

**Reduction of body weight, liver steatosis and SCD1 expression by pharmacologic administration of the isoflavone daidzein in diet-induced obesity**

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Abbreviations: ACC, acyl-CoA carboxylase; ACOX, acyl-CoA oxidase; AdipoR1/AdipoR2, adiponectin receptor 1 and 2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CB1R/CB2R, cannabinoid receptor type 1 and 2; FABP1, fatty acid binding protein 1; FAS, fatty acid synthase; GGT, gamma-glutamyltranspeptidase; GHR, growth hormone receptor; HFD, high-fat diet; PPAR $\alpha$ /PPAR $\gamma$ , peroxisome proliferator-activated receptor alpha and gamma; Rpl19, ribosomal protein 19; SCD1, Stearoyl-CoA desaturase 1; Sp1, Sp1 transcription factor; STD, standard/low-fat diet.

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## Summary

**Background and purpose.** The lack of safe and effective approved drugs against obesity has raised a great interest on natural products that may serve as alternative therapies. From this perspective we have addressed the analysis of the effects of daidzein, one of the main soy isoflavones, on diet induced obesity in rats.

**Experimental approach.** Rats made obese after exposure to a very (60%) high fat content diet were treated with Daidzein (50 mg/kg) for 14 days. The dose was selected on the basis of the acute effects of this isoflavone on a feeding test. After 14 days, animals were sacrificed and plasma, white and brown adipose tissues, muscle and liver studied for the levels and expression of metabolites, proteins and genes relevant for lipid metabolism.

**Key results.** Acute daidzein dose-dependently reduced food intake. Chronic treatment reduced weight gain and reduced fat content in the liver. This was associated with high leptin levels and low adiponectin contents in plasma. While skeletal muscle was weakly affected by treatment, both adipose tissue and liver displayed marked changes induced by daidzein that affect transcription factors and lipogenic enzymes, specially stearoyl coenzyme A desaturase-1, a pivotal enzyme in obesity. Protein expression of the uncoupling protein 1, an important enzyme involved in thermogenesis, was increased in brown adipose tissue after daidzein treatment.

**Conclusions and implications.** These results give support to the use of isoflavones in diet induced obesity, especially when hepatic steatosis is present, opening a new field of use for these natural products.

**Keywords:** Daidzein, diet-induced obesity, oxidative metabolism, SCD1, UCP1, PPAR receptor, cannabinoid receptor, skeletal muscle, liver, adipose tissue

## 1    **Introduction**

2    Epidemiologically, the prevalence of obesity and related diseases in Asian population is  
3    lower than in western countries. Such observation turned the interest on the Asian diet,  
4    which highly consists in soy products rich in phytoestrogens (Soverson *et al.*, 1989;  
5    Tham *et al.*, 1998). Recent research works have been focused on the observed beneficial  
6    effects of dietary phytoestrogens, including the estradiol (E2)-like soy isoflavones  
7    genistein, daidzein and glycitein, on obesity and diabetes (Kuiper *et al.*, 1998; Bhathena  
8    and Velasquez, 2002). Therefore, data obtained from clinical, epidemiological and  
9    nutritional studies done in animals and humans have shown that the improved metabolic  
10    consequences associated with ingestion of soy products include glucose control, insulin  
11    resistance, and plasma lipid profile (reducing serum total cholesterol, LDL cholesterol  
12    and triglycerides) (Zhuo *et al.*, 2004; Zhan and Ho, 2005; Taku *et al.*, 2007), as well as  
13    producing weight gain reduction in rats (Davis *et al.*, 2007). Such improvement exerts  
14    protective effects on disorders like hyperlipidemia, cardiovascular disease, menopausal  
15    symptoms, arteriosclerosis, osteoporosis, cancer and various forms of chronic renal  
16    disease (Kopelman, 2000; Clarkson, 2002; Messina *et al.*, 2002, Clair and Anthony,  
17    2005; Cassidy and Hooper, 2006; Zhang *et al.*, 2010), all of which associated with  
18    obesity and diabetes.

19    Current literature proposes various mechanisms through which isoflavones may exert  
20    their beneficial effects: first, and due to their structural similarities to endogenous  
21    estrogens, genistein and daidzein can act as weak estrogens after binding to and  
22    activating estrogen receptor (ERs), ER- $\alpha$  and ER- $\beta$  (Kuiper *et al.*, 1998); and thus,  
23    mimicking the effects of the hormone in a lower scale in the body (Zhu *et al.*, 2006).  
24    Estradiol (E2), among other functions, is a major regulator of adipocyte development  
25    and number, being adipocytes very important in lipid homeostasis and the maintenance

1 of the whole body energy balance (Cooke and Naaz, 2004; Rosen, 2005). E2 is also a  
2 well-known modulator of glucose homeostasis (Godsland, 2005), and it has also been  
3 suggested to protect  $\beta$ -cell biology modulating insulin sensitivity and biosynthesis (Le  
4 May *et al.*, 2006; Liu and Mauvais-Jarvis, 2010). Furthermore, ERs are expressed in  
5 various tissues cells, including adipose tissue and muscle, in a different distribution  
6 frequency which can explain their selective tissue effects (Barros *et al.*, 2006). Second,  
7 soy isoflavones also produce non-estrogen effects; recent evidence have showed that  
8 genistein and daidzein can bind to and activate both peroxisome proliferator-activated  
9 receptors, PPAR $\alpha$  and PPAR $\gamma$ , increasing  $\beta$ -oxidation, insulin sensation and improving  
10 serum triglyceride (Mezei *et al.*, 2003; Shen *et al.*, 2006; Ronis *et al.*, 2009); both  
11 isoforms have different overlapping tissue distributions and functions associated with  
12 lipid and glucose metabolism (Mezei *et al.*, 2006). Therefore, isoflavones may also act  
13 through pathways regulated by PPARs-regulated genes, and/or by other transcription  
14 factors such as the sterol regulatory element binding protein (SREBP) (Mullen *et al.*,  
15 2004; Ronis *et al.*, 2009); the latter highly involved in glucose utilization and fatty acid  
16 synthesis and metabolism in the liver by modulating the transcriptional control of  
17 specific genes (Shin *et al.*, 2007). Additionally, phytoestrogens are known to have  
18 potent antioxidative activity counteracting damaging effects of free radicals in tissues  
19 (Vedavanam *et al.*, 1999).

20 Apart from being structurally similar to E2, daidzein also resembles natural  
21 cannabinoids (See Figure 1), the lipid compounds present in *cannabis sativa*. The  
22 structural relationships between daidzein and plant cannabinoids may facilitate a  
23 possible cannabinoid-like effect of isoflavones yet to be described. Cannabinoids act  
24 through cannabinoid receptors, part of the signaling system named the endocannabinoid  
25 system (ECS). The ECS has been proven to have a high influence in the onset of obesity

1 and metabolic syndrome, mainly due to its numerous effects on appetite control and  
2 energy homeostasis. Basically, the ECS is a network of fatty acid-derived signaling  
3 molecules including the endocannabinoid ligands, anandamide (AEA),  
4 oleylethanolamide (OEA) and 2-arachidonoyl-glycerol (2-AG), all of which exert their  
5 effect mainly binding to the cannabinoid receptors type 1 (CB1), but also to  
6 cannabinoid receptor type 2 (CB2) and/or to the peroxisome proliferator-activated  
7 receptor alpha (PPAR $\alpha$ ) and gamma (PPAR $\gamma$ ) (Piomelli, 2003). Furthermore, this  
8 molecular interaction is highly affected by the nature of dietary fat (Bathena, 2000;  
9 Evans *et al.*, 2004). The major metabolic organs are also the main site of action for  
10 PPARs, and various hormones, that might contribute to the reduction in hepatic  
11 steatosis and insulin sensitization seen after soy feeding (Cederroth and Nef, 2009).

12 To date, there are few studies with isoflavones including diet-induce-obesity in their  
13 design and especially the effects caused over the main metabolic organs. Considering all  
14 the questions raised, we studied the possible changes caused by both diet and treatment  
15 with the isoflavone daidzein on the lipidic and glucidic metabolism within the three  
16 major metabolic organs: liver, adipose tissue and skeletal muscle, in order to understand  
17 the isoflavones mechanism of action into improving the metabolic parameters.  
18 Consequently, in this work, we have a) assessed the effect of daidzein treatment on food  
19 intake and body weight reduction in male Wistar rats b) investigated the impact of a  
20 very high-fat diet on lipid and glucidic metabolic-related genes, and selected proteins, in  
21 the liver, white and brown adipose tissues and skeletal muscle, c) analyzed the changes  
22 caused after treatment with daidzein on the levels of expression of these genes and  
23 proteins studied on rats being fed a standard-diet (STD 10% fat) and a high-fat diet  
24 (HFD 60% fat), and d) made a comparative study of the metabolic changes resulting  
25 after the use of a high-fat diet and daidzein treatment.

Because of the lack of information on cannabinoid-like actions of isoflavones, we have included in the present study the analysis of the effects of daidzein on the expression of cannabinoid receptors. From our work, we concluded that daidzein treatment may modulate the influence of a high fat diet on lipid and glucidic metabolic genes and proteins through various interrelated pathways; however, with an outstanding role over inhibition of lipogenesis via SCD1 and PPAR $\alpha/\gamma$ , and over activation of thermogenesis via UCP1.

## **Methods**

### *Animals*

All experimental procedures with animals were performed in compliance with the European Communities directive 86/609/ECC and Spanish legislation (BOE 252/34367-91, 2005) regulating animal research. Male Wistar rats (~250g, 10-12 weeks old) (Charles Rivers, Barcelona, Spain) were housed in groups of two in cages maintained in standardized conditions of animal facilities (Servicio de Estabulario, Facultad de Medicina, Universidad de Málaga) at 20 $\pm$ 2°C room temperature, 40 $\pm$ 5% relative humidity and a 12h light/dark cycle with dawn/dusk effect.

### *Diet-induced obesity. Measurement of food/caloric intake and body weight.*

Rats (n=16/group) were fed for 12 weeks with two different types of diets: a very high-fat diet (HFD, 60% fat diet-D12492, Brogaarden, Gentofke, Denmark) and a standard/low-fat diet (SD, 10% fat diet-D1245B) for 10 weeks in order to induce obesity. The standard/low-fat diet consisted of 3.85 kcal/g (with 20% protein, 70% carbohydrates and 10% fat) and the very high-fat diet had 5.24 kcal/g (of which, 20% of

the metabolizable energy content was protein, 20% carbohydrates and 60% fat) (see supplementary table).

The accumulated food/caloric intake by each rat and their body weight gain were measured every day for 12 weeks. Treatment was administered when the weight curves showed a clear divergence and stabilization between the two diets was reached, which lasted 10 weeks (see supplementary figure).

### *Treatment*

After 10 weeks (divergence of weight curves), diet-fed rats received one intraperitoneal (i.p.) injection or a daily i.p. injection of either vehicle (1 ml/kg of 10% Tocrisolve in saline) or daidzein (7 [hyphen] $\beta$ -glucoside; LC Laboratories (Woburn, USA) at 5 and 50 mg/kg diluted in 10% Tocrisolve for acute-dose response treatment, and at 50 mg/kg for subchronic treatment during 14 days, while the diets remained unchanged. After one administration of daidzein (acute treatment), the accumulated food intake were measured in a time course of 30, 60, 120 and 240 minutes. During subchronic treatment, we measured the accumulated food/caloric intake and the body weight gain every day during the 14 days of treatment (see supplementary figure). Finally, we generated six experimental groups for acute treatment of daidzein: STD-Vehicle, STD-Daidzein 5 mg/Kg, STD-Daidzein 50 mg/kg, HFD-Vehicle, HFD-Daidzein 5 mg/kg and HFD-Daidzein 50 mg/kg; and four experimental groups for subchronic treatment of daidzein: STD-Vehicle, STD-Daidzein, HFD-vehicle and HFD-Daidzein.

### *Sample collection*

Animals from the four experimental groups were anesthetized (sodium pentobarbital, 50 mg/kg, i.p.) two hours after the last dose of treatment in a room separate from the other

experimental animals. Blood samples were briefly collected from the orbital cavity and centrifuged (1250 *g* for 8 min, 4°C), and all plasma samples were frozen at -80°C for biochemical and hormonal analysis. Liver, white and brown adipose tissues and skeletal muscle from the abdominal wall were dissected. Part of each sample was fixated with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) by immersion until immunohistochemical analysis. The remaining of each sample, excluding brown adipose tissue, was briefly frozen at -80 until RT-PCR analysis.

#### *RNA isolation and RT-PCR analysis*

RNA from liver, skeletal muscle and adipose tissue samples was extracted using Trizol® method, according to the manufacture's instruction (Gibco BRL Life Technologies, Baltimore, MD, USA). Tissue portions of liver, skeletal muscle and adipose tissue (~100, ~100 and ~300 mg respectively) were placed into 1-3 ml of Trizol Reagent (Invitrogen, CA, USA) and homogenized with an IKA-Ultra-Turrax® T8 (IKA-Werke GmbH, Staufen, Germany). In order to ensure purity of the mRNA sequences excluding molecules smaller than 200 nucleotides, RNA samples were isolated with RNeasy minelute cleanup-kit (Qiagen, Hilden, Germany), which included digestion with DNase I column (Rnase-free DNase Set, Qiagen), according to manufacturer's instructions. Total mRNA concentration was quantified using a spectrophotometer (Eppendorf® BioPhotometer, Hamburg, Germany) to ensure A260/280 ratios of 1.8 to 2.0.

Reverse transcript reaction was carried out from 4 µg of muscle mRNA or liver mRNA and 1 µg of adipose tissue mRNA using the Transcriptor Reverse Transcriptase kit and random hexamer primers (Transcriptor RT, Roche Diagnostic GmbH, Mannheim, Germany). Negative controls included reverse transcription reactions omitting reverse

transcriptase. Quantitative real-time reverse transcription polymerase chain reaction (quantitative RT-PCR) was performed using an iCycler System (Bio-Rad, Hercules, CA, USA) or a CFX96™ Real-Time PCR Detection System (Bio-Rad), and the SYBR Green detection format (FastStart Universal Master Kit, Roche, Mannheim, Germany). Sample quantification was carried out differently in the three tissues for better performance. Skeletal muscle and liver samples were analyzed by the classic standard curve method, whereas adipose tissue samples were analyzed by relative quantification following In-Silico PCR system technology. Each reaction was run in duplicate and contained 5 µl of cDNA. For muscle and liver samples, cycling parameters were: 95°C for 3 min and 30 sec to activate DNA polymerase followed by 45 cycles at 95°C for 15 s, annealing temperature for 30 s and a final extension step of 72°C for 15 s in which fluorescence was acquired. Melting curves analysis were performed to ensure only a single product was amplified. We analyzed various housekeeping genes, selecting the most suitable for each tissue according to their homogeneity. Absolute values from each sample were normalized with regard to the housekeeping gene Actin beta for skeletal muscle and Sp1 for liver. For adipose tissue samples, cycling parameters were: 95°C for 5 min and 30 sec to activate DNA polymerase followed by 40 cycles at 95°C for 10 sec, annealing temperature for 30 sec at 60°C and a final extension step of 72°C for 15 sec in which fluorescence was acquired. The accepted amplification limit of the non-template controls was selected 5 cycles below the samples Cp value. The mean PCR amplification efficiency and quantification cycle for each gene was calculated by submitting raw fluorescence data generated with the CFX96 software (Bio-Rad) to Real-Time PCR Miner software (<http://www.miner.ewindup.info>). The qBase Excel software program was used to measure the relative expression level for each gene, and geNorm application for selecting reference genes to normalize mRNA expression

1 levels, determining that Sp1 and Rpl19 were stable for this treatment. Finally, Sp1 was  
2 the reference gene selected to normalized mRNA expression levels in adipose tissue.  
3 Primers for PCR reaction (Table 1) were designed based on NCBI database sequences  
4 of rat reference mRNA, checked for specificity with BLAST software from NCBI  
5 website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and synthesized by Invitrogen.

