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Salvia hispanica L. (chia) seed improves skeletal muscle lipotoxicity and insulin sensitivity in rats fed a sucrose-rich diet by modulating intramuscular lipid metabolism

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ABSTRACT

This study investigates the possible mechanisms involved in the beneficial effect of chia seed on skeletal muscle lipotoxicity and insulin resistance in rats fed a sucrose-rich diet (SRD). Results showed that chia seed reduced the increased lipid content in the skeletal muscle of SRD-fed rats. This was accompanied by an increase of muscle-type carnitine palmitoyltransferase 1 enzyme activity, peroxisome proliferator-activated receptors (PPARα, PPARγ) and phosphorylated AMP activated protein kinase protein levels. Moreover, the precursor and mature forms of sterol regulatory element-binding protein-1 and lipogenic enzyme activities were decreased. Fatty acid translocase FAT/CD 36 and *n*-3/*n*-6 fatty acids ratio of membrane phospholipids were increased. This work shows that an increase in the key factors levels associated with muscle oxidative capacity and a reduction in the lipogenic pathway would be some mechanisms involved in the beneficial effects of chia seed on skeletal muscle lipotoxicity in insulin resistant SRD-fed rats.

1. Introduction

Insulin resistance (IR) is one of the central etiopathogenic factors of the Metabolic Syndrome (MS) whose interrelated components increase the incidence of cardiovascular disease and type 2 diabetes, pathologies associated with high morbidity and mortality rates (O'Neill & O'Driscoll, 2015; Roberts, Hevener, & Barnard, 2013). The study of both, its physiopathology (currently not fully clarified) and possible therapeutic interventions for its prevention/improvement represent an area of great interest (Rochlani, Pothineni, Kovelamudi, & Mehta, 2017).

Skeletal muscle is considered one of the main peripheral tissues involved in the maintenance of glucose homeostasis and insulin sensitivity. It is involved in the clearance of 25% of the plasma glucose in the postabsorptive state and up to 75–85% of it in the postprandial state (insulin stimulus) (DeFronzo, 2004). In this tissue, there is a reciprocal regulation between fatty acids and glucose metabolism (metabolic flexibility). Although for many years the study of IR has focused on carbohydrate metabolism, in recent decades there has been a shift towards the study of lipid metabolism as the main promoter of this disorder (Tumova, Andel, & Trnka, 2016). Lipid accretion in skeletal muscle fibers has been related to the development of lipotoxicity and IR (Ellis et al., 2000; Itani, Ruderman, Schmieder, & Boden, 2002; McGarry, 2002). In this regard, several mechanisms that would contribute to the development of lipotoxicity in the skeletal muscle were proposed. These include a greater availability of lipids (due to an increase in the circulating levels of free fatty acids and/or triglycerides) and different defects in lipid metabolism, such as alterations in the uptake/transport, oxidation, synthesis and degradation of triglycerides, or a combination of them. Additionally, some authors have proposed that a state of "metabolic inflexibility" would be one of the features of the insulin-resistant skeletal muscle (Martins et al., 2012; Turcotte & Fisher, 2008). Moreover, changes in the activity and/or expression of transcription factors, among them, peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element-binding protein-1c (SREBP-1c) that control the aforementioned processes could also play a key role in the development of lipotoxicity in this tissue (Li et al., 2016; Sugden & Holness, 2006).

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We have previously shown that chronic administration (6 months) of a sucrose-rich diet (SRD) (60–65% of total energy) to normal rats induces IR, moderate hyperglycemia, dyslipidemia, visceral adiposity, and lipid ectopic accumulation (skeletal/cardiac muscle, liver, and pancreas) (D'Alessandro, Selenscig, Illesca, Chicco, & Lombardo, 2015; Lombardo & Chicco, 2006). In this experimental model, that mimics the phenotype of human MS, the increase in lipid content in skeletal muscle is associated with an impairment of both insulin-stimulated glucose uptake/transport (glucose transporter 4 -GLUT 4- levels) and oxidative/non-oxidative glucose metabolism. These changes are accompanied by a reduction in the protein mass levels of IRS-1 (Chicco et al., 2003; D'Alessandro, Chicco, & Lombardo, 2006, D'Alessandro, Chicco, & Lombardo, 2013). However, the mechanisms involved in the lipotoxicity and IR in the skeletal muscle have been only partially investigated.

Insulin resistance and several features of the MS could be improved or reverted by nutritional management such as an increase of dietary intake of marine *n-3* polyunsaturated fatty acids (*n*-3 PUFA): 20:5 *n-3* (eicosapentaenoic acid –EPA-) and 22:6 *n-3* (docosahexaenoic acid -DHA-) (Lombardo & Chicco, 2006; D'Alessandro et al., 2013; Flachs, Rossmeisl, & Kopecky, 2014). α-linolenic acid (ALA, 18:3 *n-3*) is another important *n*-3 PUFA, which derives from plant sources. One of the botanical oil sources rich in ALA is the seed of *Salvia hispanica* L., commonly known as chia seed. At present, only a small group of human and experimental studies analyzed some possible beneficial effects of dietary chia seed consumption on the biochemical, metabolic and functional alterations included in the MS (Ayerza & Coates, 2007; Poudyal, Panchal, Waanders, Ward, & Brown, 2012; Vuksan et al., 2017).

In previous works, we have demonstrated that dyslipidemia, IR and visceral adiposity were reversed when chia seed (*Salvia hispanica* L.) was administered as a dietary source of fat in rats chronically fed a SRD (Chicco, D'Alessandro, Hein, Oliva, & Lombardo, 2009; Rossi, Oliva, Ferreira, Chicco, & Lombardo, 2013; Ferreira, Alvarez, Illesca, Giménez, & Lombardo, 2018). Moreover, chia seed improved glucose transport and the impaired oxidative and non-oxidative glucose metabolism in the skeletal muscle (gastrocnemius) of these rats. This was accompanied by a reduction in the intracellular lipid content (Oliva, Ferreira, Chicco, & Lombardo, 2013). However, to the best of our knowledge the mechanisms underlying the beneficial effects of chia seed on the skeletal muscle lipid accretion of these rats have not been investigated.

Accordingly, the aim of the present study was to investigate the mechanisms involved in the beneficial effect of dietary chia seed on the lipotoxicity in the skeletal muscle and IR present in dyslipemic insulin-resistant rats fed a SRD for 6 months.

