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Basic nutritional investigation

# Chia oil supplementation changes body composition and activates insulin signaling cascade in skeletal muscle tissue of obese animals

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ABSTRACT

*Objective:* Chia seed oil is the richest source of plant-based ω-3 fatty acid, α-linolenic acid, but its potential and mechanisms of action to treat obesity are unclear. The aim of the study was to evaluate the effects of chia oil (ChOi) supplementation on body composition and insulin signaling in skeletal muscles of obese mice.
*Methods:* Male C57 BL/6 mice (n = 8/group) were fed regular control chow or a high-fat diet (HFD) for 135 d. Another HFD group additionally received ChOi from 90 to 135 d.
*Results:* Consumption of ChOi reduced fat mass accumulation and increased lean mass as evidenced by nuclear magnetic resonance. Moreover, obese mice treated with ChOi showed higher tyrosine phosphorylation of insulin receptor substrate 1, greater activation of protein kinase B, and increased translocation of glucose transporter type 4 in skeletal muscle tissue in response to insulin. ChOi supplementation improved glucose levels and insulin tolerance; decreased serum insulin, leptin, and triacylglycerols; and increased blood high-density lipoprotein cholesterol levels. All these effects caused by the use of ChOi seemed to be independent of the resolution of inflammation because the markers of inflammation were not altered in animals fed the HFD.
*Conclusion:* The molecular effects observed in muscle tissue together with changes in body composition may have contributed to the increased glucose tolerance and to the healthy phenotype presented by obese animals treated with ChOi.



## Introduction

Obesity is defined as abnormal or excessive fat accumulation that presents a risk to health [1]. Treating and preventing obesity should be a priority in public health policies.

Changes in body composition, such as increased lean mass and reduced fat mass, may improve the insulin sensitivity of obese individuals [2,3]. Although still controversial, the use of bioactive compounds such as ω-3 fatty acids of animal origin and polyphenols may improve insulin resistance (IR) caused by obesity [4–14].

The richest plant food source of ω-3 fatty acid is chia (*Salvia hispanica* L.), a native seed from Mexico and parts of South America, which is also rich in phenolic compounds [14,15]. It remains unclear, however, whether the observed effects on glycemia are due to the fiber content of chia or its bioactive compounds content [16–18]. Only a few studies have evaluated the potential of the oil supplementation to treat obesity-related injuries [19,20] and little is known about the possible molecular mechanisms of action triggered by the dietary supplementation with chia oil (ChOi) in obesity. Considering the limited knowledge about the physiological and molecular mechanisms that may be involved in the improved glycemic response caused by ChOi in obesity, we raised the hypothesis that supplementation with ChOi in obese animals could improve the insulin cell signaling pathway and reduce the percentage of fat mass, improving glycemic control.

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## Methods

### Animals and diets

This study was carried out in strict accordance with the ethical standards of the 1964 Declaration of Helsinki. Animal procedures were approved by the Committee on the Ethics of Animal Experimentation of the Universidade do Estado do Rio de Janeiro. C57 BL/6 N mice were obtained from the animal facilities of the National Cancer Institute, Rio de Janeiro, Brazil. Animals were housed in a temperature-controlled room (25°C ± 1°C) with 60% humidity and a 12-h artificial light/dark cycle.

Male mice (n = 8/group) were housed in cages (n = 4 animals/cage) and were fed either regular control chow (C) or a high-fat diet (HFD) for 135 d. Another HFD group was supplemented with ChOi (HFD+ ChOi) from 90 to 135 d. Details of the diets are provided in the supplementary material section. After weaning (21 d), the animals received an HFD until completing 90 d of age [21]. The amount and time of ChOi supplementation were established based on the time and equivalent amount of ω-3 fatty acids offered in the study by Oh et al. [22]. The complete chemical composition of the chia oil was previously described by Pereira da Silva et al. [23]. Body weight, food intake, and energy intake were measured throughout the treatment period.

### Body composition analysis

Body composition analysis was performed by nuclear magnetic resonance (NMR). Briefly, mice were scanned using the body composition analyzer for small animals (Minispec LF90 TD-NMR; Bruker, Billerica, MA, USA). The instrument was calibrated for these studies using NMR scans and chemical composition data from 10 mice. On the day of testing, a quality control check of internal voltages, temperature, magnets, and NMR parameters was performed using a standard provided by the manufacturer. Mice were placed in a clear plastic cylinder (50 mm diameter) and kept immobile without anesthesia by insertion of a tight-fitting plunger into the cylinder. The tube was then lowered into the sample chamber of the instrument for ~2 min, which was the duration of the scan.