#### 6 7 *Oil red O staining*

8 Liver samples were analyzed for lipid and fat depots by oil red O staining. Frozen  
9 samples were cut into 30 µm-thick sections using a sliding microtome (Leica SM200R,  
10 Wetzlar, Germany) and fixated with 10% formal calcium. Sections were washed with  
11 distilled water and rinsed with 60% isopropanol. Then, sections were stained with  
12 freshly prepared oil red O working solution for 20 minutes (Oil red O stock stain: 0.5 g  
13 of oil red O and 100 ml of isopropanol; Oil red working solution: 30 ml of the stock  
14 stain and 20 ml of distilled water). Sections were rinsed with 60% isopropanol, lightly  
15 stained with Mayer's hematoxylin, rinsed with tap water and mounted in aqueous  
16 mountant.

#### 17 18 *Immunohistochemistry*

19 Paraffined tissue microarray blocks (Manual Tissue Arrayer MTA-1, beecher  
20 Instruments, Inc., Sun Prairie, WI, USA) of rat liver and brown adipose tissue were  
21 analyzed for the presence and quantification of PPAR $\alpha$  receptor, SCD1, FAS and UCP1  
22 by immunohistochemistry, immunofluorescence and densitometry. The primary  
23 antibodies were: rabbit polyclonal antibody PPAR $\alpha$  (diluted 1:200; ABR cat. n° PA1-  
24 822A, Golden, Co, USA), goat polyclonal antibody SCD1 (diluted 1:50; Santa Cruz cat.  
25 n° sc-14720, Santa Cruz, CA, USA), rabbit polyclonal antibody FAS (diluted 1:50; Cell

Signaling cat. #3180) and goat polyclonal antibody UCP1 (diluted 1:50; Santa Cruz cat. n° sc-14720) [see supplementary material].

Digital high-resolution microphotographs of the liver and brown adipose tissue were taken under the same conditions of light and brightness/contrast by an Olympus BX41 microscope equipped with a 10x objective, an Olympus DP70 digital camera (Olympus Europa GmbH, Hamburg, Germany) and an Olympus X-Cite fluorescence system (X-Cite series 120Q, Olympus). Quantification of immunoreactivity and immunofluorescence were carried out by measuring densitometry of the images obtained from replicates and samples: two replicates per sample, eight samples per group and four groups, using the analysis software ImageJ 1.38x (National Institute of Health, Bethesda, Maryland, USA).

### *Statistical analysis*

All data are represented as mean  $\pm$  SEM (standard error of the mean) of at least eight determinations per experimental group. Kolmogorov-Smirnov normality tests indicated that all data followed a Gaussian distribution ( $P > 0.1$ ), so we selected a parametric statistical test. Differences between the diets and treatments were analyzed by two-way ANOVA for repeated measures, followed by Bonferroni *post test* for multiple comparisons. The level of statistical significance was set at  $P$  values of less than 0.05.

### **Results**

In order to control the effect of a high-fat diet and daidzein treatment; we first monitored the accumulated food/caloric intake and body weight gain after either acute (dose response) and subchronic treatment. We have also analyzed biochemical parameters such as glucose, triglycerides, cholesterol and hepatic enzymes, among

others, and plasma levels of adiponectin, insulin and leptin. In order to analyze the metabolic impact of a very high-fat diet (60% fat) and the response to daidzein treatment we studied the expression of specific genes and proteins in the liver, white and brown adipose tissues and skeletal muscle. The selected enzymes and receptors represent a selection of molecules implicated in lipid and glucidic metabolism with a putative role in energy metabolism. Clearly, the physiological differences between liver, skeletal muscle and adipose tissues determine that the molecules analyzed for gene expression are not the same in the three tissues. So, the nuclear receptors PPAR $\alpha$  and PPAR $\gamma$  and the Acetyl-CoA-oxidase (ACOX) were analyzed in these three tissues. The hormones adiponectin and leptin were analyzed in the white adipose tissue only as well as its receptors, the adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) in the skeletal muscle and liver respectively. Additional gene expressions for fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) were analyzed in liver and white adipose tissue; the growth hormone receptor (GHR) in muscle and liver; Acyl-CoA carboxylase (ACC) exclusively in the adipose tissue and fatty acid binding protein 1 (FABP-1) exclusively in liver. In order to contrast the results obtained from gene expressions, selected, relevant proteins such as PPAR $\alpha$ , SCD1 and FAS were analyzed in liver. In addition, the uncoupling protein 1 (UCP1), an enzyme involved in the regulation of body temperature and glucose metabolism, was analyzed in the brown adipose tissue. SCD1 has recently been shown to be a critical control point of lipid partitioning and body weight regulation. It catalyzes the rate-limiting step in the biosynthesis of monounsaturated fatty acids, which are required for normal rates of synthesis of triglycerides, cholesterol esters, and phospholipids (Dobrzyn *et al*, 2004). Finally, we also evaluated the gene expression of the cannabinoid CB1 and CB2 receptors in the three tissues.

1 *Effect of diet on accumulated food/caloric intake and body weight gain*

2 Figure 2 represents either accumulated food intake, caloric intake and body weight gain  
3 on the rats fed with either a standard/low-fat diet (STD, 10% fat) or with a high-fat diet  
4 (HFD, 60% fat) over the first 10 weeks of feeding period. We observed that rats fed  
5 with STD accumulated significantly more food intake than rats fed with a HFD from the  
6 day 15 on ( $P < 0.01$  between day 14-16,  $P < 0.001$  from day 17 on) (Figure 2A). On the  
7 contrary, the accumulated caloric intake was higher in HFD than in STD and such  
8 differences were also statistically significant from the day 30 on ( $P < 0.05$  in day 30,  $P$   
9  $< 0.01$  between day 34-41,  $P < 0.001$  from day 42 on) (Figure 2B). The lower food  
10 intake in HFD rats is due to the higher caloric/energy content in the HFD than the STD,  
11 as was described in the methodology. Accumulated caloric intake correlates with the  
12 body weight increase (Figure 2C). Interestingly, body weight gain also showed a  
13 significant divergence in HFD rats in comparison with STD rats from the eighth week  
14 on (56 days) ( $P < 0.05$  in day 56,  $P < 0.01$  from day 57 on). Two-way ANOVA analysis  
15 showed a very significant diet effect on accumulated food intake [Diet:  $F(1,900) =$   
16  $3092.56$ ,  $P < 0.0001$ ], accumulated caloric intake [Diet:  $F(1,900) = 447.53$ ,  $P < 0.0001$ ]  
17 and body weight gain [Diet:  $F(1,900) = 130.75$ ,  $P < 0.0001$ ]. When body weight gain  
18 began stabilizing over time (after 10 weeks in each of the diets), we scheduled both  
19 acute (5 and 50 mg/kg) and subchronic (50 mg/kg) treatments of daidzein, while the  
20 diets remained unchanged.

21  
22 *Dose response of the accumulated food intake with STD and HFD after acute treatment*  
23 *with daidzein*

24 After a feeding period of 10 weeks, rats only received one administration (acute  
25 treatment) of either vehicle or two different doses of daidzein (5 mg/kg and 50 mg/kg),

at different periods of time 30, 60, 120 and 240 minutes (Figure 3). This would help us identify the optimal dose at which treatment with daidzein would be more effective on food/caloric intake and body weight changes. We detected a very significant reduction in accumulated food intake using the 50 mg/kg dose of daidzein in both STD ( $P < 0.001$ ) and HFD ( $P < 0.01$ ) in all the time periods analyzed in the experiment (Figures 3A, B). On the contrary, the 5 mg/kg dose of daidzein did not seem to be sufficient to exert any difference on food intake in the time periods analyzed. Interestingly, two-way ANOVA analysis showed that daidzein induced a significant decrease by a dose response effect on accumulated food intake in STD [5 mg/kg:  $F(1,56) = 5.51$ ,  $P < 0.05$ ; 50 mg/kg:  $F(1,56) = 479.4$ ,  $P < 0.0001$ ], while daidzein produced an opposite dose response on accumulated food intake in HFD (raised at 5 mg/kg:  $F(1,56) = 11.98$ ,  $P = 0.001$ ; decreased at 50 mg/kg:  $F(1,56) = 122.15$ ,  $P < 0.0001$ ). These results indicate that the 50 mg/kg dose of daidzein is the most appropriate for the subchronic experiment.

#### *Effect of subchronic treatment with daidzein on accumulated food/caloric intake and body weight gain*

After a feeding period of 10 weeks, rats received a daily dose of either vehicle (1 ml/kg of 10% Tocrisolve) or daidzein (1ml/kg of 50 mg/kg in 10% Tocrisolve) during 14 days, while the diets remained unchanged (Figure 4). We observed that treatment with daidzein reduced significantly accumulated food intake in both diets from the day 11 of treatment on (Figure 4A), while daidzein reduced significantly accumulated caloric intake only in STD from the day 6 of treatment until the end of the experiment (Figure 4B). This also correlated with a significant decrease in body weight gain in both diets from the days 3-4 of treatment on, being more prominent in HFD feeding (Figure 4C). Two-way ANOVA analysis showed that daidzein induced a significant decrease on

accumulated food intake [Treatment:  $F(1,196) = 48.27$ ,  $P < 0.0001$  for STD;  $F(1,196) = 76.69$ ,  $P < 0.0001$  for HFD], accumulated caloric intake [Treatment:  $F(1,196) = 133.89$ ,  $P < 0.0001$  for STD;  $F(1,196) = 34.33$ ,  $P < 0.0001$  for HFD] and average body weight gain [Treatment:  $F(1,196) = 92.08$ ,  $P < 0.0001$  for STD;  $F(1,196) = 130.36$ ,  $P < 0.0001$  for HFD].

#### *Fat profile in skeletal muscle and liver after diet and subchronic treatment of daidzein*

To assess the magnitude of the potential physiological changes that can be influenced by accumulated fat deposits on the metabolic tissues studied in the diet-induced obese animal model; we analyzed the total fat content in both skeletal muscle and liver (Figure 5). Two-way ANOVA only showed an almost significant effect on the amount of fat in the skeletal muscle with daidzein treatment [Treatment:  $F(1,26) = 3.917$ ,  $P = 0.058$ ] (Figure 5A), however, there was a significant increase in the amount of fat in the liver after feeding the animals with a HFD [Diet:  $F(1,25) = 9.27$ ,  $P < 0.01$ ] (Figure 5B). We did not detect interaction between diet and treatment factors in any case.

The biochemical analysis of fat amount agrees with the results obtained from a specific lipid and fat depot staining, oil red O staining, in liver (Figure 6). We found a significant increase of the fat depots in the liver of rats fed with a HFD ( $P < 0.01$ ; Figure 6A-C), and a significant decrease after daidzein treatment ( $P < 0.05$ ; Figure 6A,C,E). Two-way ANOVA analysis also revealed a significant effect between diets [Diet:  $F(1,8) = 11.45$ ,  $P < 0.01$ ]. Such observation correlates with what could be expected to find in states leading to liver steatosis seen in some forms of obesity and metabolic syndrome. We also detected a significant interaction between both factors [diet vs treatment:  $F(1,8) = 14.49$ ,  $P < 0.01$ ], that is, daidzein induced a different effect on liver fat content depending on the diet. Interestingly, it seems that treatment with

daidzein reverses the amount of fat in the liver caused by high fat feeding (Figures 5B and 6A).

#### *Effect of diet and subchronic treatment with daidzein on plasma metabolites and liver enzymes*

The metabolites in plasma studied were glucose, urea, creatinine, uric acid, HDL-cholesterol, triglycerides and cholesterol; as for the liver enzymes analyzed we included the transaminases ALT, AST and GGT (Table 2). Results on diet showed a significant decrease in the circulating levels of urea ( $P < 0.05$ ) and triglycerides ( $P < 0.01$ ) in HFD compared with STD. Part of these results could be explained when considering the composition of the two diets used (supplementary table). Combining a STD and treatment with daidzein (50 mg/kg) there was a decrease in the plasma levels of creatinine ( $P < 0.001$ ), urea, HDL-cholesterol and triglycerides ( $P < 0.05$ ), compared with those in vehicle-treated animals (Table 2). However, glucose levels were very significantly increased after treatment with daidzein ( $P < 0.01$ ). As for the combination of a HFD with daidzein treatment, we observed that the levels of creatinine in plasma were decreased after treatment ( $P < 0.001$ ). Once again, glucose levels were raised after HFD and treatment with daidzein although in a lower scale than when using a STD (Table 2). Two-way ANOVA analysis revealed a significant diet effect in the plasma levels of glucose [Diet:  $F(1,27) = 10.49$ ,  $P < 0.01$ ], urea [Diet:  $F(1,27) = 10.68$ ,  $P < 0.01$ ], creatinine [Diet:  $F(1,27) = 4.69$ ,  $P < 0.05$ ] and triglycerides [Diet:  $F(1,27) = 9.2$ ,  $P < 0.01$ ] and a significant treatment effect in the plasma levels of glucose [Treatment:  $F(1,27) = 21.04$ ,  $P < 0.0001$ ], urea [Treatment:  $F(1,27) = 9.87$ ,  $P < 0.01$ ], creatinine [Treatment:  $F(1,27) = 76.56$ ,  $P < 0.0001$ ], triglycerides [Treatment:  $F(1,27) = 4.41$ ,  $P < 0.05$ ] and cholesterol [Treatment:  $F(1,27) = 3.53$ ,  $P < 0.05$ ]. We only detected

1 interaction in HDL-cholesterol [diet vs treatment:  $F(1,26) = 5.89$ ,  $P < 0.05$ ], that is,  
2 daidzein had a different effect on HDL-cholesterol depending on the diet.

3 Although plasma levels of transaminases (ALT, AST and GGT) did not differ  
4 significantly between diets or treatment of the four experimental groups, there was a  
5 tendency to the decrease in the levels of AST after treatment with daidzein using both  
6 STD and HFD (Table 2). We did not detect interaction between diet and treatment in  
7 any case. Table 3 summarizes the significance and the direction of the diet and  
8 treatment effects, including the interaction between both factors, on plasma metabolites.

#### 9 10 *Effect of diet and subchronic treatment with daidzein on plasma hormone levels*

11 Treatment with daidzein at 50mg/kg dose did not have any significant effect on the  
12 levels of insulin in plasma using neither of both diets (Figure 7A). However, the levels  
13 of adiponectin in plasma were reduced after treatment with daidzein at such dose in both  
14 STD and HFD (both at  $P < 0.05$ ) (Figure 7B). Two-way ANOVA analysis confirmed a  
15 significant decrease on adiponectin levels by daidzein [Treatment,  $F(1,27) = 14.11$ ,  $P <$   
16  $0.001$ ]. Interestingly, daidzein induced a significant increase on leptin levels [Treatment,  
17  $F(1,17) = 9.05$ ,  $P < 0.01$ ] (Figure 7C). This is, daidzein had an effect on plasma  
18 adiponectin and leptin levels but with opposite effects in each. We did not detect  
19 interaction between diet and treatment in any case. Table 3 summarizes the significance  
20 and the direction of the diet and treatment effects, including the interaction between  
21 both factors, on plasma hormone levels.

*Effect of diet on the expression of selected genes for metabolic control in white adipose tissue, skeletal muscle and liver*

After the complete feeding period (12 weeks) with either a STD or a HFD, animals were sacrificed and samples from the adipose tissue, skeletal muscle and liver extracted and processed for RT-PCR analysis. According to our results, we could observe a series of changes in gene expression that were more significant in the adipose tissue and liver, followed by skeletal muscle (Figure 8). Table 3 summarizes the significance and the direction of the diet effects on gene expression.

**White adipose tissue.** Feeding with a HFD induced a very significant increase in the levels of leptin and ACOX ( $P < 0.001$ ), and also of the endocannabinoid CB1 receptor ( $P < 0.001$ ). On the contrary, the effect of HFD feeding produced a decrease in gene expression levels for the synthesis enzymes ACC ( $P < 0.001$ ) and FAS ( $P < 0.05$ ) (Figure 8A). HFD feeding didn't seem to have any effect on the gene expression of any of the remaining molecules and enzymes studied in adipose tissue (PPAR $\alpha$ , PPAR $\gamma$ , adiponectin, SCD1 and CB2R). Two-way ANOVA analysis showed a diet effect on PPAR $\alpha$  [ $F(1,20) = 5.11$ ,  $P < 0.05$ ], leptin [ $F(1,21) = 29.34$ ,  $P < 0.0001$ ], ACOX [ $F(1,22) = 23.36$ ,  $P < 0.0001$ ], ACC [ $F(1,22) = 19.08$ ,  $P < 0.001$ ], FAS [ $F(1,22) = 20.37$ ,  $P < 0.001$ ], CB1R [ $F(1,22) = 47.91$ ,  $P < 0.0001$ ] and CB2R [ $F(1,23) = 6.02$ ,  $P < 0.05$ ] gene expressions (Table 3).

