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ORIGINAL CONTRIBUTION

Dietary Salba (*Salvia hispanica* L.) ameliorates the adipose tissue dysfunction of dyslipemic insulin-resistant rats through mechanisms involving oxidative stress, inflammatory cytokines and peroxisome proliferator-activated receptor γ

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Abstract
Purpose Rats fed a long-term sucrose-rich diet (SRD) developed adipose tissue dysfunction. In the adipose tissue of these SRD-fed rats, the present study analyzed the possible beneficial effects of dietary Salba (chia) seeds in improving or reversing the depletion of antioxidant defenses, changes in pro-inflammatory cytokines and ROS production.
Methods Wistar rats were fed a SRD for 3 months. After that, half of the animals continued with the SRD until month 6, while in the other half, corn oil was replaced by chia seeds for 3 months (SRD + chia). A reference group consumed a control diet all the time.
Results Compared with the SRD-fed rats, the animals fed a SRD + chia showed a reduction in epididymal fat pad weight; the activities of antioxidant enzymes CAT, SOD and GPx returned to control values, while GR significantly improved; mRNA GPx increased, and both mRNA SOD and the redox state of glutathione returned to control values; a significant increase in the expression of Nrf2 was recorded. These results were accompanied by a decrease in XO activity and ROS contents as well as plasma IL-6 and TNF-α levels. Chia seeds reversed the decrease in PPARγ protein mass level and increased the *n-3/n-6* fatty acids ratio of membrane phospholipids. Besides, dyslipidemia and insulin sensitivity were normalized.
Conclusion This study provides new information concerning some mechanisms related to the beneficial effects of dietary chia seeds in reversing adipose tissue oxidative stress and improving the adipose tissue dysfunction induced by a SRD.

Keywords α-linolenic acid (ALA) · Adipose tissue · Dyslipidemia · High-sucrose diet · Oxidative stress · Insulin resistance

Introduction

Excessive food ingestion and the increase in the consumption of high-fat, fructose or sucrose diets characteristic of modern society have been related to the development of visceral adiposity, systemic oxidative stress, impaired glucose homeostasis, insulin resistance (IR), type 2 diabetes, dyslipidemia and hypertension, the major components of the metabolic syndrome (MS) [1, 2]. Experimentally, our group and others have demonstrated that rats chronically fed a sucrose-/fructose-rich diet develop metabolic and physiological alterations mimicking several aspects of the metabolic syndrome in humans [3–5].

Diets can play a major role in the prevention or improvement in this syndrome and its associated pathologies. In addition to other lifestyle interventions, it has been recognized that adjustments to the quality of dietary lipids such as *n-3* polyunsaturated fatty acids (*n-3* PUFAs) from marine [fish oil; eicosapentaenoic acid (EPA) 20:5, *n-3*; docosahexaenoic acid (DHA) 22:6, *n-3*] or plant sources

M. R. Ferreira and S. M. Alvarez have contributed equally to the laboratory assays in the present study.

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[α-linolenic acid (ALA) 18:3, *n-3*] are also important in the prevention and treatment of the MS by modulating the expression of several genes involved in these chronic manifestations (e.g., SREBP1, IL-6, TNF-α, FAS,S14, L-PK,Visfatin) [6, 7]. In this regard, the seeds of *Salvia hispanica* L. commonly known as chia seeds contain the richest botanical source of ALA and a high amount of fiber and minerals. Moreover, chia seeds have antioxidant activity due to the phenolic compounds in the seed or oil, mainly quercetin, myricetin and chlorogenic acid, among others [8]. Ayerza et al. [9] demonstrated that dietary ground chia seed given to normal rats was able to reduce plasma triglyceride, total cholesterol and HDL cholesterol levels. More recently, Poudyal et al. [10] showed that the administration of dietary chia seeds induced lipid redistribution and attenuated the abnormal metabolic hepatic and cardiovascular signs developed in dyslipemic insulin-resistant rats fed a high-fat high-carbohydrate diet. In a previous work, we demonstrated that dyslipidemia and IR induced in rats chronically fed a sucrose-rich diet (SRD) were reversed when chia seed (rich in ALA) instead of corn oil (rich in LA 18:2, *n-6,*) became the dietary source of fat for the last 3 months of the experimental period [11, 12]. Besides, both the key enzyme activities of hepatic lipogenesis and mitochondrial and peroxisomal fatty acid oxidation as well as the protein mass levels of SREBP-1 and PPARα were coordinately decreased and increased by dietary chia seed [13].

On the other hand, visceral adiposity in the presence of increased plasma fatty acid levels and hyperglycemia could generate reactive oxygen species (ROS), ultimately leading to increased oxidative stress which affects insulin signaling and nitric oxide availability and contributes to IR [14, 15]. In rats fed a SRD, we have recently shown a progressive deterioration of adipose tissue function in the presence of hypertrophied epididymal fat pad, depletion of antioxidant defenses and changes in the inflammatory cytokines that contribute to the dysregulation of adipose tissue and IR [16]. Moreover, in these rats, the administration of dietary chia seeds reduced adipocyte hypertrophy and improved both lipolysis and insulin action [12]. Interestingly, as mentioned above, chia seeds contain flavonoids, among them quercetin. Vazquez Prieto et al. [17] and Rivera et al. [18] have recently shown that dietary supplementation with quercetin attenuates adipose tissue inflammation in fructose-fed rats and ameliorates MS, thus improving visceral adipose tissue inflammatory status in obese Zucker rats. However, to the best of our knowledge, no literature report has investigated whether the chia seed-induced amelioration of adipose tissue dysfunction and visceral adiposity is somehow related to mechanisms that improve or even reverse oxidative stress and bring about changes in pro-inflammatory cytokines in the adipose tissue of dyslipemic insulin-resistant SRD-fed rats.

