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# Cocoa protective effects against abnormal fat storage and oxidative stress induced by a high-fat diet involve PPARα signalling activation

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A high-fat (HF) diet increases lipid storage and oxidative stress in mouse liver and this process seems to be mediated by Peroxisome Proliferator-Activated Receptor α (PPARα). In this study we evaluated the protective effect of cocoa against hepatic steatosis induced by a HF diet. The HF diet down-regulated PPARα expression and turned off PPARα-signalling, deregulated the β-oxidation (β-Ox) system and catalase (CAT) activity, increased fat storage, reduced expression of enzymatic activity involved in oxidative defence in the liver and doubled the weight gain per calorie consumed compared to animals under the normal diet. In contrast, cocoa improved hepatic β-Ox, activated PPARα-signalling and up-regulated both gene and protein expression of SOD1. Moreover, when co-administered with the HF diet, cocoa treatment counteracted lipid storage in the liver, improved the lipid-metabolizing activity and oxidative stress defences and normalized the weight gain per calorie consumed.

## 1. Introduction

Epidemiological studies reported the benefits of a polyphenol-enriched diet especially concerning the prevention of cardiovascular diseases, cancer and other disorders, many of which are characterized by an imbalance between the production and removal of reactive oxygen species (ROS) and consequent oxidative stress.[1,2] ROS overproduction plays a pivotal role in the initiation, propagation, and development of several human diseases, such as obesity and obesity-related disorders (diabetes, cardiovascular disease, atherosclerosis, some types of cancer, hypertension), neurodegenerative diseases (Alzheimer's and Parkinson's disease) and ageing.[3–8] In animal models, administration of a HF diet shows induction of central obesity, insulin resistance, microvascular dysfunction,[9,10] and a simultaneous increase in oxidative stress parameters in the plasma, aorta wall and liver, developing hepatic

pathology.[11] The latter occurs when the maximum capacity of adipocytes to store lipids is reached and storage of lipid metabolites takes place in non-adipose tissues, e.g. the liver, muscles, pancreas, and blood. Consequently, in the liver both fatty acid input and lipogenesis increase, due to the hepatic insulin resistance. Hepatic fatty acid storage causes, in turn, an increase in fatty acid oxidation,[12,13] determining liver injury, including steatosis, glucose intolerance, oxidative and inflammatory stress and resulting in the overproduction of ROS.[14–18]

ROS is formed by the mitochondrial respiratory chain and, to a lesser extent, by some $H_2O_2$-producing peroxisomal enzymes.[19,20] Moreover, in the liver superoxide anions and hydrogen peroxide are also produced by microsomal Cytochrome P450, which is involved in the metabolism of xenobiotic compounds.[19–23]

The antioxidant defence system consists of multiple enzymatic and non-enzymatic molecules, which protect the cell against ROS. The most important enzymes are superoxide dismutases (SOD 1 and 2), which convert the superoxide anion into molecular oxygen and hydrogen peroxide; catalase, which reduces hydrogen peroxide to water and molecular oxygen; and glutathione peroxidase, which decomposes hydrogen peroxide using glutathione as a substrate.[24–28]

In addition to ROS-scavenging enzymes, polyphenols and several essential micronutrients, such as vitamins A, C, and E, zinc, selenium, play important roles in antioxidant defence.[29–32] For the former class of compounds, researchers have focused on the beneficial properties of polyphenols from food sources.[2,33]

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Various studies have highlighted the positive effects of cocoa on human health.[34–39] They are believed to result from the high amount of polyphenols (about 40–60 mg gallic acid equivalent (GAE) per g),[40] including monomeric flavan-3-ols, (+)-catechin and (−)-epicatechin, as well as oligomeric and polymeric procyanidins.[41–43] Polyphenols have several beneficial biological effects, such as anti-oxidative, anti-inflammatory and antineoplastic activities.[44–46] In particular, the antioxidant activity of flavonoids is due to the scavenging or quenching of oxygen free radicals[47] or due to direct inhibition of ROS generating enzymes.[48] Recent evidence has shown that several other substances present at low levels in cocoa may be implicated in physiological functions. This is the case of the hydrophobic compound oleoylethanoloamide (OEA), an acylethanolamide found in amounts up to 2 µg g$^{-1}$ in cocoa powder.[49] OEA, which is usually present at very low levels in many mammalian tissues and cells, plays an important role as a lipid-signalling molecule and is involved in satiety, memory consolidation and modulation of pain perception.[50,51] Moreover, it has been reported that OEA protects serum albumin against ROS-induced oxidation in seminal plasma of humans[52] and that OEA is produced in response to cellular stress.[53,54] Taken together, these observations suggest both a direct and indirect involvement of OEA in oxidative stress balance.

