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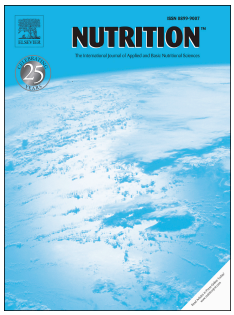

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Chia (*Salvia hispanica L.*) enhances HSP, PGC-1α expressions and improves glucose tolerance in diet-induced obese rats

RUNNING HEAD: "Protecting effects of chia in obese rats"

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R. S. M., E. A. M., S. A. L. and M. R. M.-J. designed the research; R. S. M., E. A. M. and S. A. L. conducted experimental work; C. S. M., P. C. B. L., P. N. M. and J. A-F. were involved in the analysis of western blot; R. S. M. and C. S. M. analysed the data, wrote the manuscript and discussed it with the whole group of authors. The authors declare that they have no conflict of interest.

Abstract

Objective: This study investigated the effects of chia seed and chia oil on heat shock protein (HSP) and related parameters in diet-induced obese rats. **Methods:** Animals were divided in six groups: control, high-fat and high-fructose diet (HFF), HFF with chia seed or chia oil in short (6-weeks) and long (12-weeks) treatments. Plasma indicators of glucose tolerance and liver damage, skeletal muscle expression of antioxidant enzymes and proteins controlling oxidative energy metabolism were determined. The limit of significance was set at $P < 0.05$. **Results:** HFF diet induced glucose intolerance, insulin resistance, oxidative stress, and altered parameters related to obesity complications. The consumption of chia seed or chia oil did not reduce body weight gain or abdominal fat accumulation. But, chia seed and chia oil in both treatments improved glucose and insulin tolerance. Chia oil in both treatments induced HSP70 and HSP25 expression in skeletal muscle. Chia seed short treatment increased HSP70, but not HSP25 expression. Chia oil in both treatments restored superoxide dismutase and glutathione peroxidase expression. Chia seed long treatment and chia oil short treatment restored peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) expression. **Conclusion:** Chia oil restored the antioxidant system and induced the expression of a higher number of proteins compared to chia seed. The present study contributes with new properties and molecular mechanisms associated with the beneficial effects of chia seed and chia oil consumption in diet-induced obese rats.

Keywords: Salvia hispanica**,** oxidative stress, obesity, insulin resistance, heat shock proteins.

Introduction

Excessive food ingestion and the increase in consumption of a high fat and high fructose diet (HFF) have been related with the increase in obesity, glucose intolerance, insulin resistance and type 2 Diabetes [1,2]. HFF may cause problems, mainly in controlling glycemia, insulin signaling and glucose transport. Besides, previous studies show that type 2 Diabetes reduces heat shock proteins (HSP) expression [3].

Heat shock proteins (HSP) are primarily a natural defense system, responsible for promoting higher cell resistance and tolerance against several aggressor agents and homeostasis alterations. HSPs are highly conserved and are named according to their molecular weights. HSP90, HSP70, HSP60 and HSP25 are among the main and most stress-responsive [4].

Recent studies demonstrated that induction of the HSP70 expression protects against high-fat-induced glucose and insulin intolerance [3]. HSP70 overexpression and induction have been

related with the improvement of glucose tolerance and sensitivity to insulin. It is known that increasing and activation of kinases c-Jun NH_2-terminal kinase (JNK) and inhibitor kB kinase-β (IKKβ) happen in obese animals, which cause alterations in insulin signaling and interferes in the transport and glucose uptake. HSP70 is able to inhibit JNK as well as HSP25 is able to inhibit IKKβ, that is the mechanism by which increasing and induction of HSPs improve glucose tolerance in rats fed with a high fat diet [5,6].

Current evidence suggests that increasing HSP70 and HSP25 expression can improve glucose tolerance in high fat diet-induced obese rats. Therefore, strategies that are able to induce the expression of such HSPs have received special attention [5,6]. However, little is known about the effect of HFF consumption on HSP70 and HSP90, neither regarding HSP90 nor HSP60.

Chia (*Salvia hispanica* L.) is an annual herbaceous plant that belongs to the *Lamiaceae* family native from southern Mexico and northern Guatemala [7]. The chia seed has been described as an important source of oil, protein, dietary fiber, minerals and polyphenolic compounds [8,9]. The chia oil is unique since it contains the highest proportion of omega-3-linolenic acid (C18:3) of any known natural source [7]. Many studies have provided evidence that regular consumption or dietary supplementation with long chain n-3 polyunsaturated fatty acids (PUFA) brings numerous health benefits [10].

