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ARTICLE

Chronic flavanol-rich cocoa powder supplementation reduces body fat mass in endurance athletes by modifying the follistatin/ myostatin ratio and leptin levels

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Flavanols-rich cocoa has positive effects on lipid metabolism and might enhance the performance of athletes through an improvement in their body composition. To test this hypothesis a placebo-controlled intervention study in training endurance athletes who received 5 g of cocoa daily (425 mg of flavanols) for 10 weeks was performed. Dietary intake, body composition, exercise performance and plasma levels of follistatin, myostatin and leptin were measured. Cocoa intake significantly reduced body fat percentage ($p = 0.020$), specifically in the trunk ($p = 0.022$), visceral area ($p = 0.034$) and lower limbs ($p = 0.004$). The reduction in body fat mass was accompanied by an increase in plasma follistatin and a decrease in leptin, while myostatin levels remained unchanged. The intake of cocoa reduced the percentage of body fat of athletes, without any impact on athletes' performance. The change in fat body composition did not improve athletes' performance.

Introduction

A dietary supplement is a manufactured product that is taken by mouth, containing a dietary ingredient, and that claims to improve overall health status. Many athletes (between 60 to 90%) use dietary supplements in an attempt to enhance physical performance, improve body composition or promote recovery from exercise, and these can take several forms including energy drinks, caffeine, vitamins/minerals and amino acids/proteins, among others [1].

Cocoa beans from the cocoa tree, *Theobroma cacao*, are rich in polyphenols (flavonoids) and more specifically in flavanols (procyanidins and catechins) that confer antioxidant and cardioprotective properties [2]. Flavanols may play a role in preventing obesity by increasing lipolysis and inducing changes in the lipid profile, and by decreasing the levels of low-density lipoproteins, triglycerides, total cholesterol and inflammatory markers [3,4]. On account of its numerous biological effects and its safety profile, cocoa supplementation might improve the performance of and recovery from exercise and counteract some of the physiological effects associated with exercise such as oxidative stress. Indeed, combining cocoa supplementation with exercise has shown promising effects to counteract the exercise-induced oxidative stress and to improve lipid and carbohydrate metabolism in healthy and overweight subjects [5]. In addition to flavanol monomers catechin and epicatechin, cocoa also contains oligomeric procyanidins, which together seem to be responsible for the observed positive effects of cocoa. However, it is very difficult to

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draw general conclusions on the effects of cocoa, because in many of studies the content of these compounds is not specified [5] and the composition of cocoa bioactive compounds varies considerably depending on the cocoa genotype, the fermentation degree and drying processes [6], generating cocoa products with very diverse composition [7,8].

In a pilot study in six humans, administration of 25 mg/day of epicatechin significantly elevated the plasma ratio of follistatin/myostatin and also increased handgrip strength [9]. Myostatin is a myokine produced in muscle and adipose tissue that is involved in muscle growth and metabolism, and its activation produces muscle atrophy and promotes fat accumulation [10]. Conversely, follistatin promotes the adipose tissue browning and decreases the abdominal fat content in mice, and also counteracts the myostatin blockade of muscle growth [11,12]. The loss of white adipose tissue could be beneficial for the body composition of athletes and their physical performance, as it has been described that a decrease in fat mass positively correlates with athletes´ performance [13] and predicts race time in recreational marathoners [14].

In this regard, we sought to examine whether the chronic intake of a high-flavanols characterized cocoa modifies the body composition of endurance athletes through the alteration of the circulating levels of adipokines, as a primary outcome, and whether it had an impact on exercise performance.

Materials and Methods

Materials

Hexane, acetone, formic acid, acetonitrile, methanol solvents and reagents were from J.T. Baker (JT Baker Chemical Company, Phillispburg, NJ, USA). Pure chemical standards were obtained from Sigma-Aldrich (St. Louis, MI, USA). High-flavonol cocoa (83 mg/g) was purchased in the United Kingdom market. Maltodextrin supplement was acquired from Prozis (Esposende, Portugal).

