]
Catalase (mU/mg protein)	1067 ± 135[a]	816 ± 157[a]	887 ± 294[a]	693 ± 286[a]
GPx (μU/mg protein)	3.72 ± 1.39[b]	6.83 ± 0.99[c]	1.23 ± 0.37[a]	1.24 ± 1.03[a]
GR (μU/mg protein)	3.27 ± 0.45[b]	4.43 ± 1.22[b]	1.79 ± 0.33[a]	3.53 ± 0.60[b]

GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase
* Data are presented as mean ± SD ($n = 8$). Data in a row with different superscript letters are statistically different ($P \leq 0.05$).

ish lipid peroxidation, with MDA values in hypercholesterolemic rats fed CF similar to those of normocholesterolemic animals. Moreover, CF in the normocholesterolemic group decreased serum MDA as compared with the control. Liver MDA was also enhanced in control hypercholesterolemic animals as a manifestation of the oxidative stress imposed on this organ by a cholesterol-rich diet. A protection from this dietary-derived stress by the ingestion of CF was apparent, with liver MDA values in CF hypercholesterolemic animals comparable to those of the normocholesterolemic groups.

The GSH liver concentrations were higher in the hypercholesterolemic groups than in the normocholesterolemic ones (Table 5). Addition of CF to the cholesterol-free or cholesterol-rich diets did not result in differences statistically significant when compared with the corresponding control groups.

Activity of liver antioxidant enzymes

Activities of CAT, SOD, GR, and GPx are presented in Table 6. There was a high variability in the results among animals within the same dietary group. Although the activities of SOD and CAT appeared to be lower in the group fed the high-cholesterol diet containing CF, these differences were not statistically significant. GPx activity was increased in the normocholesterolemic animals consuming CF in comparison with the controls, whereas values were comparable in both hypercholesterolemic groups and significantly lower than in the normocholesterolemic counterparts. GR showed a reduced activity in hypercholesterolemic control animals as compared with normocholesterolemic ones. However, supplementation of the cholesterol-rich diet with CF resulted in a recovery of this enzyme activity to values similar to those of normocholesterolemic groups.

Discussion

Hyperlipidemia, including hypercholesterolemia and hypertriglyceridemia, is a major risk factor for the development of cardiovascular disease. Oxidatively damaged LDLs are taken up by macrophages, which accumulate in the endothelial wall as lipid-laden foam cells in the initial phases of atherosclerotic fatty streak lesions. Therefore, reduction of circulating TGs and total and LDL cholesterol is a primary step in the prevention of vascular disease. Also, prevention of LDL oxidation by dietary antioxidants could delay the development of atherosclerosis.

In the present study, feeding rats with diets rich in cholesterol resulted in increased TG, total cholesterol, and LDL cholesterol levels, with decreased circulating HDL cholesterol levels, thus providing a model for dietary hyperlipidemia. This model was used to study the potential hypolipidemic effect of a cocoa product rich in DF that contained significant amounts of polyphenols. This CF had a strong hypotriglyceridemic action, returning serum TG to levels found in healthy, normocholesterolemic animals. It also showed a remarkable hypocholesterolemic effect, with a partial reduction of serum total and LDL cholesterol concentrations, although with no effect on HDL cholesterol. Moreover, serum and liver lipoperoxidation were significantly decreased as indicated by the lower levels of MDA. Serum total antioxidant capacity and GSH concentrations in the liver were not modified by CF, as was the general case for the enzymes of the antioxidant defense system in the liver.

Many studies in the literature have shown a protective role of cocoa products on cardiovascular health. These studies have always been performed with products such as chocolate or cocoa powder and cocoa extracts, even with purified cocoa flavonoids. However, to our knowledge this is the first study addressing the potential benefits of a CF product containing polyphenols, a product that was obtained as a byproduct of the cocoa industry and with potential uses as a functional ingredient.

