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A novel dietary multifunctional ingredient reduces body weight and improves leptin sensitivity in cafeteria diet-fed rats

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ABSTRACT

The use of a single family of dietary bioactive compounds has not been sufficient to manage highly regulated mechanisms such as body weight. Therefore, we aimed to evaluate the effect of a multifunctional ingredient (MIX) based on the co-administration of a low dose of conjugated linoleic acid with the protein hydrolysate from chicken feet, Hpp11, and a mixture of grape-seed proanthocyanidins and berry anthocyanins on body weight management in obese rats fed a cafeteria diet. The daily oral administration of the MIX for three weeks significantly reversed obesity and hyperleptinemia and increased energy expenditure and fat oxidation. In addition, animals administered the MIX exhibited greater hypothalamic activation of STAT3 in POMC-expressing neurons than non-supplemented animals. Our results strongly suggest that the co-administration of different bioactive compounds could be a promising strategy to complement the existing therapies against obesity and leptin resistance.

1. Introduction

Dietary obesity results from an altered energy regulatory mechanism in the arcuate nucleus of the hypothalamus, which gives rise to a chronic energy imbalance as a result of over nutrition, lack of physical activity or both (Di Angelantonio et al., 2016). This hypothalamic area receives signals from peripheral organs that provide information about energy status in the body (Horvath, 2005). One of these chemical signals is leptin, the key hormone in energy regulation, which is mainly produced in adipocytes, and its plasma levels are proportional to body fat stores (Friedman, 2019). Leptin activates a signal transduction pathway through the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in different neurons to suppress food intake and allow energy expenditure (Myers, Cowley, & Münzberg, 2008). In particular, leptin activates anorexigenic proopiomelanocortin (POMC) neurons that produce the α-MSH peptide responsible for binding melanocortin receptor 4 (MC4R), subsequently producing satiety signals and promoting energy expenditure (Barateiro, Mahú, & Domingos, 2017). However, in diet-induced obesity, leptin is not able to maintain energy balance despite the observation of highly increased plasmatic levels of this hormone. This loss in leptin sensitivity is known as leptin resistance, and the main molecular mechanisms involved in this phenomenon have been described in detail (Myers, 2015; Pan, Guo, & Su, 2014).

As current pharmacological treatments to control body weight and leptin sensitivity in diet-induced obesity have limited efficacy, the scientific community supports that dietary bioactive compounds could be a promising strategy to complement existing therapies. In this sense, in recent years, thousands of these compounds, such as flavonoids, fatty acids and bioactive peptides, have been investigated for their ability to prevent obesity (Konstantinidi & Koutelidakis, 2019). However, the use of a single family of bioactive compounds has not been sufficient to

Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; ALT, alanine transferase; ARA, arachidonic acid; AST, aspartate aminotransferase; CLA, conjugated linoleic acid; EPA, eicosapentaenoic acid; GSPE, grape-seed proanthocyanidin extract; Hpp11, protein hydrolysate from chicken feet; JNK, c-Jun N-terminal kinase; MC4R, melanocortin receptor 4; MIX, multifunctional ingredient; MUFA, monounsaturated fatty acids; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; POMC, proopiomelanocortin; PUFA, polyunsaturated fatty acids; RQ, respiratory quotient; STAT3, signal transducer and activator of transcription 3; VCO_2, volume of exhaled carbon dioxide; VH, vehicle; VO_2, volume of consumed oxygen; WAT, white adipose tissue

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manage body weight. Therefore, it is plausible to hypothesize that the co-administration of different bioactive compounds could promote body weight management in a much more effective way than the administration of individual bioactive compounds. In this context, we have developed a multifunctional ingredient (MIX) consisting of different natural dietary bioactive compounds that have individually been shown to exert certain beneficial effects against different targets associated with obesity. In particular, our MIX was based on the co-administration of a low dose of conjugated linoleic acid (CLA) with the protein hydrolysate from chicken feet, Hpp11, a mixture of proanthocyanidins extracted from grape seeds and anthocyanins from berries.

CLA is one of the most investigated dietary bioactive compounds in weight management, although controversial metabolic effects after its administration have been widely reported (Lamarche & Desroches, 2004; Tsuboyama-Kasaoka et al., 2000; Poirier, Shapiro, Kim, & Lazar, 2006). However, we have recently demonstrated that the daily administration of a low dose of CLA at 100 mg/kg of body weight (0.036% of the diet) caused a significant decrease in body weight gain in animals fed a cafeteria diet without any deleterious effect on insulin sensitivity or liver function (Martín-González et al., 2020). Additionally, it has also been reported that several polyphenolic-rich extracts obtained from natural sources are able to reduce adiposity and improve leptin signalling (Aragonès, Ardid-Ruiz, Ibars, Suárez, & Bladé, 2016). In particular, we demonstrated that the consumption of grape seed proanthocyanidins at 25 mg/kg of body weight for three weeks was able to reduce plasmatic leptin concentrations in obese rats and improve both hypothalamic and peripheral leptin signalling (Ibars et al., 2017). This capacity has also been observed in several pure phenolic compounds, most notably resveratrol (Ardid-Ruiz et al., 2018). Finally, chicken feet proteins have recently been demonstrated to be a great source of hydrolysates with antihypertensive properties (Bravo, Mas-Capdevila, Margalef, Arola-Arnal, & Muguerza, 2019). Although there are no reported effects of these hydrolysates on body weight management, previous studies have suggested that some angiotensin-converting enzyme inhibitors could also play a role in body fat and obesity (Weisinger et al., 2009).

