Published in final edited form as:

Food Biosci. 2020 October ; 37: . doi:10.1016/j.fbio.2020.100710.

Flavonoids from dark chocolate and (–)-epicatechin ameliorate high-fat diet-induced decreases in mobility and muscle damage in aging mice

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Abstract

Age-related muscle decline, when associated with obesity, leads to adverse outcomes with increased risks for falling, loss of independence, disability and risk of premature mortality. The aim of this study was to assess the potential beneficial effects of flavonoids in improving the age-/high-fat-diet-induced decrease in physical activity/capacity related to the onset of skeletal muscle decline. The effects of the administration of a cocoa beverage enriched with flavanols or pure (–)-epicatechin for 5 wk in a model of physical activity decline induced by the ingestion of a high-fat diet (60% fat) in middle-age mice were evaluated. The results showed that both products, the cocoa beverage enriched with flavanols and pure (–)-epicatechin, improved physical performance evaluated with the hang-wire, inverted-screen, and weight-lifting tests and dynamometry compared with the performance of the controls. The beverage and (–)-epicatechin increased the follistatin/myostatin ratio and increased the expression of myocyte enhancer factor 2A (MEF2A), suggesting an effect on molecular modulators of growth differentiation. Furthermore, the beverage and (–)-epicatechin decreased the expression of O-type fork-head transcription factor (FOXO1A)

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Conflicts of interest

Dr. Villarreal is a co-founder and stockholder of Cardero Therapeutics Inc. and Dr. Ceballos is a stockholder. All other authors declare no conflicts of interest.

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and muscle ring finger 1 (MURF1) markers of the skeletal muscle ubiquitin-proteasome degradation pathway.

Graphical Abstract

Keywords

Aging; sarcopenia; (–)-epicatechin; cocoa; dark chocolate

Introduction

The involuntary loss of muscle mass, strength and function attributable to aging, also known as sarcopenia, contributes to lower physical activity and may lead to disability and institutionalization among the elderly population (Hirani et al. 2015). The combination of sarcopenia and obesity represents an important public health risk since they share pathophysiological mechanisms that may potentiate each other (Choi, 2016). Obesity induces subclinical chronic inflammation that may inhibit the synthesis of muscle proteins, accelerate protein catabolism, and upregulate the expression of the muscle growth inhibitory factor myostatin and muscle atrophy proteins (Lippi et al. 2014).

During the progression of aging and obesity, oxidative stress increases and modulates the expression of transcription factors, such as nuclear factor- κ B, which enhances proteolytic pathways and increases the production of proinflammatory cytokines (Liu et al., 2017) which may contribute to sarcopenia (Lang et al., 2002). Together, aging and obesity contribute to decreased mobility in older individuals, increasing cardiometabolic risks. With the exception of exercise, no specific therapeutic approaches have been identified to delay aging-/obesity-related muscle loss. In the search for natural, low-cost and nontoxic alternatives for the prevention/treatment of decreased mobility related to muscle loss, flavonoids, particularly those derived from cacao, may have advantages.

It has been suggested that flavonoids may be potentially useful nutraceuticals to prevent the loss of muscle mass and function due to their promising effects on inflammation, muscle damage prevention, antifatigue, muscle atrophy prevention and muscle regeneration and differentiation (Rondanelli et al., 2016). A recent report suggested a relationship between flavonoid consumption and lower cardiovascular disease (CVD) risk (Hooper et al., 2008). A study showed that dark chocolate increases glutathione levels and decreases protein carbonylation in skeletal muscle, improving the maximum work achieved and maximal oxygen consumption (VO_2 max) (Taub et al., 2016). Additionally, (–)-epicatechin (EC), the main flavanol derived from cacao, shifts the mitochondrial-related biology of senile mice towards that of younger animals, inducing favorable changes in antioxidant defense systems, mitochondrial structure and activity (Moreno-Ulloa et al., 2015).

In the present work, a mouse model of aging through mature stages (10–49 wk) was implemented followed by obesity-induced (high-fat diet) sarcopenia (49–64 wk). Mobility changes were monitored during aging through mature stages and the high-fat diet-induced changes. The goal was to explore the effects of EC and to compare its effects to those induced by a dark chocolate beverage rich in EC and procyanidins to provide a base of information for the implementation of studies to decrease morbidity due to skeletal muscle loss in elderly populations that may be low cost and nontoxic.