### Intraperitoneal glucose tolerance test

At 120 d of life, mice were fasted for 12 h and basal glucose was measured. The mice were then administered an intraperitoneal bolus of glucose (2 g/kg body weight) and glycemia was monitored every 30 min for up to 120 min. Glycemia was measured with an Accu-Chek Active glucometer (Roche Diagnostics, Mannheim, Germany).

### Intraperitoneal insulin tolerance test

At 127 d of life, mice were weighed and fasting blood glucose levels were measured. Mice were then administered an intraperitoneal injection of insulin (0.1 U/mL, Humulin human insulin; Eli Lilly, São Paulo, Brazil) resulting in a dose of 0.5 U/kg. Blood glucose was measured 15, 30, 45, 60, and 120 min after injection.

### Sample collection

At 135 d of life, mice were fasted overnight for 10 ± 2 h and were sacrificed by withdrawing blood from the heart under anesthesia and mixing ketamine (50 mg/kg) with xylazine (20 mg/kg) (König, Buenos Aires, Argentina). Plasma was obtained by blood centrifugation (800g; 10 min) and stored at −80°C until analysis were performed [24].

### Leptin and cytokine measurements

Serum levels of leptin, interleukin (IL)-6, and tumor necrosis factor (TNF)-α were measured using enzyme-linked immunosorbent assay (ELISA) kits (Peprotech, Rocky Hill, NJ, USA). Fasting plasma insulin was measured by ELISA (Millipore, Billerica).

### Muscle protein preparation

To evaluate the insulin response, the gastrocnemius muscle was dissected (30 μg) on ice. Next, 1 μM insulin or vehicle (phosphate-buffered saline) was added to the muscle cells and the plate was incubated at 37°C for 30 min. Time and insulin concentration were determined based on a previous study published by Garcia-Souza et al. [25]. The sample was centrifuged at 1300g for 10 min, the supernatant was discarded, and the pellet was resuspended in 1 mL of HES lysis buffer (10 mM HEPES, 5 mM EDTA, 250 mM sucrose, 1 mM sodium orthovanadate, protease inhibitors [PMSF, aprotinin, and leupeptin; 40 μL: 1 mL]). The mixture was kept on ice for 30 min, sonicated with an ultrasonic convertor (VirtisVirsonic 60; The VirTis Company, New York, NY, USA) three times for a total of 45s, and then left for 30 min on ice. The tube was centrifuged at 750g for 3 min at 4°C. The supernatant was withdrawn and stored. The pellet was resuspended in 1 mL of HES and centrifuged at 750g for 3 min at 4°C. The supernatant from the second centrifugation was pooled with the first supernatant. The pooled sample was subjected to ultracentrifugation at 31 000g for 60 min at 4°C. The supernatant was the cytosolic fraction and the pellet was the membrane fraction. The samples were stored in a freezer at −80°C for subsequent protein analyses.

### Western blot analysis

Protein concentration was determined with the BCA Protein Assay kit (Pierce Biotech, Rockford, IL, USA). Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline. The membranes were incubated overnight at 4°C with antiactin (1:500), antiinsulin receptor-β (IR-β, 1:500), antiglucose transporter type 4 (GLUT4,1:500), antiprotein kinase B (Akt; 1:500), antiinsulin receptor substrate 1 (IRS-1, 1:500) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), antipIRS1 (1:500), or antipAkt (1:500) monoclonal antibodies (mAbs; the latter two from Cell Signaling Technology, Waltham, MA, USA). The membranes were washed and developed with horseradish peroxidase-coupled antimouse immunoglobulin G antibodies (Abcam, Cambridge, UK), followed by signal detection with ECL detection kits (Amersham Biosciences) and using a Gel Doc 2000 Imager (Bio-Rad Laboratories, Hercules, CA, USA). The protein bands were digitally quantified by densitometry using ImageJ 1.34s software (NIH, Bethesda. MD, USA).