Therefore, the aim of this study was to evaluate the capacity of a novel dietary multifunctional ingredient to regulate body weight in animals fed a cafeteria diet. In addition, both plasma leptin concentrations and hypothalamic leptin signalling were evaluated to investigate the primary potential mechanism by which this dietary multi-ingredient-based supplement alleviated obesity in these animals.

2. Material and methods

2.1. Multifunctional ingredient composition

CLA (Tonalin® TG 80) was purchased from BASF Chemical Company (Düsseldorf, Germany) and consisted of a mix of glycerides, of which 80% were conjugated linoleic acids. According to the manufacturer, the product was composed of equal amounts of two CLA isomers, c9,t11 and t10,c12. Grape-seed proanthocyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France), and its composition was previously characterized (Margalef et al., 2015). Anthocyanin extract (MEDOX®) was provided by Med-Palett AS (Sandnes, Norway) and consisted of 17 different natural purified anthocyanins from bilberry (*Vaccinium myrtillus*) and black currant (*Ribes nigrum*) (Qin et al., 2009). The relative content of each anthocyanin was as follows: 58% of 3-*O*-β-glucosides, 3-*O*-β-galactosides, and 3-*O*-β-arabinosides of delphinidin; 33% of 3-*O*-β-glucosides, 3-*O*-β-galactosides, and 3-*O*-β-arabinosides of cyanidin; 3% of 3-*O*-β-glucosides, 3-*O*-β-galactosides, and 3-*O*-β-arabinosides of malvidin; 2.5% of 3-*O*-β-glucosides, 3-*O*-β-galactosides, and 3-O-β-arabinosides of petunidin; 2.5% of 3-*O*-β-glucosides, 3-*O*-β-galactosides, and 3-*O*-β-arabinosides of peonidin; and 1% of 3-*O*-rutinoside of cyanidin and delphinidin. The composition and manufacturing method of the protein hydrolysate from chicken feet, Hpp11, was previously described (Bravo, Mas-Capdevila, Margalef, Arola-Arnal, & Muguerza, 2019; Mas-Capdevila, Pons, Aleixandre, Bravo, & Muguerza, 2018).

2.2. Experimental procedure

Male Wistar rats (n = 32) were purchased from Charles River Laboratories (Barcelona, Spain). The animals were housed in pairs under a 12 h light–dark cycle at 22 °C and fed a standard chow diet (Panlab A04, Barcelona, Spain) *ad libitum* during the adaptation week. Then, the animals were fed a cafeteria diet for 11 weeks as previously published (Pons, Margalef, Bravo, Arola-Arnal, & Muguerza, 2017). Briefly, the cafeteria diet was composed of 14% protein, 35% fat and 51% carbohydrates and consisted of bacon, carrots, cookies, foie gras, cupcakes, cheese and sugary milk. For the last three weeks, the animals were randomly distributed into two groups of 16 animals and orally treated with the MIX or vehicle (VH). The MIX was composed of CLA (100 mg/kg of body weight), GSPE (25 mg/kg of body weight), anthocyanins (100 mg/kg of body weight) and Hpp11 (55 mg/kg of body weight) suspended in 1 mL of glucose solution 50% (w/v). VH was composed of 400 mg maltodextrin/kg of body weight (Sigma, Madrid, Spain) diluted in the same sugary solution. Both treatments were administered daily between 8:00 a.m. and 9:00 a.m. for 3 weeks. The decision to use this combination of compounds was driven by the concept of putting together various bioactive compounds with different specific anti-obesity effects, in order to simultaneously act on the multiple processes involved in the onset and development of obesity. It should be noted that the individual doses used in this MIX were the same as we had previously used successfully (Ibars et al., 2017; Mas-Capdevila, Iglesias-Carres, Arola-Arnal, Suarez, Muguerza, & Bravo, 2019; Martín-González et al., 2020).

At the end of the experiment, the rats were fasted for 3 h after oral supplementation and then sacrificed. Blood was collected by decapitation into heparinized tubes. Serum was obtained after blood clotting and centrifugation (2,000*g*, 15 min, 4 °C) and stored at −80 °C. The liver, skeletal muscle and epididymal white adipose tissue (WAT) were excised, weighed, immediately frozen in liquid nitrogen and stored at −80 °C until further analysis, while the brain was frozen whole on dry ice to preserve morphology, embedded in Shandon M−1 embedding matrix (Thermo Fisher Scientific Inc., Bilbao, Spain) and stored at −80 °C.

The Animal Ethics Committee of University Rovira i Virgili approved all procedures (reference number 7959 by Generalitat de Catalunya). All of the abovementioned experiments were performed as authorized (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food, Madrid, Spain).

2.3. Body weight, food intake and body composition

Body weight and food intake were monitored weekly until the end of the experiment. In addition, the total body composition of live animals was assessed by proton nuclear magnetic resonance using an EchoMRI-700 system (Echo Medical Systems, Houston, TX, USA) one day before starting MIX administration and one day before sacrifice. Fat mass was directly measured in triplicate for each animal, and the results were expressed as a percentage of total body weight.

