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Weight loss enhances hepatic antioxidant status in a NAFLD model induced by high fat diet

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ABSTRACT

Purpose. Nonalcoholic fatty liver disease (NAFLD) is a benign condition that can progress to more severe liver damage in a process mediated, in part, by disturbances in redox balance. Additionally, some argue that it is set to become the main cause of end-stage liver disease in the near future. Here, we investigated whether a diet-induced weight loss is able to reverse hepatic lipid accumulation and to reduce oxidative stress in liver from C57BL/6 mice fed a high-fat (HF) diet.

Methods and results. Male C57BL/6 mice were divided into four groups: SC (standard chow, 10% energy from fat, 16 wk); HF (high fat diet, 50% energy from fat, 16 wk); SC-HF (SC 8 wk followed by HF 8 wk); and HF-SC (HF 8 wk followed by SC 8 wk). The HF diet during 8 (SC-HF) and 16 weeks (HF) downregulated mRNA levels and protein expression of Nrf2 and endogenous antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) in the liver, caused liver steatosis, affected liver function markers, increased intra-abdominal and subcutaneous adipose tissue, and induced glucose intolerance and hypercholesterolemia compared to controls (SC). Diet-induced weight loss significantly reduced the intrahepatic lipid accumulation, improved glucose tolerance, and restored both gene and protein expression of the antioxidant enzymes.

Conclusion. Our findings suggest that a dietary intervention aimed to induce weight loss may exert protective effects in NAFLD as it can reduce hepatic oxidative stress and intrahepatic lipid accumulation, which can hinder the progression of this condition to more severe states.

Keywords: fatty liver, obesity, oxidative stress, weight loss, diet

INTRODUCTION

The rising prevalence of obesity has led to the development of non-traditional deleterious health issues, and this includes non-alcoholic fatty liver disease (NAFLD), which is now the most common liver disorder in the Western world (Bellentani 2017). NAFLD is characterized by excessive lipid deposition in the hepatocyte, which in a subset of patients evolves to an inflammatory condition with hepatocyte damage (non-alcoholic steatohepatitis, NASH) that can further progress to end-stage liver disease (Chalasani et al. 2012). In addition, NAFLD is intrinsically related to hepatic insulin resistance and abnormal glucose metabolism, but it is still debated if as a cause or as a consequence (Serviddio et al. 2013). Thus, it is imperative to improve the understanding of its causative factors and therapeutic options.

Obesity may lead to NAFLD by two major pathways: (1) increased hepatic inflow of free fatty acids; and (2) in the presence of insulin resistance, there is also an increase intrahepatic synthesis of free fatty acids by *de novo lipogenesis*, as well as reduction in β -oxidation and VLDL assembly, resulting in increased triglycerides accumulation (Dietrich and Hellerbrand 2014). However, it is important to highlight that free fatty acids are also capable of causing insulin resistance through serine-kinase activation (Buzzetti et al. 2016), thus perpetuating the deleterious insult. NAFLD progression to more severe states is a complex phenomenon in which different events may be occurring in parallel in a genetically susceptible individual. Increased oxidative stress is one of these multiple factors that can contribute to NASH, as it may trigger the activation of inflammatory, apoptotic and fibrotic pathways (Buzzetti et al. 2016). Excessive lipid accumulation in the hepatocyte, as well as inflammatory cytokines released from adipose tissue or endotoxin derived from the gut, may trigger endoplasmic reticulum stress, mitochondrial dysfunction and lysosomal permeability, leading to increased reactive oxygen species (ROS) production (Feldstein and Bailey 2011). This seems to occur in a setting of reduced activity of endogenous antioxidant defences

(Videla et al. 2004; Feng et al. 2017), even though divergent findings have been reported in both humans (Perlemuter et al. 2005) and rodents (de Assis et al. 2012). In addition, it is not known whether their expression levels are altered in NAFLD. But, regardless of the expression and/or activity of antioxidant enzymes, it has been consistently shown that oxidative stress and then damage to DNA, lipids, and proteins ensues in NAFLD. Lipid peroxidation by products are extremely reactive and may lead to activation of stellate cells and a proinflammatory response from Kupffer cells (Lee et al. 1995). Both factors, in turn, contribute to the perpetuation of the inflammatory process and fibrosis development.