In mouse models, tea catechins stimulate fat oxidation through up-regulation of the β-Ox pathway.[55] PPARα transcriptionally regulates the expression of many lipid-metabolizing enzymes, such as ACOX, involved in the above pathway.[56] However, catechins are not ligands for PPARα, suggesting a β-oxidation independent-PPARα activation.[55] Matsui and co-workers found that after a three-week intake of cocoa, the expression of genes for enzymes involved in fatty acid synthesis in the liver and white adipose tissue of rats was suppressed.[57] In the nonalcoholic steatohepatitis (NASH) rat model, cocoa supplementation in the diet was associated with the increase of partial attenuation of NADPH oxidase and severity in inflammation and fibrosis.[58] Furthermore, it was observed that HF-fed obese mice supplemented with cocoa exhibited a reduction in obesity-related inflammation, insulin resistance and fatty liver disease.[59]

In our previous study,[60] we evaluated the effect of a two-week 10%-cocoa-enriched (C) diet in mouse liver on PPARα signalling and we concluded that cocoa raised the PPARα expression, mildly modulating the PPARα signalling. We also observed an increase in SOD1 gene expression, suggesting an antioxidant effect of cocoa. According to Abdelmegeed and co-workers, PPARα expression can protect male mice from high fat-induced nonalcoholic fatty liver.[16] Thus, we hypothesized that cocoa treatment could contribute to decrease the susceptibility to developing hepatic steatosis through both PPARα signalling activation and improvement of antioxidant enzyme's expression/activity. The aim of this study was to examine this hypothesis by comparing mice feeding for 4 weeks with a standard (N) diet, 10%-cocoa-enriched (C) diet, high-fat (HF) diet and high-fat 10%-cocoa enriched (HFC) diet. After cocoa powder characterization, we evaluated peroxisome proliferation, activation of PPARα signalling, steatosis and some oxidative stress-related parameters in the liver for all mentioned treatments.

# 2. Materials and methods

## 2.1. Chemicals

Ethanol, methanol, sodium carbonate, hydrochloric acid, sodium acetate and aluminum chloride were obtained from Carlo Erba (Milano, Italy). The Folin–Ciocalteu's phenol reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl), gallic acid and quercetin, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), bovine serum albumin (BSA), palmitoyl-CoA, β-NAD, FAD, nicotinamide, dithiothreitol, horse radish peroxidase, 2′,7′-dichlorofluorescein, aminotriazole, SYBR Green Taq and primers were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-catalase and Anti-SOD1 antibody were from Neo Markers (Creekwood Drive, Fremont, CA, USA); anti-GAPDH was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SYBR Green Taq was obtained from Sigma Chemical Co. (St. Louis, MO). SV Total Isolation RNA kit (SV Total RNA Isolation) and random hexamer were from Promega (Italy). Primers were acquired from Sigma-Aldrich, St Louis, MO, USA. Other chemicals were of reagent grade and used without further purification.

## 2.2. Characterization of cocoa powder

**2.2.1. Sample extraction procedure.** A three-stage extraction procedure was used to obtain samples for determination of total phenolics, total flavonoids and antioxidant activity.[61]

**2.2.2. Total phenolic content of cocoa powder.** Total phenolics were determined by the Folin–Ciocalteu's method as described by Singleton and co-workers.[62] The results were expressed as gallic acid equivalents (GAE) using a calibration curve obtained with gallic acid standards.

**2.2.3. Total flavonoid content of cocoa powder.** Total flavonoids were determined according to the procedure described by Zuorro and Lavecchia.[63] The results were expressed as quercetin equivalents (QE) using a calibration curve obtained with quercetin standards.

**2.2.4. Antioxidant activity of cocoa powder.** The antioxidant activity was determined by the DPPH radical-scavenging assay.[64] The antioxidant activity was calculated from $EC_{50}$ values (the concentration of the sample extract required to reduce the initial DPPH concentration by 50%) and expressed as Trolox equivalents (TE).

## 2.3. Diets and animal treatments

Specific diets were prepared by mixing triturated Purina chow with 10% cocoa (w/w) (C diet);[60] 5% lard, 1% cholesterol, 0.1% propylthiouracil and 0.3% taurocholic acid (w/w) (HF diet);[65] and 10% cocoa, 5% lard, 1% cholesterol, 0.1% propylthiouracil and 0.3% taurocholic acid (w/w) (HFC diet). Untreated animals were fed with Purine chow (N diet). Animal studies were performed in compliance with European Community specifications

regarding the use of laboratory animals. Swiss male mice about 35 g in weight (8-week-old) were kept at 20–22 °C with a 12 h light/12 h dark cycle. Then, they were randomly divided into 4 experimental groups and fed with different types of diets. Each group was treated with a specific diet (N, C, HF, HFC) for 4 weeks. Animals (5 for each experiment) were sacrificed after anesthesia with 500 mg kg$^{-1}$ of chloral hydrate. Livers were immediately excised and weighed. Same pieces of livers were quickly frozen in liquid nitrogen and stored at −80 °C. The residual part of the liver was used for enzyme assay and transmission electron microscopy (TEM) testing.

