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Modulating the expression of genes associated with hepatic lipid metabolism, lipoperoxidation and inflammation by cocoa, cocoa extract and cocoa flavanols related to hepatic steatosis induced by a hypercaloric diet

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ABSTRACT

Cocoa and its bioactive compounds are widely known for its beneficial effects as attenuate insulin resistance, hepatic lipid deposition, inflammation and oxidative stress induced by hypercaloric diets. The present *in vivo* study investigated 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 hepatic lipid metabolism, lipoperoxidation and inflammation associated to hepatic steatosis induced by a hypercaloric diet. This study demonstrates that oral treatment with cocoa powder, cocoa extract and cocoa flavanols significantly attenuate hepatic steatosis induced by hypercaloric feeding, in part, through downregulation of genes involved in hepatic fatty acid uptake (PPARγ, CD36) and lipogenesis (ACC) and upregulation of key regulators of mitochondrial function (PPARα, SIRT1) and FA oxidation (PGC-1α) as well as in attenuating hepatic oxidative stress and inflammation by upregulating hepatic antioxidant enzymes and decreasing gene expression of proinflammatory cytokines (TNF-α, IL-6).

1. Introduction

Cocoa and its bioactive compounds (mainly epicatechin, catechin and procyanidin B2) have increasingly attracted attention because of their beneficial effects in attenuating insulin resistance, hepatic lipid deposition, inflammation and oxidative stress induced by hypercaloric diets (Ali, Ismail, & Kersten, 2014). Some potential mechanisms that have been proposed for these effects include downregulation and inhibition of transcription factors (NF-kB, SREBP1-c), enzymes involved in oxidative stress and lipogenesis (NADPH oxidase, Fas) and proinflammatory mediators (TNF-α, IL-6, IL-1β, MCP-1), as well as upregulation and activation of endogenous antioxidant enzymes (GPx, SOD, CAT) and transcription factors (PPAR-α) (Cordero-Herrera, Martín, Goya, & Ramos, 2015; Cordero-Herrera, Martín, Fernández-Millán, et al., 2015).

Non-alcoholic fatty liver disease (NAFLD) is widely considered as the hepatic manifestation of the metabolic syndrome due to its association with central obesity, diabetes mellitus type 2, insulin resistance, hypertension and dyslipidemia (Marchesini et al., 2003). NAFLD is characterized by triglyceride (TG) accumulation in hepatocytes that results from an imbalance between the rate of fatty acids (FA) input (uptake and de novo lipogenesis) and the rate of FA output (oxidation and secretion as VLDL-TG) and hepatic inflammation (Chen, Varghese, & Ruan, 2014).

Experimental dietary models of NAFLD have demonstrated that hepatic peroxisome proliferator-activated receptor γ (PPARγ) overexpression promotes FA influx and upregulation of lipogenic genes (ACC and Fas) and its target gene CD36 (Ables, 2012; Inoue et al., 2005; Reddy & Rao, 2006). Hepatic transmembrane protein CD36 (FAT/CD36) plays an important role in long chain fatty acids uptake, intracellular trafficking and esterification into intrahepatic triacylglycerols (IHTG) (Koo, 2013).

In the setting of high caloric intake, fatty acids can be synthesized de novo from acetyl-CoA (derived from glycolysis), which is used as a substrate by acetyl-CoA carboxylase (ACC) to produce malonyl-CoA.

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The latter is considered as the major inhibitor of carnitine palmitoyltransferase 1 (CPT-1) and thus prevents the entry of FA into the mitochondrial matrix and inhibits β-oxidation (Koo, 2013).

Evidence suggests that mitochondrial dysfunction is a key player in the pathophysiology of NAFLD since its oxidative capacity becomes impaired and promotes IHTG accumulation. Peroxisome proliferator-activated receptor α (PPARα) is essential in the upregulation and activation of key enzymes involved in mitochondrial (CPT-1), peroxisomal (ACOX) and microsomal fatty acid β-oxidation (CYP4A). Therefore, downregulation of hepatic PPARα expression contributes to increased susceptibility to the development of NAFLD (Koo, 2013; Rolo, Teodoro, & Palmeira, 2012). Additionally, decreased hepatic peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and Sirtuin 1 (SIRT1) levels have also been described in a variety of models of NAFLD and have been associated with alterations in mitochondrial biogenesis (both proliferation and differentiation) and impaired mitochondrial respiratory chain and oxidative phosphorylation.

IHTG accumulation and impaired mitochondrial function increases the production of reactive oxygen species (ROS: superoxide anions, hydrogen peroxide and hydroxyl radicals) and activate alternative oxidation pathways in the peroxisomes and microsomes (Ω-oxidation), resulting in additional ROS that can attack membrane polyunsaturated fatty acids (PUFAs) and initiate lipid peroxidation within the cell. This results in the formation of trans-4-hydroxy-2-nonenal and malondialdehyde (MDA), which in turn have the potential to amplify the effects of oxidative stress. Additionally, oxidative stress decreases superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase activities and triggers proinflammatory cytokines (TNF-α and IL-6) production, leading to inflammation and further development of non-alcoholic steatohepatitis (NASH) (Browning & Horton, 2004; Rolo et al., 2012).

Most studies have focused mainly on the antioxidant and anti-inflammatory mechanisms through which cocoa powder and/or cocoa extract ameliorate hepatic steatosis; however, a deeper understanding of the molecular mechanisms regulating hepatic lipid metabolism is needed. Therefore, the aim of the present study was to investigate 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 hepatic lipid metabolism, lipoperoxidation and inflammation associated to hepatic steatosis induced by a hypercaloric diet (high fat-high sucrose).

2. Materials and methods

2.1. Chemicals

(+)-Catechin hydrate (C1251; ≥98%) and (−)-epicatechin (E1753; ≥90%) were purchased from Sigma–Aldrich (St. Louis, MO) and procyanidin B2 was purchased from Chempacific (Baltimore, Maryland).

2.2. Cocoa powder and cocoa extract

Unsweetened cocoa powder (CocoaVia®) and cocoa extract (CocoaVia®) were provided from Mars, Inc. (Hackettstown, NJ). By aluminum chloride and Folin–Ciocalteu methods were quantified the total flavonoids and polyphenols in cocoa powder and cocoa extract respectively, using (+)-catechin as a standard. (+)-Catechin, (−)-epicatechin and procyanidin B2 levels were assessed using HPLC-DAD, by a previously described method (Ortega et al., 2010). The dosage in this study of unsweetened cocoa powder (1 g/kg bw) and cocoa extract (100 mg/kg bw) contained 72.4 and 61.7 mg of total polyphenols, 55.5 and 44.1 of total flavonoids, 9.25 and 8.6 mg of (−)-epicatechin, 2.33 and 1.74 mg of (+)-catechin and 5.1 and 3.9 mg of procyanidin B2, respectively.

2.3. Animal study

Male Wistar rats (6-week-old; body weight: 180 ± 5 g) were purchased from the Animal House of Autonomous Metropolitan University, Xochimilco Campus (Mexico City) and individually housed in stainless steel cages at a controlled temperature (22 ± 2 °C), 40–60% humidity and under 12-h dark: 12-h light cycle. Rats were allowed free access to standard diet and purified water for a 5-day acclimatization period. After this period, rats were weighed and randomly assigned to the different experimental groups (n = 7 per group): standard diet (SD) (Rodent Diet 2018; Teklad Global Harlan Laboratories, Inc. Madison, WI) containing (wt/wt) 18.6% protein, 6.2% fat, and 44.2% carbohydrate, with an energy content of 3.1 kcal/g, 18% energy from fat; high-fat diet (HF) (TD. 88,137; Teklad Global Harlan Laboratories, Inc. Madison, WI) containing (wt/wt) 17.3% protein, 21.2% fat, and 48.5% of carbohydrate, with an energy content of 4.5 kcal/g, 42% of energy from fat; HF + cocoa powder (Co, 1 g/kg bw); HF + cocoa extract (Co-Ex, 100 mg/kg bw); HF + (−)-epicatechin (Epi, 10 mg/kg bw); HF + (+)-catechin (Cat, 10 mg/kg bw); HF + procyanidin B2 (PB2, 10 mg/kg bw).