2.4. Indirect calorimetry

Indirect calorimetry analysis was performed using an Oxylet Pro System (Panlab, Barcelona, Spain). Rats were put in metabolic cages for 24 h, prior to the administration of the treatment, starting at 9:00 a.m., with the change in light, and were housed under a 12:12 h day:night cycle. Measurements were collected for 16 h around the change in light, 8 h during the day and 8 h at night. The procedure was performed

4 days before sacrifice on eight animals per group. Metabolism 2.1.02 software (Panlab, Barcelona, Spain) was used to measure the volume of consumed O_2 (VO_2) and the volume of exhaled CO_2 (VCO_2) in the chambers and automatically calculated respiratory quotient (RQ) as VCO_2/VO_2 and energy expenditure using the formula: $VO_2 \times 1.44 \times (3.815 + (1.232 \times RQ)$ (Kcal/day/$kg^{0.75}$), according to the Weir equation (Weir, 1949). A nitrogen excretion rate of 135 μg/kg/min was assumed (Carraro, Stuart, Hartl, Rosenblatt, & Wolfe, 1990).

2.5. Biochemical analysis

Serum leptin concentrations were measured using an ELISA kit from Millipore (Madrid, Spain) according to the manufacturer's instructions (Ref. #EZRL-83K). The sensitivity of the assay was 0.04 ng/mL. The intra-assay and inter-assay variations were 2.5% and 3.2%, respectively. All samples were diluted 1:2 with assay buffer (0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide and 1% BSA) and tested in duplicate. The enzyme-substrate reaction was developed using 3,3′,5,5′-tetramethylbenzidine, and the optical densities were measured at 450 nm in an EON Microplate microtiter plate reader (BioTek, Vermont, USA). The concentrations were calculated from a standard curve obtained from eight dilutions of lyophilized native rat leptin (range 0.2–30 ng/mL). In addition, both serum aspartate aminotransferase (AST) and alanine transaminase (ALT) enzymatic activities were measured using QCA kits (Comercial Bellés, Tarragona, Spain).

2.6. Liver histopathological analysis

Liver pieces were thawed and fixed in 4% formaldehyde for 24 h before several subsequent dehydration steps and paraffin infiltration at 52 °C. Sections were cut at a thickness of 2 μm (Microm HM 355S, Thermo Scientific, Madrid, Spain), stained with haematoxylin-eosin and analysed by a pathologist blinded to the experimental groups to measure the degree of steatosis and fibrosis, the percentage of microsteatosis and macrosteatosis and the presence of lipogranulomas.

2.7. Faecal lipid analysis

Faeces were collected over 24 h on the last day of the experiment, weighed and frozen at −80 °C until lipid extraction. Prior to extraction, the samples were lyophilized for 3 days, weighed, and stored at −80 °C after drying until further analysis. Then, 1 g was diluted with 5 mL of 0.9% NaCl (Sigma, Madrid, Spain) and 5 mL of chloroform/methanol (2:1). The homogenate was centrifuged at 1,000*g* for 10 min. The lipidic lower phase was separated and dried with nitrogen flux. Total lipids were measured by gravimetry and subsequently dissolved isopropanol. Triglyceride (Ref. #992320) and cholesterol (Ref. #995280) levels were analysed with colorimetric kits (QCA, Barcelona, Spain).

2.8. Hepatic ^{1}H NMR spectrometry

Liver extraction was performed according to previous reports (Martín-González et al., 2020) with slight modifications. Briefly, 50 mg of liver was manually homogenized using a micropestle in 1 mL of water/acetonitrile (1/1). The homogenate was centrifuged at 15,000*g* for 30 min at 4 °C. The lipophilic pellet was subsequently mixed with 1 mL of a solution of chloroform/methanol (2:1) at 0 °C, allowed to rest at room temperature for 10 min and then vortexed and centrifuged for 15 min at 6,000*g* at room temperature. The lipophilic supernatant was isolated from the debris, dried with nitrogen flux and stored at −80 °C until further analysis. ^{1}H NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5-mm PABBO (proton-enhanced Smartprobe® (Bruker®) broadband gradient probe) and were compared to pure compound references as previously described (Vinaixa et al., 2010). After pre-processing, specific ^{1}H NMR regions identified in the spectra were integrated and quantified using the AMIX 3.9 software package using the TSP signal of buffer as an internal reference.

2.9. Leptin signalling analysis

Brain samples were cut using a Leica cryostat to obtain 10 μm-thick coronal sections. Sections were washed and prefixed in 4% paraformaldehyde solution (w/v) for 10 min and then unmasked sequentially for 10 min each in 0.3% glycine (w/v) and 0.3% SDS (w/v). Afterwards, the sections were washed and then blocked for 1 h with goat serum blocking solution. Double staining was performed by simultaneous incubation with the following primary antibodies overnight at 4 °C: mouse anti-pSTAT3 (1:100, Cell Signalling Technology Europe, B. V, Leiden, Netherlands) and guinea pig anti-ACTH (1:500, National Hormone & Peptide Program, Los Angeles, CA, USA). The sections were then washed and incubated at room temperature for 1 h with goat anti-mouse Alexa Fluor 488 and goat anti-guinea pig Alexa Fluor 594 (1:200, Thermo Fisher Scientific Inc., Bilbao, Spain). Nuclear counterstaining was performed by 4′,6-diamidino-2-phenylindole (DAPI, 1:2,000) incubation together with the secondary antibody. Sections were prepared using Vectashield (Vector Laboratories, Burlingame, CA, USA) mounting media. Images were captured with a Nikon TE2000-E microscope equipped with a Hamamatsu C8484 digital camera. Images were taken using NIS-Elements software (Nikon, Melville, NY, USA) with the same exposure time and avoiding pixel saturation. Only sections processed in the same experiments were compared. ImageJ (NIH, Bethesda, MD, USA) was used to quantify pSTAT3-positive cells. The researcher was blinded to the experimental groups of the images for the cell counting analysis. Sections were matched by anatomical landmarks according to bregma (anterior, Bregma: −2.3 mm; medial, Bregma −2,80 mm; posterior, Bregma −3.30 mm) using Paxino's and Watson's Rat Brain Atlas (Seventh Edition). At least two sections per region per animal were quantified and then averaged. To quantify the number of pSTAT3-positive cells, only signals overlapping with DAPI were included.