Polyphenolic Characterization of Cocoa Supplement

As the flavonoid content in cocoa and derived products varies significantly between different manufacturers, we analyzed seven different cocoa powders marketed in Spain and the United Kingdom using the protocol established by Alañon et al. [15], and the cocoa with the highest flavanol content was selected to perform the study. Briefly, cocoa powders were defatted with a series of successive washes in hexane (1:10 w/v) in an ultrasonic bath, followed by centrifugation at 10,000 rpm for 10 min at 4°C to obtain the residual defatted pellets. This procedure was repeated three times. The pellets were - air-dried for 24 h to remove the residual organic solvent, freeze-dried, and then submitted to three repeated extractions with 70% acetone (1:10 w/v) in an ultrasonic bath. Supernatants were recovered after centrifugation at 10,000 rpm for 10 min at 25°C, and concentrated in a roto-evaporator (Büchi Labortechnik AG, Flawil Switzerland). Samples were kept at -80°C until analysis. Analyses were carried out by ultra-high-performance liquid chromatography (Waters Corp., Milford, MA, USA) coupled with tandem photodiode array detection and electrospray ionization (ESI) triple quadrupole mass spectrometry (MS/MS) (Xevo TQS, Waters Corp., Wexford, Ireland). The chromatography system consisted of a sample manager (6°C) and a quaternary solvent manager. The polyphenolic compounds were separated on a C18, 150 mm × 2.1 mm, 1.7 µm column (Agilent Technologies, Santa Clara, CA, USA), operated at 30°C. The elution profile included two solvents: acidified Milli Q water with 7.5 mM formic acid (Solvent A) and acetonitrile (Solvent B): Initial - 3% B, 1.88 min gradient to 9% B; 5.66 min gradient to 16% B; 16.90 min gradient to 50% B; 19.62 min gradient to 3% B; 20.0 min 3% B for column stabilization at a flow rate of 210 µL/min. Ionization was carried out using methanol as co-solvent with 0.1% formic acid (v/v) at a flow of 5 µL/min with the use of an isocratic solvent manager (Waters Corp.). Multiple reactions ionization mode was used for MS/MS assays. ESI operating conditions for the negative ionization mode were as follows: capillary voltage 2.5 kV, desolvation temperature 400°C, source temperature 150°C, desolvation gas flow 800 L/h and cone gas flow 150 L/h, collision

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gas flow 0.13 mL/min, MS mode collision energy 5.0 eV and MS/MS mode collision energy 20.0 eV. For the identification and quantification of the polyphenolic profile, a mixture of standards (20 ng/µL) was used to determine retention time and m/z values and MS/MS transitions. Multiple reaction monitoring mode was used for samples and standards. The liquid chromatography and tandem Xevo TQ-S triple quadrupole MS control and data processing was done using MassLinx v. 4.1 Software (Waters Corp.). The cocoa polymerization degree (mDP) was obtained following acid-catalysis in the presence of phloroglucinol using the method previously described by Kennedy and Jones [16]. Chromatographic conditions were the same as described above.

Experimental Design, Dietary Supplementation and Control of the Intervention Compliance

This study was a randomized, parallel-group placebo-controlled trial. The Research Ethics Committee on Drug of the Comunidad de Madrid (CEIm), Spain approved the study (Ref: 07/694487.9/17). All procedures were in accordance with the 1964 Helsinki Declaration and its later amendments. Written informed consent was obtained from all participants. Inclusion criteria were male endurance cross-country athletes, 18-50 years of age, with high physical condition (VO_2 ≥ 55 ml/(kg · min) and BMI 18-25 kg/m^2; exclusion criteria were consumption of any kind of nutritional or ergogenic supplement, be vegetarian or vegan, smoke, chronic medication, gastrointestinal surgery, or any diagnosed disease. Subjects were randomized to two equal-size treatment groups for 10 weeks using the RAND function of Excel (Microsoft Office Excel 2019). The cocoa group (CO; n = 22) received 5 g of fat-reduced cocoa containing 425 mg of flavonols and the control group (CT; n = 22) received 5 g of maltodextrin. Both supplements were provided in identical single-dose paper sachets to dissolve in semi-skimmed milk which does not affect cocoa flavonoid bioavailability [17]. Maltodextrin is used as a carbohydrate supplement by athletes, however, 5 g of maltodextrin provided insufficient carbohydrate or energy bolus to adversely impact daily macronutrient and energy intake [18]. Maltodextrin was not colored of cocoa-flavored and participants were told they were taking two supplements to study their effect on body composition and performance. Sachets were dispensed by postal mail and researchers that collected and analysed the data were blinded. Supplements were consumed during breakfast before exercise tests. During the 10-week intervention period, participants were called by telephone every week to check that they were taking the corresponding supplement. All measurements were recorded before and after the 10-week endurance training intervention.

Dietary and Training Monitoring

Dietary habits of participants were recorded at the beginning and after the 10-week intervention period. A Food Frequency Questionnaire (FFQ) [19] and three 24-hour dietary recalls (two weekdays and one weekend day) were used. Data were analyzed using Dietsource software 3.0 (Novartis, Barcelona, Spain) to obtain the dietary intake of carbohydrates, protein, fats, and fiber, and the total energy. Participants were asked to maintain their normal diet throughout the training period. A single dose of cocoa (5 g) provided 13 kcal, 1.2 g of protein, 0.7 g of carbohydrates and 0.5 g of fat whereas 5 g of maltodextrin provided 19 kcal, 0 g of protein, 4.8 g of carbohydrates and 0 g of fat.

