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Aerobic interval exercise improves parameters of Non Alcoholic Fatty Liver Disease (NAFLD) and other alterations of metabolic syndrome in obese Zucker rats.

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Abstract

Metabolic syndrome (MS) is a group of metabolic alterations that increase the susceptibility to cardiovascular disease and type II diabetes. Non Alcoholic Fatty Liver Disease (NAFLD) has been described as the liver manifestation of MS. We aimed to test the beneficial effects of an aerobic interval training (AIT) protocol on different biochemical, microscopic, and functional liver alterations related to the MS in the experimental model of obese Zucker rat. Two groups of lean and obese animals (6 weeks old) followed a protocol of aerobic interval training (4 min at 65-80% of VO_2 max, followed by 3 min at 50-65% of VO_2 max, 45-60 min, 5 days/week, 8 weeks of experimental period), whereas two control groups remained sedentary. Obese rats had higher food intake and body weight ($P < 0.0001$), and suffered significant alterations in plasma lipid profile, area under the curve (AUC) after oral glucose overload ($P < 0.0001$), liver histology and functionality, and antioxidant status. The aerobic interval training protocol assayed ameliorated the severity of alterations related to glucose and lipid metabolism, and increased the liver protein expression of PPAR-γ, as well as the gene expression of Glutathione Peroxidase 4 ($P < 0.001$). The training protocol also showed significant effects on the activity of hepatic antioxidant enzymes, although this action was greatly influenced by rat phenotype. The present data suggest that AIT protocol is a feasible strategy to improve some of the plasma and liver alterations featured by the MS.

Key words: metabolic syndrome, non-alcoholic fatty liver disease, aerobic interval training, aerobic capacity, lipid metabolism, hepatic metabolic pathways, liver antioxidant status

Introduction

Metabolic syndrome (MS) is a cluster of interrelated metabolic conditions which increase the risk of developing cardiovascular disease (Kaur 2014). MS is characterized by central obesity, dyslipidemia, elevated blood pressure, and elevated plasma glucose (Grundy 2005). Patients with MS are also more susceptible to develop type 2 diabetes mellitus (Reaven 2004).

Hepatic morphology and function can be adversely affected by MS leading to the development of Non Alcoholic Fatty Liver Disease (NAFLD) (Marchesini et al. 2003) which is characterized by steatosis, lobular and portal inflammation, hepatocyte ballooning, and fibrosis (Brunt and Tiniakos 2010). Furthermore, this hepatic pathology is now considered as the liver manifestation of MS (Angelico et al. 2005). Although the exact mechanisms leading to it are not yet completely understood, insulin resistance and chronic oxidative stress have been reported to play a major role in liver damage and development of NAFLD (Polyzos et al. 2009; Rolo et al. 2012)

The effects of different types of exercise on MS have been studied. In 2009, Haram et al. (2009) reported that high-intensity aerobic interval training was more effective at reducing cardiovascular disease risk in rats with MS than moderate-intensity continuous training. Aerobic interval exercise has also been described as a feasible and efficient strategy to restore mitochondrial dysfunction in rats after myocardial infarction by inhibiting dynamic pathological remodelling (Jiang et al. 2014). With regard to liver metabolism, several authors have studied the effect of moderate or vigorous intensity exercise on different aspects of NAFLD. Moderate intensity exercise training showed beneficial effects on intrahepatic triglyceride content, although it did not improve hepatic lipoprotein kinetics in obese individuals with NAFLD (Sullivan et al. 2012). On the other hand, vigorous exercise in humans was associated with a decreased adjusted odds of having non-alcoholic steatohepatitis (NASH), whereas doubling the recommended time of vigorous exercise was associated with a decreased adjusted odds of advanced fibrosis (Kistler et al. 2011). Furthermore, Linden et al. (2015) have reported that vigorous-intensity interval exercise training (40 m/min, 15% incline, 6 × 2.5 min bouts/day, 5 days/week treadmill running) was as effective as a longer moderate intensity protocol in lowering hepatic triglycerides, serum alanine aminotransferase (ALT), perivenular fibrosis, and hepatic collagen 1α1 mRNA expression in OLETF rats.

Although a direct relationship has not been established, insufficient aerobic capacity is the basis of several cardiovascular and metabolic diseases (Tjønna et al. 2008). Therefore, an improvement in such capacity could result in health benefits reported for aerobic interval training.

Oxidative stress is responsible for part of the initiation of obesity associated co-morbidities including NAFLD and NASH (Rolo et al. 2012; Tariq et al. 2014). The obese status is characterised by oxidative stress partly caused by insulin resistance and partly by low chronic inflammation (Al Rifai et al. 2015). Conditions in which antioxidant status is altered by prevailing oxidative forces can be reflected in altered activity or expression of liver antioxidant enzymes (Soltys et al. 2001; Videla et al. 2004). The regulation of glucose and lipid metabolism at hepatic level can be significantly affected by metabolic alterations such as those related to the development of NAFLD. Several molecular pathways are involved in glucose and lipid metabolism. There are specific components of the former pathways like 5' AMP-activated protein kinase (AMPK) or Peroxisome Proliferator Activator Receptor (PPAR) that play a key role in their activation. AMPK is a regulator of energy homeostasis that down-regulate the expression of gluconeogenic and lipogenic enzymes (Galisteo et al. 2010; Lochhead et al. 2000) in energetic deficiency situations. PPARs are a family of nuclear transcription factors related to the management of lipogenic and lipolitic pathways in liver and adipose tissue (Souza-Mello 2015). The up-regulation of PPAR-γ isoform has been related to different factors such as AMPK pathway (Sakai et al. 2014) or reactive oxygen species (ROS) generation (Ristow et al. 2009). Animal experimental models are an accepted tool to study the multifactorial effects of exercise on MS associated conditions. In this context, the obese Zucker rat model shares many similarities with humans affected by MS, including obesity, dyslipidaemia, insulin resistance, hepatomegalia, altered antioxidant status, and inflammatory process (Galisteo et al. 2010; Hey-Mogensen et al. 2012). According to Kucera and Cervinkova (2014), this experimental animal model exhibits the initial stages of NAFLD mainly characterized by steatosis, but does not spontaneously progress to stage 2 of the disease. This study aimed therefore: 1) to assess the potentially beneficial effects of AIT protocol on aerobic capacity, glucose and lipid metabolism parameters, liver histology and functionality, and hepatic antioxidant status in an animal experimental model of MS, the obese Zucker rat, that presents hepatic alteration related to early stages of NAFLD, 2) to study the role of AMPK and PPAR-γ in the signaling pathways involved in exercise-derived effects.