The evaluation of the ratio of follistatin/myostatin was included, as a surrogate for skeletal muscle integrity (increase in protein synthesis and attenuation of degradation) and myocyte enhancer factor 2A (MEF2A) expression, as a marker of muscle fiber development, sarcomere integrity and postnatal muscle maturation (Anderson et al., 2015, Sepulveda et al., 2015). The expression of muscle ring finger 1 (MURF1) and muscle atrophy F-box (MAFbx) were evaluated. They are muscle-specific E3 ubiquitin ligases that increase during muscle atrophy (Bodine and Baehr, 2014) and the O-type fork-head transcription factors (FOXO) that activate MURF1 and MAFbx to induce atrophy (Bodine and Baehr, 2014).

Materials and methods

Animal treatments and sample collection

Healthy 10-wk-old C57BL/6 male mice were grouped 5/cage and maintained at room temperature (20–25°C), relative humidity (30–60%) with a 12 h light/dark cycle with *ad libitum* access to a standard commercial laboratory diet (crude protein 26.5%, fat 16.9%, carbohydrates 56.5% (3.23 kcal/g), LabDiet 5013, Purina Mills, Richmond, IN, USA). At 49 wk of age, mice were switched from a standard chow to a HFD (crude protein 20.5%, fat 36%, carbohydrates 35.7%, ash and moisture 7.8%, (5.49 kcal/g), 60% calories from fat diet, Bio-Serv Inc, Frenchtown, NJ, USA). Mice were maintained on these diets for 15 wk to develop obesity (a 20% higher body weight than that of controls) and impaired performance in physical tests. At 64 wk of age, mice were switched to a standard diet and randomly assigned to one of three interventions: 1) vehicle; 2) dark-chocolate beverage (DC) rich in flavonoids (2 mg EC + 12.8 mg procyanidins/kg bw) or 3) (–)-epicatechin (2 mg EC/kg bw). Treatments were provided once daily by gavage.

After 5 wk of treatment, the animals were anesthetized with an intraperitoneal injection of ketamine:xylazine (100:12.5 mg/kg)(Anesket and Procin, PISA agropecuaria, Guadalajara Jal. México). Skeletal muscle samples of the gastrocnemius and subcutaneous adipose tissue samples were weighed immediately after collection and stored at -80°C until use for a maximum of 12 wk. Bw was measured throughout the study with a digital scale with 0.01 g resolution. All experiments on animals were done in strict compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Council, 2011) and were approved by the Ethics and Research Committees of the Escuela Superior de Medicina of the Instituto Politecnico Nacional (Mexico City, Mexico).

Beverage treatments

The DC was a natural cocoa powder (containing monomers and oligomers) especially prepared and donated by the Hershey Co. (Hershey, PA, USA) The dry powder was reconstituted with water just before administration. The EC (90% purity) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and diluted with purified water just before administration. The control group received water as the vehicle.

Functional measurements

All functional measurements were carried out at the same time in the morning every wk, and all tests were repeated 3 times with a 15 min recovery period between repetitions.

Inverted-screen test—The inverted screen test was used as a measure of strength and muscular endurance, using the following methodology: the mouse was placed in the center of a cage-like wire grid, which was then inverted. The time that the mouse held onto the screen before falling to the padded surface at the bottom of the device was measured. The animals were allowed to be on the screen for a maximum of 120 s (this time was considered normal in young mice) (Deacon, 2013).

Weight-lifting Test

The weight test was used as a measure of the strength of the anterior limbs. The mouse was held by the tail and grasped metal rings that weighed between 20 and 98 g. If the mouse held the weight for 3 s, then it would move on to the next heavier weight. A final total score was calculated as the product of the number of links in the heaviest chain held for the full 3 s, multiplied by the time it was held (Deacon, 2013).

Hang wire test

The test consisted of measuring the longest suspension time before falling with a fixed limit of 180 s using the forelimbs to suspend the mouse's body weight on a stretched wire 60 cm above a sawdust bed. This test was used as a measure of motor coordination and forelimb strength (Li et al., 2004).

Dynamometer

Forelimb strength was measured using an electronic dynamometer (BioSeb, Chaville, France). Briefly, the mouse was held by the tail and typically dragged across a flat surface

until it grasped a bar attached to a strain gauge. The maximum force before the mouse lost its grip was measured (Contet et al., 2001).