**Skeletal muscle.** PPAR $\alpha$  and CB2 receptor gene expression was significantly reduced ( $P < 0.01$  and  $P < 0.05$  respectively) after using a HFD (Figure 8B). Although not significant, there was a tendency to the decrease for the remaining molecules analyzed in the skeletal muscle (PPAR $\gamma$ , AdipoR1, ACOX, GHR and CB1 receptor). Two-way ANOVA analysis showed a diet effect on PPAR $\gamma$  [ $F(1,25) = 7.22$ ,  $P < 0.05$ ] and CB2R [ $F(1,23) = 10.47$ ,  $P < 0.01$ ] gene expressions (Table 3).

**Liver.** Liver is the main regulatory organ with specific metabolic effects in the other peripheral tissues. In our results, HFD produced an increase in the levels of expression for PPAR $\alpha$ , PPAR $\gamma$  and ACOX ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.05$  respectively). However, the same diet induced a reduction in the levels of the synthesis enzymes SCD1 and FAS ( $P < 0.001$ ). Such decrease in synthesis enzymes agrees with results observed in adipose tissue above. As for the remaining molecules analyzed in liver (AdipoR2, GHR, CB1 and CB2 receptors) no significant changes were observed (Figure 8C). Two-way ANOVA analysis showed a diet effect on PPAR $\alpha$  [ $F(1,22) = 9.54$ ,  $P < 0.01$ ], PPAR $\gamma$  [ $F(1,23) = 9.3$ ,  $P < 0.01$ ], ACOX [ $F(1,23) = 6$ ,  $P < 0.05$ ], SCD1 [ $F(1,20) = 21.09$ ,  $P < 0.001$ ], FAS [ $F(1,21) = 30.53$ ,  $P < 0.0001$ ] and CB2R [ $F(1,21) = 4.73$ ,  $P < 0.05$ ] gene expressions (Table 3).

*Effect of diet and subchronic treatment with daidzein on the expression of selected genes for metabolic control in white adipose tissue, skeletal muscle and liver*

After feeding with STD or HFD for 10 weeks, rats received a daily i.p. injection of daidzein (50 mg/kg) for 14 days, sacrificed two hours after treatment and samples from their adipose tissue, skeletal muscle and liver extracted and analyzed by RT-PCR as described in methods (Figures 9 and 10). Table 3 summarizes the significance and the direction of the treatment effect on gene expression, including the interaction between diet and treatment.

**White adipose tissue.** Treatment with daidzein using a STD induced a reduction on the lipogenic enzyme ACC gene expression levels only ( $P < 0.05$ ), with no effect on the rest of molecules studied in this tissue (Figure 9A). We observed that feeding with a HFD and treatment with daidzein reduced the levels of some of the molecules studied: adiponectin ( $P < 0.05$ ), leptin ( $P < 0.001$ ) and ACOX ( $P < 0.001$ ) (Figure 10A).

Although not significant, the levels of SCD1 also showed a tendency to decrease. On the contrary, such treatment did not seem to have any effects on the nuclear receptor PPAR $\alpha$ , the synthesis enzyme ACC or the cannabinoid CB1 and CB2 receptors (Figure 10A). Two-way ANOVA analysis only showed a significant treatment effect on leptin gene expression [ $F(1,21) = 6.69$ ,  $P < 0.05$ ], and a tendency on ACOX and SCD1 gene expressions ( $P < 0.06$ ). Interaction between diet and treatment was detected in adiponectin [ $F(1,21) = 5.98$ ,  $P < 0.05$ ], leptin [ $F(1,21) = 21.03$ ,  $P < 0.001$ ] and ACOX [ $F(1,22) = 23.36$ ,  $P < 0.0001$ ] gene expressions, that is, daidzein had a different effect depending on the diet (Table 3).

**Skeletal muscle.** The levels of expression of the nuclear receptors PPAR $\alpha$  and PPAR $\gamma$  were reduced significantly ( $P < 0.01$  and  $P < 0.05$  respectively) after feeding with STD and treatment with daidzein. The rest of molecules showed a tendency to the decrease with this combination of diet and treatment, although not significant (Figure 9B). Feeding with a HFD and treatment with daidzein did not produce any significant change in the levels of expression in any of the genes studied in the skeletal muscle (Figure 10B). Two-way ANOVA analysis did not detect treatment effect on any of the genes analyzed, but detected interaction between diet and treatment, that is, daidzein produced a different response depending on the diet in PPAR $\alpha$  [ $F(1,26) = 11.87$ ,  $P < 0.01$ ], PPAR $\gamma$  [ $F(1,25) = 9.92$ ,  $P < 0.01$ ], ACOX [ $F(1,26) = 5.57$ ,  $P < 0.05$ ] and GHR [ $F(1,26) = 5.65$ ,  $P < 0.05$ ] gene expressions (Table 3).

**Liver.** After treatment with daidzein using a STD we observed a reduction in the mRNA levels of SCD1 and FAS ( $P < 0.01$ ), being both lipogenic enzymes in the liver. On the other hand, there were no changes on the gene expression for any of the rest of molecules (Figure 9C). We observed that the combination of treatment with daidzein and feeding with a HFD significantly reduced the levels of PPAR $\gamma$  gene expression in

liver ( $P < 0.05$ ). However, there were no changes in the levels of the remaining genes (Figure 10C). Two-way ANOVA analysis showed a significant treatment effect on PPAR $\alpha$  [ $F(1,22) = 6.05$ ,  $P < 0.05$ ], PPAR $\gamma$  [ $F(1,23) = 4.32$ ,  $P < 0.05$ ] and SCD1 [ $F(1,20) = 6.74$ ,  $P < 0.05$ ] gene expressions. Daidzein produced a different effect depending on the diet (interaction) in PPAR $\gamma$  [ $F(1,23) = 4.32$ ,  $P < 0.05$ ], ACOX [ $F(1,23) = 4.6$ ,  $P < 0.05$ ], SCD1 [ $F(1,20) = 6.38$ ,  $P < 0.05$ ] and FAS [ $F(1,21) = 12.39$ ,  $P < 0.01$ ] gene expressions (Table 3).

#### *Effect of diet and daidzein on the expression of selected proteins for metabolic control in liver and brown adipose tissue*

After evaluating the most relevant changes observed on gene expression, we analyzed selected protein expressions such as PPAR $\alpha$ , SCD1 and FAS in liver, and UCP1 in brown adipose tissue. Figure 11 shows a panel of representative high-magnification images from the liver immunostained for PPAR $\alpha$ , SCD1 and FAS in the four rat groups (standard diet, STD-daidzein, high-fat diet, HFD-daidzein). Results in the densitometrical quantification of immunoreactivity and immunofluorescence on tissue microarray blocks showed similar changes to that of gene expressions (Figure 11). PPAR $\alpha$  showed a significant increase on the immunoreactive levels in HFD rats ( $P < 0.05$ ; Figure 11E), but tended to decrease after daidzein treatment. Two-way ANOVA analysis also reveals a significant effect between diets [Diet,  $F(1,28) = 11.24$ ,  $P < 0.01$ ] and a significant effect with daidzein [Treatment,  $F(1,28) = 4.22$ ,  $P < 0.05$ ] (Table 3). SCD1 immunofluorescence showed a significant decrease in HFD rats ( $P < 0.01$ ; Figure 11J) that was accentuated in rats treated with daidzein and fed with both diets ( $P < 0.001$  for STD;  $P < 0.05$  for HFD). Two-way ANOVA analysis also revealed a significant effect between diets [Diet,  $F(1,24) = 21.45$ ,  $P < 0.001$ ] and a significant

effect with daidzein [Treatment,  $F(1,24) = 26.36$ ,  $P < 0.001$ ]. FAS also showed a very significant decrease on the immunofluorescence levels in HFD rats ( $P < 0.001$ ). Diet effect was confirmed after two-way ANOVA analysis [Diet,  $F(1,28) = 119.37$ ,  $P < 0.0001$ ] (Table 3).

Figure 12 shows the quantification of UCP1 immunofluorescence and a panel of representative high-magnification images of brown adipose tissue in the four rat groups. UCP1 immunofluorescence showed a significant increase by an effect with daidzein [Treatment,  $F(1,27) = 5.65$ ,  $P < 0.05$ ] (Figure 12A and Table 3).

## Discussion

Over the years, many data obtained from clinical trials and animal studies showed that feeding with soy isoflavones can improve blood lipid profiles and glucose metabolism, and therefore, exert beneficial effects over several chronic disorders associated with obesity and diabetes (Clarkson, 2002; Messina *et al.*, 2002; Clair and Anthony, 2005; Cassidy and Hooper, 2006; Zhang *et al.*, 2010). However, the hypolipidemic actions of isoflavones and the mechanisms involved are not totally understood being currently a subject of debate. Some new important data derived from the present study will help to clarify these questions.

Table 3 summarized the effects induced by the diet and the treatment, including the interaction between both factors, in all the parameters analyzed in this study. A first experiment showed that daidzein was capable of reducing food intake in food-deprived animals. Although we do not have clues to identity the mechanisms involved in this acute effect, such result helped us to select a dose for the subchronic treatment experiment. Because of the transcriptional properties of the estrogen receptor we do not believe that this acute effect is not only related to the E2 receptor, but to the interaction

1 with other signaling systems, involved in appetite control. After this experiment, we  
2 evaluated the effects of a high fat diet and the following response to a subchronic  
3 treatment with daidzein on body weight and several plasma parameters; as well as  
4 identify the molecular changes occurring within the physiological pathways of the main  
5 metabolic tissues.

6 As it could be expected, our first results showed that a very high-fat diet cause an  
7 important weight gain in these animals. Interestingly, acute daidzein dose-dependently  
8 modulated feeding. Acute treatment with daidzein at the 5 mg/kg dose induced a  
9 significant increase in accumulated HFD intake, whereas a significant intake reduction  
10 was observed using the 50 mg/kg dose of daidzein in both STD and HFD in all the time  
11 periods analyzed in the experiment. This biphasic dose response effect of daidzein on  
12 food intake may be explained as the result of a balance among PPARs and ERs receptor  
13 activation, as was previously described in osteogenesis and adipogenesis (Dang and  
14 Löwik, 2004). Chronic treatment with daidzein at the 50 mg/kg dose used in the  
15 experiment induced a significant weight loss in both diets. Together with body weight,  
16 food and calorie intake was also reduced after treatment with daidzein, being such  
17 reduction higher in the last days of treatment than in the remaining of the experiment.  
18 This observation could indicate that the effect of daidzein would be derived of a specific  
19 target (receptor) that gained sensitivity after several days of treatment, suggesting the  
20 induction of sensitization. The observed reduction in body weight agrees with other  
21 experiments in rodents which demonstrated that dietary isoflavones significantly  
22 decrease body weight and adipose tissue (Michael *et al.*, 2006; Davis *et al.*, 2007; Na *et*  
23 *al.*, 2008; Guo *et al.*, 2009M; Kim *et al.*, Epub ahead of print).

24 It is well known that high-fat diets increase hepatic lipids and adipose tissue, hence,  
25 overweight and obesity can lead to some forms of liver steatosis, mainly due excessive

fat accumulation in the liver. In our results we observed that daidzein treatment reverted the increased fat content in the liver produced by a HFD, this is important as it might indicate a contribution of daidzein in reducing hepatic triglycerides and steatosis which can as well induce changes in gene expression profiling, especially of genes involved in adipogenesis or fatty acid  $\beta$ -oxidation. Less hepatic fat accumulation may also improve hepatic insulin resistance.

Consequently, the main objective of this work was to monitor the response of the main genes related to lipid and glucidic metabolism after treatment with daidzein using a STD and a HFD for the three metabolic tissues: white adipose tissue, liver and skeletal muscle. We found that the effect of a HFD in the expression of genes in adipose tissue was to produce an increase in the levels of leptin and oxidative enzyme ACOX, and a decrease in the lipogenic enzymes ACC and FAS. Similar effects are found in liver where gene expression levels showed a decrease in lipogenic enzymes SCD1 and FAS and an increase in ACOX when using a HFD. Some of these effects were ratified after the analysis of SCD1 and FAS protein expression by immunofluorescence. Therefore, it seems that chronic feeding with a HFD induces an inhibition of lipogenesis via a reduction in the expression of FAS, ACC and SCD1 inducing also stimulation of fat oxidation via ACOX as well as an increase in leptin levels, the hormone that regulates food intake and impedes accumulation of fat in non adipose tissues. Interestingly, the levels of the endocannabinoid receptor CB1 were increased in white adipose tissue which implies a role of the ECS in these processes; and which agrees with results from previous literature (Osei-Hyiaman *et al.*, 2005; Crespillo *et al.*, 2011).

When treating the rats with daidzein and using a STD, we again observed a reduction in the levels of ACC expression in white adipose tissue. Accordingly, the lipogenic enzymes SCD1 and FAS also showed a significant reduction after treatment with the

isoflavone in liver, this latter agrees with other work in which FAS mRNA expression was lowered after soy feeding (Nagasawa *et al.*, 2003; Ascencio *et al.*, 2004), and with results from a recent similar work (Kim *et al.*, Epub ahead of print), pointing that daidzein might alleviate liver steatosis directly through the transcriptional control of hepatic lipogenesis and fatty acid  $\beta$ -oxidation. The same combination of STD/daidzein reduced nuclear receptors PPAR $\alpha$  and PPAR $\gamma$  levels in the skeletal muscle. As stated above, previous studies showed that genistein and daidzein can bind directly to both PPAR $\alpha$  and PPAR $\gamma$  and that these effects on the PPARs could be tissue specific. Especially in the skeletal muscle some studies in mice have associated activation of PPARs with other effect such as AMP-activated kinase and with improvement in lipid and glucose signaling (Cederroth *et al.*, 2008; Ronis *et al.*, 2009). It is known that AMPK can modulate the activities of transcription factors and coactivators resulting in either activation or repression of specific genes (Woods *et al.*, 2000). Similar effects were observed in another study in which dietary soy improved insulin sensitivity by increasing glucose uptake in skeletal muscle (Cederroth and Nef, 2009). Whichever the pathways implicated daidzein clearly has an effect on skeletal muscle oxidative metabolism and our study confirms such effect in the case in animals fed with a STD.

As for the effect of treatment with daidzein when using a HFD, we found a decrease in the levels of some of the genes studied in the adipose tissue. Thus, the hormones adiponectin and leptin were reduced as well as the specially the oxidative enzyme ACOX. Interestingly, analyzing treatment effect, PPAR $\alpha$  and PPAR $\gamma$  levels of expression were also reduced in liver where we also observed a reduction in SCD1 levels. However, no significant changes were observed in the skeletal muscle in any of the genes studied in this tissue. Once again, these results point to a possible inhibition of the lipogenic pathways in liver and white adipose tissue.

1 Clearly, excess of fat consumption after chronic feeding with HFD can influence  
2 adipogenesis (Rosen, 2005). Adipogenesis has been known to be regulated by the  
3 cooperative interaction of various hormones and nuclear receptors, including PPAR $\gamma$ ,  
4 modulating the pattern of gene expression and lipid content in the fat cells (Harp, 2004;  
5 Kota *et al.*, 2005). Besides, as high-fat diets can also provoke fat accumulation in the  
6 liver; recent studies suggested that the effects of isoflavone feeding on insulin  
7 sensitivity and lipid homeostasis might also involved, among other factors, altered  
8 signaling in the liver through PPAR $\alpha$  and PPAR $\gamma$  (Ascencio *et al.*, 2004; Shukla *et al.*,  
9 2007). In our study, daidzein as well as producing weight loss it has also shown to have  
10 a clear effect on lipid metabolism via PPAR $\alpha$ , PPAR $\gamma$  and SCD1.

