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Strength training reduces lipid accumulation in liver of obese Wistar rats

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ABSTRACT

Obesity has a positive relation to non-alcoholic fatty liver disease (NAFLD) and studies have demonstrated that strength training can regulate lipid accumulation in the hepatocytes of obese rats.

Aims: Our aim is to evaluate the effects of high fat diet and strength training on markers of oxidation and lipogenesis in the liver of Wistar rats.

Main methods: Forty Wistar rats were divided into four groups (n = 10): control (CTL), strength training (TR), high fat diet consumption (HF) and high fat diet consumption with strength training (HFT). Animals were subjected to physical strength training and high fat diet consumption for 12 weeks, 3 session per week. Then, the animals were euthanized, and liver markers were evaluated via immunolabeling.

Key findings: Our results indicated that strength training reduced the expression of adiposity as well as the accumulation of glycogen and lipids in the liver. This reduction of fatty acid (FA) stored in hepatocytes is related to reduction of proteins linked to β-oxidation such as Fas/CD95, LIMP-II and CD36, as well as other proteins linked to lipogeneses such as SREBP-1.

Significance: Finally, we observed that high fat diet can alter lipogenesis and reduce β-oxidation promoted hepatic fat accumulation. In conclusion, there was a reduction of obesity-related hepatic lipogenesis after 12 weeks of strength training.

1. Introduction

Hepatic accumulation of abdominal fat, chronic inflammation and insulin resistance are all directly linked to nonalcoholic fatty liver disease (NAFLD) [1], which is the most common chronic disease affecting 25% of the world population [2].

NAFLD is defined as > 5% fat content in the liver and can evolve into nonprogressive fatty liver disease (hepatic steatosis) to the potentially progressive nonalcoholic steatohepatitis which can lead to fibrosis, cirrhosis and hepatocellular carcinoma [2–5]. There is a growing predominance of NAFLD in countries with sedentary lifestyles and high lipid diets which has led to the consideration of obesity as a worldwide epidemic [6]. Considering that the liver is an essential organ for lipid and glycemic control, it is a necessary component of obesity that must be investigated to understand the lipid changes associated with obesity.

There are currently pharmacological therapies for NAFLD, with potential, but their unwanted side effects: weight gain, increased cardiovascular risk (long-term use of rosiglitazone), and increased risk of bladder cancer (pioglitazone), combined with their modest antidyslipidemia activity have reduced their clinical use [1]. All these pharmacological therapies reduce lipogenesis and gluconeogenesis through modulation of FA β-oxidation [2]. On the other hand, physical exercise is a side-effect free alternative strategy in controlling NAFLD [3,4].

Aerobic physical exercise has been shown to reduce weight gain while also slowing the progression and/or preventing NAFLD [3,5]. Batatinha et al. [5] verified that aerobic exercise for 8 weeks reduced triacylglycerol accumulation in the liver while high fat diet led to NAFLD, increased aminotransferase in the serum, increased peripheral insulin resistance, and higher adiposity index in obese trained PPAR-α knockout mice. However, little has been said about the chronic effects of strength training on reduction of white adipose tissue, liver fat [13] and suppression of genes responsible for hepatic lipogenesis [14]. Pereira et al. [4] verified that one session/day for 15 days of strength

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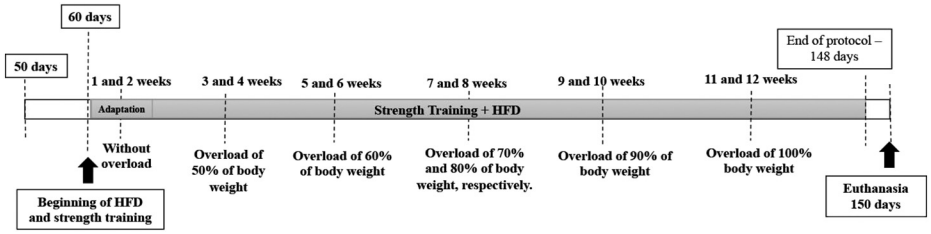

Fig. 1. Timeline of experimental design, representation of the experiments during the strength training and high-fat diet for 12 weeks.

Table 1
Characteristics of primary antibodies to immunohistochemistry.

Primary antibodies	Host species	Dilution	Code
SREBP-1	Rabbit polyclonal	1:100	sc-8984
CD36	Rabbit polyclonal	1:100	sc-9154
LIMPII	Goat polyclonal	1:100	sc-25869
Fas/CD95	Rabbit polyclonal	1:100	sc-1024

training resulted in a reduction of obesity-related hepatic lipogenesis and inflammation leading to improvements in hepatic insulin sensitivity and glycemic homeostasis in obese mice.

Recently, we demonstrated that overtraining protocol for mice based on downhill running sessions increased hepatic fat accumulation and hence, contributes to the development of hepatic steatosis [9]. Therefore, it is necessary to have a better understanding of action mechanisms of lipid reduction promoted by physical exercise and, in this way, to identify ideal type (aerobic and anaerobic), intensity (low, moderate and high) and volume (amount of stimulus for a period) of physical training.

It has not yet been possible to determine the direct effects of exercise on NAFLD, the long-term development of hepatic steatosis and modifications of autophagy proteins. Hence, the aim of this study was to investigate the influence of long-term strength training on the hepatic mechanisms of autophagy, transport and lipogenesis in obesity.

2. Materials and methods

2.1. Ethics statement and biological materials

All animal protocols were approved by the Ethics Committee on the Use of Animals (CEUA) of the School of Technology and Sciences at São Paulo State University-UNESP, campus of Presidente Prudente (Protocol Number: 02/2015). All the procedures were conducted in accordance with the guidelines for experimentation with animals according to Guide to the Care and Use of Laboratory Animals published by the US National Institutes of Health of EUA.

