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Effects of (-)-epicatechin on hepatic triglyceride metabolism

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List of abbreviations:

ACC, acetyl-CoA carboxylase; ACSL, acyl-CoA synthetase long-chain; ALT, alanine amino-transaminase; AST, aspartate amino-transaminase; ATGL, adipose triglyceride lipase; ChREBP, carbohydrate response element binding protein; COL1A1, collagen type 1 alpha 1; DGAT, diacylglycerol acyltransferases; EC, (-)-epicatechin; FAS, fatty acid synthase; PVL, phenyl-γ-valerolactone; IKK, IκB kinase; JNK, cc-Jun N-terminal kinase; MCP-1, monocyte chemoattractant protein-1; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NF-κB, nuclear factor κB; NOS, nitric oxide synthase; NOX, NADPH oxidase; PPARα, peroxisome proliferator-activated receptor α; SCD1, stearoyl-CoA desaturase 1; SMA, smooth muscle actin; SREBP-1c, sterol-regulatory element binding protein-1c; SREM, structure related (-)-epicatechin metabolites; TG, triglycerides; TGF1β, transforming growth factor 1 β; TNF-α, tumor necrosis factor-α.

Abstract:

(-)-Epicatechin (EC) consumption is associated with an improvement of hyperlipemia and other metabolic changes linked to obesity and western-style diets. This work investigated the effects of EC on triglyceride (TG) metabolism both: i) *in vivo*, mice supplemented with EC (2 and 20 mg EC/kg body weight), and ii) *in vitro*, human HepG2 hepatocytes incubated in the presence of EC and the main EC metabolites found in human plasma. Increased hepatic TG levels were only observed after 24 weeks supplementation with EC (20 mg/kg body weight), with a preserved liver structure and absence of inflammation or oxidative stress. EC caused an increased expression of diacylglycerol acyltransferases (DGAT2), key enzyme in TG synthesis, and the upregulation of PPARα, which promotes free fatty acid (FFA) oxidation. On the other hand, incubation of HepG2 cells in the presence of high concentrations of EC (1-10 μM) did not affect TG deposition nor DGAT2 expression. In summary, in mouse liver, EC upregulated mechanisms that can neutralize the potential toxicity of FFA, i.e. TG synthesis and FFA oxidation. Results in mouse liver and HepG2 cells stress the safety of EC in terms of TG metabolism and development of hepatopathies in doses within the limits given by a rational time and dose of human consumption.

1. Introduction

Triglycerides (TG) are the form in which organs store fatty acids [1]. Liver, the major organ in the modulation of TG metabolism, synthesizes TG from newly formed fatty acids or from fatty acids of dietary origin. The final step in TG synthesis is catalyzed by the rate-limiting enzymes diacylglycerol acyltransferases (DGAT), which acylate diacylglycerol to triacylglycerol by forming covalent bonds between acyl-CoA and diacylglycerol. These enzymes have major relevance not only in TG synthesis but also in the control of tissue energy metabolism [2]. While the liver normally keeps low levels of stored TG, excessive accumulation can cause steatosis and non-alcoholic fatty liver disease (NAFLD). In fact, high levels of TG accumulation in the liver can increase the production of inflammatory mediators and cause oxidative stress, which in turn ca lead to liver fibrosis and steatohepatitis [3]. On the other hand, TG synthesis is considered a protective mechanism to prevent the hepatotoxicity of free fatty acids (FFA) [4-7].

Dietary flavonoids are bioactives that are frequently associated with beneficial effects on dyslipidemia. Among flavonoids, the flavan-3-ol (-)-epicatechin (EC) is abundant in many fruits and vegetables including berries, cocoa, and tea. EC consumption mitigates several of the metabolic alterations associated to the consumption of western style diets and obesity, including hyperlipidemia [8]. In this regard, EC supplementation normalizes plasma lipids in rodent models of high fructose and high fat diet consumption [9, 10]. In rats fed a high fructose diet, EC consumption mitigated the increase in plasma and liver TG [9]. Supplementation of male mice with EC for 15 weeks also prevented the increase in plasma TG and FFA caused by consumption of a high fat diet [10]. These protective effects were observed in all the range of EC tested (2-20 mg/kg body weight (BW)) [11]. On the other hand, mice fed a control diet

supplemented with 20 mg EC/kg BW for 15 weeks showed either no changes or significantly higher plasma TG concentration [10, 11]. In addition, mice supplemented with 20 mg EC/kg BW showed a trend for higher NAFLD activity score (NAS) compared to controls [11]. In healthy humans, consumption of 2,000 mg cocoa flavanols and procyanidins for up to 3 months did not affect plasma TG and cholesterol levels [12].

Given the limited information on the potential effects of long-term EC consumption on hepatic TG homeostasis, this study investigated the effects of EC on TG metabolism both in mouse liver and in a cell culture (HepG2 cells) model of human hepatocytes. Only after a long-term feeding (24 weeks) of the highest amount of EC (20 mg EC/kg body weight (BW)) we observed the upregulation of DGAT2 and PPARα, and liver TG accumulation, without evidence of tissue inflammation or altered structure. Findings in human HepG2 hepatocytes *in vitro* did not show any effect of EC and its main human metabolites on DGAT2 expression and TG accumulation. The present findings stress the safety of EC in terms of TG metabolism and development of hepatopathies in doses within the limits given by a rational human consumption.

2. Materials and methods

2.1. Materials

Antibodies against monocyte chemoattractant protein-1 (MCP-1) (#2029), tumor necrosis factor-α (TNFα) (#11948), IκB kinase-α (IKKα) (#2682), phospho (Ser176/80)-IKKα/β (p-IKKα/β) (#2697), p65 (#8242), phospho (Ser526) p65 (p-p65) (#3033), acetyl-CoA carboxylase (ACC) (#3676), adipose triglyceride lipase (ATGL) (#2138), ubiquitin (#3936), c-Jun N-terminal

kinase-2 (JNK)2 (#9258), β-actin (#12620) and secondary antibodies anti-rabbit HRP-conjugated (#7074) or biotinylated (#14708) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies for F4/80 (sc-25830), nitric oxide synthase 2 (NOS2) (sc-649), phospho (Thr183/ Tyr185) JNK (sc-6254), transforming growth factor beta 1 (TGF1β) (sc-130348), smooth muscle actin (SMA) (sc-53015), fibronectin (sc-271098), collagen type 1 alpha 1 (COL1A1) (sc-293182), phospho (Ser 78/Ser 80) ACCα (p-ACCα) (sc-271965), fatty acid synthase (FAS) (sc-48357), stearoyl-CoA desaturase 1 (SCD1) (sc-515875), long-chain acyl-CoA synthetase 3 (ACSL3) (sc-166374), ACSL5 (sc-365478), DGAT1 (sc-271934), DGAT2 (sc-293211), sterol-regulatory element binding protein-1C (SREBP-1c) (sc-366), carbohydrate response element binding protein (ChREBP) (sc-515922), peroxisome proliferator-activated receptor alpha (PPARα) (sc-398394), α-tubulin (sc-23948), and agarose A/G beads (sc-2003) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody for NADPH oxidase 4 (NOX4) (ab216654) was from Abcam, Inc. (Cambridge, MA, USA). PVDF membranes were obtained from Bio-Rad (Hercules, CA, USA). The Enhanced chemiluminescence (ECL) Western blotting system was from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were purchased from Sigma- Aldrich Co (St. Louis, MO, USA). EC metabolites were a kind gift of Mars Inc. (Hackettstown, NJ, USA).