11 Furthermore, daidzein, whose structure resembles the steroid structure of E2, can  
12 activate ERs as well as PPAR $\alpha$ , and PPAR $\gamma$  (Dang and Lowik, 2004). Recent findings  
13 proposed that a complex balance between activated ERs and PPARs could be an  
14 important mechanism in determining the biological effects isoflavones (daidzein and  
15 genistein) (Dang and Lowik, 2004). In this respect, ERs have been shown to be  
16 expressed in muscle and various other cell types apart from adipocytes, and it is a fact  
17 that the adipose ER varies by depot and with different physiologic states (Rosen, 2005;  
18 Barros *et al.*, 2006).

19 Several studies have suggested a relevant role of PPAR $\alpha$  in uncoupling protein 1  
20 (UCP1) expression (Barberá *et al.*, 2001). In addition, PPAR $\gamma$  acts in brown adipose  
21 tissue upregulating the expression of UCP1, leading to thermogenesis. The analysis of  
22 the possible effect of daidzein on thermogenesis indicate that daidzein treatment in rats  
23 causes an important increase of UCP1 protein expression on brown adipose tissue,  
24 suggesting that daidzein could regulate uncloupling of adenosine triphosphate  
25 production as a mechanism of lower energy depots (Xue *et al.*, 2005).

Besides ERs and PPARs, other studies showed that feeding with soy protein can produce changes in hormones levels (Guo *et al.*, 2009; Ronis *et al.*, 2009). In our experiment, the levels of the hormones adiponectin and leptin in plasma were clearly affected by treatment with daidzein (50 mg/kg dose) with opposite effects in each. Kim *et al.* (Epub ahead of print) found the same results when measuring the levels leptin and adiponectin in serum of mice. Leptin also mediates specific metabolic effects, including the potent depletion of triglyceride from liver and other peripheral tissues. An interesting study assessed the identification and ranking of genes specifically repressed by leptin and showed that at the top of this list was SCD1, the rate limiting enzyme in the biosynthesis of monounsaturated fats, and it was specifically repressed during leptin-mediated weight loss (Cohen *et al.*, 2003). However, whether such effect of daidzein is due to changes on the adipose tissue physiology caused by the weight loss (both adiponectin and leptin are adipocyte-derived hormone playing a pivotal role in regulating food intake and energy expenditure) (Jiang *et al.*, 2005), or due to the hormone-like effects exerted by daidzein after activation of ERs it is not conclusively determined. We did not find significant changes on the levels of insulin in plasma. Finally, the analysis in plasma metabolites showed a significant reduction in triglycerides and cholesterol levels after treatment with daidzein. As stated above, HFD and excessive fat accumulation affects the liver, the primary organ to regulate metabolism and serum composition as most of the fatty acids and cholesterol from food are processed here before they reach other tissues. Guo and collaborators (2009) found that free fatty acid (FFA) concentration in both plasma and liver was significantly reduced by daidzein indicating that daidzein might influence the elimination of FFAs by mechanisms involving FFA oxidation in the liver and muscle. Because of the high prevalence on non alcoholic steatohepatitis, these results support for the use of

1 isoflavones as a nutritional supplement for reducing liver fat. In any case, high-fat  
2 feeding can also produce adipocyte hypertrophy and hyperplasia to demand for extra  
3 storage (García-Serrano et al., 2011), which might alter the compensatory effects of  
4 daidzein, indicating that not only daidzein but also restriction of fat in the diet had to be  
5 implemented for this purpose. In addition, the action of isoflavones on PPAR $\gamma$  can also  
6 influence adipocyte differentiation and tissue lipid accumulation, affecting also  
7 triglyceride levels in plasma.

8 Our results on diet and daidzein treatment shows a complex interaction of many  
9 different factors through various possible interrelated pathways but with an outstanding  
10 role in inhibition of lipogenesis, confirming previous results with isoflavones  
11 (Nagasawa et al., 2003; Shukla et al., 2007), and indicating a possible compensatory  
12 effect of daidzein in these processes.

13 In conclusion, our results suggest that daidzein treatment can significantly reduce body  
14 weight in rats and also ameliorate the high-fat diet-induced hyperlipidemia, which can  
15 partially explain the antisteatotic, cholesterol-lowering, and insulin-sensitizing effects  
16 of soy. Our results agree with another important related work by Kim *et al.* (Epub ahead  
17 of print) done on obese mice fed on a high-fat diet. This general physiological effect  
18 could involve one or several of the pathways suggested with synergistic results, clearly  
19 implicating receptors PPAR $\gamma$  and the SCD-1 enzyme. The size of the effect may vary  
20 depending on the physiology of the subjects (rat strains, altered leptin/ECs signaling) as  
21 well as the different procedures used (routes of administration) which could be crucial  
22 for their physiological functions. Such diversity of cellular actions of isoflavones  
23 supports the hypothesis of the benefits in their consumption. However, further  
24 investigation is needed to evaluate the long-term effects of isoflavones on obesity, type

2 diabetes and its associated disorders, and to assess whether the same mechanisms are shared in humans.

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## Legends to figures

**Figure 1.** Chemical structure of daidzein (isoflavone), present in *Glycine max* (soybean),  $17\beta$ -estradiol (estradiol),  $\Delta^9$ -tetrahydrocannabinol (THC), the main cannabinoid receptor agonist present in *Cannabis* preparations, and AM251, a synthetic CB1 cannabinoid receptor inverse agonist.

**Figure 2.** Effect of a standard/low fat diet (STD) and a very high-fat diet (HFD) on accumulated food/caloric intake and body weight gain during 10 weeks. We observed that rats fed with STD accumulated more food intake than rats fed with a HFD (**A**), in contrast with the accumulated caloric intake (**B**), and this difference was statistically significant. Body weight gain showed a significant divergence in HFD rats in comparison with STD rats from the eighth week on (**C**). When body weight gain stabilized over time (at least, for 10 weeks), daidzein was administrated, while the diets remained unchanged. Histograms represent the mean  $\pm$  SEM (16 animals per diet group). Two-way ANOVA followed by Bonferroni *post test*:  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  vs STD rats. Diet effects are summarized in Table 3.

**Figure 3.** Dose response effects of acute administration of daidzein at 5 and 50 mg/kg on accumulated food intake in male rats fed with a standard/low fat diet (STD) and a very high-fat diet (HFD) at different periods of time 30, 60, 120 and 240 minutes. We observed a significant reduction in accumulated food intake using the 50 mg/kg dose of daidzein in both STD (**A**) and HFD (**B**) in all the time periods analyzed in the experiment. On the contrary, the 5 mg/kg dose of daidzein did not seem to be sufficient to exert any difference on food intake in the time periods analyzed. Histograms represent the mean  $\pm$  SEM (8 animals per group). Two-way ANOVA followed by

1 Bonferroni *post test*: \*\*\* $P < 0.001$  vs vehicle-treated STD group; ## $P < 0.01$  vs vehicle-  
2 treated HFD group. Treatment effects are summarized in Table 3.

3  
4 **Figure 4.** Effects of a standard/low fat diet (STD) and a very high-fat diet (HFD) 10  
5 days before daidzein administration and during 14 days of daidzein treatment (50  
6 mg/kg) on accumulated food/caloric intake and body weight gain, while the diets  
7 remained unchanged. Treatment with daidzein reduced accumulated food (**A**) and  
8 caloric (**B**) intake in both diets from the day 8 of treatment on, and this correlated with a  
9 significant body weight decrease (**C**). Arrows point to the beginning of daidzein  
10 administration. Histograms represent the mean  $\pm$  SEM (8 animals per treated group).  
11 Two-way ANOVA followed by Bonferroni *post test*: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P <$   
12 0.001 vs daidzein-treated HFD group; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  vs daidzein-  
13 treated STD group. Treatment effects are summarized in Table 3.

14  
15 **Figure 5.** Effects of a subchronic treatment of daidzein (50 mg/kg) on skeletal muscle  
16 and liver fat profile in male rats fed with STD and HFD. We found no significant  
17 differences in the amount of fat in the skeletal muscle with either diet or daidzein  
18 treatment (**A**). There was only a significant increase in the amount of fat in the liver by a  
19 diet effect (**B**). Histograms represent the mean  $\pm$  SEM (8 animals per group). Two-way  
20 ANOVA followed by Bonferroni *post test*: \*\* $P < 0.05$  for diet effect.

21  
22 **Figure 6.** Effect of a subchronic treatment of daidzein (50 mg/kg) on liver fat depots in  
23 male rats fed with STD and HFD. We found a significant increase in the fat depots in  
24 the liver after HFD feeding; and a significant drop after daidzein treatment in HFD-fed  
25 rats (**A**). Representative high-magnification images from the liver stained with oil red O

in the four rat groups (**B-E**). Histograms represent the mean  $\pm$  SEM (3 animals per group). Two-way ANOVA followed by Bonferroni *post test*:  $**P < 0.01$  vs vehicle-treated STD group,  $^{\&}P < 0.05$  vs vehicle-treated HFD group,  $^{\$}P < 0.01$  for diet effect. Scale bar is shown in image B.

**Figure 7.** Effects of a subchronic treatment of daidzein (50 mg/kg) on serum insulin (**A**), adiponectin (**B**) and leptin (**C**) levels in rats fed with STD or HFD. Treatment with daidzein did not have any significant effect on the levels of insulin in plasma, but produced a reduction in the levels of adiponectin and an increase in the levels of leptin in the plasma of both STD and HFD rats. Histograms represent the mean  $\pm$  SEM (8 animals per group). Two-way ANOVA followed by Bonferroni *post test*:  $*P < 0.05$  vs vehicle-treated groups,  $**P < 0.01$  and  $***P < 0.001$  for treatment effect.

**Figure 8.** Effects of diet on gene expression of molecules implicated in adipose tissue, skeletal muscle and liver glucidic and lipidic metabolism, including cannabinoid receptors. Regarding the adipose tissue, HFD feeding induced a significant increase in the levels of Leptin, ACOX and CB1 receptor, but produced a decrease in gene expression levels of ACC and FAS. In the skeletal muscle, PPAR $\alpha$  and CB2 receptor gene expression was significantly reduced after using a HFD. In the liver, HFD produced an increase in the levels of expression for PPAR $\alpha$ , PPAR $\gamma$  and ACOX, but induced a reduction in the levels of SCD1 and FAS. Values from each sample were normalized with regard to the housekeeping gene Sp1 for adipose tissue and liver and Actin beta for skeletal muscle. Histograms represent the mean  $\pm$  SEM (8 animals per group). Two-way ANOVA followed by Bonferroni *post test*:  $*P < 0.05$ ,  $**P < 0.01$ ,

1 \*\*\* $P < 0.001$  vs STD. For abbreviation see list. Diet, treatment and interaction effects  
2 are summarized in Table 3.

3  
4 **Figure 9.** Effect of a subchronic treatment of daidzein (50 mg/kg) using a  
5 standard/low-fat diet (STD) on gene expression of molecules implicated in adipose  
6 tissue, skeletal muscle and liver glucidic and lipidic metabolism, including cannabinoid  
7 receptors. We observed a decrease in the levels of gene expression of ACC in adipose  
8 tissue (**A**), PPAR $\alpha$  and PPAR $\gamma$  in skeletal muscle (**B**), and SCD1 and FAS in liver (**C**).  
9 Values from each sample were normalized with regard to the housekeeping gene Sp1  
10 for adipose tissue and liver and Actin beta for skeletal muscle. Histograms represent the  
11 mean  $\pm$  SEM (8 animals per group). Two-way ANOVA followed by Bonferroni *post*  
12 test: \* $P < 0.05$ , \*\* $P < 0.01$  vs vehicle. For abbreviation see list. Diet, treatment and  
13 interaction effects are summarized in Table 3.

14  
15 **Figure 10.** Effect of a subchronic treatment of daidzein (50 mg/kg) using a very high-  
16 fat diet (HFD) on gene expression of molecules implicated in adipose tissue, skeletal  
17 muscle and liver glucidic and lipidic metabolism, including cannabinoid receptors. We  
18 also observed a decrease in the levels of gene expression of Adiponectin, Leptin and  
19 ACOX in adipose tissue (**A**), and PPAR $\gamma$  in liver (**C**). We did not detect any significant  
20 change in the levels of expression in any of the genes studied in the skeletal muscle.  
21 Values from each sample were normalized with regard to the housekeeping gene Sp1  
22 for adipose tissue and liver and Actin beta for skeletal muscle. Histograms represent the  
23 mean  $\pm$  SEM (8 animals per group). Two-way ANOVA followed by Bonferroni *post*  
24 test: \* $P < 0.05$ , \*\*\* $P < 0.001$  vs vehicle. For abbreviation see list. Diet, treatment and  
25 interaction effects are summarized in Table 3.

**Figure 11.** Effects of diet and daidzein treatment (50 mg/kg) on protein expression of PPAR $\alpha$ , SCD1 and FAS in liver by immunohistochemistry, immunofluorescence and densitometry. Representative high-magnification images from the liver immunostained for PPAR $\alpha$  (A-D), SCD1 (F-I) and FAS (K-N) in the four rat groups (standard diet, STD-daidzein, high-fat diet, HFD-daidzein). Quantification of densitometry showed an increase of PPAR $\alpha$  expression in HFD (E), a decrease of SCD1 expression in HFD that was accentuated after daidzein treatment in both diets (J), and a decrease of FAS expression in HFD (O). Histograms represent the mean  $\pm$  SEM (8 animals per group). Two-way ANOVA followed by Bonferroni *post test*: \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 vs vehicle-treated STD group, & $P$  < 0.05 vs vehicle-treated HFD group, \$\$ $P$  < 0.01, \$\$\$ $P$  < 0.001 for diet effect, # $P$  < 0.05, #### $P$  < 0.001 for daidzein effect. Scale bar is shown in images A, F and K. For abbreviation see list. Diet, treatment and interaction effects are summarized in Table 3.

**Figure 12.** Effects of diet and daidzein treatment (50 mg/kg) on protein expression of UCP1 in brown adipose tissue by immunofluorescence and densitometry. Quantification of densitometry only showed a significant increase of UCP1 expression after daidzein treatment (A). Representative high-magnification images from the brown adipose tissue immunostained for UCP1 in the four rat groups (standard diet, STD-daidzein, high-fat diet, HFD-daidzein; B-E). Histograms represent the mean  $\pm$  SEM (8 animals per group). Two-way ANOVA followed by Bonferroni *post test*: # $P$  < 0.05 for daidzein effect. Scale bar is shown in image B. For abbreviation see list.

