Combination of Aerobic Training and Cocoa Flavanols as Effective Therapies to Reduce Metabolic and Inflammatory Disruptions in Insulin-Resistant Rats: The Exercise, Cocoa, and Diabetes Study.

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Combination of aerobic training and cocoa flavanols as effective therapies to reduce metabolic and inflammatory disruptions in insulin-resistant rats: The ECODIA (Exercise Cocoa and Diabetes) study

ABSTRACT

We aimed to investigate the combined effects of aerobic exercise (EXE) and cocoa flavanol (COCOA) supplementation, on performance, metabolic parameters, and inflammatory and lipid profiles in obese insulin-resistant (IR) rats. Therefore, thirty-two male Wistar rats (230-250 g) were fed a high-fat diet and a fructose-rich beverage for 30 days to induce IR. Next, the rats were randomized into four groups, orally administered placebo solution or COCOA supplementation (45 $mg \cdot kg^{-1}$), and either remained sedentary or were subjected to EXE on a treadmill at 60% peak velocity for 30 min, for eight weeks. Blood samples and peripheral tissues were collected and processed to analyze metabolic and inflammatory parameters, lipid profiles, and morphological parameters. Supplementation with COCOA and EXE improved physical performance and attenuated body mass gain, adipose index, and adipocyte area. When analyzed as individual interventions, supplementation with COCOA and EXE improved glucose intolerance and the lipid profile, reduced the concentrations of leptin, glucose, and insulin, and reduced HOMA index (all effects were $p<0.001$ for both interventions), while ameliorated some inflammatory mediators in examined tissues. In skeletal muscles, both COCOA supplementation and EXE increased the expression of GLUT4 ($p<0.001$ and $p<0.001$), and combined intervention showed additive effects ($p<0.001$ *vs.* COCOA alone or EXE alone). Thus, combining COCOA with EXE represents an effective non-pharmacological strategy treating insulin resistance and could prevent type 2 diabetes mellitus by improving physical performance, glucose metabolism, neuroendocrine control, and lipid and inflammatory mediators in the liver, pancreas, adipose tissue, and skeletal muscle in obese male insulin-resistant rats.

Keywords: Type 2 diabetes. Cytokines. GLUT4. Polyphenols. Physical exercise.

1. Introduction

Type 2 diabetes mellitus (DM2) is a multifactorial disease characterized by a chronic hyperglycemic state due to reduced glucose uptake in peripheral tissues that can be associated with increased hepatic glycogenolysis and gluconeogenesis, and both of which can be attributed to insulin resistance (DeFronzo et al., 2015). Hyperglycemia, hyperinsulinemia, dyslipidemia, and low-grade inflammation are the main DM2-induced metabolic dysfunctions that have been associated with overweight and obesity (Skyler, 1979). Globally, consumption of high-calorie diets and physical inactivity are important factors underlying the development of obesity and insulin resistance (Czech,

2017). Therefore, nutritional interventions and regular physical exercise are crucial non-pharmacological strategies for preventing and treating DM2-induced metabolic dysfunctions (Raveendran et al., 2018).

Aerobic exercise improves insulin sensitivity and attenuates the hyperglycemic state in DM2 (Kumar et al., 2019). In addition, the increase in glucose uptake in peripheral tissues, independent of insulin action, has been associated with the beneficial effects of aerobic exercise (Evans et al., 2019). Long-term aerobic exercise enhances skeletal muscle and adipose tissue expression of the glucose transporter (GLUT4), reduces body weight, increases antioxidant capacity, improves the inflammatory and lipid profiles, and helps ameliorate metabolic complications observed in DM2 (Yaribeygi et al., 2019). Recent reports have revealed that moderate-intensity aerobic exercise increases physical performance and can be associated with lower mortality risks due to cardiovascular disease in patients with DM2 (Grace et al., 2017). Although the benefits of aerobic training are well described in the literature, its effects alongside nutritional interventions, such as chronic consumption of polyphenols during obesity and insulin resistance, remain poorly understood.

Polyphenols are organic compounds present in several plants and can be categorized into flavonoids, stilbenes, phenolic acids, and lignans (Fraga et al., 2019). Long-term polyphenol consumption has been associated with several health benefits (Cory et al., 2018). For example, diets rich in flavanols, a subgroup of flavonoids found in green tea, some vegetables, and abundantly present in cocoa, have proven to be an important strategy for reducing the risk of developing obesity (Coronado-Cáceres et al., 2019), diabetes mellitus (Ramos et al., 2017), and cardiovascular diseases (Ludovici et al., 2017).

The bioactive compounds present in cocoa (*Theobroma cacao*), such as (+)-catechin and (–)-epicatechin, possess several biological properties, including anti-inflammatory, antioxidant, and antimicrobial activities (Prakash et al., 2019). However, although cocoa-derived flavanols can increase insulin secretion and sensitivity in peripheral tissues, as well as prevent oxidative and inflammatory damage associated with DM2 (Flores, 2019; Ramos et al., 2017), their combined effects with aerobic training on insulin resistance and associated metabolic dysfunction have rarely been reported.

In overweight and obese individuals, aerobic exercise and high-flavanol cocoa supplementation reduced insulin resistance (HOMA-IR) and improved cardiometabolic risk factors, with no additive effects observed when combining both interventions (Davison et al., 2008).

Conversely, supplementation with flavanol (-)-epicatechin for 15 days facilitated additive effects on cardiac angiogenesis when combined with aerobic exercise in mice, leading to increases in the levels of vascular endothelial growth factor and capillary density (Ramirez-Sanchez et al., 2012). However, further studies are needed to investigate the effects of combining of cocoa flavanols and physical exercise on aerobic fitness and clinically relevant markers of metabolic dysfunction, inflammation, and dyslipidemia, as well as their underlying mechanisms. Furthermore, the physiological and molecular mechanisms underlying the potential improvements conferred by a combination strategy (aerobic exercise + cocoa flavanols supplementation), particularly on aerobic performance, metabolism, and inflammatory mediators, remain unclear in healthy or insulin-resistant populations.

Furthermore, the physiological and molecular mechanisms underlying the potential improvements conferred by a combination strategy, particularly on aerobic performance, metabolism, and inflammatory mediators, remain unclear in healthy or insulin-resistant (IR) populations. Therefore, we aimed to explore the combined effects of aerobic training and chronic supplementation with cocoa flavanols on aerobic performance, metabolism, and inflammatory and lipid mediators in obese IR rats. We hypothesized that cocoa flavanols and aerobic exercise could provide additive effects on metabolism and inflammatory mediators in obese IR rats.

2. Methods

2.1.Animal and Ethics Care

This study was approved by the Ethics Commission on the Use of Animals (CEUA-UFMG, protocol 110 / 2016) and carried out following the guidelines established by the National Council for Animal Control and Experimentation (CONCEA-Brazil). Thirty-two Wistar male rats (230-250 g) from the Biological Sciences Institute of the Federal University of Minas Gerais were used in this study. The rats were placed in collective boxes (4 rats per box) and kept in the vivarium of the Exercise Physiology Laboratory with controlled temperature (24 ± 1ºC) and light (lights on from 05:00 am until 07:00 pm) throughout the experimental protocol.

2.2.Experimental design

Insulin resistance associated with obesity was induced through the consumption of a high-fat diet combined with a fructose-rich beverage taken *ad libitum* for 30 days (Melo et al., 2021). The induction of insulin resistance through hypercaloric diets has been considered an experimental strategy with relevant ecological validity, once it promotes the metabolic alterations that are

observed in humans. Indeed, the consumption of diets that are rich in fructose and fat has had its presence increased in the nutrition habits of modern society, contributing to an increased risk of developing insulin resistance and metabolic syndrome. Therefore, the insulin resistance induction model we used in the present study proved to be a useful and effective model for studying insulin resistance in Wistar rats by mimicking insulin resistance metabolic, inflammatory, and morphological alterations observed in humans (Melo et al., 2021). Glucose intolerance was confirmed through the oral glucose tolerance test (OGTT) performed before and after the diet period (Fig. 1). Next, the rats were submitted to an incremental exercise test on a treadmill to assess their aerobic performance. Then, the rats were balanced according to the peak velocity (i.e., the highest achieved speed) (Vpeak) obtained in the incremental test and then separated into four groups (n = 8 rats/group), which were submitted to oral gavage of placebo solution (PLA) or cocoa flavanols (COCOA) and remained resting (SED) or performed treadmill running exercise at 60% Vpeak intensity for 30 minutes (EXE). PLA, COCOA and/or EXE interventions were performed once a day, five days a week for eight weeks. In the other two days per week, the rats were maintained at restat the Bioterium without any physical exercise or experimental supplementation. The rats were maintained with a high-fat diet combined with a fructose-rich beverage taken *ad libitum* during all intervention periods.

Insert Figure 1

The aerobic performance test and the OGTT were also performed at the end of the last week of intervention to assess the possible effects of chronic supplementation of cocoa flavanols and/or aerobic training on these variables. Forty-eight hours after the last intervention session, the rats were euthanized after 6-hour fasting.

2.3.Oral Glucose Tolerance Test (OGTT)

Glucose intolerance was confirmed by OGTT after 6-hour fasting. Blood samples were obtained by lancing at the distal end of the tail at 0 (baseline), 15, 30, 60, 90, and 120 minutes after oral gavage of glucose solution (1 $g \cdot kg^{-1}$, 40% solution, Sinth®). Capillary glycaemia was measured with a glucometer (Accu-Check Performa®, Roche Diagnostic, Meylan France).

2.4.Placebo or Cocoa flavanols supplementation

The supplementation of cocoa flavanols consisted in flavanol-enriched cocoa powder (compounds ($g \cdot 100g^{-1}$): 1.39g (+)-catechin; 6.10g (-)-epicatechin, 0.89g caffeine; 5.01g theobromine, NATUREX® Powder, Avignon France) dissolved in water ($18\ mg \cdot mL^{-1}$). The placebo solution was equilibrated with cocoa flavanols supplementation in caffeine and theobromine content

(compounds ($g \cdot 100g^{-1}$): 0.89g caffeine, 5.01g theobromine, SIGMA-ALDRICH®, Missouri, EUA). Both cocoa flavanols supplementation and placebo were always administered at the same time of the day (7:00-8:00 am) by oral gavage (45 $mg \cdot kg^{-1}$ body mass), one hour before physical exercise. We chose to do the gavage one hour before the start of the exercise, because the peak concentration of (+)-catechin and (-)-epicatechin in the plasma of rats occurrs between 60 and 75 minutes after oral gavage with a cocoa flavanols solution (Seigo Baba et al., 2001).

2.5.Incremental running test

The rats were initially familiarized with running on a treadmill (Panlab/Havard Apparatus, Cornella Spain) by 10 minutes during five consecutive days before the first incremental test. The velocity was adjusted daily (10, 10, 11, 13, and 15 $m \cdot min^{-1}$), with a fixed inclination (5%) and electrical stimulus of 0.2 mA.

