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Anti-obesity effect of cocoa proteins (*Theobroma cacao* L.) variety "Criollo" and the expression of genes related to the dysfunction of white adipose tissue in high-fat diet-induced obese rats

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ABSTRACT

The objective of this work was to evaluate if Cocoa Proteins (CP) from the cocoa-bean were able to reduce factors related to obesity and activated related gene targets against white adipose tissue (WAT) dysfunction in a rat model of hypercaloric diet-induced obesity. The results showed that the administration with 150 mg/kg/day of CP on a hypercaloric diet reduces body-weight gain, relative weight of WAT, serum triglycerides, NEFAs, insulin, and leptin levels, and pro-inflammatory factors, with an increase in serum HDL levels, activation (AMPK, PPAR-γ, PPAR-α, SIRT1, Plin1, and PGC-1α) and repression (TNF-α, SREBP-1c, Leptin and ACC) of the mRNA of transcription factors and proteins related to WAT dysfunction. The CP prevented dysfunction in WAT who is related to obesity by down-regulation of factors related to lipogenesis and up-regulation of those related to energy expenditure, lowering the release of triglycerides and NEFAs into peripheral tissues, thus decreasing pro-inflammatory processes.

1. Introduction

According to the World Health Organization (WHO), in 2016, the world population with overweight and obesity reached 1.9 billion, which is equivalent to 25.85% of the population. Therefore, the WHO considers obesity a pandemic of the XXI century. Obesity and overweight have been recognized as causing factors for non-communicable diseases (NCD), such as ischemic cardiopathy, cerebrovascular accidents, and type 2 diabetes mellitus that accounted for 29.8% deaths (16.8 million in 2016) of the total deaths worldwide.

It has been shown that obesity produces inflammation of the white adipose tissue (WAT), resulting from the accumulation of free-fatty acids (FFA) derived from the diet or *de novo* lipogenesis and their storage in the form of triglycerides (TG). The excess of TG in WAT leads to inflammation, increasing the blood release of reactive oxygen species (ROS) and numerous pro-inflammatory mediators, such as tumor necrosis factor alpha (TNF)-α, interleukine (IL)-6, and MCP-1 (McNelis & Olefsky, 2014; Minamino et al., 2009). This state causes hypoxia, fibrosis, necrosis, and the final dysfunction of the WAT. When WAT loses its FFA storage capacity, leads to an increase in the circulating levels of lipids which are deposited in peripheral tissues that are not specialized for their storage, provoking lipotoxicity, dyslipidemia, β-cell

Abbreviations: ACC, acetyl-CoA carboxylase; AcDP, acetone dry powder; AMPK, AMP-activated protein kinase; ApN, adiponectin; CD36, fatty acid translocase/CD36; CP, cocoa protein; CPF, cocoa polyphenols and flavanols; FAS, fatty acid synthase; FFA, free fatty acids; Glycerol-3-P, glycerol-3-phosphate; GyK, glycerol kinase; HDL, high-density lipoprotein; HF, high-fat diet; HF+CP, high-fat + CP; HOMA-IR, homeostasis model assessment insulin resistance; IL, Interleukin; IR, insulin resistance; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein 1; NCD, non-communicable diseases; NEFA, non-esterified fatty acids; PGC1-α, peroxisome proliferator-activated receptor γ coactivator 1-α; Plin1, Perilipin 1; PPAR, peroxisome proliferator-activated receptor; SIRT1, sirtuína-1; SREBP-1c, sterol regulatory element-binding protein/factor 1; STD, standard diet; TG, Triglycerides; TNF-α, tumor necrosis factor alpha; UCP-1, uncoupling protein 1; WAT, white adipose tissue; WT, wild-type

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dysfunction, insulin resistance (IR), hypertension, atherosclerosis, and a pro-thrombotic state (Mlinar & Marc, 2011; Unger & Scherer, 2010; Wang, Scherer, & Gupta, 2014). All of these factors are involved in the development of cardiovascular diseases and type 2 diabetes mellitus, which comprise major global health problems (Libby, Ridker, & Hansson, 2011). In spite of the effectiveness demonstrated against cardiovascular diseases and type 2 diabetes mellitus for pharmaceutical products, they also exert adverse effects that limit their use. Thus, statins have demonstrated to cause myotoxicity, harming and debilitating muscular tissue even after interruption of the treatment (Padala & Thompson, 2012; Sathasivam, 2012). Functional foods are being considered a promising alternative because of their efficacy and the absence of side effects. They are foods rich in proteins, peptides, and/or other phytochemicals that can exert a preventive or co-adjuvant effect in the treatment of NCD (Aluko, 2015; Girgih, Alashi, He, Malomo, & Aluko, 2014). Functional plant-protein enriched foods have demonstrated beneficial effects against obesity by inhibiting adipogenesis, (Kim, Kim, Kim, & Nam, 2015) or acting as hypolipemiant (Howard & Udenigwe, 2013).

Cocoa (*Theobroma cacao* L., Sterculiaceae family) "Criollo" variety was cultivated by the Mayas over 1500 years ago in Central America (Motamayor et al., 2002). The market for cocoa is more demanding in terms of sensory quality; thus, the "Criollo" variety is utilized for the elaboration of great quality chocolates, highly priced worldwide. In addition to its nutritional value, some studies have evidenced the beneficial effects against metabolic diseases, chronic inflammation, and hypertension of different phytochemicals present in cocoa such as polyphenols and flavonols (CPF) (Ali, Ismail, & Kersten, 2014; Rabadán-Chávez, Reyes-Maldonado et al., 2016; Rabadan-Chávez, Quevedo-Corona, Garcia, Reyes-Maldonado, & Jaramillo-Flores, 2016b; Rabadán-Chávez, Miliar Garcia et al., 2016). Furthermore, cocoa contains an elevated concentration of high-quality protein (14%), rich in hydrophobic and aromatic amino acids (de Brito et al., 2001; Ismail et al., 2012). Two storage cocoa proteins, called albumin (P32765) and vicilin (Q43358) with a protein content of 52 and 43%, respectively (Voigt, Biehl, & Wazir, 1993), have been well characterized, and some of their biological functions have been demonstrated (Rawel, Huschek, Sagu, & Homann, 2019). However, although the evidence on the role of plant protein-derived peptides against obesity has grown significantly (Howard & Udenigwe, 2013; Yao, Agyei, & Udenigwe, 2018), no data are available about the potential of cocoa proteins to release peptides with beneficial effects against this chronic disorder. Thus, the objective of this work was to evaluate the potential of cocoa (*Theobroma cacao* var. "Criollo") protein in a diet-induced obesity rat model, focusing on its effects on biochemical and molecular biomarkers associated with WAT dysfunction. The results from this stidy will allow increase the knowledge about the health benefits of Criollo cocoa proteins promoting its use as ingredient of new functional foods.