Metabolic status

After 6 h of fasting, blood samples were obtained by cardiac puncture. The glycemia and lipid profiles (total cholesterol, triglycerides, cholesterol of high-density lipoproteins [HDL-c] and cholesterol of low-density lipoproteins [LDL-c]) were determined using enzymatic colorimetric assays using commercially available kits (Randox SA, Mexico City, Mexico) using a spectrophotometer (Synergy HT, BioTek Instruments, Winooski, VT, USA). The methods for determination were according to the instructions for each kit. In brief, glucose oxidase-peroxidase and 4 amino-phenazone in Tris (pH=7.4)-phenol were used to determine glucose, the detection was done at 546 nm. The lipid profile was determined after enzymatic hydrolysis using 4-amino antipyrine, phenol, peroxidase, cholesterol esterase, cholesterol oxidase and pipes buffer (1,4-piperazinediethanesulfonate) (pH=6.8) for cholesterol quantification; 4 amino-phenazone, ATP, lipase, glycerol kinase, glycerol-3-phosphate oxidase, peroxidase, pipes buffer (pH=7.6), 4-chlorophenol and magnesium for triglycerides detection; 4 amino-phenazone, N-ethyl-N-(3-methylphenyl)N succinyl ethylene diamine, cholesterol esterase, cholesterol oxidase, pipes buffer (pH=6.8) for HDL; and cholesterol esterase, cholesterol oxidase, catalase, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline, pipes buffer (pH=7.0), 4-amino antipyrine and peroxidase for LDL. Lipid profile detection (cholesterol, triglycerides, HDL and LDL) was done at 546 nm.

Oxidative stress

Serum samples were used to measure oxidative stress levels in blood. Protein carbonylation was measured using an assay for the detection of carbonyl groups that relies on 2,4-dinitrophenylhydrazine (DNPH) (Sigma-Aldrich) as a substrate. The reaction leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product that was evaluated spectrophotometrically by measuring the absorbance at 375 nm. The concentration of malondialdehyde (MDA) was assessed as described by (Gerard-Monnier et al., 1998). This assay is based on the reaction of 1-methyl-2-phenylindole (MPI) (Sigma-Aldrich) with MDA in the presence of 4-hydroxyalkenals, with acidic conditions, to produce a blue/purple chromophore that was evaluated spectrophotometrically by measuring the absorbance at 586 nm.

Western blots

After euthanasia, tissue samples were collected from the gastrocnemius skeletal muscle and immediately frozen and stored at -80°C until analysis for a maximum of 12 wk. Samples were homogenized with a Polytron (PT1200 homogenizer, Kinematika AG, Littau, Switzerland) in 500 μL lysis buffer (1% Triton X-100 (Thermo Fisher Scientific, Waltham, MA, USA), 20 mM Tris, 140 mM NaCl, 2 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich) with protease and phosphatase inhibitor cocktails (P8340 and P5870, Sigma-Aldrich) supplemented with 0.15 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich), 5 mM Na_3VO_4 and 3 mM NaF. Homogenates were sonicated in an ice bath for 20 min (Branson 200, 120 V, 55 kHz) (Emerson Electric Co. Brookfield, CT, USA) at 4°C and centrifuged at 12,000 g for 10 min at 4°C . Protein content determination was

done with the Bradford technique (Bradford, 1976) using bovine serum albumin (Sigma-Aldrich) as a standard. The total protein samples (100 µg) were mixed with 2x Laemmli sample buffer (1:1) and heated in boiling water for 5 min and separated with a Bio-Rad Mini Protean system (Bio-Rad, Hercules, CA, USA) on a 10% polyacrylamide gel electrophoresis at 200 V (SDS-PAGE, Bio-Rad Laboratories) and electroblotted in a Trans-Blot cell (Bio-Rad) to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at 0.2 A for 90 min. Membranes were blocked for 1 h using 5% (w/v) nonfat milk (Sigma-Aldrich) in 0.1% Tween-Tris-buffered saline (T-TBS) (Tween 20, pH 7.5, Sigma-Aldrich) at room temperature (23 to 26°C). Blocked membranes were incubated overnight with the corresponding primary antibody (anti-follistatin, anti-myostatin, anti-MEF2A, anti-MURF1, anti-FOXO1A and anti-MAFbx; all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4 °C, with continuous shaking. Membranes were washed three times with T-TBS and incubated with secondary anti-mouse IgG from Santa Cruz Biotechnology for 1 h. Protein bands were visualized after incubating with ImmunoCruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology Inc) for 5 min, and their specific density was quantified using Image Studio software (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