Accordingly, the aim of the present study was to assess in the epididymal fat pad the effect of dietary chia seed on the following: (1) enzymatic and non-enzymatic cellular antioxidant defense and the genes encoding superoxide dismutase (MnSOD), glutathione peroxidase (GPx) and the NF-E2-related nuclear factor (Nrf2), (2) the expression of NAD(P)H oxidase p47phox subunit (p47NOX), xanthine oxidase (XO) activity and ROS levels (3) the protein mass level of peroxisome proliferator-activated receptor gamma (PPARγ) and pro-inflammatory cytokine (IL-6, TNF-α) plasma levels. Besides, the composition of fatty acids phospholipids of adipose tissue after the administration of chia seed was analyzed. Along this way, information concerning the possible mechanism/s involved, and their relationship with IR could be obtained. This study was conducted in rats fed a SRD during 6 months, in which permanent dyslipidemia, IR, visceral adiposity and adipose tissue dysfunction were present before the source of dietary fat, corn oil, was replaced by an isocaloric amount of chia seeds for the last 3 months of the experimental period in half of the animals.

Materials and methods

Animals and diets

Male Wistar rats ($n = 72$) initially weighing 180–190 g and purchased from the National Institute of Pharmacology (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). They were initially fed a standard non-purified diet (Ralston Purina, St Louis, MO, USA). After 1 week of acclimation period, the rats were randomly divided into two groups: control ($n = 24$) and experimental ($n = 48$) and were housed individually. The control group received a semi-synthetic diet containing corn starch (60 % energy), protein (17 % energy) and corn oil as a source of fat (23 % energy) throughout the experimental period (6 months) (control diet: CD). The experimental group received the same semisynthetic diet with sucrose as the carbohydrate source (SRD). After 3 months of treatment, the animals in the SRD group were randomly divided into two subgroups. The rats in the first subgroup continued on the SRD ($n = 24$) up to 6 months of feeding. The second subgroup ($n = 24$) received the Salba seed (chia) as the source of dietary fat for the next 3 months (SRD + chia). The carbohydrates, proteins, fibers, vitamins and mineral content in the chia seed of the SRD + chia group were balanced with those of the CD and SRD groups according to the amount of these nutrients present in the chia seed. Details on the

Table 1 Composition of experimental diets

Diet ingredients	Control diet (CD)		Sucrose-rich diet (SRD)		SRD + chia seed (SRD + chia)	
	% w/w	% energy	% w/w	% energy	% w/w	% energy
Carbohydrates						
Corn starch	58.0	60.0	2.5	2.6	–	–
Sucrose	–	–	55.5	57.4	55.5	57.4
Chia seed[a]	–	–	–	–	2.5	2.6
Fat						
Corn oil	10.5	23.0	10.5	23.0	0.1	0.2
Chia seed	–	–	–	–	10.4	22.8
Protein						
Casein (vitamin free)	16.3	17.0	16.3	17.0	8.6	9.0
Chia seed	–	–	–	–	7.7	8.0

The composition of experimental diets are based on AIN-93 M diet. All diets contain by weight: salt mix, 3.5 % (AIN-93 M-MX); vitamin mix, 1 % (AIN-93VX); choline chloride, 0.2 %; methionine, 0.3 %; fiber, 10–11 %. The SRD + chia was balanced in salt mix according to the amount of each one in the chia seed provided by the manufacturer

[a] 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

Table 2 Total fatty acid composition of the experimental diets (g/kg of diet)

Fatty acids[a]	CD and SRD	SRD + chia seed
16:0	10.92	6.96
18:0	2.73	2.42
18:1 *n-9*	33.71	7.39
18:2 *n-6*	54.10	19.85
18:3 *n-3*	0.80	67.26
20:1 *n-9*	0.47	0.36
Total saturated	13.65	9.38
Monounsaturated	34.18	7.75
Total polyunsaturated	54.90	87.11
n-6	54.10	19.85
n-3	0.80	67.26
n-3: n-6	0.015	3.388
n-3/total saturated	0.59	7.17

[a] Other minor fatty acids have been excluded

composition of each diet are given in Table 1. The fatty acid composition of each experimental diet (g/kg of food) is shown in Table 2. As can be seen the fatty acid composition of CD and SRD differ from SRD + chia seed diet in the amount of monounsaturated fatty acids, the type and amount of polyunsaturated fatty acids and to a lesser extent in the amount of saturated fatty acids.

The preparation and handling of the diets have been reported elsewhere [12]. All diets provided approximately 17.00 kJ/g of food. The body weight of each animal was recorded twice per week throughout the experimental period in all groups and subgroups of rats. In a separate experiment, the individual caloric intake and weight gain of six animals in each group and subgroup were assessed twice a week. At the end of the 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.

Rats from the three dietary groups were anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg body weight). Blood samples were obtained from the jugular vein, collected in tubes containing sodium EDTA as anticoagulant, and rapidly centrifuged. Plasma was either immediately assayed or stored at −20 °C. Retroperitoneal and omental adipose tissue were totally removed and weighed. Epididymal adipose tissue was totally removed, weighed and immediately frozen and stored at the temperature of liquid N_2. The visceral adiposity index (%) was calculated as recently described [13]. Epididymal adipose tissue lipids were extracted according to the procedure described by Folch et al. [19] and total phospholipids separated by thin-layer chromatography. Samples were esterified with boron trifluoride at 64 °C for 3 h, and the fatty acid composition of total phospholipids was determined by gas liquid chromatography of their methyl esters as previously described [20]. The experimental protocols were approved by the Human and Animal Research Committee of the School of Biochemistry, University of Litoral, Argentina.

Analytical methods

Plasma triglyceride, free fatty acids (FFA), uric acid and glucose levels were determined by spectrophotometric methods and insulin levels by immunoreactive assays as previously described [11–13]. The immunoreactive insulin assays were calibrated against rat insulin standard (novo Nordisk, Copenhagen, Denmark). Plasma IL-6, TNF-α and the TNF-α content of the cytosolic phase of adipose tissue were measured using commercial ELISA kits (Thermo Scientific, Rockford, USA; Legendmax ™ Biolegend® Inc., San Diego, USA; and Thermo Scientific rat TNF-α ELISA kit, Rockford, USA, respectively). For IL-6, the minimum detectable limit was 16 pg/mL with an intra- and inter-assay CV of less than 6.8 and 14.9 %, respectively. For TNF-α, 4.2 pg/mL in plasma and 15.0 pg/mL in adipose tissue were the minimum detectable limits with an intra- and inter-assay CV of less than 8.3 and 10.5 %, respectively. All determinations were performed in triplicate. Whole-body peripheral insulin sensitivity was measured using the euglycemic–hyperinsulinemic clamp technique as previously described in detail [21].