2. Materials and methods

2.1. Protein extraction from cocoa beans

Cocoa variety "Criollo" beans were collected in the Municipality of Tuxtla Chico, Chiapas, Mexico. The seeds were obtained from the pods, the mucilage and coat were removed, and the seeds were lyophilized and stored at −20 °C. The seeds were ground, and the flour was defatted in three phases: 1:15 (w/v) flour was dissolved in hexane:-chloroform (2:1, v/v), with three times-magnetic stirring during 90 min, and centrifugation at 4700*g* for 20 min at 4 °C. Once the supernatant was removed, the pellet was collected and allowed to dry in the extraction hood. The dry pellet was used to obtain the acetone dry powder (AcDP) that was prepared according to Voigt (Voigt et al., 1993) the cocoa protein (CP) was extracted from AcDP that was dissolved in a solution containing l0 mM Tris-HC1 (with 2 mM EDTA, pH 7.5), 0.5 M NaCl (with 2 mM EDTA and l0 mM Tris-HCl, pH 7.5), and 0.1 N NaOH. The supernatants were collected in each phase, mixed, and precipitated with 6 N HCl (pH 3.4), and centrifuged at 10,000*g* for 20 min at 4 °C. The final supernatant was discarded and the pellet was lyophilized and stored at −20 °C until further analysis.

2.2. Experimental animals

The methodology described (Grasa-López et al., 2016; Rabadán-Chávez, Reyes-Maldonado et al., 2016; Rabadan-Chávez, Quevedo-Corona et al., 2016, Rabadán-Chávez, Miliar Garcia et al., 2016) was followed to carry out the animal experiment. Male Wistar rats (180 ± 5 g of body weight) were used and randomly divided into three dietary groups (n = 7 per group) as follows: i) Standard Diet (STD) (Rodent Diet 2018, Teklad Global Harlan Laboratories, Inc., Madison, WI, USA), with a nutritional value of 3.1 kcal/g as energy density. The composition of the STD was: 44.2% carbohydrate, 18.6% protein, 6.2% fat, 3.4% polyunsaturated fatty acids (PUFA), 1.3% monounsaturated fatty acids (MUFA) and 0.9% saturated fatty acids (SFA); ii) High-Fat diet (HF) (TD. 88137; Teklad Global Harlan Laboratories, Inc.), with a nutritional value of 4.5 kcal/g and which composition was: 48.5% carbohydrate, 21.2% fat, 17.3% protein, 12.8% SFA, 5.6% MUFA, and 1.0% PUFA; and iii) HF + intragastric administration of CP in PBS solution (150 mg/kg/day) (HF + CP), using a feeding cannula. Animals were fed *ad libitum* with free access to water. Feed consumption was monitored daily and body weight was measured weekly throughout the experiment. The design of the experimental study is shown in Fig. 1. At the end of the experimental period, rats fasted for 12 h and they were anesthetized with pentobarbital sodium (35 mg/kg i.p.). The three WAT deposits were collected: retroperitoneal (rWAT), mesenteric (mWAT) and epididymal (eWAT). The excess of blood was removed by washing them with 1X phosphate buffer saline (PBS), and deposits were dried and their weight was measured. The percentage of relative weight was calculated as follows:

Relative weight (%): [WAT weight (g) × 100]/Body weight (g)

The experimental protocol was performed in accordance with the Ethics Code for Animal Studies of the Escuela Nacional de Ciencias Biológicas (ENCB-IPN, Mexico City, 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 analysis

At week 8, rats were overnight fasted and blood glucose levels were measured in venous blood using a glucometer (ACCU-CHEK® Performa, Roche Diagnostics, Indianapolis, IN, USA). In serum, the levels of cholesterol-LQ (#Ref.:41019, Spinreact CIMA Diagnostics, Spain), TG (#Ref.:41033; Spinreact CIMA Diagnostics, Spain), NEFA (#Ref.:FA115; Randox Laboratories LTD, United Kingdom;), HDL-c (#Ref.:1001097; Spinreact CIMA Diagnostics, Spain) and LDL-c (#Ref.:41023, Spinreact, CIMA Diagnostics, Spain) were determined using a semi-autoanalyzer (Ekem Control Lab, Mindray, China).

2.4. Enzyme-Linked ImmunoSorbent Assay (ELISA)

Serum levels of rat leptin, insulin, IL-4, IL-10, TNF-α, and MCP-1 were measured using commercial Enzyme-Linked ImmunoSorbent Assay (ELISA) kits, following the manufacturer's protocols (CUSABIO, Biotech Co., Ltd). Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated from the following formula:

HOMA-IR = fasting serum insulin (μU/mL) × fasting blood glucose (mg/dL)/405.

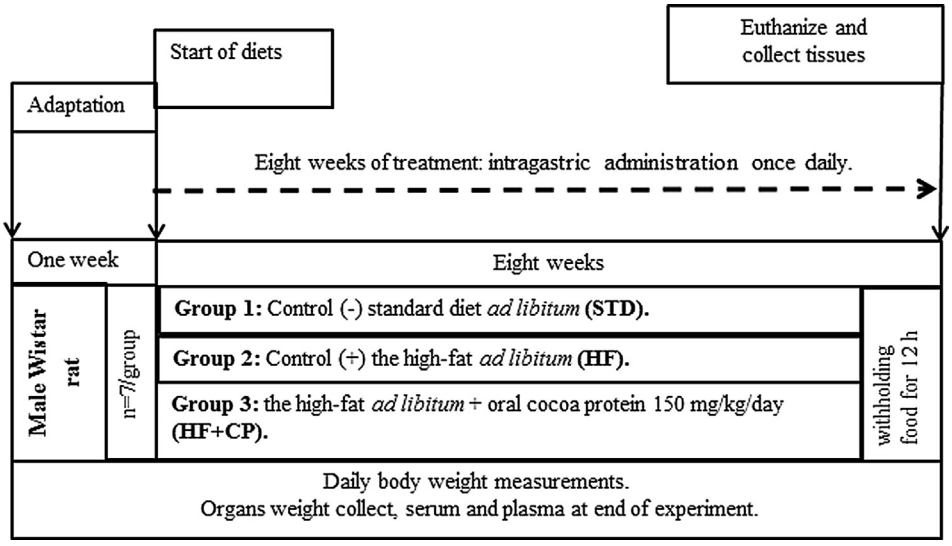

Fig. 1. Animal model experimental design.

2.5. RNA extraction and reverse-transcription polymerase chain reaction

The relative expression by Real-time PCR was carried out according to the methodology used by Rabadan-Chávez, Quevedo-Corona et al. (2016), Rabadán-Chávez, Miliar Garcia et al. (2016), using the rWAT as sample. The forward and reverse primer sequences of the selected genes are listed in Table 1. The relative expression levels of the messenger RNA (mRNA) target genes were normalized to 18S mRNA levels. The fold change or relative quantification in gene expression was determined using the $2_T^{-\Delta\Delta C}$ method (Livak & Schmittgen, 2001). Results were expressed in relation to the average expression of the STD group.