2. Materials and methods

2.1. Animals and diets

Male Wistar rats (n = 42) purchased from the Faculty of Pharmacy and Biochemistry, University of Buenos Aires (Argentina) were maintained with unrestricted access to water and food under controlled temperature (22 ± 1 °C), humidity and air flow conditions, with a fixed 12-h light/dark cycle (light on from 07:00 to 19:00 h). Adequate measures were taken to minimize the pain or discomfort of the rats and we used the smallest number of animals possible. Animal experiments were evaluated and approved by the Institutional Ethics Committee of the Faculty of Biochemistry and Biological Sciences, (Universidad Nacional del Litoral, Santa Fe, Argentina).

The animals were initially fed a standard powdered rodent commercial diet (GEPSA FEED, Buenos Aires, Argentina). When they weighted 180–200 g they were randomly divided into two groups and were housed individually. One group (n = 14) continued receiving the standard rodent commercial diet throughout the experimental period (6 months) [reference diet (RD)]. Another group (n = 28) received a semisynthetic diet with sucrose as the carbohydrate source [sucrose-rich diet (SRD)]. After 3 months of treatment, the animals were randomly divided into two subgroups. One subgroup continued on the SRD (n = 14) up to 6 months of feeding and the other (n = 14) received a SRD where 362 g of whole chia seed per kg of diet was incorporated as the source of dietary fat for the next 3 months (SRD + chia). Chia seeds (*Salvia hispanica* L., Salba variety) grown in Salta, Argentine were used for this study and provided by Agrisalba, Buenos Aires, Argentina. The composition of chia seed was provided by the supplier. The content of carbohydrates, proteins, fibers, vitamin and mineral mix in the SRD + chia and SRD was similar, taking into account the amount of these nutrients present in the chia seed. Details of the composition and energy of each diet are given in Table 1. The fatty acid composition of the diets was analyzed as previously described (Fortino, Oliva, Rodriguez, Lombardo, & Chicco, 2017). The preparation and handling of home-made SRD and SRD + chia diets have been reported elsewhere (Creus, Benmelej, Villafañe, & Lombardo, 2017; Ferreira et al., 2018; Oliva et al., 2013). In a separate experiment, the individual weight gain and energy intake of six animals in each group and subgroup were assessed twice per week throughout the experimental period.

At the end of the 6-month experimental period, food was removed at 07:00 h (end of the dark period) and, unless otherwise indicated, experiments were performed between 07:00 and 09:00 h. At least six rats from each dietary group were used in each procedure. They were

Table 1
Composition of experimental diets.

Diet ingredients (g/kg of food)		SRD[a]	SRD + chia[a]
Carbohydrates			
Corn starch		25	
Sucrose		555	555
Chia seed[b]		–	25
Protein			
Casein (vitamin free)		163	86
Chia seed		–	77
Fat			
Corn oil		105	1
Chia seed		–	104
Energy (kJ/g)		16.3	16.3
Fatty acid profile (g/kg of food)[c]	**RD**[d]	**SRD**	**SRD + chia**
16:0	11.80	10.92	6.96
18:0	11.84	2.73	2.42
18:1 *n-9*	26.94	33.71	7.39
18:2 *n-6*	5.68	54.10	19.85
18:3 *n-3*	0.12	0.80	67.26
20:*1 n-9*	0.09	0.47	0.36
Total saturated	23.64	13.65	9.38
Total Monounsaturated	27.03	34.18	7.75
Total Polyunsaturated	5.80	54.90	87.11
n-6	5.68	54.10	19.85
n-3	0.12	0.80	67.26
n-3/n-6	0.021	0.015	3.388
n-3/total saturated	0.005	0.59	7.17

[a] The home-made experimental diets SRD and SRD + chia are based on the modified AIN-93 M diet. Both diets contain by weight (g/kg of diet): salt mix, 35 (AIN-93M-MX); vitamin mix, 10 (AIN-93VX); choline chloride, 2; methionine, 3; fiber, 120. The SRD + chia was balanced in salt mix according to the amount of each one in the chia seed provided by the manufacturer.

[b] Chia seed (Salba; *Salvia hispanica* L.): 362 g/kg diet. Chia composition (g/100 g chia seed): carbohydrate, 37.45; insoluble fiber, 81% of total carbohydrate; fat, 30.23; protein, 21.19. Mineral composition (mg/100 g chia seed): Na, 103.15; K, 826.15; Ca, 589.60; Fe, 11.90; Mg, 77.0; P, 604.0; Zn, 5.32; Cu, 1.66; Mn, 1.36.

[c] Other minor fatty acids have been excluded.

[d] Rodent commercial diet (g/kg diet): carbohydrate (corn, sorghum, wheat, oats, barley) 420; protein 240; fat 60; fiber 70; minerals and vitamins 80; water 130; digestible energy 15.33 kJ/g as stated by the manufacturer.

anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg body weight). Blood samples were collected from the inferior vena cava, rapidly centrifuged and serum was either immediately assayed or stored at −20 °C until used. Gastrocnemius skeletal muscle (red and white fiber muscle mixture) was totally removed and stored at −80 °C. Epididymal, retroperitoneal and mesenteric adipose tissues were also removed, weighed, and visceral adiposity index was calculated as previously described (Rossi et al., 2013). In another group of animals, whole-body peripheral insulin sensitivity was measured in fasting rats (5 h) using the euglycemic–hyperinsulinemic clamp technique, which had been used by our laboratory in earlier studies (D'Alessandro, Chicco, Karabatas, & Lombardo, 2000). The glucose infusion rate (GIR) during the second hour of the clamp study was taken as the net steady-state of the whole-body glucose. The animals were euthanized by an overdose of sodium pentobarbital administration according to their body weight.

2.2. Analytical methods

Serum triglyceride and glucose levels were measured by spectrophotometric methods using commercial enzymatic kits according to the manufacturer's protocols (Wiener Lab., Argentina). Serum free fatty acids (FFAs) were determined using an Acyl-CoA oxidase based colorimetric kit (Randox Laboratories Limited, United Kingdom). Insulin was measured by immunoradiometric assay according to the method of Herbert, Lau, Gottlieb & Bleicher (1995). The assay was calibrated against the rat insulin standard (Novo Nordisk, Copenhagen, Denmark).