2.2. Experimental design

Forty male Wistar rats (± 200 g) were obtained from the Institute of Bioscience, IBB/UNESP, Botucatu-SP at 50 days old and transferred to the vivarium of School of Technology and Sciences at São Paulo State University-UNESP. Animals were maintained at an average temperature of 22 ± 2 °C under a 12 h:12 h light:dark cycle and had ad libitum access to filtered tap water and standard food chow (Supra Lab-Alisul Ind. Alimentos LTDA, São Leopoldo, RS), composed of 25% carbohydrates, 18% fiber, 11% minerals, 3% lipids, 2% calcium and 0.5% phosphorus.

At 60 days old, the animals were divided into four groups of 10 animals each and received their respective diet and training protocols. The high fat diet group (HF) received 59% of lipids, 28% of carbohydrates and 13% of proteins, and remained sedentary throughout the study, whereas control groups (CTL) were maintained on the standard diet (SD) and remained sedentary. The physical training groups (TR) were also maintained on standard diet but were subjected to physical strength training. The high-fat diet with physical training group (HFT) was supplemented with the high-fat diet and subject to the physical strength protocol. The high fat diet was previously used by Panveloski-Costa [10] that was composed of bacon, mortadella, sausage and wafer, and animals were provided soda instead of water. All animals had ad libitum access to food and water or soda throughout the duration of the experiment and were weighted and measured weekly until the end of the study.

2.3. Physical strength training

The physical strength training protocol was initially proposed and described by Tamaki [11]. In brief, animals were placed vertically on a metal platform using a fitted vest attached to a wooden bar. The wooden bar sustained an adjustable weight load that the animals were required to push upwards when prompted. An electrical stimulus was delivered to the tail through a metal clip attached to an electrical stimulator to prompt rats to perform a full hindleg extension through the knee and ankle, otherwise known as a *squat jump movement*. The squat jump movement has previously been tested and validated as an inducer of hypertrophy in rodents [11], three times per week, the animals performed squat jumps in three sets of 12 repetitions with a one-minute pause between sets. Weight load was adjusted weekly according to the rat's body mass as described below, and the training onset was accompanied with the high fat diet induction at 60 days old.

During the two first weeks of training, the rats were habituated to the training apparatus without weight load. A weight load equivalent to 50% of the animal's body weight was attached to the animal vest during the third week of training and increased to 60% during the fourth week of training. The overload was increased to 70% during fifth and sixth weeks. This overload was increased to 80% at the seventh week until the eighth week, 90% from the ninth until the tenth week and the overload was increased to 100% of animal's total body weight at eleven until twelve weeks. Forty-eight hours after the last physical training session the animals were euthanized at 150 days old intraperitoneally with thiopental sodium anesthetic (60 mg/kg of body weight). Following euthanasia, an abdominal pelvic laparotomy was performed to the liver and adipose tissue, which were then weighed and processed for subsequent analysis (Fig. 1).

2.4. Histology, stereological analysis and lipid accumulation

Tissue was fixed in formalin solution for 24 h, washed in 70% alcohol, dehydrated, diaphonized, para-plasticized, and cut at a thickness of 5 μm for analysis. The blades were stained with hematoxylin and eosin (H&E) to analyze histological changes by differentiating cells from surrounding connective tissue and to identify cytoplasm and nuclei. The stereology (hepatocyte cytoplasm, connective tissue and nucleus) of liver cross sections was quantified using a Weibel reticle of 160 points [12], 10 images per slides were quantified per sample. Hepatic steatosis was assessed by the point counting method [13,14]. Five slides of H&E (per sample) were performed and 10 microscopic fields were