Materials and methods

Animals and experimental design

Twenty young male obese (fa/fa) (O) and 20 lean heterozygous (fa/+) (L) Zucker rats (6 weeks old) with an initial mean body weight of 179±2.8 and 148±3.4g, respectively, were allocated to four different experimental groups (two obese and two lean groups, n=10 rats each). Two of the experimental groups (an obese and a lean one) performed aerobic interval exercise according to an established training protocol (OE, LE) while the two remaining groups were considered as sedentary groups (OS, LS). The experiment lasted for 8 weeks, during which the animals were housed in a well ventilated, thermostatically controlled room (21±2°C). A reversed 12:12 light/dark cycle was implemented so the animals would perform the training protocol in darkness. Throughout the trial, animals had free access to type 2 water and consumed the experimental diet (see below) *ad libitum*. Food intake was recorded daily whereas body weight was measured once a week. At the end of experimental period, a glucose tolerance test following the protocol described by Prieto et al. (2004) was performed 24 h after the last training session in order to re-establish the normal physiological conditions altered in response to the energetic demand induced by the aerobic interval exercise. Blood glucose concentration from the animals' tail was recorded at periods 0, 15, 30, 90 and 120 min after the glucose overload ingestion (BREEZE® 2, Bayer), and the area under the curve (AUC) was determined. The animals were allowed to recover for 24 h prior being fasted for a further 8 h, anesthetized with xylazine/ketamine and sacrificed. Blood was collected by puncture of the abdominal aorta (with heparin as anticoagulant) and centrifuged at 1458 × *g* for 15 min to separate plasma that was subsequently frozen in liquid N_2 and stored at -80°C. The liver was extracted, weighed, photographed for macroscopic studies, divided into various portions and immediately frozen in liquid N_2 and stored at -80°C. All experiments were undertaken according to Directional Guides Related to Animal Housing and Care (EUC 2010) and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada, Spain.

Experimental diet

The experimental diet was formulated following the guidelines of the American Institute of Nutrition (AIN-93M; Reeves et al. 1993), in order to meet the nutritional recommendations of adult rats (NRC 1995), with casein (70%) and whey (30%) as protein sources, to reach a 12% protein level. Dietary insoluble fiber was added as cellulose to provide a dietary level of 10%, while 4% of fat was provided as sunflower oil. There was no further addition of saturated fat or cholesterol.

Exercise Protocol

The exercise groups followed a protocol of aerobic interval training five days a week during the eight weeks of the experimental period. The training protocol was performed in a motorized treadmill specially designed for rats (Panlab Treadmills for five rats, LE 8710R), and all sessions were performed during the dark cycle of the animals (active period). This training protocol was designed based on recent studies that have demonstrated that a high intensity interval training (65-80% of VO_2max combined with periods of 50-65% of VO_2max) promotes best results on weight reduction and blood lipid profile (Donnelly et al. 2009). One week before the start of the study, the animals were adapted to the training procedures through a low intensity running protocol every day for 20 min in the treadmill at 18m/min. The running sessions of 1 h started with a 10 min warm up at 40% VO_2max, and consisted of successive 4 min exercise periods at 65-80% of VO_2 max, followed by 3 min recovery periods at 50-65% of VO_2 max. The intensities and length of the training were gradually incremented every week (Table 1). To establish the velocity that would correspond to the VO_2max of each rat, a maximal incremental test was performed at the start of the study. A final incremental test was performed 96 h prior the end of the study to test the maximal aerobic capacity and physical performance achieved by the animals as a result of the intervention. The maximal incremental test was carried out following the protocol described by Clemente et al. (2011) and Wisløff et al. (2001) with slight modifications. This protocol ran by the computer software SeDaCom V2. (Panlab. Harvard apparatus), first measures 5 min ambient air and then air within the treadmill to determine the appropriate ratio VO_2: VCO_2. The test ends when the animal is visibly exhausted and rested on the shock bar for > 5 seconds. Basal and final blood lactate concentrations were measured at the start and at the end of the incremental test in blood obtained from the animals' tail (Lactate Pro, Arkray, The Netherlands).

During the experimental trial, the sedentary groups were subjected to a 15 min of low velocity (15 m/min) training protocol twice a week, to reflect a human sedentary lifestyle (Morris et al. 2007).

Plasma and liver biochemical analysis

Biochemical parameters of glucose and lipid metabolism, and liver function were measured in plasma using a Shenzhen Midray BS-200 Chemistry Analyzer (Bio-Medical Electronics) at the Bioanalysis Unit of the Scientific Instrumentation Centre (Biomedical Research Park, University of Granada). Plasma insulin concentration was quantified using a rat insulin enzyme immunoassay kit (Spibio, Montigny le Bretonneux, France).

A portion of liver was lyophilized in order to determine the percentage of water. Hepatic lipids were extracted from the freeze-dried liver portion using the method described by Folch et al. (1957) with slight modifications (Kapravelou et al. 2013). The extracted lipids were dissolved in 1mL of 96% hexane to measure triglycerides content (Spinreact, S.A., Girona, Spain).

Macroscopic and microscopic liver study

Liver area of the macroscopic image was estimated in all the liver images of the four experimental groups assayed by morphometric study using the software Image Pro Plus 6.0. A portion of liver was fixed in 10% phosphate-buffered formalin, dehydrated in ethanol, embedded in paraffin, and sectioned for histological examination using hematoxylin-eosin (HE), and Masson's trichrome (MT) staining for general microscopy morphology and fibrosis development, respectively. Four different preparations of each staining were analyzed for each animal, and 10 animals were evaluated in each experimental group (n=40). Histological alterations were evaluated according to the following grading score: -, non-existent; +, mild; ++, mild/moderate; +++, moderate; ++++, abundant; +++++, severe.

Antioxidant activity assays

Liver was homogenized (1:10 w/v) in 50 mM phosphate buffer (pH 7.8) containing 0.1% Triton X-100 and 1.34 mM of DETAPAC using a Micra D-1 homogenizer (ART moderne labortechnik) at 18,000 rpm for 30 sec followed by treatment with Sonoplus HD 2070 ultrasonic homogenizer (Bandelin) at 50% power three times for 10 sec. Liver homogenates were centrifuged at 13 000 × *g*, 4°C for 45 min and the supernatant was used to determine the activity of antioxidant enzymes. Catalase activity was measured by the method of Aebi (1984) and the enzyme unit was defined as μmol of H_2O_2 consumption per min. Total cellular GPX activity was determined by the coupled assay of NADPH oxidation (Lawrence et al. 1974) using cumene hydroperoxyde as substrate. The enzyme unit was defined as nmol of GSH oxidized per min. Total SOD activity was measured as described by Ukeda et al. (1997). Mn-SOD activity was determined by the same method after treating the samples with 4mM KCN for 30 min. Cu,Zn-SOD activity resulted from subtracting the Mn-SOD activity from the total SOD activity. One unit of SOD activity was defined as the enzyme needed to inhibit 50% XTT reduction. Protein concentration was assayed by the method of Lowry et al. (1951).