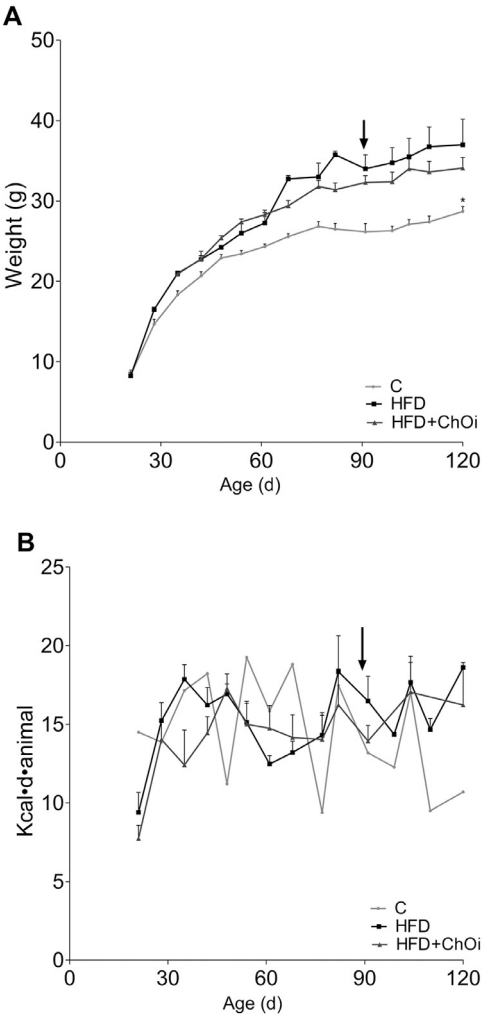


**Fig. 1.** Body weight curve (**A**) and total energy intake (**B**). C, control diet group (n = 8); H, high-fat diet group (n = 8); HFD + ChOi, high-fat diet supplemented with chia oil (n = 8). The arrows indicate the beginning of chia oil supplementation. Data are expressed as mean ± SD and statistically significant differences are established at $P < 0.05$.

*Statistical analyses*

Values were analyzed with two-tailed unpaired Student's *t* test or analysis of variance and are expressed as mean ± SD. When appropriate, individual comparisons were subsequently tested with Bonferroni's *t* test for unpaired values. Differences were considered statistically significant when $P < 0.05$. Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA).

## Results

*ChOi supplementation improves glucose tolerance and insulin sensitivity in mice*

Obese mice fed the HFD + ChOi diet had lower energy intake than mice in the HFD group. However, no significant difference was detected in the body mass between the groups (Fig. 1A, B). In addition, ChOi intake improved glucose and insulin tolerance (Fig. 2A, B, and D) and diminished serum fasting insulin levels compared with those of the HFD group, with the values approaching the values of the lean control group (Fig. 2C).

*ChOi supplementation decreases blood levels of leptin and triacylglycerols in obese mice*

Compared with the levels in the HFD group, the HFD + ChOi mice displayed reductions in serum leptin and triacylglycerol (TG) levels (Fig. 3A, D), but no significant differences in total cholesterol blood concentrations (Fig. 3E). However, the serum high-density lipoprotein cholesterol (HDL-C) level was higher in the HFD + ChOi mice than in the HFD mice (Fig. 3F). One week after assessing glucose and insulin sensitivities, when the animals were sacrificed, inflammatory markers were evaluated in adipose tissue and plasma. HFD-fed animals did not present any increase in the markers of inflammation: circulating levels of TNF-α and IL-6 (Fig. 3B, C), or epididymal mRNA expression (supplementary material).

*ChOi supplementation changes body composition*

Although ChOi treatment did not reduce the total body mass of the obese animals, a comparison of the body composition between the groups revealed the significant reduction in body fat mass (Fig. 4A) and an increase in lean body mass in the HFD + ChOi group (Fig. 4B) relative to the HFD group.

*ChOi supplementation improves insulin signaling in obese animals*

To understand where in the insulin signaling pathway the ChOi supplement acts to improve glucose tolerance, we analyzed the expression and/or activation of key proteins involved in insulin signaling in skeletal muscle from the HFD and HFD + ChOi mice. No significant differences in IR-β (Fig. 5A) or IRS1 expression were observed between the HFD and HFD + ChOi mice. Supplementation with ChOi increased the insulin-stimulated phosphorylation on Tyr989 of *IRS-1* (Fig. 5B). Furthermore, total Akt content did not