2.2. Animals and animal care

All procedures were in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals; experimental protocols were approved before implementation by the University of California, Davis Animal Use and

Care Administrative Advisory Committee. Procedures were administered under the auspices of the Animal Resource Services of the University of California, Davis.

Healthy 8-week-old male C57BL/6J mice (20-25 g) were purchased from The Jackson Laboratories (Sacramento, CA). After one week of acclimation, mice were randomly divided (15 animals/group) and fed for 24 weeks either: i) a control diet containing approximately 10% total calories from fat (C) or ii) the control diet supplemented with 2 (CE2) or 20 mg EC (CE20)/kg BW. The composition of the control diet has been previously described [13]. Body weight and food intake were measured weekly. The EC-containing diet was prepared every two weeks to adjust the amount of EC on the base of the body weight. In addition, all diets were replaced every two days and stored at −20 °C until use to avoid EC degradation. The amounts of EC used in the studies were calculated using the Reagan-Shaw et al. scaling criteria [14] to relate consumption in humans to mice. Based on this calculation, the human equivalent dose (HED) for 2 and 20 mg EC/ kg BW of EC would be 11 and 110 mg, respectively, for a 70 kg adult. Allometric scaling is an appropriate method to compare bioactive consumption in different species [15]. In humans, the equivalent of 2 mg EC/kg BW in mice can be reached by consuming a diet rich in EC [16], while 20 mg EC/kg BW in mice can be reached in humans by supplementation or consumption of select EC-rich fruits and vegetables and derivatives [17]. After 13 and 24 weeks on the dietary treatments, mice were euthanized by cervical dislocation. Blood was collected from the sub-mandibular vein into EDTA tubes, and plasma was obtained after centrifugation at 3,000 × g for 10 min at room temperature. Livers were collected, weighed, and flash-frozen in liquid nitrogen, and then stored at −80 °C until further analysis.

2.3 HepG2 cell culture

HepG2 cells were cultured at 37 °C under humidified air and 5% (v/v) CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM high glucose) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 IU/ml penicillin and 10 mg/ml streptomycin). The medium was replaced every 2 days until 80% confluency. For the experiments, confluent cells were switched to DMEM low glucose supplemented with 2% (v/v) FBS and antibiotics. Cells were then incubated in the absence or the presence of 1 and 10 µM of EC, structurally related (-)-epicatechin metabolites (SREM) and of 5-(4'-hydroxyphenil-γ-valerolactones (PVL) for 1 to 72 h. SREM is a mixture that contains (-)-epicatechin-3'-β-D-glucuronide (40%), (-)-epicatechin-3'-sulfate (30%), and 3'-*O*-methyl-(-)-epicatechin-5-sulfate (30%), which are the most abundant EC metabolites detected in human plasma after oral EC consumption (1.8 mg EC /kg BW) [18]. The PVL mix is composed of the main gut microbiota-generated EC metabolites in humans consisting of 65% and 35% of the sulfate and glucuronide PVL conjugates, respectively[19]. The lowest concentration used to incubate cells were based on evidence that consumption of 1.8 mg EC/kg BW, approximately 135 mg EC for a 70 kg individual, leads to a maximum plasma concentration of approximately 1 µM SREM [18].

2.4. Cell viability

Cell viability was assessed by the MTT assay, based on the conversion of MTT into formazan crystals by living cells. After the corresponding treatments, HepG2 cells were added with MTT solution (0.5 mg/ml in PBS) and after 45 min of incubation at 37 °C, the reaction was stopped by addition of 0.01 N HCl containing 10% (w/v) SDS. After 2.5 h incubation, absorbance

(λ570-λ690 nm) was measured using a Biotek Synergy H1 plate reader (BioTek Instruments, Winooski, VT) and values expressed as percentage of control (non-treated) cells.

2.5. Determination of liver lipids content

The determination of liver TG content was performed as previously described [20]. Briefly, after extraction and saponification, liver and cell homogenates were mixed with a KOH (30% w/v):ethanol (1:2 v:v) solution and evaporated overnight at 55°C. The following day, 1 ml of 50% (v/v) ethanol was added, and samples were centrifuged for 5 min at 10,000 × g at room temperature. The resulting supernatant was added with 1M $MgCl_2$ and incubated on ice for 10 min. After centrifugation at 10,000 × g for 5 min at room temperature, supernatants were collected and analyzed for TG content using the colorimetric enzymatic triglyceride kit TG Color GPO/PAP AA (Wiener Lab, Rosario, Argentina).

Liver cholesterol content was determined using the Bligh and Dyer method [21] with some modifications. Briefly, a 300 µl aliquot of 10% (w/v) liver homogenate was mixed with 900 µl of a chloroform:methanol (2:1, v:v) solution. After centrifugation at 3,000 × g for 10 min, the supernatant was discarded, and the lower phase was collected and evaporated for 1.5 h using an Eppendorf Vacufuge (Eppendorf, Hamburg, Germany). After evaporation, the pellet was resuspended in 50 µl of methanol with 0.05% (v/v) Tween-20 solution and sonicated. 10 µl of the lipid suspension was analyzed for cholesterol content using the enzymatic total cholesterol kit Cholestat (Wiener Lab, Rosario, Argentina).

Liver FFAs were extracted and measured following the manufacturers' protocol (Abcam Inc., Cambridge, MA). For the extraction, 10 mg of liver were homogenized in 200 µl of 1% (v/v)

Triton X-100 in chloroform, incubated on ice for 20 min and then centrifuged at 14,000 × g for 5 min at room temperature. The organic phase was collected and first air dried at 50°C and then vacuum dried for 30 min. Dried pellets were resuspended in 200 µl of fatty acid assay buffer, and vortexed for 5 min. FFA concentration was determined by fluorometric assay following the manufacturers' instruction.