The results are expressed as the mean $\pm$ standard error of the mean (SEM). Two-way ANOVA was used for intergroup analysis. When needed, the Student's paired t-test was used to assess the differences within the same group, and a t-test was used for the comparison of 2 groups. Although a $p < 0.05$ was generally used, the authors have also chosen to use 0.01 for some of the data to indicate the greater significance of the differences. All analyses were done using GraphPad Prism version 7.00 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Results

Effects of aging and HFD

Body weight—To examine the effects of aging and aging plus a HFD on body weight, the slope of the changes in body weight from 10 to 49 wk, attributable to the normal rate of weight increase during aging, were compared with the slope of changes in body weight from 49 to 64 wk for the weight gain attributable to aging plus consumption of the HFD. The results showed a significant increase (2.38x) in the rate of body weight gain (slope differences) when mice were switched to a HFD (Figure 1A).

Inverted screen test—The results showed a decline in inverted screen test performance throughout the aging process (10–49 wk), and the decline was accelerated by the consumption of the HFD. The slope of the decline from 10 to 49 wk (-0.68 ± 0.12 s/wk), indicating a loss of physical capacity attributable to the increase in age, was significantly different from the slope from 49 to 64 wk (-2.81 ± 0.7 s/wk), suggesting an additive effect of aging and the HFD (Figure 1B).

To determine whether the decrease in physical performance was related to the increase in bw, the results from the inverted screen test were normalized to bw. There was no difference

in slopes compared to the uncorrected data (data not shown). It was assumed that the decrease in performance was due to biochemical alterations induced by the consumption of a HFD.

Effects of treatments on the aging-/HFD-induced effects

Body weight and fat content—The baseline body weight values at randomization (64-wk) for the vehicle, DC and EC groups showed no significant differences. The results showed that 5 wk of administration of the DC beverage caused a significant decrease ($p<0.05$) in body weight (-11.6 g) compared to the vehicle (-2.5 g). EC induced a nonsignificant (-6.8 g) decrease in body weight.

DC and EC induced a significant decrease in fat pad content ($p<0.05$) compared to the effect of the vehicle. This was attributable to a lower visceral and subcutaneous fat depot. (Table 1)

Metabolic changes—Glycemia was slightly lower in both treated groups without significant differences. The DC significantly lowered LDL-c and triglycerides ($p=0.05$). Epicatechin had similar effects on both LDL-c and triglycerides ($p<0.05$). Total cholesterol and HDL-c remained the same for all groups (Table 2).

Oxidative stress biomarkers—Serum protein carbonylation showed a small decrease in the DC and EC groups without reaching significance (Figure 2A). The MDA concentration was significantly lower in the DC and EC groups ($p<0.05$) than in the vehicle group (Figure 2B).

Effects of DC and EC on physical performance

Inverted screen—The results showed that the DC and EC significantly increased the time on the inverted screen test compared to the results associated with the vehicle (Figure 3A).

The area under the curve showed a significant increase in mouse performance in the EC and DC groups vs. the vehicle group (Figure 3B), showing no differences between the EC and DC groups.

Weight-lifting Test—The weight-lifting test showed a decrease attributable to aging that was exacerbated by HFD consumption (Figure 3C).

Five wk of treatment showed that a significant difference existed in the EC group (change in score: 4 ± 1 , $p<0.05$) compared to the vehicle and DC groups (Figure 3D).

Hang wire test—The hang wire test showed a decrease attributable to aging and HFD consumption (Figure 3E). The results of the treatments showed that the vehicle group had a decrease in hang wiring time. On the other hand, the DC group showed significant increases in the test time compared to the vehicle and EC groups (Figure 3F).

Dynamometry—The forelimb dynamometry test was done only as an endpoint measurement at the end of the experimental phase. The results showed a significant

difference in strength in the DC and EC groups ($p<0.05$) compared to the vehicle group (Figure 4).

Muscle biomarkers—No differences were observed in gastrocnemius weight among the groups; however, the interventions significantly increased the protein expression of muscle growth and differentiation regulators, as shown in Figure 5, where DC and EC increased the follistatin/myostatin ratio ($p<0.05$, both) and MEF2A ($p<0.05$, both) (Figure 6A).

Additionally, in the ubiquitin-proteasome pathway, DC and EC significantly decreased the protein expression of FOXO1A ($p<0.05$, both) (Figure 7B) and MURF1 ($p<0.05$, both) (Figure 7C). However, the decrease in MAFbx did not reach significance with either treatment (Figure 6B).