Some studies report that Chia seed has promoted health benefits [11,12], improving biological markers related to dyslipidemia, inflammation, cardiovascular disease, glucose homeostasis and insulin resistance, without promoting adverse effects. In sucrose-fed rats, dietary chia seed improved adiposity and normalized hypertriacylglycerolaemia and insulin resistance without affecting glucose homeostasis in dyslipaemic rats [13]. However, there is little information available regarding the effects of chia seed and oil *in vivo* and, to the best of our knowledge, there are, to date, no reports on chia seed and oil effects on the HSP system.

The present study was designed to test the hypothesis that ingestion of chia (*Salvia hispanica L.*) could protect against oxidative stress, glucose intolerance and associated complications in (high-

fat/high-fructose) diet-induced obese rats, and that the HSP system can be involved in the mechanism of protection.

Material and methods

Seeds and oil

Chia seed and chia oil from FTP S.A. Santiago, Chile were purchased from R&S Blumus Comercial de Produtos Alimentícios Ltda, Brazil. According to the manufacturer's information, chia oil was obtained by cold pressing and stored at 2-8 °C until use in amber glass bottles without head space. The seeds were ground in a laboratory impact mill (Marconi MA 630/1) and passed through a 0.850 mm sieve and stored at 2-8 °C in amber glass bottles until use.

Animals, diets and interventions

This work was approved by the Ethics Commission on Animal Use (CEUA/ UNICAMP, protocol no. 2936-1) and followed the University guidelines for the use of animals in experimental studies. Forty-eight male *Wistar* rats aged 21 to 23 days were obtained from the Multidisciplinary Center for Biological Investigation, University of Campinas. The animals remained in individual cages for growth with free access to water and chow diet for 4 weeks and were maintained under controlled conditions (22 ± 1 ºC, 60-70% humidity, 12 hours light/dark cycle). After growth, animals (220.32 g ± 18.32) were randomly divided in six experimental groups (n=8/group) for 12 weeks.

Diets were based on the AIN-93M diet [14] with protein concentration of 12%. Groups: Control (lean control) received the standard diet; High-fat and high-fructose group (HFF) received a diet containing 4% (w/w) soybean oil, 31% (w/w) lard and 20% fructose (w/w) [15]; chia seed short and long treatments received HFF diet with 13.3% (w/w) of chia seed; chia oil short and long treatments received HFF diet with 4% (w/w) of chia oil. For chia seed and chia oil groups, the soybean oil was replaced by the oil content of chia seed and chia oil. The protein and dietary fiber

content in the HFF with chia seed groups was balanced taking into account the amount of such nutrients present in the chia seed [9]. The composition of the diets is presented in Table 1. There were a long (12 weeks) and a short (6 weeks) treatment with chia seed or chia oil. Animals from long treatment were fed with HFF containing chia seed or chia oil for 12 weeks. Short groups were initially fed only with HFF diet for 6 weeks, followed by 6 more weeks with HFF containing chia seed or chia oil, as shown on Figure 1. Diets were prepared monthly and packed in dark polyethylene bags and stored at -20 ºC to minimize the oxidation of fatty acids. The weight gain was monitored weekly and the food intake every 2 days.

<Insert Table 1>

<Insert Figure 1>

Intraperitoneal glucose tolerance test (iGTT) and insulin tolerance test (ITT)

An intraperitoneal glucose tolerance test (iGTT) and insulin tolerance test (ITT) were performed on food-deprived (12 h) rats after 10 and 11 weeks of experiment, respectively. Blood glucose levels were measured with an FreeStyle Lite® handheld glucometer (*Abbott Diabetes Care*, Abbott Laboratorios do Brazil LTDA) using appropriate test strips. For the iGTT, a solution of 50% D-glucose (2 g/kg body weight) was administered into the peritoneal cavity. Blood samples were collected from the tail vein at 30, 60, 90 and 120 min for the determination of glucose concentrations. The AUC of glucose was calculated. For the ITT, glucose blood levels were sampled at 5, 10, 15, 20, 25 and 30 min following intraperitoneal injection of human insulin (0.75U/kg body weight, Novolin R, Novo Nordisk® Farmacêutica do Brazil LTDA). The constant rate for glucose disappearance during the ITT (K_{ITT} %/min) was calculated using the formula [0.693 / ($t_{1/2}$)]. The glucose $t_{1/2}$ was calculated from the slope of the least-square analysis of the glucose concentrations during the linear phase [16].