2.6. Metabolic measurements

Plasma levels of alanine amino-transaminase (ALT), aspartate amino-transaminase (AST), TG, and cholesterol were determined using kits from Wiener Lab Group (Rosario, Argentina) following the manufacturers' protocols. Determination of plasma FFA was conducted using a fluorometric assay from Abcam Inc. (Cambridge, MA) following manufacturers' guidelines.

2.7. Histological analyses

Liver samples were fixed overnight in 4% (w/v) neutralized paraformaldehyde solution in PBS. Samples were subsequently washed twice in phosphate buffer saline solution, dehydrated, embedded in paraffin, cut into sections of 5 µm thickness, and placed on glass slides for histological analysis. Hematoxylin and eosin staining was performed following standard procedures to assess liver steatosis. Hepatic histological examination was performed using the NAS described by Kleiner et al [22]. Sections were examined using an Olympus BX51 microscope (Olympus America Inc., Center Valley, PA). Four randomly selected fields per animal

were assessed and analyzed using Image J (National Institutes of Health, Bethesda, MD). The researcher was blinded to the treatment groups.

2.8. Protein extraction and western blot analysis

Livers were homogenized as previously described [11] using a BeadMill24 (Thermo Fisher Scientific Inc., Piscataway, NJ). Aliquots of total homogenates containing 30 µg protein were denatured with Laemmli buffer, separated by reducing 7-15% polyacrylamide gel electrophoresis, and electroblotted to PVDF membranes. Colored and biotinylated molecular weight standards were run simultaneously. Membranes were blocked for 1.5 h in 5% (w/v) non-fat milk and subsequently incubated in the presence of the corresponding primary antibodies overnight at 4 °C (1:750 or 1:1000 v/v) in 5% (w/v) BSA in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.6), containing 0.1% (v/v) Tween-20. After incubation for 90 min at room temperature in the presence of the secondary antibodies (HRP conjugated) (1:10,000 v/v), conjugates were visualized using enhanced chemiluminescence. Images were captured using Bio-Rad ChemiDoc Imager, and bands were quantified using Image Lab Software (Bio-Rad, Hercules, CA).

2.9. Immunoprecipitation

To assess the ubiquitination status of DGAT2, tissue homogenates were prepared as described in the previous section with IP buffer (#87787), and 200 µg proteins were incubated with 0.4 µg of the secondary antibody m-IgGκ BP-HRP (sc-516102) and 15 µl agarose A/G beads for 30 min at 4 °C. After centrifugation at 500 × g for 3 min at 4 °C, 45ul of supernatant were collected and immunoprecipitated with 3 µl monoclonal mouse anti-DGAT2 antibody on

agarose A/G beads overnight at 4 °C. After centrifugation, beads were washed three times with complete lysis buffer and protease inhibitors, the pellet was resuspended in Laemmli sample buffer and PBS and heated at 95 °C for 5 min. Then, lysates and the immunoprecipitated proteins were separated by 7-10% (w/v) SDS-PAGE followed by transfer to PVDF membranes and blotting with antibodies against DGAT2 and ubiquitin. A negative control was used to validate the accuracy of the immunoprecipitation.

2.10. RNA isolation and quantitative PCR (q-PCR)

RNA was extracted from tissues and HepG2 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturers' instructions. The cDNA was generated using high-capacity cDNA Reverse Transcriptase (Applied Biosystems, Grand Island, NY). Gene expression was assessed by q-PCR (iCycler, Bio-Rad) with the corresponding primers for DGAT1, DGAT2, and PPARα described in **Supplemental Table 1**. Ct values were normalized to the β-actin housekeeping gene. Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method [23].

2.11. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Statview 5.0 (SAS Institute Inc., Cary, NC). Fisher's least significance difference test was used to examine differences between group means. A P value < 0.05 was considered statistically significant. Data are shown as means ± SE.

3. Results

3.1. Effects of 24-week EC supplementation on body weight and plasma metabolic parameters

Supplementation with 2 or 20 mg EC/kg BW for 24 weeks did not affect cumulative food intake and body weight compared to mice consuming non-supplemented diet (**Table 1**). Plasma lipid levels, including total cholesterol, TG, and FFA, were similar among groups (**Table 1**).

3.2. Effects of 13- and 24-week EC supplementation on liver lipid deposition

We next evaluated the effects of long-term (24 weeks) EC supplementation on liver lipid content and parameters of liver damage. Liver weight was similar among the different dietary groups (**Table 1**). Plasma ALT and AST activities, indicators of liver damage, were not affected by EC supplementation (**Table 1**).

While liver cholesterol and FFA content was similar among all groups (**Fig. 1A**), liver TG content was significantly higher in mice supplemented with 20 mg EC/kg BW for 24 weeks compared to C and CE2 animals (42% and 44% higher, respectively) ($p < 0.05$) (**Fig. 1A**). On the other hand, mice supplemented with 20 mg EC/kg BW for 13 weeks did not show an increase in liver TG content compared to control mice (**Fig.1 B**).

Because of the observed increase in liver TG content in mice supplemented with 20 mg EC/kg BW for 24 weeks, we next conducted a histological tissue characterization using hematoxylin/eosin staining. The NAS score represents the sum of scores for steatosis (microvesicular and macrovesicular steatosis), and hepatocyte injury (hypertrophy). Hematoxylin/eosin staining showed a preserved liver structure and similar NAS scores for all groups (**Fig. 1 C-E**). To assess liver fibrosis, TGF1β, SMA, fibronectin, and COL1A1 protein levels

were measured by Western blot (**Fig. 1F**). Supplementation with EC did not affect the protein levels of any of these liver fibrosis markers.

3.3. Effects of 24-week EC supplementation on parameters of liver inflammation and oxidative stress

According to the liver "two hits hypothesis" [24, 25], increased liver TG synthesis and accumulation can result in an increased production of inflammatory mediators and oxidative stress. Thus, we next investigated the effects of long-term EC supplementation on redox-sensitive signaling pathways (NF-κB and JNK) and select parameters of inflammation (MCP-1, TNFα, and F4/80) and oxidative stress (NOX4 and NOS2). Supplementation with EC for 24 weeks did not result in the liver activation of the redox-sensitive signals JNK (phosphorylation in Thr183/ Tyr185) and NF-κB (IKK phosphorylation in Ser176/Ser80) and p-65 phosphorylation in Ser526) (**Fig. 2A**). Furthermore, EC did not affect NOS2, NOX4, MCP-1, F4/80, and TNFα liver protein levels (**Fig. 2B**).