### 2.4. Enzyme assay

The liver was homogenized (30%, w/v) in cold 10 mM Tris-HCl buffer at pH 7.4 containing 0.25 M sucrose, 1 mM ethylenedi-aminetetracetate, 1 mM EDTA (sodium salt), 0.1% ethanol, 0.2 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride in a Thomas homogenizer, filtered through two layers of surgical gauze and frozen under nitrogen. The protein concentration, the CAT activity and the cyanide-insensitive fatty acid β-Ox system were assayed as previously reported.[66] Enzymatic activity was expressed as specific activity (mU mg$^{-1}$ of protein).

### 2.5. Isolation of total RNA and RT-PCR

Analysis of expression of mRNA was performed by RT-PCR amplification with respect to the house-keeping gene GAPDH. Total RNA was extracted using the SV Total Isolation RNA kit (SV Total RNA Isolation; Promega, Italy). RNA quality isolation was assessed using the A260/A280 ratio. RNA was stored at −70 °C. First-strand cDNA was transcribed from 2 μg RNA using random hexamer and MVL RT (Promega). Synthesized cDNA corresponding to 25 ng total RNA and SYBR Green Taq was used for amplification. Specific primers used in PCR reaction were as follows:

PPARα (forward) 5′-GAACCCAAGTTTGACTTCGC-3′; PPARα (reverse) 5′-TAATTTGCTGTGGAGATCGG-3′; CYP4a10 (forward) 5′-GTGTTTGACCCTTCCAGGTT-3′; CYP4a10 (reverse) 5′-CAAATTGTTTCCCAATGCAG-3′; PDK4 (forward) 5′-CTAGTGATGTGCGTGAGGCT-3′; PDK4 (reverse) 5′-TCTTGCTTCCCTCTCTCCAT-3′; ACOXI (forward) 5′-AAATCATTCAGGCCACCTTT-3′; ACOXI (reverse) 5′-CACAGCCTGCACTCATTCTT-3′; CATALASE (forward) 5′-ATCCAGGCTCTTCTGGACAA-3′; CATALASE (reverse) 5′-TGAGCACCGGAGTTACAGGT-3′; SOD1 (forward) 5′-CTCAGGAGAGCATTCCATCA-3′; SOD1 (reverse) 5′-CTCCCAGCATTTCCAGTCTT-3′ GAPDH (forward) 5′-ACATCATCCCTGCATCCACT-3′; GAPDH (reverse) 5′-ATCCACGACGGACACATTG-3′.

Data analysis was carried out using the iCycler Software (Bio-Rad). Amplification was made for 40 cycles, each cycle consisting of two steps: 15 s at 95 °C and 30 s at 60 °C.

### 2.6. Western blot assay

SDS polyacrylamide gel electrophoresis (about 50 μg of proteins) was performed on a 7.5% slab gel according to Laemmli.[67] Proteins from polyacrylamide gels were electro-blotted on a nitrocellulose membrane (Bio-Rad). After protein transfer, nitrocellulose membranes were incubated (1 h at room temperature) in 50 mM Tris-HCl buffer containing 150 mM NaCl and 3% BSA or 5% delipidated milk. The blots were then incubated overnight at 4 °C with anti-catalase (1 : 400), anti-SOD1 (1 : 200) and anti-GAPDH (1 : 200). Thereafter the preparations were incubated for 1 h at room temperature with a 1 : 2000 dilution of IgG alkaline phosphatase conjugate. The antigen antibody complexes were detected by alkaline phosphatase labelled protein using 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium for staining.

Densitometry was performed using the ImageJ software (NIH, http://rsb.info.nih.gov/ij/). Each band was normalized vs. the housekeeping protein GAPDH.

### 2.7. Triglyceride assay

Triglycerides were assayed using the Triglyceride Quantification Kit (Abcam, Cambridge, UK) according to manufacturer's instructions.

### 2.8. Transmission electron microscope

Small pieces of the liver from mice treated as above (three animals per group) were fixed by immersion in 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 1% calcium chloride for 1 h at 4 °C, rinsed three times in the same buffer and post-fixed in 1% OsO$_4$ in 0.1 M cacodylate buffer for 1 hour at 4 °C, dehydrated and embedded in Epon. Ultrathin sections, obtained with a Reichert Ultratome III ultramicrotome, were contrasted with uranyl acetate and observed in a Philips CM120 electron microscope equipped with a Philips Megaview III camera. Electronic images were captured using the AnalySys 2.0 software and composed in the Adobe Photoshop CS5 format.

### 2.9. Statistical analysis

Values are the means (±SD) for 5 different experiments run in duplicate. The student t-test was used to calculate statistically significant differences. For TEM images, three animals were used for each experimental condition. Assays for characterization of cocoa powder were carried out in triplicate and expressed as mean ± SD.

## 3. Results and discussion

Several studies have shown that a HF diet results in oxidative stress and inflammation in rodent liver.[68] On the other hand, Abdelmegeed and co-workers suggested that PPARα signalling may exert a protective function against HF-induced hepatic steatosis.[16] Indeed, after HF diet treatment, PPARα$^{-/-}$ animals showed higher fat storage and exacerbated oxidative stress, as compared to their wild-type counterparts.