In addition, leptin signaling in the liver, calf skeletal muscle and epididymal WAT was also assessed by calculating the activation of STAT3 using an ELISA kit with a phospho-specific antibody for STAT3 phosphorylation (pSTAT3) at tyrosine 705 (Abcam, Cambridge, UK). As cellular pSTAT3 levels are mainly attributable to leptin action, leptin sensitivity in the peripheral tissues was objectively estimated as the ratio of pSTAT3 levels in each tissue to the leptin concentration in serum as previously reported (Ardid-Ruiz et al., 2018).

2.10. Statistical analysis

The data are expressed as the means ± standard errors of the means (SEMs). Groups were compared by Student's *t*-test or paired *t*-test. Outliers were determined by Grubbs' test. Statistical analyses were performed using XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel 2017 (Addinsoft, Paris, France). Graphics were prepared using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. MIX reversed the obesity induced by the cafeteria diet without compromising liver function

As shown in Fig. 1A, the body weight of all animals at the beginning of the study was 173 ± 3 g. During the course of the experiment, the animals fed the cafeteria diet continuously and consistently gained weight (4.92 ± 0.2 g/day) until they reached a final body weight of 552 ± 14 g in the last week of the experiment. Notably, there was a

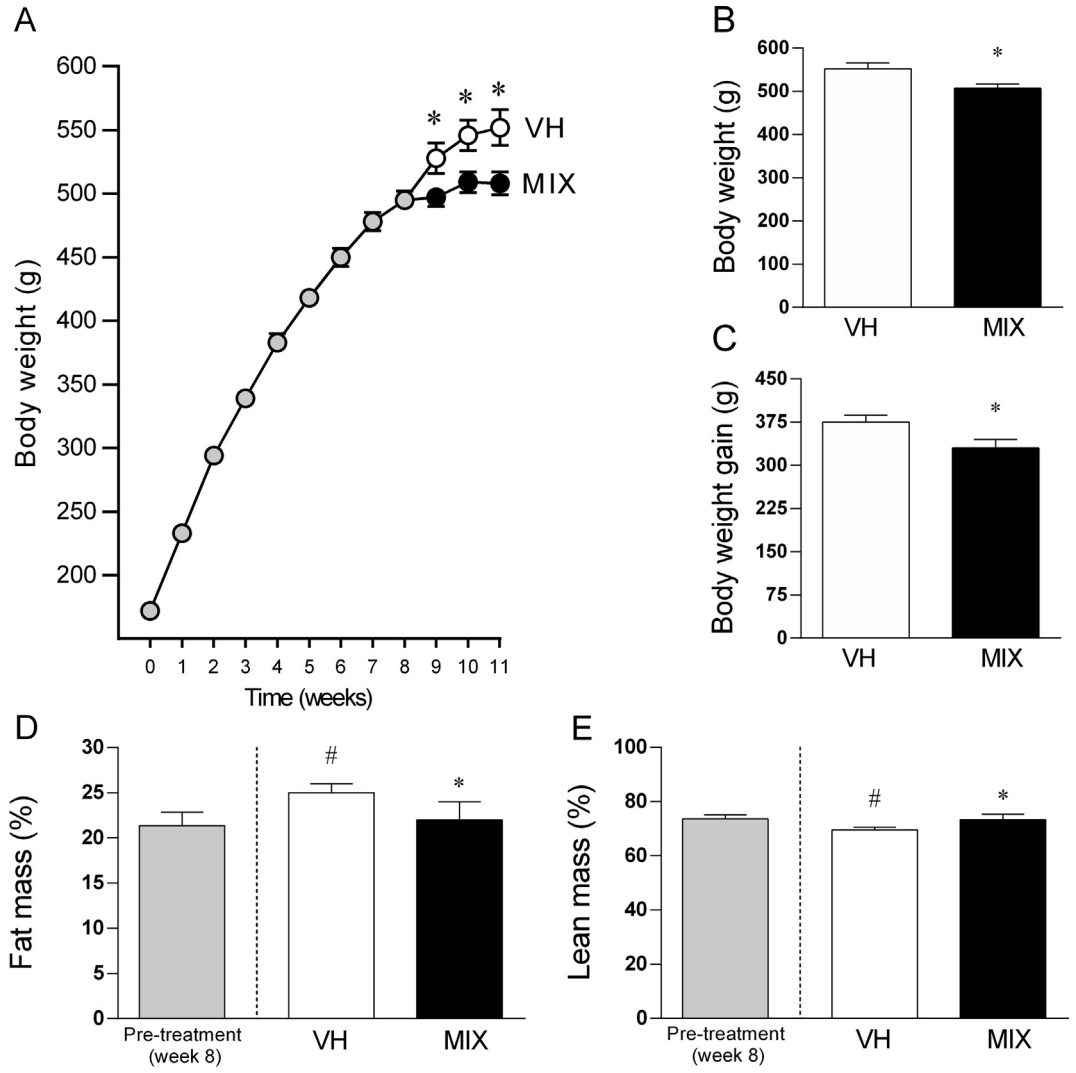

Fig. 1. Assessment of body weight and composition. Changes in body weight throughout the experiment (A), and final body weight (B) and body weight gain (C) at the end of the experiment. Pre- and post-treatment body composition assessed by ^{1}H NMR, including fat (D) and lean (E) content. The rats were fed a cafeteria diet for 11 weeks and were orally treated with VH or MIX for the last three weeks (weeks 8 to 11). Data are expressed as the mean ± SEM. * indicates $P < 0.05$ as assessed by Student's *t*-test comparing the VH group to the MIX group. # indicates $P < 0.05$ as assessed by paired *t*-test comparing pre- and post-treatment fat and lean mass. ^{1}H NMR: proton nuclear magnetic resonance; MIX: multifunctional ingredient; VH: vehicle.

strongly significant reduction of 8.1% in the final body weight of animals supplemented with the MIX compared with the animals supplemented with the VH (Fig. 1B). In addition, body weight gain was 11.8% lower in animals supplemented with the MIX than in those in the VH group (Fig. 1C), and this reduction was directly associated with a significant decrease in total body fat mass (Fig. 1D) and was not accompanied by a decrease in lean mass (Fig. 1E).