Lipid peroxidation and protein carbonyl groups determination in plasma

Lipid peroxidation in plasma was estimated by measuring thiobarbituric acid reactive substances (TBARS) according to the method of Lee and Csallany [22] as recently described [16]. Protein carbonyl formation, a marker of protein oxidative damage, was measured spectrophotometrically using the method described by Reznick and Packer [23] with minor modifications. Briefly, 250 µL of plasma were treated with 1 mL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 1 h vortexing every 15 min. Proteins in samples were precipitated by adding 1.25 mL of 20 % (w/v) trichloroacetic acid (TCA) solution to a final concentration of 10 % (w/v) TCA and centrifuged at 11,000*g* for 5 min. The supernatants were discarded and protein pellets were treated with 10 % (w/v) TCA and centrifuged. The resultant pellet was then washed three times with ethanol:ethyl acetate (1:1, V/V) to remove free DNPH and lipid contaminants.The final precipitate were suspended in 6 M guanidine hydrochloride solution at 37 °C for 15 min. The difference between the DNPH-treated and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 370 nm. The results were calculated as nmol of carbonyl groups/mL plasma, using an absorption coefficient Ɛ of 22,000 M^{-1} cm^{-1} for aliphatic hydrazones.

ROS and glutathione redox state determination in epididymal fat tissue

Intracellular ROS was measured using the dichlorodihydrofluorescein diacetate ($DCFH_2DA$) method according to Wang et al. [24] with minor modifications. Briefly, adipose tissue was homogenized at 4 °C with 0.1 M sodium phosphate buffer (pH 7.4) and centrifuged at 1000*g* for 10 min at 4 °C. The supernatant was incubated with 10-µM $DCFH_2DA$ for 30 min at 37 °C in dark. The fluorescence intensity was determined using a PerkinElmer luminescence spectrometer at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. To correct background fluorescence, samples were incubated under the same conditions but without fluorescent dyes. Results were normalized by protein concentration (fluorescence intensity/mg protein) and expressed relative to the control group. Protein concentrations were quantified by the Bradford assay (Bio-Rad reagent). Total glutathione [reduced (GSH) + oxidized form (GSSG)] and GSSG were assayed according to the method of Griffith [25] and expressed as redox state [(oxidized form/total form) × 100] as previously described [16].

Antioxidant and oxidant enzyme activities in epididymal fat tissue

Epididymal adipose tissue catalase (CAT), SOD, GPx, glutathione reductase (GR) activities and XO activity—as a source of ROS—were determined as recently described [16]. The protein content in all enzyme assays was measured by the Bradford assay (Bio-Rad reagent).

RNA isolation and semi-quantitative RT-PCR analysis

GPx, SOD, p47NOX subunit and Nrf2 mRNA levels of epididymal fat tissue were determined by RT-PCR as described by Alvarez et al. [26]. Total RNA was isolated from adipose tissue samples using Trizol reagent (Invitrogen) and following the manufacturer indications. 1 µg of total RNA was reversely transcribed, using random primer hexamers (Genbiotech, Argentina) and 200 units of MMLV Reverse Transcriptase (Promega Inc.), at 37 °C during 1 h. PCR amplification was carried out using specific oligonucleotide primers (Table 3). A cDNA aliquot (1/10 of the RT reaction product) was amplified with a PCR master mix, using Taq DNA polymerase (Invitrogen). PCR products were analyzed on 2 % agarose gels, containing GelRed (Genbiotech) and photographed under UV transillumination to visualize the bands. Band intensities were quantified using NIH Image software Scion Image. Relative amounts of mRNA were expressed as the ratio of band intensity for the target genes relative to that for 28S rRNA.

Table 3 Sequences of the primers used to amplify different genes by RT-PCR and sizes of the fragments generated

Gene name	Forward primer sequences (5′-3′)	Reverse primer sequences (5′-3′)	Size (bp)
SOD	AGCTGCACCACAGCAAGCAC	TCCACCACCCTTAGGGCTCA	191
GPx	CCACCGTGTATGCCTTCTCC	ACCGGGGACCAAATGATGTA	424
p47 phox	AGGGAACGCTCACCGAGTACT	TCTTTGGCCGTCAGGTATGTC	160
Nrf2	CGGCATTTCACTGAACACAAGT	TGGCTGTGCTTTAGGTCCATT	160
S28	GTGAAAGCGGGGCCTCACGATCC	TACTGAGCAGGATTACCATGGC	200

Western Blot analysis of epididymal fat tissue protein mass level of PPARγ

The assay of the protein mass level of PPARγ was described elsewhere [27]. Briefly, frozen epididymal adipose tissue powder was homogenized and total protein samples were resolved on 10 % SDS-PAGE and transferred to PVDF membranes. The membranes were probed with specific polyclonal rabbit anti-PPARγ antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) followed by anti-rabbit horseradish peroxidase-conjugated antibody. PPARγ was visualized by chemiluminescent detection according to the manufacturer's instructions (Super Signal West Pico Chemiluminescent detection, Pierce Biotechnology, Rockford, IL). The protein levels were normalized to β-actin. The intensity of the bands was quantified by NIH imaging software (http://rsb.info.nih.gov/nih-image).

Drugs and chemicals

Enzymes, substrates, coenzymes and all other chemicals used for experiments were of analytical grade and were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Statistical analysis

Sample sizes were calculated on the basis of measurements previously made with rats fed either a control diet or a SRD [16, 27]considering an 80 % power as described by Glantz [28]. 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 analyses. The statistical significance between groups (CD, 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 Tukey's test [29]. When appropriate, the statistical significance between two groups (CD and SRD) was determined by 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 *P* values reported were 2-sided.