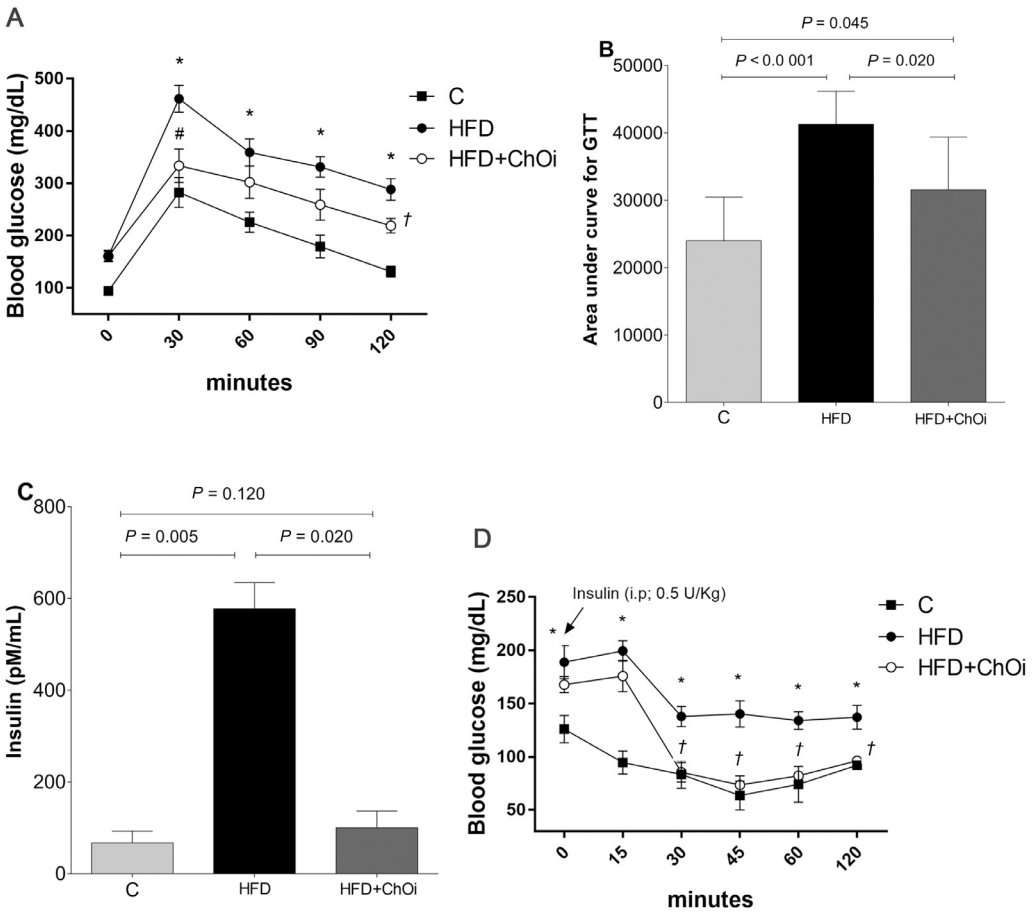


**Fig. 2.** Glucose tolerance test (GTT) (**A**), area under the curve for GTT (**B**), insulin levels (**C**), insulin tolerance test (**D**) in mice fed the C diet, HFD, or HFD supplemented with chia oil. C, control diet group (n = 8); H, high-fat diet group (n = 8); HFD + ChOi, high-fat diet supplemented with chia oil (n = 8). Data are expressed as mean ± SD and statistically significant differences are established at $P < 0.05$.