Furthermore, as rapid and severe body fat mass loss could result in fat accumulation within the liver, hepatic lipid content was quantified with ^{1}H NMR spectroscopy in all animals. As shown in Table 1, supplementation with the MIX did not significantly change the lipid profile in the liver of these animals, although the concentrations of total and esterified cholesterol, diglycerides, sphingomyelin and omega-3 fatty acids were significantly increased with respect to the animals supplemented with the VH. However, no significant changes in free cholesterol, triglycerides and total fatty acid concentrations were observed in MIX-supplemented animals with respect to VH animals.

Serum transaminase enzymatic activity and haematoxylin-eosin staining were also used to evaluate hepatic damage in these animals. Importantly, our data confirmed that supplementation with the MIX did not result in significant changes in serum transaminases and did not exacerbate the degree and type of hepatic steatosis induced by the cafeteria diet (Table 2). In addition, no significant changes in the hepatic transcripts of inflammatory genes were observed in the MIX-supplemented group compared to the VH group (Supplementary Fig. 1).

3.2. MIX improved hyperleptinemia and energy expenditure without modifying faecal lipid excretion and food intake

Decreased body fat mass could be a consequence of reduced intestinal lipid absorption. Thus, to analyse the mechanism underlying the reversed obesity observed in animals supplemented with the MIX, we directly measured faecal lipid excretion in these animals. As shown in Table 3, faecal cholesterol and triglyceride concentrations and total lipid content were not affected by MIX consumption, indicating that intestinal lipid absorption was not altered by the dietary incorporation of the MIX in animals fed a cafeteria diet. However, supplementation with the MIX for three weeks significantly reduced the hyperleptinemia induced by the cafeteria diet (28.6% lower), indicating that supplementation with the MIX during this period was able to normalize the leptin levels of cafeteria diet-fed rats (Fig. 2).

As leptin is reported to maintain energy balance in mammals, we

Table 1
Individual concentrations (arbitrary units) of lipid metabolites identified in liver.

	VH	MIX	P value*
Total cholesterol	15.66 ± 1.5	29.05 ± 3.6	< 0.01
Free cholesterol	3.8 ± 0.2	4.16 ± 0.2	*ns*
Esterified cholesterol	12.06 ± 1.5	24.79 ± 3.4	< 0.01
Triglycerides	17.76 ± 2.5	21.56 ± 2.3	*ns*
Diglycerides	5.2 ± 0.3	6.08 ± 0.3	0.05
Sphingomyelin	0.93 ± 0.03	1.02 ± 0.03	0.04
ARA + EPA	11.88 ± 0.4	12.83 ± 0.5	*ns*
Plasmalogen	0.3 ± 0.01	0.4 ± 0.01	*ns*
Total phospholipids	24.73 ± 0.9	26.05 ± 0.7	*ns*
Linoleic acid	9.21 ± 0.9	11.18 ± 1.1	*ns*
Oleic acid	44.91 ± 5	55.07 ± 4.5	*ns*
Omega-3 fatty acids	4.39 ± 0.2	5.81 ± 0.5	0.01
PUFA	51.49 ± 1.9	53.07 ± 1.5	*ns*
MUFA	62.32 ± 6.7	77.6 ± 6.4	*ns*
Total fatty acids	140.65 ± 10.4	166.95 ± 10.7	*ns*

Values are presented as the mean ± SEM. * assessed by Student's *t*-test; *ns* indicates not significant.
ARA: arachidonic acid; EPA: eicosapentaenoic acid; MIX: multifunctional ingredient; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; VH: vehicle.

Table 2
Summary of biomarkers of hepatic damage.