Results

Body weight, energy intake, fat pad weight, plasma metabolites, insulin, glucose infusion rate (GIR) and adipokine levels

As previously demonstrated and confirmed in the present work [12, 13], the altered body weight gain, energy intake, the epididymal tissue weight as well as plasma metabolites, insulin levels and peripheral insulin sensitivity (GIR) recorded in rats fed a SRD were normalized or improved by dietary chia seed administration (Table 4). Besides, a significant reduction in the visceral adiposity index was recorded in the SRD + chia group. Values were as follows: $x \pm$ SEM ($n = 6$); 3.92 ± 0.11 in CD; 6.08 ± 0.14 in SRD and 4.25 ± 0.18 in SRD + chia ($P < 0.05$ CD and SRD + chia vs. SRD).

The present study shows that the increased plasma inflammatory mediators IL-6 and TNF-α observed in rats fed a SRD were reversed in the SRD + chia group, reaching values similar to those of the CD-fed rats. Furthermore, the elevated TNF-α content in the epididymal fat of SRD-fed rats reported in previous studies [16] returned to control values when chia seed replaced corn oil as a dietary source of fat (data not shown).

Plasma lipid peroxidation and protein carbonyl groups level, adipose tissue XO activity and ROS content

Figure 1a shows that the enhanced plasma TBARS—as an estimation of lipid peroxidation—as well as the levels of protein carbonyl groups formation—a marker of protein damage of rats fed a SRD were significantly decreased reaching values similar to those of the CD group when chia seed replaced corn oil during the last 3 months of the diet. In addition, the increase in XO activity and ROS content in the fat pad of the SRD group were completely normalized in the group of rats fed a SRD + chia (Fig. 1b). Besides, no changes in the mRNA abundance of p47NOX subunit were recorded in the three dietary groups. Values of p47NOX

Table 4 Body weight, energy intake, epididymal fat pad weight, plasma metabolites, insulin, glucose infusion rate (GIR) and inflammatory cytokine levels in rats fed a control diet (CD), a sucrose-rich diet (SRD) or a SRD + chia seed (SRD + chia)

	CD	SRD	SRD + chia
Body weight (g)			
Initial	190.4 ± 3.8	193.5 ± 4.3	
3 months	370.1 ± 12.6	388.0 ± 8.2	374.2 ± 11.7
Final (6 months)	445.3 ± 8.8[a]	518.2 ± 16.3[b]	486.5 ± 11.5[b]
Energy intake (kJ/d)			
Initial to 3 months	289.5 ± 9.5	296.5 ± 9.7	
3–6 months	287.0 ± 5.3[a]	354.4 ± 15.1[b]	350.9 ± 12.1[b]
Epididymal fat pad			
Total weight (g)	7.6 ± 0.7[a]	14.7 ± 1.1[b]	9.0 ± 0.8[a]
Relative weight (g/100 g body weight)	1.71 ± 0.08[a]	2.84 ± 0.07[b]	1.85 ± 0.03[a]
Plasma metabolites and insulin levels			
Triglyceride (mM)	0.70 ± 0.03[a]	1.97 ± 0.08[b]	0.77 ± 0.04[a]
FFA (μM)	303.0 ± 12.4[a]	798.4 ± 23.4[b]	337.8 ± 33.3[a]
Uric acid (μM)	212.7 ± 22.3[a]	403.7 ± 29.4[b]	201.0 ± 4.04[a]
Glucose (mM)	6.78 ± 0.07[a]	8.31 ± 0.09[b]	7.10 ± 0.09[a]
Insulin (pM)	386.10 ± 21.45	427.86 ± 25.01	437.97 ± 32.35
GIR [μmol/(kg × min)]	59.38 ± 2.29[a]	25.56 ± 1.29[b]	56.90 ± 2.12[a]
Plasma inflammatory cytokines			
TNF-α (pg/mL)	5.79 ± 0.14[a]	15.14 ± 0.49[b]	4.30 ± 0.70[a]
IL-6 (pg/mL)	35.62 ± 8.34[a]	79.58 ± 15.96[b]	31.13 ± 5.75[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 Tukey's test. When appropriate, the statistical significance between the two groups (CD and SRD) was determined by Student's t test. For more details, see Statistical analysis in section "Materials and methods"

mRNA/28 s abundance expressed as arbitrary units relative to control rats were as follows: mean ± SEM (six animals per group); 1.00 ± 0.30 in CD; 1.11 ± 0.08 in SRD and 1.03 ± 0.04 in SRD + chia.

Antioxidant enzymes activity and expression

Figure 2 shows the effect of chia seed on the activities and transcriptional levels of antioxidant enzymes GPx and SOD. The significant decreases ($P < 0.05$) of both GPx activity and mRNA expression observed in the SRD-fed rats compared with the CD groups were completely reversed in rats fed SRD + chia, reaching values of mRNA even higher than those observed in the rats fed a CD. Dietary chia seed was able to normalize the reduction in total SOD enzymatic activity recorded in the SRD group. Interestingly, in spite of the lower SOD activity the expression of the MnSOD mRNA was significantly higher in the fat pads of SRD-fed rats. Dietary chia seed decreased MnSOD mRNA expression to a value similar to the one observed in the CD-fed rats (Fig. 2). Besides, the epididymal fat tissue of the SRD + chia group shows a normalization of CAT and a significant improvement in GR activities. Values were as follows: mean ± SEM (six animals per group); CAT: U/mg of protein; CD: 2.37 ± 0.08; SRD: 1.83 ± 0.09; SRD + chia: 2.77 ± 0.32 ($P < 0.05$, SRD vs. CD and SRD + chia). GR: mU/mg of protein; CD: 22.0 ± 0.8; SRD: 14.6 ± 0.9; SRD + chia: 17.5 ± 0.5 ($P < 0.05$, SRD vs. CD and SRD + chia and CD vs. SRD + chia). In addition, the redox state of glutathione—a non-enzymatic antioxidant defense—that significantly decreased ($P < 0.05$) in the adipose tissue of SRD-fed rats returned to normal values in the SRD + chia group. Values were as follows: mean ± SEM (six animals per group); redox state [(oxidized form/total form) × 100]; CD: 44.3 ± 0.7; SRD: 26.1 ± 1.0; SRD + chia: 39.8 ± 3.9 ($P < 0.05$, SRD vs. CD and SRD + chia).