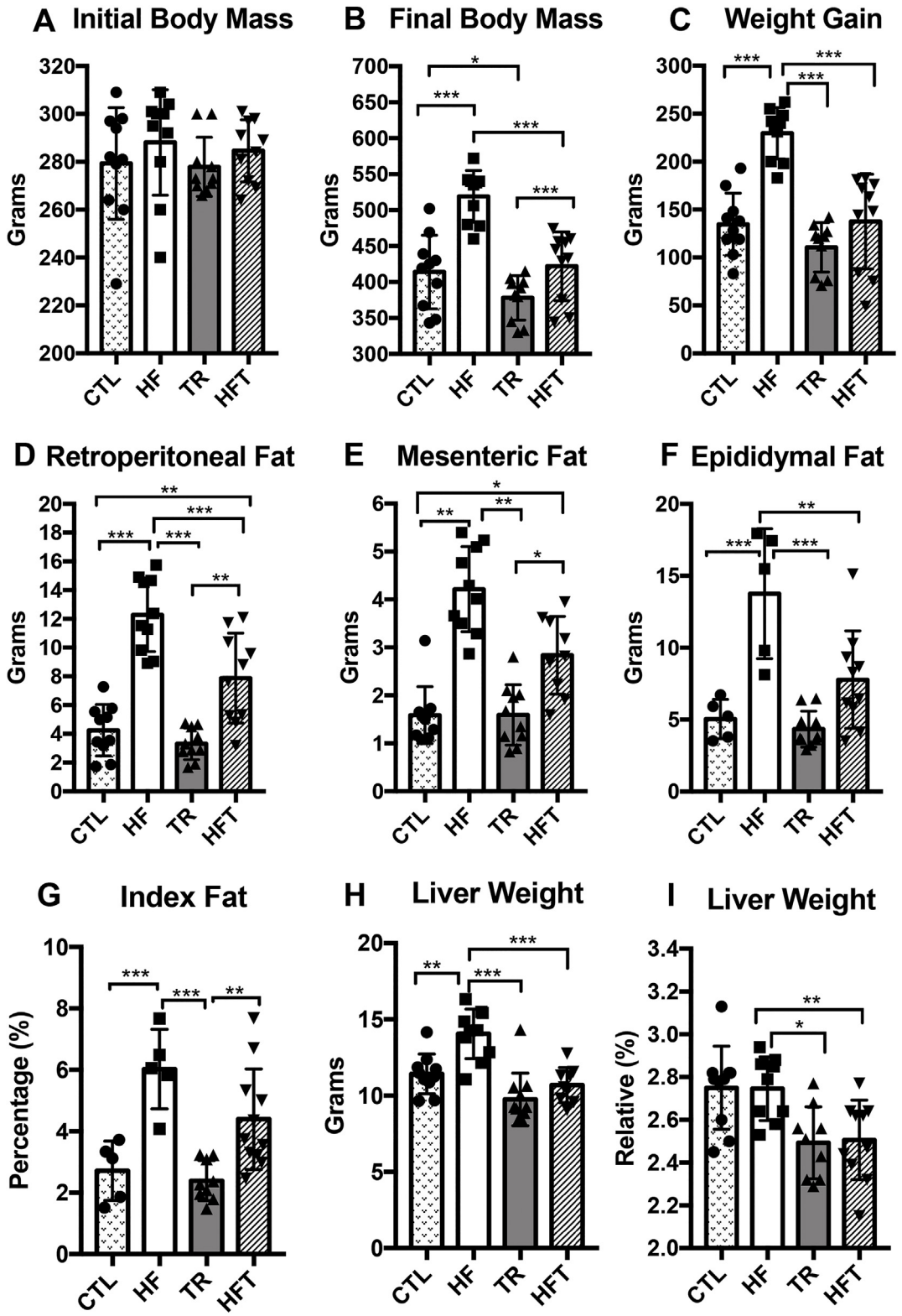

Fig. 2. The body weight, fat weight, liver weight of CTL, HF, TR and HFT groups. (A, B and C) Body mass beginning and at the end of experiment and weight gain. (D, E and F) absolute weight of retroperitoneal fat, mesenteric fat and epididymal fat. (G) index fat (%). (H and I) absolute and relative liver weight. One-way ANOVA followed by Tukey post hoc test. *$p < 0,05$, ** $p < 0,001$ and ***$p < 0,0001$.

selected at random for estimating volume density (Vv) of liver steatosis by the point-counting method. To assess hepatocyte size, we used ten randomly selected fields, H&E stained regions in each microscopic field which were counted under 400× magnification to ascertain cell numbers of hepatocytes. The findings are presented as numbers of cells per field.

Liver samples were submitted to histologic techniques to identify the collagen as masson's trichrome and periodic acid schiff (PAS) to identify the glycogen. Images were acquired at 40× resolution, the intensity of staining of glycogen and connective tissue was examined in