Serum biomarkers	VH	MIX	P value*
Aspartate aminotransferase (U/mL)	190.9 ± 16.1	177.8 ± 15.3	*ns*
Alanine transferase (U/mL)	70.3 ± 9.1	58.8 ± 8.1	*ns*
Liver histological analysis	VH	MIX	P value*
Steatosis degree (0 to 3 in severity)	1.8 ± 0.2	2.1 ± 0.2	*ns*
Microsteatosis (% of total steatosis)	82.2 ± 6	80.6 ± 6	*ns*
Microsteatosis (% of total steatosis)	11.6 ± 2	13.1 ± 2	*ns*
Fibrosis degree (0 to 4 in severity)	0	0	*ns*
Portal inflammation (0 to 2 in severity)	0	0	*ns*
Lipogranulomas (number of samples)	0/16	0/16	*ns*

Values are presented as the mean ± SEM. * assessed by Student's *t*-test; *ns* indicates not significant.
MIX: multifunctional ingredient; VH: vehicle.

Table 3
Faecal lipid and water content.

	VH	MIX	P value*
Total Lipids (mg/g collected faeces)	12.7 ± 2.3	10.15 ± 1.7	*ns*
Triglycerides (mg/g collected faeces)	0.37 ± 0.1	0.52 ± 0.1	*ns*
Total Cholesterol (mg/g collected faeces)	1.26 ± 0.2	1.11 ± 0.3	*ns*
Water Content (%)	32.51 ± 4.1	32.35 ± 4	*ns*

Values are presented as the mean ± SEM. * assessed by Student's T-test; *ns* indicates not significant.
MIX: multifunctional ingredient; VH: vehicle.

reasoned that MIX consumption could decrease food intake, enhance energy expenditure and physical activity or lead to the utilization of fat as the main energy source. Thus, we directly measured the cumulative food intake, locomotor activity, energy expenditure and respiratory quotient (RQ) of both groups of rats (Table 4). However, although food intake was decreased by MIX consumption, this difference among groups did not reach statistical significance. No significant differences were either observed in physical activity levels between the two groups. In contrast, animals supplemented with the MIX had significantly increased VO_2 values and, subsequently, energy expenditure (Supplementary Fig. 2). In addition, a significant increase in fat oxidation was also observed in the MIX group compared with the VH group, although no significant changes were observed in RQ values. Together, these results indicate that compared with VH supplementation, MIX consumption increased energy expenditure and favoured lipid use as an energetic substrate in cafeteria diet-fed rats, resulting in reduced energy balance and body fat mass percentage.

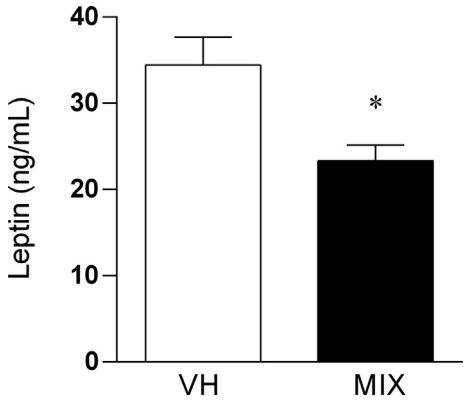

Fig. 2. Serum leptin concentrations. Serum leptin values were measured using an ELISA kit from Millipore (Madrid, Spain). The rats were fed a cafeteria diet for 11 weeks and were orally treated with VH or MIX for the last three weeks (weeks 8 to 11). Data are expressed as the mean ± SEM. * denotes P < 0.05 assessed by Student's *t*-test. MIX: multifunctional ingredient; VH: vehicle.

Table 4
Summary of food intake, substrate oxidation and energy expenditure.

	VH	MIX	P value*
Cumulative food intake (kJ/day/animal)	2099.9 ± 172	1877.6 ± 56	*ns*
RQ	0.79 ± 0.02	0.76 ± 0.01	*ns*
EE (Kcal/day/$kg^{0.75}$)	101.30 ± 3.8	111.81 ± 3.3	0.04
CH oxidation (kJ/min/$kg^{0.75}$)	321.42 ± 20.3	276.99 ± 21.9	*ns*
Fat oxidation (kJ/min/$kg^{0.75}$)	185.91 ± 23.5	230.44 ± 17.7	0.05
Activity (counts/min)	0.38 ± 0.02	0.35 ± 0.01	*ns*

Values are presented as the mean ± SEM. * assessed by Student's T-test; *ns* indicates not significant.
CH: carbohydrate; EE: energy expenditure; MIX: multifunctional ingredient; RQ: respiratory quotient; VH: vehicle.

3.3. MIX enhanced hypothalamic and peripheral leptin signalling

To address whether the decrease observed in serum leptin concentrations could indicate that supplementation with MIX affects the functionality of POMC-expressing neurons, we assessed the leptin signalling pathway through the detection of STAT3 activation in the hypothalamus using a phospho-specific antibody recognizing Tyr705-phosphorylated STAT3 (p-STAT3). Indeed, a statistically significant increase in the levels of p-STAT3 in POMC-expressing neurons was detected in the animals supplemented with the MIX (Fig. 3A and Supplementary Fig. 3), indicating that the consumption of this multifunctional ingredient for three weeks was sufficient to rescue hypothalamic leptin signalling in these animals. Alternatively, to assess the contribution of the leptin-related metabolic signals derived from peripheral tissues to the regulation of energy homeostasis, we also investigated the leptin signal transduction in the liver, skeletal muscle and epididymal WAT. Indeed, an increase in leptin sensitivity was also observed in the liver and epididymal WAT compared with the VH group (Fig. 3B, C), although no significant differences were observed in the skeletal muscle (Fig. 3D).