Blood and tissue collection for biochemical analysis

The animals were sacrificed by decapitation preceded by 12-hour-fasting after 12 weeks of the experimental period. Blood samples were collected in polyethylene tubes with EDTA anticoagulant to obtain plasma. Tubes were centrifuged at 3000 x g (4 ºC, 15 min). Plasma was separated and stored in polypropylene microtubes at -80°C until analysis. Abdominal adipose tissue was removed and weighed. Soleus muscle was removed, frozen in liquid nitrogen and stored at -80ºC for subsequent analysis.

Blood parameters

Plasma fasting insulin was analyzed using enzyme-linked immunosorbent assay (ELISA) (EZRMI-13K, *Millipore*, MA, USA) and unesterified fatty acids (NEFA) were determined using commercial enzymatic colorimetric assay kit (700310, *Cayman Chemical*, Ann Arbor, MI, USA) following manufacturer's protocol. Plasma total thiol content was assessed with 5,5’ -dithiobis (2-nitrobonzoic acid) DTNB assay. Crude plasma samples (10µL) were treated with 200µL DTNB (Sigma D8130, 0.5 mM DTNB diluted in phosphate buffer 100 mM, pH 7.4) and incubated in the dark for 30 minutes at room temperature. After incubation, the reaction was measured at 412 nm using microplate reader (Biotek, Winooski, VT). The glutathione (GSH, Sigma G4251) solution (0.5 mM) was diluted in phosphate buffer (100 mM, pH 7.4) for standard curve. The results were expressed as mM GSH/mg protein from glutathione (GSH) standard curve (0 - 0.5 mM).

Analyses for uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein and globulin were carried out using clinical kits (Laborclin, Vargem Grande, Paraná, Brazil) and albumin (Labtest, Lagoa Santa, Minas Gerais, Brazil) with spectrophotometric determination in a Biotech EPOCH microplate reader (Biotek, Winooski, VT).

Western blot

The soleus samples were subjected to SDS-PAGE (8%) and transferred using a semi-dry system (Bio-Rad, CA, USA) to a nitrocellulose membrane of 0.22 µM (Bio-Rad, cat. 162-0112).

The total protein content was determined in the supernatant using the Lowry method [17]. A molecular weight standard was used and ran concurrently on each gel for accurate determination of the proper molecular weight for each antibody (Thermo Scientific, #26634). The membranes were incubated with the appropriate primary antibodies overnight to assess the protein level of (all the following antibodies are from Stressgen, Victoria, BC, Canada): HSP90 (Ref. ADI-SPA 831 diluted 1:3000), HSP70 (Ref. ADI-SPA 810 diluted 1:2000), HSP60 (Ref. ADI-SPA 806 diluted 1:2000), HSP25 (Ref. ADI-SPA 801 diluted 1:2000), GAPDH (Ref. ADI 905734 diluted 1:2000). Catalase was from Santa Cruz (Santa Cruz, CA, USA, Ref. sc271803 diluted 1:1000). The following were from Abcam: SOD (cytosolic CuZnSOD) (Ref. ab51254 diluted 1:10.000), GPx (Ref. ab22604 diluted 1:2000), PGC-1α (Ref. ab72230 diluted 1:1000), Lipase (Ref. ab109251 diluted 1:2000), IDE (Ref. ab25733 diluted 1:1000), AMPK total (Ref. ab80039 diluted 1:2000), AMPK phosphorylated (Ref. ab39400 diluted 1:2000), DHFR (Ref. ab124814 diluted 1:2000). The appropriate secondary antibodies were used for detection. The bands were visualized using a UVITEC Cambridge instrument (model Alliance LD2) and blots were quantified using the digital program Image J.

Statistical Analysis

All data were presented as mean values ± SEM and analyzed by one-way ANOVA followed by the Duncan post-hoc test using Statistical Package for the Social Sciences (SPSS, Chicago, United States) software, version 17.0 for windows. The level of significance was set at $P< 0.05$.

Results

Body, abdominal fat weight and food intake

All the groups that consumed HFF diet had a significant increase in body weight (Figure 2 (a)) and abdominal fat weight (Figure 2 (b)) compared to control group. As expected, food intake was lower in the groups that consumed HFF, in comparison with control (Figure 2 (c)). Consuming

chia seed or chia oil did not reduce body weight gain and abdominal fat accumulation, as well as not influencing animal food intake.