Anthropometry and Body Composition

Height and body mass were measured with a stadiometer (Asimed T2, Barcelona, Spain) and a balance scale (Ano Sayol SL, Barcelona, Spain), respectively; body mass index (BMI) was calculated as body mass (kg)/height (m2). Body composition was evaluated by dual-energy X-ray absorptiometry (Hologic DXA scan, Hologic Inc, Barcelona, Spain). Quality control calibration procedures were performed on a spine phantom. Subjects wore typical athletic clothing and removed all metal jewelry. They were positioned supine on the device within the borders delineated by the scanning table. The measures of body composition were as follows: total body fat percentage (BF%), estimated visceral adipose tissue (VAT) and fat (%) and fat-free mass (kg) distribution in the trunk and upper and lower body limbs. The interday coefficient of variation was 1.78 %.

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The participants were endurance (cross-country) athletes. The athletes were training during the 10-week intervention with 5 or 6 training sessions per week. They followed a polarized distribution of training time, approximately 77% in Zone 1, ~5% in Zone 2, and 17–18% in Zone 3, similar to the protocols of Seiler and Kjerland [20] and Esteve-Lanao et al. [21]. The total training load was approximately 43 %, 7 % and 50 % (Z1-Z2-Z3, respectively). The objective load scale training load quantification method was used [22]. Participants reported the duration of the training session in each training area individually 2 h after each work session, and the HR data was recorded in each work session (Garmin Forerunner 235, Garmin Ltd., Georgetown, DC, USA).

Determination of Maximal Oxygen Uptake, First and Second Ventilatory Threshold, and Maximal Aerobic Speed

After a standardized warm-up of 10 minutes of continuous running on a treadmill (H/P/Cosmos Venus, Nussdorf-Traunstein, Germany) at 60% of their maximum heart rate (HRmax), subjects performed a maximum oxygen consumption test (VO_{2max}) with a gas analyzer (UltimaTM Series, MGC Diagnostic Corporation, St. Paul, MN, USA), which was calibrated according to the manufacturer's instructions. The volume calibration was performed at different flow rates with a 3-L calibration syringe allowing an error <3%. The calibration of gas analyzers was performed automatically using reference values of environmental gases and cylinders (16% O2, 4% CO2). During the progressive test, HR and oxygen consumption values were constantly monitored and associated with the speed at which the first ventilatory threshold, second ventilatory threshold and maximal aerobic speed (VT1, VT2 and MAS, respectively) were identified. Based on the classical 3-phase model of Skinner and McLellan [23], three main zones were differentiated: <VT1 (at or below VT1 – Zone 1), VT1-VT2 (beyond VT1 and below VT2 – Zone 2), and >VT2 (at or beyond VT2 – Zone 3). The following variables were determined: oxygen uptake (VO_2), pulmonary ventilation (VE), ventilatory equivalents for oxygen (VE/VO_2) and carbon dioxide (VE/CO_2), and end-tidal partial pressure of oxygen ($PETO_2$) and carbon dioxide ($PETCO_2$). Using the determined variables absolute maximal oxygen consumption ($VO_{2maxABS}$), relative maximum oxygen consumption ($VO_{2maxREL}$), maximal aerobic speed (MAS), first ventilator threshold (VT1) and second ventilator threshold (VT2) were calculated as is described elsewhere [21]. The protocol started with a slope of 1% at a speed of 10 km/h, with increments of 0.3 km/h every 30 s until volitional exhaustion [21]. Finally, ten minutes after the treadmill test, volunteers ran 1 kilometer (t1km) outdoor as fast as possible and the time needed to cover the distance was recorded.

Myostatin, Follistatin and Leptin Analyses

Before and after 10 weeks of supplementation, blood samples were collected in vacutainer EDTA-tubes and plasma samples were obtained by centrifugation at 3000 rpm for 10 min, and stored at -80 °C until use. Myostatin and follistatin levels were measured using RayBiotech ELISA kits (Norcross, GA, USA) and leptin analysis was performed using a Human Leptin ELISA (Peprotech, Rocky Hill, NJ, USA) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was carried out using SPSS software 21.0 (SPSS, Chicago, IL, USA). Normality of distribution (Shapiro–Wilk test) and homogeneity of variance (Levene's test) and sphericity (Mauchly's test) were checked before analyses. When sphericity was violated the Greenhouse-Geisser test was chosen. All data were analyzed with the use of a 2-factor repeated measures ANCOVA with the supplement intake (between) and time (within) as the main variables. The variables $VO_{2maxABS}$ and $VO_{2maxREL}$ were used as covariates in body composition analyses. The Wilcoxon signed-rank test or the Mann-Whitney test were used when normal distribution could not be assumed. The significance was set at $p < 0.05$. The effect size was calculated using G*Power 3.1.9.4 software. Graphs were built with SigmaPlot V14.0 (Systat Software, Inc. San Jose, CA, USA).