Purified water (control groups) and treatment compounds were administered by oral gavage daily during the experimental period. The cocoa extract and epicatechin doses used in this study were selected on the basis of their estimated content per gram of cocoa powder (116.8 mg and 9.25 mg, respectively/g cocoa powder). This epicatechin oral dose has already shown to be efficiently absorbed. Catechin and procyanidin B2 were administered at the same dose as epicatechin in order to compare their effects. Rats were allowed *ad libitum* access to purified water and their respective diets for 8 weeks. Body weight and food intake (individually) were monitored daily. At the end of the experimental period, rats were anesthetized with pentobarbital sodium (35 mg/kg IP) after withholding food for 12 h. Blood samples were collected from abdominal aorta into 3.5 mL-sterile gold BD Vacutainer test tubes for serum isolation by centrifugation at 2500 rpm for 15 min at 4 °C. Serum was aliquoted and stored at −80 °C until biochemical analysis. Liver and retroperitoneal, epididymal and mesenteric adipose tissues were removed, rinsed with phosphate buffered saline (PBS), weighed and immediately frozen in liquid nitrogen and stored at −80 °C until further use. Experimental protocol followed the Ethics Code for Animal Studies of the Escuela Nacional de Ciencias Biológicas (ENCB) and the Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care (NOM-062-ZOO-1999).

2.4. Biochemical analyses

Levels of serum TG were determined using a semi-autoanalyser (Ekem control Lab, Mindray, China). Serum total cholesterol (TC) and non-esterified fatty acids (NEFAs) were measured using commercially available enzymatic colorimetric assay kits (Randox Laboratories, Crumlin, United Kingdom and Wako Chemicals, Neuss, Germany, respectively).

2.5. Measurement of liver triglycerides and total cholesterol

Triacylglycerol and total cholesterol were measured in the liver as described by Zhou et al. Briefly, hepatic tissue (100 mg) was homogenized in 1 mL ice-cold PBS using a MagNA Lyser Instrument (Roche Diagnostics, USA). Then 2 mL chloroform/methanol (2: 1, *v*/v) were added to the homogenate and vortexed for 60 s. After standing for 12 h, each sample was centrifuged at 4000 rpm and 4 °C for 15 min. The organic phase was dried under nitrogen gas and the residue was dissolved in 3% Triton X-100. The hepatic triglyceride and cholesterol contents were analyzed using the same semi-autoanalyser as used for the serum analysis (Zhou et al., 2015).

2.6. Measurement of liver adipocytokines levels

Liver samples were homogenized 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). Homogenates were incubated for 10 min at 4 °C and centrifuged at 14,000 xg at 4 °C for 15 min. The supernatants were separated into two aliquots (500 μL each). One was immediately used for cytokine analysis, while the second was stored at −80 °C until further analysis. TNF-alpha, IL-6 and adiponectin levels were determined by ELISA in liver supernatants, as described previously. Total protein content was determined by the Bradford method (Bradford, 1976).

2.7. RNA Extraction and reverse-transcription polymerase chain reaction

Liver was homogenized in TRIzol® reagent (Invitrogen Life Technologies, Grand Island, NY) and total RNA was isolated according to the manufacturer's instructions. For quality and quantity control, RNA purity was evaluated using the GENESYSTM 10 Series (ThermoSpectronic, Thermo Fisher Scientific, Inc., USA). 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. The RNA samples were treated with amplification grade DNase I (Invitrogen™) before reverse transcription, in order to prevent trace DNA contamination. All RNA samples were stored at −70 °C in an RNA elution solution until further use (Hummon, Lim, Difilippantonio, & Ried, 2007).

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). Amplified cDNA was quantified using the spectrophotometric method (Abs 260 nm). 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: http://www.universal-probelibrary.com). Forward and reverse primers sequences of the selected genes are listed in Table 1. PCR amplification was performed in borosilicate glass capillaries using the LightCycler Nano Real-Time PCR System (Roche Diagnostics, Germany). Conditions of PCR assay 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). The data were analyzed in LightCycler software version 4.0. Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method, where

ΔΔCt = ΔCt of target sample - ΔCt of reference sample

Table 1
Sequences of primers used for RT-PCR.

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
PPARα	TTTAGAAGGCCAGGACGATCT	GCACTGGAACTGGATGACAG
PPARγ	GGGGGTGATATGTTTGAACTTG	CAGGAAAGACAACAGACAAATCA
PGC-1α	GGGTCATTTGGTGACTCTGG	GCAGTCGCAACATGCTCA
SIRT1	AACTTCACAGCATCTTCAATTGTATT	TGACACTGTGGCAGATTGTTATT
TNF-α	GCCAGAGGGCTGATTAGAGA	CAGCCTCTTCTCCTTCCTGA
IL6	ACAACATCAGTCCCAAGAAGG	CCTTCAGGAACAGCTATGAA
ACC	GATCCCCATGGCAATCTG	ACAGAGATGGTGGCTGATGTC
CD36	TCGAGACTTCTCACCAAGAGG	GGGAAAGTTATTGCGACATGA
TLR4	TGATCCATGCATTGGTAGGTAA	GGATGATGCCTCTTGCAT
GPx	CGACATCGAACCCGATATAGA	ATGCCTTAGGGGTTGCTAGG
SOD	TGGACAAACCTGAGCCCTAA	GACCCAAAGTCACGCTTGATA
18S	CGAACGTCTGCCCTATCAAC	TTGGATGTGGTAGCCGTTTC

ΔCt of target sample was the Ct value normalized to the endogenous housekeeping gene (18S), and ΔCt of reference sample was the Ct value for the calibrator also normalized to the endogenous housekeeping gene. The $\Delta\Delta CT$ is the difference in ΔCT (as described above) between the target (treated and HF groups) and reference samples (SD group). The final result of this method was presented as the fold change of target gene expression in a target sample (treated groups) relative to a reference sample (SD group), normalized to 18S ribosomal RNA gene. The relative gene expression was set to 1 for SD group because $\Delta\Delta CT$ is equal to 0 and therefore 2^0 is equal to 1.

2.7.1. Hepatic lipid peroxidation assay

Quantification of the malondialdehyde (MDA) content was assayed in the form of thiobarbituric acid-reactive substances by the method of Buege and Aust with minor modifications (Buege & Aust, 1978). Briefly, 50 mg of liver samples were homogenized in 0.5 mL cold phosphate buffer (pH 7.0), followed by the addition of 1 mL of a reagent containing trichloroacetic acid (TCA), thiobarbituric acid (TBA) and HCl (15% (*w*/*v*), 0.375% (*w*/*v*), and 0.25 N, respectively). The solution was kept in boiling water for 30 min. After cooling, the flocculent precipitate was removed by centrifugation at 1000 *xg* for 10 min. Absorbance was measured at 532 nm using a Bio-Mini Shimadzu Spectrophotometer. The MDA content was calculated using an extinction coefficient of $1.56 \times 10^{-5}\ M^{-1}\ cm^{-1}$ and expressed as nmol of malondialdehyde per mg protein. Tissue protein was estimated using the Bradford method (Bradford, 1976).

2.8. Statistical analyses

Prior to statistical analysis, data were tested for normality and homogeneity of variances by Shapiro–Wilk and Levene tests, respectively. Data were expressed as mean values ± SEM. For multiple comparisons, one-way ANOVA was performed followed by the Holm-Sidak test in all quantitative variables. Pearson correlation coefficients were calculated to determine the degree to which two continuous variables were related. We used the pooled data of all groups to calculate the correlation between the selected variables. $P < 0.05$ was considered significant. The statistical analyses were performed using SigmaPlot version 12.0 from Systat Software, Inc., San Jose California, USA.