Liver protein expression analyses

Liver samples were homogenized in 20mM Tris HCl buffer containing 0.1% Igepal, 100mM EGTA, and a cocktail of protease inhibitors (Sigma, St Louis, MO) that provided a final concentration of 100mM dichloro dipheny thrichloroethane (DDT), 100mM orthovanadate, 1mM EDTA, 2mM AEBSF, 130μM Bestatin, 14μM E-64, 1μM Leupeptin and 1μM Apoprotin. Samples were homogenized as before. Liver homogenates were centrifuged at 13 000 × *g* for 45 min, at 4°C and supernatants were aliquoted and stored at -80°C, until further use for western blot analysis. Protein concentration was measured by the method of Lowry et al. (1951). Equal amounts of total protein for each sample were loaded per lane (two samples from each experimental group were run per gel), subjected to 12% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany) by wet transfer at 90 V for 2 h using a Mini Trans-Blot cell system (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked using 5% non-fat dry powered milk dissolved in Tris-Buffered saline Tween-20 (TBST) for 90 min at room temperature. The primary antibodies for 5'-AMP-activated protein kinase (AMPK), phosphorylated-AMPK (PAMPK) (Cell Signaling Technology, Inc. Danvers, MA, USA), and proliferator activating receptor-γ (PPAR-γ) (Abcam, Cambridge, UK) were used according to the manufacturer recommended dilutions (1:1000) and were incubated overnight at 4°C. The membranes were then washed three times for 10 min with TBST, before incubation for 2 h at room temperature with secondary peroxidase conjugated goat anti-rabbit antibody (Sigma, St Louis, MO) diluted at 1:2000 in 5% nonfat dry milk–TBST. Membranes were washed as before, and the bound antibodies were visualized by an ECL Pro system (PerkinElmer, Boston, USA) using a Fujifilm Luminescent Analyzer LAS-4000 mini System (Fujifilm, Tokyo, Japan). PAMPK expression was determined in relation to AMPK expression while PPAR-γ was normalized to ponceau reagent. Results were expressed in relative density units.

Liver gene expression analyses

Total RNA was extracted from 10-20 mg of frozen liver tissue using Trizol™ reagent (Invitrogen; UK) and following the manufacturer's instructions. RNA purity was determined by the A=260/A=280 ratio, using a UV/VIS spectrophotometer (Thermo Spectronic, Helios γ). Expression of GPX1 and GPX4 genes was measured by semi-quantitative RT PCR. GAPDH gene expression was used as housekeeping gene. Total RNA (100ng) was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen, UK), 10mM of each dNTP (Promega, UK), 10-20U RNaseOUT (Invitrogen), 1mg/mL BSA (BioLabs, UK), and 500μg/ml of Random Hexamers (Promega, UK) as primers. The amplification of cDNA was performed by

adding 10x PCR buffer (w/o $MgCl_2$), 1.75mM $MgCl_2$, 1U TaqDNA polymerase and 1 μM of each specific primer for GPX1, GPX4 and GAPDH (Table 2). After a hot start (95°C) and 4 min at 94°C, 25 cycles of 1 min at 94°C, 2 min at 59°C and 2 min at 72°C, were performed. Samples were further incubated at 72°C for 8 min to complete any elongation reaction. PCR products were then separated by gel electrophoresis on a 1.5% agarose gel containing GelRed™ (1:10,000, Biotium, UK). PCR amplified gene products were visualized under UV light and, images were captured using Fusion Fx7 imaging system (PEQLAB Biotechnologies, UK). Optical density of the obtained products was quantified by Image J software. Expression of GPX1 and GPX4 was related to expression of GADPH. To test the expression stability of GAPDH, equal amounts of PCR product from liver samples of rats within each experimental group were loaded per lane and the band density of the corresponding samples measured and compared among them. The average band density of LS group was assigned with a value of 1, and relative values for the rest of samples in the three remaining experimental groups were calculated and averaged. After statistical comparisons, no significant differences were found for GAPDH expression among the four groups assayed.

Statistical analyses

Time-repeated measurement analysis was applied to weekly food intake and body weight data as well as to blood glucose content after an oral glucose overload in order to analyze within subject effects (time) or within group effects (phenotype or aerobic interval training protocol) on the above parameters. The effect of phenotype and AIT protocol on final body weight, aerobic capacity and physical performance, plasma and liver biochemical parameters, hepatic antioxidant enzyme activity, protein and gene expression was analyzed by 2 × 2 factorial ANOVA with phenotype and AIT protocol as main treatments. Results are given as mean values and pooled standard error of the mean. Bonferroni's test was used to detect differences between treatment means. The analyses were performed with SAS, version 9.0, and the level of significance was set at $P < 0.05$.

Results

Food intake and body weight

Changes observed in weekly food intake and body weight during the study are presented in Fig. 1A and 1B. Time-repeated measurement analysis revealed a significant time effect, phenotype effect, and exercise effect on food intake that was 20% higher in the obese compared to the lean Zucker rats ($P < 0.0001$) and

decreased by 5% as a result of the aerobic interval training ($P < 0.05$). Body weight was significantly affected by phenotype and the aerobic interval training, with lower values being found for lean vs obese ($P < 0.0001$), and trained vs sedentary ($P < 0.0001$) rats, respectively. The effect of exercise on body weight was more pronounced in the obese when compared to lean rats.

Aerobic capacity and physical performance

The effects of phenotype and AIT protocol on aerobic capacity and physical performance of Zucker rats during an incremental test are shown in Table 3. Blood lactate content at the end of incremental tests was lower in lean when compared to obese rats ($P < 0.0001$), whereas the opposite was observed for the total running time and maximal speed achieved ($P < 0.0001$). Exercise increased all the above parameters ($P < 0.0001$) with the exception of final blood lactate that decreased in the obese rats ($P < 0.001$) and was not affected in the lean ones. The effects of exercise on running time and maximal speed were significantly affected by phenotype, a finding that was reflected in significant phenotype × exercise interactions.

Plasma parameters

The effects of phenotype and AIT protocol on blood and plasma parameters of glucose and lipid metabolism of Zucker rats are presented in Figure 2 and Table 4. With regards to the plasma parameters related to glucose metabolism affected by phenotype (glucose, insulin, and AUC, $P < 0.0001$), the training protocol only had a significant effect on the AUC. Exercise tended to decrease both glucose and insulin concentrations in the obese rats and increase them in the lean ones, although the effects were not significant. AUC was differentially affected by exercise depending on the rat phenotype, a finding that was reflected in phenotype × exercise interaction ($P < 0.0001$). Exercise caused a 2.7-fold reduction in the AUC of obese rats, returning this index to values similar to those found in lean animals, among which no appreciable effect of this intervention was found. When blood glucose content of lean and obese Zucker rats prior to or at different time points after an oral glucose overload was represented (Fig. 2), the rise in blood glucose during the initial stages after oral administration was more pronounced in obese when compared to lean rats, and higher levels were observed among the former animals for sedentary when compared to trained individuals. Blood glucose levels of obese sedentary animals remained higher than the rest of experimental groups during the 120 min post administration period, whereas those of obese trained animals were not significantly different from the lean ones from 30 min post administration time.

The plasma parameters related to lipid metabolism (Total-, LDL-, and HDL-cholesterol, triglycerides) were all significantly affected by phenotype. There was a significant effect of exercise on Total- and LDL-cholesterol contents that were considerably diminished in the obese groups that carried out the training protocol (20 and 41%, respectively) and to a lesser extent in the lean animals, giving rise to a significant phenotype × exercise interaction. Triglyceride content was considerably reduced by exercise ($P = 0.0845$) in both the obese and lean animals (12 and 74%, respectively).