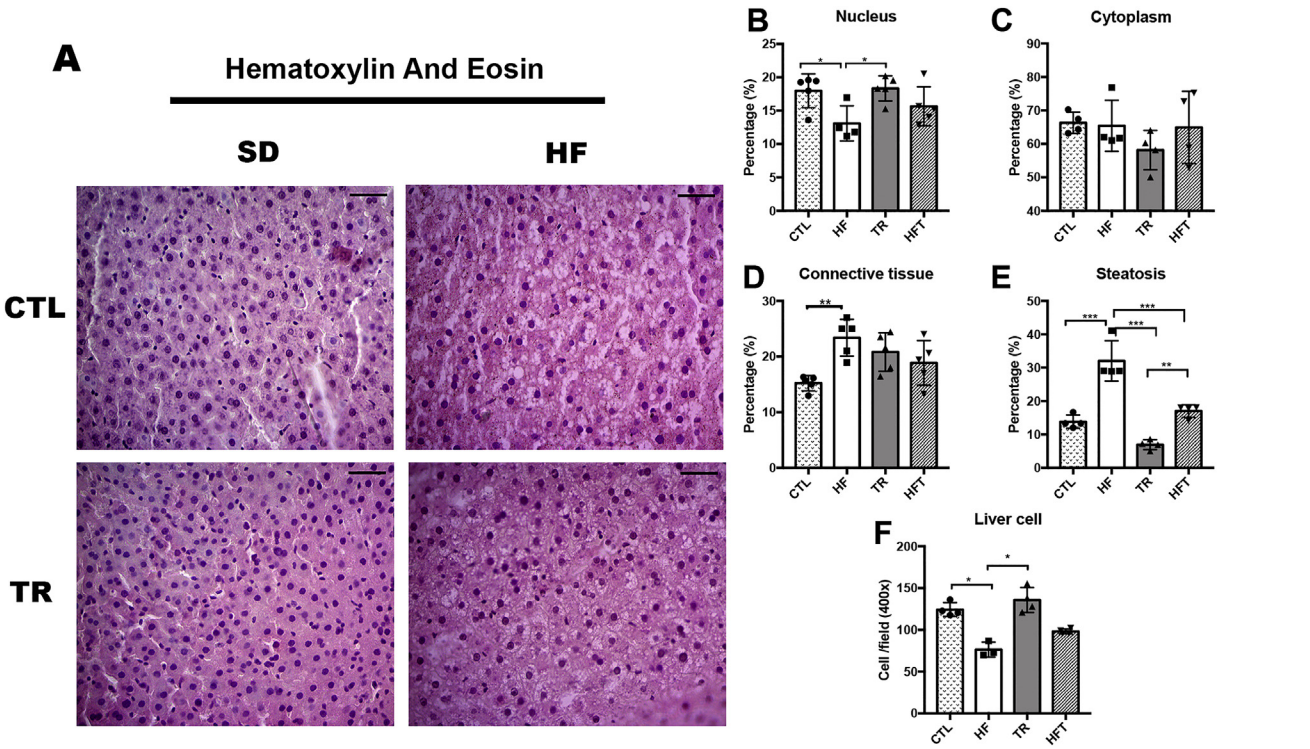

Fig. 3. The hepatic fat content and volume analysis of CTL, HF, TR and HFT groups. The diet types are represented by SD (standard diet) and HF (high fat diet). (A) Hematoxylin and eosin staining of the right lobe for the four experimental groups. Hepatic steatosis tissue volume quantitation for microvesicular fat (B), connective tissue (C) cytoplasm without fat (D) and nucleus (E) for the four treatment groups. One-way ANOVA followed by Tukey post hoc test. *p < 0,05, ** p < 0,001 and ***p < 0,0001. Magnification of 400×, Bar = 20 μm.

10 fields per animal using Image-J software (version 1.50i) and the percentage of tissue labeling was quantified for each image. Tissue was analyzed using a AxioCam ER.c 5 s (Zeiss Primo Star, Germany) photomicroscope with 40× resolution at the Department of Physical Education of São Paulo State University.

2.5. Immunohistochemistry protocol

All liver tissues were fixed in formalin and embedded in paraffin to prepare 5 μm sections for immunolabeling. Antigenic recovery was performed in pressure cooker (Electrolux Chef), and endogenous peroxidase activity was blocked with 3% of hydrogen peroxide diluted in methanol for 15 min. Subsequently, tissue was blocked with 3% bovine serum albumin (BSA), diluted in TBS-T (1% Triton X-100, 100 mM of Tris, pH 7.4), for 1 h and incubated with polyclonal primary antibodies to SREBP-1, CD-36, LIMPII and Fas/CD95 (Table 1) diluted in 1% BSA per overnight (12 h). The following day, the sections were incubated with secondary goat anti-rabbit HRP antibody (Santa Cruz sc-2030; 1:200) diluted in 1% BSA for 2 h. The liver sections of each experimental group were evaluated through the brownish precipitate of diaminobenzidine that was used as the chromogen indicating immunoreactivity and then stained with hematoxylin. For all markers positive and negative controls were performed [15].

Liver samples were acquired using photomicroscope Zeiss Axiophoto (Zeiss, Munich, Germany) at 40× resolution, the intensity of immunoreactivity of CD36, LIMP-II, SREBP- and Fas/CD95 antigens was examined in 10 fields per animal using Image-J software (version 1.50i) and the percentage of tissue marking was quantified for each image.

2.6. Statistical analysis

All data are expressed as the mean ± standard error of the mean. According to the Shapiro-Wilk test, the data were normally distributed, and homogeneity was confirmed by the Levine test. One-Way ANOVA with a post-hoc Tukey test was used to statistically analyze the data. Statistical analysis was performed using Sigma Plot 12.5 and GraphPad Prism 6.0 software. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Strength training, weight gain and adiposity

After 12 weeks of obesity induction the animals showed a significant increase in final body mass and weight (Fig. 2B–C), when compared to the other groups. After 12 weeks of strength training, the trained animals, TR and HFT, showed significant differences in body mass and weight gain (Fig. 2B–C) when compared with CTL and HF groups. There was also a significant difference between the groups in terms of adipose reserves (Fig. 2D–F). The HF group increased retroperitoneal, mesenteric and epididymal fat and consecutive increased fat index (Fig. 2D–F and G) when compared with other groups. Animals subjected to strength training and HF showed an increase in adipose tissue and fat index (Fig. 2D–F and G) compared with HF and CTL.

Long-term strength training decreased liver weight, glycogen and fatty liver accumulation.

In the next step, we evaluated the lipid deposits and TG levels in the rat livers after HF and HFT. Initially, we observed that HF group increased absolute and relative liver weight (Fig. 2H and I) when compared with TR and HFT groups. The HFT reduced absolute liver weigh by 28% compared with HF in 12 weeks. However, the TR group showed a reduction in lipid droplets size and microvesicular fat (Fig. 3), when compared with HF and HFT groups. Analyses of morphology and hepatic steatosis count with hematoxylin and eosin staining showed that the HF group had higher lipid stocks when compared with other groups,