The cardiovascular health effects reported for chocolate and cocoa products in the literature have been attributed to the antioxidant activity of polyphenols in cocoa. Thus, reduced susceptibility of LDL to oxidation has been reported in vitro [38] and in vivo [4,10,12,13,39] after consumption of cocoa products. Concerning the total antioxidant capacity of serum, short-term studies on the bioavailability of cocoa polyphenols have shown increased serum antioxidant activity concomitant with increased levels of serum epicatechin and reduced lipid peroxidation, yet these effects were transient [19,20]. In long-term feeding studies, only Wan et al. [4] reported a slight increase in serum antioxidant capacity

after prolonged consumption of cocoa and dark chocolate, providing nearly 0.5 g of flavonols daily. In the present work, neither the free radical scavenging capacity nor the reducing power of serum was affected by the intake of CF, which provided <40 mg of polyphenols daily. Most studies in the literature failed to observe an increase in the total antioxidant capacity of serum [2,10,39], which agrees with our results.

In contrast, many studies have shown that levels of biomarkers of lipid peroxidation such as F_2-isoprostanes or thiobarbituric acid-reactive substances were not modified as a consequence of cocoa consumption by healthy individuals [2,10,11,22,39]. Only in conditions that represent a challenge to the well-regulated balance between pro-oxidation and antioxidant reactions in the organism, such as hypercholesterolemia [12] or strenuous physical exercise [11], did flavonoid-rich cocoa prevent an increase of in vivo lipid peroxidation. In line with this, our study showed that MDA levels in serum and in the liver were lower in hypercholesterolemic animals when the diet was supplemented with CF. More importantly, serum MDA concentrations were lower in the normocholesterolemic animals consuming CF (Table 5). This might suggest that polyphenols in CF protect the organism against lipid peroxidation not only in situations of potential oxidative stress such as hypercholesterolemia but also in normal conditions. A similar decrease in serum and liver MDA in comparison with control animals was found in healthy rats consuming diets supplemented with polyphenol-rich fruits such as strawberry or plum [33]. It is worth noting the existence of a positive correlation between the body weight gained during the experimental period and concentrations of MDA in serum ($R^2 = 0.918$) and in liver ($R^2 = 0.813$). Increased concentrations of MDA-modified lipoproteins have been associated to body fat in humans ($R^2 = 0.646$) [40], and it has been suggested that the accumulation of fat in the body might result in a higher susceptibility to lipid peroxidation [40]. However, this correlation had not been reported in obese Zucker rats [41] or in Watanabe hyperlipidemic rabbits [42], contrary to what has been observed in the present work. It would be of interest to study further the possible relation between lower body weight (fat) and lipid peroxidation/redox status and the potential underlying mechanisms.

To further study the potential effect of CF on the antioxidant status in vivo and because the liver is the main organ involved in lipid metabolism and thus prone to potential oxidative damage in conditions of hyperlipidemia, we measured the levels of GSH as the major intracellular antioxidant and the activity of four of the enzymes constituting the enzymatic antioxidant defense system. GSH concentrations were increased in hypercholesterolemic animals in comparison with normocholesterolemic ones, which might reflect an adaptation of the liver to oxidative stress because high intracellular GSH levels promote better survival under such conditions [12,13,43]. However, although polyphenols have been shown to induce hepatic GSH [44], we found no differences in the GSH levels between controls and animals fed the CF diets in the normo- or hypercholesterolemic groups. Similarly, Orozco et al. [22] found no differences in GSH pools in the liver and testes of rats fed cocoa.