3.4. Effects of 24-week EC supplementation on liver enzymes involved in lipid de novo lipogenesis and lipolysis.

The observed increase in TG liver deposition upon supplementation with 20 mg EC/kg BW could be due to an increase in de novo lipogenesis (DNL) or decreased TG lipolysis. Thus, we next evaluated the effects of EC supplementation on the levels of proteins involved in these processes, i.e. malonyl-CoA synthesis (ACC), fatty acid synthesis (FAS), fatty acid desaturation (SCD1), fatty acid activation (ACSL3 and ACSL5), and TG synthesis (DGAT1, DGAT2) and lipolysis

(ATGL). Phosphorylation of ACCα (Ser78/Ser80) and protein levels of FAS, ACSL3 and ACSL5 were not affected by EC supplementation (**Fig. 3A**). Regarding DNL, mice supplemented with 20 mg EC/kg BW showed increased SCD1 protein levels compared to CE2 and C mice (**Fig. 3A**). Furthermore, while 24-week supplementation with 2 mg EC/kg BW did not affect DGAT2 protein and mRNA levels, supplementation with 20 mg EC/kg BW led to higher levels of DGAT2 protein (84 %) (**Fig. 3A**) and mRNA (27 %) (**Fig. 4A**) compared to the C group. EC supplementation 2 and 20 mg EC/kg BW) did not affect DGAT1 protein levels (**Fig. 3A**). In alignment with no effects on liver TG content (**Fig. 1B**), supplementation with 20 mg EC/kg BW for a shorter period of time (13 weeks) did not affect SCD1 and DGAT2 protein levels (**Fig. 3B**)

3.5. Effects of 24-week EC supplementation on transcription factors involved in DNL and lipolysis.

We next investigated the modulation of transcription factors that are involved in TG and fatty acid synthesis (SREBP-1c, ChREBP) and fatty acid β-oxidation (PPARα). Liver protein levels of SREBP1-c (precursor and mature forms) and ChREBP were similar among groups, as evaluated by Western blot (**Fig. 5A**). On the other hand, PPARα mRNA levels were higher in the CE20 group compared to C, while PPARα protein levels were significantly higher in CE2 compared to C mice (**Fig. 5B,C**).

3.6. Effects of 24-week EC supplementation on DGAT2 post-translational regulation

DGAT2 is post-translationally regulated via degradation through the endoplasmic reticulum-associated degradation (ERAD) pathway. To investigate whether the increased

protein levels of DGAT2 associated with EC supplementation were mediated through an impairment of DGAT2 degradation, we next evaluated DGAT2 ubiquitination levels. DGAT2 was immunoprecipitated from liver homogenates and, its ubiquitination levels were subsequently assessed by Western blot. Supplementation with 20 mg EC/kg BW led to higher levels of DGAT2 ubiquitination compared to the C group (**Fig. 4B**).

3.7. Effects of EC and SREM on TG content and DGAT1 and DGAT2 mRNA levels in HepG2 cells

We next investigated the potential effects of EC and its metabolites that appear in human plasma after EC oral consumption, i.e. the main structurally related EC metabolites (SREM) (-)-epicatechin-3′-β-D-glucuronide, (-)-epicatechin-3′-sulfate, and 3′-*O*-methyl-(-)-epicatechin-5-sulfate at a ratio and concentration similar to that found in human plasma, and the main microbiota metabolite phenyl-γ-valerolactone (PVL), on TG content and DGAT1 and DGAT2 mRNA levels in human HepG2 cells. Human HepG2 cell cultures have demonstrated their reliability as a valuable system for studying TG metabolism in hepatocytes. We initially assessed the effects of EC, SREM, and PVL on HepG2 cell viability using the MTT assay (**Supplemental Fig. 1 A,B**). HepG2 cell incubation with 1 or 10 μM EC for 24, 48, and 72 h did not affect cell viability. Treatment of HepG2 cells for up to 72 h with 1 and 10 μM EC did not affect cell TG content (**Fig. 6A**). Incubation of HepG2 cells with 1 or 10 μM EC, SREM, and PVL for up to 24 h did not affect DGAT2 or DGAT1 mRNA levels (**Fig. 6B,C** and **Fig. 7A,B**).

4. Discussion

Only long-term (24 weeks) consumption of EC at the highest dose (20 mg/kg BW) resulted in the upregulation of liver DGAT2 and PPARα, and TG increased deposition. On the other hand, incubation of human HepG2 cells with high concentrations of EC and the EC human plasma metabolites did not affect cell TG levels and/or DGAT2 gene expression. While results support the safety of rational EC supplementation in humans, current results point to the need of understanding potential negative effects of long-term and pharmacological doses of polyphenols supplementation on human health.

Consumption of EC or EC-rich foods has been linked to improvements in plasma and hepatic lipids [8]. Consumption of enriched bread containing 0.05% of a 1:1 mixture of EC and quercetin for three months significantly decreased plasma total cholesterol, LDL-cholesterol, TG, and fasting glucose levels in humans who had at least three of the risk factors for metabolic syndrome [26]. Daily supplementation with 50 mg EC for four weeks resulted in a decrease (75 mg/dl) in plasma TG levels in subjects with hypertriglyceridemia [27]. Additionally, the consumption of 993 mg of cocoa flavanols for 8 weeks significantly reduced circulating levels of TG, total cholesterol, and LDL cholesterol in elderly individuals [28]. In fact, a meta-analysis of randomized controlled trials found that chocolate or cocoa products consumption significantly reduces TG levels [29]. On the other hand, both acute (2 h) and chronic (4 weeks) consumption of isolated apple monomeric flavanols, primarily EC, by humans at doses of 70 mg or 140 mg, did not exhibit a significant impact on blood lipids, including TG and total cholesterol [30]. In mice, supplementation for 15 weeks with 20 mg EC/kg BW mitigated high fat diet-induced increase in plasma TG concentrations, not preventing the increase in FFAs, and total cholesterol [10]. EC (20 mg/kg BW) also mitigated the increase in plasma and liver TG caused by consumption of a high

fructose diet in rats [9]. Administration of 1 mg EC/kg BW twice daily for 2 weeks to male offspring of obese rats reduced their serum TG levels [31]. Overall, findings show that EC consumption decreases circulating and liver TG in humans and/or rodents. In this study we found that a longer term (24 weeks) consumption of EC (20 mg/kg BW) increased TG deposition in the liver.

EC-mediated liver TG increase was associated with elevated protein levels of DGAT2 in mouse liver, while DGAT1 was not affected. DGAT2 is responsible for catalyzing the final and limiting step in TG biosynthesis. DGAT2 possesses two transmembrane domains, and its active site is oriented towards the cytosol. This positioning suggests that DGAT2 plays a role in catalyzing the synthesis of TG specifically intended for storage within cytosolic lipid droplets [32]. In contrast, DGAT1 exhibits three transmembrane domains, and its active site is situated in the lumen of the endoplasmic reticulum, which directs TG towards nascent lipoproteins [33]. The regulation of DGAT2 involves not only transcriptional control but also protein stability [34], in particular degradation. Findings that EC supplementation increased DGAT2 protein ubiquitination, an indication of protein degradation, and increased DGAT2 mRNA and protein levels, suggest that EC could be modulating DGAT2 at the level of transcription, not post translationally. Besides TG synthesis, liver TG levels are regulated by lipolysis. The central enzyme in TG degradation is ATGL. Findings that ATGL liver levels were not affected by EC supplementation supports an effect at the level of TG synthesis, not degradation.