Liver surface, lipid composition, functionality and antioxidant status

The effects of phenotype and AIT protocol on liver weight and surface, lipid composition, and functionality, are presented in Table 5 and Fig. 3. There was a significant effect of phenotype on liver weight and surface, total fat, and triglyceride content that was higher in obese compared to lean rats. The liver of obese rats showed clear signs of hepatomegalia and steatosis compared to their lean counterparts (Fig. 3). The training protocol caused 8.6 and 9.3% decrease in liver weight and surface, respectively, and significant reductions in hepatic total fat and triglyceride contents in the obese animals (35 and 50%, respectively). Such AIT-induced improvements in the hepatic outcomes of lipid metabolism were associated to a lower body weight exhibited by trained animals (Fig. 1B) and, to a lesser extent, to the lower weight of their abdominal fat pad (6.4±0.2 vs 5.7±0.2 g in OS and OE groups, respectively)

Liver function was measured as plasma AST, ALT, ALP, GGT activities. All of these parameters were affected by phenotype, showing a significant increase in the obese animals. The training protocol was effective at reducing the activity of AST in both lean and obese animals, and ALP in obese but not in lean rats, thus giving rise to a significant phenotype × exercise interaction. However, it did not have any major effect in ALT and GGT activity.

With regard to the hepatic antioxidant enzyme activities, there was a significant phenotype effect on SOD activity reflected by lower values for Cu/Zn-SOD and higher values for Mn-SOD in obese compared to lean rats. The training protocol caused a 40% increase in Cu/Zn-SOD activity of obese but no appreciable effect on lean rats, and a 43% increase in Mn-SOD activity of lean rats that in contrast was significantly reduced in their obese counterparts (20%). Such differential effects of exercise depending on rat phenotype gave rise to significant phenotype × exercise interactions ($P=0.049$ and $P<0.0001$, respectively). GPX activity was significantly affected by phenotype, with lower values in the obese when compared to the lean sedentary

rats. The training protocol caused a 17% decrease in GPX activity of lean compared to a 100% increase in obese rats. Such differential effects of the training protocol gave rise to a strong phenotype × exercise interaction ($P < 0.0001$). Exercise also exhibited a differential effect on catalase activity depending on rat phenotype ($P = 0.0053$), decreasing as a result of the training protocol in the obese rats whereas it increased in their lean controls.

The effects of phenotype and AIT protocol on the liver expression of GPX1 and GPX4 genes are shown in Fig. 4. The expression of GPX1 and GPX4 genes, two major redox enzymes that take part in the antioxidant defence system of the liver of Zucker rats, was not affected by phenotype. Exercise had only a significant enhancing effect on the expression of liver GPX4 gene.

Liver histology

Several phenotype-related changes in liver histology were observed under the experimental conditions of the present study (Fig. 5, Table 6). The obese sedentary rats exhibited clear signs of microvesicular steatosis and fatty droplets (Fig. 5C), lipogranulomas and portal inflammation (Table 6) when compared to their lean counterparts. The training protocol improved microvesicular steatosis, reduced the number of fatty droplets (Fig. 5D), and decreased the amount of lipogranulomas and portal inflammation. However, it caused the appearance of multinucleic cells and necrosis (Table 6) followed by the development of fibrosis (Fig. 5H).

Liver protein expression

The effects of phenotype and AIT protocol on the liver expression of AMPK, PAMPK, and PPAR-γ are shown in Fig. 6. Western blot analysis indicated a significantly lower expression and activation of AMPK (shown by the ratio PAMPK/AMPK) (Figure 6A) in the liver of obese compared to lean animals, and the training protocol did not induce major effects on AMPK phosphorylation. No significant differences in PPAR-γ expression were observed between obese and lean rats, whereas the training protocol caused 1.7-fold increment in the obese and lean phenotypes (OE, LE), respectively (Figure 6B).

Discussion

The study of MS and the development of strategies for its prevention and treatment has attracted increasing attention in recent years due to its growing prevalence and associated comorbidities exemplified by cardiovascular disease and NAFLD (Kaur 2014; Marchesini et al. 2003). Changes in lifestyle, i.e. caloric restriction and physical activity, are the primary interventions chosen to improve this condition. However, the type and intensity of exercise are still a matter of debate. In this study, the influence of an aerobic interval

training protocol consisting of successive 4 min periods at 65-80% of VO_2max, followed by 3 min recovery periods at 50-65% of VO_2max on plasma and liver biochemical parameters, was studied in obese and lean Zucker rats. Obese rats exhibited higher food intake and body weight, and suffered significant alterations in MS-associated parameters such as plasma lipid profile, OGTT and AUC after oral glucose overload, liver histology and functionality, and antioxidant status. Exercise increased the aerobic capacity of both rat phenotypes and diminished the severity of MS alterations, especially those related to glucose and lipid metabolism, affecting the levels and activity of proteins involved in metabolic and antioxidant pathways and the gene expression of GPX4, a key antioxidant enzyme, in the liver. The effects of exercise on glucose and lipid metabolism were independent of hepatic AMPK activation, but matched significant increments in the protein expression of PPARγ.

Zucker obese rats are known to present a genetic defect in leptin receptor that causes the development of hyperphagia and other metabolic disturbances leading to obese phenotype (Galisteo et al. 2008). The anorectic effects of exercise on Zucker rats have been described by (Kibenge and Chan 2002) that related such effects to an increased production of corticotrophin-releasing hormone. Such anorectic effects would in turn lead to a lower weight gain both in obese and lean animals. Decrease in weight gain is among the most widespread recommendations for the treatment of metabolic syndrome and has been associated to significant improvements in cardiovascular health and metabolic disorders intrinsic to that disease.

Physical performance was always lower in obese when compared to lean Zucker rats due to the severe metabolic disturbances, impaired skeletal muscle perfusion, and muscular atrophy inherent to this experimental model. Low intrinsic aerobic capacity in rats has been related to lower energy expenditure and reduced whole body and hepatic mitochondrial lipid oxidation, which in turn made the animals more susceptible to dietary-induced hepatic steatosis (Morris et al. 2014). Our results show a clear improvement in the aerobic capacity of lean and obese rats that followed the aerobic interval training protocol although the effect of exercise on VO_2max did not reach statistical significance. The adaptation changes in blood lactate, maximal speed, and running time were significantly improved in trained rats. The enhancement in aerobic capacity derived from aerobic interval exercise has been reported by other authors (Haram et al. 2009; Tjønna et al. 2008) that related such changes to amelioration in several risk factors of MS associated cardiovascular disease. Under our experimental conditions, the higher physical performance of trained

353 Zucker rats was related to significant changes in glucose and lipid metabolism as well as to improved hepatic
354 histology and function altered in NAFLD.

355 The experimental model of obese Zucker rat has been described to exhibit impaired lactate transport by
356 the skeletal muscle that can be alleviated by endurance exercise (Metz et al. 2005). The aerobic training
357 protocol tested in our study achieved a consistent reduction in blood lactate after the incremental oxygen
358 consumption test. Since lactate release under exercise conditions is mostly related to skeletal muscle
359 metabolism, our results suggest that the benefits of the AIT protocol on lactate uptake and metabolism are
360 clear. Such improvement represents an important benefit on glucose metabolism in relation to
361 hyperlactatemia and aggravation of insulin resistance (Juraschek et al. 2013; Souto et al. 2011).