Peroxisomes, which mediate a wide variety of biosynthetic and biodegradative reactions, are involved in the metabolism of very long fatty acids (VLCFAs) and ROS (markedly hydrogen peroxide), thus defining the cellular oxidative state. Moreover,

the redox imbalance in cells potentiates inflammatory responses, which can ultimately trigger human diseases.[69] Our previous results showed a moderate modulation of PPARα signalling in mouse liver and an increase in SOD1 gene expression after a two-week 10%-cocoa-enriched diet.[60] Based on these results, here we investigated the possible protective effect of cocoa in mouse liver following a four-week HF diet treatment, focusing on lipid storage, oxidative stress and PPARα signalling.

We first characterized the polyphenol content of the cocoa used in our study. The total phenolic content of cocoa powder was 40.86 ± 1.69 mg GAE per g and the total flavonoid content was 3.36 ± 0.27 mg QE per g. These values are comparable to those determined by Miller and co-workers.[40] The Trolox equivalent antioxidant capacity (TEAC) was 152.3 ± 7.7 μmol TE per g. As a result, the average intake of antioxidants for C and HFC diet treated animals was about 91.4 and 89.8 μmol TE per d, respectively.

As shown in Table 1, although differing in the macronutrient composition, chows for N and C diet groups and HF and HFC diet groups contained comparable calories per gram, respectively. In line with this, as reported in Table 2, although there was a slight difference in the average amount of food consumed, the energy intake was comparable for N and C diet groups and HF and HFC diet groups, respectively. Cocoa did not seem to affect the taste of food, since both C and N groups and HF and HFC groups consumed the same amount of chow. Conversely, the HF diet group showed a greater hunger while the HFC diet group did not. The weight gain after four weeks in N, C and – surprisingly – in HFC diet-treated animals was similar, while the HF diet group showed an increase in body

**Table 1** Chow composition

| | Proteins (%) | Lipids (%) | Carbohydrates (%) | Calories (kcal g$^{-1}$) |
|---|---|---|---|---|
| Standard | 18.4 | 6.2 | 48.0 | 2.97 |
| HF | 17.3 | 11.8 | 45.1 | 3.35 |
| C | 18.9 | 7.7 | 45.0 | 3.01 |
| HFC | 17.8 | 13.3 | 42.2 | 3.39 |

**Table 2** Weight gain, mean food consumption and energy intake

| | Weight gain (WG) (g) | Mean food consumption (g d$^{-1}$) | Energy intake (EI) (kcal d$^{-1}$) | Ratio WG : (EI × 28) |
|---|---|---|---|---|
| N | 3.00 ± 0.52 | 5.80 ± 0.25 | 17.23 ± 0.50 | 0.006 |
| HF | 7.00 ± 0.63* | 6.20 ± 0.10* | 20.77 ± 0.31* | 0.012 |
| C | 3.20 ± 0.40 | 6.00 ± 0.21 | 18.07 ± 0.40 | 0.006 |
| HFC | 3.10 ± 0.41# | 5.90 ± 0.07# | 20.01 ± 0.35* | 0.006 |

N, animals fed with a standard diet; HF, animals treated for 4 weeks with a high-fat diet; C, animals treated for 4 weeks with a cocoa-enriched diet; HFC, animals treated for 4 weeks with a high-fat cocoa-enriched diet. The ratio expresses the weight gain per intake of calorie during the treatment. * and ** significantly different from the N group, respectively, $P < 0.05$ and $P < 0.01$. # Significantly different from the HF group, $P < 0.05$.
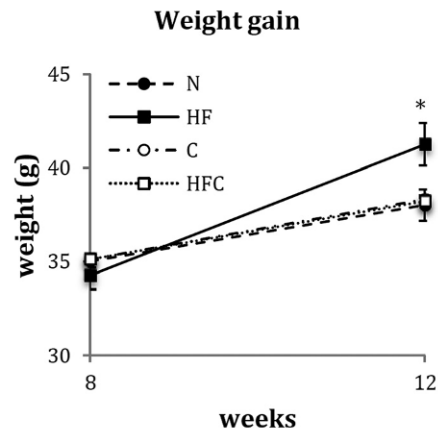


**Fig. 1** Weight gain during diet treatments. N, animals fed with a standard diet; HF, animals treated for 4 weeks with a high-fat diet; C, animals treated for 4 weeks with a cocoa-enriched diet; HFC, animals treated for 4 weeks with a high-fat cocoa-enriched diet. *Significantly different from the N group, $P < 0.05$.

mass that was 2.3-fold higher than that of the N group (4 grams, N *vs.* HF, $P < 0.05$) (Fig. 1). Both HF and HFC groups consumed more calories per day than the N group (3.54 and 2.78 kcal d$^{-1}$, N *vs.* HF and N *vs.* HFC, $P < 0.05$), which were equivalent to 99.12 and 77.84 calories at the end of treatment, respectively. This difference (21.28 calories) in caloric intake can explain, at least in part, the greater weight gain of the HF group. Interestingly, by comparing N and C groups a comparable difference was observed in caloric intake (23.52 calories) which did not coincide with the weight gain (Fig. 1). Indeed, from a comparison between the weight gain and the total calories consumed during the treatment (ratio weight gain/(kcal per day × 28 days)), it can be seen that in the HF group the weight gain per calorie consumed per day was twice that of other groups (Table 2). This is consistent with recent evidence regarding nutrition and the role played by the quality – rather than the quantity – of calories.[38]