1 **Table 1.** Primers sequences used for real time polymerase chain reaction (RT-PCR).

| Genes                                                             | GenBank code   | Tissue                   | Oligonucleotide primers (forward, reverse)                | Product size (bp) |
|-------------------------------------------------------------------|----------------|--------------------------|-----------------------------------------------------------|-------------------|
| Cannabinoid receptor 1 (CB1R or Cnr1)                             | NM_012784.3    | Adipose                  | 5'ACAGCCAGCATGCACAGGGC<br>5'CGGCGGACGTGTCTGTGGAC          | 94                |
|                                                                   |                | Muscle / Liver           | 5'AGACCTCTCTACGTGGGCTCG<br>5'GTACAGCGATGGCAGCTGCTG        | 314               |
| Cannabinoid receptor 2 (CB2R or Cnr2)                             | NM_020543.4    | Adipose                  | 5'AGGATAAGCAGGAGTTGGGAGGAGA<br>5'TGAATCTGCCAGAGACAGCATGGG | 80                |
|                                                                   |                | Muscle / Liver           | 5'GCAGCCTGCTGCTGACTGCTG<br>5'TGCTTTCCAGAGGACATACCC        | 284               |
| Peroxisome proliferator-activated receptor alpha (Ppar $\alpha$ ) | NM_013196.1    | Adipose / Muscle / Liver | 5'TGCTGTCTCTCCTTGATGAAC<br>5'GCTTGAGCACGTGCACAATC         | 270               |
| Peroxisome proliferator-activated receptor gamma (Ppar $\gamma$ ) | NM_013124.1    | Adipose / Muscle / Liver | 5'GACCACTCCCATTCTTTTGA<br>5'CGCACTTTGGTATTCTTGGAG         | 153               |
| Adiponectin hormone (Adipoq)                                      | NM_144744.2    | Adipose                  | 5'AGGGATTACTGCAACCGAAG<br>5'TCCTGTCTATTCCAGCATCTC         | 211               |
| Adiponectin receptor 1 (AdipoR1)                                  | NM_207587.1    | Muscle                   | 5'TGATTATGGGGAGCTTCGTC<br>5'ACAACACCACTCAAGCCAAG          | 196               |
| Adiponectin receptor 2 (AdipoR2)                                  | NM_001037979.1 | Liver                    | 5'AATGACAACCACCGGAGA<br>5'CCTTCCCACACCTTACAAAC            | 200               |
| Leptin hormone (Lep)                                              | NM_013076.1    | Adipose                  | 5'AGGAAAATGTGCTGGAGACC<br>5'ATACCGACTGCGTGTGTGAA          | 160               |
| Acyl-CoA oxidase (Acox1)                                          | NM_017340.1    | Adipose                  | 5'CACCTTCGAGGGAGAGAACA<br>5'CGCACCTGGTCGTAGATTTT          | 75                |
|                                                                   |                | Muscle / Liver           | 5'GGACCATGGTTTTTGTGAGG<br>5'AGTGAAGGCATAGGCAGTG           | 201               |
| Growth hormone receptor (Ghr)                                     | NM_017094.1    | Muscle / Liver           | 5'ACAAATGGTGATTGTGTTGGA<br>5'TCACTTGGATATCTCCACGGA        | 130               |
| Stearoyl-CoA desaturase 1 (Scd1)                                  | NM_139192.1    | Adipose                  | 5'GAAGCGAGCAACCGACAG<br>5'GGTGGTCGTGTAGGAAGTGG            | 71                |
|                                                                   |                | Liver                    | 5'TGGGTTGGCTGCTTGTG<br>5'GTGTGGGCAGGATGAAG                | 150               |
| Acyl-coA carboxilase (ACC $\alpha$ or Acaca)                      | NM_022193.1    | Adipose                  | 5'TGTGGGCTGGCTGGGGTCAT<br>5'CGCCACATGGCCTGACTCG           | 104               |
| Fatty acid synthase (FAS or Fasn)                                 | NM_017332.1    | Adipose / Liver          | 5'AGTTTCCGTGAGTCCATCCT<br>5'TCAGGTTTCAGCCCCATAGA          | 182               |
| Fatty acid binding protein 1 (Fabp1)                              | NM_012556.1    | Liver                    | 5'CTCATCCAGAAAGGGAAGGA<br>5'GTCACCTCCATCTTAACCA           | 183               |
| <b>Housekeeping genes</b>                                         |                |                          |                                                           |                   |
| Sp1 transcription factor (Sp1)                                    | NM_012655.2    | Adipose                  | 5'GCTATAGCAAACACCCAGGT<br>5'GATCAGGGCTGTTCTCTCCTT         | 115               |
|                                                                   |                | Liver                    | 5'AGCAGGATGGTTCTGGTCAA<br>5'AGGTGATGTTCCCATTCAGG          | 207               |
| Ribosomal protein L19 (Rpl19)                                     | NM_031103.1    | Adipose                  | 5'TGCCGGAAGAACACCTTG<br>5'GCAGGATCCTCATCCTTCG             | 121               |
| Actin, beta (Actb)                                                | NM_031144.2    | Muscle                   | 5'CAGGCTGTGTTGTCCCTGTA<br>5'GCTGTGGTGGTGAAGCTGTA          | 203               |

2

1 **Table 2.** Effect of diet and daidzein on plasma metabolites of obese male Wistar rats<sup>1</sup>

|                              | <b>Standard diet</b> |                 | <b>High-fat diet</b> |                           |
|------------------------------|----------------------|-----------------|----------------------|---------------------------|
| <b>Plasma metabolites</b>    | <b>Vehicle</b>       | <b>Daidzein</b> | <b>Vehicle</b>       | <b>Daidzein</b>           |
| <b>Glucose (mg/dl)</b>       | 105.25±8.95          | 163.87±12.16**  | 75±10.89             | 120.57±13.34 <sup>#</sup> |
| <b>Urea (mg/dl)</b>          | 39.50±1.79           | 32.62±2.43*     | 33.86±0.74*          | 26.71±1.87                |
| <b>Creatinine (mg/dl)</b>    | 0.82±0.02            | 0.58±0.02***    | 0.86±0.02            | 0.65±0.04 <sup>###</sup>  |
| <b>Úric Acid (mg/dl)</b>     | 0.57±0.17            | 0.66±0.07       | 0.43±0.07            | 0.60±0.11                 |
| <b>HDL-C (mg/dl)</b>         | 27.37±1.21           | 22.37±1.28*     | 22.71±1.56           | 24.57±1.63                |
| <b>Triglycerides (mg/dl)</b> | 200.75±33.10         | 113.37±11.36*   | 93.50±14.8**         | 91.43±18.02               |
| <b>Cholesterol (mg/dl)</b>   | 92.00±7.36           | 71.62±5.14*     | 81.62±8.71           | 76.14±5.17                |
| <b>ALT (U/L)</b>             | 41.12±3.88           | 36.63±6.65      | 48.12±4.75           | 37.85±2.53                |
| <b>AST (U/L)</b>             | 112.57±14.04         | 77.13±6.41      | 112.38±20.60         | 95.43±30.34               |
| <b>GGT (U/L)</b>             | 6.125±0.74           | 5.75±0.56       | 5.86±0.43            | 5.53±0.67                 |

2 <sup>1</sup> Levels of plasma metabolites in rats fed with STD or HFD and treated with vehicle or  
3 daidzein (50 mg/kg). Values represent the mean ± SEM (8 animals per treated group).  
4 Two-way ANOVA and Bonferroni post test for multiple comparisons: \**P* < 0.05, \*\**P* <  
5 0.01, \*\*\**P* < 0.001 *versus* STD rats treated with vehicle; <sup>#</sup>*P* < 0.05, <sup>###</sup>*P* < 0.001 *versus*  
6 HFD rats treated with vehicle. For abbreviation see list. Diet, treatment and interaction  
7 effects are summarized in Table 3.

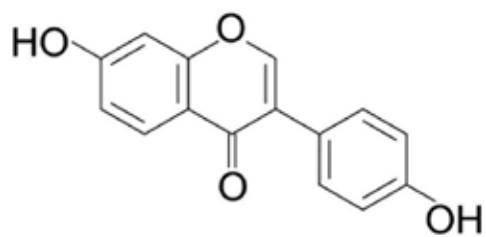
- 1 **Table 3.** Summary of the effects produced by diet, treatment and interaction on each  
2 parameter analyzed<sup>1</sup>.

| Diet effects                              |             |                  |             |
|-------------------------------------------|-------------|------------------|-------------|
| Accumulated food intake                   | *** ↓       |                  |             |
| Accumulated caloric intake                | *** ↑       |                  |             |
| Body weight gain                          | *** ↑       |                  |             |
| Acute treatment effects                   |             |                  |             |
|                                           | STD         | HFD              |             |
| 5 mg/kg daidzein                          | * ↓         | ** ↑             |             |
| 50 mg/kg daidzein                         | ** ↓        | *** ↓            |             |
| Subchronic treatment effects (50 mg/kg)   |             |                  |             |
|                                           | STD         | HFD              |             |
| Accumulated food intake                   | *** ↓       | *** ↓            |             |
| Accumulated caloric intake                | *** ↓       | *** ↓            |             |
| Body weight gain                          | *** ↓       | *** ↓            |             |
| METABOLITES IN PLASMA                     | Diet effect | Treatment effect | Interaction |
| Glucose                                   | ** ↓        | *** ↑            | ns          |
| Urea                                      | ** ↓        | ** ↓             | ns          |
| Creatinine                                | * ↑         | *** ↓            | ns          |
| Uric acid                                 | ns          | ns               | ns          |
| HDL-C                                     | ns          | ns               | *           |
| Triglycerides                             | ** ↓        | * ↓              | ns          |
| Cholesterol                               | ns          | * ↓              | ns          |
| ALT                                       | ns          | ns               | ns          |
| AST                                       | ns          | ns               | ns          |
| GGT                                       | ns          | ns               | ns          |
| FAT CONTENT                               | Diet effect | Treatment effect | Interaction |
| Fat in skeletal muscle                    | ns          | ns               | ns          |
| Fat in liver                              | ** ↑        | ns               | ns          |
| Oil red O in liver                        | ** ↑        | ns               | **          |
| HORMONES                                  | Diet effect | Treatment effect | Interaction |
| Insulin                                   | ns          | ns               | ns          |
| Adiponectin                               | ns          | *** ↓            | ns          |
| Leptin                                    | ns          | ** ↑             | ns          |
| WHITE ADIPOSE TISSUE<br>(gene expression) | Diet effect | Treatment effect | Interaction |
| CB1R                                      | *** ↑       | ns               | ns          |
| CB2R                                      | * ↑         | ns               | ns          |
| PPARα                                     | * ↓         | ns               | ns          |
| PPARγ                                     | ns          | ns               | ns          |
| Adiponectin                               | ns          | ns               | *           |
| Leptin                                    | *** ↑       | * ↓              | ***         |
| ACOX                                      | *** ↑       | ns               | ***         |
| SCD1                                      | ns          | ns               | ns          |
| ACC                                       | *** ↓       | ns               | *           |
| FAS                                       | *** ↓       | ns               | ns          |
| SKELETAL MUSCLE                           | Diet effect | Treatment effect | Interaction |

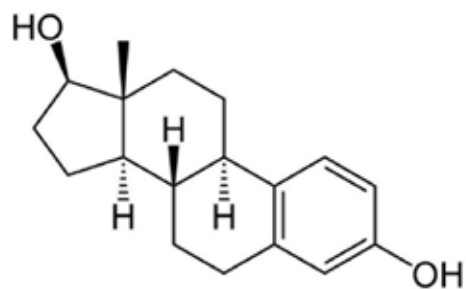
| (gene expression)                            |             |                  |             |
|----------------------------------------------|-------------|------------------|-------------|
| CB1R                                         | ns          | ns               | ns          |
| CB2R                                         | ** ↓        | ns               | ns          |
| PPAR $\alpha$                                | ns          | ns               | **          |
| PPAR $\gamma$                                | * ↑         | ns               | **          |
| AdipoR1                                      | ns          | ns               | ns          |
| ACOX                                         | ns          | ns               | *           |
| GHR                                          | ns          | ns               | *           |
| LIVER<br>(gene expression)                   | Diet effect | Treatment effect | Interaction |
| CB1R                                         | ns          | ns               | ns          |
| CB2R                                         | * ↓         | ns               | ns          |
| PPAR $\alpha$                                | ** ↑        | * ↓              | ns          |
| PPAR $\gamma$                                | ** ↑        | * ↓              | *           |
| AdipoR2                                      | ns          | ns               | ns          |
| ACOX                                         | * ↑         | ns               | *           |
| GHR                                          | ns          | ns               | ns          |
| SCD1                                         | *** ↓       | * ↓              | *           |
| FABP1                                        | ns          | ns               | ns          |
| FAS                                          | *** ↓       | ns               | **          |
| LIVER<br>(protein expression)                | Diet effect | Treatment effect | Interaction |
| PPAR $\alpha$                                | ** ↑        | * ↓              | ns          |
| SCD1                                         | *** ↓       | *** ↓            | ns          |
| FAS                                          | *** ↓       | ns               | ns          |
| BROWN ADIPOSE TISSUE<br>(protein expression) | Diet effect | Treatment effect | Interaction |
| UCP1                                         | ns          | * ↑              | ns          |

- <sup>1</sup>Diet effect (STD vs HFD), treatment effect (vehicle vs daidzein) and the interactions
- between both factors (diet vs treatment), analyzed by two-way ANOVA and Bonferroni
- post-test: ns means no statistical significance, \* $P < 0.05$ ; \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .
- Arrows indicate the direction of the effect. For abbreviation see list.