Results

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Cocoa Characterization

Quantitative analysis showed that the cocoa employed in this study contained mostly flavonoids with 99.6% of flavanols (Table 1) and small amounts of flavonols (quercetin-derivatives), the flavanone naringin and the flavone luteolin (data not shown). Within the flavanol group, the major compound was procyanidin B2 (81%), followed by (-) epicatechin (9.5%), catechin (4.58%) and procyanidin B1 (3.79%) (Table 1). The analysis of the degree of polymerization showed that the cocoa employed had an mDP = 2.45. Cocoa also contained 4 mg/g of caffeine and 25 mg/g of theobromine (indicated by the manufacturer).

Table 1. Flavanols content of the cocoa powder used in the study.

Compound	RT (min) PDA	RT (min) QqQ	λ max	[M-H]- m/z	Transitions m/z	Cone voltage	Collision energy	Content (mg/g cocoa)	Content per serving (mg)
Gallocatechin	5.58	6.39	273	305.33	125.02	56	26	0.085 ± 0.004	0.424
Procyanidin B1	7.09	7.89	278	577.53	407.21, 289.18	64	22, 24	3.368 ± 0.349	16.84
Catechin	8.08	8.88	278	289.24	245.12, 123.03	2	16, 32	3.852 ± 0.136	19.26
Procyanidin B2	8.91	9.68	278	577.44	407.21, 289.18	64	22, 24	68.507 ± 0.665	343.54
(Epi)-catechin	9.44	10.22	278	289.24	245.12, 123.03	2	16, 32	8.008 ± 0.255	40.04
Total flavanol [DP1-2]								83.819	419.09

DP1-2, with a degree of polymerization up to 2; RT, retention time; PDA, photodiode array detector; QqQ, triple quadrupole detector. Values are means ± standard deviation.

Characteristics of Subjects and Dietary Intake

A total of 44 subjects were enrolled in the study and 32 finished the study: 15 in the CO group and 17 in the CT group (Figure 1). Seven subjects dropped out from the CO group and 5 from the CT group because of illness (not related to the study), muscular injury, or because they did not follow the training program (Figure 1). After drop-out, the effect size for the primary outcome (body fat percentage) was computed, obtaining an effect size dz = 1.06 with an $\alpha = 0.05$ and power $(1-\beta) = 0.095$, indicating that observed effect in this parameter is large. The 95% confidence interval was (0.331 – 2.13). Age and BMI were similar between the two groups (age: CO = 33 ± 7 years; CT = 36 ± 8 years; p = 0.315) (BMI: CO = 21.80 ± 1.30 kg/m2; CT = 22.35 ± 2.13 kg/m2; p = 0.401).

Macronutrient data obtained from the FFQ and the three 24 h recalls were not significantly different (data not shown). Both dietary assessment methods indicated that dietary intake was maintained along the study (Table 2).

Table 2. Macronutrients, total energy and fiber composition of the athletes' diet.

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	CO			CT					
	t = 0	t = 10 w	p	t = 0	t = 10 w	p	P*	P**	Time x group
Energy (kcal)	2073 ± 538	2049 ± 533	0.887	2159 ± 633	2117 ± 635	0.806	0.423	0.760	0.272
Carbohydrates (%)	44.15 ± 7.84	45.23 ± 11.05	0.723	46.56 ± 6.84	44.43 ± 8.69	0.604	0.385	0.830	0.766
Protein (%)	20.69 ± 2.87	20.00 ± 3.65	0.248	18.12 ± 2.96	19.81 ± 3.86	0.130	0.155	0.895	0.104
Fat (%)	35.30 ± 6.81	34.76 ± 10.25	0.858	36.00 ± 4.18	35.60 ± 4.94	0.804	0.963	0.797	0.839
Carbohydrates (g/kg b.m)	3.33 ± 1.08	3.43 ± 1.51	0.738	3.91 ± 1.70	3.40 ± 0.97	0.421	0.302	0.934	0.161
Protein (g/kg b.m)	1.53 ± 0.45	1.43 ± 0.23	0.774	1.58 ± 0.89	1.54 ± 0.53	0.128	0.858	0.519	0.777
Fat (g/kg b.m.)	1.20 ± 0.49	1.15 ± 0,50	0.745	1.49 ± 1.26	1.25 ± 0.52	0.356	0.443	0.624	0.353
Fiber (g)	27.27 ± 12.02	31.16 ± 17.23	0.196	31.47 ± 18.56	28.72 ± 12.55	0.088	0.605	0.380	0.067

Values are means ± standard deviation. CO: cocoa group; CT: control group. b.m.: body mass; p* intergroup comparison at t = 0; p** intergroup comparison at t = 10 w.

In general, carbohydrate intake was in the lower range of the recommendations of the European Food Safety Authority (EFSA), which recommends an intake of 45 to 60% of total energy intake. The intake of fiber was adequate (above the recommended 25 g/d) [24]. Protein intake was within the recommendations for endurance and strength-trained athletes of the American Dietetic Association, the Dietitians of Canada, and the American College of Sports (recommend intake 1.2–2 g/kg body mass/day) [25]. Fat intake was close to the upper level of EFSA recommendations for fat intake (range 20–35 % of total energy intake) [24].