To date, changes in lifestyle are considered the mainstay of the management of NAFLD. Weight loss, induced by dietary restriction or increased physical activity, usually results in a reduction in hepatic steatosis (strength 1, level of evidence A, according to the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology position statement) (Chalasani et al. 2012). In addition, diet- or surgery- induced weight loss has been shown to reduce systemic (Razavi Zade et al. 2016) and hepatic (Bell et al. 2010) lipid peroxidation markers. To our knowledge, it is not known, however, whether diet-induced weight loss may improve liver redox state and the complications associated with an excess of intrahepatic lipid accumulation.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Ethics Committee for the Care and Use of Experimental Animals of the State University of Rio de Janeiro (CEA/ 033/2011). Forty male C57BL/6 mice were housed in a room with controlled temperature ($21 \pm 1^{\circ}\text{C}$) and

humidity (60 ± 10 %), with a 12 h light/ dark cycle (lights on: 01:00 AM to 01:00 PM) and free access to food and water.

At three months of age, mice were randomly divided into four groups ($n = 10$, each), according to the diet received: (a) standard chow (SC, 16 weeks of standard chow); (b) high fat (HF, 16 weeks of high fat diet); (c) SC-HF (8 weeks of SC followed by 8 weeks of HF diet); and (d) HF-SC (8 weeks of HF followed by 8 weeks of SC). A detailed diet description is given below.

Experimental diets

Diets were manufactured by PragSoluções (PragSolucoes, SP, Brazil) in accordance with the AIN-93M recommendations (Reeves et al. 1993). The standard chow (SC) was composed of 76% energy from carbohydrates, 14% energy from protein, and 10% energy from lipids (total energy 15 KJ/g). The high-fat diet (HF) was composed of 36% energy from carbohydrates, 14% energy from protein, 50% energy from lipids (43% lard, 7 % soybean oil) (total energy 21 KJ/g). The mineral and vitamin contents were identical in both diets.

Body mass and energy intake

Body mass was measured every week and dietary intake was monitored daily. Food was freely allowed and fresh chow was provided daily. Food intake was determined as the difference between the amount of food supplied (g) and the amount of food left in the grid (g), divided by the number of animals per cage. Energy intake was calculated as the product of food intake (g) and the respective energy content of the diet (KJ/g).

Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was conducted two days before sacrifice. Glucose (1.0 g/kg) was administered by orogastric gavage after a six-hour fasting period. Glycemia was measured with a glucometer (Accu-Chek Performa, Roche Diagnostic, Mannheim, Germany) from blood collected from the tail vein in the fasting state (0 min) and 15, 30, 60 and 120 minutes after glucose administration.

Euthanasia

On the day before euthanasia, animals were deprived of food for 6 hours prior to being anesthetized with thiopental sodium (0.42 mg/g, i.p.) and then blood samples were obtained by cardiac puncture through the right atrium. The blood was collected in tubes containing heparin, which was further centrifuged for plasma obtainment. The liver was dissected and weighed, fixed in Millonig formalin and processed for light microscopy. Liver fragments were also frozen at -80°C for Western blotting, RT-PCR and enzymatic assays. The inguinal, retroperitoneal and epididymal adipose tissue were dissected and weighed to calculate the index of adiposity.

Adiposity index

The inguinal fat pad, located between the lower part of the rib cage and the mid-thigh, was considered subcutaneous fat. Retroperitoneal fat (connected to the posterior abdominal wall near the kidneys and the abdominal portion of the ureters) and epididymal fat (located in the lower part of the abdomen and connected to the epididymis) were considered the intra-abdominal fat pad. The adiposity index was determined as the ratio between the sum of intra-abdominal and subcutaneous adipose tissue divided by total body mass, presented as a percentage.

Biochemical measurements

Total cholesterol and triglyceride serum levels were measured by a colorimetric assay (Bioclin, Belo Horizonte, MG, Brazil). In order to assess liver function, alanine aminotransferase serum levels were measured using an automated chemistry analyzer (Cobas 6000TM, Roche, Switzerland).