2.6. Statistical analysis

Data were expressed as mean values ± Standard Error of the Mean (SEM). All data were tested for normality and equality of variance using the Shapiro-Wilks and Levene test. One-way ANOVA was performed followed by the Holm-Sidak test (Sigma Plot ver. 12.0; SYSTAT Software, San Jose, CA, USA), for multiple comparisons in all quantitative variables. A value of $p < 0.05$ represented a significant difference. Figure constructions were performed using GraphPad Prism ver. 6 statistical software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effects of cocoa protein on energy intake, body weight, and the relative weight of WAT

According to the experimental design proposed (Fig. 1), energy consumption and weight of male Wistar rats were measured daily and weekly, respectively. The increase in weight of the group fed HF-diet was 301.6 ± 4.60 g, significantly greater ($p < 0.001$) than that measured in the group fed HF + CP-diet (278.5 ± 6.50 g) (Fig. 2A and B). However, energy consumption was similar in both groups (Fig. 2C and D). In Fig. 2E, the relative WAT weight of intra-abdominal fat mass of three deposits: retroperitoneal WAT (rWAT); epididymal WAT (eWAT), and mesenteric WAT (mWAT) is shown. The group fed the STD-diet showed the lowest intra-abdominal fat mass content (3.80 ± 0.19%), compared with the group fed the HF-diet (7.50 ± 0.17%; $p < 0.001$), and the group fed the HF + CP-diet (6.80 ± 0.27%) that were also significantly different ($p < 0.001$). Additionally, the relative intra-abdominal fat mass of eWAT and mWAT in animals fed HF + CP-diet were 2.50 ± 0.13% and 1.26 ± 0.08%, respectively, significantly lower than those determined in the group fed HP ($p < 0.001$). However, the percentage of fat present in rWAT was similar in HF + CP (2.97 ± 0.12%) and HF (3.00 ± 0.06%) groups. Both groups fed the HF diet showed a higher percentage of fat in the three depots in comparison with the STD group.

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
IL-10	TCATGGCCTTGTAGACACCTT	AGTGGAGCAGGTGAACAATGA
lep	AATGAAGTCCAAACCGGTGA	CCAGGATCAATGACATTTCACA
ApN	TGGTCACAATGGGATACCG	CCCTTAGGACCAAGAACACCT
CD36	TCGAGACTTCTCACCAAGAGG	GGGAAAGTTATTGCGACATGA
SREBP-1c	ACAAGATTGTGGAGCTCAAGG	TGCGCAAGACAGCAGATTTA
LPL	GTCTTGGAGCCCATGCTG	GGGGCTTCTGCATACTCAAA
ACC	GATCCCCATGGCAATCTG	ACAGAGATGGTGGCTGATGTC
AMPK-α	TCTTACCTCATCCGTGACCTC	CCTGGTCTTGGAGCTACGTC
PLIN1	TGTACAGGGTGCCAGCAA	GCAGGCCAACTCATTAGCAG
18S	CGAACGTCTGCCCTATCAAC	TTGGATGTGGTAGCCGTTTC

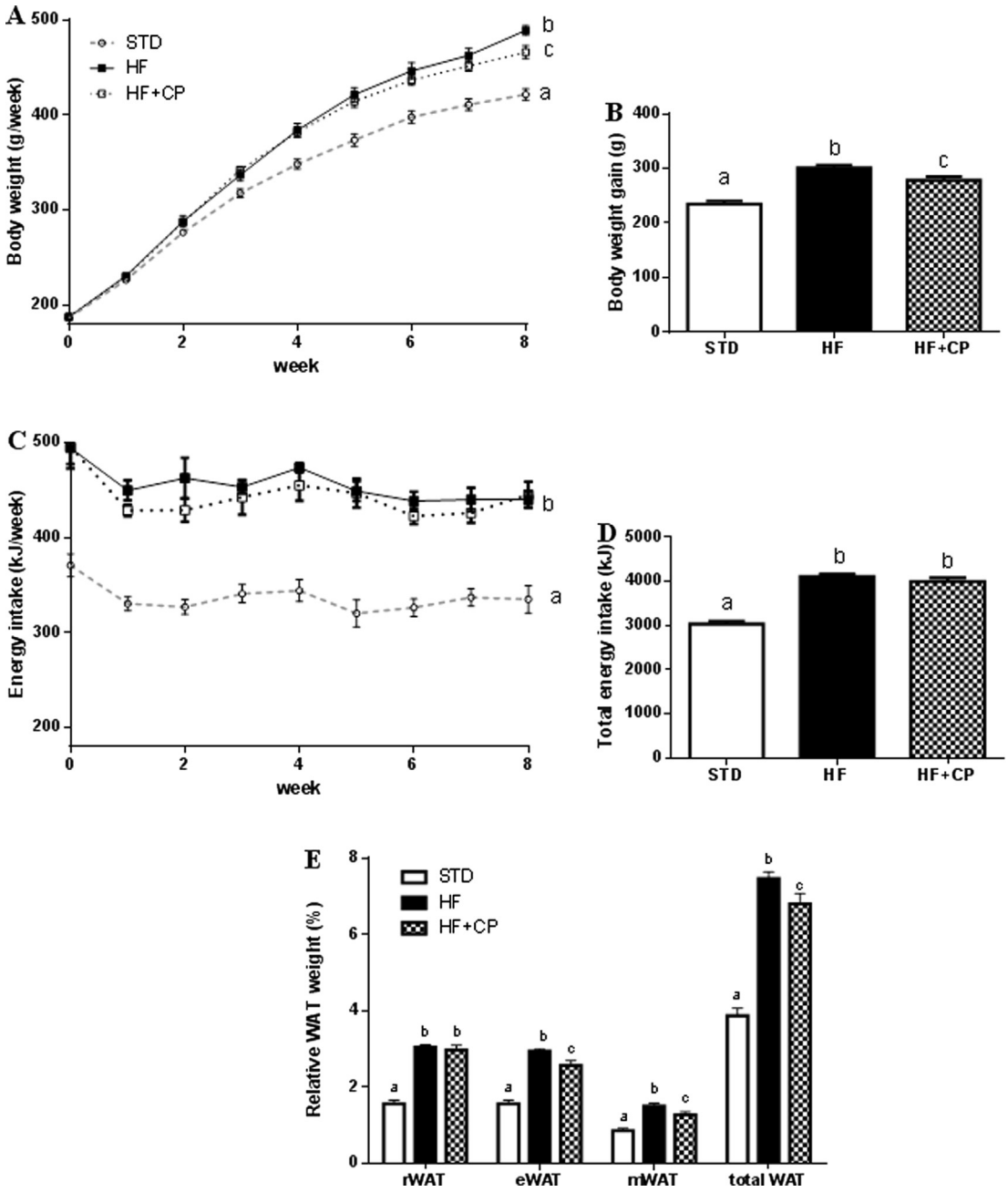

Fig. 2. Effect of "Criollo" cocoa proteins on (A) body weight, (B) body weight gain, (C, D) energy intake, and (E) relative weight of white adipose tissue (WAT). Data represented as mean ± Standard Error of the Mean (SEM) (n = 7). ^{a,b,c} indicate significantly different values ($p < 0.001$) by one-way ANOVA analysis.