Nrf2 expression in epididymal fat pads

From the above results, we analyzed the expression of the Nrf2—a key molecule of the endogenous antioxidant system within the body. No changes in the expression of the mRNA Nrf2 level were observed in the SRD group compared with CD group, while a significant increase ($P < 0.05$) of this nuclear factor was recorded in rats fed SRD + chia at the end of the experimental period (Fig. 3).

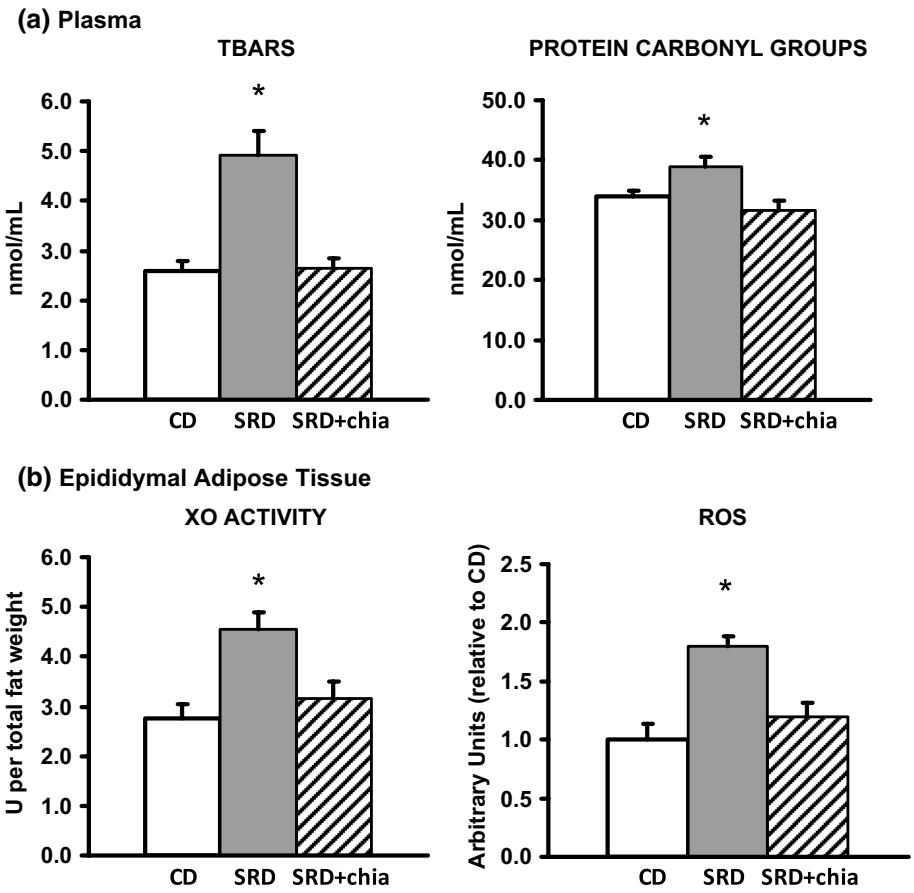

Fig. 1 **a** Plasma levels of TBARS and protein carbonyl groups in rats fed a control diet (CD); a sucrose-rich diet (SRD) or a SRD + chia seed (SRD + chia). **b** Xanthine oxidase (XO) activity and ROS content in epididymal fat pad. Values are expressed as mean ± SEM ($n = 6$), with their standard errors represented by *vertical bars*. *Asterisk* mean values were significantly different from those of the CD and SRD + chia groups ($P < 0.05$) (one-way ANOVA followed by Tukey's test)

Protein mass level of PPARγ

Since increased levels of TNF-α down regulated PPARγ—a major modulator of adipogenesis—we examined the effect of dietary chia seed on the protein mass level of this nuclear receptor. The immunoblotting of adipose tissue revealed a single 67 kDa band consistent with PPARγ. Each gel contained an equal number of samples from rats fed CD, SRD and SRD + chia (Fig. 4, top panel). After the densitometry of immunoblots, the PPARγ of the CD group was normalized to 100 % and both SRD and SRD + chia were expressed relative to this. Confirming previous results, quantitative analyses of Western blot showed that the relative abundance was significantly decreased in the fat pad of the SRD group compared with rats fed a CD (Fig. 4, bottom panel).The present data demonstrated that the chia seed enriched diet normalized the PPARγ protein mass level, reaching values similar to those of the CD group.

Fatty acid composition of adipose tissue phospholipids

Table 5 depicts the adipose tissue fatty acid membrane phospholipids and the ratios of *n-3* PUFAs to total fatty acids, *n-3* to *n-6* and *n-3* to total saturated fatty acids. Compared with the CD-fed rats, a decrease in saturated and polyunsaturated fatty acids can be observed in the SRD-fed rats, while monounsaturated fatty acids significantly increased in the latter group. The *n-3*/*n-6* ratio was similar in both groups. The addition of chia seed to the SRD significantly decreased monounsaturated fatty acids compared with both CD and SRD, while an enhancement of *n-3* PUFAs was observed due to an increase in ALA, *22:5 n-3* and *22:6 n-3*. Besides, a greater increase in the *n-3*/*n-6* ratio was also recorded.

Discussion

The present study provides new information on the biochemical and molecular mechanisms associated with the consumption of chia seed and their effects upon the reversion/improvement in preexisting oxidative stress and adipocytokines involved in the dysregulation of adipose tissue of dyslipemic insulin-resistant rats.