Discussion

Age-related loss of muscle mass leads to physical decline, disability, falls, loss of independence and a higher mortality rate in elderly populations (Metter et al., 2002). The changes in physical performance throughout normal maturation (aging) from 10 to 49 wk of age (equivalent to 39 human yr) (Dutta et al., 2016) were assessed using the accelerating effect of obesity (induced by HFD consumption) on physical performance decline in 64-wk-old mice (~51 human years) (Dutta and Sengupta, 2016). The use of a HFD was done to combine the effect of aging and lipotoxicity as it occurs in sarcopenic obesity. Once the sarcopenic obesity model was established, the effects induced by DC or EC were evaluated.

The administration of DC or EC started in 64-wk-old mice considering this age as the beginning of muscle-power decline (Graber et al., 2015) and when adipose tissue remodeling occurs, switching to a fat-storing phenotype (Gonçalves et al., 2017).

The approach was based on the lack of a prevention strategy for middle-aged adults to preserve mobility and health in later years. Dietary modifications and long-term exercise are considered the first-line treatment strategies for managing and preventing age-related muscle wasting (White et al., 2016). There is evidence suggesting that flavonoids contribute to skeletal muscle welfare, and since (–)-epicatechin is reported to mimic the effects of sustained training (Lee et al., 2015), the hypothesis is that DC and EC would decrease the decline in physical performance induced by aging and obesity.

It has been reported that flavonoids prevent the loss of muscle mass and function and exert anti-fatigue, muscle-atrophy-prevention and muscle-regeneration effects (Rondanelli et al., 2016).

In the recent past, Taub et al. (2016) showed that dark chocolate increases glutathione levels and decreases protein carbonylation in skeletal muscle, improving maximum work achieved and VO₂ max. They also reported that EC shifts the mitochondrial-related biology of senile mice towards that of younger animals, and found favorable changes in antioxidant defense systems and mitochondrial structure and activity (Moreno-Ulloa et al., 2015).

In the present work, the effects of flavanols from DC and “free” (–)-epicatechin were evaluated with respect to delaying or limiting muscle damage and the slope of the decrease in physical performance induced by aging and obesity was assessed.

The effects of the treatments on muscle damage induced by oxidative stress, was evaluated using lipid peroxidation by measuring MDA (Gawel et al., 2004) which showed that the administration of flavanols from DC and (–)-epicatechin significantly reduced MDA.

The study also explored biomarkers involved in muscle growth, differentiation, cell death and degradation. Follistatin antagonizes myostatin, increases protein synthesis and decreases muscle degradation; its modulation is a promising therapeutic approach for age-related muscle loss. MEF2A, an important regulator of muscle fiber development and glucose uptake metabolism, is also involved in sarcomere integrity and postnatal muscle maturation (Anderson et al., 2015). The results showed that the DC and EC treatments induced similar increases in the follistatin:myostatin ratio and in MEF2A expression (Sepulveda et al., 2015).

On the other hand, MURF1 and MAFbx, muscle-specific E3 ubiquitin ligases, increased during muscle atrophy (Bodine and Baehr, 2014). The FOXO are able to activate both MURF1 and MAFbx to induce atrophy (Bodine and Baehr, 2014). The results showed that DC and (–)-epicatechin treatments induced similar significant decreases in MURF1 and FOXO1A expression and a non-significant decrease in MAFbx.

The results may be relevant at the human level since elderly individuals with lower functionality are more susceptible to specific adverse effects of sarcopenic obesity, such as a frail phenotype (Jarosz and Bellar, 2009). Elderly individuals also experience decreases in strength and physical performance (Moreira et al., 2016).

The main results of this work were as follows: 1) A HFD induced (biochemical) changes that accelerated the slope of the decline in physical performance related to aging, although bw by itself did not seem to be the cause of those changes; 2) DC and EC induced similar metabolic improvements; 3) DC and EC induced similar improvements in physical performance, and these changes seemed to be related to skeletal muscle quality/capacity enhancements measured as increases in the follistatin/myostatin ratio and MEF2A and to decreases in FOXO1A, MURF1 and MAFbx.

Conclusions

A simple approach of 5 consecutive wk of administration of (–)-epicatechin, either as a flavanol mixture (monomers and oligomers from cacao) or as the pure form, to obese middle-age mice significantly improved physical performance consistent with the modulation of the muscle biomarkers for sarcopenia. The results suggested a flavonoid-induced protective effect against skeletal muscle loss and physical performance decline induced by the lipotoxicity state associated with obesity. These results encourage the further examination of the potential clinical application of flavonoids in obesity and age-related muscle loss disorders.