As for the antioxidant enzymes, their activity in the liver has been reported to decrease as a response to stress situations elicited by alcohol [45] or by drugs such as hexachlorocyclohexane [46], although acetaminophen did not cause any modifications [35]. Conversely, increased activity of these enzymes as a result of polyphenol intake has been reported in the literature [46,47], suggesting an enhanced protection of the liver against oxidative stress situations by these antioxidants. This hypothesis would be supported by observations on the prevention of age-related decline on enzyme activities [48] or their recovery by antioxidant supplementation in animals subjected to oxidative stress [45,46,49]. However, other investigators found no effect derived from antioxidant consumption [35,50,51] or showed opposite effects, with decreased activity of GPx, GR, and CAT after polyphenol intake [52]. In line with these contradictory results, we have observed no effect (SOD, CAT), or decreased (GPx, GR) enzyme activities as a consequence of hypercholesterolemic stress. Intake of CF as a source of polyphenols had no effect on SOD and CAT but preserved GR activity in hypercholesterolemic animals. In control animals consuming CF, the activity of GPx was increased in comparison with the controls consuming cellulose, but it had no effect in the hypercholesterolemic groups, where the activity of this enzyme was lower. Considering that GPx is involved in the homeostasis of intracellular GSH levels through its oxidation in the GPx-catalyzed reduction of reactive oxygen species and other oxidized substrates, the decreased activity of GPx observed in the livers of hypercholesterolemic animals might account for the increased levels of GSH observed in these groups. The observed variable effects of the experimental diets on the activity of the studied enzymes indicates that the response of these enzymes involved in antioxidant defense in the liver seems to vary greatly depending on the enzyme, the origin of the oxidative stress, and the type or source of dietary antioxidants. Deeper knowledge on the subjacent mechanisms regulating these enzyme activities is necessary to understand the physiological implications of their variable response to different stressors and dietary antioxidants.

As for the effect of cocoa products on serum lipid levels, there is a dearth of information on this subject. Some investigators [4,39] have reported increased HDL cholesterol levels in healthy humans consuming dark chocolate. In diabetic rats, with enhanced serum TG and cholesterol concentrations, intake of a cocoa extract increased HDL cholesterol and decreased TG and total and LDL cholesterol concentrations [13]. This is in agreement with the observed effects elicited by CF in our animal model of dietary-induced hyperlipemia. Similar hypocholesterolemic and hypotriglyceridemic effects have been reported for other

polyphenol-rich foods and beverages such as red wine [53], tea [54], or apple [55]. Lower serum TG levels by polyphenol-rich foods has been associated with decreased intestinal absorption of TG by inhibition of pancreatic lipase [54] or with a lower microsomal transfer protein activity and apolipoprotein B secretion and increased lipoprotein lipase activity [53,56,57]. This could result in modified LDL levels, which in turn would lead to decreased LDL cholesterol concentrations. Also, upregulation of hepatic LDL receptor expression and activity by polyphenols might account for their hypocholesterolemic effect [56,58].

All these potential mechanisms of action explaining the hypolipidemic effects of cocoa products are related to the polyphenolic fraction. Nevertheless, it is noteworthy that the CF used in this study is rich in DF, mainly insoluble DF, but also contains significant amounts of soluble DF [27]. DFs, especially viscous soluble polysaccharides, are well known for their effect lowering total and LDL cholesterol, thus attenuating or preventing hypertriglyceridemia [reviewed in 59]. Viscous soluble fibers hinder digestion and absorption of dietary fats, resulting in lower cholesterol delivery to the liver by chylomicron remnants, with a concomitant upregulation of LDL receptor and decreased lipoprotein secretion to maintain cholesterol homeostasis in the liver. Further, bile salts are trapped in the viscous matrix formed by soluble DF polysaccharides in the gut. To compensate for the increased fecal excretion of bile salts, cholesterol is derived to the synthesis of bile acids. Moreover, soluble fibers are fermented by the colonic microflora generating short-chain fatty acids (acetic, propionic, and butyric acids). Hypolipidemic effects have been associated with propionate by inhibition of cholesterol and FA synthesis in the liver. Moreover, insoluble DF through its effect diluting gastrointestinal contents may hinder digestion and absorption of dietary fats, thus contributing to the effects of soluble DF. All these mechanisms lead to lower serum levels of cholesterol and TG, subjacent to the reduced risk of cardiovascular disease associated to DF intake [59–61]. Therefore, not only polyphenols in CF but also the soluble and insoluble fractions of DF may account for the observed hypolipidemic effects elicited by CF.

Conclusion

Cocoa fiber obtained from cocoa husks, an important byproduct of the cacao industry, has a remarkable hypolipidemic action in an animal model of dietary-induced hypercholesterolemia. This effect might be attributed to its high DF content and to the natural presence of antioxidant polyphenols that seem to prevent lipid peroxidation. This suggests that CF might contribute to a reduction of cardiovascular risk and thus would put forward the potential application of this CF as a functional food ingredient.

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