The adipose tissue is especially susceptible to the damage of oxidative stress. Different studies in rodents including our own have shown that the consumption of diets rich in sucrose, fructose or fat induced adipocyte hypertrophy

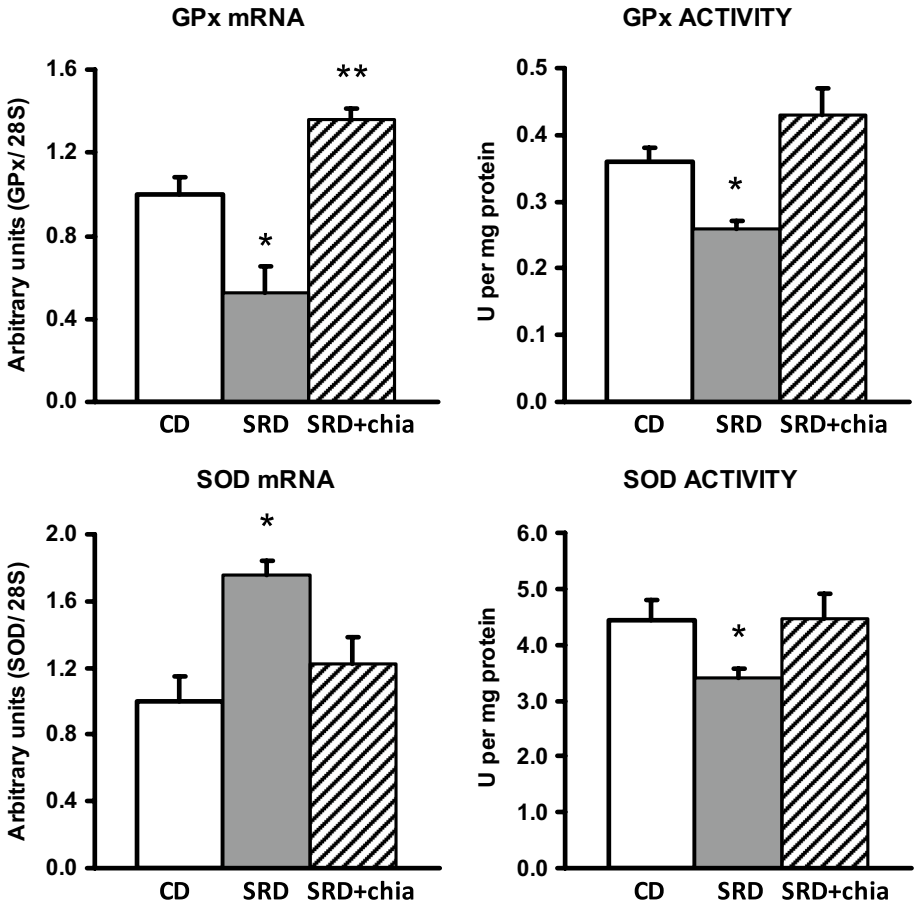

Fig. 2 Glutathione peroxidase (GPx) and superoxide dismutase (SOD) mRNA expression and activity in epididymal fat pad in rats fed a control diet (CD), a sucrose-rich diet (SRD) or a SRD + chia seed (SRD + chia). GPx and SOD transcript levels were measured by RT-PCR, normalized against 28S rRNA and expressed in arbitrary units relative to the CD group. Values are expressed as mean ± SEM ($n = 6$), with their standard errors represented by *vertical bars*. *Asterisk* mean values were significantly different from those of the CD and SRD + chia groups ($P < 0.05$). *Double asterisks* mean values were significantly different from those of the CD and SRD groups ($P < 0.05$) (one-way ANOVA followed by Tukey's test.)

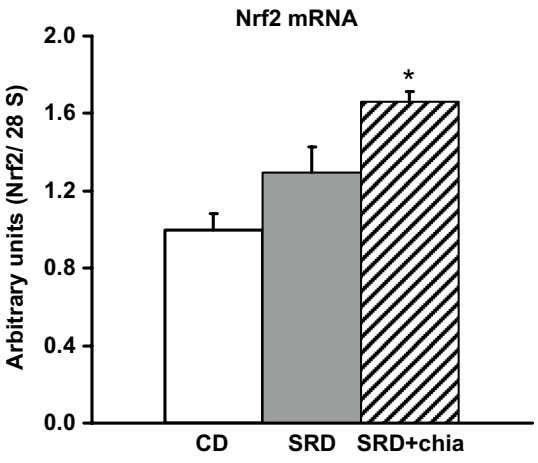

Fig. 3 Nrf2 expression in epididymal fat pad in rats fed a control diet (CD), a sucrose-rich diet (SRD) or a SRD + chia seed (SRD + chia). Nrf2 transcript levels were measured by RT-PCR, normalized against 28S rRNA and expressed in arbitrary units relative to the CD group. Values are expressed as mean ± SEM ($n = 6$), with their standard errors represented by *vertical bars*. *Asterisk* mean values were significantly different from those of the CD and SRD groups ($P < 0.05$, one-way ANOVA followed by Tukey's test)

with increased triglyceride storage and dysfunction [5, 16, 30]. The present data demonstrate that the replacement of dietary corn oil by chia seed in the SRD-fed rats restored the activities of key enzymes involved in the antioxidant system (e.g., CAT, GR) and was able to increase both the activity and gene expression of GPx, which reached values higher than those recorded in the CD-fed group. Besides, our results show that the increases of the MnSOD expression recorded in the SRD-fed rats do not correlate with a decreased level of the SOD enzyme activity. In this regard, the recorded SOD data do not differentiate between the activities of Cu–Zn–SOD and MnSOD isoforms. Along this line, a lack of correlation between activity and expression was also demonstrated by Busserolles et al. [31] in the heart of rats fed a high-sucrose diet. These animals showed a decrease in Cu–Zn-SOD without changes in the MnSOD activities and a significant increase in Cu–Zn–SOD mRNA. Moreover, both the Cu–Zn–SOD and MnSOD isoforms could have posttranslational modifications (i.e., nitration, phosphorylation) that could alter their enzymatic activities. Besides, nitration and phosphorylation of SOD have