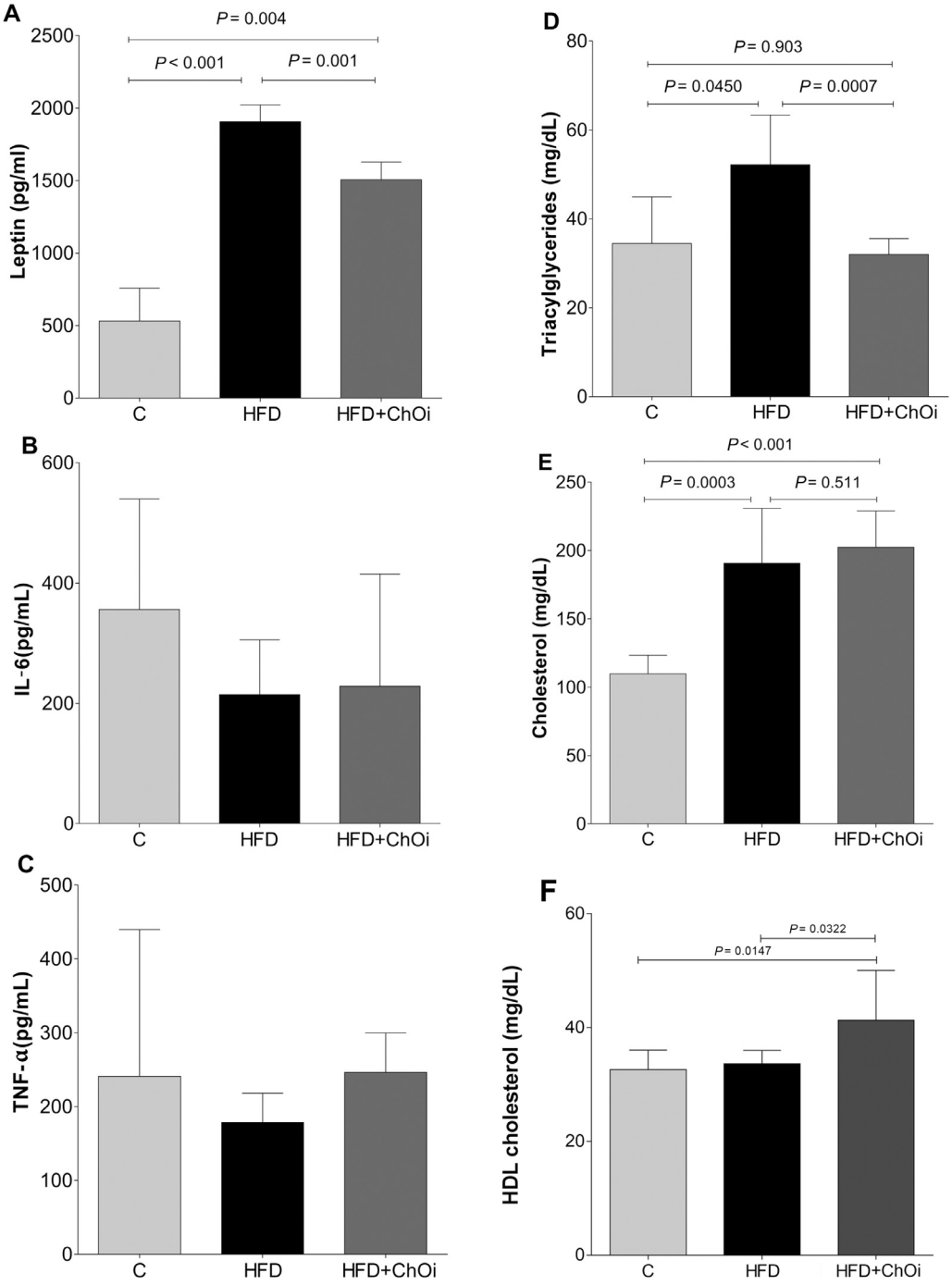


**Fig. 3.** Serum levels of leptin (**A**), TNF-α (**B**), IL6 (**C**), triacylglycerols (**D**), cholesterol (**E**), and HDL cholesterol (**F**) in mice fed the C diet, HFD, or HFD supplemented with chia oil. C, control diet group (n = 8); H, high-fat diet group (n = 8); HFD + ChOi, high-fat diet supplemented with chia oil (n = 6–8/group) Data are expressed as mean ± SD and statistically significant differences are established at $P < 0.05$.

