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Cocoa powder, cocoa extract and epicatechin attenuate hypercaloric diet-induced obesity through enhanced β-oxidation and energy expenditure in white adipose tissue

Griselda Rabadan-Chávez [a], *Lucia Quevedo-Corona* [b], *Angel Miliar Garcia* [c], *Elba Reyes-Maldonado* [d], *María Eugenia Jaramillo-Flores* [a,*]

[a] *Graduados e Investigación en Alimentos, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Carpio y Plan de Ayala S/N, Col. Casco de Santo Tomas, Mexico City, CP 11340, Mexico*
[b] *Departamento de Fisiologia, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Carpio y Plan de Ayala S/N, Col. Casco de Santo Tomas, Mexico City, CP 11340, Mexico*
[c] *Laboratorio de Biología Molecular, Escuela Superior de Medicina, Instituto Politécnico Nacional, Plan de San Luis y Diaz Miron s/n, Col. Casco de Santo Tomás, Mexico City, CP 11340, Mexico*
[d] *Departamento de Morfología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Carpio y Plan de Ayala S/N, Col. Casco de Santo Tomas, Mexico City, CP 11340, Mexico*

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ABSTRACT

Cocoa flavan-3-ols have been shown to exert a positive influence on obesity-related metabolic risk factors. This study evaluated the effects of cocoa powder (Co), cocoa extract (Co-Ex) and its main flavanols (Epi, Cat and PB2) on the expression of genes involved in WAT lipid metabolism in a rat model of hypercaloric diet-induced obesity. Co, Co-Ex and Epi are associated with adipogenesis, β-oxidation and energy expenditure in WAT linked to upregulating the expression of peroxisome-proliferator-activated receptor γ (PPARγ), PPARα, PPARγ coactivator 1α (PGC1α), sirtuin 1 (SIRT1) and uncoupling protein 1 (UCP1). Additionally, these treatments are associated with decreases in body weight gain and total fat mass and insulin resistance, reduced lipogenesis, and inflammation related to downregulating acetyl-CoA carboxylase gene expression, decreasing TNF-α and increasing ApN concentrations in WAT. Co, Co-Ex and Epi may be considered to be potential agents for the treatment of obesity-related metabolic disorders.

* *Corresponding author.* Graduados e Investigación en Alimentos, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Carpio y Plan de Ayala S/N, Casco de Santo Tomás, México City, CP 11340, Mexico. Tel.: +52 55 57 296000 ext.62458; fax: +52 55 57 296000 ext. 62359.
E-mail address: jaramillo_flores@hotmail.com (M.E. Jaramillo-Flores).
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1. Introduction

Cocoa (*Theobroma cacao*) and cocoa-based products are among food sources with high content of a subgroup of flavonoids known as flavan-3-ols, mainly composed of monomers (epicatechin and catechin), oligomers (such as procyanidin B2) and polymers. A growing number of studies have suggested that consumption of cocoa-derived products (cocoa powder, cocoa extract, cacao liquor, chocolate) have a positive influence on metabolic risk factors such as blood pressure, cholesterol levels, insulin resistance and inflammatory markers (Dorenkott et al., 2014; Gu, Yu, & Lambert, 2014; Osakabe, 2013).

Obesity has reached epidemic proportions globally (600 million obese adults in 2014), with at least 2.8 million people dying each year as a result of obesity-related complications (OMS, 2015). An obesogenic environment, characterised by a sedentary lifestyle and excessive intake of high-sucrose and high-fat diets (HFD), has precipitated an alarming rise in obesity and related metabolic disorders worldwide (Jung & Choi, 2014). The accumulation of white adipose tissue (WAT), especially in the visceral compartment, favours the development of insulin resistance, a key factor in developing type 2 diabetes mellitus, dyslipidemia and hypertension (Harms & Seale, 2013; Langin, 2010).

Two types of adipose tissue (AT) have been described to coexist in humans and animals: white adipose tissue (WAT) and brown adipose tissue (BAT). The former stores triacylglycerols (TG) in periods of positive energy balance, while it releases glycerol and fatty acids (FA) into the bloodstream during fasting or low energy intake in order to meet body energy demand (Rutkowski, 2015). The capacity for the expansion of WAT is considered to be a mechanism to avoid lipotoxicity and metabolic disturbances in peripheral organs (especially liver and skeletal muscle). In this regard, peroxisome-proliferator-activated receptor γ (PPARγ) and fatty acid translocase (FAT/CD36) play an important role mediating adipose tissue expansion in response to positive energy balance (Goudriaan et al., 2005; Medina-Gómez et al., 2007). Moreover, hypertrophic WAT produces high quantities of adipocytokines (particularly adiponectin, leptin, TNF-α and IL-6) that can contribute to chronic low-grade inflammation and to the development of obesity-related metabolic diseases (Barnea, Shamay, Stark, & Madar, 2006; Jung & Choi, 2014; Langin, 2010).

Conversely, BAT specialises in fatty acid oxidation and thermogenesis due to the large number of mitochondria that express uncoupling protein 1 (UCP1). UCP1 allows for the dissipation of the proton electrochemical gradient generated by the mitochondrial respiratory chain and promotes energy dissipation as heat (Harms & Seale, 2013; Langin, 2010; Seale & Lazar, 2009). UCP1 was thought to be expressed exclusively in BAT; however, its expression in WAT has been confirmed in many recent studies. These WAT UCP1-expressing adipocytes have been named "beige" adipocytes and share the complete thermogenic programme of BAT adipocytes, including peroxisome-proliferator-activated receptor α (PPARα), PPARγ coactivator 1α (PGC-1α) and deacetylase SirT1 (Langin, 2010; Qiang et al., 2012; Seale et al., 2007).

Promoting BAT-like features in WAT can open up new opportunities for the development of novel and safe therapeutic strategies aimed to reduce the burden of obesity and its metabolic complications. *In vitro* and *in vivo* studies have shown that flavonoids from natural foods can act as potential agents to modulate obesity and associated disorders by regulating metabolic pathways, including lipid absorption, energy intake and expenditure, lipolysis, lipogenesis, and the differentiation and proliferation of preadipocytes (González-Castejón & Rodriguez-Casado, 2011; Meydani & Hasan, 2010).

Currently, some proposed mechanisms through which cocoa flavan-3-ols have been shown to exert their beneficial effects include decreased intestinal fat absorption, increased glucose uptake by stimulating the insulin signalling pathway, suppressing anabolic metabolism, stimulating catabolic pathways, and reducing the chronic inflammation associated with adiposity (Gu, Yu, Park, Harvatine, & Lambert, 2014; Gutiérrez-Salmeán et al., 2014; Min et al., 2013; Yamashita, Okabe, Natsume, & Ashida, 2012). However, most of the investigations have focused on liver and skeletal muscle, and there is a lack of knowledge regarding the underlying lipid-lowering and anti-obesity mechanisms of specific cocoa flavanols.

In this context, the present study examines the effects of cocoa powder, cocoa extract and its main flavanols (epicatechin, catechin and procyanidin B2) on the expression of genes involved in the regulation of lipid metabolism and browning in retroperitoneal adipose tissue (rWAT) and changes in metabolic parameters associated with hypercaloric diet-induced obesity in rats.

2. Materials and methods

2.1. Diets and chemicals

(-)-Epicatechin (E1753; ≥ 90%) and (+)-catechin hydrate (C1251; ≥ 98%) were purchased from Sigma-Aldrich Co. Ltd (Toluca, México). Procyanidin B2 (51656; 95.25% purity) was purchased from Chempacific Corp. (Baltimore, Maryland, USA). Unsweetened cocoa powder (CocoaVia®) and cocoa extract (CocoaVia®) were purchased from Mars, Inc. (Hackettstown, NJ., USA). Total polyphenols and flavonoids in cocoa powder and extract were quantified by using the Folin–Ciocalteu method and the Aluminium chloride (AlCl3) colorimetric method, respectively, using (+)-catechin as a standard (Chang, Yang, Wen, & Chern, 2002). The contents of (–)-epicatechin, (+)-catechin and procyanidin B2 were determined by HPLC-DAD, as described by a previous method (Ortega et al., 2010). Table 1 shows the content of phenolic compounds in the doses of cocoa powder and cocoa extract. Standard diet (commercially available: 2018), with nutritional value of 3.1 kcal/g as energy density and macronutrient composition of 18.6% of total protein, 6.2% of fat and 44.2% of carbohydrate, and high-fat diet (commercially available as adjusted calories diet: TD. 88137) with a total energy content of 4.5 kcal/g and macronutrient composition 17.3% of total protein, 21.2% of fat, and 48.5 of carbohydrate were purchased from Harlan Laboratories, Inc. (Teklad Global Diets, Madison, WI., USA).