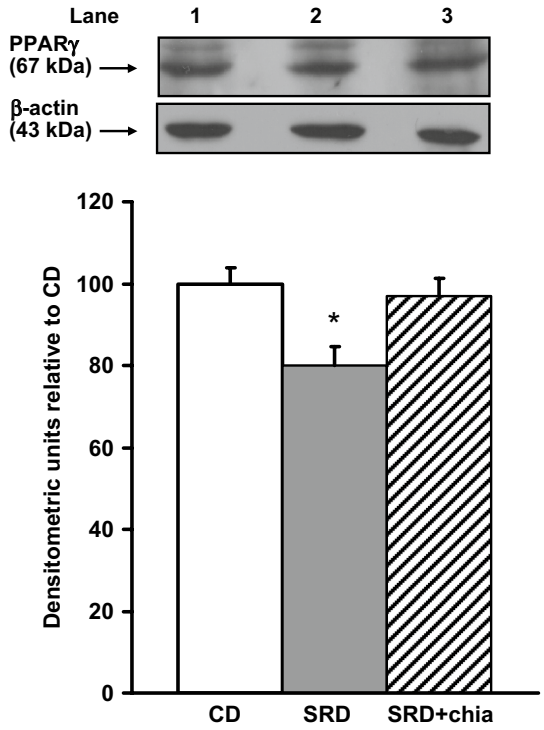

Fig. 4 Epididymal fat pad protein mass levels of PPARγ in rats fed a control diet (CD), a sucrose-rich diet (SRD) or a SRD + chia seed (SRD + chia). *Top* a representative immunoblot of PPARγ from the CD, SRD and SRD + chia-fed rats. Molecular marker is shown on the right. *Lane 1* CD, *lane 2* SRD, *lane 3* SRD + chia. *Bottom* densitometric immunoblot analysis of PPARγ protein mass levels in epididymal fat pad of rats fed a CD, SRD or SRD + chia. Values are expressed as mean ± SEM ($n = 6$), with their standard errors represented by *vertical bars*, and expressed as a percentage relative to CD. *Mean values were significantly different from those of the CD and SRD + chia groups ($P < 0.05$, one-way ANOVA followed by Tukey's test)

been shown to have possible reversible processes, and these modifications may be related to a redox signaling process in the cells [32]. Therefore, we cannot discard the possibility that posttranslational modifications and/or changes in individual SOD isoform activities could be involved in the absence of the correlation observed in the adipose tissue of the SRD-fed rats. Nevertheless, a limitation of this study arises from the semi-quantitative RT-PCR analysis used for the gene expression determinations. The administration of chia seed, which normalized adipose tissue ROS levels, returned to normal values both the activity of SOD as well as the MnSOD expression.

On the other hand, the increase in the epididymal fat tissue weight and the enzymatic activity of XO observed in the SRD-fed rats was significantly reduced in the SRD + chia group. Although XO is not the only enzyme involved in the generation of ROS in the adipose tissue, recent studies by Tsushima et al. [33] in the epididymal fat of ob/ob mice fed a high-fat high-sucrose diet showed a marked increase in XO and XOR (XO + XDH) activities when compared with control mice. In addition, they reported that adipose tissue can secrete uric acid and that the production of this metabolite is augmented in obesity [33]. In this regard, our data show that dietary chia seed was able to normalize plasma uric acid levels and this was accompanied by a significant reduction in the visceral adiposity index. Several studies in rodents have indicated a complex modulation of gene expression in white adipose tissue by long chain fatty acids, especially EPA and DHA [6, 34, 35]. An increase in intracellular *n-3* PUFAs has also resulted from the ingestion of vegetable sources (e.g., chia seed, flax seed) rich in ALA via elongase/desaturase activities [7]. In agreement with our results, Baranowski et al. [36] demonstrated a reduction in adipocyte hypertrophy and protein levels of several inflammatory markers in obese Zucker rats fed a dietary flax seed. Moreover, in 1 % cholesterol-fed rats, flax seed and pumpkin seed supplementation improved the efficiency of the antioxidant enzyme system and decreased plasma and hepatic malondialdehyde levels [37].

The regulation of the redox state of the cell and the removal of ROS play a pivotal role in the regulation of energy homeostasis within the body. In this vein, dietary chia seeds were able to both reverse the decreased redox state of glutathione and normalize ROS levels within the fat pads of SRD-fed rats. Interestingly, these were accompanied by a significant increase in the mRNA Nrf2 expression—a master key regulator of redox balance—[38] and as mentioned above, by a higher activity and gene expression of GPx, one of their target antioxidant enzymes.

Our results do not provide data concerning the mechanism underlying the effect of chia seeds and/or ALA upon the increase in the Nrf2 gene expression in the adipose tissue of SRD-fed rats. However, some clues have been provided by recent publications. For instance, a recent study by Kusunoki et al. [39] in 3T3-L1 adipocyte cells treated with EPA, DHA or 4-HHE dose dependent demonstrated that an induction of heme-oxygenase-1 (HO-1) expression through the activation of Nrf2 inhibits H_2O_2 induced ROS generation preventing oxidative stress. Besides, Gao et al. [40] demonstrated that *n*-3 fatty acid oxidation products reacted directly with the negative regulator of Nrf2, Keap1, initiating Keap1 dissociation with Cullin 3 and thereby inducing Nrf2–directed gene expression. Interestingly compared to corn oil, chia seed contains appreciable amounts of *n-3* ALA (0.8 vs. 64.6 g/kg of diet) [13] and the dietary inclusion of ALA could be a valuable source of *n*-3 long chain fatty acids (EPA, DHA). Therefore, we cannot discard the possibility that EPA, DHA or their products might activate the Nrf2 pathway. Further studies are needed to evaluate this issue.

Table 5 Fatty acid composition of epididymal adipose tissue phospholipids in rats fed a control diet (CD), a sucrose-rich diet (SRD) or a SRD + chia seed (SRD + chia)