2.3. Lipid content and fatty acid analysis

Triglycerides, long-chain acyl-CoA (LCA-CoA) and diacylglycerol (DAG) content was determined in gastrocnemius muscle homogenates, as described in detail in previous researches (D'Alessandro et al., 2006). Gastrocnemius muscle lipids were extracted according by Folch, Lees, and Sloane Stanley (1957) and the fatty acid composition of total phospholipids was determined by gas liquid chromatography of their methyl esters, as previously described (D'Alessandro et al., 2013). All chemicals used for these and subsequent experiments were of analytical grade and were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.4. Enzymatic activity assays

The activities of muscle-type carnitine palmitoyltransferase 1 (M-CPT1), M-CPT-2 and total M-CPT were determined spectrophotometrically according to Ling, Aziz, and Alcorn (2012) with some modifications. Frozen muscle was homogenized at 4 °C in a homogenization buffer containing: 20 mM HEPES (pH 7.4); 140 mM KCl; 10 mM EDTA; 5 mM $MgCl_2$ and centrifuged at 500*g* at 4 °C for 10 min. The supernatant was centrifuged at 4 °C at 9000*g* for 45 min and the resulting mitochondrial pellet was resuspended in the homogenization buffer. To determine the total M-CPT activity, an aliquot of the mitochondrial extract was added to a reaction medium comprising: 20 mM HEPES (pH 7.4); 1 mM EGTA; 220 mM sucrose; 40 mM KCl; 0.1 mM 5,5′-dithio-bis (2-nitrobenzoic acid) DTNB; 1.3 mM bovine serum albumin and 40 μM palmitoyl-CoA. The reaction was initiated with the addition of 1 mM L-carnitine. The rate of appearance of TNB-CoAS was monitored at 412 nm for 5 min at 37 °C. The total M-CPT activity was calculated based on the variation of absorbance per minute and the molar extinction coefficient of the DTNB (ε = 13.6 mM). The activity of M-CPT2 was determined under the same assay conditions in the presence of 10 μM malonyl-CoA in the reaction medium. The M-CPT1 activity was calculated as the difference between total M-CPT and M-CPT2 activities. For lipogenic enzyme activities assays, frozen tissue was homogenized at 4 °C in a buffer (pH 7.4) containing 250 mM sucrose; 1 mM DTT; 1 mM EDTA and centrifuged at 4 °C at 10 500*g*. Fatty acid synthase (FAS) and glucose-6 phosphate dehydrogenase (G-6-P DH) activities were determined in the supernatant (cytosolic fraction) as we described previously (Rossi, Lombardo, & Chicco, 2010). Protein concentration was determined by the Bradford method (Bradford, 1976).

2.5. Preparation of whole cell, plasma membrane, cytoplasmic and nuclear protein extracts

Whole cell protein extracts were prepared for PPARα, PPARγ, total and phosphorylated AMP activated protein kinase (AMPK and $pThr^{172}$AMPK) protein mass levels determination. Frozen gastrocnemius muscle was homogenized in a lysis buffer and centrifuged at 4 °C as we detailed previously (Creus et al., 2017). Plasma membrane fractions from skeletal muscle were prepared according to Creus, Ferreira, Oliva, and Lombardo (2016) for fatty acid translocase FAT/CD 36 levels determination. Nuclear and cytoplasmic extracts were obtained according to Dimauro, Pearson, Caporossi, and Jackson (2012) for mature and precursor forms of SREBP-1 quantification. Briefly, frozen muscle tissue was homogenized in a STM buffer containing 250 mM sucrose; 50 mM Tris-HCl (pH 7.4); 5 mM $MgCl_2$ and protease inhibitor cocktail (Sigma- Aldrich Co., St. Louis, MO, USA). The homogenate was maintained on ice for 30 min and then centrifuged at 800*g* for 15 min at 4 °C. The supernatant was collected and stored at −80 °C for precursor form of SREBP-1 determination. The pellet was resuspended in STM buffer and centrifuged at 500*g* for 15 min at 4 °C. To increase nuclear fraction purity, the pellet was washed again with STM buffer and centrifuged. The washed pellet was resuspended in a buffer containing 20 mM HEPES (pH 7.9); 1.5 mM $MgCl_2$; 500 mM NaCl; 0.2 mM EDTA; 20% glycerol; 1% Triton-X-100 and protease inhibitors, and then it was vortexed at maximum speed, incubated on ice for 30 min, and centrifuged at 9000*g* for 30 min at 4 °C. The resultant supernatant was the nuclear fraction. Protein concentration was determined by the Bradford method (Bradford, 1976).

2.6. Western blot analysis

PPARα, PPARγ, $pThr^{172}$AMPK, AMPK, SREBP-1 and FAT/CD36 protein levels were determined by western blot. Proteins were separated by SDS-PAGE in a 10% gel and electrotransferred onto PVDF membranes. The membranes were probed with rabbit primary polyclonal antibodies against PPARα, PPARγ, $pThr^{172}$AMPK, AMPK, SREBP-1 and FAT/CD36 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and then they were incubated with goat anti-rabbit IgG conjugated to horseradish-peroxidase antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The specific signals were visualized using a chemiluminescent detection system (Super Signal West Peak Chemiluminiscent Substrate, Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The intensity of the bands was quantified by the National Institute of Health (NIH) imaging software (Bethesda, MD, USA). After the densitometry of immunoblots, values of RD group were normalized to 100% and both SRD and SRD + chia were expressed relative to this. The protein levels were normalized to β-actin.

2.7. Statistical analysis

Results were expressed as mean values with their standard errors. Statistical comparisons were made transversely between different dietary groups. Data were tested for variance using Levene's test and normality by Shapiro–Wilk's test. Variables that were not normally distributed were transformed (using log 10 function) prior to the statistical analysis. The statistical difference between groups (RD, SRD and SRD + chia) was determined by one-way ANOVA, with one factor (diet) followed by the inspection of all differences between pairs of means by Newman-Keuls' test. When appropriate, the statistical significance between two groups (RD and SRD) was determined by

Table 2
Body weight gain, energy intake, adipose tissues weight, visceral adiposity index, serum metabolites and insulin levels in rats fed a reference diet (RD), sucrose-rich diet (SRD) or SRD with chia seed (SRD + chia).