3.2. Effect of cocoa protein on serum biomarkers and lipid inflammation mediators

The obesity-associated inflammatory markers were measured in serum (Fig. 3). The concentration of anti-inflammatory IL-10 in the STD group (50.05 ± 5.20 pg/mL) was significantly greater than that determined in the groups fed HF (18.26 ± 3.16 pg/mL) and HF + CP (13.21 ± 2.3 pg/mL), without significant differences between them (Fig. 3A). Similarly, no differences were observed in IL-10 mRNA levels in rWAT between HF and HF + CP groups (Fig. 3F). However, the reductions in the anti-inflammatory IL-4 levels caused by the HF-diet (38.00 ± 0.50 pg/mL) were partially reverted through the administration of CP (42.42 ± 1.9 pg/mL), reaching values similar to that determined in the STD group (44.9 ± 2.04 pg/mL) (Fig. 3B).

The serum concentration of the TNF-α is shown in Fig. 3C, where the levels increased significantly ($p < 0.001$) in the HF group (13.30 ± 0.12 pg/mL) in comparison with the control group (6.25 ± 0.02 pg/mL). The administration of CP significantly decreased the levels of TNF-α up to 12.40 ± 0.05 pg/mL ($p < 0.001$), compared with the HF group. In Fig. 3D, the level of the pro-inflammatory chemokine MCP-1 was increased after ingestion of the HF-diet (65.80 ± 1.70 pg/mL) while the co-administration of CP reduced it up to 45.60 ± 0.90 pg/mL. The mRNA levels of TNF-α measured in rWAT (Fig. 3E) of the group fed HF-diet were significantly higher than that measured in the STD and HF + CP groups (4.21- and 1.5- times greater, respectively).

3.3. Effect of cocoa protein on the serum lipid profile and leptin levels

In Fig. 4A and B, the levels of total cholesterol and LDL-c in the

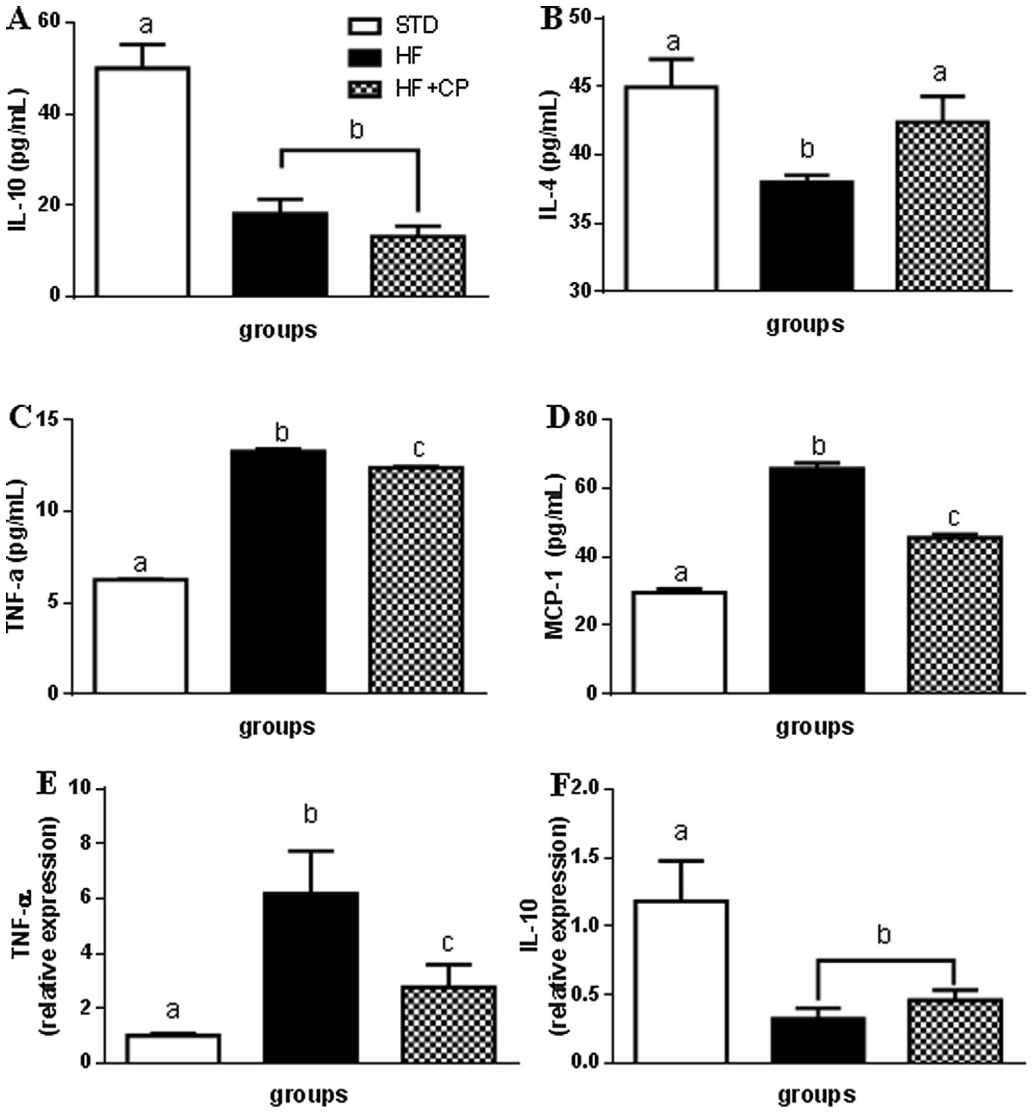

Fig. 3. Effect of "Criollo" cocoa proteins on the levels of inflammation-associated cytokines: (A) interleukin (IL)-10, (B) IL-4, (C) tumor necrosis factor (TNF-α), (D) MCP-1, and the relative expression of (E) TNF-α and (F) IL-10. Data represented as mean ± Standard Error of the Mean (SEM) (n = 7). [a,b,c] indicate significantly different values ($p < 0.001$) by one-way ANOVA analysis.

serum of animals showed no statistical differences among the three groups ($p < 0.05$). However, HDL-c levels decreased significantly in the HF group (52.1 ± 1.7 mg/dL) compared with STD (57.5 ± 3.6 mg/dL) and HF + CP groups (59.8 ± 1.9 mg/dL) without significant difference between these groups (Fig. 4C). Administration of CP also reverted the increase in TG levels (127.6 ± 4.1 mg/dL) caused by the HF diet (149.1 ± 11.7 mg/dL) although the value was still higher than that determined in the STD group (69.4 ± 5.2 mg/dL) (Fig. 4D). This reversion was also observed for NEFAs levels (0.92 ± 0.04 mEq/L in HF group and 0.70 ± 0.03 mEq/L in HF + CP group) (Fig. 4E). The levels of serum leptin in the animals fed HF-diet (4.92 ± 0.53 ng/mL) was 4.1- and 1.5-times higher than those determined in animals fed STD-diet (1.2 ± 0.06 ng/mL) and HF + CP diet (3.14 ± 0.26 ng/mL), respectively (Fig. 4F).