The incremental test running on a treadmill (Panlab/Havard Apparatus, Cornella Spain) started at a velocity of 10 $m \cdot min^{-1}$ and consisted of increments of 1 $m \cdot min^{-1}$ every three minutes. The treadmill inclination and electrical stimulation were the same as those used in the familiarization sessions. Fatigue was determined if the animals stayed on the electrical stimulation grid for 10 uninterrupted seconds. The oxygen uptake was continuously measured during the incremental test by open-flow indirect calorimetry (Gas Analyzer Panlab/Havard Apparatus, Cornella Spain). The values of the last 30 seconds at the end of each stage was used to determine oxygen uptake values. The peak oxygen uptake ($\dot{V}O_{2peak}$) was considered the highest value obtained during the last stagy of the incremental protocol. The total external work was calculated using the following equation: $W\ (Joules) = bm \times g \times s \times \sin\theta \times t$, where bm is the animal's body mass (kg), g is the acceleration of gravity (9.8 $m \cdot s^{-2}$), s is the treadmill speed ($m \cdot min^{-1}$), θ is the angle of treadmill inclination, and t is the time spent in each stage (Soares et al., 2019).

2.6.Post-mortem analyses

Forty-eight hours after the last session of intervention, the rats were killed by decapitation following 6 hours of fasting. Samples of blood were collected using a standardized protocol. Immediately after decapitation, 5 mL of trunk blood were collected at the decapitation site. The samples of blood were processed (30 min at ambient temperature and centrifugated by 15 min at 3500 rpm) and stored at -80°C for posterior serum analyzes. The euthanasia by decapitation was approved by the local Ethics Commission and represents an adequate euthanasia method for metabolic serum biomarkers collection in Wistar rats (Pierozan et al., 2017). The liver,

gastrocnemius muscles, epididymal (EAT), retroperitoneal (RAT), and mesenteric adipose tissues (MAT) were dissected, totally removed, weighed (SHIMADZU® Model BL320H, the accuracy of 0.001g), and stored at -80°C until further analysis. The epididymal, retroperitoneal, and mesenteric adipose tissues were used to calculate the adiposity index: Adiposity index = {[(EAT + RAT + MAT) ÷ body mass] x 100}.

2.7.*Serum dosages*

Serum samples were used for the analysis of glucose (enzymatic kit, Gold Analisa®, Belo Horizonte, Brazil), insulin (enzyme-linked immunosorbent assay, ELISA; EMD Millipore Corporation® – EZRMI-13K, MA, USA), leptin, and adiponectin (ELISA kits R&D Systems), total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL) concentrations by enzymatic colorimetric assays (Lab Test® Diagnostic S.A. Brazil). Insulin resistance was assessed using the homeostasis assessment model (HOMA2-IR). HOMA2-IR was calculated using fasting plasma glucose and insulin concentrations using the HOMA2 model calculator (https://www.dtu.ox.ac.uk/homacalculator).

2.8.Pro and anti-inflammatory cytokine: inflammatory mediators

The concentrations of interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 1β (IL-1β), and tumor necrosis factor-alpha (TNF-α) were evaluated in the liver, pancreas, gastrocnemius muscle, and epididymal adipose tissue by ELISA (R&D Systems, Inc., Minneapolis, MN, USA; DuoSet kits DY506, DY522, DY501, and DY510, respectively) following all the manufacturer's recommendations.

2.9.Immunohistochemical analysis

Samples of the gastrocnemius muscle were obtained in different areas of its parenchyma and fixed in paraformaldehyde. After fixation, the fragments were dehydrated, diaphanized, and embedded in paraffin. The paraffin blocks were cut into 5 µm sections, mounted on histological slides (3 sections/slide), and subjected to the immunoperoxidase assay using the primary GLUT4 antibody (NOVUS BIOLOGICALS, NBP1-49533, France). For exposure of specific epitopes, the sections were submitted to antigenic recovery in citrate buffer pH 6.0 at 95ºC for 30 min. The endogenous peroxidase activity was blocked with a solution of hydrogen peroxide (3%) in PBS. The blocking of nonspecific bonds was performed using a 2% bovine serum albumin solution (BSA). The sections were incubated with primary antibody (dilution 1:200) overnight at 4ºC in a humid chamber. For the negative control, the primary antibody was omitted in a section of each slide analyzed. The biotinylated secondary antibody (Daki EnVision™ + Dual Link System-HRP)

was used for 30 min. The 3'3 diaminobenzidine (DAB) was the substrate used for the reactions, and the sections of the liver and skeletal muscle were counterstained with hematoxylin.

To measure the effect of interventions on the pattern of GLUT4 distribution in the gastrocnemius muscle, 15 fields per animal were photographed using an optical microscope (BX-60 Olympus) with a digital camera (UC-50) coupled. The images were evaluated using the ImageJ program (Abràmoff et al., 2004), and the number of marked cells was obtained from a total of 1000 cells counted.

2.10. Morphometric analyzes

Pancreas and adipose tissue were sectioned in different regions of their parenchyma, and the fragments obtained were fixed by immersion in a 4% glutaraldehyde solution in 0.05 M phosphate buffer, pH 7.3 for 24 hours. Following the process, they were dehydrated and included in histological plastic resin (LEICA®-HistoResin). Sections (4 μm) were inserted into histological slides, stained with toluidine-borate sodium blue, and photographed (UC-50 camera) under a light microscope (BX-60 Olympus) in 40X magnification. The quantification of the area corresponding to the islets of Langerhans and adipocytes was performed using the ImageJ software, using 8 rats per group and 15 photos per experimental animal.

2.11. Statistical Analysis

The normality and homoscedasticity of the data were analyzed by the Shapiro-Wilk and Levene tests, respectively. The results are expressed as mean values ± standard deviations except when noted in the figures. Capillary glucose concentrations in response to OGTT obtained before and after the insulin resistance induction period (30 days) were compared between *moments* (before vs. after) and over *time* using two-way ANOVA with repeated measurements over time. The area under the curve (AUC) of glucose in response to OGTT was compared between moments by Tukey's T-test.

The effects of aerobic training and chronic supplementation of cocoa flavanols on parameters of aerobic performance (delta, from 4 to 12 weeks, of $\dot{V}O_{2peak}$, total exercise time (TET), total work, and Vpeak), metabolism, inflammatory mediators, and lipid profile were compared using two-way ANOVA considering *supplementation* (PLA or COCOA) and *activity* (SED or EXE) as the variation's parameters. Glucose concentrations in response to OGTT obtained before and after the intervention period with supplementation of cocoa flavanols and/or aerobic training were compared using three-way ANOVA considering *supplementation* (PLA or COCOA), *activity* (SED or EXE), and *time* as the variation's parameters, with repeated measures on *time*. The area under the

curve (AUC) of capillary blood glucose was compared using two-way ANOVA considering *supplementation* (PLA or COCOA) and *activity* (SED or EXE) as the variation's parameters.

For all the aforementioned ANOVAs, when significant interactions were found, Tukey's post hoc test was performed to compare differences between pairs. Similarly, additive effects were observed when a significant interaction was reported, with values in the COCOA+EXE group being higher or lower than values in both the PLA+EXE and COCOA+SED groups. All statistical analyzes were performed using the SigmaPlot software (version 11.0, SYSTAT SOFTWARE, USA), adopting a significance level of $\alpha = 5\%$ ($p < 0.05$).

The sample size a priori was calculated by G*Power 3 software (Faul et al., 2007), adopting $\alpha = 0.05$, power $(1-\beta) = 0.80$, number of groups = 4. The $\dot{V}O_{2peak}$ results from experimental pilot project was used to calculate the partial eta squared ($\eta^2 = 0.06$) and effect size (0.25). Therefore, the total sample size calculated was n = 8 rats per group and actual power = 0.81.

3. Results

After thirty days consuming a high-fat diet ingested with a fructose-rich beverage, the rats showed a significant increase in body mass (375.57 ± 10.24 g after *vs*. 256.42 ± 6.36 g before; $p < 0.001$; t = -119.50) and glucose intolerance (Fig. S_1A-B) when compared to baseline (before-diet moment).

3.1 – Body mass gain, adiposity index and hormone levels

The descriptive analysis is shown in table S_1. The cocoa-supplemented rats and trained rats attenuated body mass gain ($p = 0.034$ and $p = 0.049$), reduced the adiposity index ($p = 0.022$ and $p = 0.034$; Fig. 2A-B), promoted an increase in adiponectin concentrations ($p = 0.018$ and $p = 0.025$; Fig. 2C) and in the adiponectin/leptin ratio ($p = 0.029$ and $p = 0.009$; Fig. 2E) when compared to placebo supplemented and sedentary rats, respectively. Besides, COCOA and EXE per se reduced leptin concentrations ($p < 0.001$ and $p < 0.001$, respectively), however, they did not show additive effects when combined (Fig. 2D).

Insert Figure 2

3.2 – Glucose homeostasis and lipid profile

COCOA and EXE improved glycemic metabolism during OGTT (Fig. S_2A-B). The rats supplemented with flavanols showed an attenuated increase in glucose concentration 60 minutes

after gavage when compared to rats supplemented with placebo. Besides, trained rats showed an attenuated increase in glucose concentration for up to 120 minutes after exposure to glucose, when compared to sedentary rats (Fig. S_2A).

COCOA and EXE per se reduced fasting blood concentrations of glucose ($p < 0.001$ and $p < 0.001$), insulin ($p < 0.001$ and $p < 0.001$), and consequently the HOMA index ($p < 0.001$ and $p < 0.001$; Fig. 2F-H). The two separate interventions also reduced blood concentrations of total cholesterol ($p = 0.001$ and $p = 0.001$), LDL ($p = 0.002$ and $p = 0.002$), and triglycerides ($p < 0.001$ and $p < 0.001$; Fig. 2I, K-L), respectively. However, the combination of both interventions did not result in additive effects when compared to the interventions per se. Trained rats also showed a significant increase in HDL concentrations ($p < 0.001$) when compared to sedentary rats, regardless of the supplementation of cocoa flavanols (Fig. 2J).

3.3 – Aerobic performance

Trained rats showed an increase in aerobic performance, including $\dot{V}O_{2peak}$ ($p = 0.016$), TET ($p < 0.001$), Vpeak ($p < 0.001$), and total work ($p < 0.001$) when compared to sedentary rats regardless of supplementation with cocoa flavanols. However, cocoa supplementation attenuated the reduction in the total exercise time ($p = 0.004$) and peak velocity ($p = 0.007$) when compared to rats supplemented with placebo independent of aerobic training (Fig. 3B-C).

Insert Figure 3

3.4 - Liver

When we looked at the inflammatory profile in the liver, cocoa-supplemented rats ($p < 0.001$) and trained rats ($p < 0.001$) reduced the concentration of IL-6 in the studied rats (Fig 4A). Besides, the trained animals showed a reduction in the hepatic concentrations of TNF-α ($p = 0.005$) and IL-1β ($p = 0.009$) and an increase in the concentration of IL-10 ($p = 0.004$) when compared to sedentary rats independent of cocoa flavanols supplementation (Fig. 4B-D).

Insert Figure 4

3.5 – Gastrocnemius muscle

In gastrocnemius muscle (a muscle largely made up of fast glycolytic fibers), cocoa-supplemented rats ($p = 0.039$) and trained rats ($p < 0.001$) had a reduction in the concentration of TNF-α (Fig. 5D). Moreover, an increase in the number of GLUT4 positive cells in skeletal muscle was observed in the groups supplemented with flavanols ($p < 0.001$) or submitted to training ($p <$

0.001; Fig. 5A-B). However, it is noteworthy that the combined interventions had an additive effect ($p < 0.001$) on this skeletal muscle's glucose transport capacity, resulting in a significantly greater number of GLUT4 expressing cells (Fig. 5A-B). Moreover, trained rats reduced the concentration of IL-6 ($p = 0.039$) when compared to sedentary rats was independent of cocoa flavanols supplementation (Fig. 5C).