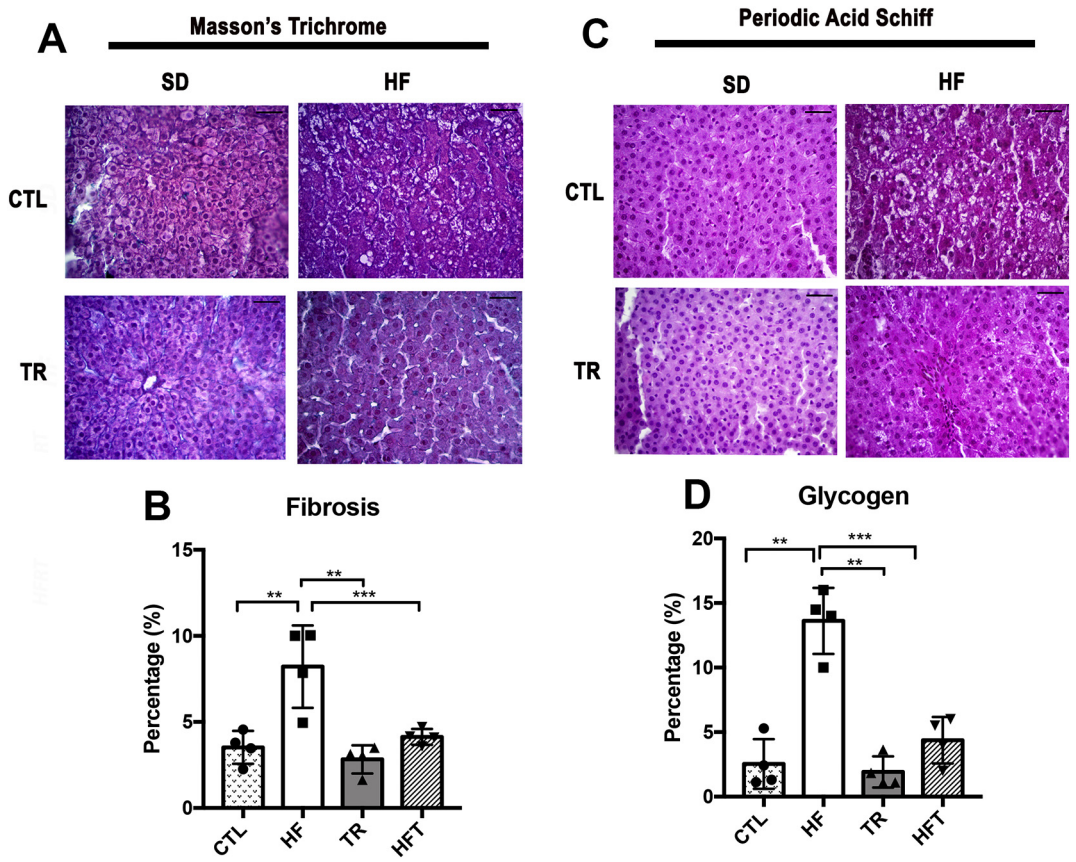

Fig. 4. Data of liver fibrosis where the diet types is represented by SD (standard diet) and HF (high fat diet). Photomicrograph of cells with collagen staining (masson's trichome). Liver histological cross sections are from four groups (A) and a quantification of percentage of fibrosis (B). Photomicrograph of cells with glycogen (Periodic acid schiff). Liver histological cross sections are from four groups with glycogen stain (C) and a quantification of percentage of glycogen (D). One-way ANOVA followed by Tukey post hoc test. *$p < 0,05$, ** $p < 0,001$ and ***$p < 0,0001$. All photomicrographs magnification 400×, bar = 20 μm.

and lipids accumulation is reduced with strength training in the HFT group when compared with the HF group (Fig. 3A). The quantification of cell volume showed an increase in connective tissue volume and reduction in nucleus volume resulting in hepatocyte hypertrophy in the HF group, the treatment with the lowest number of hepatocytes per area (Fig. 3A–E) when compared with TR and CTL groups. These results were confirmed with masson's trichome staining, for identifying connective tissue for fibrosis, and was verified HF group presented more fibrosis compared with other groups (Fig. 4A and B). Similar data were observed for the hepatic glycogen levels, in which the strength training reduced the glycogen levels stored in the liver of obese animals (Fig. 4B and D) when compared with HF group.

Strength training reduces lipogenesis, increases lipid oxidation.

Finally, we tested the role of hepatic Fas/CD95 activation in obesity-induced metabolic dysregulation. We observed HF group increased immunoreactivity of Fas while strength training reduced this expression when associated with HF (Fig. 5A and B). These results indicate that hepatic Fas depletion protects rats at least partly from obesity-induced lipid accumulation and possibly insulin resistance in the liver. On the other hand, CD36 expression in normal hepatocytes is low, but is elevated with HFD (Fig. 5C and D). Strength training was also shown to reduce the obesity-related increase in CD36 as shown via immunohistology (Fig. 5C and D).

Immunohistochemistry of LIMP-II (Fig. 6A and B) showed an increased expression in HF group, suggesting that the vesicle accumulation was a result of an alteration in triglyceride transporters. Morphological analysis showed that loss of autophagic vacuoles and LIMP-II reduction occurred across long-term strength training and suggests that autophagy may be suppressed and promote liver regeneration. In addition, we found increased SREBP-1 levels in HF group, and long-term strength training resulted in an approximately 33.3% reduction in its activity, reducing lipogenesis and consecutively hepatic triglycerides (Fig. 7).

4. Discussion

Dysregulated hepatic lipid metabolism can be induced with a high fat diet and plays an important role in various metabolic diseases. The HFD increases the accumulation of lipids and may impair hepatocyte function promoting liver injury and insulin resistance [16]. The deposition of triglycerides in hepatocytes, modification in lipogenesis and mitochondrial failure due to high concentration of FAs are environmental factors that can promote metabolic diseases such as NAFLD [17]. We demonstrated that 12 weeks of exposure to HFD was efficient to promote environment of obesity and NAFLD. The present study identifies strength training as a regulator of hepatic function reducing transport, and accumulation of hepatic fat and glycogen reflecting improved insulin sensitivity. We observed the reduction of hepatic lipids was accompanied by reduction of CD36, Fas/CD95, SREBP1 and LIMP-2 levels.