Acknowledgements

Dr. Villarreal was supported by NIH DK98717 and Dr. Ceballos by a Conacyt #253769 grant. Levy Munguia acknowledges support from CONACYT in the form of a graduate scholarship.

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Figure 1.

A. Changes in body weight in C57BL/6 mice fed a normal chow diet (10–49 wk) and the change in slope when mice were fed with high-fat diet (HFD) for 15 wk (49–64 wk). **B.** Comparison of slopes of the time-dependent decrease in physical performance (effect of aging) and the slope of aging plus the HFD in the inverted screen test with the normal chow diet and the change in slope after switching to HFD. Data are shown as the means $\pm$ SEM.

Figure 2. Oxidative stress biomarkers. **A.** Evaluation at the endpoint of the protein oxidation measured as free carbonyls in the 3 treatment groups. **B.** Evaluation at the endpoint of lipoperoxidation measured as malondialdehyde in the 3 treatment groups. Data are shown as the means $\pm$ SEM.

Figure 3.

A. Evolution of the physical performance in the inverted screen test in the 3 treatment groups. **B.** Area under the curve of the effect of the treatments. **C.** Evolution of strength in the weight-lifting test associated with the normal chow diet and the HFD. **D.** Change in the strength score in the 3 treatment groups. **E.** Evolution of the physical performance in the hanging wire test associated with the normal chow diet and the HFD. **F.** Change in the performance score in the 3 treatment groups. Data are presented as the means $\pm$ SEM.

Figure 4.
Dynamometer test: Evaluation at the endpoint of forelimb strength in the 3 treatment groups.
Data are presented as the means $\pm$ SEM.

Figure 5. Evaluation of the follistatin/myostatin ratio in the 3 treatment groups. Data are presented as the means $\pm$ SEM.

Figure 6.

A. A representative Western blot of the myocyte enhancer factor 2A (MEF2A) and the effect of the 3 treatment groups. **B.** A representative Western blot of the muscle atrophy F-box (MAFbx) and the effect of the 3 treatment groups. Data are presented as the means $\pm$ SEM of 3 independent experiments ($n = 3$). Linearity of the load-dependent densitometry was done using the square of the correlation coefficient (R^2).

Figure 7.

A. A representative Western blot of the O-type fork-head transcription factor 1 (FOXO1A) and muscle ring finger 1 (MURF1) **B.** Effect of the 3 treatment groups on FOXO1A. **C.** Effect of the 3 treatment groups on MURF1. The same GAPDH expression was used in **7 B and C.** Data are presented as the means $\pm$ SEM of 3 independent experiments ($n = 3$). Linearity of the load-dependent densitometry was done using the square of the correlation coefficient (R^2).

Table 1.

Body and organ weights of obese mice supplemented with vehicle, DC or (-)-epicatechin.

	Vehicle	DC	EC	p
Initial weight (g)	39±5	43±5	37±5	p 0.05
Final weight (g)	36±3	31±1	30±2	p 0.05
Change	3±2	12±4	7±3	p<0.05
p	p 0.05	p<0.05	p 0.05	
Total fat pad (g)	2.3±0.3	1.2±0.3	1.2±0.2	p<0.05
Gastrocnemius (mg)	370±10	397±10	392±10	ns

Data are shown as the means ± SEM. The total fat pad represents the sum of the subcutaneous and visceral fat. Statistical analysis was done using ANOVA (intergroup differences) and by a paired "t"-test (intragroup differences). DC (dark chocolate beverage), EC ((-)-epicatechin)

Table 2.

Concentrations of serum glucose and lipid panel of obese mice supplemented with vehicle, DC or (-)-epicatechin.

	Vehicle	DC	EC	p
Glucose (mg/dL)	136±4	125±4	127±4	p 0.05
Cholesterol (mg/dL)	100±10	101±1	97±7	p 0.05
LDL-c (mg/dL)	53±3	41±2	41±3	p<0.05
HDL-c (mg/dL)	34±1	39±1	36±1	p 0.05
Triglycerides (mg/dL)	130±10	113±4	115±5	p<0.05

Data are shown as the means ± SEM. Statistical analysis was done using ANOVA (intergroup differences). DC (dark chocolate beverage), EC ((-)-epicatechin)