2.2. Rat model of obesity and treatments

Six-week-old male Wistar rats (180 ± 5 g of body weight) were purchased from the Animal House of Autonomous

Table 1 – Content of phenolic compounds (mg) in the experimental doses used in the experiment.

	CocoaVia® cocoa powder	CocoaVia® cocoa extract	(–)-Epicatechin	(+)-Catechin	Procyanidin B2
Total polyphenols	72.4	61.7	–	–	–
Total flavonoids	55.5	44.1	–	–	–
(–)-epicatechin	9.25	8.6	10	–	–
(+)-catechin	2.33	1.74	–	10	–
Procyanidin B2	5.1	3.9	–	–	10

Note: Total polyphenol, flavonoids and flavanols content are given based on each treatment dose: cocoa powder (1 g/kg bw), cocoa extract (100 mg/kg bw), epicatechin (10 mg/kg bw), catechin (10 mg/kg bw) and procyanidin B2 (10 mg/kg bw).

Metropolitan University, Xochimilco Campus (Mexico, D.F., Mexico) and individually housed in stainless steel cages with mesh bottoms at 22 ± 2 °C and 40–60% humidity, on a 12-h light–dark cycle. The rats were acclimatised for a 5-day period with free access to food and water. Thereafter, the rats were weighed and randomly divided into 7 groups (n = 7) as follows: standard diet control group (SD); high fat diet control group (HF); HF + intragastric administration of cocoa powder (Co, 1 g/kg bw); HF + intragastric administration of cocoa extract (Co-Ex, 100 mg/kg bw); HF + intragastric administration of (–)-epicatechin (Epi, 10 mg/kg bw); HF + intragastric administration of (+)-catechin (Cat, 10 mg/kg bw); and HF + intragastric administration of procyanidin B2 (PB2, 10 mg/kg bw). The oral dose of cocoa extract was based on the estimated content per gram of cocoa powder (exactly, 116.8 mg cocoa extract/g cocoa powder). The doses of epicatechin, catechin and procyanidin B2 were selected based on the approximate content of epicatechin in the doses selected for both cocoa powder (9.25 mg/g) and cocoa extract (8.6 mg/100 mg), adjusting the doses to 10 mg/kg bw in order to facilitate comparison between individual compounds. Rats were administered orally, once daily between 10 and 12 a.m., and had access to purified water and their respective diets for 8 weeks. Body weight (individually) was monitored daily and food intake was recorded every other day. Energy intake was calculated by multiplying the feed mass by its respective caloric density factor. These results were then converted to kilojoules (kJ) by multiplying the kilocalorie value (kcal) by 4.184. Feed efficiency was expressed as body weight gain in grams per kilojoules consumed × 100 (Prieto-Hontoria, Pérez-Matute, Fernández-Galilea, Martínez, & Moreno-Aliaga, 2013).

At the end of the experimental period (week 8), rats were anaesthetised with pentobarbital sodium (32 mg/kg IP) after withholding food for 12 h. Blood samples were harvested from abdominal aorta into 3.5 mL sterile gold BD Vacutainer SST™ test tubes (Cat. No.: 367983, New Jersey, USA) to determine the serum biomarkers. Retroperitoneal adipose tissue was removed, rinsed with 1X Phosphate Buffered Saline (PBS Buffer), weighed, immediately frozen in liquid nitrogen and stored at –80 °C until further use. The experimental protocol was performed in accordance with the Ethics Code for Animal Studies of the Escuela Nacional de Ciencias Biológicas (ENCB-IPN, Mexico) and the Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care (NOM-062-ZOO-1999).

2.3. *Biochemical analyses*

At week 8, fasting blood glucose levels were monitored in venous blood drawn from the tail vein using a glucometer (ACCU-CHEK® Performa, Roche Diagnostics, USA). Blood samples in test tubes (Vacutainer SST™) were allowed to clot for 15 minutes and centrifuged at 1465 ×g for 15 minutes, and then the serum was aliquoted and stored at –80 °C until further use. The levels of serum triacylglycerols (TG), high-density lipoprotein (HDL-c) and low-density lipoprotein (LDL-c) were determined using a semi-autoanalyser (Ekem Control Lab, Mindray, China). Enzymatic assays for serum total cholesterol and non-esterified fatty acids (NEFAs) were performed using commercially available kits (Catalogue No. CH201, Randox S.A de C.V., Mexico; and NEFA-HR (2), Wako Chemicals, Neuss, Germany).

2.4. *Measurement of insulin sensitivity*

Insulin sensitivity was evaluated by means of the insulin tolerance test (ITT) and Homeostasis Model Assessment of IR (HOMA-IR). At week 7, rats were submitted to ITT after 4 hours of fasting. Briefly, blood was collected from the tail vein for determination of baseline glucose level. Thereafter, 0.6 IU/kg bw of human recombinant insulin (Humulin R; from Eli Lilly, Mexico City) was injected intra-peritoneally and blood samples were taken at 15, 30, 60, 90, 120 and 150 minutes post-insulin injection. The results were analysed by calculating the area under the curve (AUC) for blood glucose with the trapezoidal rule. The HOMA-IR index was calculated from the following formula: fasting insulin (μIU/ml) × fasting glucose (mmol/ml)/22.5 (Zheng, Nanhui, Shuanghui, & Guohua, 2013).

2.5. *Measurements of serum insulin and leptin levels*

Serum levels of insulin (Catalogue No. EZRMI-13K, EMD Millipore, St. Charles, Missouri 63304, USA) and leptin (Catalogue No. EZRL-83K, EMD Millipore, St. Charles, Missouri 63304, USA) were measured according to the manufacturer's protocols using commercial enzyme-linked immunosorbent assay (ELISA) kits (Bikman et al., 2012; Manneras et al., 2010).

2.6. *Determination of cytokine levels in retroperitoneal white adipose tissue*

Retroperitoneal white adipose tissue samples were homogenised in ice-cold lysis buffer (0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide and 0.05% Triton X-100) supplemented with protease inhibitor cocktail (P1860, Sigma-Aldrich Co. St Louis, MO, USA). Homogenates were incubated for 10 minutes at 4 °C and then centrifuged at 14,000

× g at 4 °C for 15 minutes. The supernatants were divided into two 500-μL aliquots. One was processed immediately for cytokine analysis, while the second was stored at –80 °C until further use. Adiponectin, leptin, TNF-alpha and IL-6 concentrations in homogenate supernatants of retroperitoneal adipose tissue were determined by ELISA, as described previously. Total protein concentration was determined by the Bradford method (Borovikova et al., 2000; Bradford, 1976).

2.7. *RNA extraction*

Total RNA was extracted from retroperitoneal adipose tissue using TRIzol® reagent according to the manufacturer's protocol (Life Technologies, USA). RNA quantity (OD-260) and quality (OD-260/OD-280) were determined using the GENESYS 10 Series (ThermoSpectronic, Thermo Fisher Scientific, Inc., Madison, WI, USA). The integrity of RNA was determined by denaturing agarose gel electrophoresis. An aliquot of the RNA sample (5 μg) was separated on a 1% agarose gel containing ethidium bromide in MOPS buffer. The running buffer and gel contained 0.2 M formaldehyde. To prevent trace DNA contamination, the RNA samples were treated with amplification grade DNase I (Invitrogen™, Life Technologies, NY, USA) before reverse transcription. All of the RNA samples were stored at –70 °C in an RNA elution solution until further use (Hummon, Lim, Difilippantonio, & Ried, 2007).