Insert Figure 5

3.6 – Pancreas and adipose tissue

The area of the Langerhans' islets and the pro and anti-inflammatory cytokines concentrations in the pancreas are shown in figure 6. When compared to sedentary rats, trained animals showed increased areas of the islets of Langerhans ($p < 0.001$; Fig. 6A-B). COCOA and EXE per se reduced the concentration of TNF-α ($p < 0.001$ and $p < 0.001$; Fig. 6D) and increased the concentration of IL-10 in the pancreas ($p < 0.001$ and $p < 0.004$; Fig. 6F), however, without presenting an additive effect when the interventions were combined. Rats supplemented with cocoa flavanols also showed in the pancreatic tissue a reduction in the concentration of IL-6 ($p = 0.024$) when compared to rats supplemented with placebo, independent of aerobic training (Fig 6C). Cocoa-supplemented rats ($p = 0.001$) and trained rats ($p < 0.001$) reduced the area of adipocytes after eight weeks of intervention (Fig. 6G-H). Besides, COCOA and EXE *per se* also reduced the concentration of IL-6 ($p = 0.005$ and $p = 0.002$; Fig 6I) and TNF-α ($p = 0.002$ and $p = 0.001$; Fig. 6J) in adipocytes, however, without showing additive effects when combining interventions.

*** Insert Figure 6 ***

4. Discussion

In the present study in IR rats, chronic supplementation with cocoa flavanols and aerobic training performed at 60% V_{peak} for eight weeks afforded the following benefits: i) attenuated body mass gain and reduced the adiposity index; ii) improved glucose tolerance and lipid profile; iii) improved the inflammatory milieu in the liver, pancreas, adipose tissue, and skeletal muscle of IR rats, when compared to sedentary, placebo supplemented group. In addition, individual supplementation with cocoa flavanols and aerobic training increased the expression of GLUT4 in skeletal muscle, and additive effects on GLUT4 expression were observed by combining these interventions. Collectively, our data revealed the effectiveness of cocoa flavanol supplementation and aerobic training in modulating key physiological and molecular responses for preventing and

treating insulin resistance. Our findings indicated that the combination of these interventions could be an effective non-pharmacological treatment strategy for DM2.

GLUT4, a key protein that determines glucose metabolism, is primarily expressed in adipose tissue and skeletal muscle and is responsible for the insulin-dependent transport of glucose across the cell membrane in these tissues (Vargas et al., 2021). Although the effects of aerobic training on GLUT4 expression have been previously reported (Evans et al., 2019), to the best of our knowledge, this is the first study to reveal the additive effects of cocoa flavanol supplementation and aerobic training in increasing the number of cells expressing GLUT4 in skeletal muscle under obese and IR conditions.

Although mechanisms underlying the enhanced GLUT4 synthesis in skeletal muscle during aerobic training need to be comprehensively elucidated, accumulated evidence suggests that the increase in cytosolic Ca^{2+} from the sarcoplasmic reticulum in response to muscle contraction is a key factor mediating this process. Activation of the calcium/calmodulin complex (CaMK), mediated by increased cytosolic Ca^{2+}, activates mitogen-activated protein kinase (p38MAPK), which promotes GLUT4 synthesis by activating transcriptional coactivator myocyte enhancer factor 2 (MEF2) (Wright, 2007). At rest, MEF2 is inhibited by the transcriptional repressor II histone deacetylase (HDAC) (McGee & Hargreaves, 2006); however, aerobic training can induce GLUT4 transcription by inactivating HDAC4/5 in an AMPK-dependent manner (Niu et al., 2017). Increased AMPK phosphorylation promotes HDAC5 phosphorylation at serine 259 and 498, thus inhibiting it; this favored the binding of MEF2 to the GLUT4 gene promoter, increasing its gene expression in several tissues (McGee et al., 2008).

Attenuated body weight gain and the reduced adiposity index following COCOA and/or EXE have been attributed to several physiological mechanisms, depending on dosage and the total duration of intervention. These include the suppression of factors related to lipogenesis, such as SREBP-1c and acetyl Co-A carboxylase (Coronado-Cáceres et al., 2019) and the activation of the factors involved in energy expenditure, including peroxisome proliferator-activated receptor (PPAR)-γ, sirtuin 1, uncoupling protein 1, and PPAR-gamma coactivator (PGC)-1α (Rabadan-Chávez et al., 2016). In addition, the beneficial effects of COCOA and/or EXE may be mediated via appetite suppression through different hormones, such as increased GLP-1 (Strat et al., 2016) and decreased ghrelin concentrations (Massolt et al., 2010), as well as enhanced leptin and reduced adiponectin levels (Flores, 2019).

Reduced levels of circulating adiponectin have been observed in conditions of obesity and insulin resistance and are reportedly associated with lipotoxicity and ectopic lipid accumulation in several tissues (Forny-Germano et al., 2019). In the present study, we demonstrated that COCOA and EXE increased adiponectin concentrations and the adiponectin/leptin ratio in IR rats, potentially improving the neuroendocrine control of energy homeostasis and adipose tissue dysfunction, respectively. Our results also revealed that chronic supplementation with COCOA and EXE effectively reduced leptin concentrations in obese IR rats.

The reduction in circulating leptin concentrations mediated by COCOA and EXE is associated with changes in body composition, specifically lowering the percentage of body fat and reducing the adipocyte size (Coronado-Cáceres et al., 2019; Fedewa et al., 2018). Moreover, leptin has known pleiotropic effects, such as neuroendocrine regulation, glucose homeostasis, as well as modulation of the reproductive and hematopoietic system, inflammation, and immune system (Abella et al., 2017). Accordingly, the decrease in circulating leptin levels due to aerobic training and flavanol supplementation may have contributed to the improved glycemic metabolism, attenuated body weight gain, reduced adiposity index, and overall improvement of the inflammatory milieu in different tissues. This emphasizes the importance of combining both interventions, i.e., aerobic training and supplementation with polyphenols such as cocoa flavanols, for neuroendocrine control during obesity and insulin resistance conditions.

Regarding the inflammatory profile, we observed an improved inflammatory profile in all peripheral tissues (liver, pancreas, adipose tissue, and skeletal muscle) of IR rats on combining the COCOA and EXE. To the best of our knowledge, this is the first study investigating the combined effects of these interventions on the inflammatory milieu in different peripheral tissues under obesity and insulin resistance conditions.

In the present study, physiological mechanisms through which COCOA and/or EXE may potentially reduce the concentration of inflammatory cytokines were not explored. However, accumulated evidence suggests that cocoa flavanols may inhibit the activation of transcriptional factors involved in synthesizing inflammatory cytokines, including NF-kβ and complex IKK (IKK1/IKK2/IKβ), and reduce the activity of key inflammatory enzymes, such as cyclooxygenase 2 (Ellinger & Stehle, 2016). Furthermore, these interventions have been shown to decrease the expression of vascular cell adhesion molecule and intercellular adhesion molecule, and promote the suppression of redox-sensitive signals, such as the MAPK pathway (ERK/JNK/p38) (Goya et al., 2016). In addition, the cytoprotective role of cocoa flavanols has been observed in pancreatic beta

cells, which showed reduced oxidative stress, cell apoptosis, muscle mass loss following exposure to this compound, thus preventing the development of DM2 (Ghorbani et al., 2019).

In the present study, EXE, in addition to improving the inflammatory profile, increased the area of pancreatic islets by possibly reducing the pancreatic concentration of IL-1β and TNF-α. IL-1β is an important inflammatory mediator and a marker of cell damage in pancreatic beta cells. Inhibition of IL-1β reportedly improves beta-cell dysfunction and insulin secretion in individuals presenting insulin resistance (Westwell-Roper et al., 2014). Furthermore, the increase in TNF-α levels due to M1 macrophage accumulation in pancreatic tissues contributes to inflammation, apoptosis, and dysfunction of beta cells (Eguchi & Manabe, 2013). Experimental studies have shown that three main mechanisms can mediate the increased β-cell mass in response to aerobic training: increased β-cell proliferation, reduced β-cell apoptosis, and increased β-cell viability (Curran et al., 2020). Thus, our results reinforce the importance of aerobic training in preserving pancreatic islets, possibly attributed to its anti-inflammatory action in obesity and insulin resistance conditions.

As expected, aerobic training performed at 60% V_{peak} effectively increased the aerobic performance in trained rats. Supplementation with cocoa flavanols for eight weeks effectively mitigated the reduced total exercise time and V_{peak}, as observed in non-trained rats supplemented with a placebo solution. The enhanced aerobic capacity owing to moderate-intensity physical training has been associated with increased cardiac output, oxidative enzymes, and the size and density of mitochondria, in addition to improved endothelial function and consequently cardiorespiratory fitness (Colberg et al., 2016). In addition, chronic supplementation with cocoa flavanols can increase the antioxidant capacity, PGC-1α expression, and enzymatic capacity of citrate synthase, thus contributing to increased mitochondrial biogenesis and aerobic capacity, respectively (Watanabe et al., 2014); this could explain the ameliorative effects of cocoa flavanols on reduced total exercise time and V_{peak} in non-trained IR rats.

Mitochondrial dysfunction and reduced cardiorespiratory fitness, known to occur during obesity and insulin resistance, contribute to the increased risk of mortality from cardiovascular diseases in patients with DM2 (Sivitz & Yorek, 2010). Our findings indicate that the benefits of physical performance following COCOA and/or EXE can be attributed to the improvement and/or attenuation of diminished cardiorespiratory capacity observed during DM2.

Although there is a huge similarity in several physiological insulin resistance-related mechanisms, the potential transferability of the results found in the rats of our study to humans must be considered with caution. In addition, the impact of sex on metabolic dysfunctions depends on the

animal model of DM2 chosen (Franconi et al. 2008). In the current study, the male rats were selected since the method we used to induce insulin resistance was recently developed in male Wistar rats (Melo et al., 2021). To our knowledge, there is no data in literature about sexual dimorphism in rats made insulin-resistant using a high-fat and -fructose diet. However, it is worth mentioning that the ECODIA (Exercise, Cocoa and Diabetes) study is a collaborative project developed in Federal University of Minas Gerais (Belo Horizonte, Brazil), Université de Lille (Lille, France), and Vrije Universiteit Brussel (Brussels, Belgium) in which the objective is to investigate, in a translational approach, the combined effects of physical exercise and cocoa flavanol supplementation on DM-induced metabolic dysfunction, with controlled-randomized trials being performed in humans with diabetes (studies still in progress) and more mechanistic experiments in rats.

Conclusion

In obese IR rats, coupling COCOA and EXE provided additive effects on skeletal muscle GLUT-4 expression, along with benefits afforded by each intervention, including improved aerobic power and increased regions of Langerhans after exercise training and decreased pancreatic IL-6 levels after cocoa flavanol supplementation. Thus, combining COCOA with EXE represents an effective non-pharmacological strategy to potentiate the health status in patients with obesity and insulin resistance by improving physical performance, glycemic metabolism, neuroendocrine control, and lipid and inflammatory mediators in the liver, pancreas, adipose tissue, and skeletal muscle.