Mitochondrial FA oxidation is the dominant oxidative pathway for the removal of FAs under normal physiological conditions and the literature provides evidence that aerobic training is an effective method for reducing liver fat deposits by increasing β-oxidation and activating genes linked a lipogenesis [18–20]. In the review by Berzigotti et al. [21], it was found that aerobic physical exercise combined with caloric restriction elevated mitochondrial β-oxidation, oxidative enzymatic function, reduced glucose tolerance, hepatic suppression and

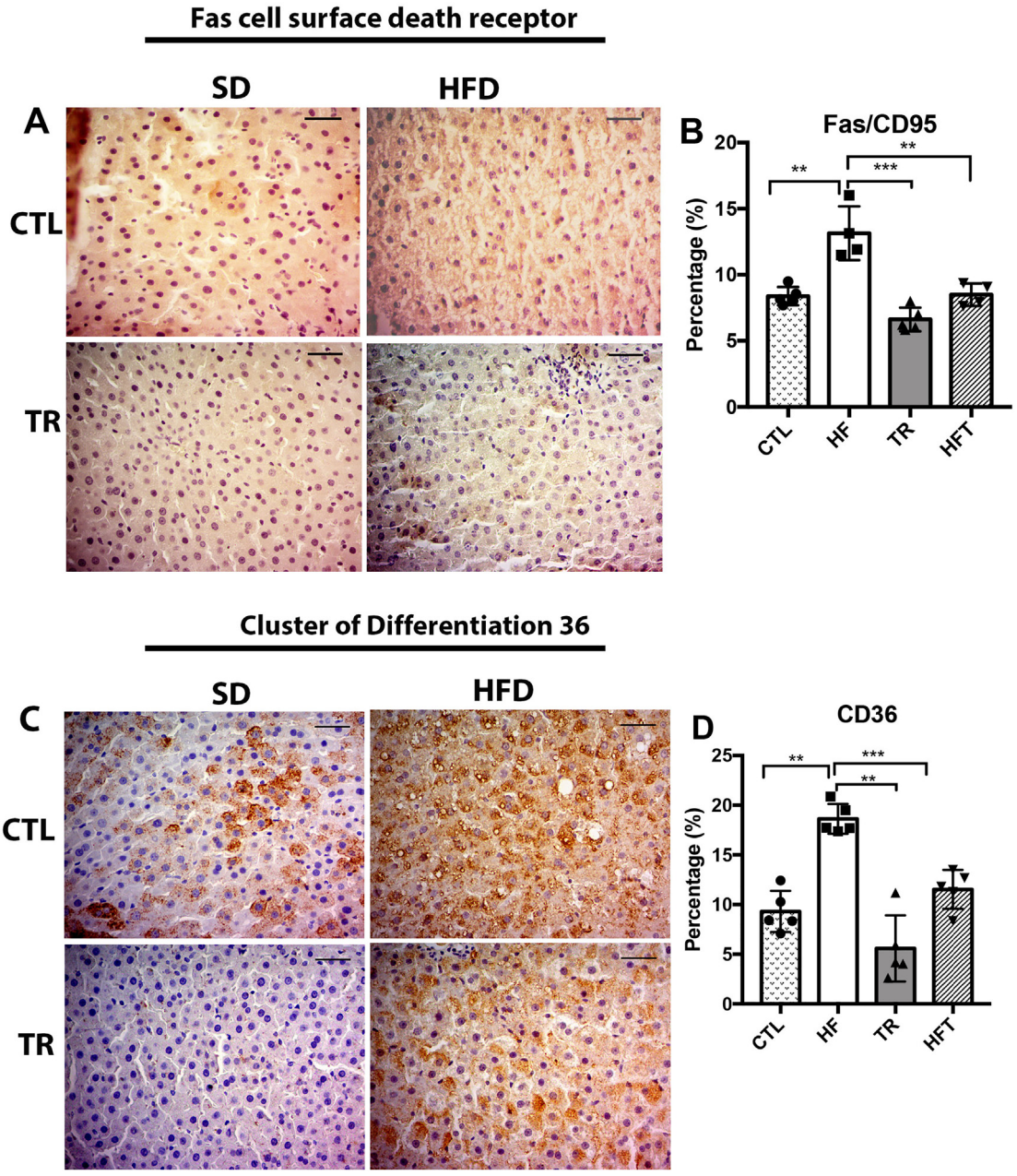

Fig. 5. Immunoreactivity in liver of Fas/CD95 and CD36 of CTL, HF, TR and HFT groups. The diet types is represented by SD (standard diet) and HF (high fat diet). (A and B) Photomicrograph of cells with Fas/CD95 staining and quantification of staining. (C and D) Photomicrograph of cells with CD36 staining and quantification of staining. One-way ANOVA followed by Tukey post hoc test. *p < 0,05, ** p < 0,001 and ***p < 0,0001. All photomicrographs magnification 400×, bar = 20 μm.

lipogenesis and was efficient at treating NAFLD hepatocellular carcinoma and cirrhosis. However, effects of strength training and potential effects on hepatic adiposity have been little explored in the literature. Our study showed that strength training was effective in reducing the size of fat droplets and reducing the percentage of steatosis and the amount of glycogen stored in the liver. These results reinforce that strength training may be considered another strategy for the treatment of NAFLD.

In the present study, we observed that body weight, body weight gain, liver weight, relative liver weight and visceral fat, were markedly increased in HFD-fed rats. The increase of visceral adipose tissue (VAT) in HFD group was linked to increased hepatic insulin sensitivity. Abdominal VAT volume increased with obesity and is consistent with a higher risk for type 2 diabetes [22,23]. Whole-body and hepatic insulin resistance correlates positively with volume of VAT [22]. Insulin inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis and indirectly reduces free FAs generated from visceral fat [27,28]. Enhanced cytoplasmic FA delivery to the liver, resulting from higher catecholamine response in VAT, may promote hepatic and whole-body insulin resistance, ectopic fat deposition and result in hepatic steatosis [23,26]. In this study we observed increased microvesicular fat in liver HF rats related with increased VAT. Little is known about the effects of physical strength training on variables linked to hepatic steatosis. Recently, Pereira et al. [4] observed that short-term strength training reduced the levels of hepatic steatosis and inflammation in the liver of rodents fed with high fat diet. In this context, it was observed that long-term strength training promoted greater metabolic protection, lowering fat levels in the liver and VAT.