Compared to the N group, C treated animals did not show any significant difference in the hepato-somatic index (HSI) (*i.e.* liver weight as a percentage of body weight) or hepatic triglyceride content, indicating that cocoa treatment does not affect these parameters. In contrast, the HF group showed a significant increase in HSI and hepatic triglyceride content, as compared to N (HSI: 173%, N *vs.* HF, $P < 0.01$; triglycerides: 113%, N *vs.* HF, $P < 0.05$), and C (HSI: 167%, C *vs.* HF, $P < 0.01$; triglycerides: 112%, C *vs.* HF, $P < 0.01$) groups. Furthermore, HFC treated mice exhibited a 32%-reduced increase in HSI with respect to the HF group (HF *vs.* HFC, $P < 0.01$) and their hepatic triglyceride content was comparable to that of N and C groups. These data suggest that the C diet does not affect the mentioned parameters and that cocoa in combination with the HF diet can modulate fat storage in mouse liver (Table 3). Notably, electron microscopy analysis (Fig. 2) shows that the liver of the HF-treated mouse contains more numerous lipid droplets and peroxisomes compared to N. Also, hepatocytes of

**Table 3** HSI, triglyceride content, peroxisomal β-oxidation system and catalase specific activities in mouse liver

|  | HSI | TG (mg g$^{-1}$) | β-Oxidation system (β-Ox) (SA) | Catalase (CAT) (SA) | Ratio CAT : β-Ox |
|---|---|---|---|---|---|
| N | 5.70 ± 0.50 | 26.8 ± 4.1 | 5.90 ± 0.60 | 22.60 ± 2.40 | 3.80 |
| HF | 9.90 ± 0.90**,$$ | 30.4 ± 3.9*,$$ | 12.10 ± 1.50** | 18.20 ± 1.90* | 1.50 |
| C | 5.90 ± 0.60 | 27.2 ± 3.6 | 11.80 ± 2.10* | 28.30 ± 3.10* | 2.40 |
| HFC | 8.10 ± 0.92*,$ | 27.4 ± 3.5 | 13.90 ± 1.50** | 28.90 ± 2.90*,# | 2.10 |

N, animals fed with a standard diet; HF, animals treated for 4 weeks with a high fat diet; C, animals treated for 4 weeks with a cocoa-enriched diet; HFC, animals treated for 4 weeks with a high-fat cocoa-enriched diet. * and ** significantly different from the N group, respectively, $P < 0.05$ and $P < 0.01$. # Significantly different from the HF group, $P < 0.05$; $ and $$ significantly different from the C group, respectively, $P < 0.05$ and $P < 0.01$.
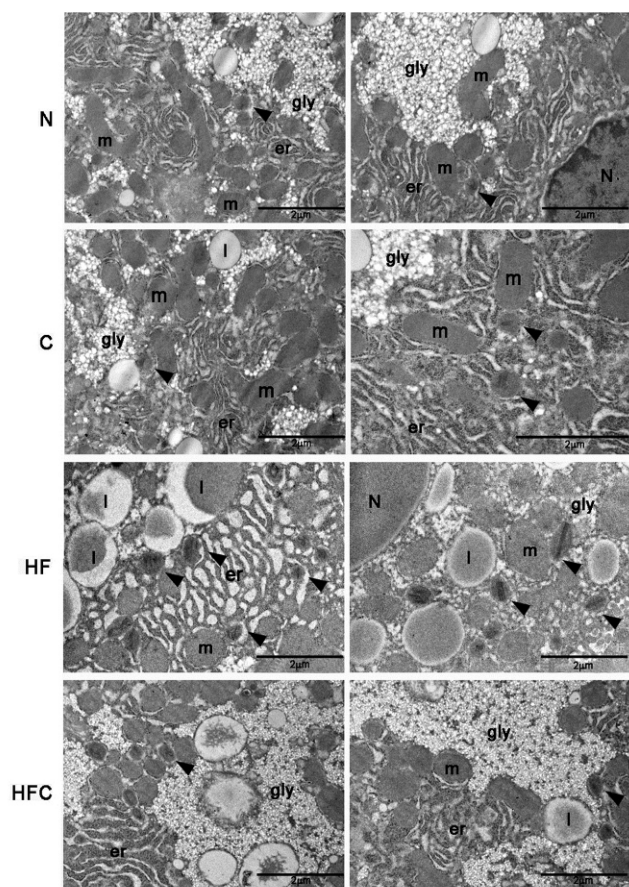


**Fig. 2** Electron microscopy analysis of mouse liver. The figure shows two representative ultrathin sections for each treatment. N, animals fed with a standard diet; HF, animals treated for 4 weeks with a high-fat diet; C, animals treated for 4 weeks with a cocoa-enriched diet; HFC, animals treated for 4 weeks with a high-fat cocoa-enriched diet. Legend: arrow (peroxisome), m (mitochondrion), N (nucleus), er (endoplasmic reticulum), l (lipid droplet), gly (glycogen).

the HF group displayed an enlarged endoplasmic reticulum, while lacking glycogen storage areas. Conversely, HFC-treated animals showed dramatically reduced lipid droplets, compared to the HF group, even though fat storage was slightly higher than under normal conditions. Liver peroxisomes of HFC animals appeared less numerous than in the HF group and still increased when compared to the N group.