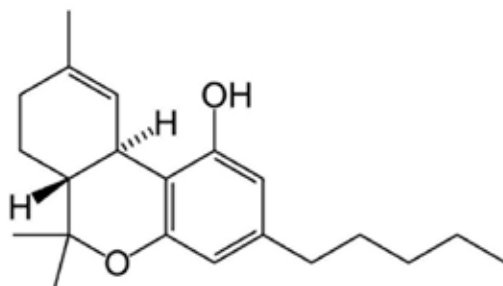
Daidzein



Estradiol



Delta-9-tetrahydrocannabinol



AM251

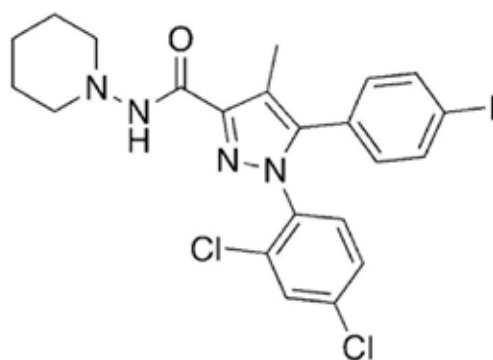


Figure 1.tif

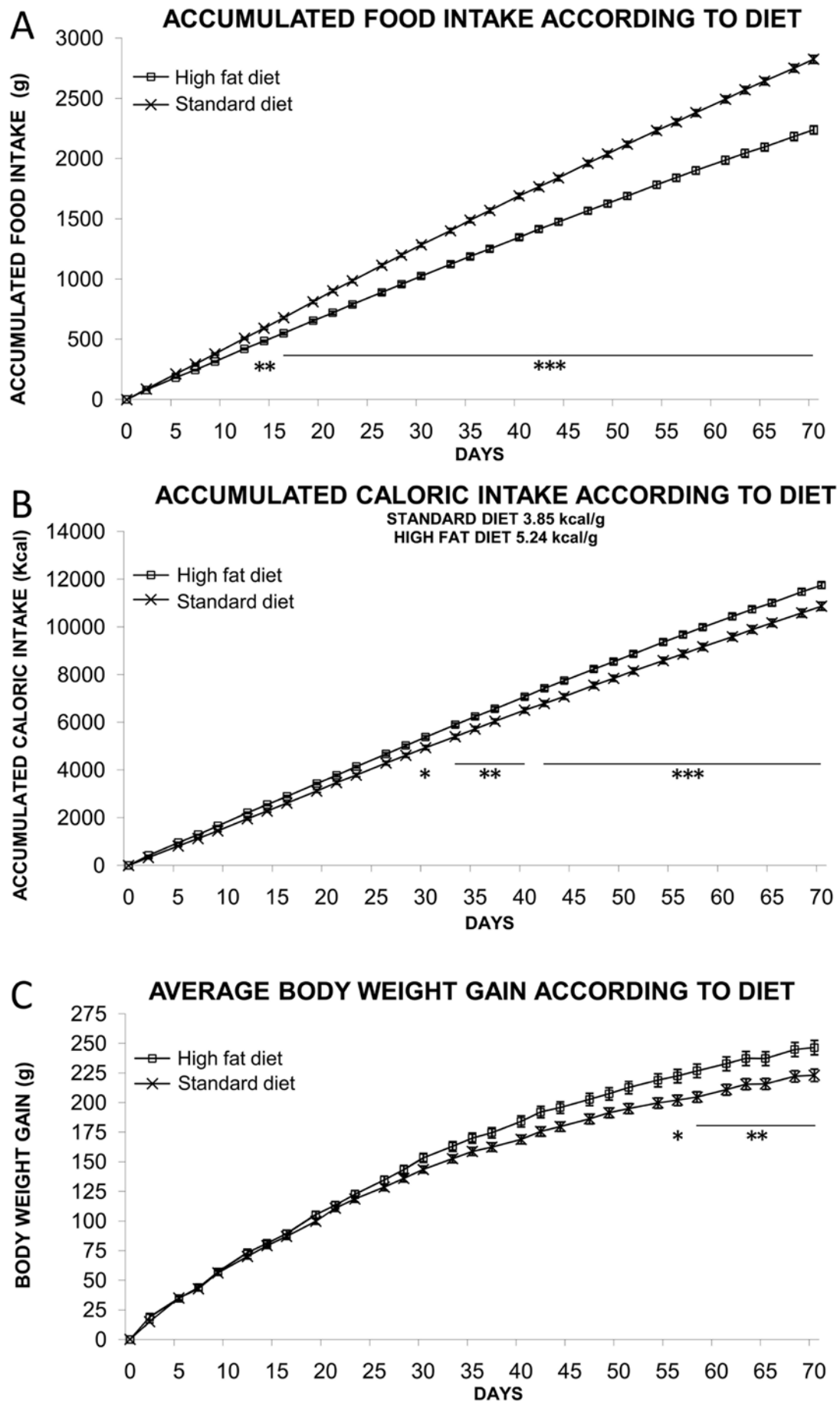
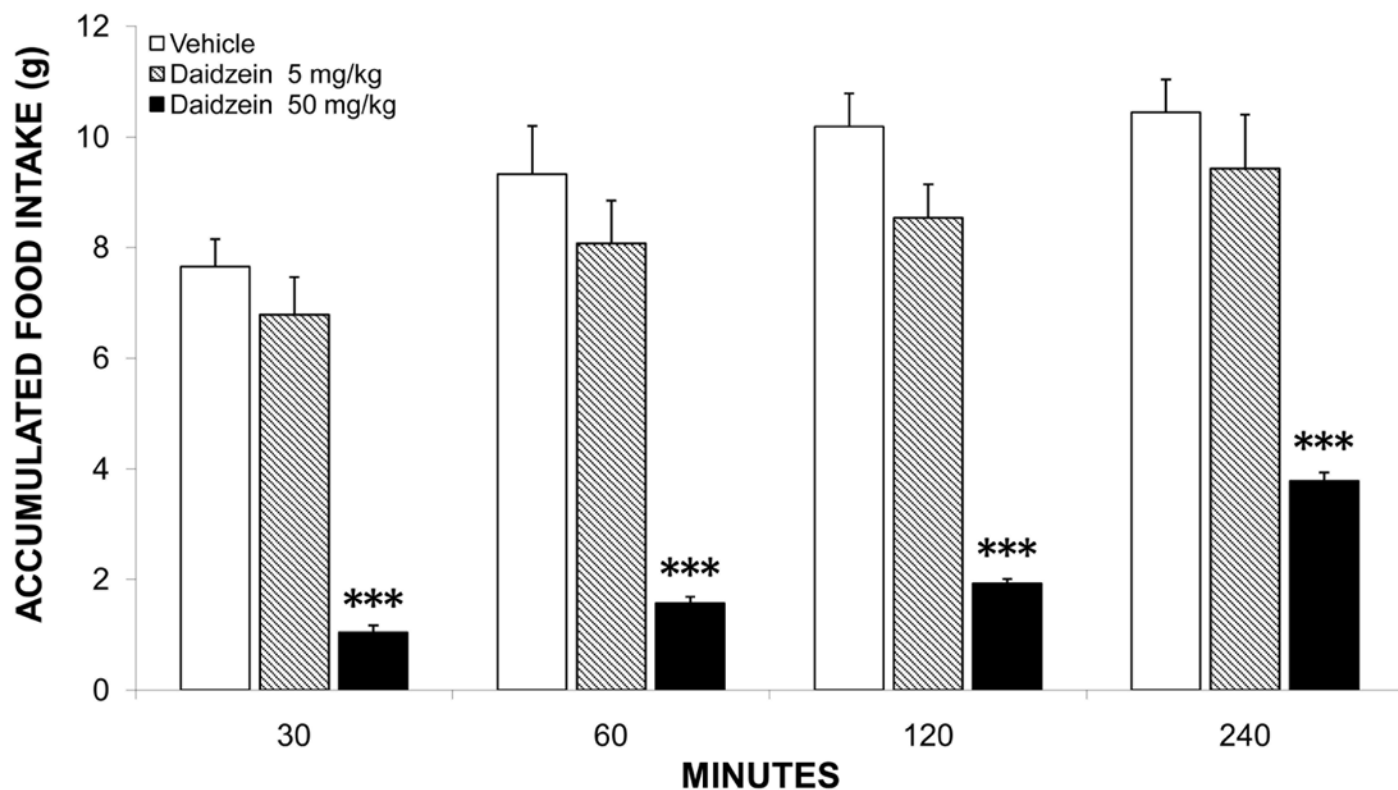


Figure 2.tif

## A ACCUMULATED STD INTAKE ACCORDING TO ACUTE TREATMENT



## B ACCUMULATED HFD INTAKE ACCORDING TO ACUTE TREATMENT

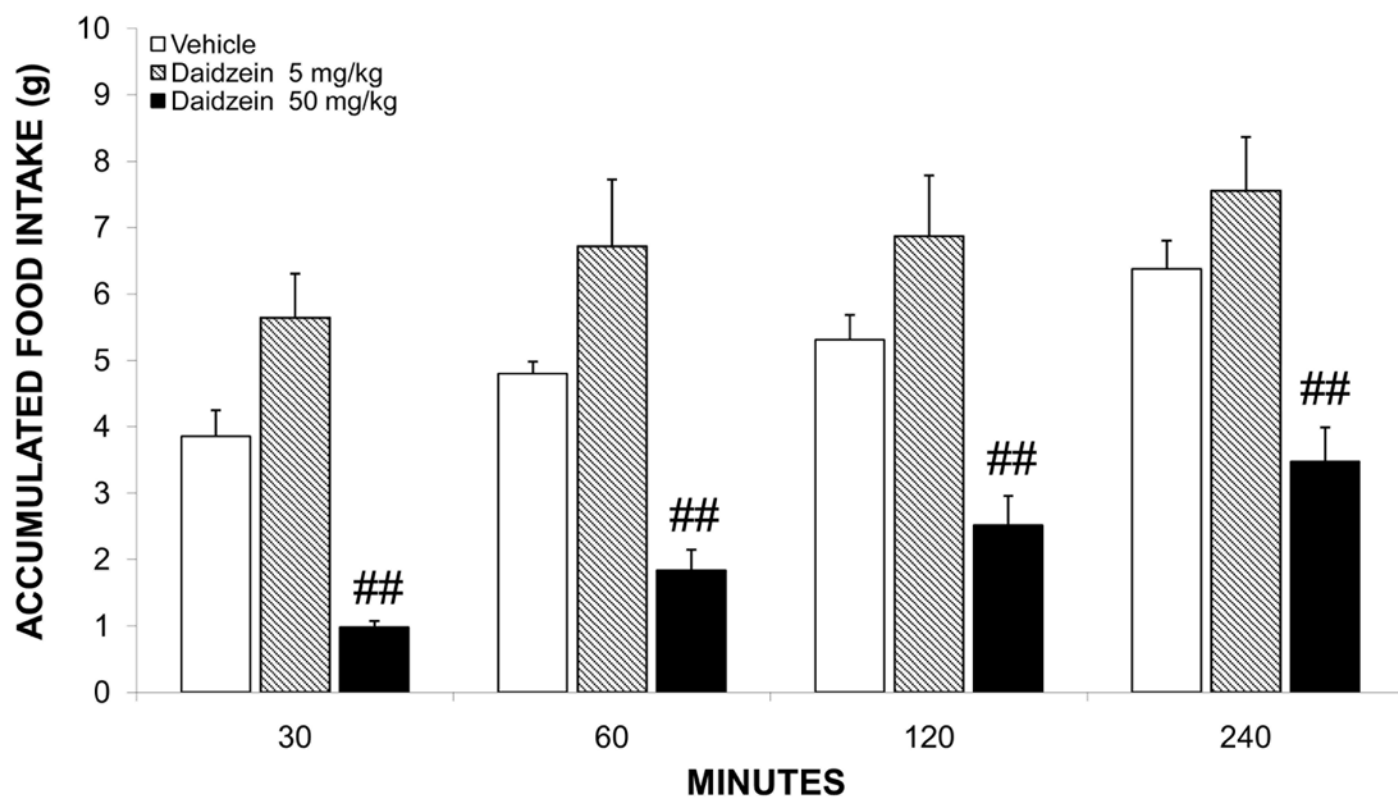


Figure 3.tif

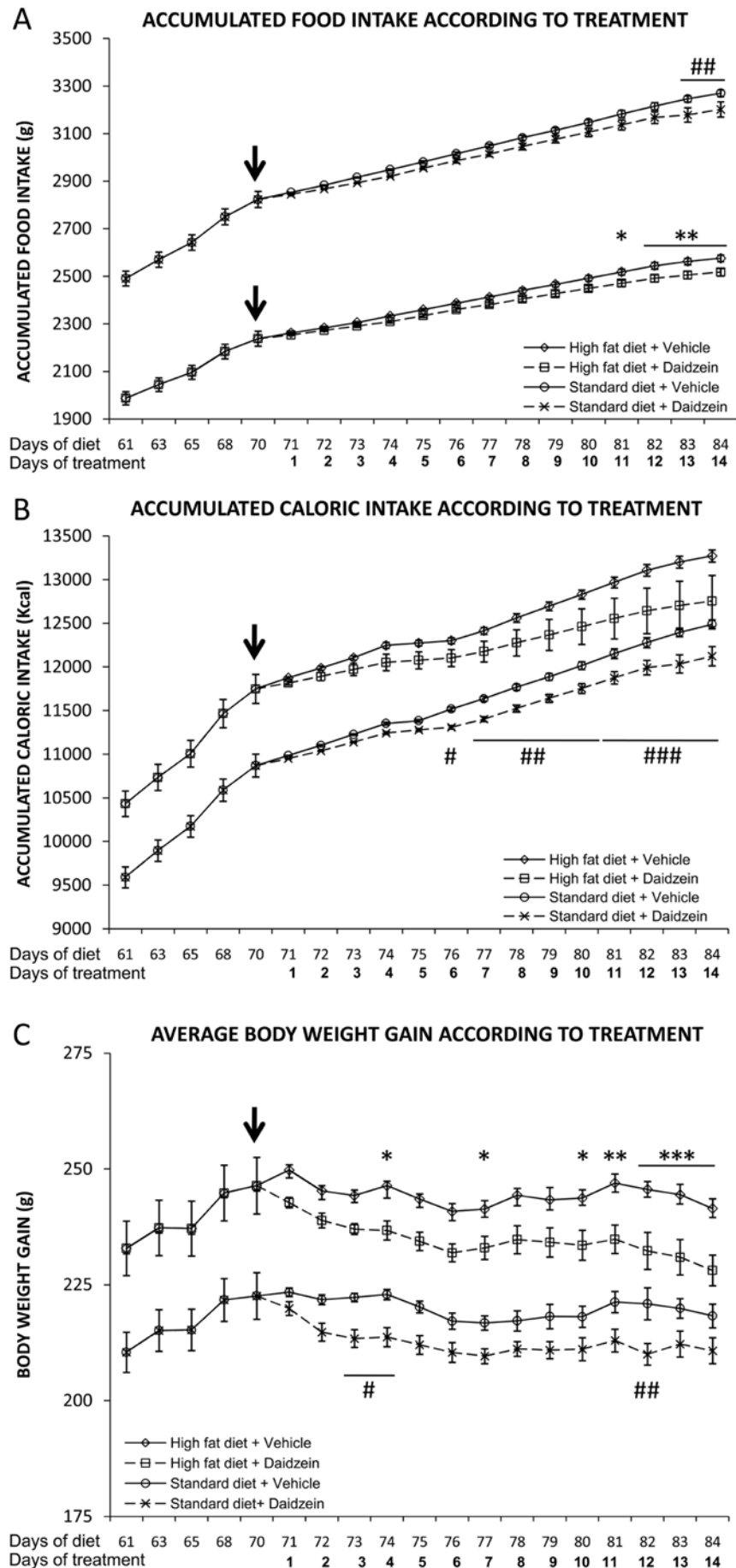
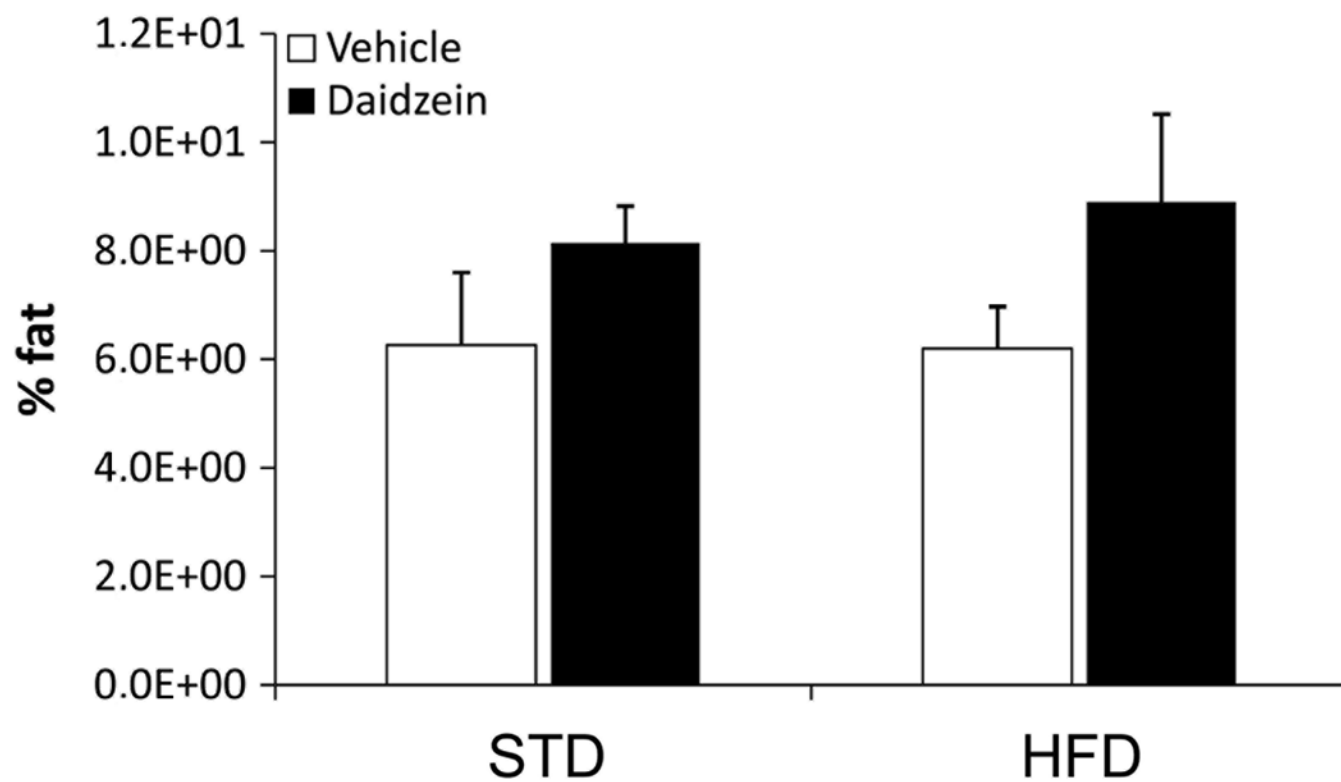