3.4. Effect of cocoa protein on glucose homeostasis and insulin resistance

The serum glucose levels are shown in Fig. 4G. A significant increase was observed when animals were fed HF diet alone or in combination with intragastric CP-treatment in comparison with animals fed STD. However, increased insulin levels in HF animals were reverted by CP (Fig. 4H). Similarly, HOMA-IR was reduced by CP administration (Fig. 4I) without reaching the values shown by the STD group.

3.5. Effects of cocoa protein on gene expression in retroperitoneal white adipose tissue (rWAT)

The ingestion of HF-diet supplemented with CP induced epigenetic changes on the rWAT mRNA level, as it is observed in Fig. 5. Thus, the expression of PPAR-α mRNA in the animals fed HF-diet decreased 3.5- and 9-times in comparison with HF + CP and STD groups, respectively. For the PPAR-γ gene, the expression in the HF + CP group was 11.6-times higher than that of the STD group and 1.6-times compared to the HF group (Fig. 5A and B). The mRNA expression levels of ApN were increased in animals fed HF diet, and more notably in animals treated with CP (Fig. 5C). However, CP was able to revert the increase in leptin gene expression provoked by HF diet, and thus, these levels were 2-times lower than that determined in animals fed HF diet (Fig. 5D). A 1.7-time reduction of HF-induced SREBP-1c gene expression was also observed in the HF + CP group (Fig. 5E). However, in the case of the relative expression of CD36, although increased in both groups fed the HF diet, there were no significant differences ($p < 0.05$) between them

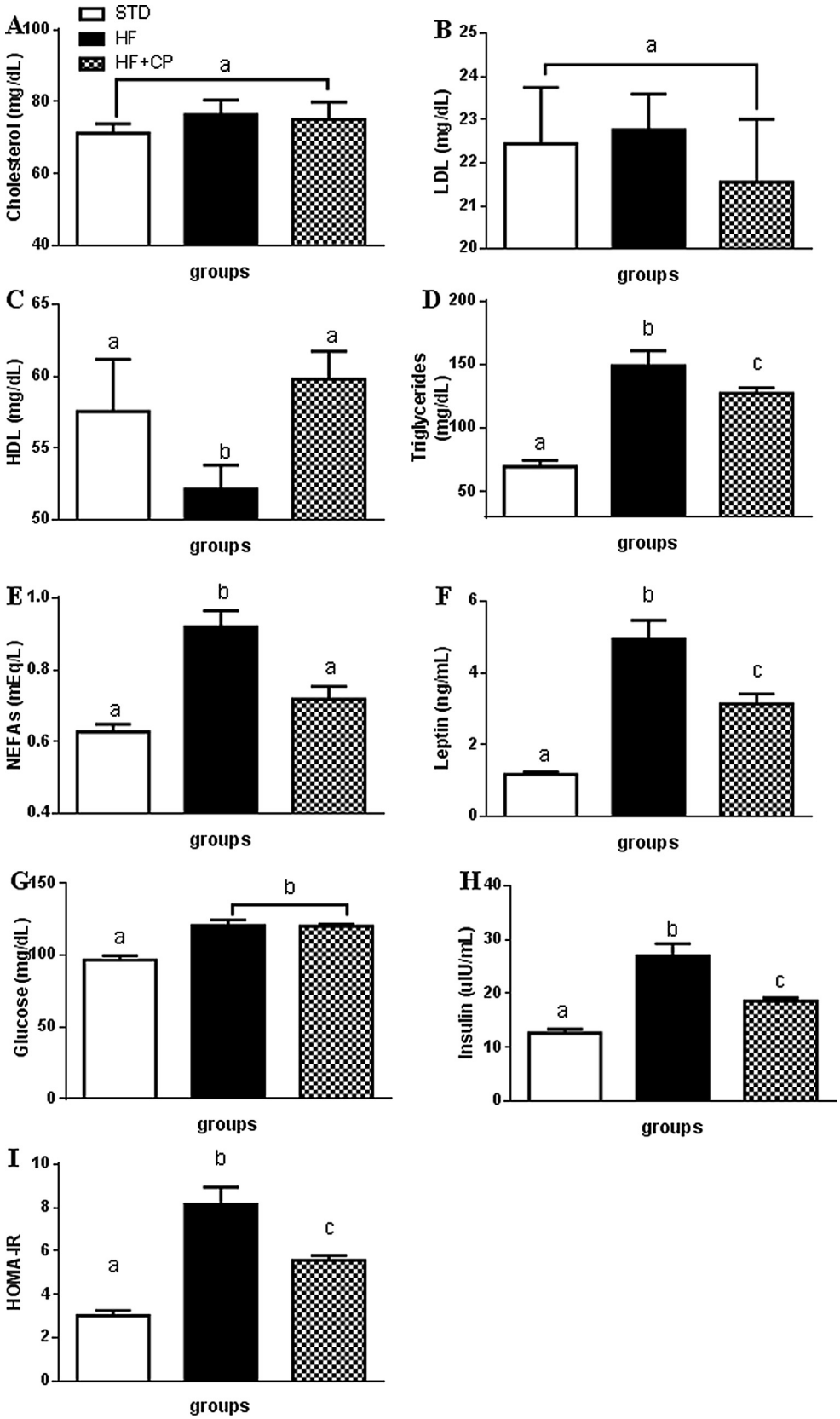

Fig. 4. Effect of "Criollo" cocoa proteins on the serum levels of (A) total cholesterol, (B) LDL-cholesterol (LDL-c), (C) HDL-cholesterol (HDL-c), (D) triglycerides (TG), (E) non-essential fatty acids (NEFAs), (F) leptin, (G) glucose, (H) insulin, and (I) HOMA-1R. Data represented as mean ± Standard Error of the Mean (SEM) (n = 7). ^{a,b,c} indicate significantly different values ($p < 0.05$) by one-way ANOVA analysis.