Gene expression of Keap1, Nrf2 and antioxidant enzymes

The liver was homogenized under liquid nitrogen using a polytron, and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined using Nanovue (GE Life Sciences) spectroscopy, and 1 µg of RNA was treated with DNase I (Invitrogen). Synthesis of the first strand cDNA was performed using Oligo (dT) primers for mRNA and Superscript III reverse transcriptase (both from Invitrogen). Quantitative real-time PCR (qPCR) was performed using a BioRad CFX96 cycler and SYBR Green mix (Invitrogen). Primers for qPCR were designed using Primer3 online software (Table 1). Beta-actin was used as the endogenous control to normalize expression of the selected genes. The efficiency of qPCR for the target gene and endogenous control were approximately equal and were calculated through a cDNA dilution series. The PCR reactions were conducted as follows: after a pre-denaturation and polymerase activation program (4 min at 95°C), forty-four cycles, each consisting of 95°C for 10s and 60°C for 15s, were performed according to a melting curve program (60 to 95°C with a heating rate of 0.1°C/s). Negative controls consisted of wells in which cDNA was substituted for deionized water. The relative expression ratio of mRNA was calculated by the $2^{-\Delta\Delta C_t}$ equation where $-\Delta C_t$ represents the difference between the number of cycles (C_t) of the target genes and the endogenous control.

Western Blot for superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR)

Liver fragments (100 mg) were added to a lysis buffer containing protease inhibitors, homogenized and centrifuged (4500 rpm during 20 minutes at 4 °C). Protein concentration was then determined using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). After denaturation, proteins were separated by electrophoresis on a polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane. Membranes were then blotted with primary antibodies for SOD, catalase, glutathione peroxidase (GPx) and glutathione reductase (GR), followed by incubation with proper horseradish peroxidase-conjugated secondary antibody. Bands were detected by chemiluminescence (ECL Prime, Amersham, UK) using the ChemiDoc system (Bio-Rad, Hercules, CA, USA). The intensity of the bands was quantified using ImageJ software (NIH, imagej.nih.gov/ij, USA). The expression of the structural protein β -actin was used to correct the blot data. Both primary and secondary antibodies were purchased from SantaCruz Biotechnology, CA, USA.

Antioxidant enzyme activity assays

SOD, catalase and GPx activity were determined in liver homogenate by spectrophotometry (Genesys™ 10S UV-Vis Spectrophotometer, Thermo Scientific, CA, USA). Catalase activity was measured by the rate of decrease in hydrogen peroxide concentration (Aebi 1984). SOD activity was assayed by measuring adrenochrome concentration which results from adrenaline autoxidation (Sun and Zigman 1978). GPx activity was measured by monitoring the oxidation of NADPH at 340 nm in the presence of hydrogen peroxide (Flohe and Gunzler 1984). The total protein content of each sample was determined by BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

Oxidative damage

Lipid peroxidation was assessed by thiobarbituric acid reactive substances (TBARS) assay, as previously described by Draper and co-workers (1993). In brief, liver samples were mixed with 10% trichloroacetic acid and 0.67% thiobarbituric acid, followed by heating in a boiling water bath for 30 minutes. TBARS levels were determined by absorbance at 532 nm (Genesys™ 10S UV-Vis Spectrophotometer, Thermo Scientific, CA, USA) using 1,1,3,3-tetramethoxy propane as a standard. In addition, we measured 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT) modified proteins by Western Blot, using the same procedures described before in 'Western Blot', except for the use of primary antibody against 4-HNE (1:500, EMD Millipore, CA, USA) or 3-NT (1:1000, Santa Cruz Biotechnology, CA, USA).

Liver structure

The liver was weighed on a digital analytical balance to an accuracy of 0.001 g (Shimadzu, Japan) and its volume was determined by the method of Scherle (1970). Livers were fixed in Millonig formalin, processed by routine histological procedures, and embedded in Paraplast Plus (Sigma-Aldrich, St Louis, MO, USA). The material was sectioned at 5 µm and stained with hematoxylin-eosin. A piece of each of the five lobes of each animal was selected for processing, in order to assure that the organ was analysed as a whole. Digital images were acquired at random using a Leica DMRBE microscope (Leica Microsystems, Wetzlar, Germany) and an Infinity 1-5c camera (Lumenera, Ottawa, ON, Canada). Five random microscopic fields per animal were analyzed. A test system with 36 points (PT) was produced by the web-based program STEPanizer (www.stepanizer.com) and superimposed on photomicrographs. We estimated the volume density of hepatic steatosis (V_v [steatosis]) as: V_v [steatosis]: = P_p [steatosis] / P_t , where P_p is the number of points counted from the test system in the intra-hepatocyte (Tschanz et al. 2011).