362 The beneficial effects of different types of aerobic exercise on glucose and lipid metabolism have been
363 extensively reported in the literature (Johnson et al. 2009; Rosety-Rodriguez et al. 2012). Our results confirm
364 such positive actions of AIT protocol, and point out to training-induced enhanced insulin sensitivity in the
365 obese animals as seen by changes in blood glucose levels and AUC after an oral glucose load, rather than to
366 changes in insulin secretion. Moreover, the specific action of the training protocol at decreasing total- and
367 LDL-cholesterol, while leaving HDL-cholesterol unchanged, suggests a direct protection against well known
368 cardio-metabolic risk factors. Such effects on the plasma lipid profile could be explained by a lower free
369 fatty acid uptake and lipogenesis in the adipose tissue (Haram et al. 2009). In addition, it has been reported
370 that physical exercise can elicit a significant improvement in the content and functionality of mitochondria
371 measured by increased citrate synthase activity, and palmitate oxidation (Linden et al. 2015). Moreover,
372 physical exercise is a successful strategy to prevent and mitigate NASH-induced mitochondrial bioenergetics
373 impairment, thus improving lipid metabolism in liver (Gonçalves et al. 2014).

374 The aerobic interval training triggered a clear improvement in liver lipid composition (lower total fat and
375 triglyceride content) as described by other authors in different human and animal models (Johnson et al.
376 2009; Linden et al. 2015). AIT can lead to such improvements in lipid composition through increases in
377 mitochondrial content and oxidative phosphorylation, or greater lipid and carbohydrate oxidation (Barker et
378 al. 2014; Larsen et al. 2015). Indeed, a long term aerobic training, for 3 months, at 60-75% of VO_2max has
379 been shown to induce a decrease in intrahepatic lipids in obese female adolescents (Lee et al. 2013), whereas
380 a 7-day aerobic training protocol during 1 h at 80-85% of maximum heart rate in obese individuals with
381 hepatic steatosis resulted in increased resting fat oxidation and favourable effects in hepatic lipid

composition by increasing polyunsaturated lipid index (Haus et al. 2013). Furthermore, the beneficial effect of a 12 week interval training on lipid oxidation was also proven in healthy, sedentary subjects (Astorino et al. 2013).

Fatty liver has been associated to high plasma AST and ALT activities, resulting from hepatic damage mediated by inflammation and oxidative stress reflected in higher levels of hepatic nitrate and malondialdehyde (Jung and Kim 2013; Linden et al. 2015). Significant improvements of hepatic plasma parameters have been observed under our experimental conditions related to the fat composition changes in the obese Zucker rats. It is worth mentioning that our exercise training protocol has been beneficial both in acute and chronic hepatic markers (AST and ALP activities, respectively) of altered functional status.

Oxidative stress is one of the main factors involved in the development of NAFLD (Rolo et al. 2012; Tariq et al. 2014). Indeed, the "two-hit" hypothesis on NASH development points out to oxidative stress as one of the factors directly promoting the progress from steatosis to the advanced stages of the pathology. A decrease in antioxidant defence system has been described as a major promoting factor in the development of oxidative stress in patients with NASH (Videla et al. 2004), whereas obese Zucker rats with fatty liver have been described to exhibit an altered antioxidant status as shown by the decrease in liver content of GSH, tocopherol, and catalase activity (Soltys et al. 2001). Exercise is a useful lifestyle intervention strategy to improve oxidative stress in the muscle of type 2 diabetic rats (Qi et al. 2011; Rosety-Rodriguez et al. 2012) and plasma of obese middle-age women (Shin et al. 2008). Furthermore, in obese individuals with hepatic steatosis, short-term aerobic exercise has proved to favourably alter hepatic lipid composition, insulin resistance and oxidative stress, risk factors that influence the severity of NAFLD (Haus et al. 2013). However, the effects of exercise on oxidative stress may vary depending on parameters such as age, health status, severity of pathology of the individual, and/or type and intensity of the exercise protocol applied. Although, the effect of exercise on SOD, GPX, and catalase activities differed between obese and lean rats under our experimental conditions, a finding that can be attributed to the compromised antioxidant status of the obese animals associated to their fatty liver condition, exercise was in general terms an effective strategy to lower oxidative stress and balance SOD and GPX activities that were altered in obese sedentary rats, returning them to levels closer or even higher than those of lean animals. Of particular interest was the increment in GPX activity attained by trained obese rats that nearly doubled that of their sedentary counterparts and led us to conduct further experiments to confirm if such increments were related to the

induced expression of two relevant genes belonging to the GPX group of selenoenzymes such as GPX1 and GPX4. Nevertheless, neither exercise nor phenotype had a significant effect on the hepatic GPX1 gene expression under our experimental conditions. Similar findings have been observed in pediatric patients with NASH that underwent liver biopsy (Desai et al. 2014) or mononuclear cells isolated from peripheral blood samples of active or inactive healthy participants after completing a 30-min treadmill run at 75–80% VO_2max (Jenkins et al. 2009). A possible explanation for this lack of coincidence between enzymatic activity and gene expression pattern is the existence of different members within the group of GPX selenoenzymes that are not taken into account when the total GPX activity is measured. Furthermore, Bermano et al. (1995) reported that both the activity of the selenoenzymes and the abundance of their respective mRNAs are not regulated in a similar manner in the liver of rats with different Se status.

While GPX4 deficiency has been linked to disorders associated with reactive oxygen species and lipid peroxides generated in mitochondria (Imai and Nakagawa 2003), its overexpression is associated with the inhibition of atherosclerosis development in $ApoE^{-/-}$ mice (Guo et al. 2008) and lipid peroxidation in endothelial cells (Sneddon et al. 2003). In our study, hepatic GPX4 gene expression was up-regulated by exercise in both lean and obese groups. Similar results were obtained by (Daussin et al. 2012) in the expression of GPX4 after endurance training for 10 days.

The improvement in liver histological features associated to changes in lipid composition and function exerted by the training protocol in the obese Zucker rat shows the prospective benefits of this type of exercise in ameliorating hepatic morphological and histological alterations present in the early stages of NAFLD characteristic of the experimental animal model selected for this study. Nevertheless, although the training protocol has shown interesting results on glucose and lipid parameters in plasma, as well as lower lipid content and decreased fatty droplet accumulation and microvesicular steatosis in liver of obese rats, the potentially deleterious effects of that intensive type of exercise on individuals prone to liver damage (e.g. suffering from MS), should be considered, since necrosis and fibrosis were detected in the liver of trained rats, especially in the obese animals. It has been described that the obse Zucker rat does not spontaneously progress from steatosis to steatohepatitis but needs an additional intervention (Kucera and Cervinkova 2014). It seems that the experimental training protocol assayed was protective against steatosis but triggered some distinctive features of NASH. In fact, exhaustive or strenuous exercise has been shown to increase certain biomarkers of liver damage like AST and ALT and cause oxidative damage to nuclear DNA (Ogonovszky et

al. 2005; Ramos et al. 2013). Moreover, some authors have used exhaustive acute exercise to induce liver injury in experimental animal models (Huang et al. 2013; Praphatsorn et al. 2010). Histopathological lesions described in such models were mediated by pro-inflammatory cytokines and consisted on extensive nuclear pyknosis, severe necrosis with haemorrhage and neutrophil infiltration, edema and necroinflammation, as well as accelerated apoptosis. Under conditions of demanding physical exercise blood flow is preferentially derived to skeletal muscle at the expense of other tissues like the liver in which decreased blood flow may induce ischemic hypoxia-reperfusion of hepatocytes and lead to necrosis.