Normal glycogen storage areas were seen in hepatocytes of HFC-treated mice. Livers from animals treated with cocoa exhibited ultrastructural features similar to controls. These data are in accordance with HSI and hepatic triglyceride content and suggest that cocoa in the HFC diet can reduce the triglyceride content in the liver, while weakly affecting peroxisome proliferation, thus keeping the HSI at high values. A recent work performed by Kozawa and coworkers[70] reports that the HF diet induces the peroxisomal lipid metabolism in the mouse liver. The peroxisomal β-oxidation system is made up of acyl-CoA oxidase, L-bifunctional protein and thiolase, and catalyzes the degradation of Very Long Fatty Acids (VLFAs) in peroxisomes producing acyl-CoA and $H_2O_2$, which is then decomposed by catalase into oxygen and water.[71] Hence, the importance of evaluating the ratio of the β-oxidation system to catalase activity, which can describe the oxidative stress generated by the mentioned pathway following diet treatment. Moreover, the genes encoding the β-oxidation pathway in the liver of the rodent are transcriptionally regulated by PPARα.[56] After 4 weeks of cocoa treatment, the specific activity of both CAT and fatty acyl β-Ox systems increased (2- and 1.2-fold, respectively, N *vs.* C, $P > 0.05$). In contrast, in the HF diet we found an increased activity of β-Ox (2-fold, N *vs.* HF, $P < 0.01$) and a decrease in CAT (0.8-fold, N *vs.* HF, $P < 0.05$). This imbalance may lead to accumulation of hydrogen peroxide and the consequent production of oxidative stress. Relevantly, the HFC group showed increased activities of both β-Ox (2.4-fold with respect to N, $P < 0.01$; and a comparable value for HF and C) and CAT (1.3-fold with respect to N, $P < 0.05$; 1.6-fold for HF, $P < 0.05$; and a comparable value for C). Considering the ratio of CAT/β-Ox activity, C and HFC groups show a value closer to N than HF, suggesting that cocoa can concertedly modulate CAT and β-Ox activities and counteract the imbalance induced by excessive fat dietary intake. Interestingly, Murase and coworkers[55] have shown that supplementation of tea catechins is able to improve hepatic β-Ox during high-fat diet in mice independently from PPARα activation. Thus, the enhancement of enzymatic activity reported by Murase[55] and observed by us in mouse liver under the C diet may be due, at least in part, to the high content of catechins in cocoa powder (3–4 mg g$^{-1}$ (ref. 59)). Surprisingly, in our previous work we observed that cocoa up-regulates PPARα expression and activates PPARα-signalling.[60] This effect may be imputable to the up-regulation of PPARα expression, which in turn amplifies the effect of

endogenous ligands, or to substances that are present in low amounts in cocoa, such as OEA (2 µg g$^{-1}$ (ref. 49)), which has been recently identified as a potent PPARα activator.[51] To address the involvement of PPARα signalling during HFC treatment, we checked the mRNA expression of PPARα and typical PPARα target genes: cytochrome P450 4A10 (Cyp4a10), mitochondrial pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4) and peroxisomal acyl-CoA oxidase 1 (ACOX1) (Fig. 3). These genes have been characterized and found to bear in their promoter a peroxisome proliferator response element (PPRE) recognized by PPARα which, after its activation, triggers mRNA up-regulation.[72,73] Animals fed with a cocoa-enriched diet showed increased expression of the above mentioned genes (Cyp4a10, 32-fold, ACOXI, 2,6-fold, and PDK4, 4-fold, N vs. C, P < 0.01), suggesting a moderate activation of PPARα. For comparison, it is worth recalling that ciprofibrate, a strong peroxisome proliferator, was able to up-regulate Cyp4a10, ACOXI and PDK4 about 800-, 15- and 300-fold, respectively.[60] After cocoa-enriched diet administration, we also observed up-regulation of PPARα gene expression which likely results in an increase of protein levels. The HF group showed a weak down-regulation of Cyp4a10 (−33%, N vs. HF, P < 0.05), an up-regu-