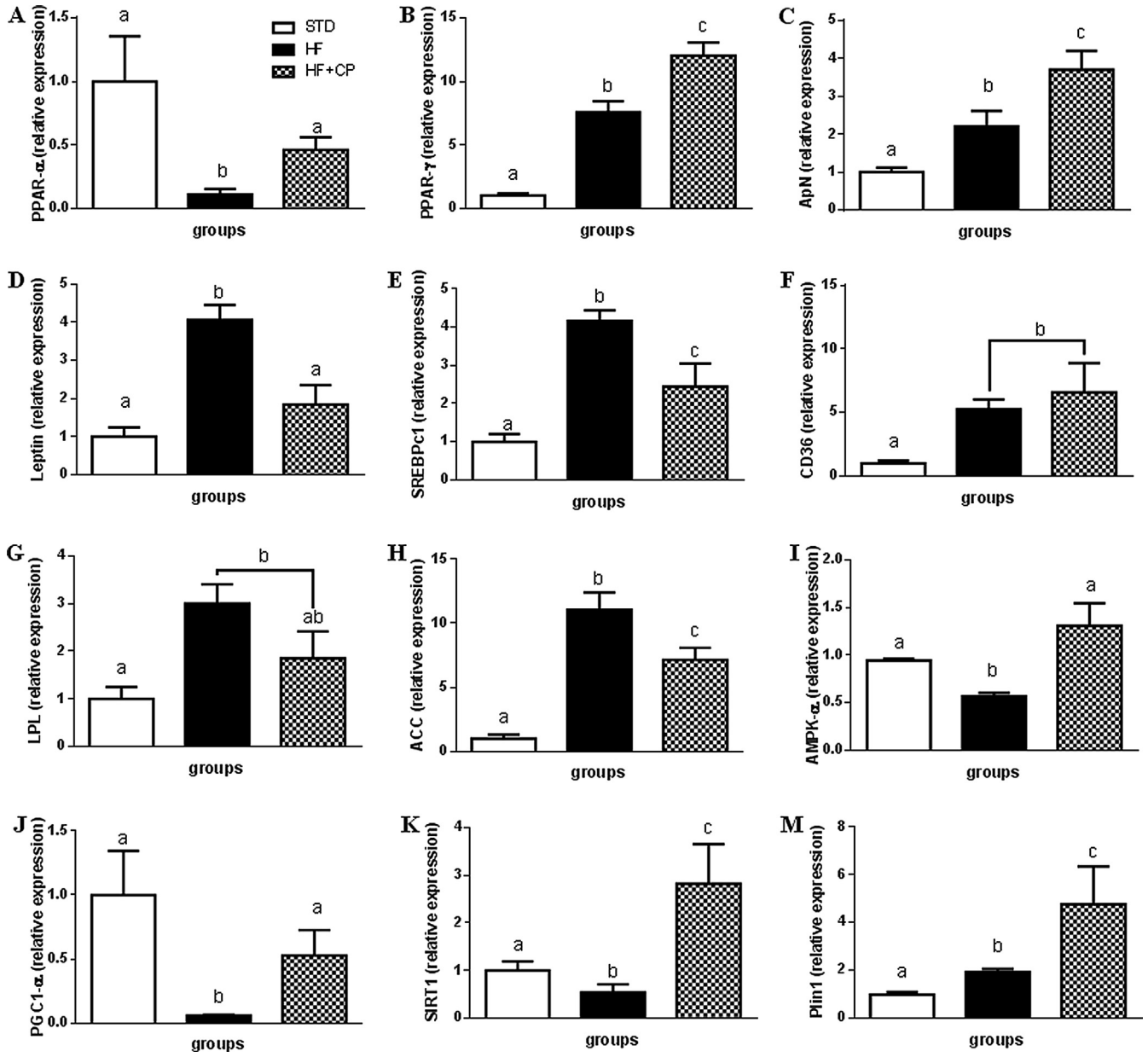

Fig. 5. Effect of "Criollo" cocoa proteins on the expression of genes associated with WAT dysfunction. (A) PPAR-α, (B) PPAR-γ, (C) ApN, (D) leptin, (E) SREBPc, (F) CD36, (G) LPL, (H) ACC, (I) AMPK-α, (J) PGC1-α, (K) SIRT1, and (L) Plin1. Data represented as mean ± Standard Error of the Mean (SEM) (n = 7). ^{a,b,c} indicate significantly different values ($p < 0.05$) by one-way ANOVA analysis.

(Fig. 5F).

The levels of LPL expression in HF and HF + CP groups did not presented significant differences among them, but were significantly higher than in STD group ($p < 0.05$, Fig. 5G) while the expression of ACC was increased in HF and reduced in CP group by 1.54-times compared with the HF group (Fig. 5H). Effects of CP were also observed in the expression of AMPK-α which was reduced to values similar to those determined in the animals fed STD ($p < 0.05$, Fig. 5I). In the case of PGC-*1α*, the reduced expression of this biomarker due to the HF diet was normalized by CP administration (Fig. 5J). Fig. 5K shows the relative expression of SIRT1 gene that was reduced in the group fed HF diet. However, no significant differences were observed between groups fed STD and HF + CP-diets ($p < 0.05$). The expression of Plin1 gene (Fig. 5M) was 7.4-times increased in the HF + CP group compared with the HF group.

4. Discussion

The present study examined the functional effects of the variety "Criollo" cocoa proteins on WAT dysfunction and obesity-associated serum parameters in a diet-induced obesity rat model. In this study, the protective effect of CP against the weight gain caused by the ingestion of HF-diet was demonstrated, without affecting energy consumption. Similar effects have been described when mice models were fed soybean protein isolates (Aoyama et al., 2000; Jang et al., 2008). In WAT, CP helped to decrease the mass of mesenteric and epididymal fat. Studies conducted with black soy proteins revealed a diminution of weight in the eWAT of C57Bl/6 mice (Jang et al., 2008). In our study, it is important to consider that the retroperitoneal fat determined in both groups fed HF diet did not exhibit differences, although total fat content was significantly lower in the group treated with CP. On the other hand, similar effects have been observed for CPF, where weight gain and intra-abdominal fat mass were reduced in rats fed fat-rich diets

(Cordero-Herrera, Martín, Goya, & Ramos, 2015; Gu, Yu, & Lambert, 2014; Gu, Yu, Park, Harvatine, & Lambert, 2014; Rabadan-Chávez, Quevedo-Corona et al., 2016). Obesity is a chronic disease characterized by the accumulation of lipids with an increase in the levels of pro-inflammatory cytokines and a reduction of the levels of anti-inflammatory cytokines surrounding the WAT (Castoldi, De Souza, Saraiva Câmara, & Moraes-Vieira, 2016). This condition has been associated with the development of NCD (Aroor, McKarns, Demarco, Jia, & Sowers, 2013; De Pergola & Silvestris, 2013; Rose, Gracheck, & Davis, 2015). In the group fed HF diet, a greater amount in total WAT was observed, resulting in a TNF-α increase, in comparison with the STD and HF + CP groups. TNF-α is a pro-inflammatory cytokine which induction contributes to the development of various inflammatory diseases, while its inhibition favors the decrease of obesity-induced IR (Andrade-Oliveira, Câmara, & Moraes-Vieira, 2015). In this work, the administration of HF + CP diet decreased serum insulin levels compared to HF diet, which results in the decrease of HOMA-IR index. Similar effects were observed in mice fed HF diet and treated with casein glycomacropeptide hydrolyzates (Song, Gao, Du, & Mao, 2018).