Body composition

No significant changes were found in terms of body mass and body composition along the study before and after 10 weeks of supplementation. However, significant changes were found in several body composition parameters when intragroup analyses were performed (Table 3). In the analysis of body composition, $VO_{2maxABS}$ and $VO_{2maxREL}$ covariables were analyzed as possible factors that could impact the observed results, but no influence of these variables was detected. A decrease in the percentage of total body fat (BF%), visceral fat, as well as the percentage of fat in the lower limbs was observed in the CO group after the intervention (Table 3). By contrast, no significant changes were observed for any of the analyzed fat-free mass parameters.

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Table 3. Body composition parameters.

	CO				CT					
	CO (t = 0)	CO (t = 10 w)	p	η 2	CT (t = 0)	CT (t = 10 w)	p	p *	p **	Time x group
Body mass (kg)	68.68 ± 6.03	68.55 ± 5.62	0.672	0.006	68.17 ± 7.19	68.08 ± 6.63	0.938	0.836	0.837	0.981
BMI	21.80 ± 1.30	21.76 ± 1.32	0.856	0.021	22.35 ± 2.13	22.32 ± 1.92	0.873	0.441	0.436	0.898
Upper Limbs Fat (%)	17.62 ± 4.19	17.02 ± 4.22	0.193	0.026	18.88 ± 6.70	18.96 ± 5.76	0.840	0.859	0.801	0.813
Lower Limbs Fat (%)	18.48 ± 4.43	17.75 ± 4.35	0.004	0.258	19.32 ± 4.36	19.16 ± 4.23	0.404	0.985	0.084	0.361
VAT (cm3)	277.14 ± 53.18	251.78 ± 69.32	0.034	0.151	295.56 ± 110.23	286.75 ± 114.81	0.690	0.565	0.329	0.325
Trunk Fat (%)	17.71 ± 2.42	16.44 ± 2.25	0.022	0.173	18.56 ± 4.94	18.33 ± 4.80	0.369	0.568	0.191	0.534
BF (%)	18.63 ± 3.25	17.40 ± 2.90	0.020	0.178	19.06 ± 4.52	19.00 ± 4.22	0.468	0.767	0.244	0.472
Upper Limbs Fat - free mass (kg)	3.25 ± 0.32	3.20 ± 0.33	0.427	0.014	3.13 ± 0.45	3.13 ± 0.42	0.880	0.647	0.737	0.906
Lower Limbs Lean mass (kg)	10.15 ± 0.76	10.32 ± 0.81	0.131	0.031	9.97 ± 1.06	9.93 ± 0.99	0.582	0.710	0.223	0.704
Trunk Fat - free mass (kg)	23.89 ± 1.79	24.41 ± 1.88	0.060	0.121	23.75 ± 2.36	24.08 ± 2.28	0.263	0.859	0.682	0.979
Total Body Fat - free mass (kg)	54.36 ± 3.55	55.08 ± 3.80	0.077	0.108	53.58 ± 5.04	53.83 ± 4.97	0.375	0.752	0.463	0.875

BMI, body mass index; BF %, body fat percentage; VAT, estimated visceral fat. Values are means ± standard deviation. CO: cocoa group; CT: control group; p* intergroup comparison at t = 0; p** intergroup comparison at t = 10 w

Training load and Performance

Training load, volume and time spent in training were not significantly different between groups. The 10 weeks training did not produce significant t differences between groups. There was an improvement in $VO_{2maxABS}$, $VO_{2maxREL}$, VT1, VT2, MAS and the t1km ($p < 0.001$) in both groups after the intervention (Table 4).

Table 4. Peak oxygen consumption test determinations and performance measurement.

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	CO (t = 0)	CO (t = 10 w)	p	CT (t = 0)	CT (t = 10 w)	p	p *	p **	Time x group
VO2maxABS (mL/min)	4058 ± 308	4210 ± 418	0.008	3975 ± 428	4173 ± 462	<0.001	0.803	0.981	0.728
VO2maxREL (mL/kg·min)	58.72 ± 4.17	60.80 ± 4.65	0.007	58.69 ± 3,37	60.41 ± 3.83	<0.001	0.984	0.809	0.711
MAS (km/h)	18.14 ± 1.02	18.48 ± 1.00	<0.001	17.85 ± 1.04	18.40 ± 1.21	<0.001	0.447	0.833	0.256
VT1 (km/h)	16.08 ± 0.83	16.49 ± 0.90	<0.001	15.69 ± 0.82	16.18 ± 0.93	<0.001	0.221	0.235	0.438
VT2 (km/h)	13.31 ± 0.71	13.69 ± 0.88	<0.001	13.21 ± 0.74	13.74 ± 0.74	<0.001	0.716	0.847	0.325
t1km (min)	3.21 ± 0.24	3.15 ± 0.21	<0.001	3.28 ± 0.26	3.19 ± 0.27	<0.001	0.459	0.945	0.500