3. Results

3.1. Effects of cocoa powder, cocoa extract and its main flavanols on physiological and biochemical parameters

Table 2 shows that initial body weights of all experimental groups were similar (184.6 ± 0.25), whereas after 8 weeks of high-fat diet consumption, final body weight of the HF group was 24.7% higher ($p < 0.05$) than that of the SD group. All treated groups significantly reduced final body weight compared to HF group; though Co-Ex and Co were the most effective treatments in reducing final body weight by 7.9% ($p < 0.05$). The mean daily energy intake of all high-fat diet-fed groups was significantly higher than that of SD group. However, Co treatment showed a significantly lower energy intake (7.8%, $p < 0.05$) than HF group. Liver and intra-abdominal WAT weights were significantly increased in all high-fat fed groups compared to SD group. However, Co-Ex, Co and PB2 treatments significantly decreased liver weight by 18.6, 15.8 and 11% ($p < 0.05$), respectively, compared to HF group. Additionally, Co-Ex, Co and Epi treatments significantly attenuated visceral adiposity (25.5, 22.2 and 12.8%, respectively; $p < 0.05$) compared to HF group, whereas Cat and PB2 treatments showed no effect. At the end of the experimental period, HF group showed significantly increased serum triacylglycerols (3-fold, $p < 0.001$) and total cholesterol (TC) levels compared to SD group (162.26 ± 2.03 vs. 84.4 ± 1.39 mg/dL, respectively; $p < 0.001$). All treatments improved lipid profile; though, Co-Ex and Co groups showed the greatest decrease in serum TG (25.6

Table 2
Effects of cocoa powder, cocoa extract and their main flavanols on physiological and liver/serum lipids associated with lipid metabolism.

	SD	HF	Co	Co-Ex	Epi	Cat	PB2
Initial weight (g)	184.9 ± 0.25	185.1 ± 0.24	184 ± 0.3	185 ± 0.27	184.1 ± 0.4	184.4 ± 0.24	184.9 ± 0.32
Final weight (g)	375.7 ± 2.2^{a}	468.6 ± 2.2^{e}	431.9 ± 1.1^{b}	431.1 ± 1.7^{b}	450.2 ± 1.9^{c}	455.4 ± 1.3^{cd}	458.3 ± 2.4^{d}
Energy intake (kJ/day)	325 ± 2.5^{a}	432.1 ± 2.7^{cd}	398.2 ± 3.8^{b}	442.8 ± 3.2^{d}	430.6 ± 3.6^{c}	426.5 ± 2.2^{c}	422.3 ± 2.5^{c}
Intra-abdominal fat pads weight (g)	15.9 ± 1.2^{a}	48.3 ± 3.8^{d}	37.6 ± 1.6^{b}	35.1 ± 1.7^{b}	42.1 ± 1.06^{c}	45.9 ± 2.9^{d}	48.6 ± 3.3^{d}
Liver weight (g)	9.4 ± 1.01^{a}	14.5 ± 1.29^{d}	12.2 ± 1.1^{b}	11.8 ± 1.04^{b}	13.9 ± 1.3^{cd}	14 ± 1.3^{cd}	12.9 ± 1.3^{bc}
Liver TC (mg/g tissue)	1.9 ± 0.04^{a}	7.5 ± 0.12^{c}	5.5 ± 0.09^{b}	5.5 ± 0.08^{b}	5.9 ± 0.12^{b}	5.6 ± 0.06^{b}	5.9 ± 0.08^{b}
Liver TG (mg/g tissue)	0.28 ± 0.02^{a}	20 ± 0.6^{f}	14.8 ± 0.31^{c}	15.5 ± 0.37^{d}	12.8 ± 0.48^{b}	15.1 ± 0.55^{cd}	16.9 ± 0.47^{e}
Serum TG (mg/dL)	39.7 ± 2.03^{a}	122 ± 1.11^{d}	83.2 ± 1.18^{b}	90.7 ± 1.39^{c}	92.2 ± 2.06^{c}	93.3 ± 1.63^{c}	93 ± 1.54^{c}
Serum FFA (mEq/L)	0.51 ± 0.02^{ab}	0.63 ± 0.02^{c}	0.46 ± 0.01^{b}	0.54 ± 0.02^{ab}	0.55 ± 0.01^{abc}	0.58 ± 0.02^{ac}	0.61 ± 0.02^{c}

Final body weight, energy consumption and lipid profile in serum and in liver of rats fed with standard (SD), high-fat diet (HF) and high-fat diet + cocoa (Co) or cocoa extract (Co-Ex) or epicatechin (Epi) or catechin (Cat) or procyanidin B2 (PB2) for 8 weeks. Data are presented as the means ± SEM; n = 7 per treatment group. All data were compared using one-way ANOVA followed by Holm-Sidak test for multiple comparisons. Values with different superscript letters in the same row are significantly different ($p < 0.05$).

and 31.8%, respectively; $p < 0.05$) compared to HF group. Additionally, Epi, Co and Co-Ex were the most effective treatments in decreasing serum TC compared to HF group (TC: ~136.6 ± 1.7 vs. 162.26 ± 2.03 mg/dL; $p < 0.05$). FFA serum levels increased significantly in HF group. Co-Ex treatment significantly decreased FFA levels (14.3%; $p < 0.05$) compared to HF group, whereas Co group showed FFA levels similar to those of the SD group ($p < 0.001$). Hepatic triglyceride and cholesterol contents in HF group were higher (71.7- and 3.9-fold, respectively; $p < 0.001$) than those in SD group. In contrast, all treated groups significantly decreased hepatic cholesterol content by 24.4% ($p < 0.001$) compared to HF group. Epi was the most effective treatment in lowering hepatic triglyceride levels (35.6%; $p < <0.001$) compared to HF group, followed by Co, Co-Ex and Cat groups (24.7%; $p < 0.001$). Positive correlations between both hepatic cholesterol and triglyceride content with total intra-abdominal fat pads weight ($r = 0.95$ and $r = 0.94$, respectively; $p < 0.001$) and liver weight ($r = 0.87$ and $r = 0.85$, respectively; $p < 0.001$) were observed.

3.2. Effect of cocoa powder, cocoa extract and its main flavanols on hepatic lipogenic gene expression

Fig. 1(A – C) shows that the HF group significantly upregulated the hepatic mRNA expression of PPARγ (1.9-fold, $p < 0.001$), CD36/FAT (4.6-fold, $p < 0.001$) and ACC (8.4-fold, $p < 0.001$) compared to SD group. Fig. 1A shows that all treated groups significantly decreased ($p < 0.05$) PPARγ mRNA expression compared to HF group, though Co-Ex group showed the greatest effect (2.3-fold decrease, $p < 0.001$). Similarly, Fig. 1B shows that Co-Ex, Epi and Co were the most effective treatments in decreasing CD36 mRNA levels compared to HF group (1.5-, 1.4- and 1.3-fold, respectively; $p < 0.001$). Moreover, compared to HF group, all treatments significantly decreased ($p < 0.001$) hepatic expression of ACC, though Co, Epi and Co-Ex showed the greatest effect (1.9- and 1.7-fold, respectively; $p < 0.001$) (Fig. 1C).

3.3. Effect of cocoa powder, cocoa extract and its main flavanols on mRNA expression of genes involved in hepatic fatty acid oxidation

As shown in Fig. 2A, hepatic mRNA expression of PPAR-α was upregulated in all high-fat fed groups compared to SD group, though, Co and Co-Ex groups showed significantly higher PPAR-α expression levels (1.5-fold, $p < 0.05$) than HF group. Fig. 2B shows that HF group significantly downregulated PGC1-α expression (3.1-fold; $p < 0.001$) compared to SD group, whereas all treated groups showed significantly higher expression levels ($p < 0.001$) compared to HF group. Co and Co-Ex groups showed the highest levels of hepatic PGC1-α expression (3.4-fold increase compared to HF group; $p < 0.001$). Similarly, Fig. 2C shows that even though all treated groups, with the exception of PB2, significantly upregulated ($p < 0.05$) SIRT1 expression compared to HF group, Co and Co-Ex showed to be the most effective treatments (1.7-fold, $p < 0.001$).