Activation of AMPK depends on the ADP:ATP ratio, and it is reduced in the liver of obese Zucker rat due to an excess of energetic substrates entering this tissue (Galisteo et al. 2010). Furthermore, AMPK activity has been shown to be inhibited by insulin and glucose in several tissues, a finding that would be supported by the hyperinsulinemia characteristic of this experimental animal model. Although exercise can activate AMPK in the skeletal muscle, and subsequently up-regulate PPAR-γ expression (Sasaki et al. 2014), the aerobic interval protocol tested under our experimental conditions was not able to ameliorate high plasma insulin levels of obese rats or show any effect on liver PAMPK expression. In contrast, liver AMPK activity in obese Zucker rats has been described to be activated by different nutritional and pharmacological strategies like diet supplementation with *Plantago ovata* or chronic treatment with polyphenols (Galisteo et al. 2010; Rivera et al. 2009). On the other hand, it has been reported that activation of liver PPARγ improves insulin sensitivity and NASH in human patients (Neuschwander-Tetri et al. 2003) and this correlates with the significant increase of liver PPAR-γ protein expression by our exercise protocol. Therefore PPAR-γ activation under our experimental conditions appeared to be independent of the AMPK pathway.

In conclusion, the AIT protocol used in this study is a feasible intervention strategy to improve plasma and hepatic biochemical parameters as well as hepatic histological alterations inherent to early stages of NAFLD in obese Zucker rats, although it caused the development of fibrosis. The training protocol was especially efficient to improve insulin sensitivity and decrease the hepatic lipid content, as well as ameliorating the oxidative stress conditions in this organ. Such effects run in parallel to an increased expression of liver PPAR-γ.

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672

673 **Table 1.** Details of the AIT protocol.

Week (5 days/week)	Work Time (min/day)	% VO_2max
1	45´	50%→3 min
		65%→4 min
2	50´	55%→3 min
		70%→4 min
3	50´	60%→3 min
		75%→4 min
4	55´	60%→3 min
		75%→4 min
5-8	60´	65%→3 min
		80%→4 min

674

675

676 **Table 2.** Sequence of primers used in RT-PCR

Gene	Primer's 5'-3' sequence	
GPX1	Forward	CACCGAAATGAATGATCTGC
	Reverse	TGTATCTGCGCACTGGAACA
GPX4	Forward	CCGGCTACAATGTCAGGTTT
	Reverse	CGGCAGGTCCTTCTCTATCA
GAPDH	Forward	ATGGGAAGCTGGTCATCAAC
	Reverse	GTGGTTCACACCCATCACAA

677 GPX1: Glutathione peroxidase 1; GPX4: Glutathione peroxidase 4.

678 **Table 3.** Effect of AIT protocol on aerobic capacity and physical performance of lean and obese Zucker rats.

	Lean		Obese						
	Sedentary	**Exercise**	**Sedentary**	**Exercise**	**SEM**	**R^2**	**Phenotype Effect**	**Exercise Effect**	**Phenotype × Exercise**
Exercise Lactate (mmol/L)	6.8^{A}	6.2^{A}	15.7^{C}	10.4^{B}	0.79	0.7822	P < 0.0001	P = 0.0003	P = 0.0076
VO_2max (mL/min/$kg^{0.75}$)	18.7^{A}	19.9^{A}	17.7^{A}	19.5^{A}	1.01	0.1049	P = 0.3318	P = 0.1380	P = 0.7525
Running Time (min)	13.3^{B}	23.6^{C}	7.6^{A}	11.2^{B}	0.72	0.9204	P < 0.0001	P < 0.0001	P < 0.0001
Maximal Speed (cm/sc)	55.8^{B}	85.6^{C}	38.9^{A}	49.8^{B}	2.2	0.9161	P < 0.0001	P < 0.0001	P = 0.0002

679 ^{A,B,C} Results are mean of 8-10 rats. Means within the same row with different superscripts differ significantly (P < 0.05). SEM, pooled standard error of the mean.

Table 4. Effect of AIT protocol on plasma parameters of lean and obese Zucker rats.

	Lean		Obese						
	Sedentary	**Exercise**	**Sedentary**	**Exercise**	**SEM**	**R^2**	**Phenotype Effect**	**Exercise Effect**	**Phenotype × Exercise**
Glucose (mg/dL)	177.6^{A}	238.3^{A}	400.2^{B}	341.9^{B}	30.5	0.5114	P<0.0001	P=0.9679	P=0.0607
Insulin (ng/mL)	0.062^{A}	0.126^{A}	0.685^{B}	0.558^{B}	0.081	0.6929	P < 0.0001	P = 0.6687	P = 0.1992
AUC (arbitrary units)	2417^{A}	2516^{A}	7054^{B}	2599^{A}	301	0.8470	P < 0.0001	P < 0.0001	P < 0.0001
T-Cholesterol (mg/dL)	74.9^{A}	74.2^{A}	209.2^{C}	167.5^{B}	6.1	0.9224	P<0.0001	P=0.0016	P=0.0021
LDL-Cholesterol (mg/dL)	4.9^{A}	3.4^{A}	19.9^{C}	11.8^{B}	1.27	0.7772	P<0.0001	P=0.0007	P=0.0148
HDL-Cholesterol (mg/dL)	27.5^{A}	29.4^{A}	51.0^{B}	45.3^{B}	3.88	0.4619	P<0.0001	P=0.6331	P=0.3395
Triglycerides (mg/dL)	100.2^{A}	26.5^{A}	279.3^{B}	246.2^{B}	29.9	0.6025	P<0.0001	P=0.0845	P=0.5051

^{A,B,C} Results are mean of 8-10 rats. Means within the same row with different superscripts differ significantly (P < 0.05)**.** AUC, Area under the curve. SEM, pooled standard error of the mean.

Table 5. Effect of AIT protocol on liver weight, composition, and function of lean and obese Zucker rats.

	Lean		Obese						
	Sedentary	**Exercise**	**Sedentary**	**Exercise**	**SEM**	**R^2**	**Phenotype Effect**	**Exercise Effect**	**Phenotype × Exercise**
Weight (g FW)	8.2^{A}	9.3^{A}	17.5^{B}	16.0^{B}	0.5	0.8738	P < 0.0001	P = 0.7508	P = 0.0255
Surface (cm^2)	12.5^{A}	13.2^{A}	22.6^{B}	20.5^{B}	0.5	0.8450	P < 0.0001	P = 0.293	P = 0.048
Fat (g/100 g DM)	7.1^{A}	4.3^{A}	19.6^{C}	12.7^{B}	1.2	0.7412	P < 0.0001	P = 0.0005	P = 0.1040
Triglycerides (mg/g DM)	5.3^{A}	4.1^{A}	26.9^{B}	13.4^{A}	2.5	0.6139	P < 0.0001	P = 0.0096	P = 0.0204
Liver function plasma markers									
AST (U/L)	98.4^{A}	66.9^{A}	182.3^{B}	107.3^{A}	14.7	0.5226	P<0.0001	P=0.0010	P=0.1494
ALT (U/L)	25.7^{A}	31.8^{A}	61.0^{B}	59.7^{B}	4.7	0.5917	P<0.0001	P=0.6134	P=0.4287
ALP (U/L)	98.2^{A}	100.7^{A}	202.6^{B}	137.6^{A}	10.5	0.6823	P<0.0001	P=0.0055	P=0.0030
GGT (U/L)	0.10^{A}	0.70^{A}	13.9^{B}	9.3^{B}	1.56	0.6440	P<0.0001	P=0.2162	P=0.1098
Antioxidant enzymes									
Cu/Zn-SOD (Units/mg protein)	223.8^{C}	233.4^{C}	112.5^{A}	157.7^{B}	8.5	0.7987	P < 0.0001	P = 0.0047	P = 0.0494
Mn-SOD (Units/mg protein)	26.8^{A}	38.2^{B}	85.8^{D}	68.9^{C}	2.6	0.9019	P < 0.0001	P = 0.4839	P < 0.0001
Catalase (µmol H_2O_2/min/mg protein)	487.1^{AB}	551.8^{B}	503.9^{AB}	461.7^{A}	19.4	0.2803	P = 0.0630	P = 0.4260	P = 0.0053
GPX (nmol NADPH/min/mg protein)	9.2^{B}	7.6^{AB}	6.4^{A}	13.0^{C}	0.5	0.7040	P=0.03	P< 0.0001	P< 0.0001