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Author contributions

MELO, BP: Conceptualization, Methodology, Investigation, Formal analysis, Project administration, Roles/Writing - original draft, Writing - review & editing, **ZACARIAS AC:** Investigation, Formal analysis, Writing - review & editing, **OLIVEIRA JCC**: Investigation, Formal analysis, **DE SOUZA CORDEIRO LM**: Conceptualization, Methodology, Formal analysis, Writing - review & editing, **WANNER SP**: Methodology, Formal analysis, Writing - review & editing, **DOS SANTOS ML**: Formal analysis, Resources, **AVELAR GF**: Methodology, Formal analysis, Resources, Writing - review & editing, Supervision, Funding acquisition, **MEEUSEN R:** Methodology, Formal analysis, Project administration, Funding acquisition, **HEYMAN E**:

Conceptualization, Resources, Project administration, Writing - review & editing, Funding acquisition, **SOARES DD**: Conceptualization, Methodology, Formal analysis, Resources, Project administration, Roles/Writing - original draft, Writing - review & editing, Funding acquisition, Supervision.

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Competing interest

The authors declare that they have no competing interests.

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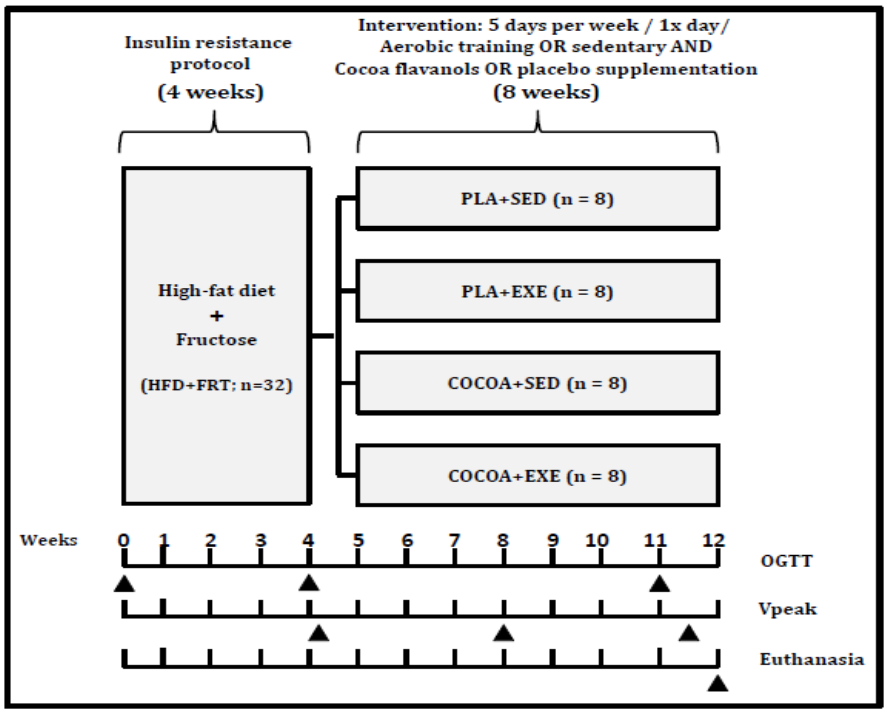
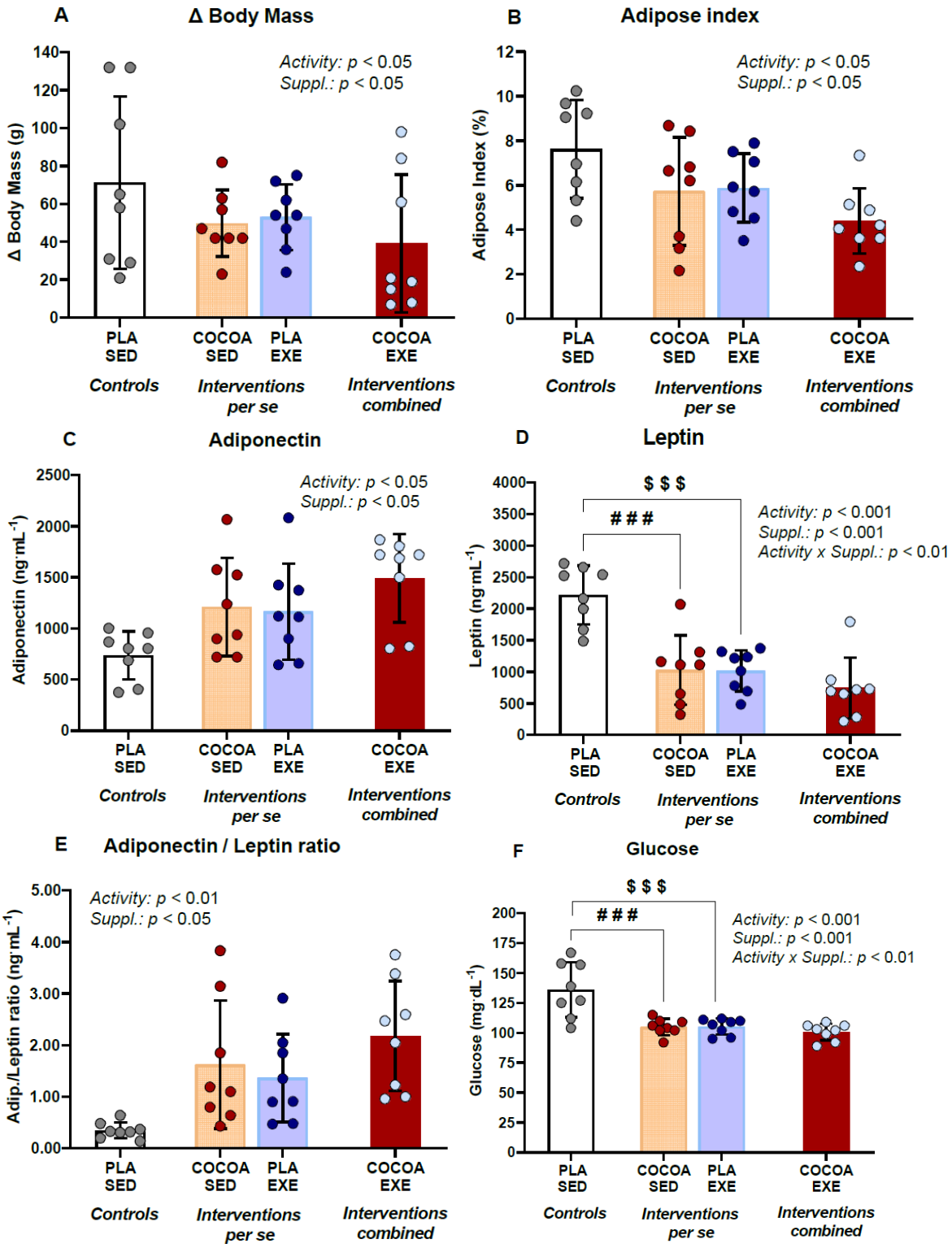


Figure 1. Experimental design. OGTT: Oral Glucose Tolerance Test; PLA: Placebo; SED: Sedentary; EXE: Physical Exercise; COCOA: Cocoa flavanols; Vpeak: Peak velocity.


A
Δ Body Mass
Δ Body Mass (g)
Activity: p < 0.05
Suppl.: p < 0.05
B
Adipose index
Adipose Index (%)
Activity: p < 0.05
Suppl.: p < 0.05
C
Adiponectin
Adiponectin (ng·mL-1)
Activity: p < 0.05
Suppl.: p < 0.05
D
Leptin
Leptin (ng·mL-1)
$ $ $
# # #
Activity: p < 0.001
Suppl.: p < 0.001
Activity x Suppl.: p < 0.01
E
Adiponectin / Leptin ratio
Adip./Leptin ratio (ng·mL-1)
Activity: p < 0.01
Suppl.: p < 0.05
F
Glucose
Glucose (mg·dL-1)
$ $ $
# # #
Activity: p < 0.001
Suppl.: p < 0.001
Activity x Suppl.: p < 0.01
PLA SED
COCOA SED
PLA EXE
COCOA EXE
Controls
Interventions per se
Interventions combined

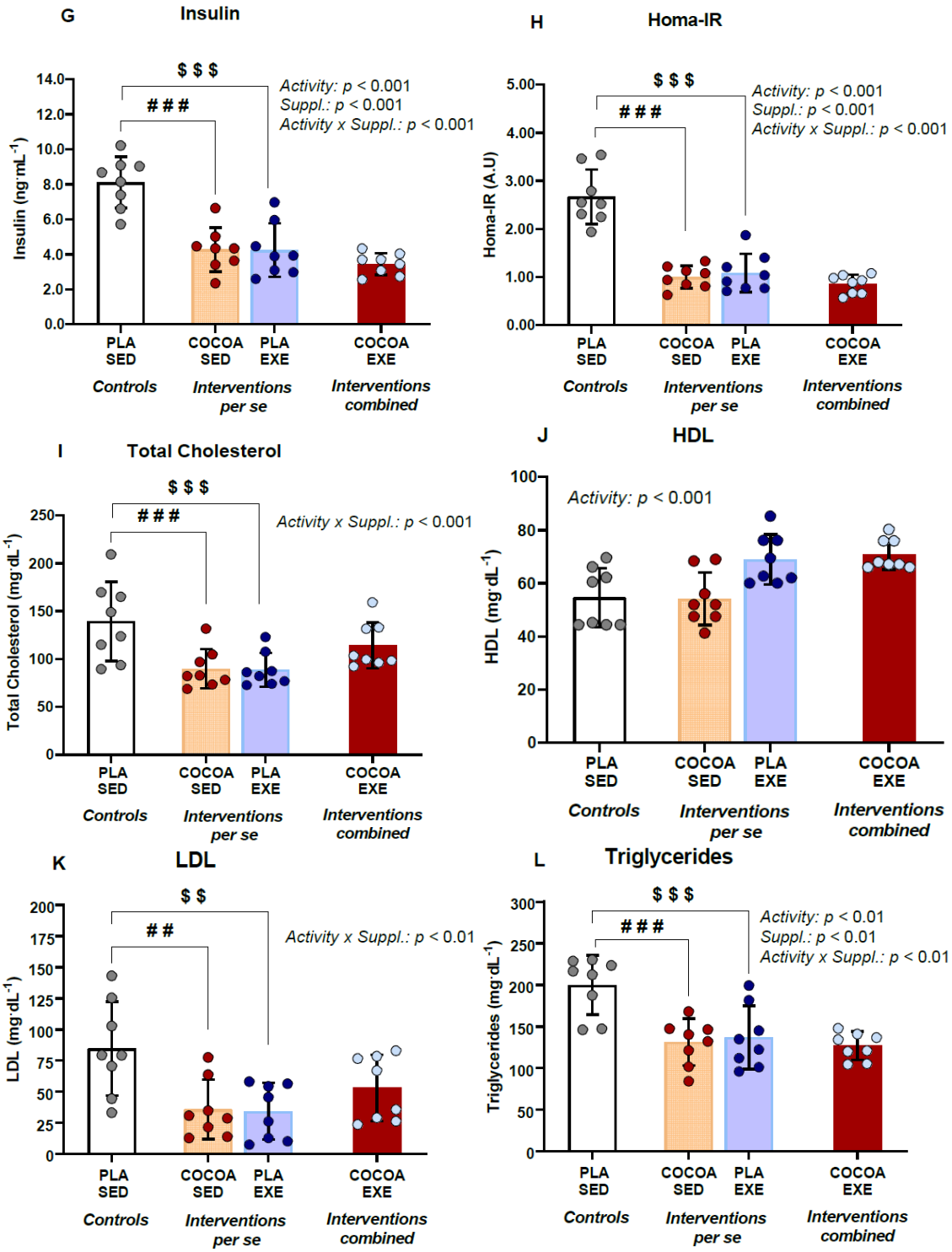


Fig. 2. Body mass change (Delta Δ) (A), adiposity index (B), adiponectin concentration (C), leptin (D), adiponectin/leptin ratio (E), glucose (F), insulin (G), Homa-IR (H), Total cholesterol (I), HD (J), LDL (K), and Triglycerides (L) after eight weeks of intervention with cocoa flavanols supplementation and/or aerobic training. Data are expressed as the mean and standard error of the mean. Figures 2A-L: Only the significant main effects of ANOVA are shown in the graphs. Post hoc analysis for activity effect: $$$ $p < 0.001$ for PLA.SED vs. PLA.EXE; Post hoc analysis for supplementation effect: ### $p < 0.001$ for PLA.SED vs. COCOA.SED.