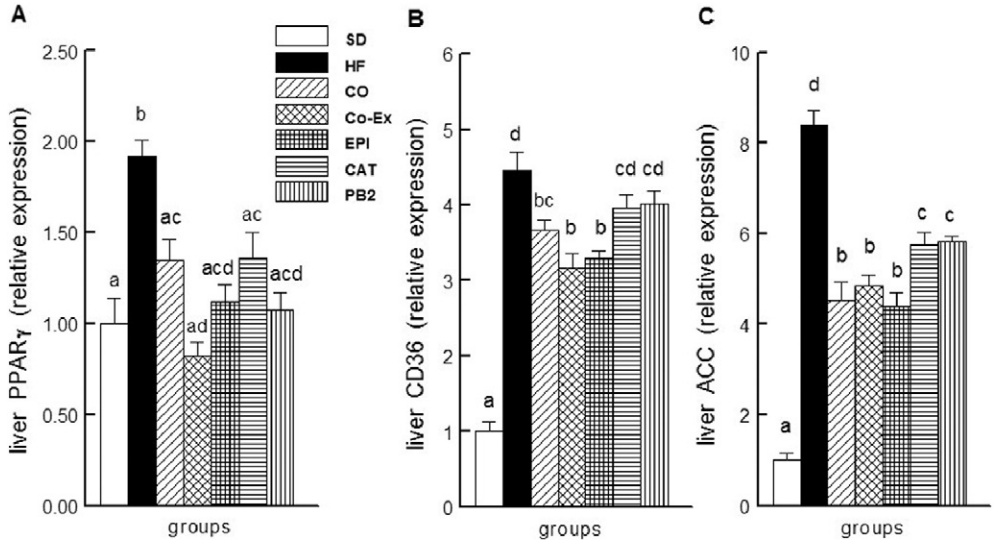

Fig. 1. Effect of cocoa powder, cocoa extract and their main flavanols on mRNAs encoding proteins involved in hepatic fatty acid uptake and accumulation. Quantitative real-time PCR assays of the genes encoding (A) PPARγ (B) CD36 (C) ACCα. The relative expression level of each gene was quantified and normalized to 18S ribosomal RNA for each sample. Data are expressed as the fold change compared with SD group that was arbitrarily set to 1 (means ± SEM, n = 7/group). Data were compared using one-way ANOVA followed by Holm-Sidak test for multiple comparisons. Different letters indicate statistical significance ($p < 0.05$). 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).

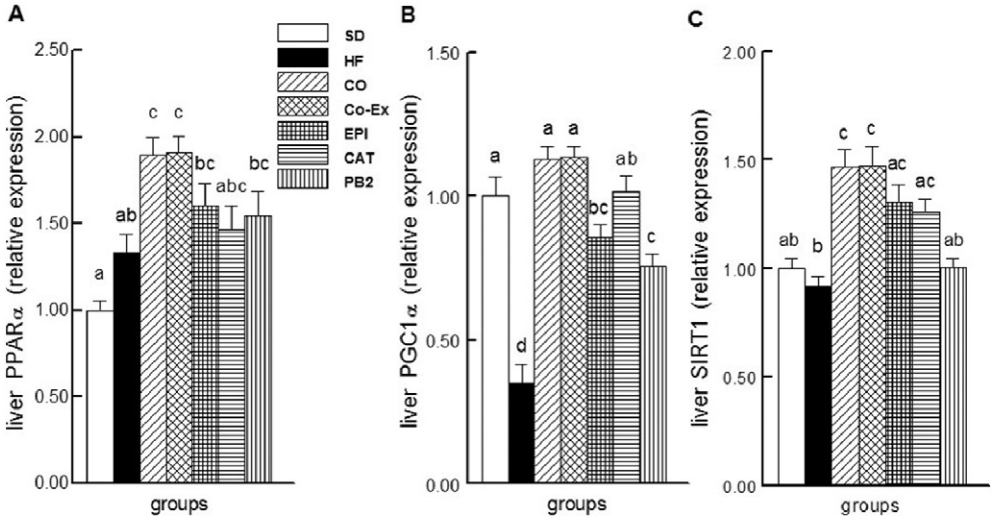

Fig. 2. Effect of cocoa powder, cocoa extract and their main flavanols on mRNAs encoding proteins involved in hepatic fatty acid oxidation. Quantitative real-time PCR assays of the genes encoding (A) PPARα (B) PGC1α (C) SIRT1. The relative expression level of each gene was quantified and normalized to 18S ribosomal RNA for each sample. Data are expressed as the fold change compared with SD group that was arbitrarily set to 1 (means ± SEM, n = 7/group). Data were compared using one-way ANOVA followed by Holm-Sidak test for multiple comparisons. Different letters indicate statistical significance ($p < 0.05$). 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).

3.4. Effect of cocoa powder, cocoa extract and their main flavanols on hepatic lipid peroxidation and mRNA levels of antioxidant enzymes

Fig. 3A shows that hepatic MDA levels were significantly increased in HF group (51.2%, $p < 0.001$) compared to SD group; whereas all treatment groups significantly decreased MDA levels compared to HF group. As shown in Fig. 3B, all treated groups exhibited significantly higher GPx mRNA expression levels than the HF group. Co-Ex was the most effective treatment in increasing GPx mRNA levels (1.6-fold, $p < 0.001$) compared to HF group, followed by Co, Epi and Cat groups (~1.4-fold, $p < 0.05$). Likewise, Fig. 3C shows that all treatments significantly upregulated SOD mRNA expression by ~1.8-fold ($p < 0.001$) compared to HF group.

3.5. Effect of cocoa powder, cocoa extract and its main flavanols on hepatic inflammatory markers

Fig. 4(A – C) shows that mRNA expression levels of TNF-α, IL6 and TLR4 were higher (7.5-, 3.9- and 5.2-fold, respectively; $p < 0.001$) in HF group than in SD group. TNF-α mRNA levels in Co-Ex, Epi and Co groups were markedly lower (2.8-, 2.6- and 2.3-fold, respectively; $p < 0.001$) than those in HF group. Additionally, Co-Ex, Epi and Co treatments significantly downregulated hepatic IL-6 levels (1.6- and 1.4-fold, $p < 0.001$) compared to HF group. All treated groups showed a significant reduction of TLR4 mRNA expression (~1.7-fold, $p < 0.05$) compared to HF group. Fig. 4D and E show a significant increase in hepatic TNF-α (2-fold, $p < 0.001$) and IL-6 (2.4-fold, $p < 0.001$) levels in the HF group

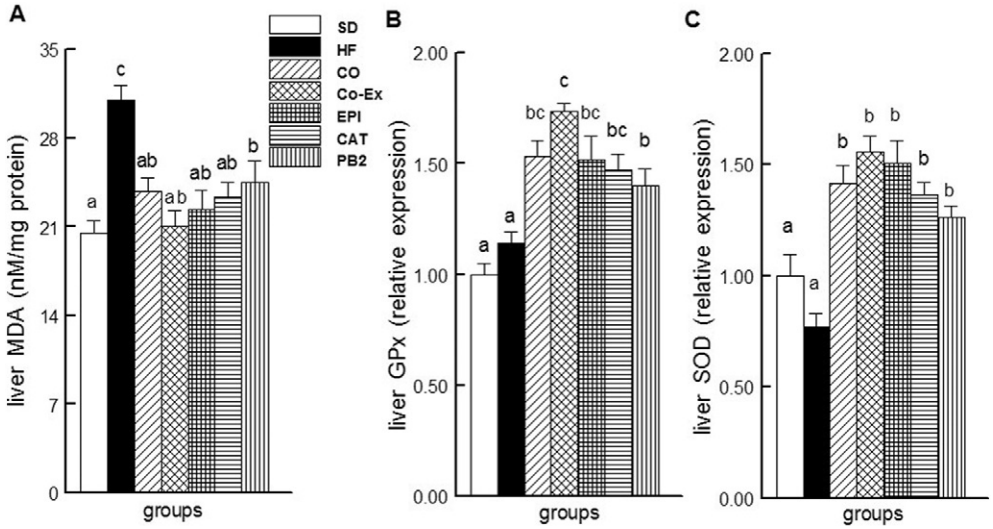

Fig. 3. Effect of cocoa powder, cocoa extract and their main flavanols on (A) liver lipoperoxidation and quantitative real-time PCR assays of the genes encoding (B) GPx and (C) SOD. The relative expression level of each gene was quantified and normalized to 18S ribosomal RNA for each sample. Data are expressed as the fold change compared with SD group that was arbitrarily set to 1 (means ± SEM, n = 7/group). Data were compared using one-way ANOVA followed by Holm-Sidak test for multiple comparisons. Different letters indicate statistical significance ($p < 0.001$). 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).