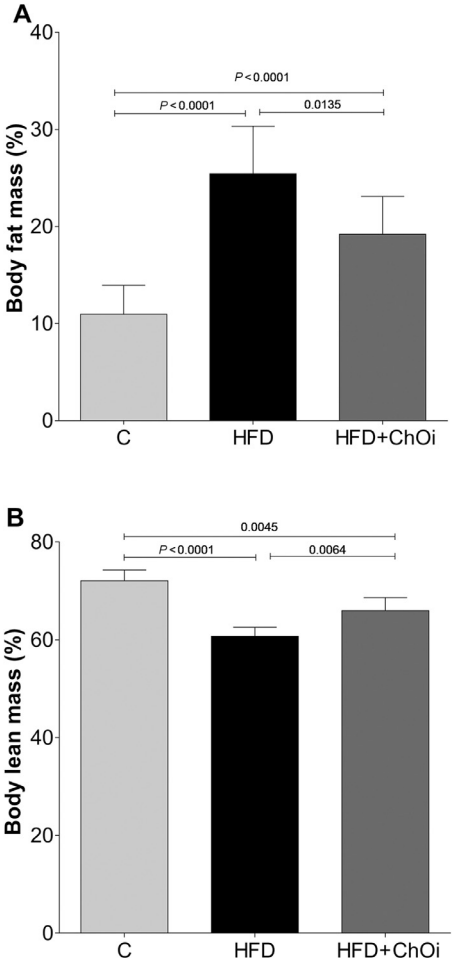


**Fig. 4.** Percentage of body fat mass (**A**) and body lean mass (**B**) in mice fed a C diet (n = 6), HFD (n = 6) or HFD supplemented with chia oil (n = 6). C, control diet group; H, high-fat diet group; HFD + ChOi, high-fat diet supplemented with chia oil. Data are expressed as mean ± SD and statistically significant differences are established at $P < 0.05$.

differ between the two groups, but Akt was highly phosphorylated in the skeletal muscle of HFD + ChOi mice after stimulation with insulin (Fig. 5C). Ultimately, we evaluated the translocation of GLUT4 to the plasma membrane after insulin stimulation in skeletal muscle from the HFD and HFD + ChOi groups. Upon insulin stimulation, GLUT4 was readily translocated to the membrane in skeletal muscle from the HFD + ChOi mice. However, the treatment with insulin failed to induce GLUT4 translocation in the HFD mice (Fig. 5D).

## Discussion

The greatest novelties brought by the present study were the identification of mechanisms responsible for the beneficial effects of the use of ChOi while treating obese animals. In this condition, the oil could reduce fat mass, increase muscle mass, and improve the activation of the insulin signaling pathway in muscle tissue. The change in body composition itself is a mechanism that may contribute to improve glycemic response. Another novelty was that the oil attenuated the symptoms of obesity regardless of resolving inflammation because the obese animals did not show any signal of inflammation. What the most recent literature indicates is that the IR caused by an HFD leads to inflammation, and not the opposite [26]. This is an innovative aspect of the mechanism of action of chia oil. The effects brought by ChOi supplementation were metabolic syndrome (MetS) attenuation, with improved glucose tolerance and IR, reduced serum levels of TGs and increased HDL-C cholesterol. These results show potential health benefits exhibited by an oil of plant origin, which is source of phenolic compounds and ω-3 fatty acid [14,15,23].

Although there are many controversies, some studies indicate a potential effect of bioactive compounds, such as phenolic compounds or ω-3 fatty acids, on the resolution or minimization of symptoms of MetS [4–13,27]. Regarding fatty acids, marine oils are considered more potent because they do not depend on genetic or dietary factors for the bioconversion of α-linolenic acid (ALA; 18:3, ω-3) into eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) [4–7,28]. In the present study, it was observed that obese animals supplemented with ChOi exhibited increased accumulated amount of EPA and DHA in the liver (supplementary material). Possibly, these data result from the sum of genetic factors, treatment time, dosage, and ω-6/ω-3 fatty acid ratio and other bioactive compounds also present in ChOi. Despite this, there is a body of evidence indicating that ALA can be effective regardless of its bioconversion to EPA and DHA. ALA is the most abundant ω-3 fatty acid in human adipose tissue, accounting for ~1% of total fatty acids, whereas there are only traces of DHA and EPA [29,30], suggesting that ALA should not necessarily be used for the synthesis of EPA and DHA, and may be oxidized or stored in adipose tissue [31]. Supplementation with ALA mostly increases EPA in various tissues. But the physiological responses induced by ALA seems to be different from those induced directly by EPA and DHA, especially in cases of obesity. A study using EPA, DHA, and ALA supplementation in animals with MetS induced by a diet rich in fats and carbohydrates, demonstrated that ALA did not alter total body fat but promoted a lipid redistribution of the abdominal area, improved glucose tolerance and insulin sensitivity, attenuated dyslipidemia, and improved cardiovascular parameters. Supplementation with EPA and DHA showed similar results; however, it did not improve glucose tolerance. The authors concluded that ALA responses in MetS are independent of their conversion to EPA and DHA [32].