VO2maxABS, absolute maximal oxygen consumption; VO2maxREL, relative maximum oxygen consumption; MAS: maximal aerobic speed; VT1: first ventilatory threshold; VT2: second ventilator threshold; t1km: time to run 1 km; CO: cocoa group; CT: control group. p* intergroup comparison at t = 0; p** intergroup comparison at t = 10 w

Circulating Follistatin, Myostatin and Leptin

Intergroup comparison did not detect any differences before and after supplementation between groups (T0: p = 0.423, p = 0.182, p = 0.187 and T = 10: p = 0.380, 0.987 and 0.418) for myostatin, follistatin and follistatin/myostatin ratio respectively. Time per group interaction was also no significant (p = 0.741, p = 0.242 and p = 0.741) for myostatin, follistatin and follistatin/myostatin ratio. There were no significant changes over time in myostatin levels with either treatment (CO t = 0 = 4.83 ± 2.71 ng/mL, CO t = 10 w = 4.12 ± 1.72 ng/mL, p = 0.390; CT t = 0 = 4.12 ± 1.72 ng/mL; CT t = 10 w = 3.99 ± 1.42 ng/mL, p = 0.741); however, follistatin levels increased significantly after 10 weeks of cocoa consumption (Figure 2A, B). The change in the levels of follistatin also produced a significant change in the follistatin/myostatin ratio in the CO group (Figure 2C). Lastly, a significant decrease in leptin was detected in the CO group after 10 weeks of supplementation (Figure 2D) that was also significant when time per group interaction was analyzed (p = 0.030).

Discussion

Our results show that cocoa improves the body composition of endurance athletes by decreasing body fat levels, without impacting or improving performance. The observed body fat changes could be mediated, at least in part, by the modification of the follistatin/myostatin ratio and by the decrease in leptin levels. Indeed, previous studies in animals have shown that cocoa supplementation counteracts the effects of diets high in fat, preventing weight gain and total fat mass increase, by modulating lipid metabolism [26–28]. The effect of cocoa on body composition in a healthy animal model has been addressed very recently and showed that cocoa decreased body mass, BMI, Lee's index (which evaluates the degree of obesity) and retroperitoneal fat content [29]. The positive effects of cocoa on body composition are believed to be attributed to its flavanols content, particularly the monomeric flavanol epicatechin [30], because of its high bioavailability and potential to exert systemic effects [31]. Likewise, the body fat-lowering effects of cocoa have been ascribed to the presence of oligomeric procyanidins [32], which although fairly non-absorbable, can exert their effects at the intestinal level by inhibiting the intestinal absorption of cholesterol and bile acids and modifying the enzymatic activity of α-glucosidase[28].

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The majority of studies using cocoa or its food derivatives do not carry out a detailed analysis of its composition, which can be key to explain the observed effects. Indeed, there is great variability in cocoa flavonoid content, both in quality and quantity, and there are many factors that influence its composition, for example, the variety and the geographical location of the cocoa beans, and the manufacturing process of the cocoa (fermentation, roasting, alkalization) [7,33,34]. This variability makes it necessary to perform detailed analyses of the cocoa used in studies. The cocoa supplement used in the present study consisted mainly of procyanidin B2 and approximately 10% epicatechin, with a degree of polymerization of 2.45, in addition to caffeine and theobromine. The epicatechin and catechin contents of the cocoa employed here were 10 - fold and 8 - fold higher, respectively, than those reported in a study in which 11 cocoa powders were analyzed [7], and the dose used was similar to that employed in other human studies assessing endothelial function and vascular stiffness [35,36].

Although studies in animals show a clear effect of cocoa on body fat composition, the relationship in humans is not so clear. The epidemiological data point to an effect of cocoa on fat mass, however, the results of intervention studies are heterogeneous. In a study on 2734 healthy female twins, it was observed that higher intake of flavanols, the principal polyphenols in cocoa, was associated with a lower fat mass independently of genetic or other environmental factors [37]. Likewise, in European adolescents, high chocolate consumption was associated with lower central fatness [38], and in a pilot study of normal weight obese women (excessive body fat but a normal BMI), intake of dark chocolate promoted a decrease in the abdomen circumference [39]. By contrast, chronic supplementation with cocoa (that provided 451 mg of flavanols per day) along with exercise failed to change body composition in a study of overweight and obese subjects despite improving numerous cardiometabolic risk factors [40]. In a recent study carried out in college students who performed light-to-moderate intensity exercise, the consumption of high-cocoa chocolate (508 mg/day of polyphenols) for 4 weeks failed to produce any changes in body composition, but the study was not placebo-controlled and its duration was shorter than our present study [41], which might have had an impact on the results. Recently, it has been described that the main flavanols in cocoa, epicatechin, catechin and procyanidin B2, increase beta-oxidation and energy expenditure of white adipose tissue [30]. In fact, in a study carried out with a catechin-rich green tea extract, a 1.6% decrease in body fat percentage caused an 11% improvement in performance, probably not due to fat loss itself, but to increased fatty acid oxidation [42].