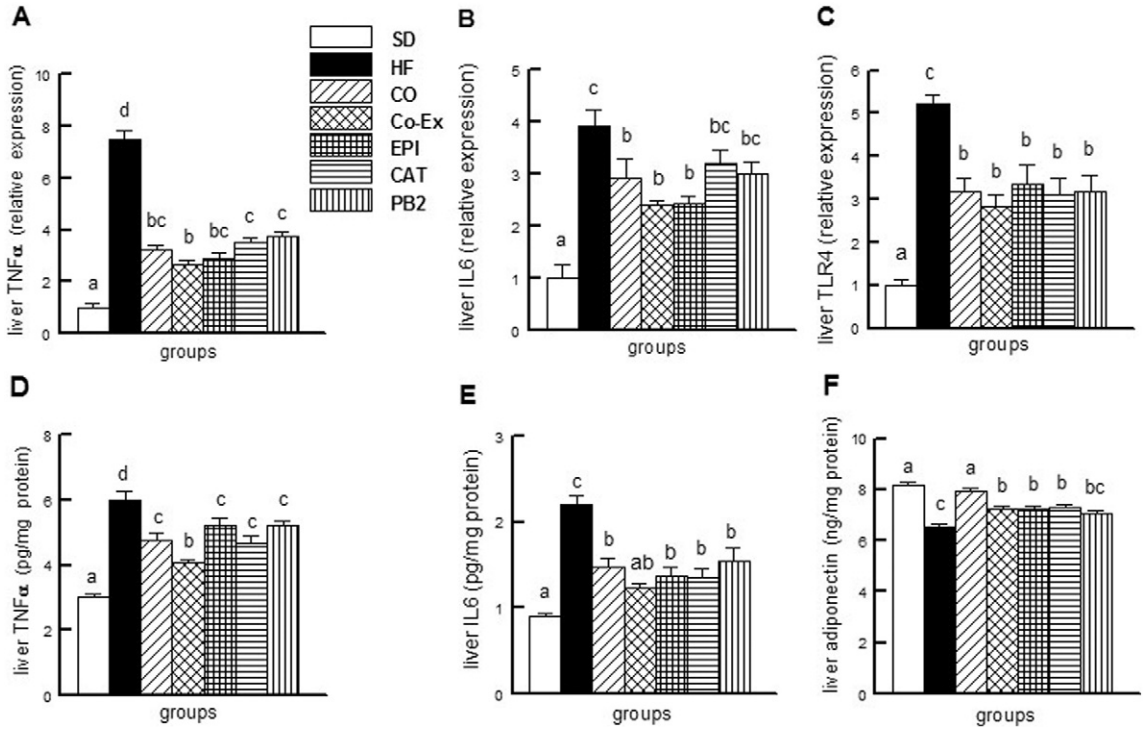

Fig. 4. Effects of cocoa powder, cocoa extract and their main flavanols on hepatic markers of inflammation. Hepatic mRNA expression of genes encoding (A) TNF-α, (B) IL-6 and (C) TLR4. The relative expression level of each gene was quantified and normalized to 18S ribosomal RNA for each sample. Data are expressed as the fold change compared with SD group that was arbitrarily set to 1 (means ± SEM, n = 7/group). Concentrations of proinflammatory and anti-inflammatory cytokines in liver samples: (D) TNF-α, (E) IL-6 and (F) ApN. Data are presented as the mean ± SEM (n = 7/group). All data were compared using one-way ANOVA followed by Holm-Sidak test for multiple comparisons. Different letters indicate statistical significance ($p < 0.001$). 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).

compared to the SD group. TNF-α levels were significantly reduced in Co-Ex, Cat and Co groups by 33.5, 23.5 and 21.9%, respectively ($p < 0.001$) compared to HF group. All treatments decreased IL-6 hepatic levels compared to HF group; however, Co-Ex was the most effective treatment in reducing IL-6 levels (45.4%, $p < 0.001$). Hepatic adiponectin levels were significantly lower (17.9%, $p < 0.001$) in HF group than in SD group, whereas Co-Ex, Epi and Cat treatments significantly increased ApN levels by 7.4% ($p < 0.001$) compared to HF group. Co treatment showed ApN levels similar to those of the SD group (Fig. 4F).

4. Discussion

Clinical and epidemiologic studies have suggested a direct association between abdominal obesity and development of hepatic steatosis, which occurs when the rate of hepatic fatty acid uptake from serum and *de novo* fatty acid synthesis is greater than the rate of fatty acid oxidation and export (as VLDL particles).

In order to face the epidemic increase in obesity and associated dysregulation of hepatic lipid homeostasis, experimental studies have focused on using food sources with high content of polyphenolic compounds like cocoa powder to enhance lipid metabolism (Aguirre, Portillo, Hijona, & Bujanda, 2014).

The molecular mechanisms by which cocoa polyphenols ameliorate obesity-related fatty liver in human and animal studies, have not yet been fully defined. In this study, we demonstrated that oral administration of cocoa powder, cocoa extract and its main flavanols (Epi, Cat and PB2) to rats fed a hypercaloric diet, modulate the expression of genes involved in the regulation of lipid metabolism (PPARγ, CD36, ACC, PPARα, PGC1α, SIRT1), inflammation (TNF-α, IL-6 and TLR4) and oxidative stress (GPx and SOD) in liver. In agreement with previous studies, our results show that Co, Co-Ex and Epi treatments attenuate body weight gain, intra-abdominal fat mass accumulation and serum dyslipidemia in rats fed a high-fat diet (Cordero-Herrera, Martín, Fernández-Millán, et al., 2015; Cordero-Herrera, Martín, Goya, et al., 2015; Gu, Yu, & Lambert, 2014).

PPARγ is a critical transcription factor highly expressed in adipose tissue and less expressed in many other tissues such as the liver. Several murine models of obesity and diabetes have shown that hepatic overexpression of PPARγ and its target gene (FAT)/CD36 play a key role in the development of liver steatosis via enhanced lipid uptake and upregulation of genes encoding lipogenic enzymes, such as Fas and ACC (Morán-Salvador et al., 2011; Zhang et al., 2006). It has been clearly established that hepatic *de novo* lipogenesis (DNL) is very active in rodents fed with hypercaloric diets (high-fat and high-sucrose). ACC is a rate-limiting enzyme for FA biosynthesis and also a regulator of FA oxidation via malonyl-CoA synthesis, a potent inhibitor of CPT-1 (Koo, 2013).

Exposure of the liver to elevated levels of FFA, in response to high fat diets and obesity-associated insulin resistance, contributes to increased fatty acid uptake into the liver and subsequent IHTG accumulation that leads to the pathogenesis of NAFLD (Kawano & Cohen, 2013).

In the present study, overexpression of hepatic PPARγ, CD36 and ACC in response to a hypercaloric diet was significantly reduced in all treated groups, supporting significantly decreased hepatic lipid accumulation. These results are also in line with decreased serum NEFA levels in all treated groups, which have shown to be an important source of IHTG content (Liu, Bengmark, & Qu, 2010). Besides the effect of all treatments on PPARγ gene expression, it has been demonstrated that several bioactive compounds, including cocoa powder and epicatechin, downregulate hepatic SREBP-1c gene expression, which is a more potent transcriptional activator of lipogenic genes (Cordero-Herrera, Martín, Fernández-Millán, et al., 2015; Yogalakshmi, Sreeja, Geetha, Radika, & Anuradha, 2013).

Further complementary studies are needed to evaluate the effect of our treatments in the protein expression and/or activity of key regulators of hepatic lipogenic pathway.