<Insert Figure 2>

Glucose and Insulin Tolerance tests (GTT / ITT)

HFF diet induced glucose intolerance and insulin resistance, compared to control group, as shown in Figure 3. Chia seed and chia oil consumption, both long and short treatments improved glucose and insulin tolerance, regressing to lean control level in obese rats (Figure 3).

<Insert Figure 3>

Proteins expression

Results show that consuming chia oil, both long and short treatments, induced the increase in HSP70 and HSP25 expression. Chia seed short treatment, but not long, increased HSP70 expression, but had no effect on HSP25 expression (Figure 4 (a) and (b)).

HSP60 reduction was observed only in short treatment with chia seed in comparison with control (Figure 4 (c)). None of the diets showed any effect on HSP90 expression (Figure 4 (d)).

HFF diet decreased copper- and zinc-containing superoxide dismutase (cytosolic CuZnSOD) and glutathione peroxidase (GPx) expression compared to control. Chia oil, both long and short treatments, restored SOD expression to control levels. Chia seed long and short treatments increased SOD expression compared to HFF group; however, it is still in lower level when compared to the control group (Figure 4 (e)). Chia oil long treatment restored GPx expression, followed by chia oil short treatment and no improvement was observed in GPx expression from chia seed groups, which were equally reduced as HFF when compared to control (Figure 4 (f)). Regarding catalase, HFF group did not reduce its expression compared with control group. The highest expression was found in chia seed short treatment and the lowest values in chia seed long and chia oil short treatment compared to control group (Figure 4 (g)).

Lipase expression showed no response to any of the diets, except to the chia oil short treatment (Figure 4 (h)). HFF diet reduced peroxisome proliferator-activated receptor-γ coactivator-

1α (PGC-1α) expression when compared with control. Chia seed long and chia oil short treatment restored PGC-1α expression (Figure 4 (i)). Insulin-degrading enzyme (IDE) expression was increased in chia seed and chia oil, both short treatments (Figure 4 (j)). AMP-activated protein kinase (AMPK) expression was reduced by all HFF intakes in comparison with control (Figure 4 (k)). Finally, no alteration in dihydrofolate reductase (DHFR) expression was found (Figure 4 (l)).

<Insert Figure 4>

Blood parameters

The results for the analysis of blood parameters are illustrated in Figure 5. All the groups that consumed HFF diet, including the ones with chia seed or chia oil exhibited high fasting insulin compared to control group (Figure 5 (a)). Although, both chia seed and chia oil improved insulin tolerance from ITT, they did not reduce HFF-induced hyperinsulinemia. NEFA were increased in HFF group, whereas chia seed and chia oil both long treatments showed to reduce NEFA levels compared to HFF group and match the control group (Figure 5 (b)). Results indicate that the effects of seed or oil on NEFA reduction are effective in the long treatment, but not in short.

For markers of liver damage, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), data show that HFF promoted the increase in AST and ALT levels compared to the control. All groups with chia seed and chia oil reduced the concentration of markers of liver damage to base levels (AST, ALT), except for chia seed short treatment group (Figure 5 (c) and (d) respectively).

No alteration was observed for uric acid, total protein, albumin, globulin and total thiol content, (Figure 5 (e), (f), (g), (h), (i) respectively).

<Insert Figure 5>

Discussion

The present data can be considered novel because few studies have been published on the functional properties of chia and because no work was found in the literature to date dealing with

the effect of chia on the expression of HSP in obese rats. Heat shock protein (HSP) induction improves glucose tolerance in high fat diet-induced obese rats, which HSP70 and HSP25 induction inhibit JNK and IKKB kinases, which are increased by obesity [5,6]. Chia oil, both long and short treatments, induced the increase in HSP70 and HSP25 expression, whereas chia seed (only for short treatment group) induced HSP70 expression, but did not induce HSP25. In contrast, both chia seed and chia oil (short and long) equally improved glucose tolerance. Only the chia oil, however, induced HSP70 and HSP25 expression. Those results indicate that chia seed may improve glucose tolerance by some mechanism, other than by HSP induction. It may be related to the expression of proteins directly from insulin signaling pathway, such as insulin receptor, for instance.

Zhang et al. [18] observed that high fructose intake reduces HSP70 expression in liver of hamsters. However, data have not been reported about the effect of high fat and high fructose together (HFF diet) on HSP expression, or for the use of skeletal muscle in rat model.