The Fas (CD95/Apo-1) glycoprotein belongs to the tumor necrosis

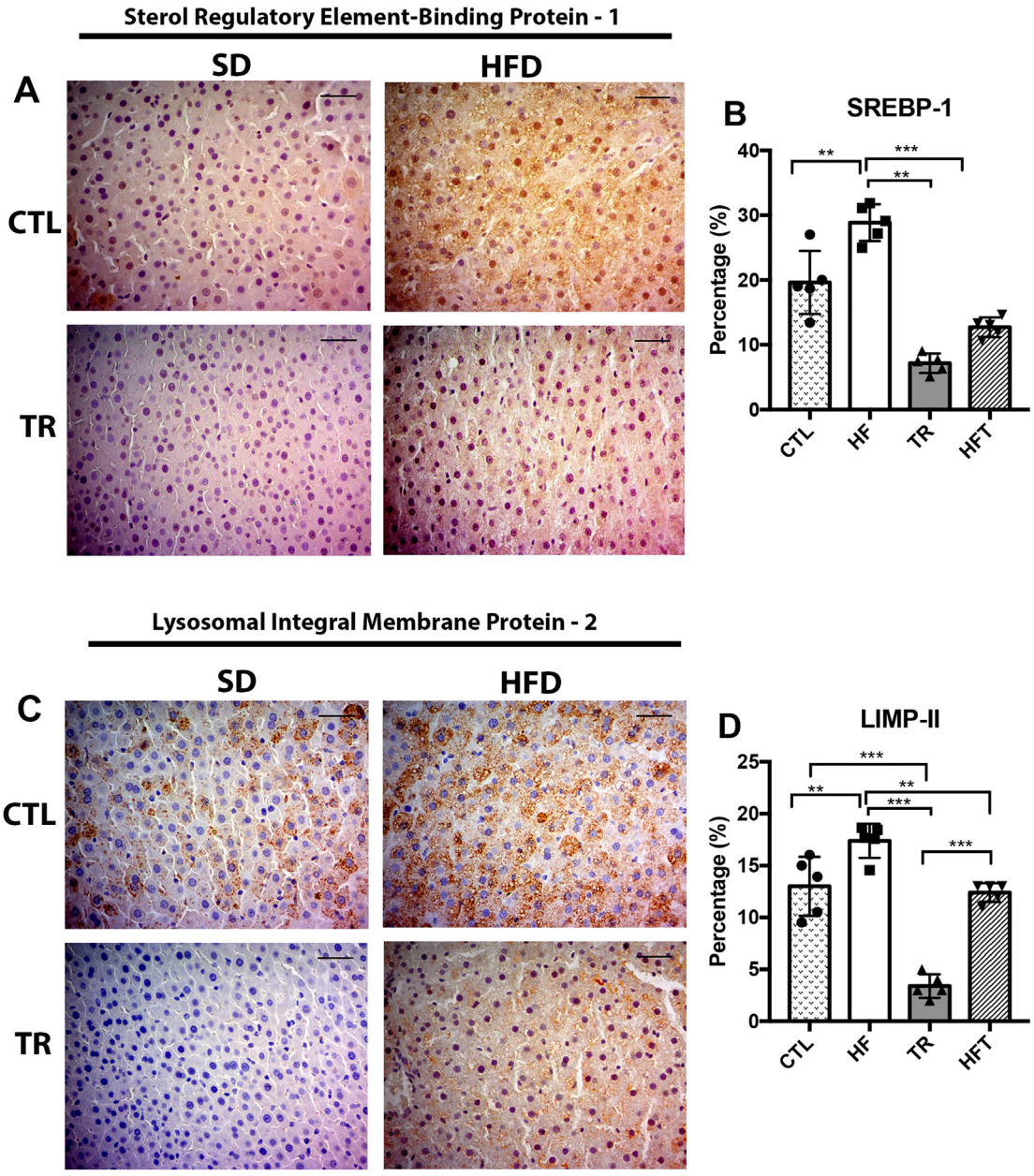

Fig. 6. Immunoreactivity of SREBP-1 and LIMP-II of CTL, HF, TR and HFT groups. The diet types is represented by SD (standard diet) and HF (high fat diet). (A and B) Photomicrograph of cells with SREBP-1 staining and quantification of staining. (C and D) Photomicrograph of cells with LIMP-II staining and quantification of staining. One-way ANOVA followed by Tukey post hoc test. *p < 0,05, ** p < 0,001 and ***p < 0,0001. All photomicrographs magnification 400×, bar = 20 μm.

factor (TNF) receptor superfamily. Ligation on the Fas receptor initiates proteolytic cleavage of intracellular caspase and results in apoptosis [27], and it is identified as another key protein in the development of NAFLD [28]. Consistently, Fas may regulate the hepatic mitochondrial caspase-8/BID/Bax pathway resulting in membrane permeabilization and cytochrome *c* release [29]. Furthermore, it has been suggested that increased hepatic Fas expression contributes to obesity-associated fatty liver disease and insulin resistance by impairing mitochondrial FA oxidation, respiration, and the abundance of respiratory complexes [28]. Recent studies have shown that HFD-fed FasL knockout mice reduced triglyceride levels in hepatocytes [27]. Our findings showed that HFD promoted increased Fas in the liver, affirming the potential of NAFLD. The downregulation of Fas is a promising treatment of hepatic steatosis and insulin resistance [28]. In this study, strength training reduced Fas levels, reinforcing the potential effect of exercise on lipid regulation.