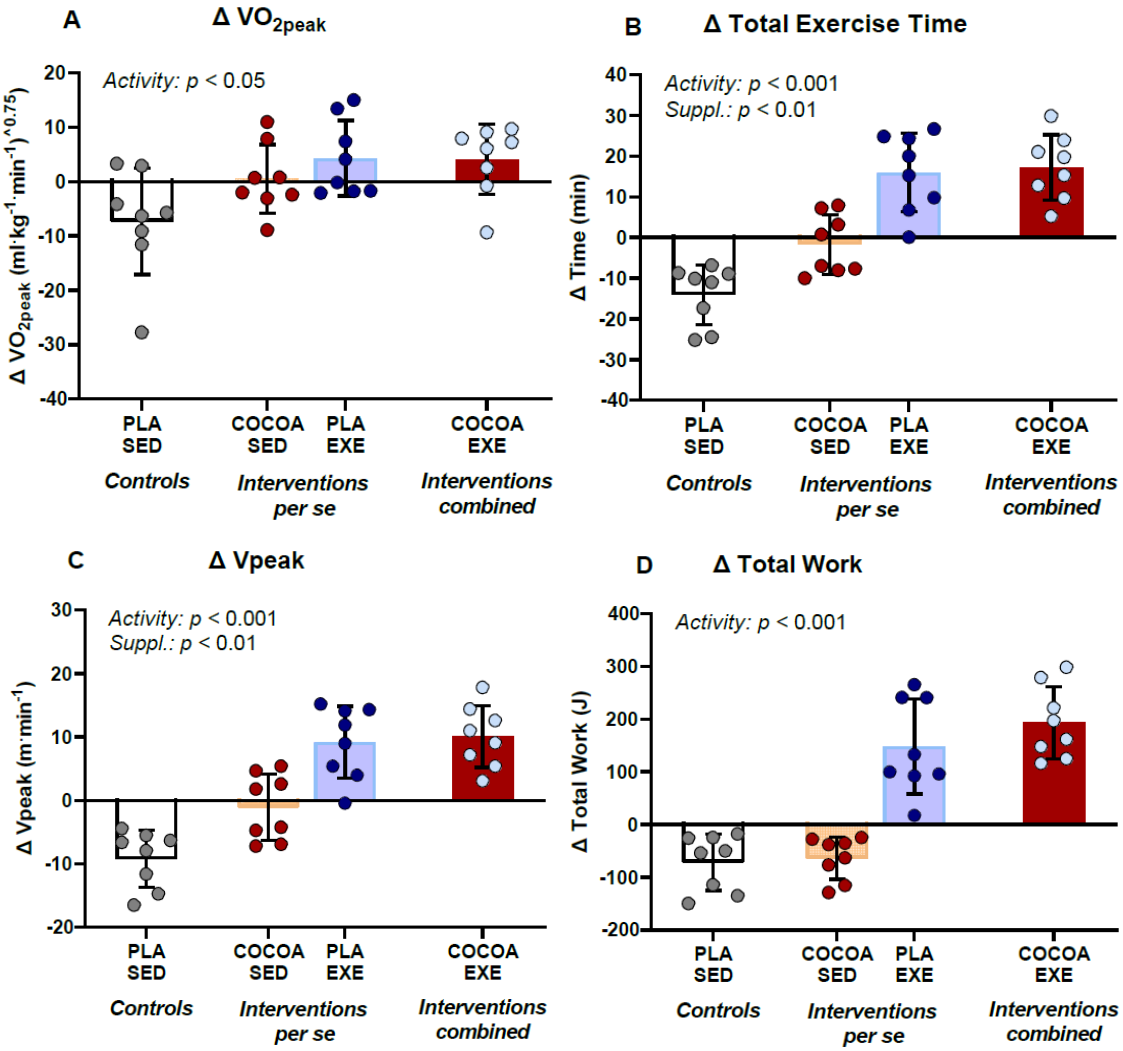


Fig. 3. Delta (Δ) of peak oxygen consumption peak (A), total exercise time (B), peak velocity (C), and total work (D) obtained during the incremental exercise test calculated from post-pre, eight-week intervention with cocoa flavanols supplementation and/or aerobic training values. Figure 3A-D: Only the significant main effects of ANOVA are shown in the graphs.

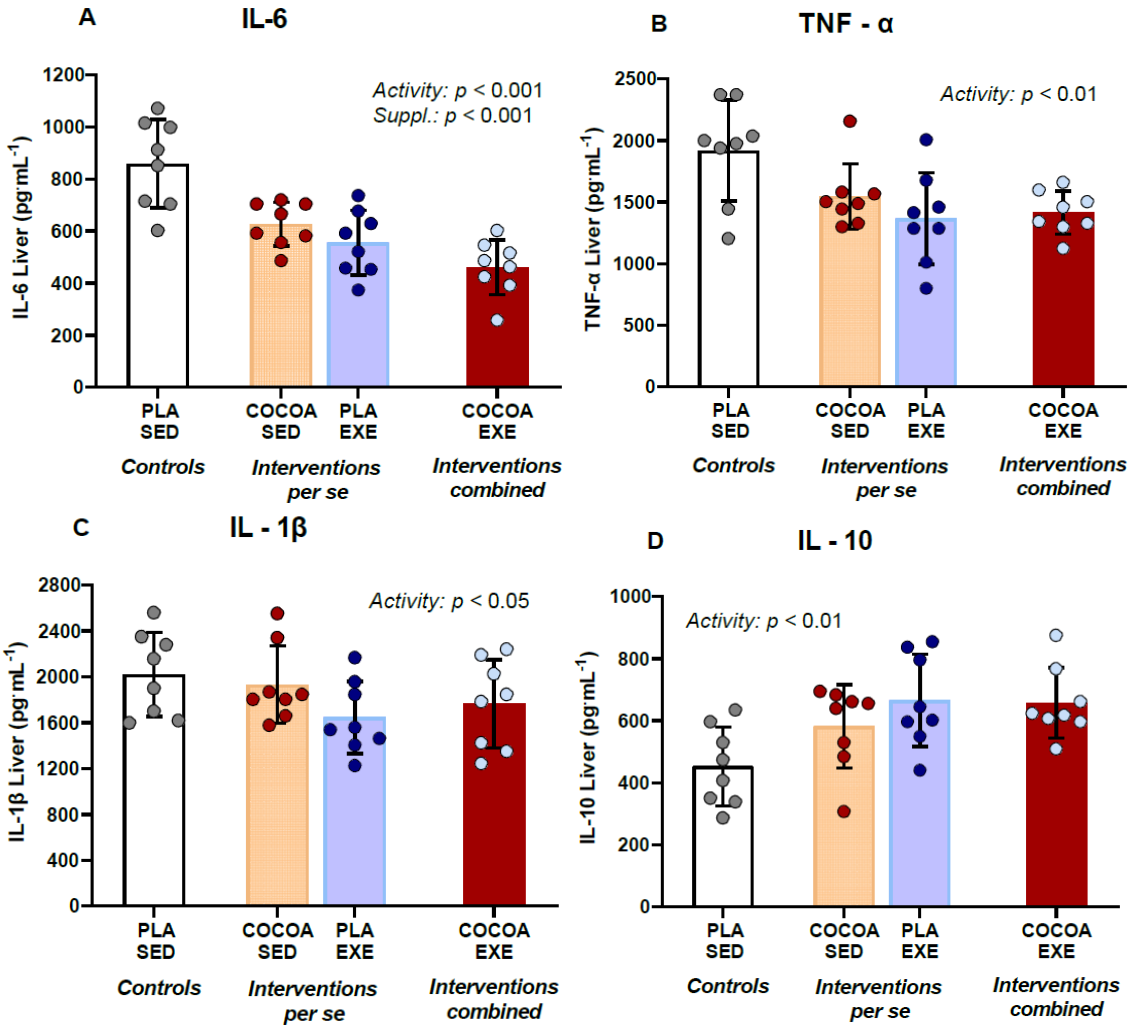


Fig. 4. Pro and anti-inflammatory cytokines concentrations on the liver tissue after eight weeks of intervention with supplementation of cocoa flavanols and/or aerobic training. Data are expressed as the mean and standard error of the mean. Figure 4A-D: Only the significant main effects of ANOVA are shown in the graphs.