	RD	SRD	SRD + chia
Body weight gain (g)			
Initial – 3 months	235.0 ± 9.2	243.7 ± 5.0	
3–6 months	70.5 ± 1.6^{b}	91.1 ± 2.6^{a}	93.1 ± 1.1^{a}
Energy intake (kJ/day)			
Initial – 3 months	296.3 ± 11.3	290.3 ± 7.3	
3–6 months	298.4 ± 11.3^{b}	386.5 ± 15.8^{a}	368.9 ± 12.1^{a}
Adipose tissues weight (g)			
Epididymal adipose tissue	8.98 ± 0.45^{b}	13.24 ± 0.75^{a}	9.42 ± 0.75^{b}
Retroperitoneal adipose tissue	5.97 ± 0.24^{b}	13.09 ± 0.93^{a}	7.25 ± 0.65^{b}
Mesenteric adipose tissue	5.82 ± 0.24^{b}	8.79 ± 0.54^{a}	6.52 ± 0.45^{b}
Visceral adiposity index (%)	3.46 ± 0.22^{b}	6.10 ± 0.26^{a}	4.09 ± 0.28^{b}
Serum metabolites			
Glucose (mM)	6.62 ± 0.14^{b}	7.70 ± 0.34^{a}	6.65 ± 0.11^{b}
Insulin (pM)	395.0 ± 30.0	413.8 ± 29.4	437.97 ± 32.3
Triglyceride (mM)	0.96 ± 0.04^{b}	1.34 ± 0.05^{a}	0.93 ± 0.07^{b}
Free fatty acids (μM)	361.3 ± 32.8^{b}	575.2 ± 54.0^{a}	358.1 ± 48.5^{b}

Values are expressed as mean ± SEM, n = 6. Values in a line that do not share the same superscript letter are significantly different ($P < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls' test. When appropriate, the statistical significance between the two groups (RD and SRD) was determined by Student's *t* test. For more details, see Statistical analysis in section "Materials and methods".

Student's *t* test. Differences having *P* values lower than 0.05 were considered to be statistically significant (SPSS 17.0 for Windows, SPSS INC. Chicago, Illinois). All reported *P* values were 2-sided.

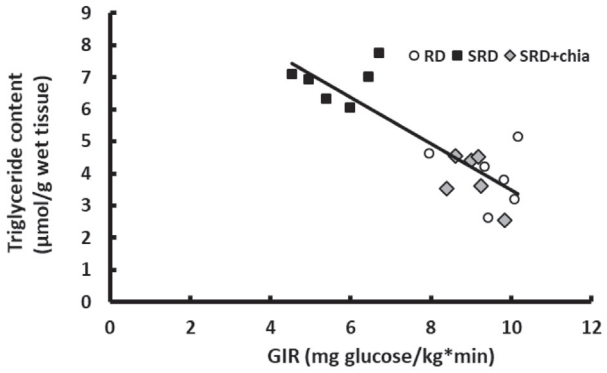
3. Results

3.1. Body weight gain, energy intake, adipose tissue weight, visceral adiposity index, serum metabolites and insulin levels

The increase in body weight gain and energy intake recorded in rats fed a SRD from 3 to 6 months was still present for SRD + chia group ($P < 0.05$). However, SRD + chia fed rats showed a significant reduction ($P < 0.05$) in fat pad weights and visceral adiposity index (Table 2). Besides, chia seed corrected the moderate hyperglycemia and dyslipidemia (triglyceride and free fatty acids serum levels) without any changes in insulinemia.

3.2. Whole-body peripheral insulin sensitivity and gastrocnemius muscle lipid content

Whole-body peripheral insulin sensitivity was estimated by GIR (clamp study) at the end of the experimental period. Fig. 1 shows the high negative correlation (Pearson r: −0.8431, $P < 0.05$) between skeletal muscle triglyceride content and GIR. In SRD-fed rats, triglyceride content was significantly increased compared with RD-fed rats, whereas GIR was significantly lower. When chia seed was incorporated as the source of fat in the SRD for the last 3 months of the experimental period, both skeletal muscle triglyceride content and GIR reached values similar to those observed in the RD-fed animals. Furthermore, in the SRD-fed rats the enhanced skeletal muscle LCA-CoA content was reduced after chia seed administration, reaching values similar to those of the RD group. DAG levels recorded in the SRD group were also significantly decreased when chia seed was present in the diet, although the values were still higher than those in the RD group (Table insert in Fig. 1).



	RD	SRD	SRD+chia
LCA-CoA (μmol/g wet tissue)	5.9 ± 0.5^{b}	11.9 ± 0.8^{a}	6.8 ± 0.4^{b}
DAG (ηmol/g wet tissue)	109.2 ± 12.5^{c}	204.3 ± 19.4^{a}	135.6 ± 5.5^{b}

Fig. 1. Correlation between whole-body peripheral insulin sensitivity (GIR) and skeletal muscle triglyceride content in rats fed a reference diet (RD), sucrose-rich diet (SRD) or SRD with chia seed (SRD + chia). The table inserted in Fig. 1 shows LCA-CoA and DAG content in the skeletal muscle. Values are expressed as mean ± SEM, n = 6. Values in a line that do not share the same superscript letter are significantly different ($P < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls' test. LCA-CoA: Long-chain Acyl-CoA, DAG: Diacylglycerol.

Table 3
Muscle-type carnitine palmitoyltransferase enzyme activities in rats fed a reference diet (RD), sucrose-rich diet (SRD) or SRD with chia seed (SRD + chia).

mU/mg protein	RD	SRD	SRD + chia
M-CPT1	4.03 ± 0.34^{a}	2.86 ± 0.22^{b}	4.18 ± 0.24^{a}
M-CPT2	7.95 ± 0.53	7.05 ± 0.35	7.87 ± 0.45
Total M-CPT	12.18 ± 0.33^{a}	10.41 ± 0.48^{b}	12.05 ± 0.47^{a}

M-CPT: Muscle-type carnitine palmitoyltransferase.
Values are expressed as mean ± SEM, n = 6. Values in a line that do not share the same superscript letter are significantly different ($P < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls' test.

3.3. Muscle-type carnitine palmitoyltransferase enzyme activities

The M-CPT activities (key enzymes of fatty acid mitochondrial oxidation) were measured in gastrocnemius muscle and are depicted in Table 3. SRD-fed rats showed a significant decrease ($P < 0.05$) of both M-CPT1 and total M-CPT activities when compared with the RD group. The above parameters were increased in the SRD + chia group, showing similar values than those observed in the RD-fed animals. M-CPT2 activity remained similar in the three dietary groups.

3.4. PPARα, PPARγ, AMPK and pThr^{172} AMPK protein mass levels

The immunoblotting of the gastrocnemius skeletal muscle revealed a single 55 kDa band consistent with PPARα, 68 kDa band for PPARγ and 63 kDa band for AMPK and pAMPK. Each gel contained an equal number of samples from RD, SRD and SRD + chia groups. The qualitative and quantitative analysis of the western blot showed that the relative abundance of PPARα, PPARγ, and pAMPK was significantly lower ($P < 0.05$) in the skeletal muscle of the SRD-fed rats compared with the RD group (Fig. 2). The addition of chia seed to the SRD significantly increased ($P < 0.05$) the protein mass levels of PPARα, PPARγ, and pAMPK, which reached values similar to those of the RD group. Changes in the total AMPK protein mass levels were not observed among the three dietary groups (Fig. 2).