2.8. *Real-time PCR*

Reverse transcription of total RNA (1 μg) was performed using Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics) in the presence of random hexamers. RT reactions were carried out in a thermal cycler (Eppendorf Mastercycler, Germany). The amplified cDNA was quantified using the spectrophotometric method (Abs 260 nm). The real-time PCR reaction mixture (20 μL) contained 1X LightCycler TaqMan Master (Roche Diagnostics), 200 nM forward primer, 200 nM reverse primer, 100 nM hydrolysis probe (Rat Universal Probe Library, Roche Diagnostics), 0.5 U LightCycler Uracil-DNA glycosylase, and 2 μL cDNA. Specific oligonucleotide primers were originally generated using the online assay design software (ProbeFinder: https://lifescience.roche.com). The PGC-1α primers were 5′ GGGTCATTTGGTGACTCTGG 3′ as the forward primer and 5′ GCAGTCGCAACATGCTCA 3′ as the reverse primer. SIRT1 was detected using 5′ AACTTCACAGCATCTT CAATTGTATT 3′ as the forward primer and 5′ TGACACTG TGGCAGATTGTTATT 3′ as the reverse primer. Peroxisome proliferator-activated receptor-alpha (PPARα) was detected using 5′ TTTAGAAGGCCAGGACGATCT 3′ as the forward primer and 5′ GCACTGGAACTGGATGACAG 3′ as the reverse primer. The peroxisome proliferator-activated receptor-gamma (PPARγ) primers were5′ GGGGGTGATATGTTTGAACTTG 3′ as the forward primer and 5′ CAGGAAAGACAACAGACAAATCA 3′ as reverse primer. TNF-α gene expression was determined using 5′ GCCAG AGGGCTGATTAGAGA 3′ as the forward primer and 5′ CAGCCTCTTCTCCTTCCTGA 3′ as the reverse primer. For IL-6, 5′ ACAACATCAGTCCCAAGAAGG 3′ and 5′ CCTTCAGGAACA GCTATGAA 3′ were used as forward and reverse primers, respectively, and the primers used for leptin detection were 5′ AATGAAGTCCAAACCGGTGA 3′ as the forward primer and 5′ CCAGGATCAATGACATTTCACA 3′ as the reverse primer. For UCP1, the forward primer (5′- 3′) was TGGCCTTCACCTTGGATCT and the reverse primer (5′- 3′) was GCCTGCCTAGCAGACATCAT. For ACC, the forward and reverse primers were GATCCC CATGGCAATCTG and ACAGAGATGGTGGCTGATGTC, respectively. For fatty acid translocase/CD36 (CD36) detection the forward primer was 5′TCGAGACTTCTCACCAAGAGG 3′ and the reverse primer 5′ GGGAAAGTTATTGCGACATGA 3′. PCR amplification was performed in borosilicate glass capillaries using the LightCycler Nano Real-Time PCR System (Roche Diagnostics, Germany). PCR assay conditions consisted of an initial incubation at 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C, 10 s), annealing (50 °C, 30 s) and extension (72 °C, 1 min). Relative expression levels of the target genes' mRNA were normalised to 18S mRNA levels. The relative quantification or fold change in gene expression was determined using the 2 – ΔΔCT method. All mRNA expressions were expressed in relation to the average expression of the SD group.

3. Statistical analyses

Data are expressed as mean values ± SEM. Statistically significant differences between groups were determined by one-way or two-way repeated measures analysis of variance (ANOVA), followed by the Holm-Sidak test for multiple comparisons. Pearson correlation coefficients were calculated to describe associations between selected variables (bivariate correlation). A value of $p < 0.05$ represented a significant difference.

4. Results

4.1. *Effect of cocoa powder, cocoa extract and its main flavanols on body weight, body fat mass and energy intake*

Fig. 1A shows that the body weight at baseline (week 0) was 185.08 ± 2.41 g in all groups. After 3 weeks of treatment, all HFD groups showed a rapid weight gain ($p < 0.001$) compared with the SD group, and continued thereafter to be progressively higher until the end of the study. Compared to the SD group, the Co-Ex group showed a significant increase in body weight gain until week 5; however, it was significantly lower than that of the HF group over the 8-week study period. The body weight gain of the Co group was similar to that of the HF group until week 6. Afterwards, a lower rate of gain ($p < 0.001$) was observed until the end of the experiment (week 8). Fig. 1B shows that body weight gain in HF group was 1.5-fold higher than that of SD group. Co and Co-Ex were the most effective treatments in decreasing body weight gain (12.4 and 13.3%, respectively) compared to HF group, followed by Epi group (5.97%). As shown in Fig. 1C, the energy intake was higher in all HFD groups compared with SD group throughout the study period, although Co group showed a lower energy intake from week 4 until the end of the study compared to the HF group. There was no difference in total energy intake between the HFD groups, except for Co group which showed a significantly lower energy intake (7.89%) compared to HF group (Fig. 1D). Fig. 1E shows that the consumption of an HFD resulted in a

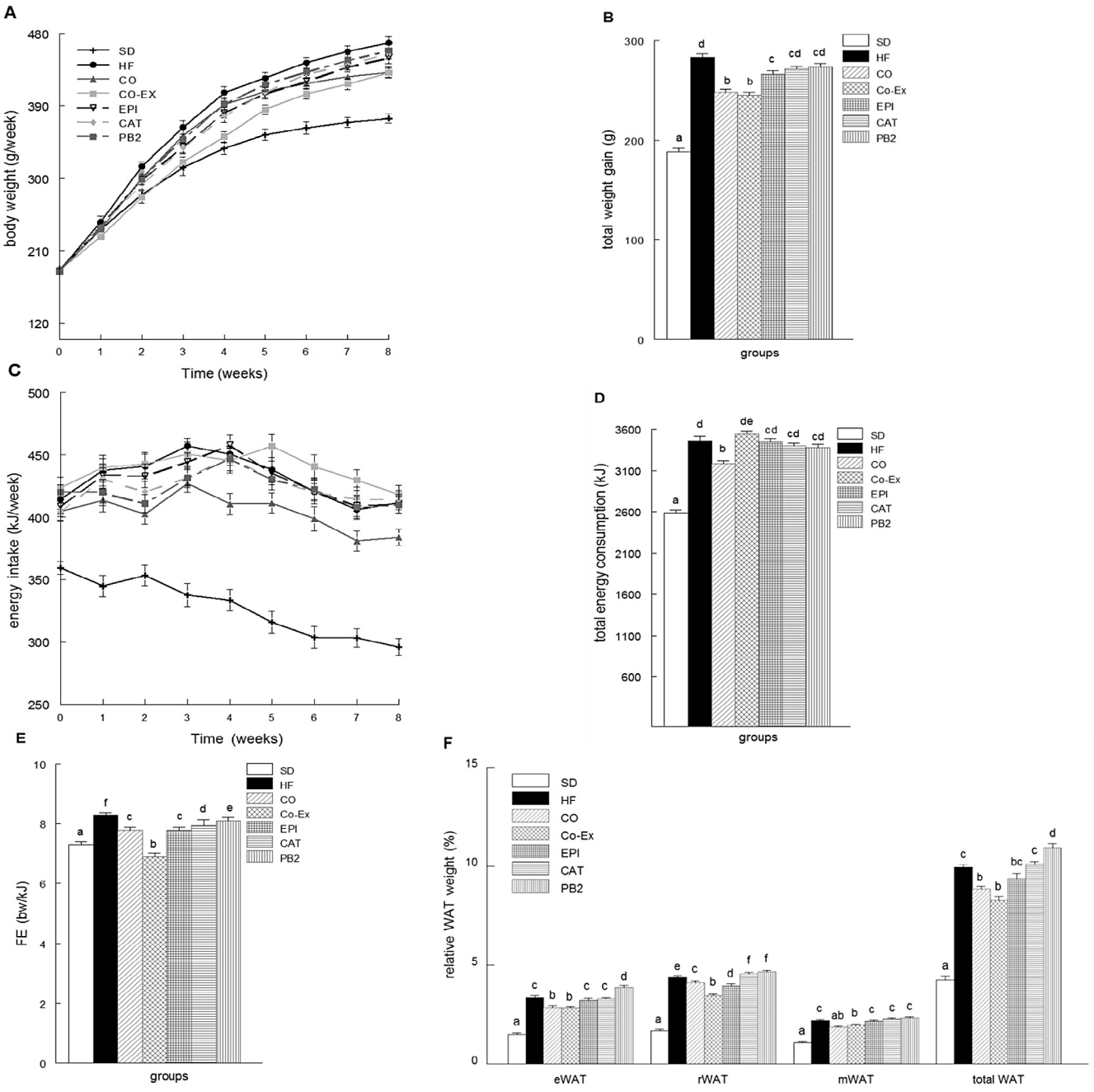

Fig. 1 – Effect of cocoa powder and its main flavanols on body weight gain (g/day), energy intake (kJ/day), feed efficiency (FE) and visceral fat accumulation. Body weight (A) and energy intake (C) over the course of 8 weeks. (B) Total body weight gain, (D) total energy consumption, (E) feed efficiency and (F) relative weight of white adipose tissue (WAT): epididymal (eWAT), retroperitoneal (rWAT) and mesenteric (mWAT). Groups are abbreviated as standard diet (SD), high-fat diet (HF), HF + cocoa powder (Co), HF + cocoa extract (Co-Ex), HF + epicatechin (Epi), HF + catechin (Cat), HF + procyanidin B2 (PB2). Values are expressed as mean ± SE, n = 7. Body weight and energy intake were compared by two-way repeated measures ANOVA with Holm-Sidak test for multiple comparisons. ǂ $p < 0.001$ all high-fat-fed groups vs. SD group; * $p < 0.001$ compared to HF group; # $p < 0.001$ compared to HF group. Total body weight gain, total energy consumption, FE values and relative weight of 3 WAT depots were compared by one-way ANOVA with Holm-Sidak test for multiple comparisons. Means with different superscripts are significantly different ($p < 0.001$).

significant increase in feed efficiency (FE) compared to the SD group. Compared to the HF group, the Co-Ex group lowered FE by 15.85%, followed by the Epi and Co groups (5%). Fig. 1F shows that the relative total WAT weight was significantly increased in all HFD groups compared to the SD group. Co and Co-Ex treatments attenuated fat mass accumulation by 11 and 16.8%, respectively, compared to the HF group. In contrast, PB2 treatment increased the relative WAT weight (7.3%)

Table 2 – Effects of cocoa powder, cocoa extract and its main flavanols on biochemical parameters.