Current evidence suggests that obesity, hyperglycemia, lipid excess and high-fat diet reduce mitochondrial oxidative capacity and increase reactive oxygen species (ROS) production, leading to oxidative stress. It has been proposed that oxidative stress caused by obesity may contribute to impaired glucose uptake and insulin resistance in skeletal muscle [19,20]. Together, investigators have reported that obesity, high-fat diet, hyperglycemia and hyperlipidemia reduce antioxidant defense [21]. Previous studies also showed that high-fat diet decreased SOD and GPx expression [22], consistent with our results for HFF, which reduced SOD and GPx expression when compared to control. Consequently, strategies, which can upregulate antioxidant system expression show to attenuate high-fat-induced oxidative stress [22], besides, the use of antioxidant agents prior to or after the onset of obesity may ease glucose intolerance [19,21]. Our results show that the consumption of chia oil (long and short treatments) restored SOD and GPx expression in skeletal muscle. Data indicate that chia oil restores the antioxidant system and may help reducing HFF-induced oxidative stress.

In addition, chia seed long and short treatments increased SOD expression compared to HFF

group. However, no improvement was observed in the seed groups regarding GPx expression, independent of the length of treatment. It is not possible at this time to understand this apparent inconsistency, but one possibility is that the difference may lie in the diversity of bioactives (phenolics, for instance) in the two chia products [9]. Aside from this, the presence of such substances should not necessarily affect all enzymes of the GPx redox system in the same fashion and to the same extent. Antioxidant enzymes are expressed and respond independently to different inducing radicals [23] and therefore we should not expect these enzymes to be expressed identically. Furthermore, the potential effect of daily chia seed and oil consumption on this biomarker has not been sufficiently studied, and a mechanism that can account for these findings cannot be readily outlined and needs to be further explored.

The catalase expression has shown to be increased in several tissues during high-fat diet intervention [24-26]. According to Meng et al. [24], after the administration of an antioxidant, catalase returned to the control level. In our findings we did not observe the increase in catalase expression caused by HFF, whereas the lowest levels, even lower than the control, were observed in chia oil short and chia seed long treatments.

It has been proposed that oxidative stress also stimulates JNK kinase that promotes inhibition of insulin signaling, which may result in insulin resistance [21]. We once more may suggest that the effect of chia oil in increasing HSPs and restoring antioxidant defense expression is an important factor in the improvement of glucose tolerance, either coming from oxidative stress or from mitochondrial dysfunction that obesity may cause, as it is a multifactorial illness.

One of the mechanisms suggested for the increase in the production of oxidants by the mitochondria is the excess of energy [19]. However, the exact mechanism to explain how obesity causes oxidative stress is not elucidated. It has been suggested that excessive caloric consumption may generate more substrates to enter mitochondrial respiration. The number of electrons increase in electron transport chain, however once the potential membrane reaches threshold voltage, such electron excess may accumulate at complex III and by keeping oxygen donation constant it may

generate high levels of superoxide. Another mechanism is related to the increase in glycation due to high glucose in bloodstream. Such increase may activate NADPH oxidase, which generates superoxide, besides antioxidant defense reduction itself [21].

It has been reported that obesity, high-fat diet and type 2 Diabetes promote downregulation of PGC-1α expression; such reduction may favor problems in mitochondrial function, biogenesis and oxidative capacity in skeletal muscle [1,27,28]. Our results are in agreement with this concept, in which HFF diet reduced PGC-1α expression compared to control. In contrast, Hancock et al. [29] found that rats fed with high-fat diet showed a gradual increase in muscle mitochondria, measured by the increase in PGC-1α expression. Such findings are against the concept that insulin resistance is mediated by a deficiency of muscle mitochondria.

PGC-1α is a key regulator involved in energy homeostasis control, thermogenesis and glucose metabolism control, besides being related to the regulation of biogenesis and mitochondrial oxidation/respiration in skeletal muscle. The functions and capacity of PGC-1α in interfering in metabolism may turn it into a target to compounds with attribution of antiobesity function [30]. Our results show that the chia seed long and chia oil short treatments made it possible to recover and increase PGC-1α expression that may favor restoring of PGC-1α functions. Previous studies have reported that deleterious effects in mitochondrial capacity caused by high-fat were reverted with PGC-1α overexpression [31].