Another plant source of ω-3 fatty acid is flaxseed. However, Mohammadi-Sartang et al., in their systematic review and meta-analysis, concluded that whole flaxseed, but not flaxseed oil, has significant effects on improving glycemic control [33].

To our knowledge, this was the first time that the insulin signaling pathway in skeletal muscle tissue has been investigated in obese animals in response to ChOi supplementation. When challenged with insulin, the mice consuming the diet supplemented with ChOi showed increased Akt phosphorylation and phosphorylation of IRS-1 on Tyr 989, and the decreased content of GLUT4 in the cytosol. In a previous study, in which corn oil was replaced by chia seed in the diet of non-obese animals, muscle tissue metabolism was altered [16]. However, that study was not able to clarify whether the effects were due to the lipid content, dietary fiber content, or both, and there was no information regarding the fatty acid profile present in those animal tissues. Because of the potential of fibers to reduce fatty acid uptake and improve glycemic response, we chose to evaluate the effects of the oil supplementation.

The obese mice maintained on a hypercaloric and HFD with ChOi supplementation showed incremental changes in lean mass and reduced fat mass, as determined using NMR. Several studies have concluded that the content of fat mass is directly related to IR [34,35]. In addition, other studies have indicated that a greater percentage of lean mass is positively associated with an improvement in the glycemic