Importantly, despite the increased liver TG deposition associated with the long-term high dose of EC supplementation in mice, there was no associated inflammation or evidence of fibrosis. In fact, studies show that TG accumulation *per se* is not inherently hepatotoxic and can

actually serve as a defense mechanism to counterbalance excess FFA availability [4, 5, 35, 36]. Liver DGAT2, which expression was increased by EC supplementation, plays a crucial role in safeguarding the endoplasmic reticulum against the lipotoxic effects induced by high fat diets [37]. Additionally, decreased DGAT2 expression reduces hepatic TG, which subsequently increases FFA oxidation and exacerbates steatohepatitis in mice [4].

In terms of mechanism of action, EC and cocoa polyphenols can regulate the expression of transcription factors involved in TG and FFAs homeostasis, including PPARγ [38], PPARα, ChREBP and SREBPs [39]. PPARα is recognized for its central role in modulating FFA homeostasis in liver, heart, and brown adipose tissue [40], also exerting anti-inflammatory properties [41]. PPARα regulates FFAs and TG metabolism through its direct effects on fatty acid transport and oxidation genes and its induction of lipolysis, respectively [40]. The transcriptional regulation of fatty acid synthesis, elongation, and desaturation involves SREBP-1c and ChREBP [42]. In terms of fatty acid synthesis, both ChREBP and SREBP-1c stimulate the expression of ACC and FAS [42-46]. We currently observed that EC chronic supplementation did not affect SREBP-1c and ChREBP protein levels, but significantly increased PPARα gene and protein levels in mouse liver. These findings suggest a potential positive effect of EC in regulating lipogenesis and/fatty acid oxidation, and subsequently attenuating the accumulation of lipids in the liver. In support of this, daily administration of 1 mg EC/kg BW [47] or 10-40 mg EC/kg BW prevented the upregulation of SREBP-1c in the liver of rats fed a high-fat diet [39].

There are notable differences in the metabolism of EC among various species, such as those observed between mice and humans [48]. There is no 3'-*O*-methyl-EC-7-sulfate or EC-3'-O-β-D-glucuronide appearing in mouse plasma upon EC consumption. Among the four major

SREMs identified in humans, only two, EC-3'-sulfate and 3'-*O*-methyl-EC-5-sulfate, were found in mice. In addition, the conjugation of PVL is different in rodents and in humans [19]. As an initial step in understanding a potential negative effect of EC supplementation in humans on liver TG homeostasis, we evaluated the effects of the main human EC metabolites on DGAT2/1 expression and/or TG accumulation. Evidence that exposure to very high levels of EC and its metabolites did not affect these parameters is relevant initial evidence suggesting that the liver TG accumulation observed in mice does not seem to occur in an *in vitro* model of human hepatocytes.

Flavan-3-ols have been recognized for their numerous beneficial properties [49-51]. Nevertheless, it should be noted that they can also have toxicity effects when consumed as pure compounds, at high doses and during long periods of time. While the safety implications of supplementing with high doses of EC in humans have not been thoroughly studied, available studies have not found adverse effects. The COcoa Supplement and Multivitamin Outcomes Study (COSMOS), which is the most extended trial to date (with a median duration of 3.6 years) investigating the effects of long-term cocoa extract supplementation on clinical events in older individuals in the United States, revealed no safety issues related to cocoa flavanol supplementation (500 mg flavanols/d, including 80 mg EC) [52]. Other human study showed that consumption of cocoa flavanols up to 2000 mg per day for a duration of 12 weeks was well tolerated [12]. It should be noted that the specific amount of EC provided in that study is unknown as it primarily represents the intake of flavanol monomers. The inclusion of the evaluation of parameters of liver damage in future clinical studies supplementing chronically

with pure EC or EC-rich foods will help to rule out a potentially damaging accumulation of TG in the liver.

In summary, chronic EC consumption promotes changes in liver lipid homeostasis. An elevation in hepatic TG content in mice was associated with an upregulation of DGAT2. While excess TG deposition may have negative consequences in the long-term, the upregulation of DGAT2 can protect the tissue from lipotoxicity, by incorporating FFA into TG. Additionally, EC upregulated PPARα which plays a major role in the regulation of fatty acid oxidation. *In vitro* experiments in human HepG2 cells, did not replicate the effects of EC on TG accumulation in mouse liver. Considering the limited available literature, our study provides valuable initial insight for future research on the effects of EC on TG metabolism. Results also suggest that while consumption of EC-rich foods is safe, supplementation with large doses of pure EC for long periods of time should be avoided and consider potential liver toxicity.

Conflicts of interest

There are no conflicts to declare.

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Legend to Figures

Figure. 1. Effects of long-term EC supplementation on liver lipids, steatosis, and hepatic fibrosis in mice. Mice were fed the different diets as described in methods. After 24 weeks on the corresponding diets the following parameters were measured: **A-** liver cholesterol, TG, and FFA content were assessed as described in methods; **C-** NAFLD activity score (NAS) and inflammation scores were evaluated as described in methods; **D-** images of liver hematoxylin/eosin tissue staining; and **E-**proteins involved in fibrosis: TGF1β, SMA, fibronectin, and COL1A1 were assessed by Western blot and values were normalized to β-actin (TGF1β) or α-tubulin levels (SMA, fibronectin, and COL1A1) as loading controls. Results for CE2 and CE20 were referred to control group values (C). Results are shown as mean ± SE of 10-13 animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA test). **B**- liver TG were also measured in mice that were fed for 13 weeks the control diet without EC supplementation (C), and the control diet supplemented with 20 mg EC/kg BW (CE20) as previously described [53]. Results are shown as mean ± SE of 6-7 animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA test).

Figure. 2. Effects of long-term EC supplementation on parameters of liver inflammation and oxidative stress in mice. Mice were fed the different diets as described in methods. After 24 weeks on the corresponding diets, the following parameters were measured by Western blot: **A-** activation of NF-κB, i.e. phosphorylation of IKK at Ser176/Ser80 and of p65 at Ser526, and of

JNK, measuring its phosphorylation at Thr183/Tyr185; **B-** inflammation and oxidative stress, i.e. NOS2, NOX4, MCP-1 and F4/80 protein levels. Bands were quantified and values referred to total protein levels (IKK, p65, and JNK), to α-tubulin (NOS2, NOX4, and F4/80), or to β-actin (MCP-1) levels as loading controls. Results for CE2 and CE20 were referred to control group values (C). Results are shown as mean ± SE of 8-10 animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA test).