Figure 4.tif

## A Fat in skeletal muscle



## B Fat in liver

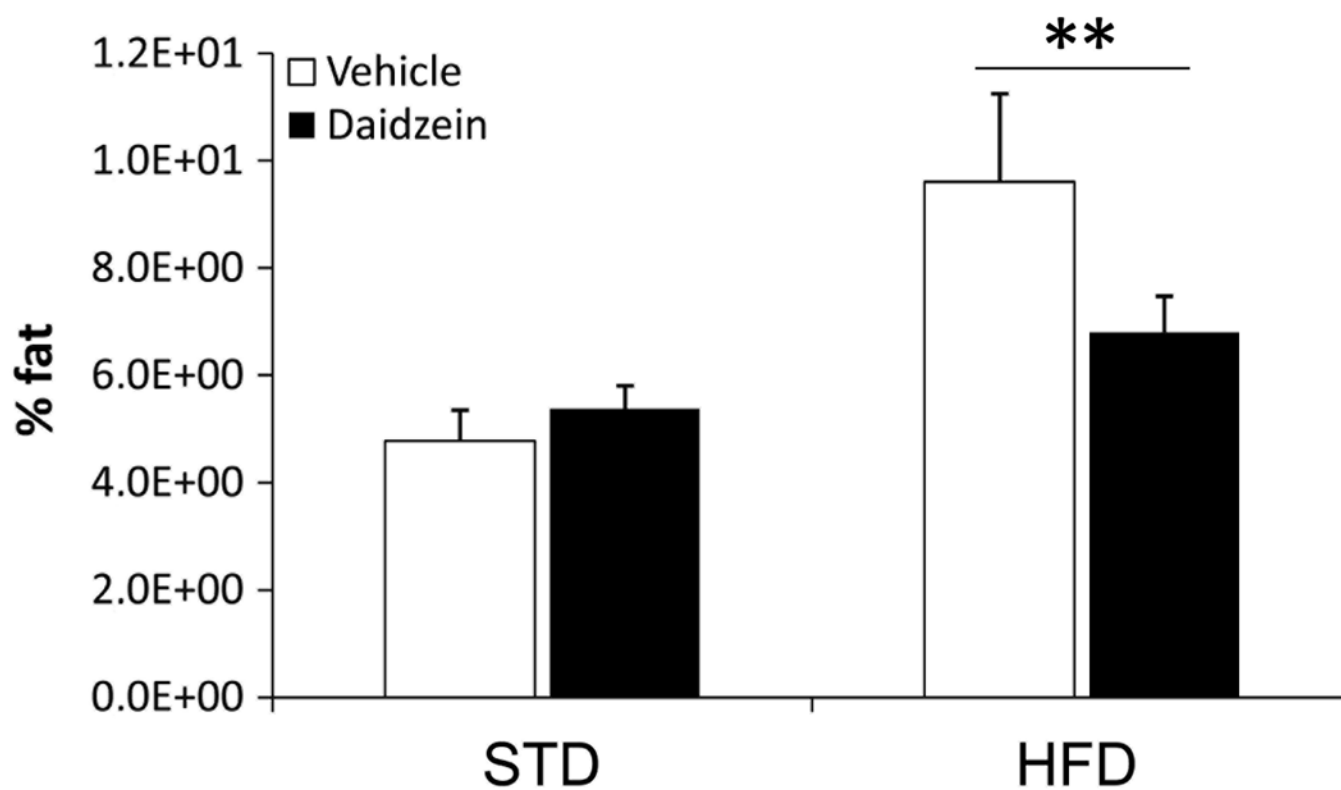


Figure 5.tif

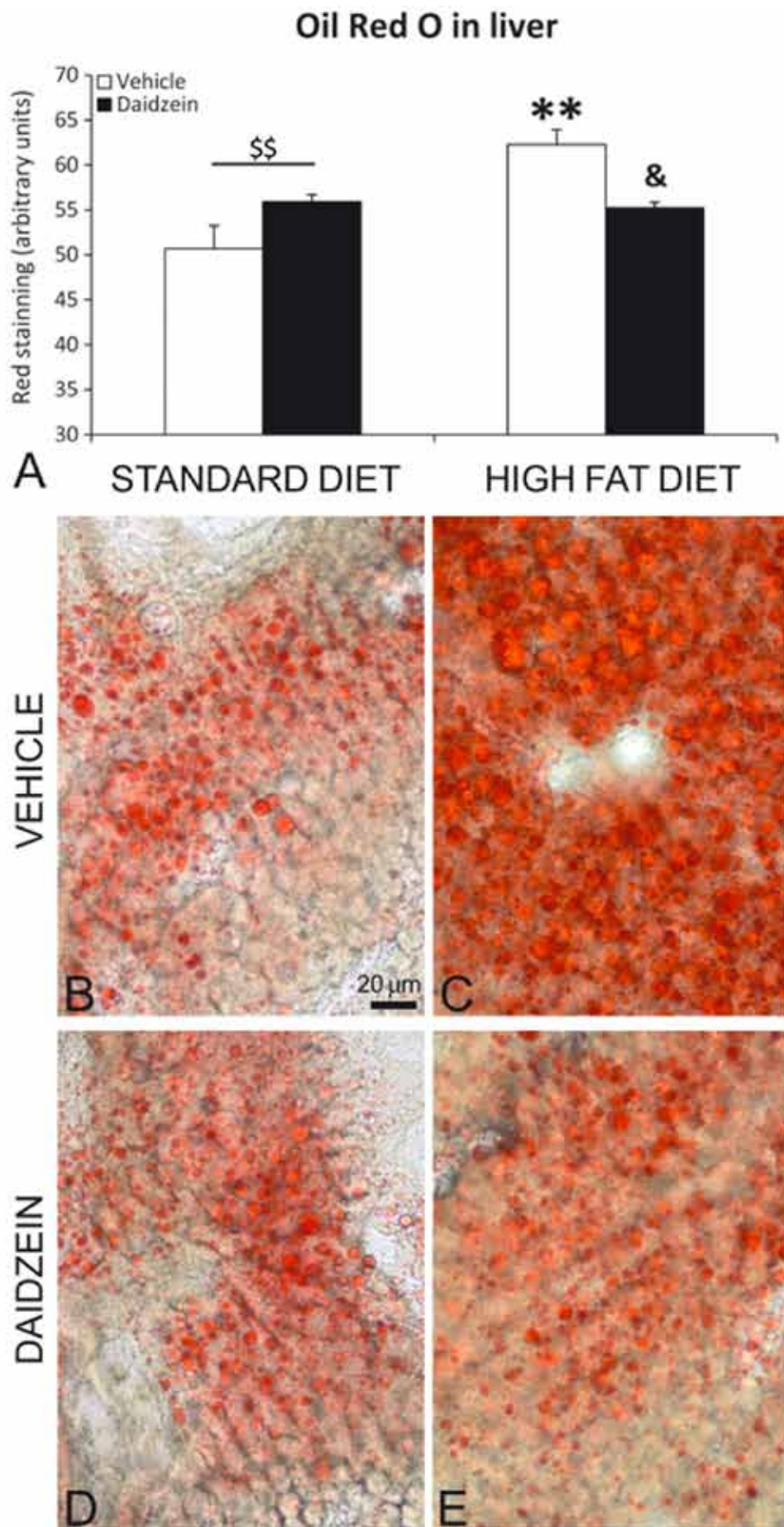


Figure 6.tif

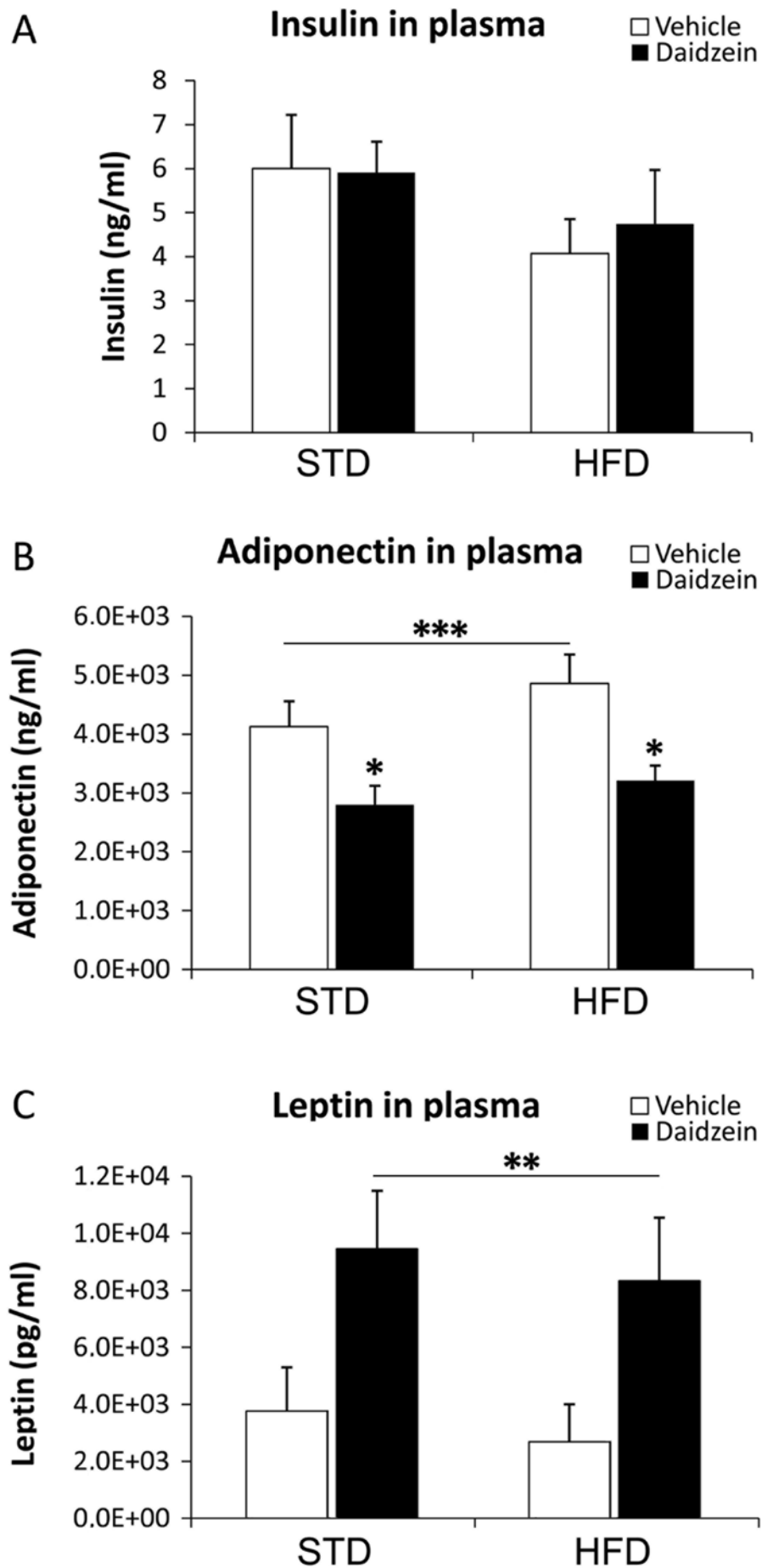


Figure 7.tif

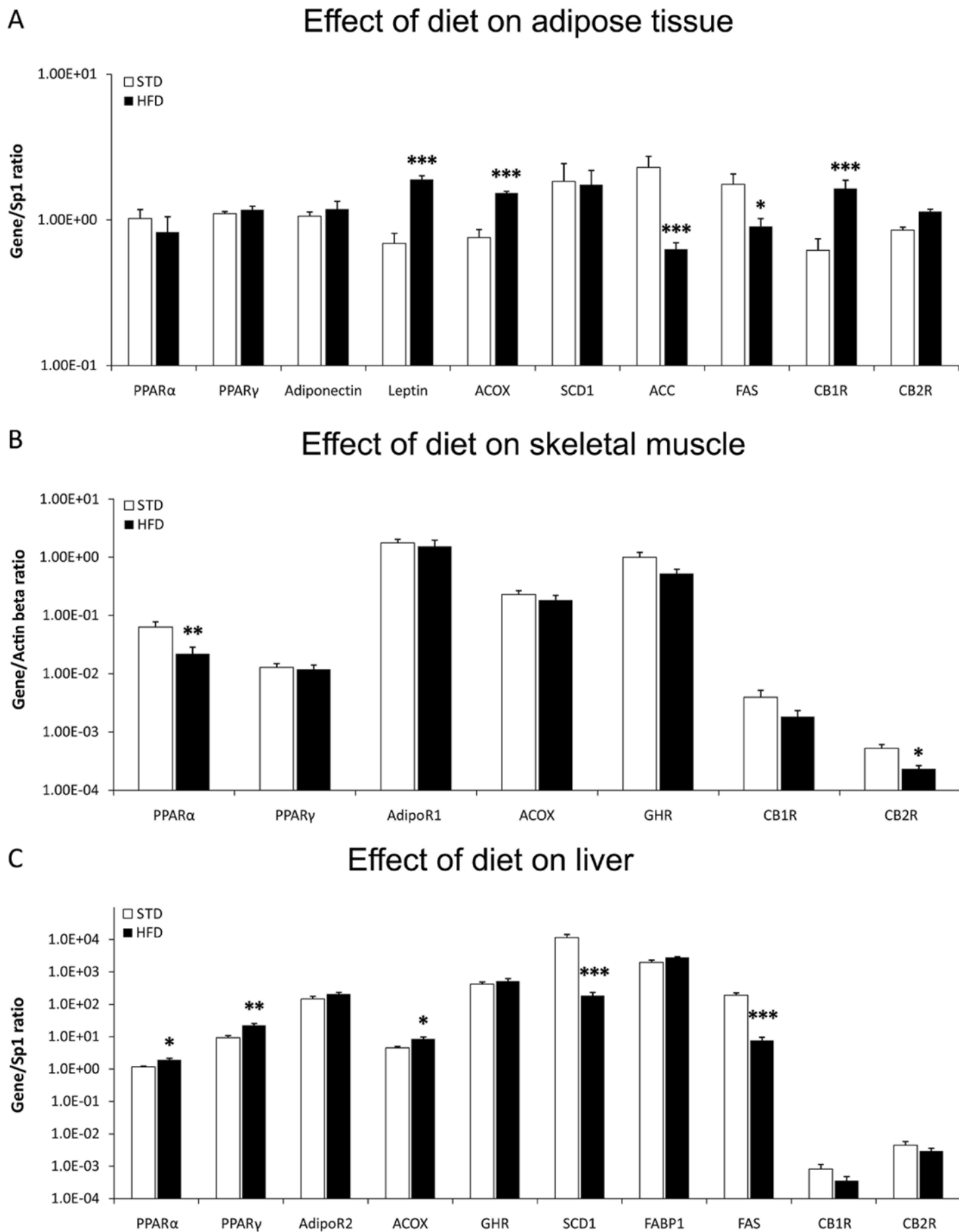


Figure 8.tif

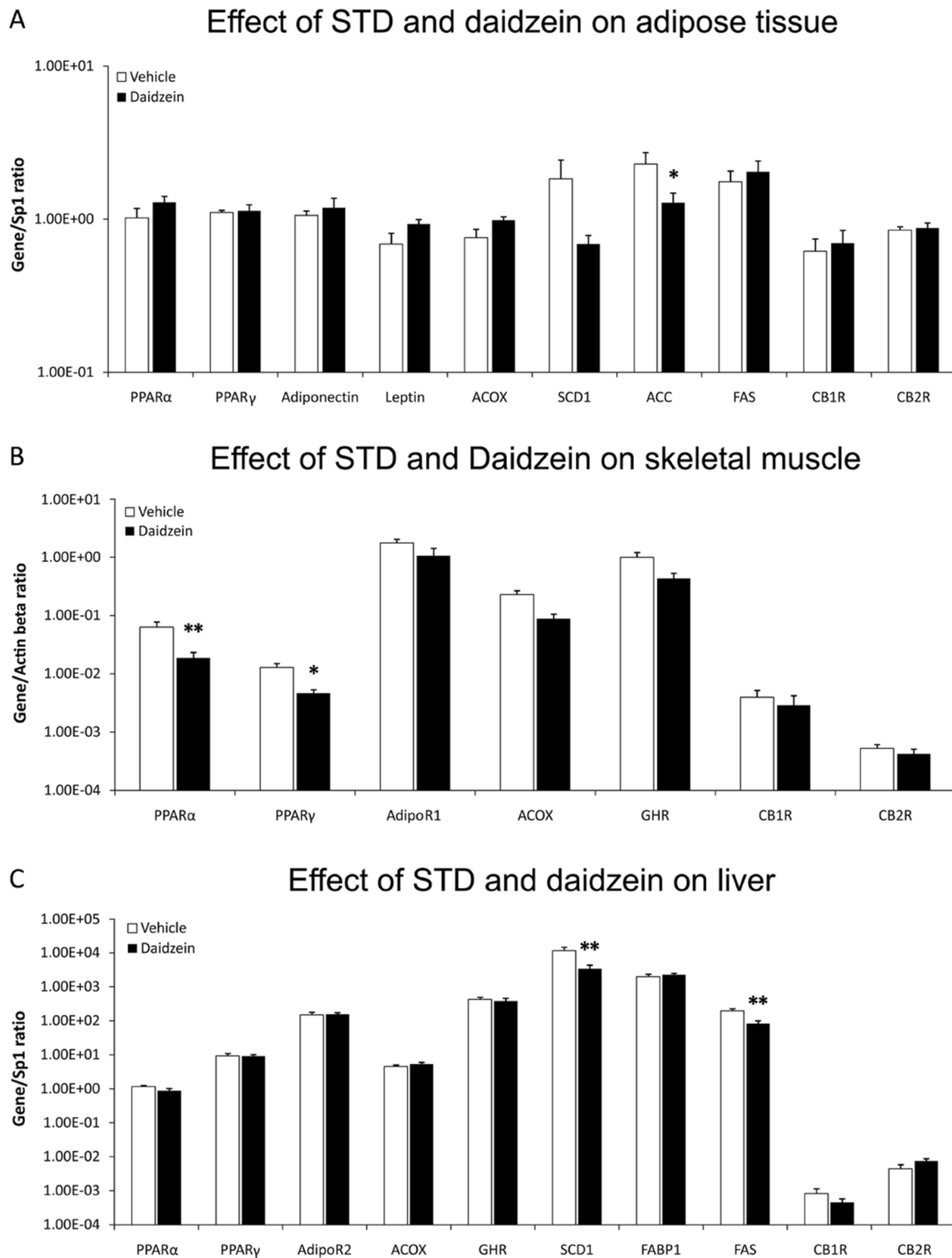
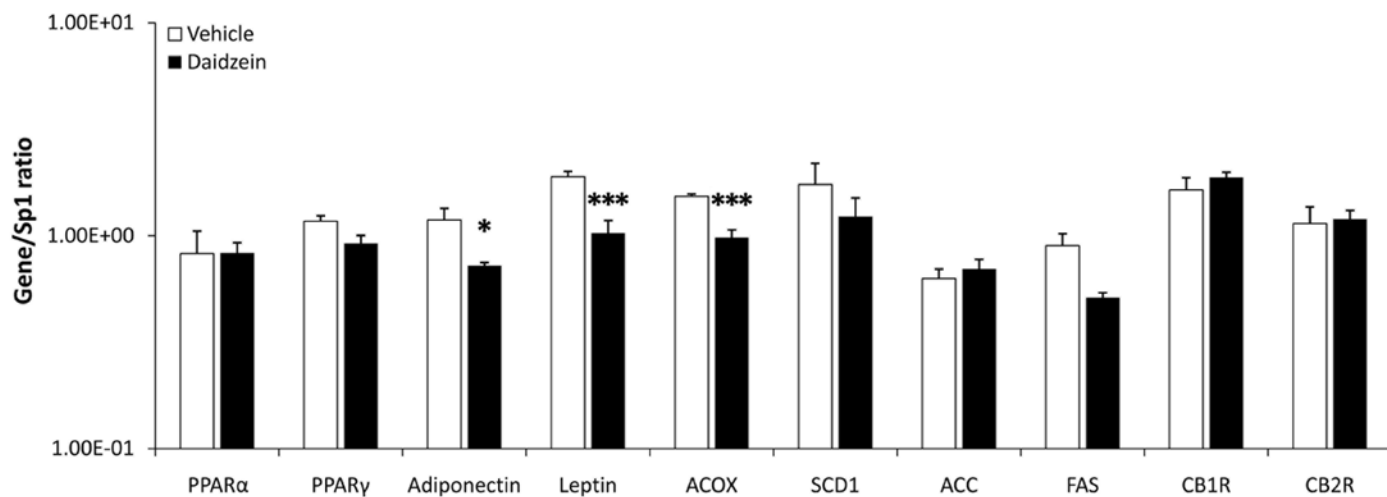