The concentration of the chemokine MCP-1 was higher in the HF group than in HF + CP and STD groups. It is clearly observable that CP decreased MCP-1, accelerating the infiltration of pro-inflammatory macrophages involved in adiposity and cancer development (Li, Knight, Snyder, Smyth, & Stewart, 2013; Panee, 2012). Therefore, the inducible effects of CP on MCP-1 and TNF-α levels, and TNF-α expression might contribute on the delay of the development of obesity-related inflammatory diseases.

The concentrations of TG and NEFAs were lower in STD and HF + CP groups while higher content of HLD-c was observed. This results is similar to previous studies carried out in mice fed soybean β-Conglycinin or Wistar rats fed goby fish protein and *Thornback ray* protein, in which an increase of HDL levels were observed (Lassoued et al., 2018; Nasri et al., 2018; Tsuchida et al., 2005). In other study, in mice fed a mixture of black soy peptides, a diminution of TG levels without changes in total cholesterol levels were described (Jang et al., 2008).

It is known that leptin regulates the energy equilibrium and the neuroendocrine function, producing satiety. In obesity, high levels of leptin in serum induce a state of resistance to this hormone, interrupting the delivery of signals to the hypothalamus (Ahima & Osei, 2004; Bates & Myers, 2003; Spiegelman & Flier, 2001). In this study, CP were able to decrease the mRNA expression of this hormone in adipose tissue, and its levels in serum. Similar results were described in mice fed β-Conglycinin and soybean glycinin, where the authors observed an decrease of leptin in serum (Moriyama et al., 2004) This hormone was also reduced in 3T3-L1 adipocytes treated with soybean peptide lunasin (Hsieh, Chou, & Wang, 2017).

With respect to the expansion of adipose tissue, *PPAR*-γ plays an important role when positive energy balance occurs (i.e. after ingestion of a HF diet) because the increase of the capacity for FFA absorption and storage in the adipocytes, reducing the deposition of ectopic lipids, especially in the liver and skeletal muscle (Goudriaan et al., 2005; Vroegrijk et al., 2013) In this work, it was found that the treatment with CP resulted in greater expression of PPAR-γ levels compared to HF group, with the concomitant diminution of serum TG and NEFAs levels. On the opposite way, a previous study had demonstrated inhibitory effects of tuna-derived peptides on *PPAR*-γ expression, (Kim et al., 2015) being these differences due to the use of 3T3-L1 cells instead of animals. It was demonstrated that *PPAR*-γ promote apoptosis in large and mature hypertrophic adipocytes while it stimulates the production of small insulin-sensitive adipocytes, performing an important role in the sensitivity to insulin (Leonardini, Laviola, Perrini, Natalicchio, & Giorgino, 2009; Medina-Gomez et al., 2007). This was described for Val-Pro-Pro and Ile-Pro-Pro lactotripeptides, whose capacity for inducing adipocytic differentiation and the positive regulation of *PPAR*-γ in 3T3-F442A cells, employing insulin as a positive control, was demonstrated (Chakrabarti & Wu, 2015). *PPAR-α* promotes lipolysis, and mitochondrial and peroxisomal β-oxidation of FFA (Vega, Huss, & Kelly, 2000). The up-regulation of *PPAR-α* in the WAT of animals treated with CP concurred with the low NEFAs levels found in serum. The normalization of *SIRT1* after treatment with CP suggests the protection exerted by these proteins in WAT. It has been demonstrated that *SIRT1* protects against the obesity-induced metabolic dysregulation acting by antagonizing metabolic deterioration in different cell lines (Dixon, Lane, MacPhee, & Philips, 2014; Mariani et al., 2015; Ramírez, 2013; Svensson, LaBarge, Martins, & Schenk, 2017). These results have also been demonstrated in mice, in which elimination of *SIRT1* in their adipocytes produced a greater degree of obesity-induced metabolic dysregulation (Hui et al., 2017).

The increase of mRNA expression of PGC-1α observed after administration of CP agree the results obtained by feeding rats with fish (*Pollachius virens*) protein hydrolyzates (Liaset et al., 2009). There are data that support that *SIRT1* interacts with PGC1-*α* promoting its transcriptional activity via deacetylation. Together, they regulate energy homeostasis and increase energy expenditure in WAT (Feige et al., 2008; Qiang et al., 2012). Previous studies showed that the metabolism of fatty acids in WAT is regulated by the interaction of PGC1-α and PPAR-α. This occurs in the lipolysis of TG (lipid drops) form NEFAs, which can accumulate in peripheral tissues. In order to avoid this, PGC1-α and PPAR-α are capable of inducing the synthesis of glycerol kinase (GyK), which produces glycerol-3-phosphate that react with NEFAs to newly form TG. Therefore, the activity of GyK allows the creation of a futile cycle of NEFAs mobilization and re-esterification. The interaction of PPAR-α and PGC1-α also stimulates the β-oxidation of NEFAs. Additionally, PGC1-α induces mitochondriogenesis and the expression of Krebs-cycle enzymes, the respiratory-chain proteins, and UCP-1, finally rising the utilization of NEFAs (Guan, Ishizuka, Chui, Lehrke, & Lazar, 2005; Langin, 2010; Mazzucotelli et al., 2007; Newsholme & Parry-Billings, 1992; Ryall & Goldrick, 1977). The positive regulation of PPAR-α, PGC1-α, and *SIRT1* in WAT have been proposed as a potential mechanism to reduce adiposity, and to improve metabolic disorders such as IR and dyslipidemia. Recent studies have demonstrated that the positive regulation and activation of these molecules increase the metabolic rate by promoting the β-oxidation of fatty acids (Rodgers, Lerin, Gerhart-Hines, & Puigserver, 2008; Rutanen et al., 2010; Tsuchida et al., 2005). Additionally, the diminution of the expression of ACC and SREBP-1c produced in the WAT of animals treated with CP could be an indicator of the reduction of the synthesis of fatty acids, thus reducing lipogenesis (Kang et al., 2012). The gene expression of transcription factor SREBP-1c regulates the lipid homeostasis by controlling the expression of enzymes such as FAS and HMG-CoA reductase used for the endogenous synthesis of cholesterol, NEFAs, and triacylglycerols (Eberlé, Hegarty, Bossard, Ferré, & Foufelle, 2004; Fajas et al., 1999).