Besides DNL, impaired FA oxidation has been considered as a major mechanism underlying NAFLD. PPARα is highly expressed and has been identified as a key regulator of genes involved in hepatic mitochondrial and peroxisomal β-oxidation (CPT-1 and ACOX) and microsomal Ω-oxidation (CYP4A subfamily) (Monsalve, Pyarasani, Delgado-Lopez, & Moore-Carrasco, 2013; Nassir & Ibdah, 2014). Additionally, mitochondrial content and function have shown to play a protective role in hepatic steatosis. PGC-1α is a master transcription coactivator involved in the regulation of the expression of genes involved in hepatic FA oxidation and mitochondrial oxidative phosphorylation (Liang & Ward, 2006; Nassir & Ibdah, 2014). It is well-established that PGC-1α is deacetylated and activated by SIRT1, a NAD^+-dependent deacetylase that also plays an important role in hepatic lipid and glucose homeostasis and energy balance (Gillum, Erion, & Shulman, 2011).

In the current study we observed that all groups fed on a hypercaloric diet showed upregulation of PPARα in response to an increased hepatic FA uptake compared to SD group. However, HF group significantly downregulated PGC-1α and SIRT1 expression supporting that mitochondrial dysfunction leads to impaired mitochondrial oxidative capacity and increased IHTG content (Gusdon, Song, & Qu, 2014). Conversely, all treatments showed to attenuate these effects by upregulating PPARα, PGC-1α and SIRT1 gene expression in the liver, thus resulting in a significant decrease in IHTG content.

These results are in agreement with several studies demonstrating that plant-derived polyphenols reduce hepatic lipid droplets in high fat-fed animals via upregulation of hepatic PPARα and FA oxidation enzymes gene expression (Wan et al., 2013; Yang et al., 2014; Yogalakshmi et al., 2013).

Hepatic fatty acid overload leads to impairment of mitochondrial β-oxidation and activation of alternative pathways in the peroxisomes (β-oxidation) and endoplasmic reticulum (Ω-oxidation) which increase the hepatocyte ROS load and therefore, hepatic vulnerability to oxidative stress and proinflammatory damage.

In the present study, hepatic steatosis in HF group enhanced oxidative stress, as evidenced by increased content of lipid peroxidation products (MDA), even though the gene expression of antioxidant enzymes (SOD and GPx) was not significantly decreased compared to SD group. Conversely, all treated groups showed to reinforce hepatic antioxidant defense system by significantly upregulating antioxidant enzymes mRNA expression and decreasing MDA levels. As SOD and GPx play a major role in detoxification of superoxide and hydrogen peroxide radicals, respectively, upregulation of both hepatic antioxidant enzymes, together with decreased IHTG accumulation by all treatments may protect the liver against ROS-mediated damage.

Hepatic oxidative stress and mitochondrial dysfunction constitute potent mechanisms underlying the central role of TNF-α in the pathogenesis of NAFLD (Carter-Kent, Zein, & Feldstein, 2008; Rolo et al., 2012). Our results suggest that decreased gene and protein expression of TNF-α in all treated groups may attenuate TNF-α-induced ROS production and inflammatory response associated with NAFLD. Previous studies have demonstrated that cocoa-derived monomeric (Epi and Cat) and dimeric flavanols (PB2) reduce NF-κB activation by reducing ROS overproduction, enhancing antioxidant enzyme gene expression and activity and by specific bonding to proteins involved in the NF-κB pathway (Fraga & Oteiza, 2011; Mackenzie et al., 2004; Vázquez-Agell et al., 2013).

Consistent with decreased hepatic TNF-α levels, hepatic gene and protein levels of IL-6 were decreased by all treated groups compared to HF group. Our results agree with previous *in vitro* studies showing that epicatechin and cocoa extract decreased gene expression and macrophage release of proinflammatory cytokines, such as TNF-α and IL-6 (Vázquez-Prieto, Bettaieb, Haj, Fraga, & Oteiza, 2012). In addition, several *in vitro* and *in vivo* studies have demonstrated that IL-6 stimulates lipogenesis (via STAT3 activation), leading to exacerbation of hepatic steatosis in HF-fed rodents (Kinoshita et al., 2008; Vida et al., 2015; Yamaguchi et al., 2011). Emerging evidence suggests that IL-6 is an important mediator of hepatic lipid metabolism, though some controversy exists related to the role of IL-6 in obesity-related hepatic steatosis. On the one hand, the administration of IL-6 has shown to alleviate diet-induced hepatic steatosis in IL-6 deficient mice by exerting a positive effect on PPARα (El-Assal, Hong, Kim, Radaeva, & Gao, 2004; Hong et al., 2004; Vida et al., 2013), but on the other hand the elevation of plasma and tissue IL-6 concentrations in diet-induced NAFLD has been associated with the upregulation of ACC and FAS expression (via STAT3 activation) and thereby with increased lipogenesis and steatosis aggravation (Glund & Krook, 2008; Kinoshita et al., 2008; Vida et al., 2015).

Based on these evidences, our results suggest that decreased levels of IL-6 in all treatments may be associated with downregulation of ACC expression and, therefore, with decreased hepatic lipid accumulation (TC and TG). However, more studies are needed to fully elucidate the role of cocoa and its main flavanols in the regulation of hepatic IL-6 signaling and its association with lipogenesis regulation on hepatic steatosis induced through a high-fat diet.

Recent experimental studies have shown that circulating FFA are important modulators of inflammatory pathways in hepatocytes through toll-like receptor 4 (TLR4) signaling. The activation of TLR4 triggers NF-kB activation, proinflammatory cytokine production (mainly TNF-α) and upregulation of genes involved in *de novo* lipogenesis (Jia et al., 2014; Miura, Seki, Ohnishi, & Brenner, 2010). In the present study, we found that all administered compounds significantly downregulated liver expression of TLR4 compared to HF group. These results were associated with decreased serum FFA, hepatic TG and cholesterol content and proinflammatory cytokines levels, which support that reduced hepatic TLR4 mRNA expression is related to attenuated HF-induced inflammation, lipotoxicity and insulin resistance.

ApN has been shown to protect against NAFLD through its anti-inflammatory and antilipogenic effects (Finelli & Tarantino, 2013; Liu et al., 2012; Ye & Scherer, 2013). In the current study, increased hepatic ApN levels in all treatment groups suggests that it may be involved in PPARα upregulation and reduced IHTG content. It has been suggested that PGC-1α acts as an important mediator of ApN effects on hepatic FA oxidation by providing a link between ApN and PPARα, thus PGC-1α upregulation by all treated groups may be associated with PPAR-α upregulation in the liver (Kong et al., 2010).

In addition, it has been shown that ApN exerts a potent lipid-lowering effect in liver by increasing phosphorylation of ACC via activation of AMPK (Liu et al., 2012; Xu et al., 2003). Hence, our results showed that increased ApN levels in all treated groups may contribute to ACC downregulation and decreased hepatic lipid accumulation. An inverse relationship has been shown to exist between TNF-α and adiponectin, as both are tightly regulated by a negative feedback mechanism.

In genetic and diet-induced murine models of obesity, increased TNFα-levels have shown to be partially responsible for the decreased adiponectin production. However, when anti-obesity therapeutic agents are administered, it is difficult to determine whether an increase in adiponectin levels suppress the production of TNF-α or vice versa.

However, considering the anti-inflammatory and regulatory effects of ApN on glucose and lipid metabolism (mainly through AMPK activation), it has been suggested that the increased production and secretion of adiponectin constitutes a potential mechanism for decreased expression and production of proinflammatory cytokines, such as TNF-α (Finelli & Tarantino, 2013; Tishinsky, Dyck, & Robinson, 2012; Yuji, 2010).

Additionally, it has been shown that ApN attenuates proinflammatory cytokine production by decreasing the translocation of nuclear factor kappa B (NF-κB) to the nucleus, suppressing the activation of Kupffer cells and hepatic stellate cells (main sources of IL-6 and TNF-α) and downregulating toll-like receptor-4 (TLR-4) gene expression in

macrophages (Fukushima et al., 2009; Huang, Park, McMullen, & Nagy, 2008; Jarrar et al., 2008; Wang, Wang, & Lee, 2011).