Fatty acids	CD	SRD	SRD + chia
16:0	18.30 ± 0.80	16.20 ± 0.90	17.60 ± 0.70
16:1	1.40 ± 0.10[a]	2.30 ± 0.10[b]	1.20 ± 0.10[a]
18:0	4.50 ± 0.20[a]	3.20 ± 0.10[b]	4.50 ± 0.10[a]
18:1	34.30 ± 1.80[a]	42.40 ± 1.50[b]	21.60 ± 1.10[c]
18:2 *n-6*	38.30 ± 2.0[a]	34.20 ± 1.80[a]	28.20 ± 1.90[b]
18:3 *n-3*	0.60 ± 0.10[a]	0.50 ± 0.20[a]	25.80 ± 0.90[b]
20:2 *n-6*	0.80 ± 0.10[a]	0.40 ± 0.10[b]	Traces
20:3 *n-6*	0.20 ± 0.02[a]	0.10 ± 0.02[b]	Traces
20:4 *n-6*	1.60 ± 0.10[a]	0.50 ± 0.10[b]	0.20 ± 0.03[c]
20:5 *n-3*	Traces	Traces	Traces
22:5 *n-3*	–	0.20 ± 0.01[a]	0.60 ± 0.10[b]
22:6 *n-3*	–	–	0.30 ± 0.03
Total			
Saturated	22.80 ± 0.50[a]	19.40 ± 0.50[b]	22.10 ± 0.40[a]
Monounsaturated	35.70 ± 0.95[a]	44.70 ± 0.80[b]	22.80 ± 0.60[c]
Polyunsaturated	42.50 ± 0.46[a]	35.90 ± 1.10[b]	55.10 ± 0.60[c]
n-6	40.80 ± 1.04[a]	35.20 ± 0.94[b]	28.40 ± 0.96[c]
n-3	0.60 ± 0.10[a]	0.70 ± 0.15[a]	26.7 ± 0.80[b]
n-3/total fatty acids	0.06 ± 0.01[a]	0.07 ± 0.01[a]	0.36 ± 0.09[b]
n-3/*n-6*	0.015 ± 0.002[a]	0.020 ± 0.004[a]	0.940 ± 0.020[b]
n-3/total saturated fatty acids	0.026 ± 0.004[a]	0.036 ± 0.006[a]	1.210 ± 0.020[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 Tukey's test

On the other hand, Zhao et al. [41] demonstrated that ALA and DHA decreased the expression of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 in human monocyte (TH1P) 1 cells, via the inhibition of nuclear factor NF-kB induced binding activity. Additionally, ALA could elicit its anti-inflammatory effects mediated PPARγ activation by inhibition of nuclear factor NF-kB activation [41]. A significant decrease in PPARγ protein levels was observed in 3T3-L1 adipocytes incubated for 24 h in the presence of TNF-α [42]. The present results showed that dietary chia seed was able to normalize the significant increase in plasma and adipose tissue TNF-α levels and the reduction in PPARγ protein levels in the fat pad of SRD-fed rats. Moreover, chia seed induced a significant reduction in plasma IL-6. This cytokine positively correlated with fat mass, plasma FFA levels and IR [43]. Interestingly, this was accompanied by a decrease in visceral adiposity and both normalization of plasma FFA levels and whole-body insulin sensitivity and glucose homeostasis. In addition, plasma TBARS and protein carbonyl groups levels were significantly decreased in the SRD + chia group. All these findings would favor an improvement in the adipose tissue dysfunction.

In addition to ALA, chia seed also contains antioxidant activity due to the presence of phenolic compounds in the seed or oil (e.g., quercetin, among others) [8]. In the adipose tissue of rats fed a fructose-rich diet supplemented with quercetin Vazquez Prieto et al. [17] showed that the protective effect of this compound on inflammation is in part associated with its capacity to decrease the activation of the mitogen-activated kinase (MAPKs) JNK and p38 and to prevent the down regulation of PPARγ. Furthermore, quercetin prevents the TNF-α-induced increase in several parameters of inflammation and oxidative stress and the decrease in insulin sensitivity in 3T3-L1 adipocytes. Therefore, it is possible that the presence of quercetin in the dietary chia seed could be involved in the present findings.

Another mechanism by which dietary chia seed might exert its favorable effect on adipose tissue dysfunction is the incorporation of long chain *n-3* fatty acids into the lipid membrane phospholipids fraction of the adipocytes. It is well known that the degree of unsaturation in the membrane phospholipids is associated with increased insulin sensitivity and insulin stimulated glucose uptake [44]. In the present study, the consumption of dietary chia seed during the last 3 months of the experimental period was sufficient to induce a significant increase in the incorporation of ALA into the phospholipids of the epididymal adipose tissue of SRD-fed rats. Moreover, an increase in 22:5 and 22:6 *n-3* fatty acids as well as the *n-3*/*n-6* ratio was also recorded after chia administration. Poudyal et al. [10] showed an increase in ALA and *n-3*/*n-6* ratio in the retroperitoneal adipose tissue of rats fed a high-fat high-fructose diet supplemented with 5 % of chia seed during 8 weeks compared with those fed the same diet without chia. González-Mañán et al. [45] in Sprague-Dawley rats fed chia seed during 3 weeks showed an increase in ALA, EPA and DHA and a decrease in the *n-6*/*n-3* ratio in total lipids and phospholipids of adipose tissue. Therefore, the changes recorded in the profile of fatty acid composition of membrane phospholipids in the adipose tissue of SRD + chia-fed rats might be one of the possible mechanisms responsible for the improvement in insulin sensitivity and action.

In the present experimental design, the isocaloric replacement of corn oil by chia seed as a dietary source of fat in SRD-fed rats shows a moderate reduction in the amount of saturated fatty acids (13.6 vs. 8.9 g/kg diet) and a higher increase in both the n-3 fatty acids (ALA) and the ALA/LA ratio. The present data as well as previous studies with the same experimental protocol [11, 13] suggest that the high content of ALA in the chia seed could play a key role in the effects mentioned above. In this regard it has been demonstrated by our group and others [9, 11] that

chia seed administration change the plasma fatty acid profile increasing ALA, EPA, DPA and DHA levels as well as the *n-3*/*n-6* ratio. These *n-3* fatty acids are important regulator of cell metabolism through multiple mechanisms. Moreover, chia seed is a complex mixture of proteins, fibers, minerals and antioxidants; therefore, we cannot attribute all the observed effects only to the increased content of ALA. Besides, the reduction in saturated fatty acids in the SRD + chia diet might also contribute to these findings.

In brief, this study provides new information on the beneficial effects of chia seed to improve or reverse the depletion of antioxidant defenses, the changes of pro-inflammatory cytokines and oxidative stress triggered by a sucrose-rich diet in the adipose tissue of a dyslipemic insulin-resistant rat model. Thus, these findings suggest that dietary chia seeds might have potential benefits in mitigating adipose tissue dysfunction, dyslipidemia and insulin resistance associated with the metabolic syndrome. Caution is warranted before extrapolating these results to humans, especially considering the few studies published so far and the different quantities of chia seed that have been used (0.36–0.72 g daily/kg of body weight in human—average body weight 70 kg—versus 20 g daily/kg body weight in rats) [46, 47].

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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