4. Discussion

Previous results from our group and other authors indicated that the

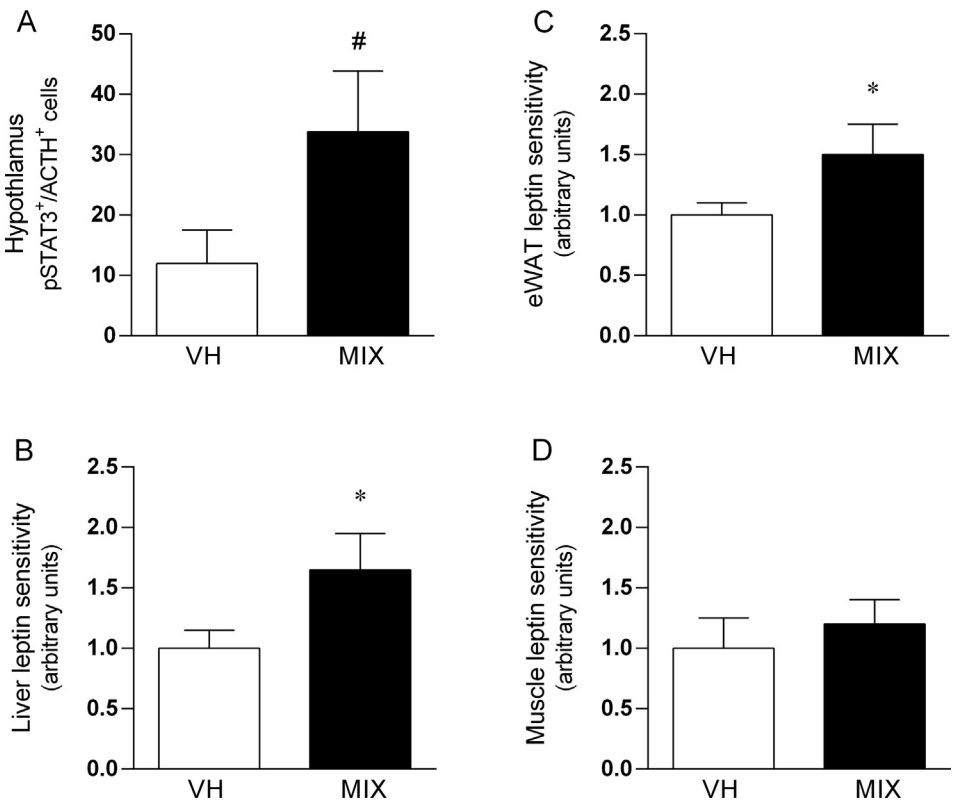

Fig. 3. Leptin signalling pathway. Leptin sensitivity in hypothalamic POMC neurons (A), liver (B), epididymal WAT (C) and skeletal muscle (D). Rats were fed a cafeteria diet for 11 weeks and were orally treated with VH or MIX for the last three weeks (weeks 8 to 11). Data are expressed as the mean ± SEM. * denotes $P < 0.05$ and # denotes $P < 0.01$ as assessed by Student's *t*-test comparing the VH group to the MIX group. WAT: white adipose tissue; MIX: multifunctional ingredient; VH: vehicle.

individual consumption of different families of bioactive compounds is able to exert certain beneficial effects against different metabolic alterations induced by a cafeteria diet (Casanova et al., 2014; Pons, Margalef, Bravo, Arola-Arnal, & Muguerza, 2017; Ardid-Ruiz et al., 2018; Martín-González et al., 2020; Guirro et al., 2020). However, several studies over the past few years have reported that the use of a single family of bioactive compounds at physiological doses is usually not sufficient to effectively correct highly regulated processes such as body weight (Abidov, Ramazanov, Seifulla, & Grachev, 2010; Rondanelli et al., 2013; Maunder, Bessell, Lauche, Adams, Sainsbury, & Fuller, 2020). Thus, the objective of this study was to determine whether the co-administration of a novel dietary multifunctional ingredient was able to properly manage body weight in animals fed a cafeteria diet.

Our results demonstrated that the co-administration of different bioactive compounds in a single multifunctional ingredient was successful in promoting body weight management and fat loss. In this sense, the co-administration of 100 mg of CLA /kg of body weight with the protein hydrolysate from chicken feet, Hpp11 (55 mg/kg of body weight), and a mixture of grape-seed proanthocyanidins (25 mg/kg of body weight) and berry anthocyanidins (100 mg/kg of body weight) resulted in a marked decrease in body weight in cafeteria diet-fed rats. In particular, our results showed that the administration of MIX to cafeteria diet-fed animals for three weeks resulted in a marked reduction in fat mass without any impact on lean body mass. This protection against lean body mass loss is of value since several studies reported that the loss of lean body mass could have multiple negative health implications (Willoughby, Hewlings, & Kalman, 2018), and several studies have incorporated exercise or dietary supplement products in conjunction with a weight loss program in an effort to offset this lean body mass.