	SD	HF	Co	Co-Ex	Epi	Cat	PB2
Total cholesterol (mg/dL)	84.4 ± 1.39^{a}	162.26 ± 2.03^{d}	136.17 ± 2.35^{b}	137.21 ± 1.8^{b}	136.63 ± 1.20^{b}	142.84 ± 1.67^{c}	142.72 ± 1.47^{c}
LDL-cholesterol (mg/dL)	40.83 ± 2.28^{a}	105.19 ± 0.96^{f}	95.16 ± 1.68^{c}	98.77 ± 0.89^{d}	92.48 ± 2.34^{b}	100.83 ± 0.79^{e}	102.1 ± 0.65^{e}
HDL-cholesterol (mg/dL)	35.80 ± 0.83^{a}	25.3 ± 1.10^{d}	30.02 ± 0.97^{b}	29.27 ± 0.78^{bc}	28.90 ± 1.11^{c}	29.76 ± 0.85^{bc}	29.09 ± 0.90^{bc}
Triacylglycerols (mg/dL)	39.77 ± 2.13^{a}	122.04 ± 1.11^{e}	83.22 ± 1.62^{b}	90.72 ± 1.39^{c}	92.19 ± 2.06^{cd}	93.34 ± 1.63^{d}	93.07 ± 1.54^{d}
NEFAs (mEq/L)	0.51 ± 0.024^{ab}	0.63 ± 0.021^{c}	0.46 ± 0.017^{a}	0.54 ± 0.02^{abc}	0.54 ± 0.02^{abc}	$0.\ 57 \pm 0.02^{bc}$	0.60 ± 0.024^{bc}
Leptin (ng/mL)	1.91 ± 0.31^{a}	$23.\ 24 \pm 1.18^{d}$	13.12 ± 1.02^{b}	11.97 ± 1.09^{b}	19.13 ± 1.95^{c}	22.06 ± 2.39^{d}	24 ± 1.86^{d}
Glucose (mg/dL)	84.6 ± 2.55^{a}	102.5 ± 1.25^{f}	93.8 ± 2.34^{bc}	92.5 ± 2.75^{b}	97.8 ± 1.97^{d}	96.8 ± 2.08^{cd}	99.8 ± 1.77^{ef}
Insulin (ng/mL)	0.70 ± 0.13^{a}	4.42 ± 0.1^{e}	2.61 ± 0.35^{b}	2.93 ± 0.18^{c}	2.49 ± 0.39^{b}	2.84 ± 0.30^{bc}	3.68 ± 0.26^{d}
HOMA	4.35 ± 0.99^{a}	32.15 ± 1.49^{e}	17.5 ± 2.01^{b}	19.3 ± 1.45^{c}	17.47 ± 2.09^{b}	19.7 ± 2.5^{c}	26.06 ± 2.04^{d}

Data represent means ± SEM (n = 7 per group). SD, standard diet; HF, high-fat diet; Co, HF + cocoa powder; Co-Ex, HF + cocoa extract; Epi, HF + epicatechin; Cat, HF + catechin; PB2, HF + procyanidin B2. Values without a common letter differ significantly ($p < 0.001$).

compared to the HF group. Co and Co-Ex treatments markedly suppressed the relative weights rWAT (6 and 20.8%, respectively), eWAT (14.3 and 14.6%, respectively) and mWAT (15 and 10.9%, respectively) compared to the HF group. Additionally, epicatechin treatment resulted in decreased rWAT relative weight (9.6%) compared to the HF group. Fat accumulation in the 3 WAT depots remained unaffected by Cat and PB2 treatments.

4.2. *Effect of cocoa powder, cocoa extract and its main flavanols on serum lipid profile and leptin levels*

Table 2 shows an increase in serum total cholesterol (20-fold), LDL-cholesterol (2.5-fold) and triacylglycerols (3-fold) levels in the HF group compared to the SD group. Conversely, all treated groups significantly improved the lipid profile compared to the HF group; however, Epi, Co and Co-Ex were the most effective treatments in decreasing serum CT (16.7%) and LDL-c (12.1, 9.5 and 6.1%, respectively). TG levels were significantly decreased by the Co group (1.4-fold) compared to the HF group. Moreover, all treatments showed higher levels of serum HDL-c than the HF group. There were no differences in serum NEFA levels between the HF and treated groups, with the exception of Co, which showed NEFA levels similar to those of the SD group.

A linear correlation was observed between serum leptin and body weight gain ($r = 0.97$, $p < 0.001$) and fat mass ($r = 0.88$, $p < 0.001$) in all groups. HF and PB2 groups showed higher levels (11.5- and 12.5-fold, respectively) compared to the SD group. Conversely, the Co-Ex, Co and Epi groups showed a significant decrease (48.4, 43.5 and 17.7%, respectively) in leptin levels compared to the HF group.

4.3. *Effects of cocoa powder, cocoa extract and its main flavanols on glucose homeostasis and insulin resistance (IR)*

At the end of experimental period, the HF group developed fasting hyperglycaemia (>100 mg/dL), hyperinsulinaemia and IR measured by the HOMA-IR index (6.3- and 7.3-fold higher values, respectively, than the SD group). Table 2 shows that all treated groups exhibited decreased fasting glucose and insulin levels compared to the HF group. The Epi and Co groups showed the greatest decrease (43.6 and 40%, respectively, compared to the HF group) in serum insulin levels, whereas PB2 treatment was the least effective (16.7% decrease compared to the HF group). IR was determined using both HOMA and the insulin tolerance test (ITT). As shown in Fig. 2A, after intraperitoneal insulin injection, blood glucose level recorded a time-dependent decrease at 15, 30 and 60 min in all groups. SD group showed lower ($p < 0.05$) glucose levels at every point, compared to HF group. In SD group, the lowest glucose level was observed at 120 min post-insulin injection, and at 150 min glucose level showed a gradual increase towards the baseline. In contrast, all high-fat-fed groups showed the lowest blood glucose level at 90 min post-insulin injection and a gradual increase towards baseline glucose level at 120 min. Compared to HF group, Co group showed lower blood glucose concentrations at 90, 120 and 150 min time points. Fig. 2B shows that the AUC was significantly higher in the HF group than the SD group; however, the Epi, Co and Co-Ex groups showed decreased AUC values (6.8, 6.1 and 4.7%, respectively). Similar results were observed for the HOMA-IR index, which showed a significant decrease in the Epi, Co (1.8-fold) and Co-Ex groups (1.6-fold) compared to the HF group.

4.4. *Effect of cocoa powder, cocoa extract and its main flavanols on adipocytokine levels in retroperitoneal adipose tissue (rWAT) (leptin, ApN, IL-6 and TNF-α)*

As shown in Table 3, the HF, Cat and PB2 groups exhibited significantly increased leptin levels in rWAT by 2.86-, 2.82- and 2.92-fold, respectively, compared to the SD group; however, the Co-Ex and Co groups exhibited significantly decreased leptin levels (14.64 and 6.1%, respectively) compared to the HF group. Additionally, the HF group decreased ApN levels in rWAT (28.8%) but increased IL-6 and TNF-α levels (28.8 and 51.7%, respectively) compared to the SD group. Compared to the HF group, the Co and Co-Ex groups exhibited significantly increased ApN levels (19.8 and 23.5%, respectively) and were the most effective treatments in decreasing IL-6 (17%) and TNF-α levels (27.7 and 31.8%, respectively) in rWAT. Additionally, the Epi group showed increased ApN levels by 15% and decreased IL-6 and TNF-α levels (13.7 and 25.9%, respectively) compared to the HF group. There was a negative correlation between ApN levels in rWAT and TNF-α ($r = -0.75$, $p < 0.001$) and IL-6 levels ($r = -0.78$, $p < 0.001$).