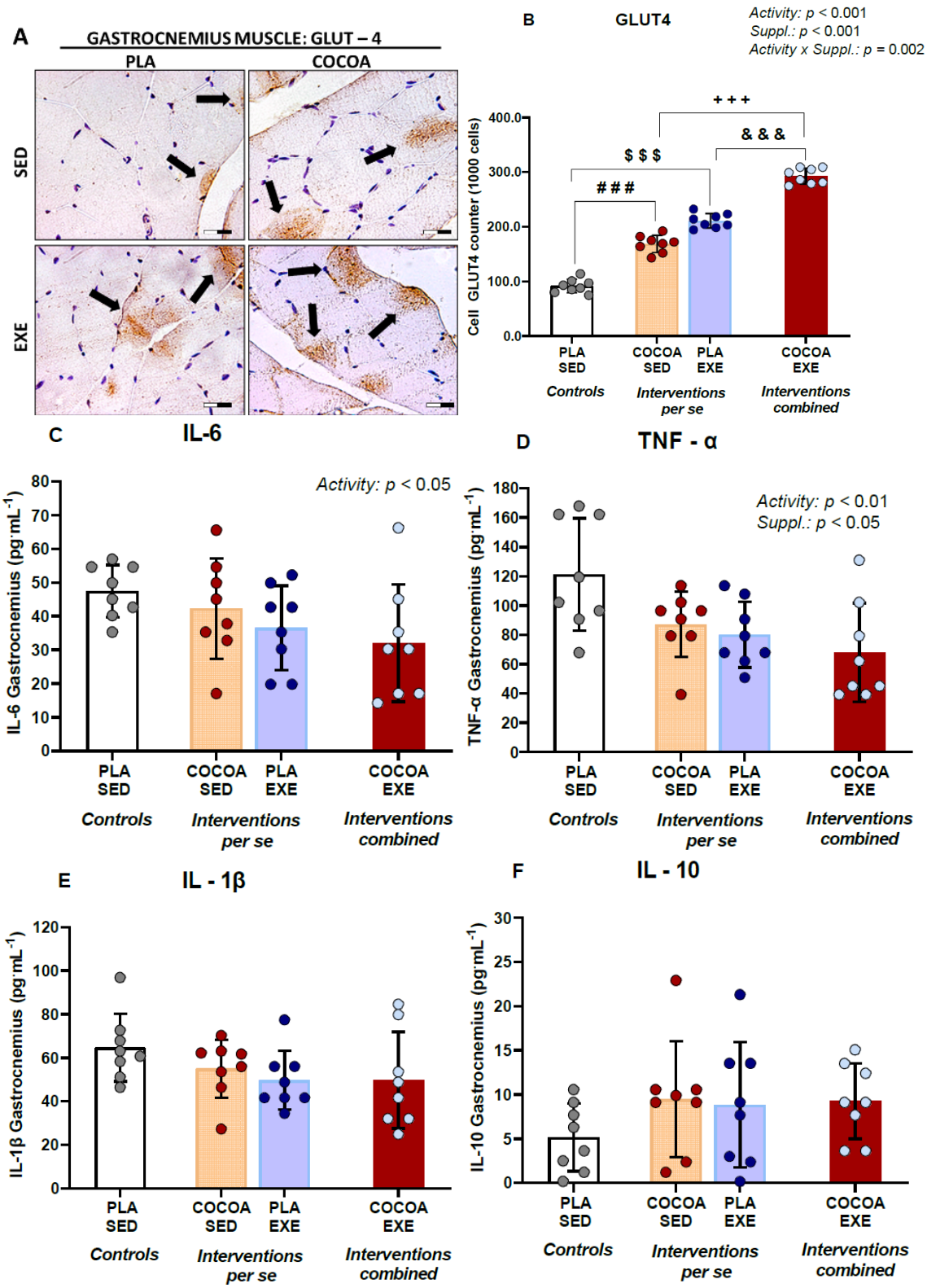
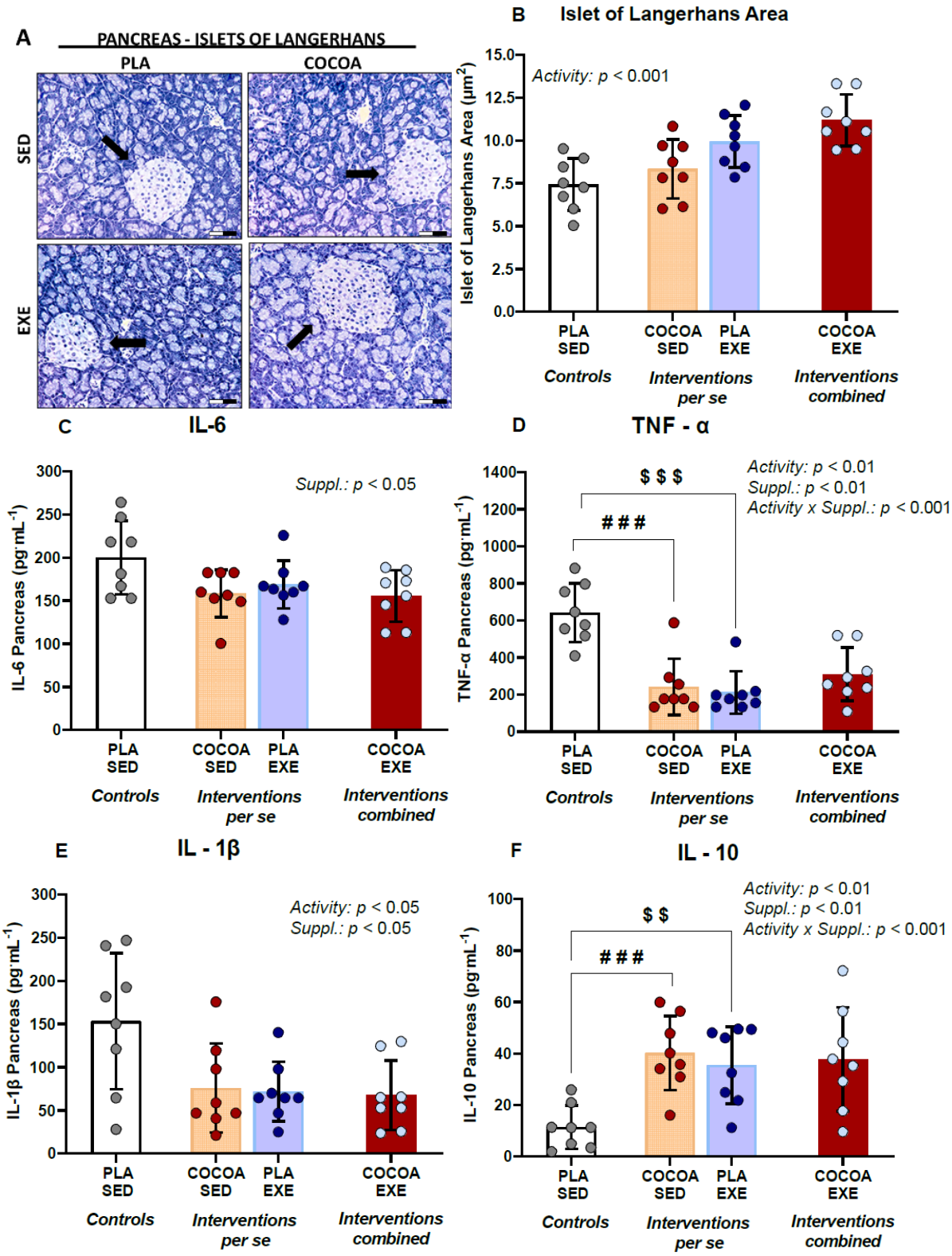


Fig. 5. GLUT4 and pro and anti-inflammatory cytokines in the gastrocnemius muscle after eight weeks of intervention with cocoa flavanols supplementation of and/or aerobic training. Data are expressed as the mean and standard error of the mean. Bars: 50µm; (➡): GLUT4; Figure 16b-f: Only the significant main effects of ANOVA are shown in the graphs. Post hoc analysis for activity effect: $$$ $p < 0.001$ for PLA.SED vs. PLA.EXE; Post hoc analysis for supplementation effect: ### $p < 0.001$ for PLA.SED vs. COCOA.SED; Post hoc analysis for activity effect: +++: $p < 0.001$ for COCOA.SED vs. COCOA.EXE; Post hoc analysis for supplementation effect: &&&: $p < 0.001$ for PLA.EXE vs. COCOA.EXE.


A
PANCREAS - ISLETS OF LANGERHANS
PLA
COCOA
SED
EXE
B
Islet of Langerhans Area
Islet of Langerhans Area (µm²)
Activity: p < 0.001
PLA SED
COCOA SED
PLA EXE
COCOA EXE
Controls
Interventions per se
Interventions combined
C
IL-6
IL-6 Pancreas (pg·mL⁻¹)
Suppl.: p < 0.05
D
TNF - α
TNF-α Pancreas (pg·mL⁻¹)
Activity: p < 0.01
Suppl.: p < 0.01
Activity x Suppl.: p < 0.001
$ $ $
# # #
E
IL - 1β
IL-1β Pancreas (pg·mL⁻¹)
Activity: p < 0.05
Suppl.: p < 0.05
F
IL - 10
IL-10 Pancreas (pg·mL⁻¹)
Activity: p < 0.01
Suppl.: p < 0.01
Activity x Suppl.: p < 0.001
$ $
# # #

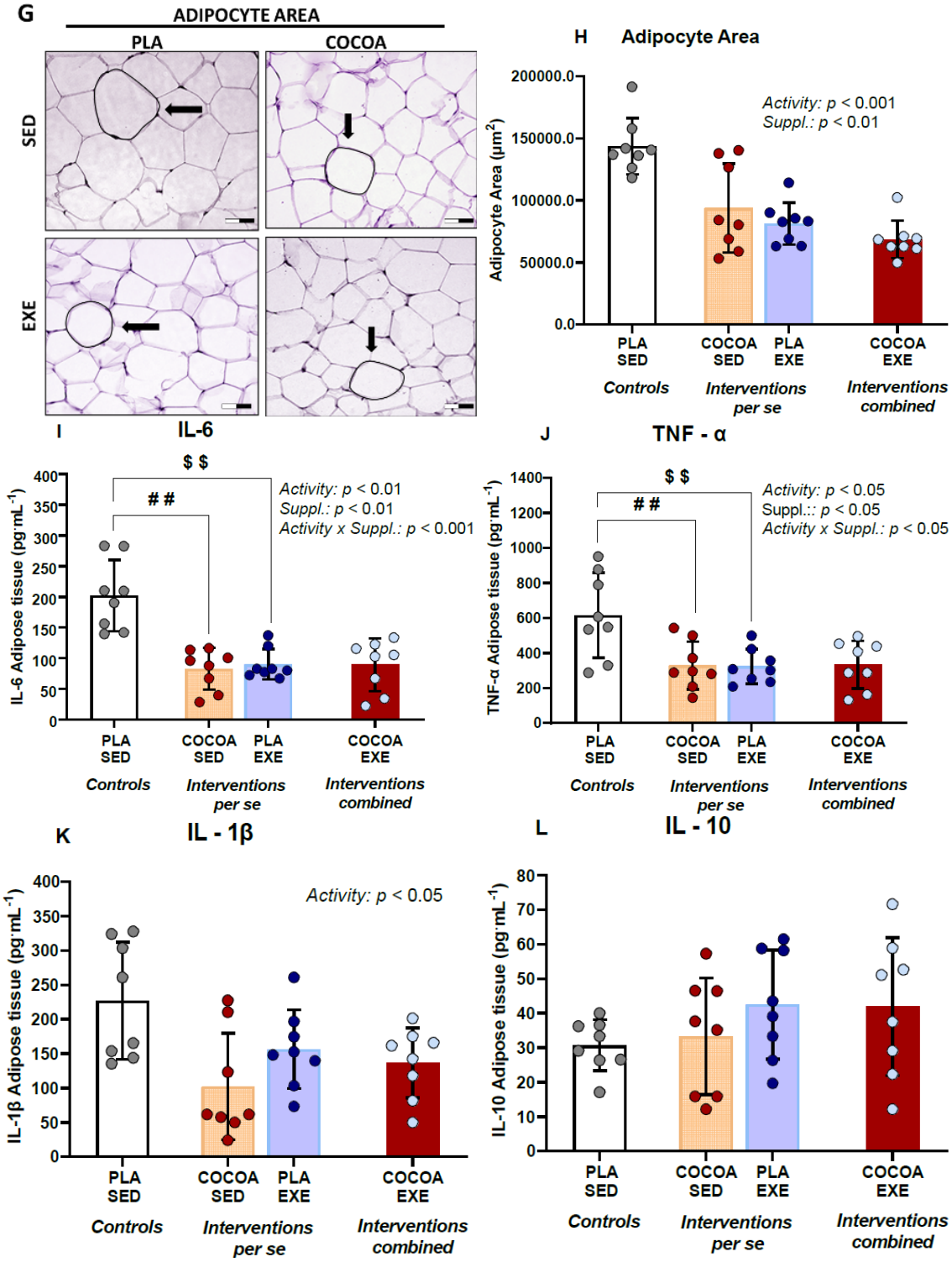


Fig. 6. Area of the islets of Langerhans, area of adipocytes, and pro and anti-inflammatory cytokines in the pancreas and epididymal adipose tissue of rats after eight weeks of intervention with supplementation of cocoa flavanols and/or aerobic training. Data are expressed as the mean and standard error of the mean; Bars: 50µm. Figure 6B-L: Only the significant main effects of ANOVA are shown in the graphs. Post hoc analysis for activity effect: $$ $p < 0.01$; $$$ $p < 0.001$ for PLA.SED vs. PLA.EXE; Post hoc analysis for supplementation effect: ### $p < 0.001$ for PLA.SED vs. COCOA.SED.