In the present study, we cannot ensure whether increased hepatic ApN levels contribute to decrease gene and protein expression of TNF-α or viceversa. However, since hepatic ApN-mediated signaling pathways lead to enhanced fat oxidation and reduced *de novo* lipogenesis, we suggest that increased ApN levels may represent a potential mechanism whereby cocoa, cocoa extract and its main flavanols modulate hepatic expression of molecules related to hepatic lipid metabolism and decrease TNF-α gene and protein expression.

Further studies are required to better understand and clarify the possible mechanisms through which cocoa and its flavanols regulate adiponectin synthesis and secretion and how they are associated with decreased levels of inflammatory markers (specially, TNF-α).

It has been proposed that this effect is mediated in part by decreasing ROS formation and attenuating nuclear translocation of NF-κB (Finelli & Tarantino, 2013). Thus, we propose that increased ApN levels reduce liver inflammation by reducing TNF-α and IL-6 levels through upregulation of SIRT1, PGC-1α and PPARα, which enhance hepatic mitochondrial function and attenuate lipid peroxidation.

In conclusion, the present study demonstrates that even though all treatments effectively attenuated hepatic lipid accumulation induced by hypercaloric feeding, cocoa powder and cocoa extract treatments produced more pronounced effects, as evidenced by the upregulation of key regulators of mitochondrial function and FA oxidation (PPARα, SIRT1 and PGC-1α) and downregulation of genes involved in hepatic fatty acid uptake and lipogenesis (PPARγ, CD36 and ACC). These effects contributed to reduced hepatic oxidative stress and inflammation by decreasing hepatic MDA levels, upregulating hepatic antioxidant enzymes (GPx and SOD) and decreasing gene and protein expression of proinflammatory cytokines (TNF-α and IL-6). Overall, these results suggest that the synergistic interactions between the major bioactive compounds in cocoa and cocoa extract (such as flavanols and methylxantines) may contribute to the beneficial effects of both treatments on diet-induced hepatic steatosis. In addition, epicatechin treatment demonstrated a significant improvement in hepatic lipid content, suggesting that it might be an important mediator of cocoa and cocoa extract effects. However, other bioactive substances present in cocoa need much further investigation to fully understand the health-promoting properties of cocoa.

Interestingly, our data suggest that increased hepatic ApN levels may play a significant role in mediating the beneficial effects of cocoa flavanols on hypercaloric diet induced-fatty liver disease, through the enhancement of the above-mentioned pathways. However, further investigations should evaluate the gene and protein expression of other transcription factors and key enzymes involved in hepatic lipid metabolism.

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References

Ables, G. P. (2012). Update on Pparγ and nonalcoholic fatty liver disease. *PPAR Research*, *2012*, 5.

Aguirre, L., Portillo, M. P., Hijona, E., & Bujanda, L. (2014). Effects of resveratrol and other polyphenols in hepatic steatosis. *World Journal of Gastroenterology: WJG*, *20*, 7366–7380.

Ali, F., Ismail, A., & Kersten, S. (2014). Molecular mechanisms underlying the potential antiobesity-related diseases effect of cocoa polyphenols. *Molecular Nutrition & Food Research*, *58*, 33–48.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry*, *72*, 248–254.

Browning, J. D., & Horton, J. D. (2004). Molecular mediators of hepatic steatosis and liver injury. *The Journal of Clinical Investigation*, *114*, 147–152.

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid, Peroxidation. In S. Flesicher, & L. Packer (Eds.), *Methods in Enzymology. Vol. 52*. (pp. 302–310). New-York: Academic Press.

Carter-Kent, C., Zein, N. N., & Feldstein, A. E. (2008). Cytokines in the pathogenesis of fatty liver and disease progression to steatohepatitis: implications for treatment. *Am J Gastroenterol*, *103*, 1036–1042.

Chen, Y., Varghese, Z., & Ruan, X. Z. (2014). The molecular pathogenic role of inflammatory stress in dysregulation of lipid homeostasis and hepatic steatosis. *Genes & Diseases*, *1*, 106–112.

Cordero-Herrera, I., Martín, M.Á., Fernández-Millán, E., Álvarez, C., Goya, L., & Ramos, S. (2015a). Cocoa and cocoa flavanol epicatechin improve hepatic lipid metabolism in in vivo and in vitro models. Role of PKCζ. *Journal of Functional Foods*, *17*, 761–773.

Cordero-Herrera, I., Martín, M.Á., Goya, L., & Ramos, S. (2015b). Cocoa intake ameliorates hepatic oxidative stress in young Zucker diabetic fatty rats. *Food Research International*, *69*, 194–201.

El-Assal, O., Hong, F., Kim, W. H., Radaeva, S., & Gao, B. (2004). IL-6-deficient mice are susceptible to ethanol-induced hepatic steatosis: IL-6 protects against ethanol-induced oxidative stress and mitochondrial permeability transition in the liver. *Cellular & Molecular Immunology*, *1*, 205–211.

Finelli, C., & Tarantino, G. (2013). What is the role of adiponectin in obesity related non-alcoholic fatty liver disease? *World Journal of Gastroenterology: WJG*, *19*, 802–812.

Fraga, C. G., & Oteiza, P. I. (2011). Dietary flavonoids: role of (−)-epicatechin and related procyanidins in cell signaling. *Free Radical Biology and Medicine*, *51*, 813–823.

Fukushima, J., Kamada, Y., Matsumoto, H., Yoshida, Y., Ezaki, H., Takemura, T., & Hayashi, N. (2009). Adiponectin prevents progression of steatohepatitis in mice by regulating oxidative stress and kupffer cell phenotype polarization. *Hepatology Research*, *39*, 724–738.

Gillum, M. P., Erion, D. M., & Shulman, G. I. (2011). Sirtuin-1 regulation of mammalian metabolism. *Trends in Molecular Medicine*, *17*, 8–13.

Glund, S., & Krook, A. (2008). Role of interleukin-6 signalling in glucose and lipid metabolism. *Acta Physiologica*, *192*, 37–48.

Gu, Y., Yu, S., & Lambert, J. (2014). Dietary cocoa ameliorates obesity-related inflammation in high fat-fed mice. *European Journal of Nutrition*, *53*, 149–158.

Gusdon, A. M., Song, K. -X., & Qu, S. (2014). Nonalcoholic fatty liver disease: pathogenesis and therapeutics from a mitochondria-centric perspective. *Oxidative Medicine and Cellular Longevity*, *2014*, 20.

Hong, F., Radaeva, S., Pan, H. -N., Tian, Z., Veech, R., & Gao, B. (2004). Interleukin 6 alleviates hepatic steatosis and ischemia/reperfusion injury in mice with fatty liver disease. *Hepatology*, *40*, 933–941.

Huang, H., Park, P. H., McMullen, M. R., & Nagy, L. E. (2008). Mechanisms for the anti-inflammatory effects of adiponectin in macrophages. *Journal of Gastroenterology and Hepatology*, *23*, S50–S53.

Hummon, A. B., Lim, S. R., Difilippantonio, M. J., & Ried, T. (2007). Isolation and solubilization of proteins after TRIzol extraction of RNA and DNA from patient material following prolonged storage. *BioTechniques*, *42*, 467–470.

Inoue, M., Ohtake, T., Motomura, W., Takahashi, N., Hosoki, Y., Miyoshi, S., & Okumura, T. (2005). Increased expression of PPARγ in high fat diet-induced liver steatosis in mice. *Biochemical and Biophysical Research Communications*, *336*, 215–222.

Jarrar, M. H., Baranova, A., Collantes, R., Ranard, B., Stepanova, M., Bennett, C., & Younossi, Z. M. (2008). Adipokines and cytokines in non-alcoholic fatty liver disease. *Alimentary Pharmacology & Therapeutics*, *27*, 412–421.

Jia, L., Vianna, C. R., Fukuda, M., Berglund, E. D., Liu, C., Tao, C., & Elmquist, J. K. (2014). Hepatocyte toll-like receptor 4 regulates obesity-induced inflammation and insulin resistance. *Nature Communications*, *5*, 3878.

Kawano, Y., & Cohen, D. E. (2013). Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. *Journal of Gastroenterology*, *48*, 434–441.