A very important target in this study was the AMPK pathway because its relation to the synthesis and uptake of fatty acids, mitochondrial biogenesis, and synthesis of lipogenic enzymes (Hardie, 2018). Thus, the drugs that activate this pathway can comprise the most promising anti-obesity agents. AMPK improves mitochondrial biogenesis, (Zong et al., 2002) activating or stabilizing PGC1-α, and thus, favoring the synthesis of new mitochondrial components (Cantó et al., 2009; Jager, Handschin, St.-Pierre, & Spiegelman, 2007). AMPK also represses the expression of lipogenic enzymes, in part by SREBP-1c phosphorylation, (Li et al., 2011) and promotes the uptake of fatty acids in cells through fatty-acid transporter CD36 (Habets et al., 2009). The expression of AMPK was similar in both HF + CP and STD groups, indicating that this pathway could positively activate other targets related to the decrease of fatty acids levels found in this study. Thus, the administration of CP was able to increase the expression of AMPK and decrease the expression of ACC, which causes a reduction in malonyl-CoA synthesis, a target performing a crucial role in the metabolism of fatty acids, principally in lipogenic tissues such as liver, adipose tissue, and

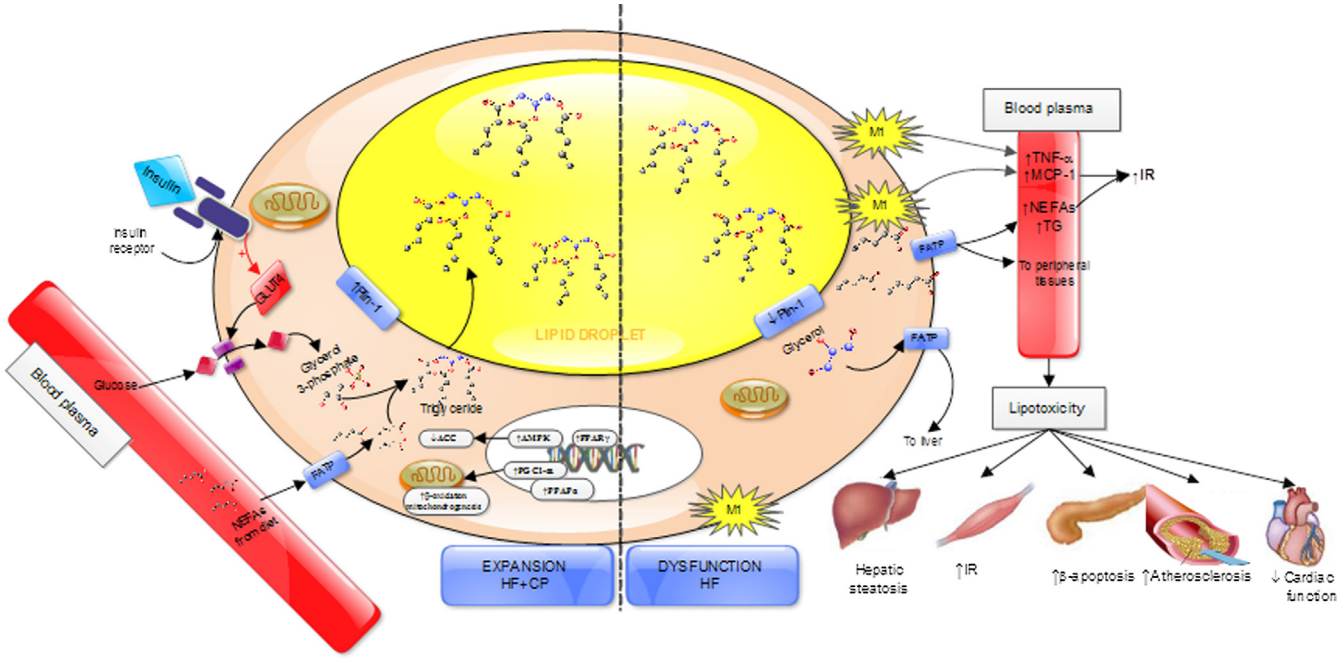

Fig. 6. Possible mechanism of action of "Criollo" cocoa proteins on white adipose tissue (WAT) dysfunction. HF + CP-diet represents the expansion capacity of WAT. Insulin can activate the insulin receptor allowing the activation of GLUT4 and the subsequent transport of glucose for becoming glycerol 3-phosphate. NEFAs from the diet can enter the WAT via fatty acid transport protein (FATPs). Three NEFAs are esterified to glycerol to produce triglycerides (TG) through the endoplasmic reticulum (ER) and storage within the lipid droplet. The expression of PCG1-α and PPAR-α favors the β-oxidation and re-esterification of NEFAs to TG and PCG1-α, producing an increase in mitochondrion genesis. Increased expression of AMPK may decrease the expression of ACC and PPAR-γ, allowing the development of new WAT capable of store TG, thus preventing storage in peripheral tissues. On the other hand, the HF diet represents the dysfunction of WAT. The excess of lipids generates an inflammatory process, the TG storage capacity of WAT is lost and TG is hydrolyzed to NEFAs, which are sent to peripheral tissues, increasing the lipotoxicity and the development of non-communicable diseases (NCD).

the mammary glands (Brownsey, Zhande, & Boone, 1997). Similar results were described for a mixture of isoflavone-free soy peptides, that increased the expression of AMPK, also inhibiting the expression of ACC in eWAT, resulting in an increase of fatty acids β-oxidation in the adipose tissue (Jang et al., 2008).

Plin1 is the major lipid droplet coating-protein, playing a central role in lipolysis regulation. In basal state, lipolysis is inhibited by Plin1. However, phosphorylation of Plin1 by PKA initiate TG lipolysis, activating the coactivator of adipose triglyceride lipase (ATGL) (Lass, Zimmermann, Oberer, & Zechner, 2011; Rutkowski, Stern, & Scherer, 2015). In this study, the increase in Plin1 levels observed in the group HF + CP compared with the HF group could reduce the lipolysis of TG, decreasing serum NEFAs and possibly their accumulation in peripheral tissues. As a summary, the potential mechanism of action of CP in WAT dysfunction is shown in Fig. 6.

5. Conclusions

This study demonstrates that CP were able to normalize WAT function altered by HF ingestion through activation of mechanisms that prevented the excessive synthesis and accumulation of lipids, and reduced inflammation and insulin resistance. Thus, these findings evidence the beneficial effects exerted by proteins from cocoa var. "Criollo" on obesity and associated metabolic disorders. Future research should be performed in order to identify the compounds responsible for the observed effects, confirming their activity and bioavailability.

Authors contributions

E.L.C., and G.R.C. proposed the project; G.R.C. designed the experiments; L.C.C., and G.R.C. performed the experiments; L.C.C. developed and wrote the manuscript; G.R.C., L.Q.C., B.H.L., A.M.G., and L.M. provided scientific guidance throughout the research and revised and edited the manuscript. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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