APPENDICES

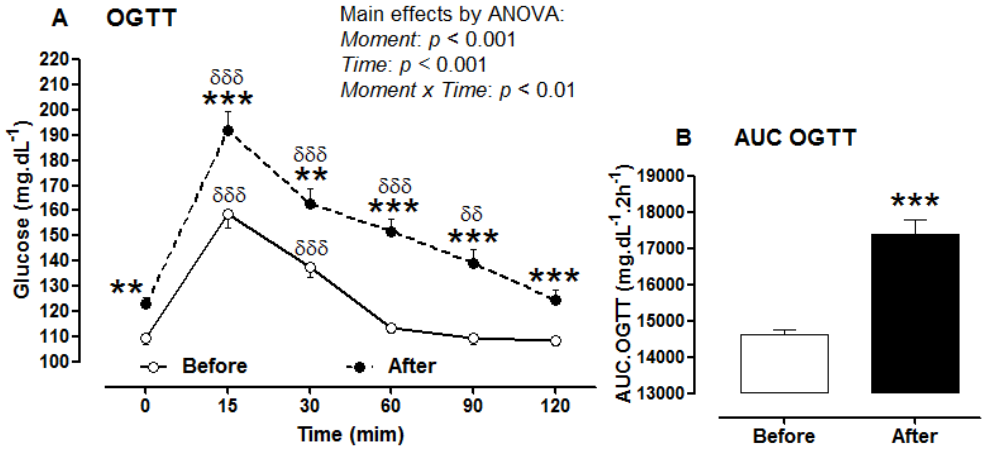


Figure S1. Oral glucose tolerance test performed before and after consumption of high-fat diet and fructose-rich beverage (A). Area under curve of OGTT (B). Data are expressed as the means and standard error of the mean. Figure S1A: only the significant main effects of ANOVA are shown in the graphs. Post hoc analysis for moment effect: ** $p < 0.01$; *** $p < 0.001$ to Before vs. After. Post hoc analysis for time effect: δδ $p < 0.01$; δδδ $p < 0.001$, vs. 0 (baseline). Figure S1B: Tukey's T-test, *** $p < 0,001$ vs. Before.

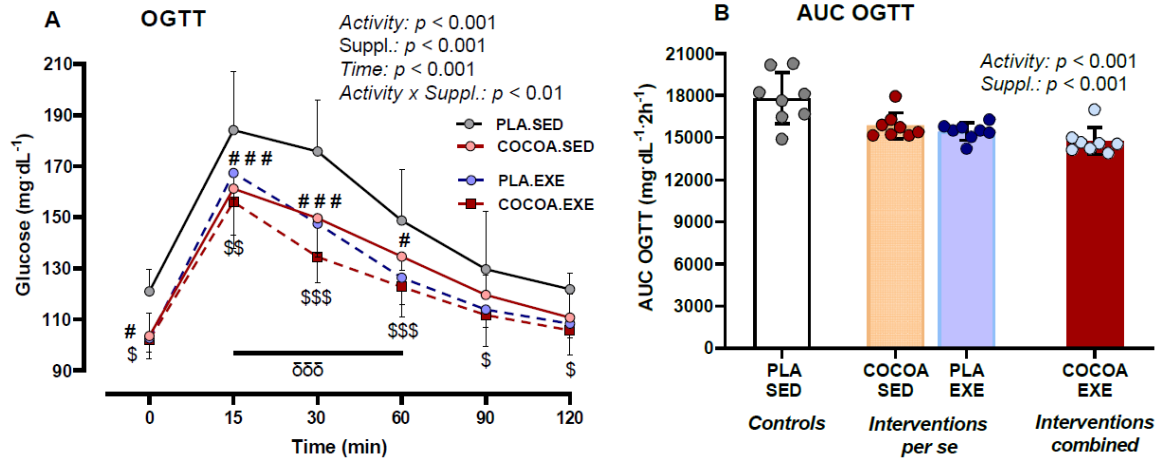


Figure S2. Oral glucose tolerance test OGTT (A) and area under the curve (B) after eight weeks of intervention with supplementation of cocoa flavanols and/or aerobic training. Data are expressed as the mean and standard error of the mean. Figure 4a-b: Only the significant main effects of ANOVA are shown in the graphs. Post hoc analysis for activity effect: $ $p < 0.05$; $$ $p < 0.01$; $$$ $p < 0.001$ for PLA.SED vs. PLA.EXE; Post hoc analysis for supplementation effect: # $p < 0.05$; ### $p < 0.001$ for PLA.SED vs. COCOA.SED; Post hoc analysis for time effect: δ $p < 0.05$; δδδ $p < 0.001$, vs. 0 (baseline).

Table S_1: Combined effects of aerobic training and chronic supplementation with cocoa flavanols on aerobic performance, metabolism, and inflammatory and lipid mediators in obese insulin resistant rats.

	PLA.SED			COCOA.SED			PLA.EXE			COCOA.EXE			Two-way ANOVA		
	Mean	CI 95%		Mean	CI 95%		Mean	CI 95%		Mean	CI 95%		a	s	a x s
Δ Body mass	71.3	39.7	102.8	49.8	37.5	62.0	53.0	41.0	65.0	39.1	13.9	64.3	**0.049**	**0.034**	0.746
Δ Adiposity Index	7.6	6.1	9.2	5.7	4.0	7.4	5.9	4.8	6.9	4.4	3.4	5.4	**0.021**	**0.034**	0.754
Glucose Homeostasis															
Glucose ($mg \cdot dL^{-1}$)	136.1	120.2	1520	105.0	100.2	109.8	105.3	100.6	109.9	100.8	95.9	105.6	**<0.001**	**<0.001**	**0.007**
Insulin ($ng \cdot dL^{-1}$)	8.1	7.1	9.1	4.3	3.4	5.1	4.2	3.2	5.3	3.4	3.0	3.9	**<0.001**	**<0.001**	**<0.001**
HOMA-index	2.7	2.3	3.1	1.0	0.8	1.2	1.1	0.8	1.4	0.9	0.7	1.0	**<0.001**	**<0.001**	**<0.001**
Hormone Levels															
Adiponectin ($ng \cdot mL^{-1}$)	737.5	574.3	900.7	1210.0	877.3	1542.7	1164.6	838.1	1491.1	1491.2	1191.7	1789.8	**0.025**	**0.018**	0.585
Leptin ($ng \cdot mL^{-1}$)	2219.9	1896.0	2543.9	1028.4	646.0	1410.8	1015.7	788.6	1242.8	745.2	411.6	1078.8	**<0.001**	**<0.001**	**0.009**
Ratio Adipon. / Leptin	0.3	0.2		1.6	0.8		1.4	0.8	2.0	2.2	1.4	2.9	**0.009**	**0.029**	0.707
Lipids Profile															
Total Cholesterol ($mg \cdot dL^{-1}$)	139.4	110.7	168.1	89.9	75.6	104.2	88.8	76.6	101.1	114.3	97.6	130.3	0.191	0.224	**<0.001**
HDL cholesterol ($mg \cdot dL^{-1}$)	54.6	47.0	62.3	54.2	47.4	61.1	69.0	62.5	75.5	70.8	66.9	74.7	**<0.001**	0.296	0.242
LDL cholesterol ($mg \cdot dL^{-1}$)	84.8	58.6	110.9	35.7	19.1	52.2	34.4	18.6	50.1	53.0	34.5	71.5	0.112	0.142	**0.002**
Triglycerides ($mg \cdot dL^{-1}$)	199.9	175.2	224.6	131.4	111.8	151.0	137.0	110.5	163.4	127.1	115.2	139.0	**0.001**	**0.005**	**0.009**
Aerobic Performance															
Δ $\dot{V}O_{2peak}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)^0.75	-7.3	-14.1	-0.5	0.5	-3.9	4.9	4.3	-0.6	9.1	4.1	-0.4	9.1	**0.016**	0.193	0.243
Δ Total Exercise Time (min)	-14.3	-19.2	-9.0	-1.7	-6.8	3.4	16.0	9.3	22.7	17.2	11.7	22.8	**<0.001**	**0.004**	0.165
Δ V_{peak} ($m \cdot min^{-1}$)	-9.3	-12.3	-6.1	-1.1	-4.7	2.6	9.2	5.2	13.1	10.1	6.7	13.4	**<0.001**	**0.007**	0.215
Δ Total Work (J)	-71.4	-108.5	-34.2	-63.6	-95.2	-35.6	148.5	86.3	210.7	193.7	146.2	241.2	**<0.001**	0.536	0.781
Liver															
IL-6 ($pg \cdot mL^{-1}$)	859.2	741.1	977.1	628.8	568.3	685.3	555.4	469.2	641.7	461.6	388.3	534.8	**<0.001**	**<0.001**	0.129
TNF-α ($pg \cdot mL^{-1}$)	1917.8	1634.7	2201.0	1546.4	1361.7	1731.2	1368.0	1109.5	1626.8	1414.9	1293.9	1536.0	**0.005**	0.162	0.074
IL-1β ($pg \cdot mL^{-1}$)	2021.0	1766.7	2275.2	1932.5	1699.1	2166.0	1646.4	1427.9	1864.9	1764.2	1496.9	2031.6	**0.009**	0,907	0,415
IL-10 ($pg \cdot mL^{-1}$)	452.6	364.5	540.7	582.2	489.4	675.0	665.5	562.37	768.7	657.6	579.9	736.6	**0.004**	0.201	0.150
Gastrocnemius Muscle															