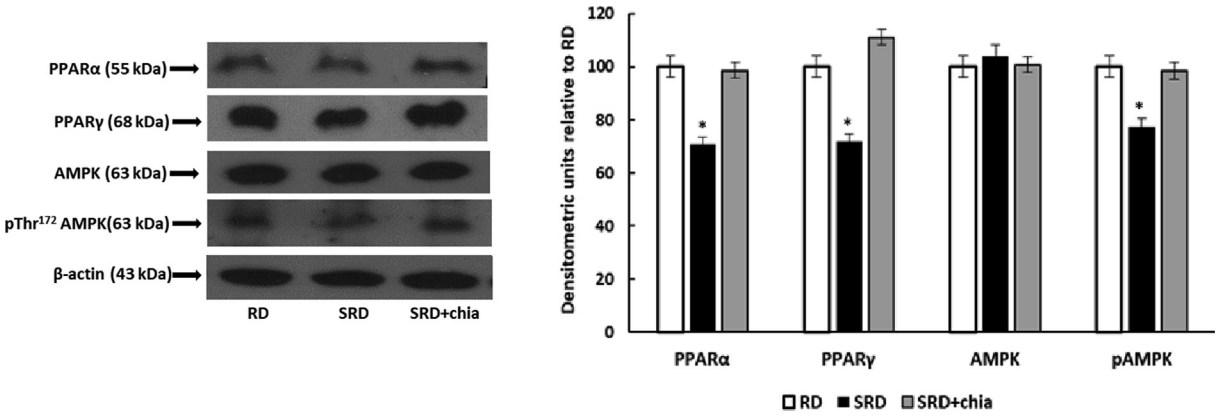


Fig. 2. Skeletal muscle protein mass levels of PPARα, PPARγ, AMPK and pThr^{172}AMPK in rats fed a reference diet (RD), sucrose-rich diet (SRD) or SRD with chia seed (SRD + chia). *Left:* Representative immunoblot of the skeletal muscle of PPARα, PPARγ, AMPK and pAMPK from the RD-, SRD- or SRD + chia-fed rats. *Right:* Densitometric immunoblot analysis of PPARα, PPARγ, AMPK and pAMPK protein mass levels in the muscle tissue of rats fed a RD, SRD or SRD + chia. Values are expressed as mean (n = 6), with their standard errors represented by vertical bars, and expressed as a percentage relative to each RD, respectively. *Mean values were significantly different from those of the RD and SRD + chia groups ($P < 0.05$) (one-way ANOVA followed by Newman-Keuls' test).

3.5. Lipogenic enzyme activities and SREBP-1 protein mass levels in the membrane fraction (precursor form) and nuclear extracts (mature form)

Fig. 3(A) and (B) depict the activity of key enzymes involved in *"de novo"* lipogenesis in the skeletal muscle. Compared with the RD-fed rats, a significant increase ($P < 0.05$) in FAS and G-6-P DH enzyme activities were observed in the rats chronically fed a SRD. The presence of chia seed as the principal source of dietary fat in the SRD reduced the activity of both enzymes, which achieved similar values to the RD-fed group.

From the above results, we examined the protein mass levels of the precursor and mature forms of SREBP-1 in the skeletal muscle of RD-, SRD- and SRD + chia-fed rats by immunoblotting. The 125 and 68 kDa bands observed represent the precursor and mature forms of SREBP-1 respectively. Each gel contained an equal number of samples from the RD, SRD and SRD + chia groups (Fig. 3C). The qualitative and quantitative analysis of the western blot showed no differences in the relative abundance of the precursor form of SREBP-1 between RD and SRD groups. Interestingly, when chia seed was present in the SRD diet a significantly decrease ($P < 0.05$) of the precursor form of SREBP-1 was recorded. Moreover, the presents results show that the relative abundance of the mature form of SREBP-1 protein was significantly