The decrease in body fat mass in our study occurred both in the trunk and the lower limbs, highlighting that visceral fat mass, a cardiovascular risk factor, is a target of cocoa. However, the fat loss in the CO group was not accompanied by an improvement in sports performance, as there were no significant differences between the two groups. In contrast to these results, a chronic study (3 months) using dark chocolate (175 mg of flavanols; 26 mg of epicatechin) found an improvement in cycling exercise capacity (VO_{2max} and power) [43], although the study was performed in sedentary people with an initial aerobic capacity much lower than the aerobic capacity of the athletes of our study (24 mL/ kg·min VO_{2max} vs 58 mL / kg ·min VO_{2max}) and the supplementation was not accompanied by exercise training. Studies employing an acute intake of cocoa have generally failed to show improvements in physical performance (reviewed in [5]). Similar to those results, we did not detect any improvement in performance after cocoa supplementation. Low body fat composition is desirable in athletes to reduce energy expenditure, increase running economy and improve exercise performance. In contrast to our results, the intake of 25 g of cocoa for 7 days was found to improve the physical performance in soccer players by 4% (Cooper Test) [44]. Further, the intake of 25 mg of epicatechin for seven days was found to increase handgrip strength and the plasma follistatin / myostatin ratio in humans [9]. Because an increase in this ratio has been associated with an increase in skeletal muscle mass and a decrease in fat accumulation [45], we measured these proteins in plasma to test whether they were potentially responsible for the effect of cocoa on body fat composition (athletes in our study ingested 40 mg / day of epicatechin). Our results show that the chronic intake of cocoa increased the plasma ratio of follistatin / myostatin by modifying the levels of follistatin, without changes to myostatin. These data are in agreement with a recent human study by Schwarz et al. who reported no changes in muscle gene expression of myostatin after aerobic and anaerobic exercise training and supplementation with 200 mg daily of epicatechin for 4 weeks [46]. By contrast, skeletal muscle myostatin expression in sedentary men was found to decrease after acute and long-term exercise [47]. We found no changes in serum myostatin levels in our study, although physical

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improvements were detected in both intervention groups. These results are, however, tempered by the fact that we measured myostatin in blood and not in muscle.

The changes of the follistatin/myostatin ratio were accompanied by a decrease in plasma leptin levels in the CO group. Leptin is a hormone secreted by adipose tissue that promotes lipolysis and depresses lipogenesis. Accordingly, the decrease in the levels of leptin after 10 weeks of supplementation could be a consequence of the loss of body fat. Indeed, the results of an in vitro study showed that a cocoa polyphenol powder decreases fat accumulation and the release of leptin in adipocytes [48], and similar effects have been reported for theobromine [49]. The *in vivo* effects of cocoa consumption on leptin and associated mechanisms are not yet clear. It has been observed that cocoa powder, cocoa extract and epicatechin reduces body fat mass and leptin levels in a rat model of obesity [30], whereas in healthy rats cocoa failed to modify leptin levels after 3 weeks of supplementation, although a tendency for a decrease was observed [50]. To the best of our knowledge, our study is the first in humans to demonstrate an effect of chronic consumption of cocoa on leptin levels. Consistent with our results, it has been observed that a diet enriched in flavonoids (provided by dark chocolate, dehydrated apple and green tea; giving an approximate daily dose of epicatechin of 425 mg), added to antihypertensive treatment, decreases leptin levels in young hypertensive patients [51]. By contrast, acute intake of dark chocolate failed to modify leptin levels in postmenopausal women [52].

A limitation of our study is that we have not carried out measurements in short-term periods to study the behavior of the determined parameters over time. Considering together our present findings and those of previous studies, it is clear that more work is needed to clarify the effect of cocoa on adipose and muscle tissue in humans, and this would likely benefit from the use of standardized preparations or individual cocoa components, to determine the underlying mechanisms of the observed effects. Similarly, regarding exercise performance, future research is necessary to assess whether cocoa supplementation, through its effect on body fat, could be beneficial in other exercise modalities.

Conclusions

Our study shows that consumption of a cocoa extract rich in polyphenols can lower total body and visceral fat mass in endurance athletes, whose fat composition is already intrinsically low. The change in body composition did not equate to an improvement in exercise performance. The modification of the body fat -could be mediated by an increase of follistatin and therefore of the follistatin/myostatin ratio concomitant with a decrease in circulating leptin levels.