PGC-1α may also induce skeletal muscle glucose transporter (GLUT4) and favor glucose uptake, without altering or increasing sensitivity to insulin [30]. Glucose tolerance test (iGTT) of the groups that consumed both chia seed or chia oil (short and long treatments) showed that glycemia was reduced to control level, although there was no strong reduction in glycemia to the groups that increased PGC-1α expression. However, the increase in PGC-1α expression may favor other parameters, such as the increase in energy expenditure, besides reducing the risk of developing insulin resistance. It has been hypothesized that insulin resistance in rats and humans with impaired glucose tolerance or diabetic may be mediated by the mitochondrial dysfunction and

deficiency in skeletal muscle, however such mechanisms were yet not elucidated [1,32].

NEFA at high levels may potentiate the reduction and inhibit PGC-1α expression [31]. In our results, that was true for HFF group that showed high levels of NEFA and reduced PGC-1α expression. Similarly, chia seed long treatment exhibited reduced levels of NEFA compared to HFF group and increased PGC-1α expression. However, the relation between NEFA and PGC-1α was not observed to the chia oil short treatment, because this group displayed higher levels of NEFA compared to the control, without inhibiting PGC-1α expression. Such results may indicate that chia oil short treatment blocked the inhibition that NEFA would provoke on PGC-1α or that in the presence of other compound besides of the high-fat alone, this relation may not be so direct.

Insulin-degrading enzyme (IDE) is involved in insulin degradation, but little is known about the factors that influence and control its expression besides its effects on glycemic control [33,34]. Obesity and cafeteria diet showed to decrease IDE expression in skeletal muscle [35]. Reduction in IDE expression caused by HFF was not found in our study, whereas chia seed and chia oil both short treatments increased IDE expression. Otherwise, whether increasing or decreasing IDE expression contributes or not to glucose intolerance is yet undefined [35]. Studies show that knockout rats for IDE exhibit glucose intolerance and hyperinsulinemia [36]. If inhibition of IDE could increase half-life and potentiate the action of insulin in the bloodstream promoting hypoglycemic, on the other hand, the reduction of IDE is found in glucose intolerance and hyperinsulinemia conditions, which characterizes a diabetic phenotype [33]. If we consider the studies that show that obesity and high-fat diet promote reduction in IDE expression, we could suggest that restoring its expression could be beneficial. However we did not observe improvement of the hyperinsulinemia condition from chia seed or chia oil consumption in our study.

AMP-activated protein kinase (AMPK) is an energy deprivation sensor that plays a key role in regulating energy balance. In our results all HFF groups reduced AMPK expression in comparison with control, consistent to the data that show that high-fat diet reduces AMPK [37].

Dihydrofolate reductase (DHFR) is an enzyme that catalyzes the formation of cofactors to

key reactions, such as aminoacid biosysthesis and nucleotides. HSP70 and DHFR connection prevent oxidation of thiol group, protecting DHFR from oxidative injury thus preserving its functions [38]. However, no alteration in DHFR expression or thiol content was found.

The reason why chia oil induced and restored a higher number of proteins expression analyzed compared to chia seeds, remains unclear.

In conclusion, data indicate that: 1) Chia oil induced the increase in HSP70 and HSP25 expression, 2) Chia seed and chia oil improved glucose and insulin tolerance, but did not reduce hyperinsulinemia, 3) HFF diet reduced SOD and GPx expression, 4) Chia oil restored antioxidant system expression, 4) Chia seed long and chia oil short treatments restored PGC-1α expression reduced by HFF diet, 5) IDE expression was increased in chia seed and chia oil both short treatments, 6) Consumption of chia seed or chia oil did not reduce body weight gain or abdominal fat weight, 7) Except for chia seed short treatment, chia seed long treatment and chia oil reduced AST and ALT parameters. The present study contributes with new properties and molecular mechanisms associated with the consumption of chia seed and chia oil in HFF-induced obese rats.

Acknowledgments

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References

[1] Sparks LM, Xie H, Koza RA *et al.* (2005) A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. *Diabetes* **54**, 1926-1933.

[2] Samuel VT (2011) Fructose induced lipogenesis: from sugar to fat to insulin resistance. *Trends Endocrinol Metab* **22**, 60-65.

[3] Chung J, Nguyen Anh-Khoi, Henstridge DC *et al.* (2008) HSP72 protects against obesity-induced insulin resistance. *Proc Natl Acad Sci U S A* **105**, 1739–1744.