In addition, the effect of MIX administration on hepatic lipid metabolism was also examined in this study since marked body weight loss could promote hepatic fat accumulation. In this sense, it is well-stablished that more than 30% of hepatic triglycerides in non-alcoholic fatty liver disease are derived from the circulating free fatty acids released by lipolysis in white adipose tissue (Donnelly et al., 2005). Our results showed that the administration of the MIX did not cause liver injury or hepatic triglyceride accumulation, despite reduced body fat mass. Supporting these results, an improvement in the hepatic lipid profile caused by the same doses of CLA and GSPE supplementation used in this study has been previously reported in cafeteria diet-fed rats (Martín-González et al., 2020; Bladé et al., 2016). In addition, the histopathological analyses performed in this study showed no signs of liver damage aggravation in rats supplemented with the MIX, and even lower serum AST and ALT enzymatic activities were observed in MIX-supplemented animals than in VH-supplemented animals, suggesting a slight improvement in the hepatic damage associated with the cafeteria diet. Nevertheless, a significant increase in the esterified cholesterol content after MIX supplementation was observed in these animals. Increased cholesterol concentrations in the liver haven been recently described for CLA supplementation (Martín-González et al., 2020) and could be related to aggravation of hepatic oxidative stress (Enríquez-Cortina et al., 2017). Thus, further research is needed to assess the effects of MIX administration on esterified cholesterol deposition in the liver.

Our results also showed an increase in energy expenditure and fat utilization as energetic substrates in animals supplemented with the MIX, resulting in a reduced energy balance compared to the control animals. Remarkably, food intake and intestinal lipid absorption were not affected by the administration of the MIX. Supporting these results, different studies with CLA, anthocyanins and GSPE demonstrated the ability of these bioactive compounds to induce fat oxidation in obese animals (Guo & Ling, 2015; Casanova et al., 2014; Terpstra et al., 2002). However, only CLA supplementation increased energy expenditure in obese animals (Terpstra et al., 2002; Den Hartigh et al., 2017). Thus, it is plausible to mainly attribute the observed differences in energy expenditure to the CLA, although the effects on fat oxidation could be caused by a synergic effect of the co-administration of CLA with the other functional ingredients.

Because of the increased energy expenditure and lipid oxidation observed in the animals supplemented with MIX, we also studied hypothalamic leptin signalling, which is reported to maintain energy balance in mammals. Our results showed that MIX administration restored serum leptin values and increased peripheral and hypothalamic leptin sensitivity. This improvement in central leptin sensitivity could mediate the decreased body weight and fat mass and the increased energy expenditure observed in animals supplemented with the MIX. Nevertheless, other possibilities cannot be discarded and, consequently, further studies are needed to better elucidate the mechanisms by which MIX promotes body weight management and fat loss.

The induction of central leptin resistance in diet-induced obesity has been mainly attributed to hypothalamic inflammation as a result of the induction of pro-inflammatory signalling molecules such as JNK and NF-κB (Benzler et al., 2015; de Git & Adan, 2015). Remarkably, previous results demonstrated that GSPE supplementation reduced hypothalamic inflammation (Ibars et al., 2017), suggesting that this local anti-inflammatory activity of proanthocyanidins in this tissue could be one of the mechanisms by which MIX administration restored hypothalamic leptin signalling. In addition, sirtuin-1 activity has also been highlighted as a mediator of hypothalamic leptin action (Sasaki et al., 2014; Sasaki, 2015). Therefore, the hypothalamic overexpression of sirtuin-1 induced by GSPE supplementation could be another mechanism by which MIX administration reduced hypothalamic leptin resistance. However, further studies are warranted to elucidate the molecular mechanism by which MIX administration re-establishes central and peripheral leptin sensitivity in cafeteria diet-fed animals.

Our study does have some limitations including the relatively short-duration period of the MIX supplementation and that we did not test the efficacy of the individual components of the MIX, although there is a substantial quantity of scientific literature available relating to the activities of the individual components within this combination of bioactive ingredients (Ibars et al., 2017; Mas-Capdevila, Iglesias-Carres, Arola-Arnal, Suarez, Muguerza, & Bravo, 2019; Martín-González et al., 2020).

5. Conclusions

In summary, our results demonstrated that the co-administration of different bioactive compounds in a single multifunctional ingredient was effective in promoting body weight management in cafeteria diet-fed animals. The effect of the MIX on body weight, which could be partially mediated by improved hypothalamic leptin sensitivity, was directly associated with a significant increase in energy expenditure and fat oxidation but not with changes in dietary fat absorption. In addition, MIX administration was not accompanied by any adverse hepatic effect associated with weight loss, although a note of caution concerning the increased values of esterified cholesterol observed in the liver is warranted. Thus, the MIX could be a good candidate to be included in functional foods to complement existing therapies for the management of obesity. Nevertheless, more studies are needed in order to further investigate the mechanisms of their possible effect.

6. Ethics statements

The study was conducted in accordance with the European Directive 2010/63/EU for animal experiments and was approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 7959 by Generalitat de Catalunya).

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CRediT authorship contribution statement

Miguel Z. Martín-González: Methodology, Formal analysis, Writing - original draft. **Héctor Palacios-Jordan:** Methodology. **Maria Ibars:** Methodology, Formal analysis. **Andrea Ardid-Ruiz:** Methodology. **Albert Gibert-Ramos:** Methodology. **Miguel A. Rodríguez:** Formal analysis. **Manuel Suárez:** Conceptualization. **Begoña Muguerza:** Conceptualization, Writing - review & editing, Funding acquisition. **Gerard Aragonès:** Conceptualization, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.104141.

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