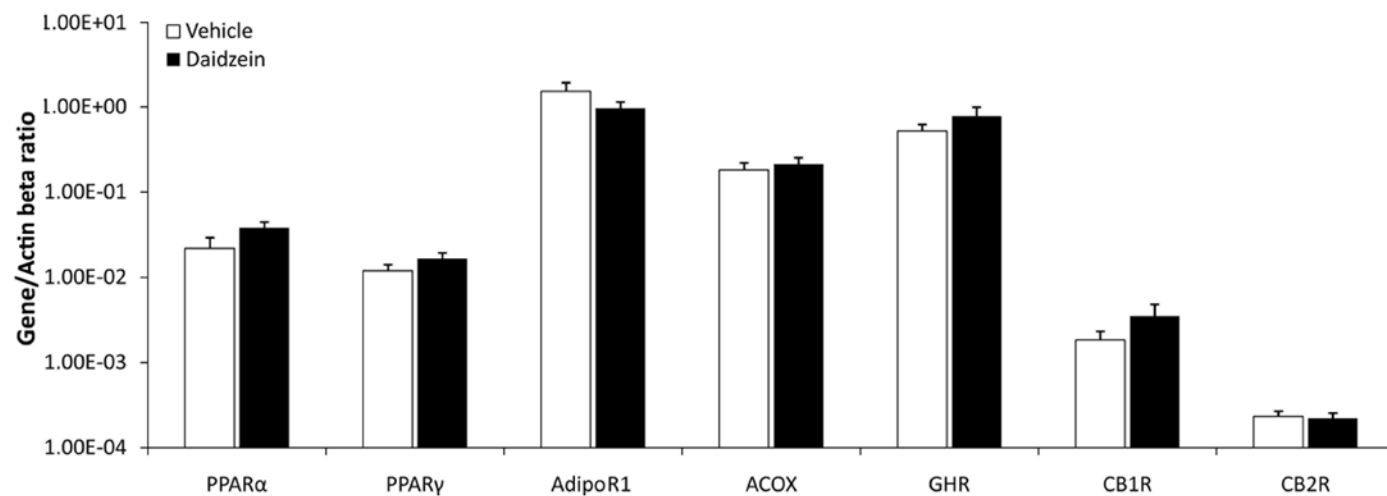


Figure 9.tif

## A Effect of HFD and daidzein on adipose tissue



## B Effect of HFD and daidzein on skeletal muscle



## C Effect of HFD and daidzein on liver

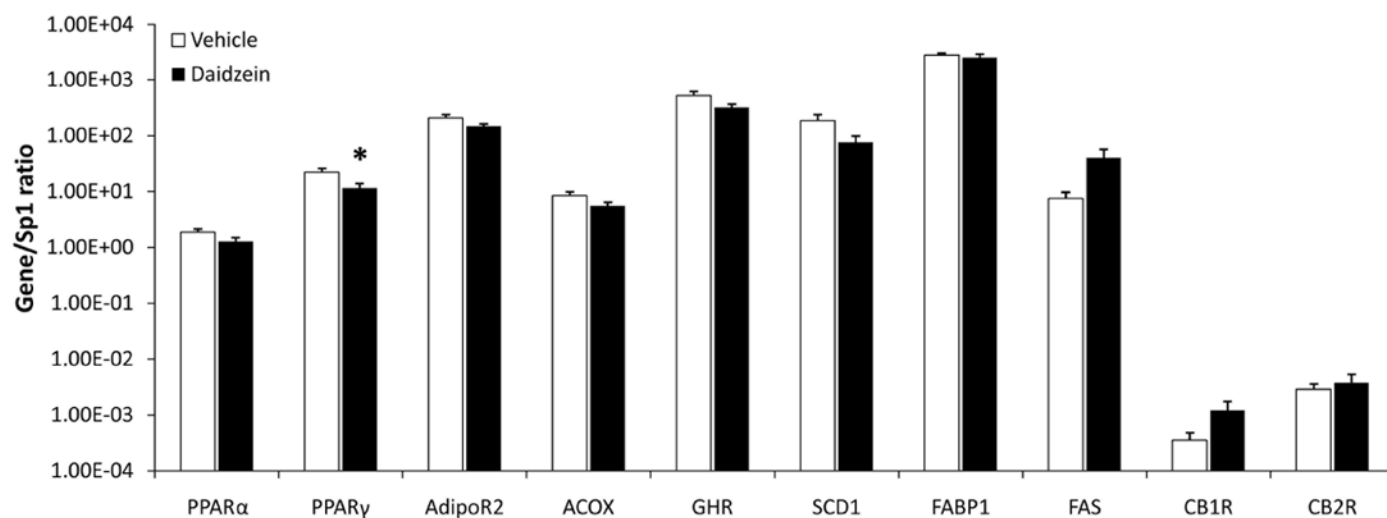


Figure 10.tif

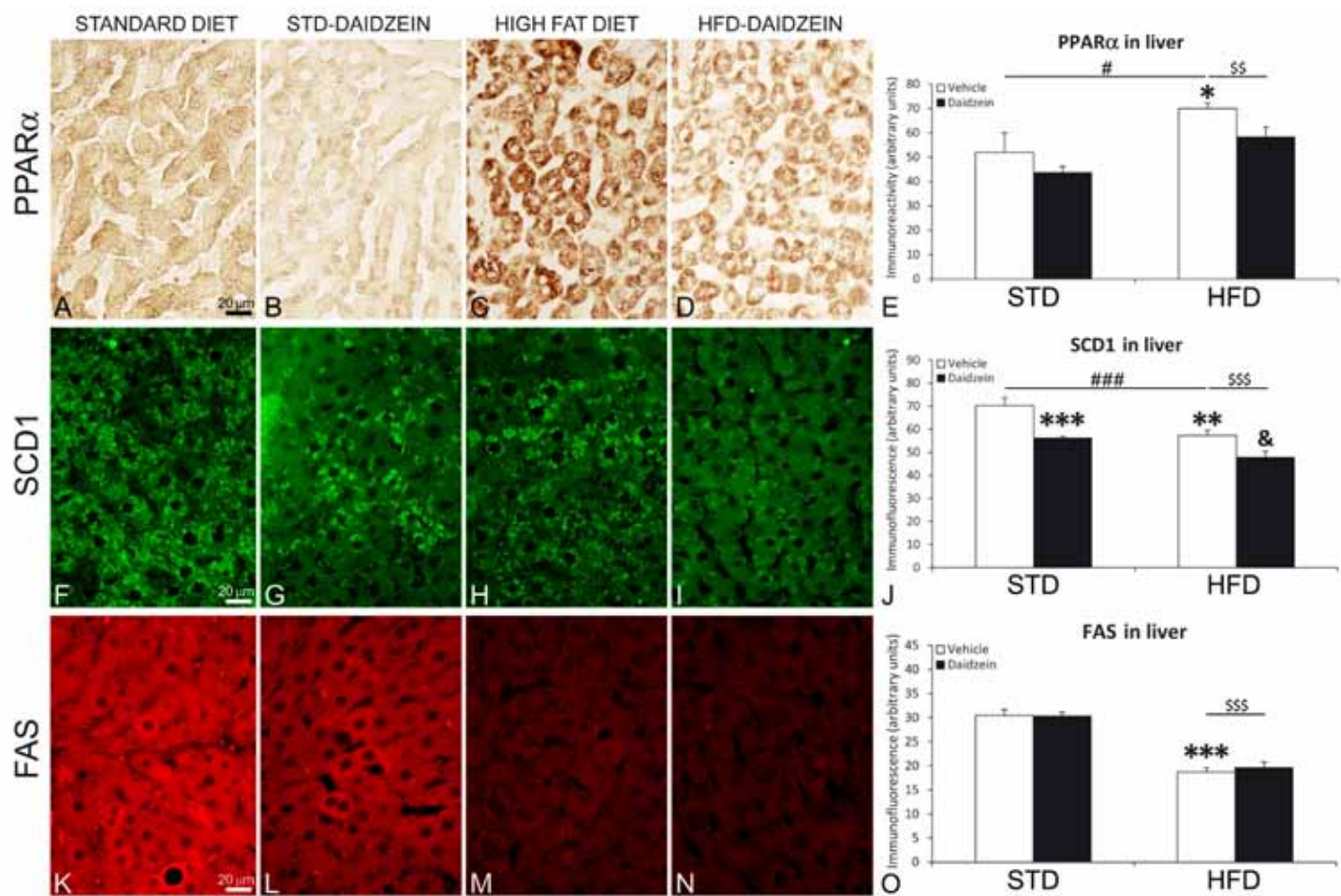


Figure 11.tif

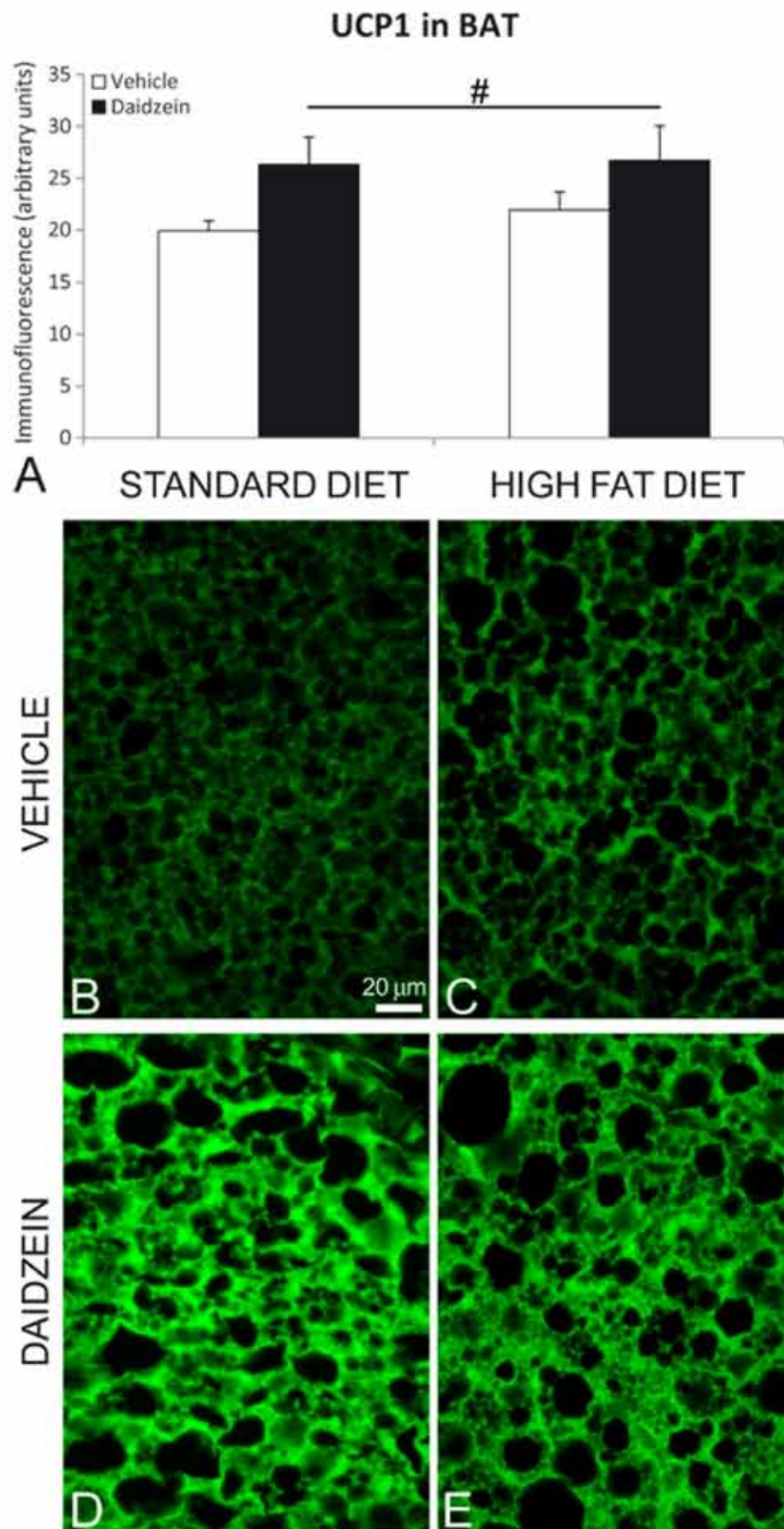


Figure 12.tif