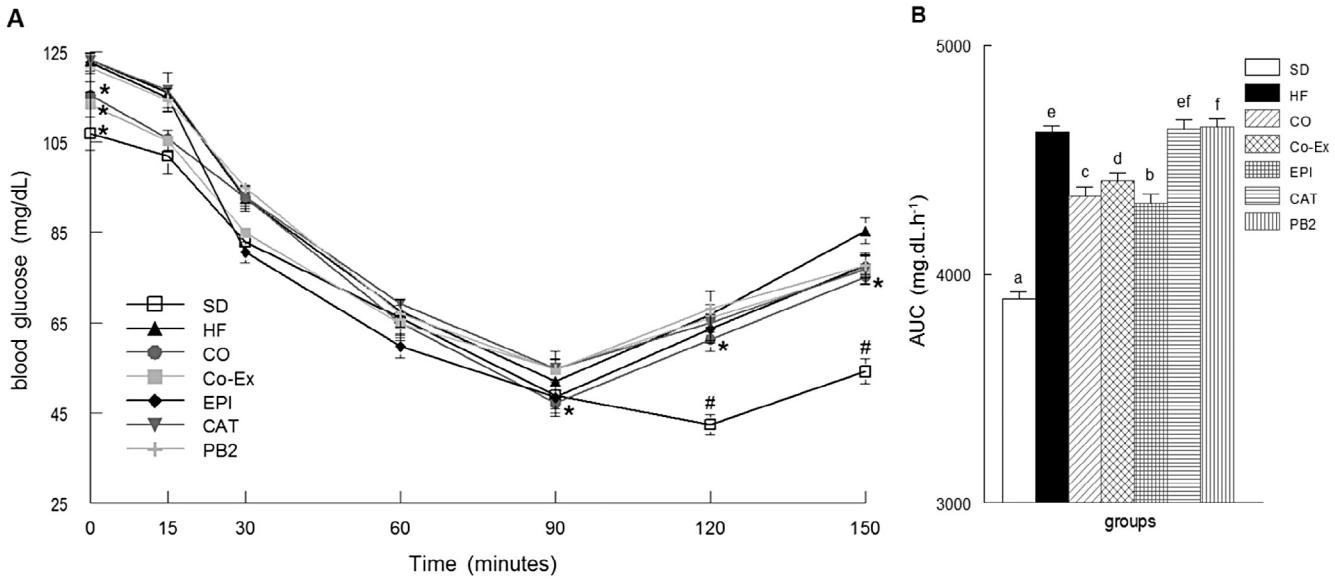

Fig. 2 – Effects of cocoa powder, cocoa extract and its main flavanols on insulin resistance. Insulin tolerance test (ITT) blood glucose levels (A) and area under the curve (AUC) during ITT (B). Values are presented as mean ± SE, n = 7. Treatment groups: standard diet (SD), high-fat diet (HF), HF + cocoa powder (Co), HF + cocoa extract (Co-Ex), HF + epicatechin (Epi), HF + catechin (Cat), HF + procyanidin B2 (PB2). Means with different superscripts are significantly different ($p < 0.001$). * $p < 0.001$ vs. HF group; # $p < 0.001$ vs. all HFD groups.

4.5. *Effect of cocoa powder, cocoa extract and its main flavanols on retroperitoneal adipose tissue (rWAT) adipocytokine gene expression*

Fig. 3A shows that all high-fat-fed groups showed significantly increased leptin mRNA expression in rWAT compared to the SD group; however, as observed in serum and rWAT cytokine levels, Co treatment showed lower levels of leptin expression (2-fold) than the HF group. Fig. 3B shows that the HF group exhibited increased TNF-α expression, 4-fold higher than that of the SD group. Epi group showed the lowest expression levels (1.6-fold), followed by the Co-Ex and Co groups (1.3-fold). In contrast, Fig. 3C shows that IL-6 mRNA levels were upregulated in all treatment groups, with the exception of the Co group, which decreased IL-6 mRNA levels by 24.7% compared to the HF group. Surprisingly, the Cat and Epi groups showed a significant increase (2.2- and 1.75-fold, respectively) in IL-6 expression compared to the HF group.

4.6. *Effect of cocoa powder, cocoa extract and its main flavanols on the mRNA levels of genes involved in lipid metabolism in retroperitoneal adipose tissue (rWAT)*

As shown in Fig. 4A, the expression of PPARγ in rWAT was upregulated in all treated groups compared to the HF group; however, Co-Ex, PB2 and Epi treatments showed the highest levels of PPARγ mRNA expression (3.5-, 3.3- and 3.2-fold, respectively), followed by the Co group (2-fold). Similarly, Fig. 4B shows that CD36 mRNA levels were significantly increased in all treated groups. Compared to the HF group, the Co and Co-Ex groups exhibited increased CD36 expression by 12- and 11-fold, respectively, followed by PB2 and Epi treatments (7- and 6-fold, respectively). Fig. 4C shows that ACC mRNA expression in rWAT was increased (4.3-fold) in the HF group compared to the SD group. Compared to the HF group, all treated groups exhibited significantly decreased ACC expression; however, the Co, Cat and Epi groups showed expression levels even lower

Table 3 – Effect of cocoa powder, cocoa extract and its main flavanols on retroperitoneal adipose tissue (rWAT) adipocytokines.

	SD	HF	Co	Co-Ex	Epi	Cat	PB2
Leptin (pg/mg protein)	82.48 ± 2.20^{a}	232.06 ± 3.52^{c}	221.62 ± 1.98^{c}	201.49 ± 4.24^{b}	223.57 ± 3.44^{c}	233.30 ± 3.56^{c}	241.35 ± 5.17^{c}
Adiponectin (ng/mg protein)	37.52 ± 0.35^{a}	26.72 ± 0.43^{d}	32.12 ± 0.22^{bc}	33.26 ± 0.39^{b}	30.71 ± 0.37^{bc}	29.56 ± 0.40^{c}	29.84 ± 0.27^{c}
IL-6 (pg/mg protein)	0.23 ± 0.008^{a}	0.29 ± 0.004^{d}	0.24 ± 0.009^{ab}	0.23 ± 0.004^{a}	0.25 ± 0.005^{bc}	0.26 ± 0.004^{bc}	0.27 ± 0.006^{c}
TNF-α (pg/mg protein)	1.45 ± 0.01^{a}	2.20 ± 0.03^{e}	1.59 ± 0.06^{c}	1.5 ± 0.04^{b}	1.63 ± 0.06^{d}	1.57 ± 0.08^{c}	1.65 ± 0.07^{d}

Data represent means ± SEM (n = 7 per group). SD, standard diet; HF, high-fat diet; Co, HF + cocoa powder; Co-Ex, HF + cocoa extract; Epi, HF + epicatechin; Cat, HF + catechin; PB2, HF + procyanidin B2. Values without a common letter differ significantly ($p < 0.001$).