[4] De Moura CS, Lollo PCB, Morato PN *et al.* (2013) Whey protein hydrolysate enhances the

exercise-induced heat shock protein (HSP70) response in rats. *Food Chem* **136,** 1350–1357.

[5] Gupte AA, Bomhoff GL, Morris, JK *et al.* (2009) Lipoic acid increases heat shock protein expression and inhibits stress kinase activation to improve insulin signaling in skeletal muscle from high-fat-fed rats. *J Appl Physiol* **106**, 1425–1434.

[6] Gupte AA, Bomhoff GL, Swerdlow RH *et al.* (2009) Heat treatment improves glucose tolerance and prevents skeletal muscle insulin resistance in rats fed a high-fat diet. *Diabetes* **58**, 567-578.

[7] Ayerza R (1995) Oil content and fatty acid composition of chia (Salvia hispanica L.), from five northeastern locations in northwestern Argentina. *J Am Oil Chem Soc* **72**, 1079-1081.

[8] Reyes-Caudillo E, Tecante A & Valdivia-López MA (2008) Dietary fibre content and antioxidant activity of phenolic compounds present in Mexican chia (Salvia hispanica L.) seeds. *Food Chem* **107**, 656–663.

[9] Marineli RS, Moraes EA, Lenquiste SA *et al.* (2014) Chemical characterization and antioxidant potential of Chilean chia seeds and oil (*Salvia hispanica L.*). *LWT- Food Sci Technol* **59**, 1304-1310.

[10] Albert CM, Oh K, Whang W *et al.* (2005) Dietary alpha-linolenic acid intake and risk of sudden cardiac death and coronary heart disease. *Circulation* **112**, 3232–3238.

[11] Vuksan V, Whitham D, Sievenpiper JL *et al.* (2007) Supplementation of conventional therapy with the novel grain Salba (Salvia hispanica L.) improves major and emerging cardiovascular risk factors in type 2 diabetes: results of a randomized controlled trial. *Diabetes Care* **30**, 2804–10.

[12] Vuksan V, Jenkins AL, Dias AG *et al.* (2010) Reduction in postprandial glucose excursion and prolongation of satiety: possible explanation of the long-term effects of whole grain Salba (Salvia Hispanica L.). *Eur J Clin Nutr* **64**, 436–438.

[13] Chicco AG, D'alessandro ME, Hein GJ *et al.* (2009) Dietary chia seed (Salvia hispanica L.) rich in alpha-linolenic acid improves adiposity and normalises hypertriacylglycerolaemia and insulin resistance in dyslipaemic rats. *Br J Nutr* **101**, 41–50.

[14] Reeves PG, Nielsen FH & Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**, 1939-1951.

[15] Shapiro A, Tumer N, Gao Y *et al.* (2011) Prevention and reversal of diet-induced leptin resistance with a sugar-free diet despite high fat content. *Br J Nutr* **106**, 390-397.

[16] Bonora E, Manicardi V, Zavaroni I *et al.* (1987) Relationships between insulin secretion, insulin metabolism and insulin resistance glucose intolerance. *Diabete Metab* **13**, 116–121.

[17] Lowry OH, Rosebrough NJ, Farr AL *et al.* (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.

[18] Zhang L, Perdomo G, Kim DH *et al.* (2008) Proteomic analysis of fructose-induced fatty liver in hamsters. *Metabolism* **57**, 1115–1124.

[19] Henriksen EJ, Diamond-Stanic MK & Marchionne EM (2011) Oxidative stress and the

etiology of insulin resistance and type 2 diabetes. *Free Radic Biol Med* **51**, 993–999.

[20] Boudina S, Sena S, Sloan C *et al.* (2012) Early mitochondrial adaptations in skeletal muscle to diet-induced obesity are strain dependent and determine oxidative stress and energy expenditure but not insulin sensitivity. *Endocrinology* **153**, 2677–2688.

[21] Styskal JL, Remmen HV, Richardson A *et al.* (2012) Oxidative stress and diabetes: What can we learn about insulin resistance from antioxidant mutant mouse models? *Free Radic Biol Med* **52**, 46–58.

[22] Cui J, Xiao Y, Shi Yong-Hui *et al.* (2012) Lipoic acid attenuates high-fat-diet-induced oxidative stress and B-cell–related immune depression. *Nutrition* **28**, 275–280.

[23] Kohen R, Nyska A. (2002) Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol* **30**, 620–650.