Statistical analysis

Data are shown as mean and S.E.M and they were tested for normality and homogeneity of variances. Statistical differences were assessed by a one-way analysis of variance (ANOVA) followed by Holm Sidak post-test. All analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, CA, USA). *P* values less than or equal to 0.05 were accepted as statistically significant.

RESULTS

Body composition and energy intake

Energy intake depended on the type of diet given to the animals, and, as such, it was higher when animals were fed the HF diet. The mean daily energy intake during the first half of the experimental period was significantly higher in HF and HF-SC groups (SC, 46.2 ± 2.6 ; HF, 67.0 ± 2.4 ; SC-HF, 49.6 ± 2.5 ; HF-SC, 56.5 ± 3.8 KJ/animal/day). And, in the second half of the experimental period, in HF and SC-HF groups (SC, 50.6 ± 1.7 ; HF, 66.1 ± 1.7 ; SC-HF, 60.2 ± 2.5 ; HF-SC, 43.1 ± 2.5 KJ/animal/day).

Figure 1 shows the body mass evolution during the whole experimental period. Due to the higher energy density of the diets, animals from both HF and HF-SC groups presented a continuous increase in body mass during the first eight weeks compared to SC and SC-HF groups. From week 8, there was a significant decrease in body mass in HF-SC group due to diet exchange, and at the end of experiment, body mass did not differ between HF-SC and SC groups. On the other side, animals from SC-HF group showed an increase in body mass after week 8, and at the end of experiments, body mass was significantly higher than SC and HF-SC, but significantly lower than HF.

In addition to increased body mass, mice from HF and SC-HF groups presented significantly higher adiposity index, as well as absolute amounts of visceral and subcutaneous fat pads (Table 2).

Biochemical analysis

At the end of the study, fasting glucose levels of the HF group were significantly higher than the other groups. Moreover, mice from HF group presented reduced glucose tolerance, assessed by AUC calculation following an OGTT, compared to SC, SC-HF and HF-SC. Mice from the HF and SC-HF groups had higher total cholesterol levels than the SC and HF-SC groups. In contrast, triglyceride serum levels did not differ among groups (Table 3).

In addition, serum levels of alanine aminotransferase, a marker of liver function, was significantly lower in HF-SC compared to HF (Table 3).

Biomarkers of oxidative stress in liver

Gene expression of Nrf2 was significantly reduced by prolonged HF diet, and upregulated after weight loss. Weight loss restored both gene (Figure 2) and protein expression (Figure 3) of the antioxidant enzymes SOD, catalase, GPx and glutathione reductase in liver homogenate. In addition, weight loss significantly reduced hepatic lipid peroxidation, as evidenced by reducing MDA levels in both SC and HF-SC groups compared to HF and SC-HF groups. However, 4-HNE and 3-NT protein adducts levels did not differ among groups (Figure 4). No significant difference was observed in the activity of SOD, catalase and GPx among groups (Figure 5).

Hepatic steatosis

All groups exhibited some degree of hepatic steatosis, as shown in Figure 5. Compared to SC group, steatosis level was 418 % higher in HF group, 268 % in SC-HF group, and 188 % in HF-SC group (Figure 6).

DISCUSSION

The main findings of the present study were that a dietary intervention aimed to induce weight loss resulted in a significant reduction in hepatic steatosis and in an upregulation of both mRNA and protein expression of the main intracellular antioxidant enzymes. These results are important as they can hinder the progression to more severe liver damage.