Figure. 3. Effects of long-term EC supplementation on liver enzymes involved in lipid *de novo* lipogenesis and lipolysis in mice.

A-Mice were fed the different diets as described in methods. After 24 weeks on the corresponding diets, the following proteins involved in de novo lipogenesis and lipolysis were measured by Western blot: phosphorylation of ACCα at Ser78/Ser80, and protein levels of ACC, FAS, SCD1, ACSL5, DGAT1, DGAT2, and ATGL. Bands were quantified and values referred to total protein levels (ACC), to α-tubulin (FAS, SCD1, ACSL5, DGAT2, and ATGL) or to β-actin (DGAT1) levels as loading controls. Results for CE2 and CE20 were referred to control group values (C). **B**- SCD1 and DGAT2 protein levels were also measured, as described above, in mice that were fed for 13 weeks the control diet without EC supplementation (C), and the control diet supplemented with 20 mg EC/kg BW (CE20) as previously described [53]. Results are shown as mean ± SE of 8-10 animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA test).

Figure. 4. Effects of long-term EC supplementation on mouse liver DGAT2 mRNA levels and ubiquitination. Mice were fed the different diets as described in methods. After 24 weeks on the corresponding diets the following parameters were measured: **A-** mRNA levels of liver DGAT2 were assessed by qPCR. Values were normalized to β-actin (housekeeping gene); **B-** DGAT2 ubiquitination was measured after DGAT2 immunoprecipitation from total protein tissue fractions as described in methods. DGAT2 and its ubiquitination levels were subsequently measured by Western blot. Ubiquitinated bands between 37 and 75 kDa were quantified and values referred to those of DGAT2. A sample that underwent the whole procedure of immunoprecipitation in the absence of anti-DGAT2 antibody was used as negative control. Right panel: Values for CE2 and CE20 were referred to control group value. Results are shown as mean ± SE of 8-10 animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA test).

Figure. 5. Effects of long-term EC supplementation on transcription factors involved in liver *de novo* lipogenesis and lipid β-oxidation in mice. Mice were fed the different diets as described in methods. After 24 weeks on the corresponding diets the following parameters were measured: **A-** master transcription factors involved in de novo lipogenesis regulation, SREBP1-c and ChREBP protein levels were measured by Western blot; **B,C-** transcription factor involved in lipid β-oxidation, PPARα gene expression and protein levels were measured by qPCR and Western blot, respectively. Bands were quantified and values referred to SREBP1-c precursor (SREBP1-c mature) or α-tubulin (SREBP1-c mature, ChREBP, and PPARα) (loading controls). mRNA levels of PPARα were normalized to β-actin (housekeeping gene). Results for CE2 and

CE20 were referred to control group values (C). Results are shown as mean ± SE of 8-10 animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA test).

Figure. 6. Effects of EC on TG content and DGAT2 and DGAT1 gene expression in HepG2 cells. HepG2 cells were incubated in the absence or the presence of 1 and 10 µM EC (EC1 and EC10, respectively) for 1-72 h as indicated in each corresponding graph. **A-** TG content were measured as described in methods and values were normalized to total protein concentration. **B-C-** mRNA levels of DGAT1 and DGAT2 were assessed in HepG2 cells by qPCR. Values were normalized to β-actin (housekeeping gene). Results for EC1 and EC10 were referred to control values (C) (non-added cells). Results are shown as mean ± SE of 10 (for TG) and 3-4 (for qPCR) independent experiments. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA test).

Figure. 7. Effects of SREM and PVL on DGAT1 and DGAT2 gene expression in HepG2 cells. HepG2 cells were incubated in the absence or the presence of 1 and 10 µM SREM (SREM 1 and SREM10; respectively) or PVL (PVL1 and PVL10; respectively) for 1 or 24 h as described in methods. **A-B-** mRNA levels of DGAT1 and DGAT2 were measured by qPCR. Values were normalized to β-actin (housekeeping gene). Results for SREM and PVL were referred to control values (C) (non-added cells). Results are shown as mean ± SE of 3-4 independent experiments. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA test).