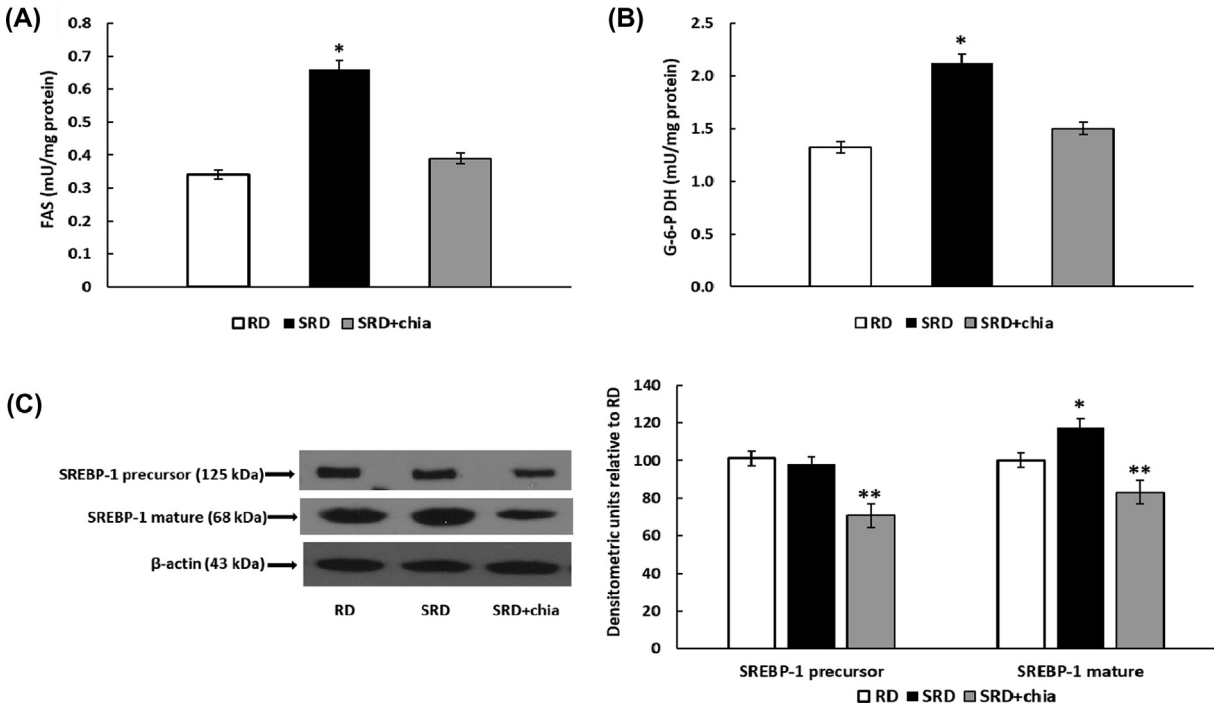


Fig. 3. Skeletal muscle lipogenic enzymes activities and SREBP-1 protein mass levels in membrane fraction (precursor form) and nuclear extracts (mature form) in rats fed a reference diet (RD), sucrose-rich diet (SRD) or SRD with chia seed (SRD + chia). (A) FAS and (B) G-6-P DH activities. Values are expressed as mean (n = 6), with their standard errors represented by vertical bars. (C) *Left.* Representative immunoblots of the skeletal muscle precursor and mature forms of SREBP-1 from the RD-, SRD- or SRD + chia-fed rats. *Right.* Densitometric immunoblot analysis of the precursor and mature forms of SREBP-1 protein mass levels in the skeletal muscle tissue of rats fed a RD, SRD or SRD + chia. Values are expressed as mean (n = 6), with their standard errors represented by vertical bars, and expressed as a percentage relative to each RD, respectively. *Mean values were significantly different from those of the CD and SRD + chia groups ($P < 0.05$) and **Mean values were significantly different from those of the CD and SRD groups ($P < 0.05$) (one-way ANOVA followed by Neuman-Keuls' test).

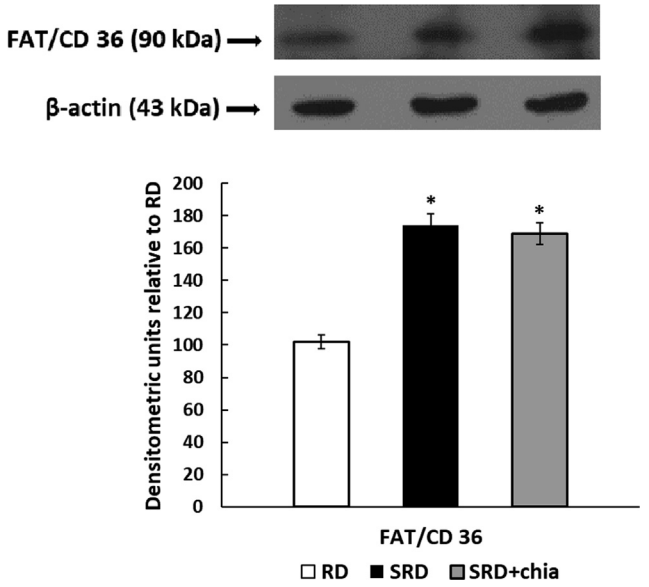


Fig. 4. Skeletal muscle protein mass levels of FAT/CD 36 (plasma membrane fraction) in rats fed a reference diet (RD), sucrose-rich diet (SRD) or SRD with chia seed (SRD + chia). *Upper.* Representative immunoblot of the muscle FAT/CD 36 from the RD-, SRD- or SRD + chia-fed rats. *Bottom.* Densitometric immunoblot analysis of the FAT/CD 36 protein mass levels in the skeletal muscle tissue of rats fed a RD, SRD or SRD + chia. Values are expressed as mean (n = 6), with their standard errors represented by vertical bars, and expressed as a percentage relative to RD. *Mean values were significantly different from those of the RD group ($P < 0.05$) (one-way ANOVA followed by Neuman-Keuls' test).

increased ($P < 0.05$) in SRD-fed rats. Dietary chia seed decreased protein mass levels of the mature form of SREBP-1, reaching values even lower than those observed in the RD-fed rats.

3.6. Protein mass levels of FAT/CD 36

Fig. 4 shows the protein mass level of FAT/CD 36 in plasma membrane fraction of skeletal muscle. The immunoblotting revealed a single 90 kDa band consistent with FAT/CD 36. Each gel contained an equal number of samples from rats fed a RD, SRD and SRD + chia. The qualitative and quantitative analysis of the western blot showed that the relative abundance of FAT/CD 36 was significantly increased ($P < 0.05$) in both the SRD and SRD + chia groups compared with the rats fed a RD.

3.7. Fatty acid composition of gastrocnemius muscle phospholipids

Table 4 shows the fatty acid composition of muscle phospholipids and *n*-3/*n*-6 PUFA ratio. The muscle phospholipids of SRD + chia group were enriched with 18:3 *n*-3, 20:5 *n*-3 and 22:6 *n*-3 fatty acids compared with the SRD-fed group and this was accompanied by a significantly decrease in linoleic and arachidonic acids levels in this animals. Moreover, *n*-3/*n*-6 ratio in the skeletal muscle of SRD + chia fed rats was increased.

4. Discussion

In the present study, we analyzed the possible beneficial effects of substitution of the source of dietary fat- corn oil by chia seed- in SRD-fed rats, particularly focusing on the mechanisms underlying the skeletal muscle lipid accumulation and its relationship with IR.

Rats fed a SRD for a long period of time (3–6 months) develop dyslipidemia (increased levels of triglycerides and free fatty acids) and IR (Chicco et al., 2003). It is well known that increased availability of

Table 4
Fatty acid composition (g/100 g total fatty acids) of skeletal muscle phospholipids in rats fed a reference diet (RD), a sucrose-rich diet (SRD) or SRD with chia seed (SRD + chia).

Fatty acids	RD	SRD	SRD + chia
14:00	0.43 ± 0.09^{b}	0.54 ± 0.02^{b}	0.80 ± 0.08^{a}
16:00	24.66 ± 0.54	25.83 ± 2.01	26.76 ± 1.16
17:00	0.57 ± 0.01^{a}	0.28 ± 0.02^{b}	0.22 ± 0.03^{b}
18:00	17.35 ± 0.59	15.45 ± 0.45	15.63 ± 0.94
24:00	1.63 ± 0.11^{b}	1.31 ± 0.03^{b}	3.43 ± 0.56^{a}
Σ SFA	46.82 ± 0.50	42.36 ± 1.47	47.64 ± 1.39
16:1 *n*-7	0.51 ± 0.07^{c}	1.49 ± 0.50^{b}	2.92 ± 0.27^{a}
18:1 *n*-9	5.88 ± 0.87^{b}	6.40 ± 0.84^{b}	8.96 ± 0.46^{a}
18:1 *n*-7	3.12 ± 0.37^{b}	3.43 ± 0.19^{b}	4.85 ± 0.29^{a}
ΣMUFA	9.51 ± 1.15^{b}	11.08 ± 1.37^{b}	16.73 ± 0.77^{a}
18:2 *n*-6	17.70 ± 0.77^{a}	16.71 ± 0.78^{a}	14.34 ± 0.40^{b}
22:5 *n*-6	0.49 ± 0.06^{b}	2.21 ± 0.58^{a}	0.26 ± 0.05^{b}
20:2 *n*-6	ND	ND	ND
20:3 *n*-6	0.44 ± 0.02^{b}	0.54 ± 0.07^{ab}	0.63 ± 0.03^{a}
20:4 *n*-6	16.99 ± 0.69^{b}	19.34 ± 0.31^{a}	9.31 ± 0.81^{c}
ΣPUFA *n*-6	33.84 ± 1.54^{a}	36.46 ± 1.62^{a}	24.69 ± 0.74^{b}
18:3 *n*-3	ND	ND	0.38 ± 0.05
20:5 *n*-3	ND	ND	0.80 ± 0.05
22:5 *n*-3	1.03 ± 0.31^{b}	2.37 ± 0.49^{a}	0.40 ± 0.07^{b}
22:6 *n*-3	10.45 ± 0.78^{a}	5.91 ± 0.97^{b}	9.88 ± 1.53^{a}
ΣPUFA *n*-3	11.24 ± 1.07	8.15 ± 1.14	10.68 ± 1.46
ΣPUFA	45.09 ± 2.57^{a}	45.74 ± 2.50^{a}	35.99 ± 1.16^{b}
PUFA *n*-3/*n*-6 ratio	0.33 ± 0.02^{ab}	0.25 ± 0.04^{b}	0.43 ± 0.05^{a}