Conflicts of interest

There are no conflicts to declare

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Author contributions

Jose Ángel García-Merino: investigation, Diego Moreno-Pérez: investigation, Beatriz de Lucas: investigation and writing, Maria Gregoria Montalvo-Lominchar: investigation, Elsa Muñoz: resources, Lara Sánchez: resources, Fernando Naclerio: supervision,

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Karen Marlene Herrera-Rocha: investigation, Martha Rocío Moreno-Jiménez: investigation, Nuria Elisabeth Rocha-Guzmán: investigation, Mar Larrosa: Conceptualization, Methodology, Funding acquisition, Supervision, Writing - Original Draft

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Fig 1. Flow chart of the study

Fig 2. Adipokines levels before and after 10 weeks of placebo or cocoa supplementation. (a) Follistatin plasma levels in the CO group (b) Follistatin plasma levels in the CT group (b) Follistatin / myostatin ratio in the CT and the CO groups (d) Circulating leptin levels. Data are mean ± SD.

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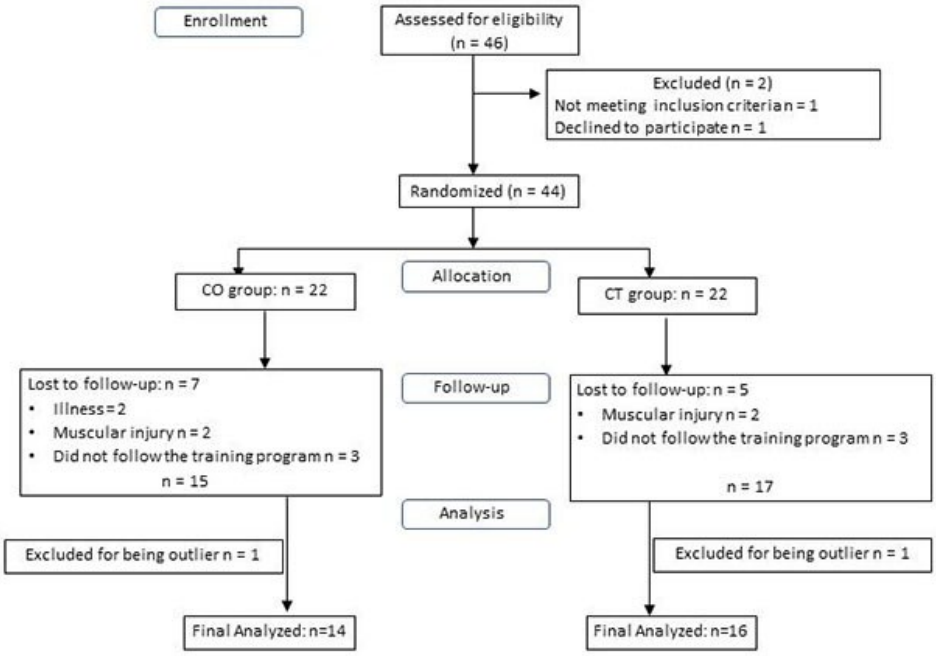

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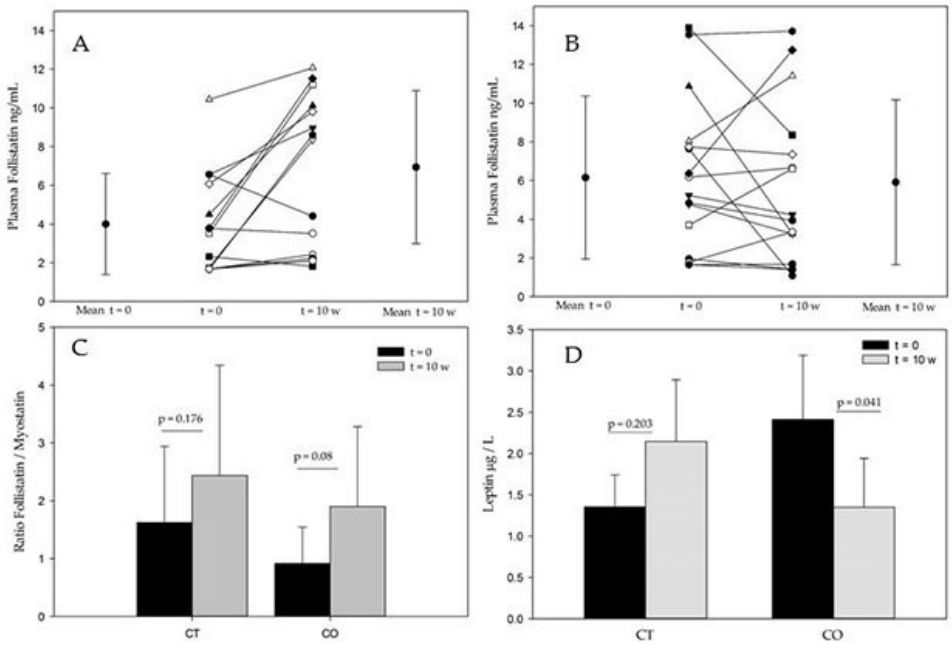

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