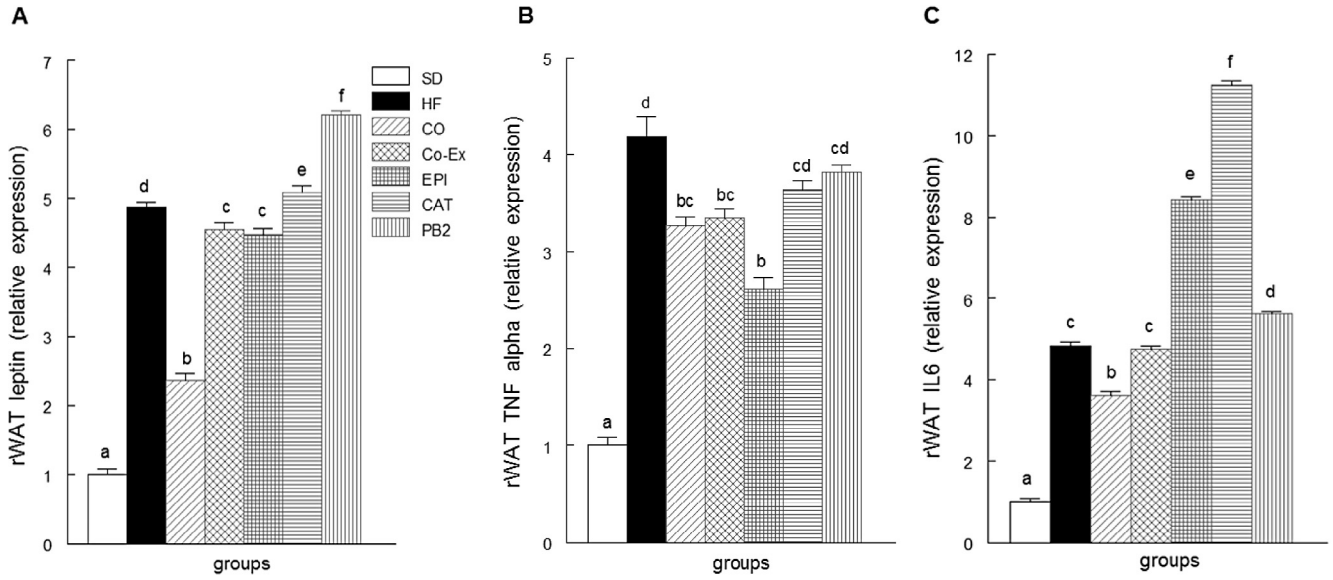

Fig. 3 – Effect of cocoa powder, cocoa extract and its main flavanols on cytokine mRNA expression in retroperitoneal adipose tissue (rWAT). Quantitative real-time PCR assays of the genes encoding (A) Leptin (B) TNF-α (C) IL-6. Samples of rWAT were collected from rats and total RNA was extracted as described in the "Materials and methods" section. The relative expression level of each gene was quantified and normalised to 18S ribosomal RNA for each sample (n = 3). Values are expressed as the fold change compared with SD group that was arbitrarily set to 1. Groups are abbreviated as standard diet (SD), high-fat diet (HF), HF + cocoa powder (Co), HF + cocoa extract (Co-Ex), HF + epicatechin (Epi), HF + catechin (Cat), HF + procyanidin B2 (PB2). Values with different superscripts are significantly different ($p < 0.001$).

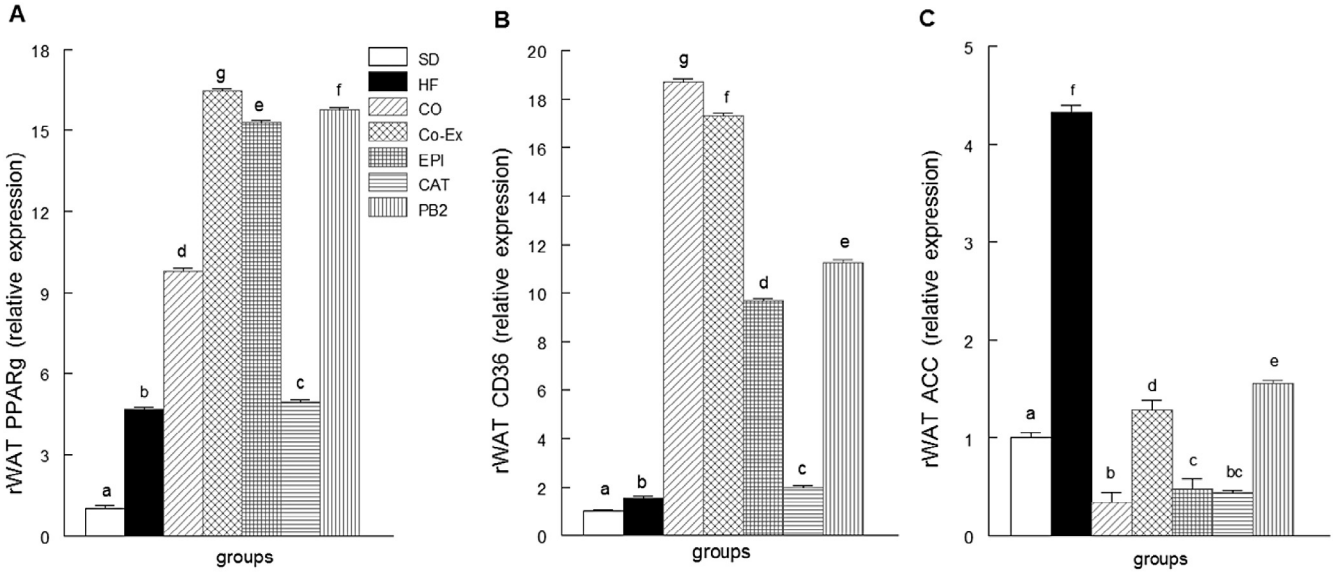

Fig. 4 – Effect of cocoa powder, cocoa extract and its main flavanols on mRNA levels of genes involved in lipid metabolism in retroperitoneal adipose tissue (rWAT). (A)The PPARγ, its downstream gene involved in fatty acid uptake (B) CD36, and the lipogenic related gene (C) ACC were measured by quantitative real-time PCR assay. Samples of rWAT were collected from rats and total RNA was extracted as described in the "Materials and methods" section. The relative expression level of each gene was quantified and normalised to 18S ribosomal RNA for each sample (n = 3). Values are expressed as the fold change compared with SD group that was arbitrarily set to 1. Groups are abbreviated as standard diet (SD), high-fat diet (HF), HF + cocoa powder (Co), HF + cocoa extract (Co-Ex), HF + epicatechin (Epi), HF + catechin (Cat), HF + procyanidin B2 (PB2). Values with different superscripts are significantly different ($p < 0.001$).

(2.9-, 2.2- and 2-fold, respectively) than those observed in the SD group.

The retroperitoneal adipose tissue (rWAT) was chosen because it has been reported that this tissue is highly responsive to nutritional conditions (overfeeding/fasting). It has been shown that under high-fat feeding conditions, rWAT exhibits a significant increase in the expression of transcription factors and enzymes involved in *de novo* lipogenesis (PPARγ, SREBP1c, ACC), as well as proteins providing substrates (glucose and fatty acids) for TG synthesis and storage (LPL, CD36 and GLUT4). Moreover, rWAT has shown increased expression of genes encoding enzymes involved in lipid mobilisation (HSL and ATGL) and reduced expression levels of genes involved in mitochondrial fatty acid oxidation (CPT1), indicating that free fatty acids resulting from TG mobilisation are mainly released into blood circulation instead of being oxidised (Barbosa de Queiroz et al., 2014; Palou et al., 2010; Sackmann-Sala, Berryman, Munn, Lubbers, & Kopchick, 2012). Overall, these results showed that rWAT could be a representative WAT depot with a high capacity to store TG and to mobilise free fatty acids into the portal circulation, impairing liver metabolism and leading to insulin resistance and dyslipidemia. Therefore, we assumed that any change produced by treatments could provide insights into their capacity to regulate lipid metabolism in rWAT and to attenuate metabolic-related disorders.

4.7. *Effect of cocoa powder, cocoa extract and its main flavanols on the expression of molecules involving fatty acid combustion and energy expenditure in retroperitoneal adipose tissue (rWAT)*

Fig. 5A and 5B shows that the HF group exhibited significantly decreased (2.5- and 2.3-fold, respectively) PGC-1α and SirT1 mRNA expression levels in rWAT compared to the SD group; however, this effect was blunted in all treatment groups. Compared to the HF group, the Co and Co-Ex groups showed the highest expression levels of PGC1α (9.6- and 10.5-fold, respectively) and SIRT1 (12.5- and 11.6-fold, respectively), followed by Epi treatment (3.2- and 7.7-fold increase in PGC1α and SIRT1 expression, respectively). Fig. 5C shows that PPARα mRNA expression in rWAT was up-regulated in all treatment groups compared to the HF group; however, Co-Ex treatment showed the highest expression levels of PPARα (10-fold), followed by the Epi and Cat groups (8.7-fold). Additionally, Fig. 5D shows the effect of cocoa and its main flavanols on energy expenditure in rWAT. A high-fat diet alone induced an increase (3-fold) in UCP1 expression compared to the SD group. Compared to the HF group, Co and Epi treatments showed a higher expression of UCP1 (43%), followed by the Co-Ex group (20%).

5. Discussion

To our knowledge, this is the first study to evaluate the effects of cocoa powder together with its main flavanols (epicatechin, catechin and procyanidin B2) and a flavanol-rich cocoa extract on the expression of genes involved in the regulation of lipid metabolism (PPARγ, CD36, ACC, PPARα, PGC1α, SIRT1, UCP1) and inflammation (TNF-α, IL-6, ApN y Lep) in white adipose tissue (WAT), and whether these changes attenuate hypercaloric diet-induced obesity and metabolic alterations in rats.

Available clinical and experimental studies examining the mechanisms of action of cocoa flavanols against obesity-related metabolic alterations are still scarce and remain to be fully elucidated. Additionally, the heterogeneity of the types and doses of cocoa-derived products (cocoa powder, cacao liquor, cocoa extract, chocolate, cocoa husk) used for clinical investigations makes it difficult to determine which bioactive compounds are responsible for the beneficial effects of cocoa on hypercaloric diet-induced obesity.