Oxidative stress has been postulated to be a central component in the deleterious progression of hepatic steatosis to steatohepatitis and, in some cases, to cirrhosis and liver failure (Spahis et al. 2017). Oxidative stress and damage occur in a setting of increased ROS production, reduced buffer capacity of antioxidant enzymes, or both (Halliwell 2011). Here, we demonstrated that obesity induced intrahepatic lipid oxidative damage and that weight loss was able to reduce it to levels similar to controls. This is a relevant finding since lipid peroxidation products, such as malondialdehyde, are extremely reactive and may activate hepatic stellate cells (Lee et al. 1995). When activated, these cells are the main source of collagen type I, which, in turn, is a major contributor to hepatic fibrosis (Friedman and Arthur 1989). Here, there was a significant reduction in hepatic lipid peroxidation after weight loss, and we believe that this finding may result from two main factors. Firstly, weight loss reversed the downregulation in gene and protein expression of the main intracellular antioxidant enzymes, namely SOD, catalase, GPx and glutathione reductase. These enzymes are important components of endogenous antioxidant defense and, to our knowledge, this is the first evidence demonstrating that their expression is reduced in NAFLD, and that their

expression is restored after weight loss. Of note, there was no significant change in the activity of these enzymes. Divergent findings are reported in the literature regarding the activity of antioxidant enzymes in NAFLD, with some authors demonstrating a decrease, others no change or an increase in the activity of these enzymes (Videla et al. 2004; Perlemuter et al. 2005; de Assis et al. 2012; Feng et al. 2017). The differences might be due to the type and duration of the diet given, as well as the clinical condition of the patient. Therefore, here, changes in redox state were mostly due to the amount of antioxidant enzymes than due to their activity.

NAFLD, in all its spectrum of histological changes - from simple steatosis to cirrhosis, seems to be a particular case that does not meet the expected physiological regulatory response. In a setting of increased levels of pro-oxidants, it would be expected an upward adaptation of the antioxidant defense system, and vice versa. NAFLD and NASH are associated not only with an increase in ROS production, but also with a reduction in the activity of the main intracellular antioxidant enzymes, as well as coenzyme Q10 and glutathione levels (Rolo et al. 2012). For instance, gene expression of SOD, glutathione peroxidase and catalase is significantly decreased in liver from patients with cirrhosis secondary to NASH, but not secondary to hepatitis C infection or primary biliary cirrhosis (Sreekumar et al. 2003). The mechanisms beyond this “inadequate” adaptation are still unknown. One possible explanation may be related to the activation of nuclear factor erythroid 2-related factor 2 (Nrf2), one of the most important transcription factor for the induction of many antioxidant enzymes, including SOD, glutathione peroxidase and catalase (Menegon et al. 2016). At least in obese animals fed a high fat diet, liver mRNA levels of Nrf2 and its downstream targets are reduced (Vomhof-Dekrey and Picklo 2012), which is in accordance with our findings. One important factor is that diet induced suppression of Nrf2 is reversed with a reduction in saturated fat ingestion and weight loss, as we showed in the

present study. Increased activation of Nrf2 *per se* might contribute to the reduction of hepatic steatosis. Beyond its important role in the antioxidant system, Nrf2 possess regulatory function on metabolism (Vomhof-Dekrey and Picklo 2012). It has been demonstrated to decrease lipid synthesis as well to increase energy metabolism, resulting in a diminished hepatic triglycerides storage.

Secondly, it is possible that the dietary intervention resulted in a reduction in ROS production due to reduced intake of saturated fatty acids *per se* and to reduced total fat and energy intake leading to both weight loss and subsequent intrahepatic lipid accumulation. Saturated fatty acids as well as liver steatosis alters intracellular redox state, promoting uncoupling between glycolysis and TCA cycle, and the formation of reactive oxygen intermediates (Serviddio et al. 2013). So, weight loss reduced oxidative stress by diminishing ROS production as well as increasing endogenous antioxidant defenses.