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Figure 1

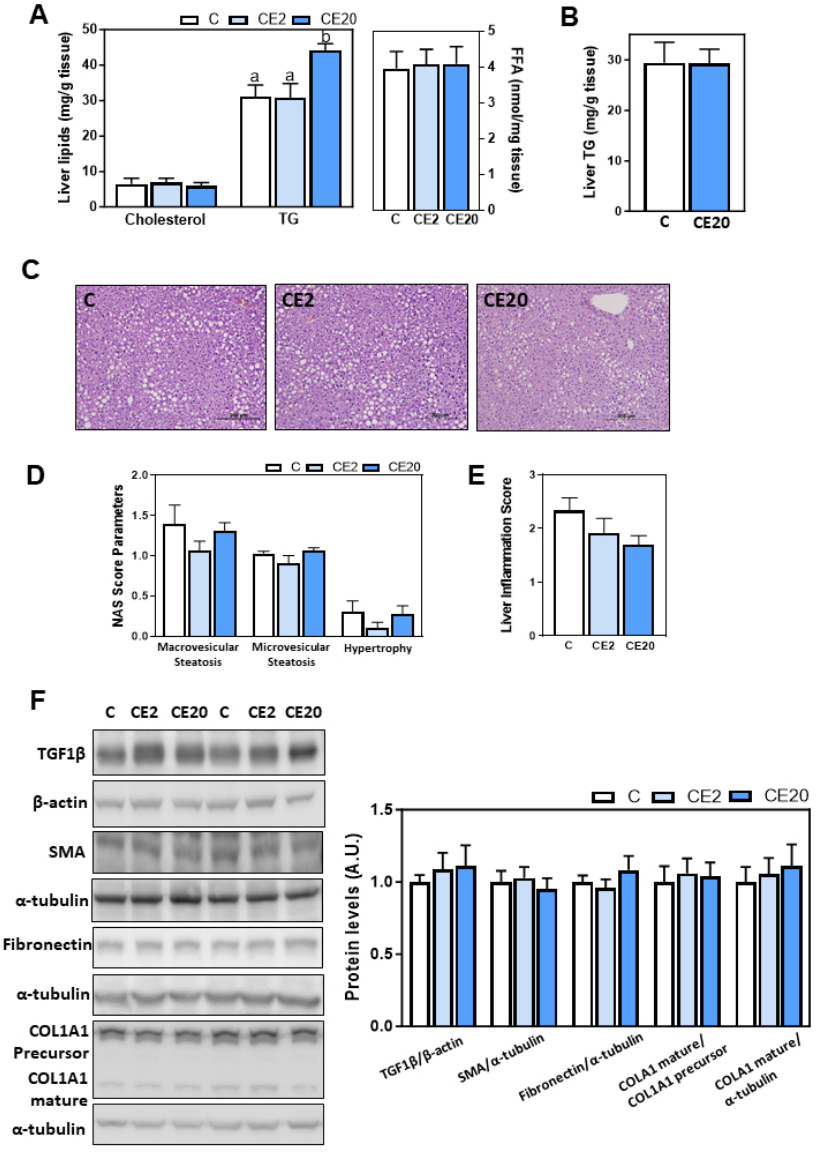

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Figure 2

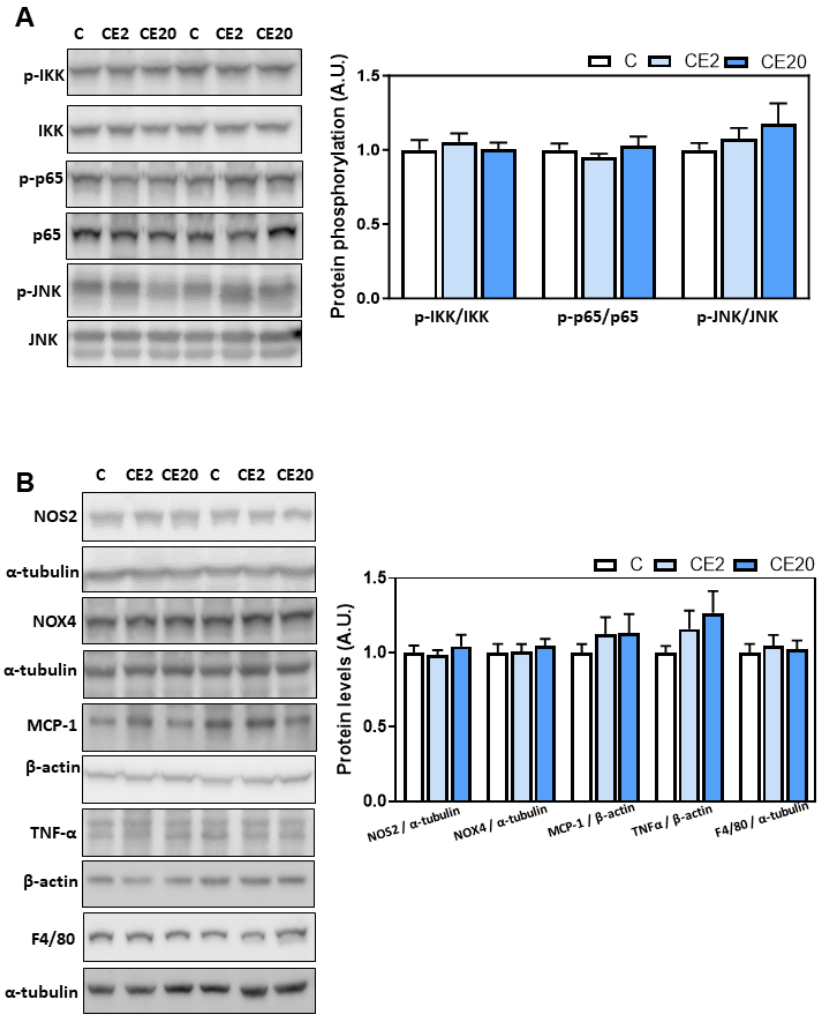

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Figure 3

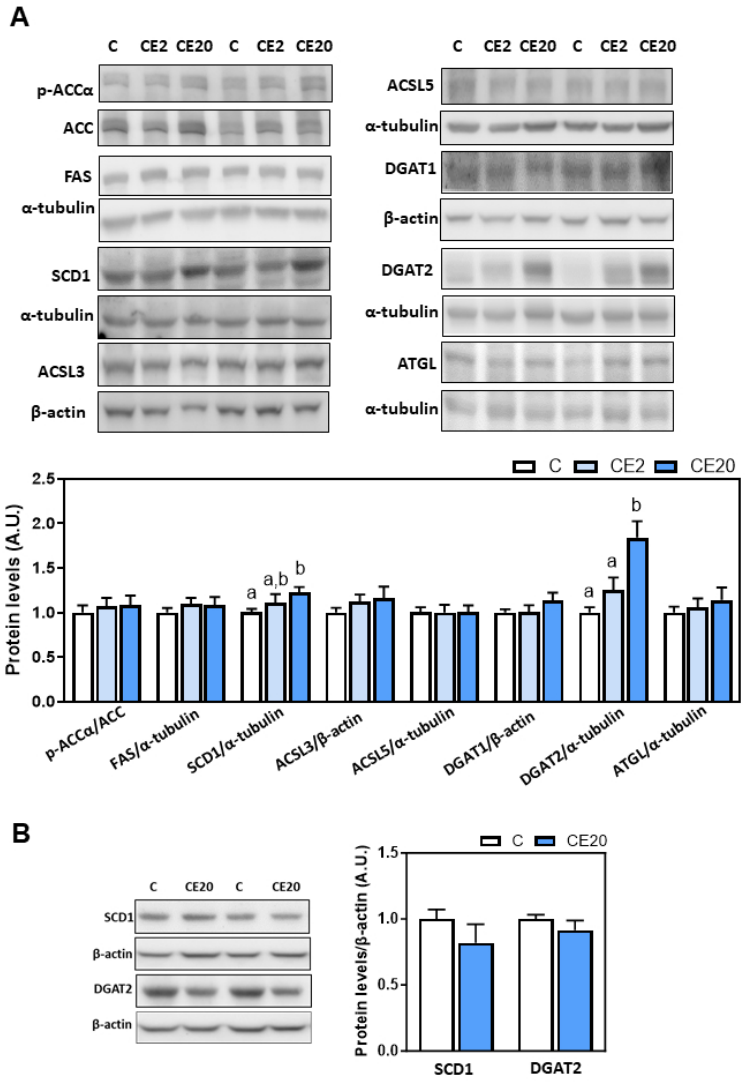

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Figure 4