lation of PDK4 (4-fold, N vs. HF, P < 0.05) and no change in PPARα and ACOXI gene expression. These data suggest that the HF diet does not affect PPARα signalling but increases the β-Ox system. Indeed, the detected enzymatic activity is involved in VLCFA degradation and its increase in response to the HF diet could be probably due to the rise in enzymatic substrate to counteract hepatic fat accumulation. Remarkably, as previously discussed, the CAT activity was decreased by the HF diet thus reducing the antioxidant defence capacity. When animals were treated with the HFC diet, an up-regulation of PPARα-target mRNA (Cyp4a10, 7-fold, N vs. HFC, P < 0.05; ACOXI, 2-fold, N vs. HFC, P < 0.05; and PDK4, 2-fold, N vs. HFC, P < 0.05) and a weak induction of the PPARα gene were detected thus suggesting a PPARα signalling activation under these treatment conditions. As mentioned above, the HF diet increases oxidative stress in mouse liver.[16] Thus, we wonder whether cocoa in mouse liver, besides having intrinsic antioxidant properties, can modulate endogenous enzymatic antioxidant defence systems when co-administered with the high-fat diet. To this aim, we studied catalase and SOD1 expression. The HF diet lowers both catalase and SOD1 mRNA (−54% and 48%, P < 0.05) and protein levels (−30% and −40%, P < 0.05)
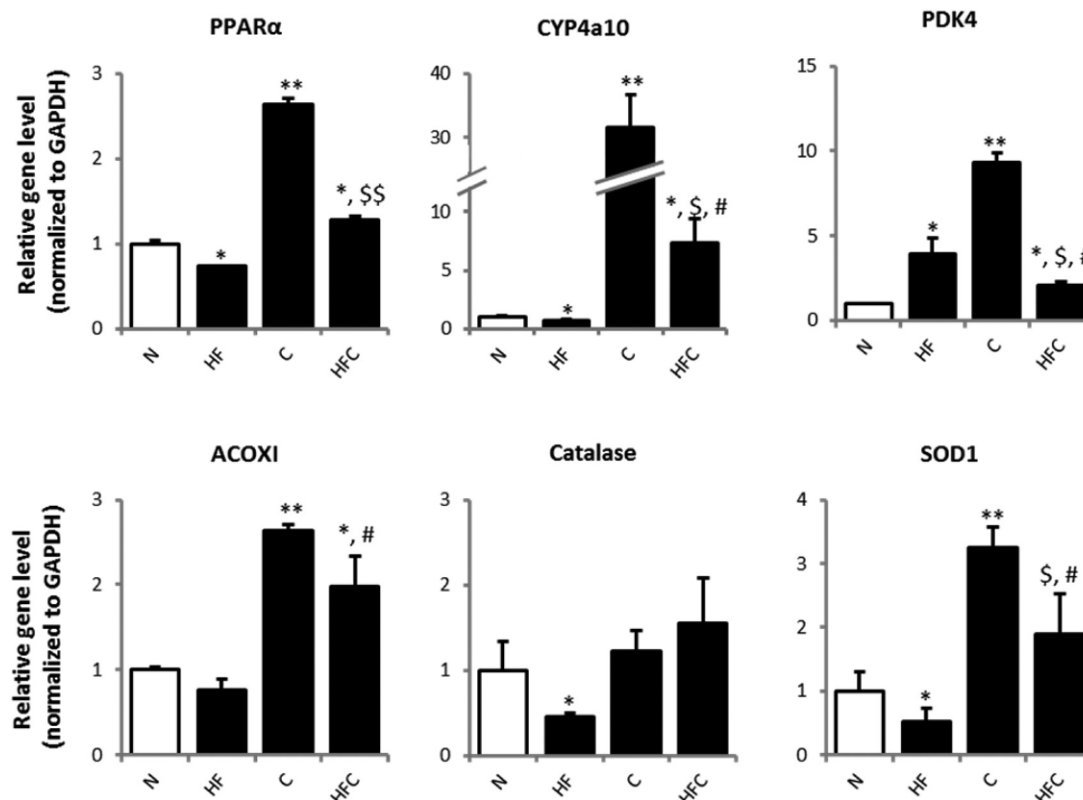