Values are expressed as mean ± SEM, n = 6. Values in a line that do not share the same superscript letter are significantly different ($P < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls' test. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

lipids promotes the ectopic accumulation in non-adipose tissues and lipotoxicity (Schaffer, 2003). In this regard, different studies have demonstrated that skeletal muscle lipid accretion (triglycerides, LCA-CoA, DAG, and ceramides) is strongly correlated with IR, by promoting insulin signaling cascade defects (Abdul-Ghani & DeFronzo, 2010; Amati et al., 2011; Chavez, Holland, Bär, Sandhoff, & Summers, 2005). Along this line, and in agreement with previous reports, the present data demonstrate that insulin-resistant rats fed a SRD have increased levels of intramuscular triglycerides and lipid intermediates (DAG, LCA-CoA). Moreover, a reduction in intramuscular lipid accumulation and a normalization of dyslipidemia, moderate hyperglycemia, visceral adiposity and insulin sensitivity was recorded when chia seed was administered as a source of fat in the SRD during the last 3 months of feeding (D'Alessandro et al., 2006; Oliva et al., 2013). In this vein, we have previously showed that dietary chia seed was able to normalize the increased nPKCθ protein mass levels in the membrane fraction of skeletal muscle in the SRD experimental model, and this was associated with an improvement of the GLUT 4 translocation to the plasma membrane (Oliva et al., 2013). Interestingly, Fonte-Faria et al. (2019) recently shown that chia oil supplementation to obese mice fed a high-fat diet was able to improved glucose tolerance and insulin sensitivity. This was associated with an increase in phosphorylation on tyrosine of IRS-1, Akt phosphorylation and plasma membrane GLUT 4 translocation in gastrocnemius muscle after insulin stimulation.

A deteriorated muscle oxidative capacity and, more specifically, the inability of muscle to oxidize fatty acids would be associated with intramyocellular fat accumulation and IR (Turcotte & Fisher, 2008). In this regards, M-CPT1 expression – a key enzyme in fatty acid β-oxidation, that carries fatty acids across the mitochondrial membrane- was found significantly reduced in the skeletal muscle of IR models (Bi et al., 2009; Song, Wang, Ren, & Zhao, 2014). Perdomo et al. (2004) observed in rat L6 myotubes incubated in the presence of palmitate, elevated lipids levels and reduced insulin sensitivity. Overexpression of

CPT1 in these cells increased the rate of β-oxidation and enhanced insulin sensitivity. In the present research, we found a significant reduction of both M-CPT1 and total M-CPT activities in SRD-fed rats. When chia seed was administered as a source of dietary fat in these rats, M-CPT1 and total M-CPT activities showed similar values to RD group.

PPARα is a key transcription factor that controls genes involved in fatty acid oxidation including M-CPT1 (Mandard, Müller, & Kersten, 2004). In this regards, Li et al. (2016) recently showed an increase of intramuscular triglycerides associated with reduced *Pparα* and *Cpt1* mRNA levels in hamster models of IR and type 2 diabetes. Besides, Song et al. (2014) observed that mRNA and protein levels of CPT1 and PPARα were downregulated in insulin-resistant rats fed a high-fat diet. In the present study, we found reduced protein mass levels of PPARα in the SRD-fed rats, so this could be a mechanism involved in the decrease of M-CPT1 activity observed in these rats. It is well known that *n*-3 PUFA are strong PPARs ligands (Calder, 2012). In a recent study, Martins et al. (2018) showed that *n*-3 PUFA administration (fish oil) upregulated mRNA expression of *Cpt1* and *Pparα* in the skeletal muscle of insulin resistant C57BL/6 mice fed a high-fat diet, improving skeletal muscle mitochondrial function and insulin sensitivity. In our study, chia seed (rich in ALA) markedly increased the PPARα protein mass levels. Similarly, we previously demonstrate that chia seed reduce the hepatic triglyceride accretion by mechanisms that include an increment in CPT1 and fatty acid oxidase enzymes activities and PPARα protein mass levels (Rossi et al., 2013). In addition, our results showed a significant decrease in skeletal muscle PPARγ protein mass levels in rats chronically fed a SRD. Chia seed administration was able to increase PPARγ protein levels. Although PPARγ expression is very low in skeletal muscle cells, it plays a crucial role in the maintenance of insulin sensitivity by regulating the expression of genes involved in insulin signaling and lipid metabolism (Hevener et al., 2003). A reduced expression of PPARγ and its target genes (*Pgc-1α, Lpl,* among others) in skeletal muscle was observed in animal models of IR (Li et al., 2016). Moreover, Verma, Singh, and Dey (2004) working with C2C12 skeletal muscle cells have shown that the inhibition of PPARγ expression induce a reduction in glucose uptake and IR.