GLUT4 counter (1000 cells)	91.6	83.0	100.3	168.6	157.7	179.5	210.8	201.6	219.6	292.9	283.1	302.7	**<0.001**	**<0.001**	**0.002**
IL-6 (pg·mL^{-1})	47.4	42.0	52.8	42.3	32.0	52.6	36.6	27.9	45.3	32.0	19.9	44.0	**0.039**	0.603	0.780
TNF-α (pg·mL^{-1})	121.1	94.6	147.7	87.2	71.7	102.7	80.1	64.6	95.5	68.0	44.6	91.4	**0.008**	**0.039**	0.313
IL-1β (pg·mL^{-1})	64.7	53.9	75.4	55.1	45.9	64.3	49.7	40.4	59.1	49.7	34.4	65.1	0.093	0.418	0.419
IL-10 (pg·mL^{-1})	5.2	2.5	7.8	9.5	4.9	14.0	8.8	3.9	13.8	9.3	6.3	12.2	0.274	0.322	0.234
Pancreas															
Islet Langerhans Area (μm^2)	7.4	6.4	8.5	8.3	7.2	9.5	9.9	8.9	11.0	11.2	10.1	12.2	**<0.001**	0.097	0.473
IL-6 (pg·mL^{-1})	200.2	170.5	229.9	158.4	139.4	177.3	168.8	149.6	188.0	155.6	134.9	176.3	0.149	**0.024**	0.226
TNF-α (pg·mL^{-1})	642.1	532.1	752.1	241.3	136.8	345.7	211.5	132.3	290.7	308.9	209.1	408.6	**0.001**	**0.006**	**<0.001**
IL-1β (pg·mL^{-1})	153.3	98.7	207.8	76.0	40.3	111.6	71.8	47.9	95.6	67.5	39.8	95.3	**0.026**	**0.041**	0.066
IL-10 (pg·mL^{-1})	11.4	5.5	17.2	40.1	30.2	50.0	35.4	25.1	45.8	37.8	23.8	51.8	**0.009**	**0.007**	**<0.001**
Adipose Tissue															
Adipocyte area (μm^2)	143704	128072	159336	93841	69054	118628	81452	69771	93133	68510	57987	79033	**<0.001**	**0.001**	0.100
IL-6 (pg·mL^{-1})	202.2	161.9	242.6	82.8	59.0	106.5	89.9	72.7	107.1	89.6	59.7	119.4	**0.009**	**0.007**	**<0.001**
TNF-α (pg·mL^{-1})	615.4	447.3	783.6	329.0	234.6	423.5	323.2	254.6	391.8	333.2	238.6	427.8	**0.018**	**0.023**	**0.016**
IL-1β (pg·mL^{-1})	227.0	167.8	286.8	102.0	48.4	155.6	156.3	116.7	196.0	137.0	101.8	172.2	**0.246**	0.056	0.156
IL-10 (pg·mL^{-1})	30.7	25.6	35.8	33.4	21.7	45.1	42.6	31.6	53.5	41.9	28.0	55.8	0.232	0.602	0.943

Δ: Variation (delta); IL: Interleukin; HDL: High-; LDL: Low-density lipoprotein; a: activity; s: supplementation; Vpeak: Peak velocity.

Supplementary File

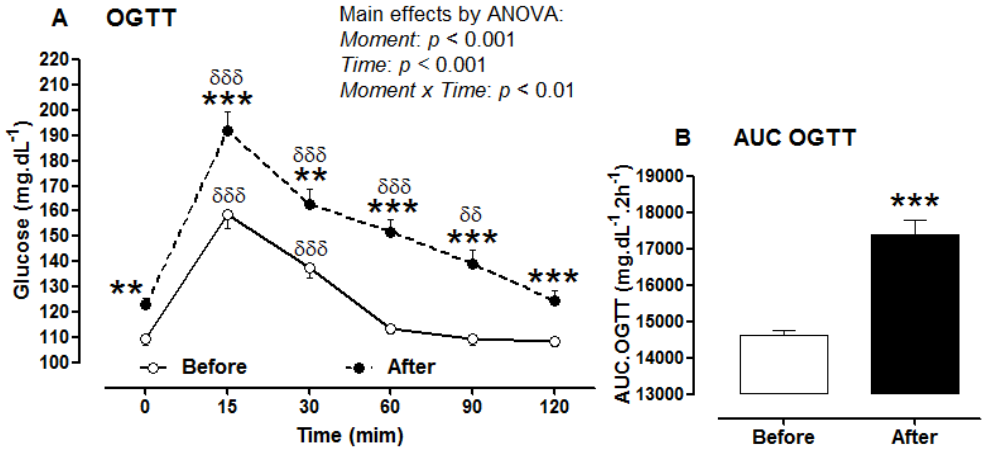


Figure S1. Oral glucose tolerance test performed before and after consumption of high-fat diet and fructose-rich beverage (A). Area under curve of OGTT (B). Data are expressed as the means and standard error of the mean. Figure S1A: only the significant main effects of ANOVA are shown in the graphs. Post hoc analysis for moment effect: ** $p < 0.01$; *** $p < 0.001$ to Before vs. After. Post hoc analysis for time effect: δδ $p < 0.01$; δδδ $p < 0.001$, vs. 0 (baseline). Figure S1B: Tukey's T-test, *** $p <$ 0,001 vs. Before.

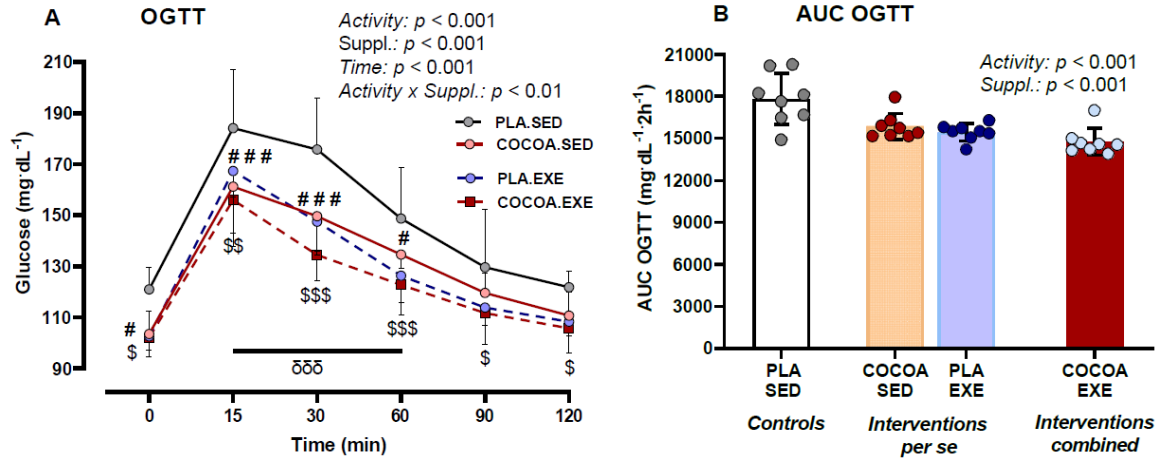


Figure S2. Oral glucose tolerance test OGTT (A) and area under the curve (B) after eight weeks of intervention with supplementation of cocoa flavanols and/or aerobic training. Data are expressed as the mean and standard error of the mean. Figure 4a-b: Only the significant main effects of ANOVA are shown in the graphs. Post hoc analysis for activity effect: $ $p < 0.05$; $$ $p < 0.01$; $$$ $p < 0.001$ for PLA.SED vs. PLA.EXE; Post hoc analysis for supplementation effect: # $p < 0.05$; ### $p < 0.001$ for PLA.SED vs. COCOA.SED; Post hoc analysis for time effect: δ $p < 0.05$; δδδ $p < 0.001$, vs. 0 (baseline).

Table S1: Combined effects of aerobic training and chronic supplementation with cocoa flavanols on aerobic performance, metabolism, and inflammatory and lipid mediators in obese insulin resistant rats.

	PLA.SED			COCOA.SED			PLA.EXE			COCOA.EXE			Two-way ANOVA		
	Mean	CI 95%		Mean	CI 95%		Mean	CI 95%		Mean	CI 95%		a	s	a x s
Δ Body mass	71.3	39.7	102.8	49.8	37.5	62.0	53.0	41.0	65.0	39.1	13.9	64.3	**0.049**	**0.034**	0.746
Δ Adiposity Index	7.6	6.1	9.2	5.7	4.0	7.4	5.9	4.8	6.9	4.4	3.4	5.4	**0.021**	**0.034**	0.754
Glucose Homeostasis															
Glucose ($mg \cdot dL^{-1}$)	136.1	120.2	1520	105.0	100.2	109.8	105.3	100.6	109.9	100.8	95.9	105.6	**<0.001**	**<0.001**	**0.007**
Insulin ($ng \cdot dL^{-1}$)	8.1	7.1	9.1	4.3	3.4	5.1	4.2	3.2	5.3	3.4	3.0	3.9	**<0.001**	**<0.001**	**<0.001**
HOMA-index	2.7	2.3	3.1	1.0	0.8	1.2	1.1	0.8	1.4	0.9	0.7	1.0	**<0.001**	**<0.001**	**<0.001**
Hormone Levels															
Adiponectin ($ng \cdot mL^{-1}$)	737.5	574.3	900.7	1210.0	877.3	1542.7	1164.6	838.1	1491.1	1491.2	1191.7	1789.8	**0.025**	**0.018**	0.585
Leptin ($ng \cdot mL^{-1}$)	2219.9	1896.0	2543.9	1028.4	646.0	1410.8	1015.7	788.6	1242.8	745.2	411.6	1078.8	**<0.001**	**<0.001**	**0.009**
Ratio Adipon. / Leptin	0.3	0.2		1.6	0.8		1.4	0.8	2.0	2.2	1.4	2.9	**0.009**	**0.029**	0.707
Lipids Profile															
Total Cholesterol ($mg \cdot dL^{-1}$)	139.4	110.7	168.1	89.9	75.6	104.2	88.8	76.6	101.1	114.3	97.6	130.3	0.191	0.224	**<0.001**
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Aerobic Performance															
Δ $\dot{V}O_{2peak}$ $(ml \cdot kg^{-1} \cdot min^{-1})^{\wedge 0.75}$	-7.3	-14.1	-0.5	0.5	-3.9	4.9	4.3	-0.6	9.1	4.1	-0.4	9.1	**0.016**	0.193	0.243
Δ Total Exercise Time (min)	-14.3	-19.2	-9.0	-1.7	-6.8	3.4	16.0	9.3	22.7	17.2	11.7	22.8	**<0.001**	**0.004**	0.165
Δ V_{peak} $(m \cdot min^{-1})$	-9.3	-12.3	-6.1	-1.1	-4.7	2.6	9.2	5.2	13.1	10.1	6.7	13.4	**<0.001**	**0.007**	0.215
Δ Total Work (J)	-71.4	-108.5	-34.2	-63.6	-95.2	-35.6	148.5	86.3	210.7	193.7	146.2	241.2	**<0.001**	0.536	0.781
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IL-6 ($pg \cdot mL^{-1}$)	859.2	741.1	977.1	628.8	568.3	685.3	555.4	469.2	641.7	461.6	388.3	534.8	**<0.001**	**<0.001**	0.129
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IL-1β ($pg \cdot mL^{-1}$)	64.7	53.9	75.4	55.1	45.9	64.3	49.7	40.4	59.1	49.7	34.4	65.1	0.093	0.418	0.419
IL-10 ($pg \cdot mL^{-1}$)	5.2	2.5	7.8	9.5	4.9	14.0	8.8	3.9	13.8	9.3	6.3	12.2	0.274	0.322	0.234
Pancreas															
Islet Langerhans Area (μm^2)	7.4	6.4	8.5	8.3	7.2	9.5	9.9	8.9	11.0	11.2	10.1	12.2	**<0.001**	0.097	0.473
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