[A,B,C,D] Results are mean of 8-10 rats. Means within the same row with different superscripts differ significantly ($P < 0.05$). FW, fresh weight, DM, dry matter, AST, aspartate aminotransferase, ALT, alanine transaminase, ALP, Alkaline Phosphatase, GGT, Gamma-glutamyl transpeptidase, GPX, Glutathione peroxidase. SEM, pooled standard error of the mean.

Table 6. Effect of AIT protocol on liver histology of lean and obese Zucker rats.

	Microvescicular steatosis	Fatty droplets	Multinucleic cells	Lipogranulomas	Portal inflammation	Necrosis	Fibrosis
LS	-	-	-	-	-/+	-	-
LE	-	-	-	+	+	-/+	-
OS	++++	++++	-	+++	+++	-	-
OE	+++	+++	++	++/+++	++/+++	++	++

LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing a protocol of aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing a protocol of aerobic interval exercise. Grading score of the histological alterations: -, non existent; +, mild; ++, mild/moderate; +++, moderate; ++++, abundant; +++++, severe.

Fig. 1. Effect of AIT protocol on food intake and body weight of lean and obese Zucker rats. (A) Weekly food intake (grams Dry Matter/day). (B) Weekly body weight (grams). Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing aerobic interval exercise. Values are means ± SEM depicted by vertical bars (n = 8-10).

Fig. 2. Effect of AIT protocol on blood glucose levels of lean and obese Zucker rats prior to or at different time points after oral glucose overload. Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing aerobic interval exercise. Values are means ± SEM depicted by vertical bars (n = 8-10). The following notation is used to express significant differences ($P < 0.05$) between groups pointed out by Dunnet's t-test: a, OS *vs* LS, b, OE *vs* LS, c, LE *vs* LS.

Fig. 3. Effect of AIT protocol on liver morphology of lean and obese Zucker rats. Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing aerobic interval exercise. Photographs are representative of livers of 8-10 different rats for each experimental group.

Fig. 4. Effect of AIT protocol on GPX1 and GPX4 mRNA levels in liver of lean and obese Zucker rats. Hepatic GPX1 and GPX4 mRNA relative expression. Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing aerobic interval exercise. GPX1 and GPX 4 levels are expressed as percentage of the mean value obtained from liver of the LS group (100%). Results represented in the graphs are means ± SEM depicted by vertical bars (n = 10). Means within the same gene expression without a common letter differ, $P < 0.05$. Image of gel used for determination of GPX1 and GPX4 expression by semiquantitative RT-PCR is representative of RNA samples of 8-10 rats for each experimental group; all samples were derived at the same time and processed in parallel. The samples were analyzed for expression of GAPDH, GPX1, and GPX4. GAPDH expression was not different among the experimental groups.

Fig. 5. Effect of AIT protocol on liver histology of lean and obese Zucker rats. (A) Histological view of control LS liver HE stain, (B) Histological view of LE liver HE stain, (C) Histological view of OS liver HE stain with clear signs of microvesicular steatosis (mv) and fatty droplet accumulation (fd), (D) Histological view of OE liver HE stain with diminished signs of microvesicular steatosis (mv) and fatty droplet accumulation (fd), (E) Histological view of control LS liver MT stain, (F) Histological view of LE liver MT

stain, (G) Histological view of OS liver MT stain, (H) Histological view of OE liver MT stain with signs of fibrosis (fb). Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing aerobic interval exercise. Photographs are representative of livers of 8-10 different rats for each experimental group.

Fig. 6. Effect of AIT protocol on AMPKα/PAMPKα and PPARγ protein expression in the liver of lean and obese Zucker rats. Western blot analysis of (A) AMPKα/PAMPKα and (B) PPARγ expression. Groups: LS, Lean (fa/+) sedentary rats, OS, Obese (fa/fa) sedentary rats, LE, Lean (fa/+) rats performing aerobic interval exercise, OE, Obese (fa/fa) rats performing aerobic interval exercise. Immunoblots are representative of liver homogenates from eight different rats for each experimental group; two samples of each experimental group were loaded per gel and processed in parallel. The amount of sample loaded per lane was 100 µg of protein for AMPKα/PAMPKα and 80 µg of protein for PPARγ. Levels of PAMPK were normalized to the total AMPK. Levels of PPARγ were normalized to ponceau reagent. Densitometric analysis values represented in the graphs are means ± SEM depicted by vertical bars (n = 8). Means without a common letter differ, $P < 0.05$.