As expected, albeit not less important, weight loss improved glucose tolerance. Indeed, weight loss is considered one of the most effective intervention to reduce both NAFLD and hepatic insulin resistance (Petersen et al. 2005), which has been confirmed in the present study. To date, there are many hypotheses trying to explain the link between these two entities. One hypothesis states that excess intrahepatic lipid accumulation leads to increase diacylglycerol content, which, in turn, translocates PKC isoform ϵ to the membrane where it binds and inhibits the insulin receptor (Perry et al. 2014). A second hypothesis includes oxidative stress as a key mediator. As discussed before, oxidative stress is associated with the deleterious morphological liver changes caused by NAFLD. In addition, increased ROS production by a dysfunctional mitochondria and NADPH oxidase seems to precede insulin resistance development (Anderson et al. 2009) possibly via redox modifications of phosphatases and kinases involved in insulin signaling, such as p38 MAPK (Besse-Patin and Estall 2014). Once again, the normalization of both protein and mRNA expression of the main

antioxidant enzymes in the liver caused by weight loss might have exerted beneficial effects for the prevention of insulin resistance and diabetes mellitus associated with obesity.

In conclusion, our findings emphasize the importance of body mass loss by dietary intervention and provide a framework for understanding the possible mechanistic processes by which NAFLD is developed.

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Conflict of interest

We declare that we have no conflict of interest.

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Table 1. Sequence of primers used in quantitative real time RT-PCR.

Target gene	5'-3'	Primers
β -actin	FW	CTCCGGCATGTGCAA
	RV	CCCACCATCACACCCT
SOD	FW	AACCATCCACTTCGAGCAGA
	RV	GGTCTCCAACATGCCTCTCT
Catalase	FW	ACATGGTCTGGGACTTCTGG
	RV	CAAGTTTTTGTATGCCCTGGT
GPx	FW	GTCCACCGTGTATGCCTTCT
	RV	TCTGCAGATCGTTCATCTCG
GR	FW	CACGACCATGATTCCAGATG
	RV	CAGCATAGACGCCTTTGACA
Keap1	FW	GGACGGCAAACTGATTC
	RV	TCGTCTGATCTGGCTCATA
Nrf2	FW	CACATCCAGACAGACACCAGT
	RV	CTACAAATGGGAATGTCTCTGC

GPx, glutathione peroxidase; GR, glutathione reductase; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor, erythroid derived 2, like 2; SOD, superoxide dismutase

Table 2. Adipose tissue distribution and adiposity index in the different experimental groups.

Variables	SC	HF	SC-HF	HF-SC
Intra-abdominal fat (g)	0.9 ± 0.1	3.0 ± 0.2 ^a	2.6 ± 0.2 ^{a,b}	0.9 ± 0.1 ^{b,c}
Subcutaneous fat (g)	0.30 ± 0.05	1.71 ± 0.09 ^a	0.87 ± 0.07 ^{a,b}	0.34 ± 0.03 ^{b,c}
Adiposity index (%)	3.1 ± 0.3	6.9 ± 0.4 ^a	7.2 ± 0.4 ^a	3.2 ± 0.2 ^{b,c}

Values expressed as mean ± SEM; SC, standard chow; HF, high-fat diet. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC group, [b] vs. HF group, [c] vs. SC-HF.

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Table 3. Biochemical analysis in the different experimental groups.

Variables	SC	HF	SC-HF	HF-SC
Glucose (mmol/L)	9.0 ± 0.7	15.6 ± 0.5 ^a	11.2 ± 0.5	11.6 ± 3.2
OGTT (a.u.c.)	1104 ± 36	1481 ± 112 ^a	1095 ± 28 ^b	956 ± 10 ^b
Total cholesterol (mmol/L)	3.5 ± 0.2	5.8 ± 0.2 ^a	4.6 ± 0.1 ^{a,b}	3.4 ± 0.3 ^{b,c}
Triglycerides (mmol/L)	0.54 ± 0.07	0.62 ± 0.02	0.53 ± 0.10	0.67 ± 0.04
ALT (U/L)	11 ± 2	49 ± 10 ^a	18 ± 4 ^b	13 ± 4 ^b

Values expressed as mean ± SEM; SC, standard chow; HF, high-fat diet. OGTT, oral glucose tolerance test; ALT, alanine aminotransferase; a.u.c, area under the curve. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC group, [b] vs. HF group, [c] vs. SC-HF.