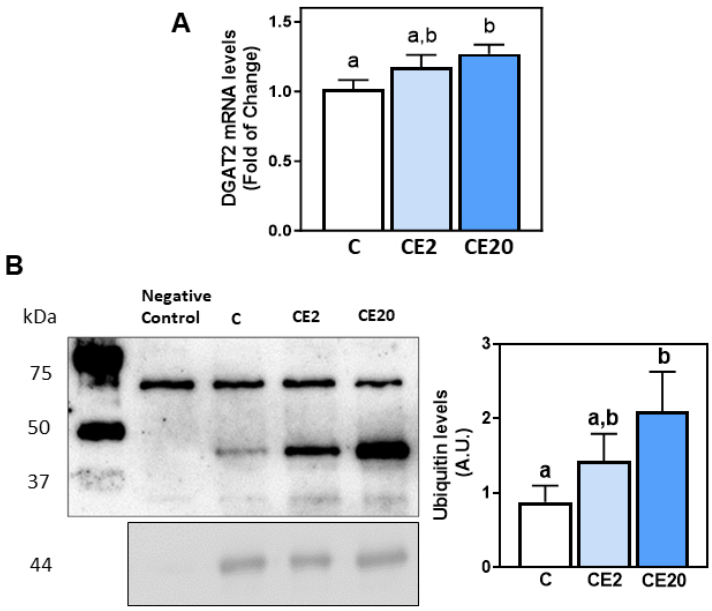

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Figure 5

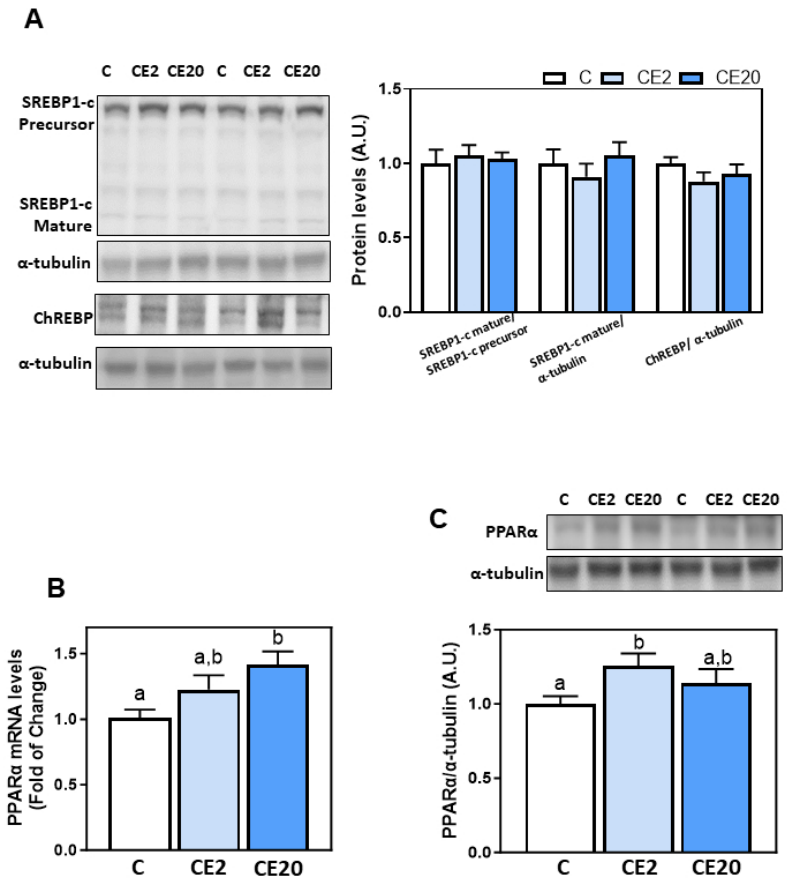

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Figure 6

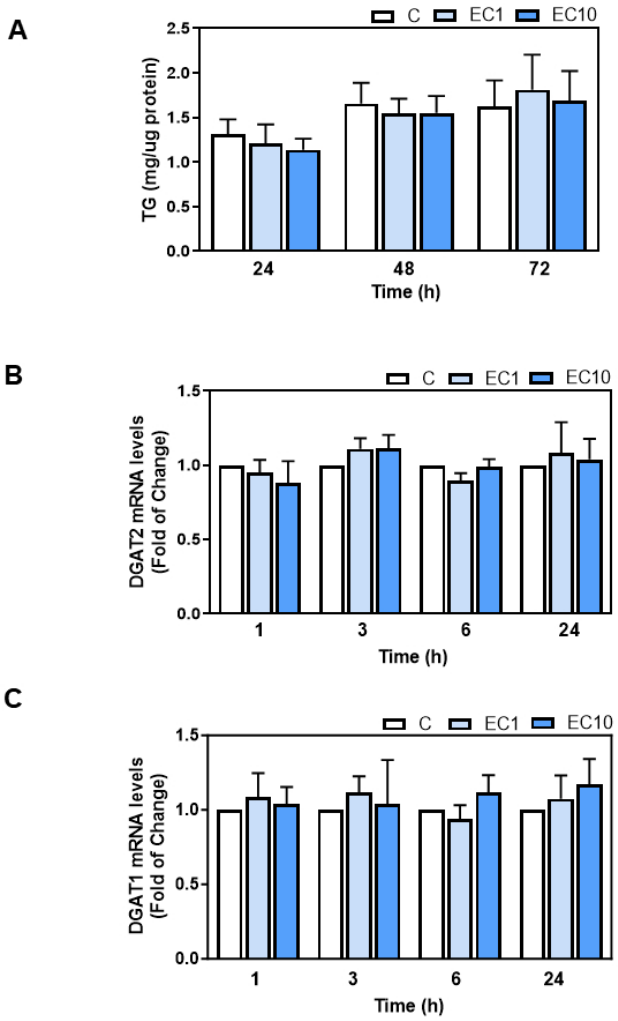

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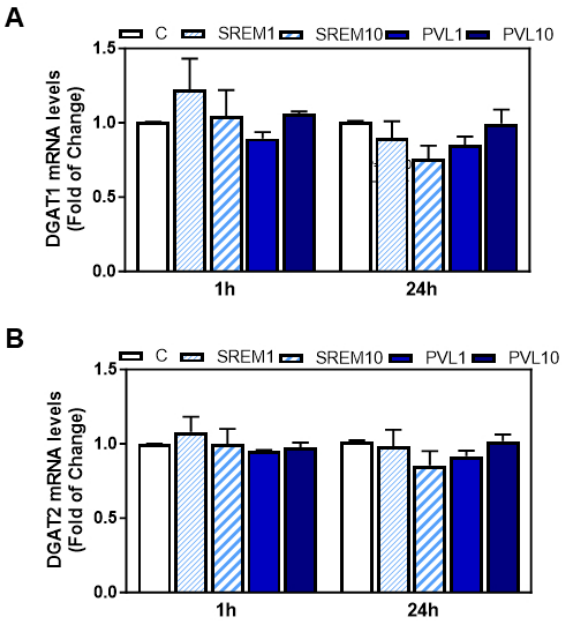
Figure 7



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