Figure 1

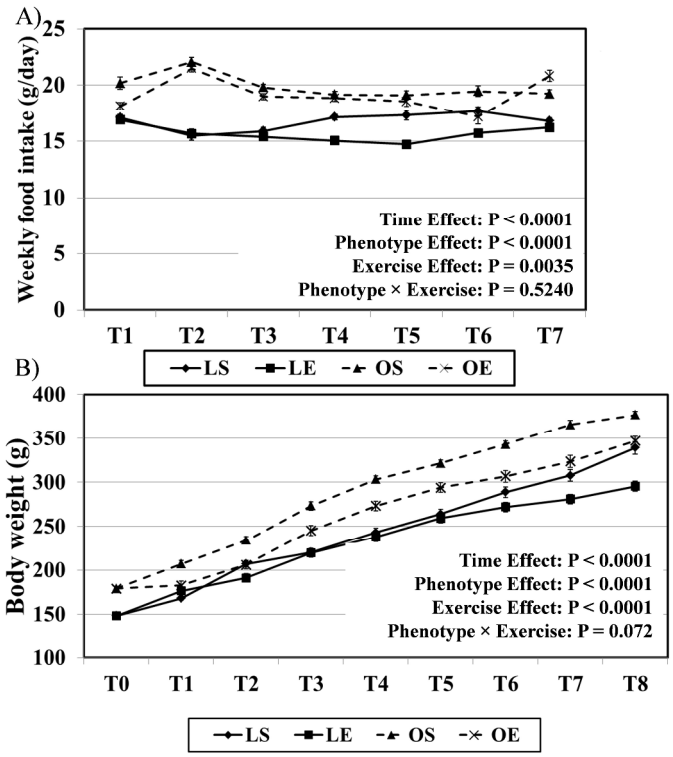

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Figure 2

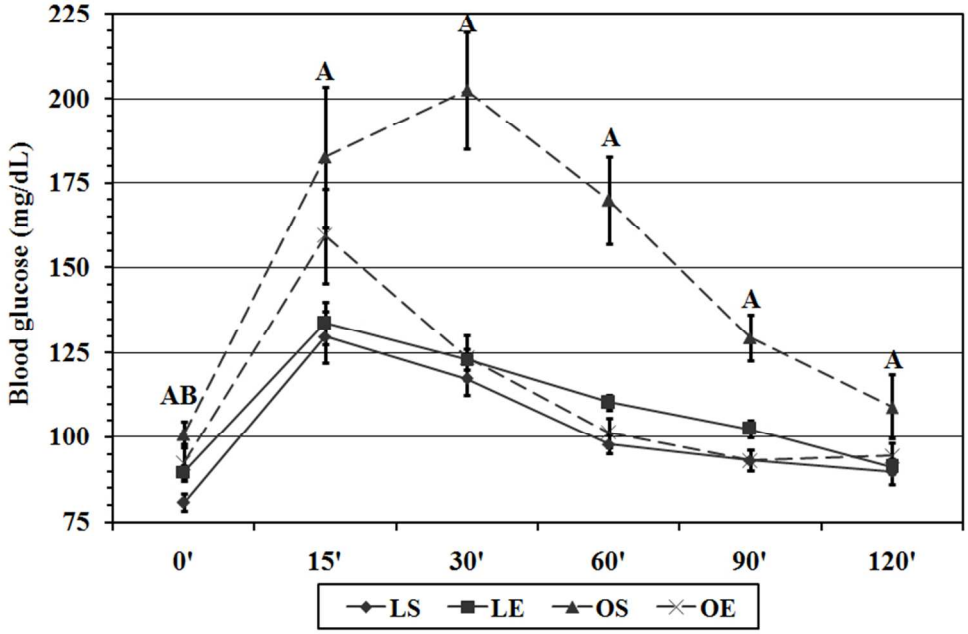

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Figure 3

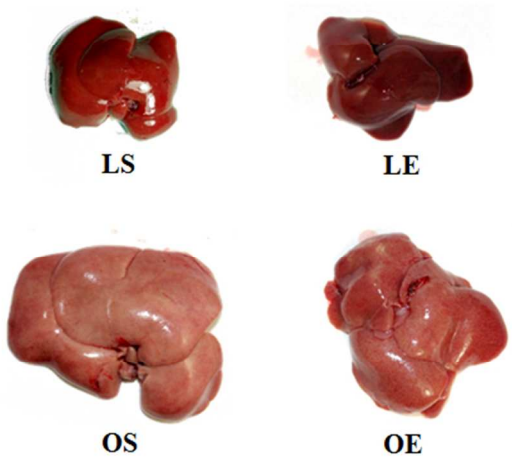

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Figure 4

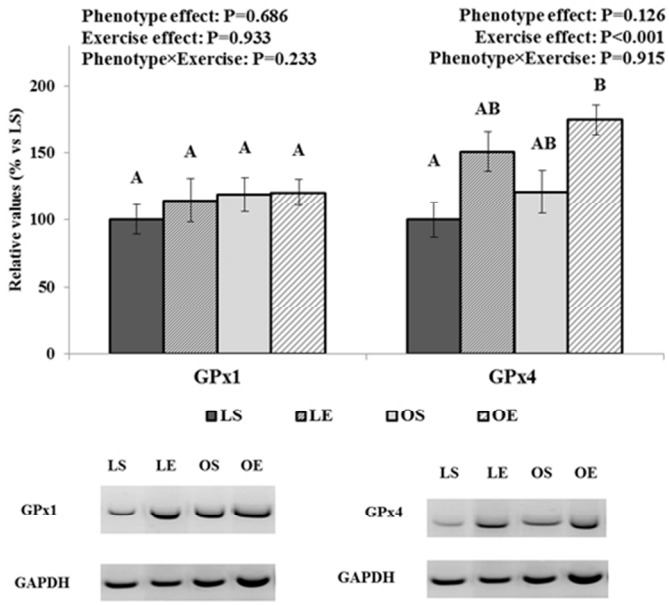

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Figure 5

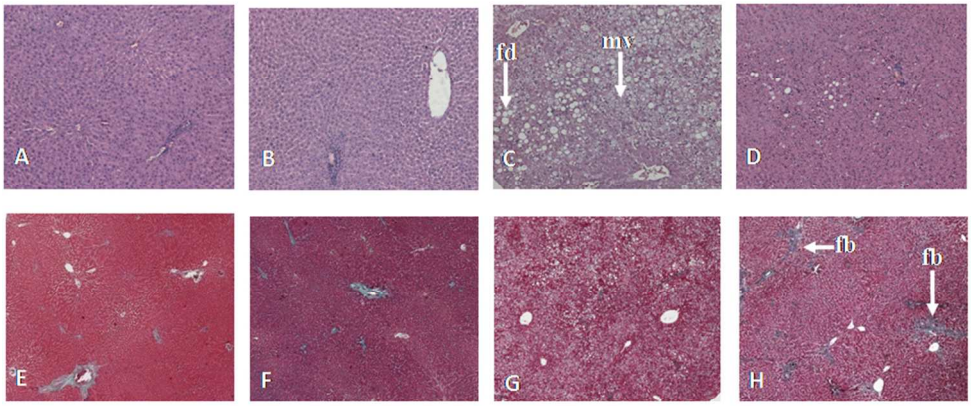

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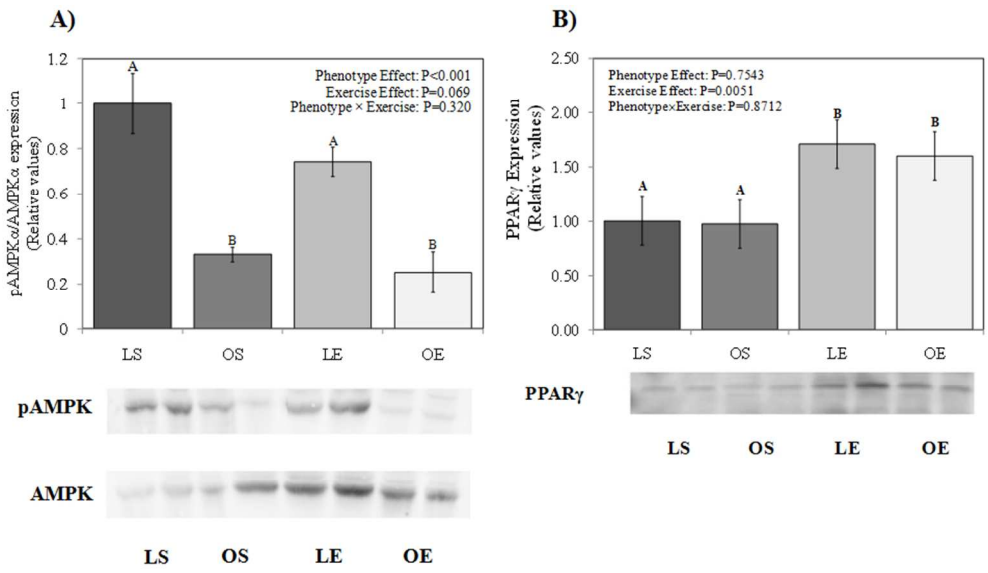
Figure 6



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