Another major regulator of skeletal muscle fatty acid metabolism is the AMPK. Its activation leads to an augmented LCA-CoA flux into the mitochondria by the acetyl-CoA carboxylase/malonyl-CoA/M-CPT1 pathway, promoting fatty acid oxidation (Thomson & Winder, 2010). Several authors have demonstrated a reduction in AMPK activity in the skeletal muscle of obese and type 2 diabetic subjects and in rodent models of IR (Bandyopadhyay, Yu, Ofrecio, & Olefsky, 2006; Ruderman, Carling, Prentki, & Cacicedo, 2013). In the present study, we found a reduction in pAMPK protein mass levels in the skeletal muscle of IR rats fed a SRD that accompanies M-CPT1 activity decrease. Interestingly, Lee et al. (2006) showed in cultured muscle C2C12 cells and mouse skeletal muscle that AMPK activation increases fatty acid oxidation by activating PPARα, in addition to the well-known allosteric regulation of CPT1. Along this line, our findings showed that by shifting dietary corn oil to chia seed in the SRD-fed group, the pAMPK protein levels were significantly augmented. This was accompanied by an increase of both PPARα protein levels and M-CPT1 activity as mentioned above.

Although skeletal muscle is not thought a lipogenic tissue, evidence suggests that an enhanced lipogenic pathway in this tissue could be a potential mechanism involved in intramuscular lipid accretion and the impaired insulin action. SREBP-1c, a major transcription factor that regulates cellular lipogenesis, is expressed in skeletal muscle (Shimomura, Shimano, Horton, Goldstein, & Brown, 1997). Recent studies have shown that an increase of muscle SREBP-1c might lead to fat storage, contributing to the pathogenesis of IR. Our results demonstrated that SRD-feeding induced an increase in the mature form of SREBP-1. Since the antibody of SREBP-1 used in this study reacted with both SREBP-1a and SREBP-1c forms, we could not distinguish these two forms and thus used the non-committal term SREBP-1 (in the skeletal muscle, SREBP-1c transcript predominates over SREBP-1a) (Shimomura et al., 1997). In addition, a greater FAS and G-6-P DH enzyme activities were recorded in these animals compared with RD group. Interestingly, Bi et al. (2014) demonstrated that SREBP-1c suppress muscular insulin signaling by binding to the *Irs-1* promoter and repressing its gene transcription, expanding the role of SREBP-1c in controlling IR beyond its lipogenic action. On the other hand, several pieces of evidence have shown that *n*-3 PUFA reduce hepatic SREBP-1c nuclear levels by different mechanisms including suppression of the proteolytic processing of the precursor protein as well as down-regulation of gene expression (Jump, Tripathy, & Depner, 2013). However, the effect of *n*-3 PUFA on skeletal muscle SREBP-1c is less known. The present data demonstrate that the replacement of dietary corn oil by chia seed in the SRD-fed rats reduce the protein levels of both the precursor and mature forms of SREBP-1 and this was accompanied by restored activities of the lipogenic enzymes. This interesting finding could be related to the reduction of muscle lipotoxicity and improvement of the insulin sensitivity observed with the administration of chia seed.

The increase in fatty acids movement across the plasma membrane of the skeletal muscle could be another mechanism contributing to the increase of intramuscular lipids depots. FAT/CD 36 is a key fatty acid transporter in skeletal muscle, that traffic between the plasma membrane and intracellular compartment (Holloway, Schwenk, Luiken, & Glatz, 2010). In human obese and type 2 diabetic subjects, Bonen et al. (2004) showed that triglyceride accumulation in skeletal muscle is associated with increased rates of fatty acid transport and increased sarcolemmal FAT/CD36 abundance. Moreover, Luiken et al. (2001) observed an increase in plasma membrane protein levels of FAT/CD 36 and fatty acids uptake in the skeletal muscle of insulin-resistant obese Zucker rats. In agreement with these studies, we found that plasma membrane FAT/CD 36 protein mass levels were significantly augmented in SRD-fed rats. When chia seed was administered as a source of fat in the SRD, FAT/CD 36 levels were also increased. In this regards, Chorner et al. (2016) found an increased sarcolemmal palmitate transport rates and plasma membrane FAT/CD36 abundance in rats fed a standard diet supplemented with flaxseed oil high in ALA (10%). Although we have found increased levels of sarcolemmal FAT/CD 36 in SRD- and SRD + chia-fed rats, this was accompanied by different fatty acids metabolic pathways inside the skeletal muscle tissue. Our results suggest that in SRD-fed rats an imbalance between fatty acid oxidation and lipogenesis could be involved, resulting in the increase of lipid storage within the skeletal muscle cells. On the other hand, changes in the disposal of lipids from synthesis toward oxidation could be at least a mechanism contributing to the reduced muscle fat accretion observed in the SRD + chia group.

Changes in the composition of cell membrane phospholipids could be another important mechanism by which chia seed might exert its beneficial effects on skeletal muscle lipotoxicity and insulin sensitivity. In this regards, a marked increase in *n*-3 PUFA within whole muscle and sarcolemmal was found in rats fed a standard diet supplemented with flaxseed oil (Chorner et al., 2016). Furthermore, Poudyal, Panchal, Ward, and Brown (2013) demonstrated that chia oil increased the proportions of ALA, docosapentaenoic acid (DPA) and DHA and augmented *n*-3/*n*-6 ratio in the skeletal muscle of rats fed a high-fructose high-fat diet. This was accompanied by an improvement in glucose and insulin tolerance. We previously demonstrated that chia seed administration to rats chronically fed a SRD increase ALA, EPA, DPA and DHA plasma levels and *n*-3/*n*-6 ratio (Chicco et al., 2009). In the present study, the administration of chia seed during 3 months was sufficient to induce an augmented in *n*-3/*n*-6 ratio as a consequence of marked increase in the incorporation of ALA, EPA and DHA and a reduction in 18:2 *n*-6 and 20:4 *n*-6 fatty acids into the phospholipids of the skeletal muscle of SRD-fed rats.

5. Conclusions

Expanding previous research of our group this work provides for the first time and to the best of our knowledge, novel information on the beneficial effects of ALA-rich chia seed to improve altered lipid metabolism and insulin sensitivity present in the skeletal muscle of rats chronically fed a sucrose-rich diet. It is important to highlight that we cannot rule out the possibility that others components of chia seed as protein, fibers, minerals and antioxidants could also contribute to the beneficial effects found in the present research.

Finally, although care must be taken in extrapolating these results from rats to humans, this study shows some mechanisms underlying the effects of nutrients in the development and management of metabolic diseases.

6. Ethics statements

Animal experiments complied with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and were evaluated and approved by the Institutional Ethics Committee of the Faculty of Biochemistry and Biological Sciences, (Universidad Nacional del Litoral, Santa Fe, Argentina).

CRediT authorship contribution statement

María del Rosario Ferreira: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. María Eugenia Oliva: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. Victoria Aiassa: Methodology, Data curation, Formal analysis. María Eugenia D'Alessandro: Funding acquisition, Conceptualization, Investigation, Data curation, Formal analysis, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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