Other important protein that predicts lipid over-accumulation is CD36 a hallmark of NAFLD. CD36 is an fundamental membrane glycoprotein facilitating FA uptake [30]. Furthermore, CD36 plays an important role in mediating signal transduction, modulating FA β-oxidation, which contributes to the maintenance of cellular FA homeostasis [17,31]. Little is known about the effects of strength training on NAFLD and its impact on the regulation of CD36. Our study is the first to demonstrate that strength training can decrease CD36 levels and reduce the severity of hepatic steatosis.

The LIMP-II lysosomal integral membrane protein is considered a member of the CD36 gene family [32]. Overexpression of LIMP-II impaired the endocytic membrane traffic out of these enlarged compartments, likely caused an accumulation of cholesterol, and resulted in large swollen vacuoles [32,33]. We observed that the HF diet promoted increased expression of LIMP-II as well as more accumulation of cholesterol and glycogen in hepatocytes. Singh et al. [34] showed evidence

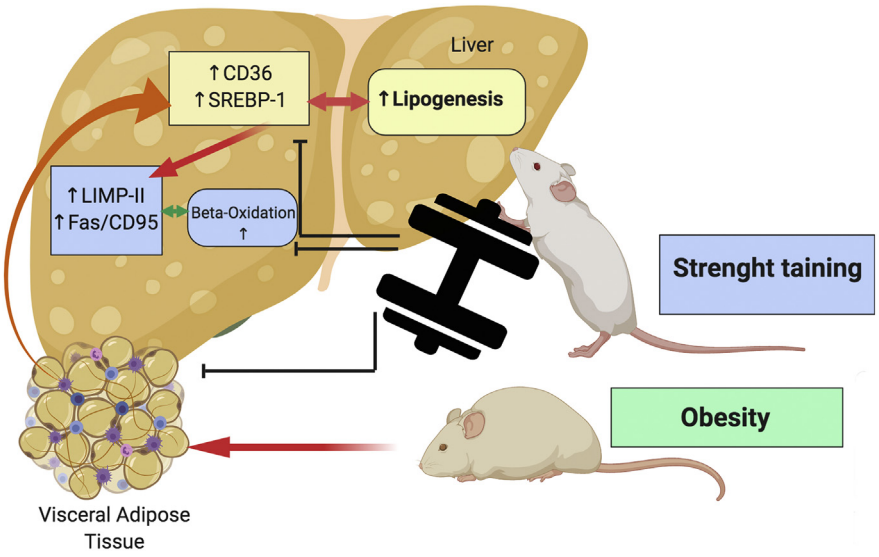

Fig. 7. Role of strength training per 12 weeks on lipid metabolism in the liver, associated the HFD. Obesity provides insulin resistance, fasting hyperglycemia and increase of lipid synthesis mechanisms in the liver, increasing protein level of CD36, LIMP-II Fas/CD95 and SREBP-1 activity. On the other hand, strength training provided the opposite effects together reduction in body adiposity reduced the lipogenesis and transport of FA increased oxidation of lipids. Therefore, we demonstrated that the exercise protocol could be an important tool in the combat and/or treatment of NAFLD.

supporting the hypothesis that lipid droplets in hepatocytes can enter the autophagic degradation pathway in the same manner as damaged proteins or organelles via formation of autophagosomes that carry the cargo for final degradation to the lysosomes. Some studies support the idea that triglycerides are degraded in lysosomes and require greater autophagic flow for this process [35,36]. If overexpression of lysosomal membrane proteins (LIMP-II) causes morphological changes in late endosomes and lysosomes by perturbing membrane equilibrium between these compartments, it could be possible that lysosomal membrane proteins themselves are involved in the membrane traffic between late endosomes and lysosomes. We observed that strength training, directly or indirectly, regulates components of the machinery regulating the late endosomal/lysosomal compartment.

Additionally, an increase of β-oxidation was observed to improve mitochondrial function and lipid transport, however, it was verified that a reduction in the transcription of genes involved in lipogenesis reduced the levels of SREBP-1 protein in the liver of trained animals. SREBP-1 is a key transcriptional factor that can regulate the expression of hepatic lipogenesis and glycolysis pathways genes, such as L-pyruvate kinase, acetyl-CoA carboxylase (ACC), FA synthase (FAS), stearoyl-CoA desaturase 1 (SCD-1), and mitochondrial glycerol-3-phosphate acyltransferase 1(mtGPAT1) [37]. Numerous studies suggest that an increase in immunoreactivity of SREBP-1 has been observed in hepatic steatosis [38]. Consequently, the reduction of lipogenesis may be considered another positive effect by which strength training acts against HF induced NAFLD.

5. Conclusion

It was observed that strength training regulated Fas/CD95, proteins involved in hepatic mitochondrial function and FA oxidation, reduced the FA levels of transporters such as CD36, and LIMP-2 protein responsible for lysosome/endosome transport of lipids impacting the accumulation of lipids for reduced SREBP-1, and consequently reduced the hepatocyte size. In conclusion, our results reveal an important and unique role of strength training that may regulate hepatic mitochondrial function, lipid metabolism, contribute to the reduction of obesity and NAFLD. This study provided new evidence supporting long-term strength training as a promising new avenue in the treatment of hepatic steatosis and problems related the obesity.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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