FIGURE CAPTIONS

Figure 1. Body mass evolution during the experimental period. Dashed line indicates diet exchange. Data are presented as mean $\pm$ SEM. SC, standard chow; HF, high-fat. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC group, [b] vs. HF group, [c] vs. SC-HF group.

Figure 2. mRNA expression of Keap1, Nrf2, and the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase in liver. Results are normalized by β -actin, which was used as loading control. Data are shown as mean $\pm$ SEM. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC, [b] vs. HF, [c] vs. SC-HF. HF, high fat; SC, standard chow.

Figure 3. Protein levels of the antioxidant enzymes superoxide dismutase (A), catalase (B), glutathione peroxidase (C), and glutathione reductase (D) in liver. Results are normalized by β -actin, which was used as loading control. Data are shown as mean $\pm$ SEM. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC, [b] vs. HF, [c] vs. SC-HF. HF, high fat; SC, standard chow.

Figure 4. Oxidative stress biomarkers in liver homogenate. (A) malondialdehyde, (B) 4-hydroxynonenal protein adducts, and (C) 3-nitrotyrosine protein adducts. Data are shown as mean $\pm$ SEM. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC, [b] vs. HF, [c] vs. SC-HF. HF, high fat; SC, standard chow.

Figure 5. Activity of the antioxidant enzymes (A) superoxide dismutase, (B) catalase, and (C) glutathione peroxidase in liver homogenate. Data are shown as mean $\pm$ SEM. Significant

differences are represented by letters ($p \leq 0.05$): [a] vs. SC, [b] vs. HF, [c] vs. SC-HF. HF, high fat; SC, standard chow.

Figure 6. Liver steatosis. (A) bar graph of the volume density of the liver steatosis (mean $\pm$ SEM). Photomicrographs of liver tissue stained with hematoxylin-eosin in (B) SC group, (C), HF group, (D) SC-HF group, and (E) HF-HF group (bar = 50 μm , same magnification in all photomicrographs). Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC, [b] vs. HF, [c] vs. SC-HF. HF, high fat; SC, standard chow.

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Figure 1. Body mass evolution during the experimental period. Dashed line indicates diet exchange. Data are presented as mean ± SEM. SC, standard chow; HF, high-fat. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC group, [b] vs. HF group, [c] vs. SC-HF group.

101x62mm (300 x 300 DPI)

Figure 2. mRNA expression of Keap1, Nrf2, and the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase in liver. Results are normalized by β -actin, which was used as loading control. Data are shown as mean $\pm$ SEM. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC, [b] vs. HF, [c] vs. SC-HF. HF, high fat; SC, standard chow.

198x96mm (300 x 300 DPI)

Figure 3. Protein levels of the antioxidant enzymes superoxide dismutase (A), catalase (B), glutathione peroxidase (C), and glutathione reductase (D) in liver. Results are normalized by β -actin, which was used as loading control. Data are shown as mean $\pm$ SEM. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC, [b] vs. HF, [c] vs. SC-HF. HF, high fat; SC, standard chow.

189x187mm (300 x 300 DPI)

Figure 4. Oxidative stress biomarkers in liver homogenate. (A) malondialdehyde, (B) 4-hydroxynonenal protein adducts, and (C) 3-nitrotyrosine protein adducts. Data are shown as mean $\pm$ SEM. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC, [b] vs. HF, [c] vs. SC-HF. HF, high fat; SC, standard chow.

53x15mm (300 x 300 DPI)

Figure 5. Activity of the antioxidant enzymes (A) superoxide dismutase, (B) catalase, and (C) glutathione peroxidase in liver homogenate. Data are shown as mean $\pm$ SEM. Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC, [b] vs. HF, [c] vs. SC-HF. HF, high fat; SC, standard chow.

69x20mm (300 x 300 DPI)

Figure 6. Liver steatosis. (A) bar graph of the volume density of the liver steatosis (mean $\pm$ SEM). Photomicrographs of liver tissue stained with hematoxylin-eosin in (B) SC group, (C), HF group, (D) SC-HF group, and (E) HF-HF group (bar = 50 μ m, same magnification in all photomicrographs). Significant differences are represented by letters ($p \leq 0.05$): [a] vs. SC, [b] vs. HF, [c] vs. SC-HF. HF, high fat; SC, standard chow.