The results from the current study show that Co-Ex, Co and Epi were the most effective treatments in attenuating body weight gain, intraabdominal fat mass accumulation, dyslipidemia, hyperglycaemia and IR induced by hypercaloric diet intake (high fat-high sucrose). Our results are in agreement with previous studies that demonstrated that cocoa and polyphenol-rich cocoa extracts decrease body weight gain and intra-abdominal adipose tissue mass in rats fed high-fat diets (Cordero-Herrera, Cordero-Herrera, Martín, Goya, & Ramos, 2015; Dorenkott et al., 2014; Gu, Yu, & Lambert, 2012; Gu, Yu, & Lambert, 2014; Matsui et al., 2005; Min et al., 2013; Yamashita et al., 2012).

Considering that visceral obesity provides an important link between inflammation, insulin resistance and dyslipidemia, this study aimed to evaluate the effects of Co, Co-Ex, Epi, Cat and PB2 treatments on the expression of genes involved in adipogenesis, lipogenesis, fatty acid oxidation, energy expenditure and inflammation in WAT (Makki, Froguel, & Wolowczuk, 2013; Mlinar, Marc, Janez, & Pfeifer, 2007; Rutkowski, 2015; Siriwardhana et al., 2013).

PPARγ transcription factor and fatty acid translocase (FAT)/CD36 play a key role in the regulation of WAT expansion in response to a positive energy balance because fatty acid uptake and storage in adipocytes will reduce ectopic lipid deposition, especially in liver and skeletal muscle (Goudriaan et al., 2005; Vroegrijk et al., 2013). Our results showed that all treatment groups upregulated PPAR?? gene expression, compared to the HF group, which has been shown to promote the apoptosis of hypertrophic mature adipocytes and to stimulate the production of small insulin-sensitive adipocytes encoding proteins involved in the insulin signalling cascade (Irs1, Irs2, PI3K p85 and Glut4). Thus, PPARγ plays an important role in the maintenance of systemic insulin sensitivity (Leonardini, Laviola, Perrini, Natalicchio, & Giorgino, 2009; Sugii et al., 2009).

Previous studies have evaluated the effects of cocoa flavanols on the expression of PPARγ and its association with fat mass accumulation, although there have been some controversial results. A recent study showed that the administration of a cocoa polyphenol-rich extract (600 mg/kg bw) significantly decreased body weight gain, hyperglycaemia and hyperinsulinaemia in diet-induced Ob-db rats, partially through the activation and upregulation of PPARγ gene expression in WAT (Aminudin et al., 2015). In contrast, Matsui et al. showed that cocoa supplementation (12.5%) in high fat-fed rats significantly reduced body weight gain and TG accumulation and downregulated the expression of PPARγ and fatty acid (FA) transport molecules in mesenteric WAT. In this study, it was suggested that the decrease in PPARγ expression might be related to a decrease in serum TG, which leads to a decrease in TG uptake and accumulation in WAT (Matsui et al., 2005).

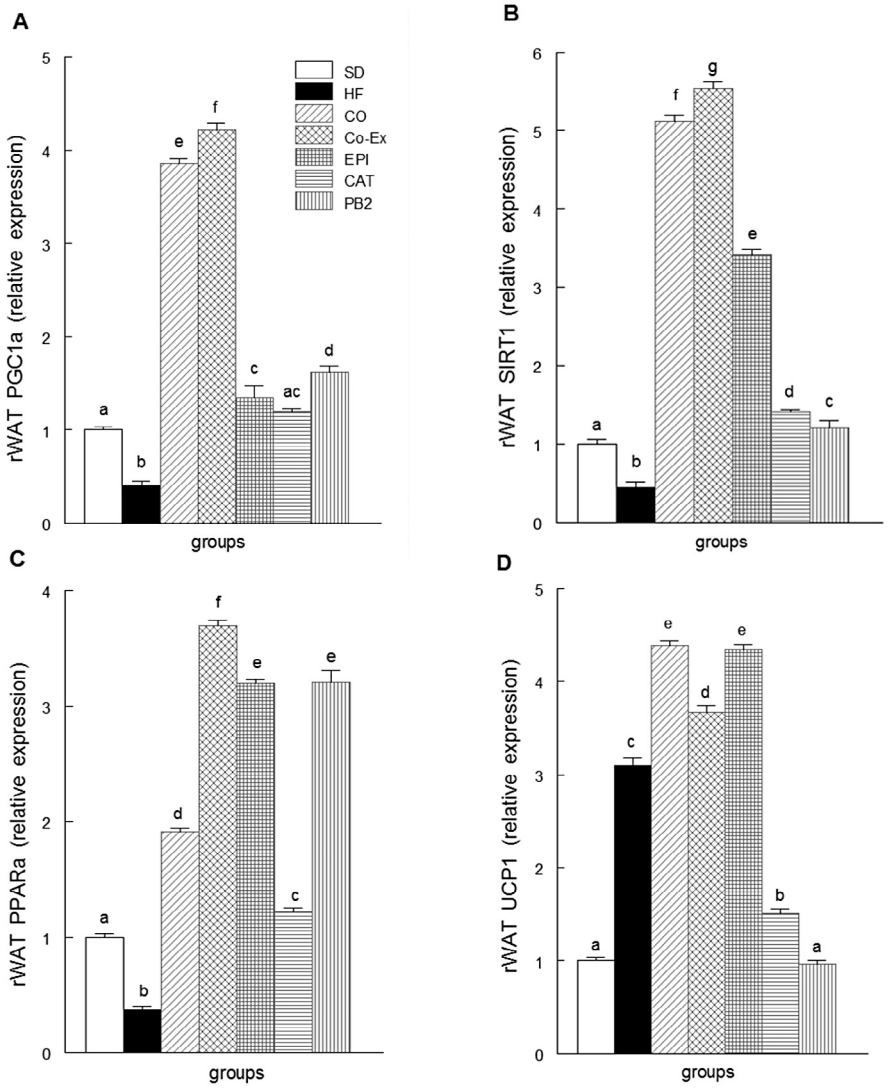

Fig. 5 – Effect of cocoa powder, cocoa extract and its main flavanols on expression of molecules involving fatty acid combustion and energy expenditure in retroperitoneal adipose tissue (rWAT). (A) PGC-1α, (B) SIRT1, (C) PPARα and (D) UCP1 mRNA expressions were determined by quantitative real-time PCR assay. Samples of rWAT were collected from rats and total RNA was extracted as described in the "Materials and methods" section. The relative expression level of each gene was quantified and normalised to 18S ribosomal RNA for each sample (n = 3). Values are expressed as the fold change compared with SD group that was arbitrarily set to 1. Groups are abbreviated as standard diet (SD), high-fat diet (HF), HF + cocoa powder (Co), HF + cocoa extract (Co-Ex), HF + epicatechin (Epi), HF + catechin (Cat), HF + procyanidin B2 (PB2). Values with different superscripts are different ($p < 0.001$).

Given the key role that PPARγ plays in the regulation of lipid uptake, transport, and storage in adipose tissue, we proposed that its upregulation also promoted TG-lowering effect of treated groups. Moreover, several studies have reported that increased PPARγ-mediated adipocyte differentiation is an important adaptive response to overnutrition that leads to a larger number of small and insulin-sensitive fat cells (Unger & Scherer, 2010; Yu & Ginsberg, 2005). Based on these reports we proposed that even though PPARγ upregulation contributed to WAT expansion, it promoted a decrease in systemic insulin resistance as indicated by the HOMA-IR values. Nonetheless, further studies are needed to expand our knowledge on the molecular mechanisms underlying such effects. Additionally, analysis of adipose tissue cellularity (histological analysis using optical or electron microscopy, flow cytometry, etc.) should be performed to support molecular data.

Studies utilising CD36 null mice (cd36-/-) have shown that this translocase plays an important role in preventing ectopic lipid deposition because increased FA uptake in WAT leads to a decreased flux of free fatty acids and TG to peripheral tissues (Goudriaan et al., 2005; Vroegrijk et al., 2013). In accordance with previous experimental studies and considering that adipose tissue is the major site for CD36-mediated uptake of dietary FA, after LPL-mediated hydrolysis of chylomicron TG, we suggest that its upregulation enhanced adipocytes fatty acid trapping (released from chylomicron-TG), and thus clearance

of TG-enriched remnant lipoproteins from the circulation. Furthermore, reduced insulin resistance in adipose tissue by all treatments may be associated with increased uptake and storage of fatty acids and decreased hydrolysis of TG within the adipocyte, contributing to decreased serum TG and NEFAs levels.