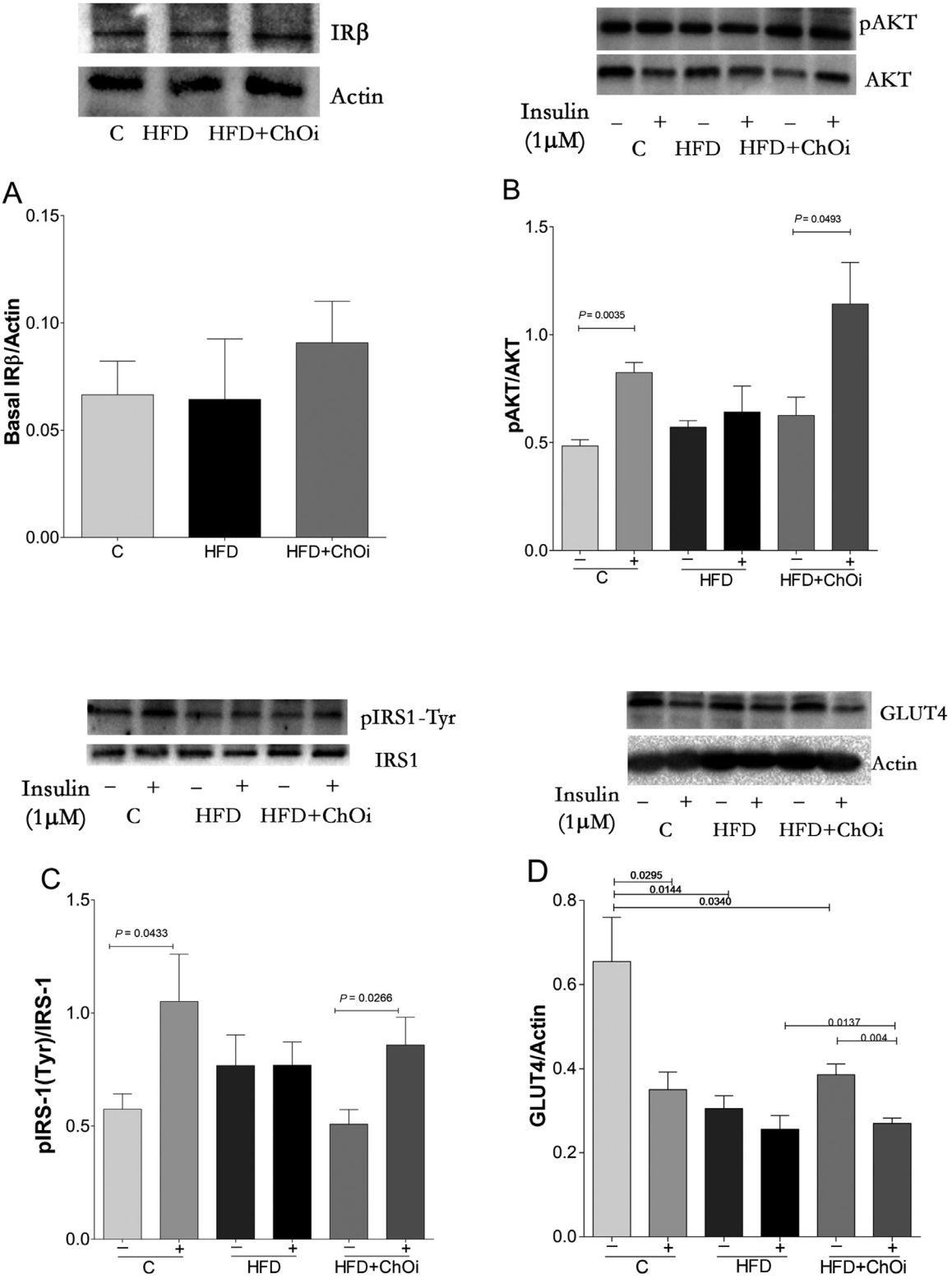


**Fig. 5.** Activation and expression of proteins involved in insulin signaling pathway. Protein expression levels of IR-β, pIRS-1, IRS-1, pAkt, Akt, GLUT4, and actin in muscle and densitometric quantitation of IR-β/actin, pIRS-1/IRS-1, pAkt/Akt, and GLUT4/actin ratio. Skeletal muscle strips were isolated and stimulated (in vitro) in the absence (−) or presence (+) of insulin (1 μM) for 30 min. H, high-fat diet group; HFD + ChOi, high-fat diet supplemented with chia oil (n = 6–8) Data are expressed as mean ± SD and statistically significant differences are established at $P < 0.05$.

response, possibly because of an increase in glucose uptake sites [2,3,36]. Increased muscle mass associated with greater efficiency in insulin signaling was probably responsible for the observed reduction of glycemia and the improvement of IR. Other studies using chia seed [16–18,37,38] or oil [19] also failed to reduce total body mass. Future studies should investigate whether the lean mass increase is caused by the activation of signaling pathways of muscle synthesis.

ChOi supplementation did not affect the circulating levels of total cholesterol in mice fed to HFD + ChOi diet, but was able to increase the HDL concentration. Obese mice treated with ChOi showed a 30% reduction in their serum TG concentration, even without decreasing the amount of carbohydrates offered in the diet. Because increased plasma TG concentration is directly related to the development of cardiovascular diseases, the use of polyunsaturated fatty acid ω-3 is an option for the treatment of hypertriacylglycerolmia [39].

It seems that the ChOi supplementation effects observed in obese mice did not depend on the inflammation resolution as the HFD-induced obesity model used in this study did not result in an increase in systemic inflammatory markers, as was apparent from the assessments of IL-6 and TNF-α serum concentrations. Recent studies have questioned the direct and independent association between inflammation and obesity. Kim et al. demonstrated that an increase in the size of adipocytes can lead to IR, regardless of inflammation [40]. The authors described some lines of evidence to support this claim, such as Cushing syndrome, in which patients have high blood levels of glucocorticoids and develop central obesity and IR while being immunosuppressed. Another line of evidence presented refers to the reduction in early B-cell factor-1, which increases the size of adipocytes and causes IR but does not influence inflammatory pathways [41]. Another study showed that HFD induces adipocyte hypertrophy and IR independent of the inflammatory response. To demonstrate that the effect was independent of inflammation, the authors used c-Jun N-terminal kinase 1 and recombination activating gene 1 knockout mice (which do not produce mature T and B cells in the immune system) and mice treated with clodronate (to deplete macrophages) [42]. More recently, Shimobayashi et al. demonstrated that is not the inflammation that determines IR [26]. Thus, we understand that the obese animal used in our study exhibited IR independently of presenting inflammation. Therefore, the beneficial effect presented by the use of ChOi seems to be independent of the resolution of the inflammation.

## Conclusion

The molecular signaling effects observed in muscle tissue together with changes in body composition may have contributed to the healthy phenotype presented by the obese animals treated with ChOi. According to the concept of obesity presented by the World Health Organization, which considers obesity to be an excess of adiposity that causes damage to health, the use of ChOi reduced the degree of obesity in the mice we studied, as it reduced fat mass and reversed the symptoms of MetS. The results of this preclinical mechanistic study provided evidence that the use of ChOi could improve obese comorbidities even when offered along with an obesogenic diet.

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## Supplementary materials

Supplementary data related to this article can be found at doi:10.1016/j.nut.2018.08.011.

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