Fig. 3 Effects on gene expression of a standard diet, high-fat diet, cocoa-enriched diet and co-administered cocoa-enriched and high-fat diet. Histograms show expression of mRNA in mouse liver. N, animals fed with a standard diet; HF, animals treated for 4 weeks with a high-fat diet; C, animals treated for 4 weeks with a cocoa-enriched diet; HFC, animals treated for 4 weeks with a high-fat cocoa-enriched diet. PPARα (peroxisome proliferator- activated receptor α), Cyp4a10 (cytochrome P450, family 4, subfamily a, polypeptide 10), PDK4 (pyruvate dehydrogenase lipoamide kinase isozyme 4), ACOXI (acyl-CoA oxidase), SOD1 (superoxide dismutase 1). * and ** significantly different from the N group, respectively, P < 0.05, P < 0.01. # Significantly different from the HF group, P < 0.05. $ and $$ significantly different from the C group, respectively, P < 0.05, P < 0.01.
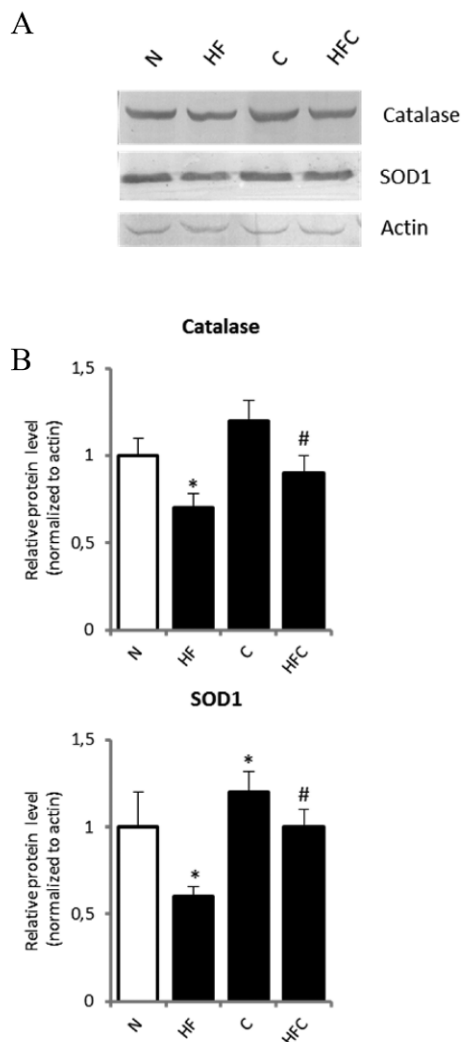
A



B



**Fig. 4** Effects on catalase and SOD1 protein expression of a high-fat diet, cocoa-enriched diet and co-administered cocoa-enriched and high-fat diet. (A) Western blot results. (B) Histograms show the expression of mRNA in mouse liver. N, animals fed with a standard diet; HF, animals treated for 4 weeks with a high-fat diet; C, animals treated for 4 weeks with a cocoa-enriched diet; HFC, animals treated for 4 weeks with a high-fat cocoa-enriched diet. SOD1 (superoxide dismutase 1). * Significantly different from the N group, $P < 0.05$. # Significantly different from the HF group, $P < 0.05$.

(Fig. 3 and 4). Consistently, we observed a significant decrease in catalase activity (Table 3), thus confirming a general down-regulation in the antioxidant defence system in mouse liver after the HF diet. In contrast, animals fed with the C diet showed, as discussed before, an increase in catalase activity (without affecting its gene or protein level) and the induction of both SOD1-mRNA and protein levels, suggesting a ROS protective effect. When cocoa was co-administered with the HF diet, both catalase and SOD1 mRNA and protein levels were similar to control animals, but the catalase activity was strongly induced as observed in animals treated with cocoa (Fig. 3 and 4, Table 3). These data suggest that cocoa can counteract the adverse effects on gene and protein expression due

to the HF diet and induce enzymatic activity, at least for the considered parameters.

## 4. Conclusions

The aim of this work was to evaluate whether cocoa treatment protects against hepatic steatosis during the HF diet and to investigate possible involvement of PPARα-signalling activation and putative changes in antioxidant defences. At the end of the treatment, animals fed with the HF diet with respect to controls showed a double weight gain per calorie consumed per day, enhanced HSI, peroxisomal β-Ox system and CAT hepatic activity. Hepatic triglyceride content assay and electronic microscopy analysis revealed increased fat storage in the liver. Moreover, PPARα-mRNA was down-regulated, PPARα-signalling turned off (probably as a consequence of the decrease in the receptor concentration) and the endogenous enzymatic antioxidant defence system went down. Fat storage and oxidative stress in a short term is linked to PPARα expression. The involvement of PPARα in fat storage and oxidative stress was recently clarified by Abdelmegeed and co-workers.[16] They showed that in mouse liver an increase in fat storage and oxidative stress occurred to a greater extent in PPARα$^{-/-}$ mice under the HF diet with respect to wild-type ones under the same treatment conditions. Conversely to the HF diet, cocoa treatment did not affect weigh gain nor HSI and hepatic triglyceride contents while it harmoniously increased the β-Ox system and CAT activity, suggesting an improvement in lipid-metabolizing activity, activation of PPARα signalling and increased antioxidant enzymatic defence. These data are in agreement with our previous results which showed that 2-week treatment with the C diet up-regulates PPARα gene expression, modulates PPARα signalling and improves oxidative stress.[60] These outcomes strongly suggest the possible protective effect of cocoa on the liver when co-administered with the HF diet. Although cocoa did not affect HSI, the β-Ox system under the HFC diet, compared to HF treatment, it prevented weight gain and allowed normalization of hepatic triglyceride levels and remarkably increased CAT activity, SOD1 and catalase expression, suggesting an improvement in lipid-metabolizing activity and oxidative stress state. Also, as shown by electron microscopy analysis of the liver, fat storage and normal glycogen areas were restored, and no hepatic steatosis was evident. These results correlate with the weak PPARα-mRNA up-regulation and middle PPARα-signalling activation induced by cocoa during the HFC diet. Taken together, our results show, for the first time to our knowledge, that the protective effect of cocoa against abnormal fat storage and oxidative stress induced by the HF diet on the liver involves PPARα-signalling activation.

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