Kinoshita, S., Ogawa, W., Okamoto, Y., Takashima, M., Inoue, H., Matsuki, Y., & Kasuga, M. (2008). Role of hepatic STAT3 in the regulation of lipid metabolism. *The Kobe Journal of Medical Sciences*, *54*, E200–E208.

Kong, X., Wang, R., Xue, Y., Liu, X., Zhang, H., Chen, Y., & Chang, Y. (2010). Sirtuin 3, a new Target of PGC-1α, plays an important role in the suppression of ROS and Mitochondrial biogenesis. *PloS One*, *5*, e11707.

Koo, S. -H. (2013). Nonalcoholic fatty liver disease: molecular mechanisms for the hepatic steatosis. *Clinical and Molecular Hepatology*, *19*, 210–215.

Liang, H., & Ward, W. F. (2006). PGC-1alpha: a key regulator of energy metabolism. *Advances in Physiology Education*, *30*, 145–151.

Liu, Q., Yuan, B., Lo, K. A., Patterson, H. C., Sun, Y., & Lodish, H. F. (2012). Adiponectin regulates expression of hepatic genes critical for glucose and lipid metabolism. *Proceedings of the National Academy of Sciences of the United States of America*, *109*, 14568–14573.

Liu, Q., Bengmark, S., & Qu, S. (2010). The role of hepatic fat accumulation in pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Lipids in Health and Disease*, *9*, 42.

Mackenzie, G. G., Carrasquedo, F., Delfino, J. M., Keen, C. L., Fraga, C. G., & Oteiza, P. I. (2004). Epicatechin, catechin, and dimeric procyanidins inhibit PMA-induced NF-kappaB activation at multiple steps in jurkat T cells. *The FASEB Journal*, *18*, 167–169.

Marchesini, G., Bugianesi, E., Forlani, G., Cerrelli, F., Lenzi, M., Manini, R., & Rizzetto, M. (2003). Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology*, *37*, 917–923.

Miura, K., Seki, E., Ohnishi, H., & Brenner, D. A. (2010). Role of toll-like receptors and their downstream molecules in the development of nonalcoholic fatty liver disease. *Gastroenterology Research and Practice*, *2010*, 9.

Monsalve, F. A., Pyarasani, R. D., Delgado-Lopez, F., & Moore-Carrasco, R. (2013). Peroxisome proliferator-activated receptor targets for the treatment of metabolic diseases. *Mediators of Inflammation*, *2013*, 549627.

Morán-Salvador, E., López-Parra, M., García-Alonso, V., Titos, E., Martínez-Clemente, M., González-Periz, A., & Claria, J. (2011). Role for PPARgamma in obesity-induced

hepatic steatosis as determined by hepatocyte- and macrophage-specific conditional knockouts. *The FASEB Journal*, *25*, 2538–2550.
Nassir, F., & Ibdah, J. (2014). Role of Mitochondria in Nonalcoholic Fatty Liver Disease. *International Journal of Molecular Sciences*, *15*, 8713.
Ortega, N., Romero, M. -P., Macià, A., Reguant, J., Anglès, N., Morelló, J. -R., & Motilva, M. -J. (2010). Comparative study of UPLC–MS/MS and HPLC–MS/MS to determine procyanidins and alkaloids in cocoa samples. *Journal of Food Composition and Analysis*, *23*, 298–305.
Reddy, J. K., & Rao, M. S. (2006). Lipid metabolism and liver inflammation. II. fatty liver disease and fatty acid oxidation. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *290*, G852–G858.
Rolo, A. P., Teodoro, J. S., & Palmeira, C. M. (2012). Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radical Biology & Medicine*, *52*, 59–69.
Tishinsky, J. M., Dyck, D. J., & Robinson, L. E. (2012). Chapter One - Lifestyle Factors Increasing Adiponectin Synthesis and Secretion. In L. Gerald (Ed.), *Vitamins & Hormones*, *Volume 90*. (pp. 1–30). Academic Press.
Vázquez-Agell, M., Urpi-Sarda, M., Sacanella, E., Camino-Lopez, S., Chiva-Blanch, G., Llorente-Cortes, V., & Estruch, R. (2013). Cocoa consumption reduces NF-kappaB activation in peripheral blood mononuclear cells in humans. *Nutrition, Metabolism, and Cardiovascular Diseases*, *23*, 257–263.
Vázquez-Prieto, M. A., Bettaieb, A., Haj, F. G., Fraga, C. G., & Oteiza, P. I. (2012). (−)-epicatechin prevents TNFalpha-induced activation of signaling Cascades involved in inflammation and insulin sensitivity in 3 T3-L1 adipocytes. *Archives of Biochemistry and Biophysics*, *527*, 113–118.
Vida, M., Gavito, A. L., Pavón, F. J., Bautista, D., Serrano, A., Suarez, J., & Baixeras, E. (2015). Chronic administration of recombinant IL-6 upregulates lipogenic enzyme expression and aggravates high-fat-diet-induced steatosis in IL-6-deficient mice. *Disease Models & Mechanisms*, *8*, 721–731.
Vida, M., Serrano, A., Romero-Cuevas, M., Pavón, F. J., González-Rodriguez, Á., Gavito, A. L., & Baixeras, E. (2013). IL-6 cooperates with peroxisome proliferator-activated receptor-α-ligands to induce liver fatty acid binding protein (LFABP) up-regulation. *Liver International*, *33*, 1019–1028.
Wan, C. W., Wong, C. N., Pin, W. K., Wong, M. H., Kwok, C. Y., Chan, R. Y., & Chan, S. W. (2013). Chlorogenic acid exhibits cholesterol lowering and fatty liver attenuating properties by up-regulating the gene expression of PPAR-alpha in hypercholesterolemic rats induced with a high-cholesterol diet. *Phytotherapy Research*, *27*, 545–551.
Wang, S. N., Wang, S. T., & Lee, K. T. (2011). The potential interplay of adipokines with toll-like receptors in the development of hepatocellular carcinoma. *Gastroenterology Research and Practice*, *2011*.
Xu, A., Wang, Y., Keshaw, H., Xu, L. Y., Lam, K. S., & Cooper, G. J. (2003). The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. *The Journal of Clinical Investigation*, *112*, 91–100.
Yamaguchi, K., Itoh, Y., Yokomizo, C., Nishimura, T., Niimi, T., Umemura, A., & Yoshikawa, T. (2011). Blockade of IL-6 signaling exacerbates liver injury and suppresses antiapoptotic gene expression in methionine choline-deficient diet-fed db/db mice. *Laboratory Investigation*, *91*, 609–618.
Yang, Y., Li, W., Liu, Y., Sun, Y., Li, Y., Yao, Q., & Zhao, J. (2014). Alpha-lipoic acid improves high-fat diet-induced hepatic steatosis by modulating the transcription factors SREBP-1, FoxO1 and Nrf2 via the SIRT1/LKB1/AMPK pathway. *The Journal of Nutritional Biochemistry*, *25*, 1207–1217.
Ye, R., & Scherer, P. E. (2013). Adiponectin, driver or passenger on the road to insulin sensitivity? *Molecular Metabolism*, *2*, 133–141.
Yogalakshmi, B., Sreeja, S., Geetha, R., Radika, M. K., & Anuradha, C. V. (2013). Grape seed proanthocyanidin rescues rats from steatosis: A comparative and combination study with metformin. *Journal of Lipids*, *2013*, 11.
Yuji, M. (2010). Adiponectin: A key player in obesity related disorders. *Current Pharmaceutical Design*, *16*, 1896–1901.
Zhang, Y. -L., Hernandez-Ono, A., Siri, P., Weisberg, S., Conlon, D., Graham, M. J., & Ginsberg, H. N. (2006). Aberrant hepatic expression of PPARγ2 stimulates hepatic lipogenesis in a mouse model of obesity, insulin resistance, dyslipidemia, and hepatic steatosis. *Journal of Biological Chemistry*, *281*, 37603–37615.
Zhou, C., Zhou, J., Han, N., Liu, Z., Xiao, B., & Yin, J. (2015). Beneficial effects of neomangiferin on high fat diet-induced nonalcoholic fatty liver disease in rats. *International Immunopharmacology*, *25*, 218–228.