It has been demonstrated that the consumption of hypercaloric diets results in an increased de novo fatty acid synthesis in WAT due to an excess of acetyl-CoA units derived from glycolysis, which can be carboxylated in the cytosol by acetyl-CoA carboxylase (ACC) and give rise to malonyl-CoA (Oh et al., 2005). In our study, results showed that all treatments reduced the mRNA expression of ACC in rWAT, although not all groups showed a decrease in fat mass accumulation (Cat and PB2 groups). Therefore, we should evaluate the effects of our treatments on the gene and protein expression of other enzymes and transcription factors involved in the lipogenic pathway of adipose tissue (DGAT, FAS, SREBP-1c, SCD1) in order to investigate other molecular targets regulating fat mass accumulation in these groups. In Co, Co-Ex and Epi groups, downregulation of ACC may contribute to reduce adiposity, but it may not be the main mechanism. Previous studies have demonstrated other anti-obesity mechanisms of cocoa polyphenols that could be influencing our results, especially those regarding reduced lipid digestion and/or absorption by inhibiting activity of gastrointestinal enzymes (pancreatic lipase, phospholipase A2) (Gu, Yu, & Lambert, 2014).

During periods of positive energy balance, the upregulation of PPARα, PGC1α and SIRT1 in WAT has been proposed as a potential mechanism to reduce adiposity and ameliorate metabolic disorders such as IR and dyslipidemia. Although a very low expression of PPARα, PGC1α and SIRT1 in WAT has been reported, recent studies have shown that the upregulation and activation of these molecules increase metabolic rate by promoting fatty acid oxidation (Hiuge et al., 2007; Rodgers, Lerin, Gerhart-Hines, & Puigserver, 2008; Rutanen et al., 2010; Tsuchida et al., 2005).

PGC1α is considered to be a master regulator of mitochondrial biogenesis and oxidative capacity that plays a key role in cellular energy metabolism. There is considerable data supporting that SIRT1, a NAD+-dependent histone deacetylase, interacts with PGC1α and promotes its transcriptional activity through deacetylation. Together, SIRT1 and PGC1α have been shown to regulate energy homeostasis and increase energy expenditure over energy storage in WAT (Feige et al., 2008; Qiang et al., 2012). Moreover, it was recently identified that PGC1α is capable of coactivating PPARα in the transcriptional control of genes involved in fatty acid oxidation (ACO, CPT1), thereby enhancing mitochondrial oxidative capacity and regulating cellular lipid and energy metabolism. In addition, recent studies have demonstrated that the SirT1-dependent deacetylation of PGC1α promotes the "browning" of WAT, which is characterised by the production of beige cells with higher mitochondrial mass and increased expression of PGC1α, genes related to fatty acid oxidation, and UCP1, a brown adipose tissue (BAT) hallmark gene responsible for energy expenditure by uncoupling oxidative phosphorylation and promoting the dissipation of cellular energy as heat (Castillo-Quan, 2012; Harms & Seale, 2013; Liang & Ward, 2006; Lo & Sun, 2013; Qiang et al., 2012).

In our study, Co, Co-Ex and Epi treatments were shown to be highly effective in inducing the expression of PGC1α, SIRT1, PPARα and UCP1 in WAT, suggesting that an increase in energy expenditure is a potential mechanism by which these treatments attenuate hypercaloric diet-induced body weight gain and fat mass accumulation.

Obesity is associated with a chronic state of inflammation characterised by the abnormal production of pro-inflammatory cytokines (TNF-α and IL-6) and anti-inflammatory adipokines (ApN) linked to the development and progression of obesity-related disorders such as IR and dyslipidemia (Lumeng & Saltiel, 2011).

Leptin is a hormone secreted by white adipocytes that plays a central role in the regulation of body weight through its effects on food intake and energy expenditure. Obese humans and rodents fed a HF diet develop a leptin-resistant state characterised by hyperleptinaemia and attenuated leptin's ability to suppress food intake, and particularly in WAT, to modulate adipocytes metabolic function (promoting fat oxidation and decreasing lipogenesis) (Unger & Scherer, 2010).

Our results showed a positive correlation between serum leptin levels and body weight gain and fat mass accumulation in all treatment groups. Co, Co-Ex and Epi treatments resulted in lower serum leptin levels associated with a lower body weight gain and adipose tissue accumulation. A previous study showed that HF-diet feeding in male rats induced a significant decline in OB-Rb mRNA in the retroperitoneal adipose tissue compared to inguinal and mesenteric depots. The latter was linked with a significant increase in retroperitoneal fat mass, upregulation of lipogenesis and lipolysis-related genes, and downregulation of mitochondrial fatty acid oxidation genes (Priego, Sánchez, Picó, & Palou, 2008).

In line with these findings, our results showed that cocoa-treated group showed the highest downregulation of leptin gene expression in rWAT, and that this effect was associated with a significant decrease in energy intake, reduction in rWAT mass and upregulation of SIRT1, PGC-1α and UCP-1, suggesting that cocoa powder attenuates hypercaloric diet induced-leptin resistance in this tissue. Although the existence of leptin resistance is well accepted, its underlying mechanisms have not yet been well characterised, although leptin receptor (OB-Rb) mRNA downregulation and inhibition of leptin-induced JAK/STAT pathway have received much attention (Unger & Scherer, 2010; White et al., 2009). Therefore, further investigations are needed to understand the precise mechanisms through which cocoa powder may improve leptin signalling, and consequently the development of obesity.

In obesity, adipose tissue expansion is associated with increased macrophage infiltration and increased levels of pro-inflammatory cytokines. TNF-α and IL-6 have emerged as key inflammatory markers that play a central role in the development of several metabolic disorders in WAT. Increased levels of pro-inflammatory cytokines induce insulin resistance through increased serine phosphorylation of insulin receptor and insulin receptor substrate-1 (IRS-1), and inhibit adipocyte differentiation by downregulating the expression of PPAR?? and genes involved in lipid uptake and storage expression (Cawthorn & Sethi, 2008; Lee & Lee, 2014; Makki et al., 2013).

In our study, we observed an increase in TNF-α rWAT levels and a higher fat mass accumulation in the HF group, and these

results agree with previous studies that have shown a positive correlation between obesity and TNF-α levels (Greenberg & Obin, 2006). Interestingly, Co and Co-Ex treatments showed decreased gene and protein levels of TNFα and IL-6 in rWAT, suggesting an anti-inflammatory effect in this tissue. We did not observe an association between mRNA levels and protein levels of IL-6 in rWAT, which suggests that decreased protein levels of IL-6 may be regulated at a post-translational level. Moreover, our results suggest that the decrease in TNF-α levels allowed an increase in adipogenesis and fatty acid uptake via the upregulation of PPARγ and CD36 expression in rWAT. Considering that individual flavanols did not show conclusive evidence for inflammatory markers in retroperitoneal adipose tissue (rWAT), we suggest that interactions between flavanols and methylxanthines (especially theobromine) may mediate anti-inflammatory effects of cocoa powder and cocoa extract in this tissue.

The inverse correlation between APN and obesity, IR, TNF-α and IL-6 levels has been well established (Makki et al., 2013; Mlinar et al., 2007; Vega, Huss, & Kelly, 2000). It is well known that ApN regulates glucose uptake, lipogenesis, lipolysis, and fatty acid oxidation in various tissues, including WAT. AMPK?? is the main molecular mediator of ApN effects in all target tissues. Therefore, AMPK?? activation allows ApN to stimulate the metabolic remodelling of WAT by lowering its lipid storage potential and increasing its oxidative capacity. However, data regarding adiponectin's effects in white fat tissue are still limited. In addition to adiponectin/AMPK?? signalling, it has been reported that increased ApN levels enhance fatty acid oxidation via PGC1α and PPARα activation in WAT (Goto et al., 2011; Wu et al., 2003).

In agreement with previous results, increased levels of ApN in rWAT in all treatment groups suggest that this adipokine might be involved in reducing lipogenesis and increasing β-oxidation in this tissue. Moreover, we found a negative correlation between ApN and TNF-α and IL-6 levels, suggesting that ApN can attenuate TNF-α's action in obesity-associated metabolic complications in WAT and exert anti-inflammatory effects in this tissue.

6. Conclusions

In conclusion, these results provide evidence that Co and Co-Ex have potential beneficial effects on obesity and associated metabolic disorders in rats fed a hypercaloric diet. Additionally, the effects of the three main cocoa flavanols were studied, and the results indicate that Epi could be an important mediator of most of the beneficial effects of both cocoa powder and extract in adipose tissue. Although other anti-obesity mechanisms have been reported for cocoa polyphenols, our results show that Co, Co-Ex and Epi are associated with the upregulation of the expression of genes involved in FA acid uptake, β-oxidation and energy expenditure in rat WAT, suggesting an improvement in adipose tissue function with a better capacity to store nutritional overload and to attenuate the production of inflammatory cytokines. More molecular targets need to be investigated in order to fully understand the mechanisms underlying the beneficial effects of cocoa and its potential bioactive compounds on the lipid metabolism in adipose tissue.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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