[24] Meng R, Zhu Da-Long, Bi Y *et al.* (2011) Anti-oxidative effect of apocynin on insulin resistance in high-fat diet mice. *Ann Clin Lab Sci* **41**, 236-243.

[25] Rindler PM, Plafker SM, Szweda LI *et al.* (2013) High dietary fat selectively increases catalase expression within cardiac mitochondria. *J Biol Chem* **288**, 1979-1990.

[26] Feillet-Coudray C, Sutra T, Fouret G *et al.* (2009) Oxidative stress in rats fed a high-fat high-sucrose diet and preventive effect of polyphenols: Involvement of mitochondrial and NAD(P)H oxidase systems. *Free Radic Biol Med* **46**, 624–632.

[27] Lowell BB & Shulman GI (2005) Mitochondrial dysfunction and type 2 diabetes. *Science* **307**, 384-387.

[28] Soyal S, Krempler F, Oberkofler H *et al.* (2006) PGC-1α: a potent transcriptional cofactor involved in the pathogenesis of type 2 diabetes. *Diabetologia* **49**, 1477–1488.

[29] Hancock CR, Han Dong-Ho, Chen M *et al.* (2008) High-fat diets cause insulin resistance despite an increase in muscle mitochondria. *Proc Natl Acad Sci U S A* **105**, 7815–7820.

[30] Puigserver P & Spiegelman BM (2003) Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α): transcriptional coactivator and metabolic regulator. *Endocr Rev* **24**, 78–90.

[31] Crunkhorn S, Dearie F, Mantzoros C *et al.* (2007) Peroxisome proliferator activator receptor γ coactivator-1 expression is reduced in obesity: potential pathogenic role of saturated fatty acids and p38 mitogen-activated protein kinase activation. *J Biol Chem* **282**, 15439-15450.

[32] Civitarese AE & Ravussin E (2008) Minireview: Mitochondrial energetics and insulin resistance. *Endocrinology* **149**, 950–954.

[33] Abdul-Hay SO, Kang D, McBride M *et al.* (2011) Deletion of insulin-degrading enzyme elicits antipodal, age-dependent effects on glucose and insulin tolerance. *Plos one* **6**, 1-6.

[34] Hamel FG, Upward JL & Bennett R (2003) *In vitro* inhibition of insulin-degrading enzyme by long-chain fatty acids and their coenzyme A thioesters. *Endocrinology* **144**, 2404–2408.

[35] Brandimarti P, Costa-Júnior JM, Ferreira SM *et al.* (2013) Cafeteria diet inhibits insulin clearance by reduced insulin-degrading enzyme expression and mRNA splicing. *J Endocrinol* **219**, 173-182.

[36] Castell-Auví A, Cedó L, Pallarès V *et al.* (2012) The effects of a cafeteria diet on insulin production and clearance in rats. *Br J Nutr* **108**, 1155–1162.

[37] Lindholm CR, Ertel RL, Bauwens JD *et al.* (2013) A high-fat diet decreases AMPK activity in multiple tissues in the absence of hyperglycemia or systemic inflammation in rats. *J Physiol Biochem* **69**, 165–175.

[38] Musch MW, Kapil A & Chang EB (2004) Heat shock protein 72 binds and protects dihydrofolate reductase against oxidative injury. *Biochem Biophys Res Commun* **313**, 185–192.

[39] Maia EL & Rodriguez-Amaya DB (1993) Evaluation of a simple and inexpensive method for the methylation of fatty acid with lipids of various fish species. *Rev Inst Adolfo Lutz* **53**, 27–35.

[40] Kramer JKG, Fellner V, Dugan MER *et al.* (1997) Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. *Lipids* **32**, 1219–1228.

Table 1. Diets composition

Ingredients (g/kg diet)	Control	HFF	Chia seed	Chia oil
Casein (HL12034) [a]	139.53	139.53	106.53	139.53
Maltodextrin	155.0	45.40	45.40	45.40
Corn starch	465.69	136.44	136.44	136.44
Sucrose	100.0	29.32	29.32	29.32
Soybean oil	40	40	-	-
Chia oil	-	-	-	40
Chia seed	-	-	133	-
Lard	-	310	310	310
Fructose	-	200	200	200
Cellulose	50	50	3.6	50
Mineral mixture	35	35	35	35
Vitamin mixture	10	10	10	10
L-Cystine	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5
tert-Butyl hydroquinone	0.008	0.008	0.008	0.008
Energy density (kJ/g diet)	15.73	24.64	24.35	24.56
Fatty acids [b]				
Linoleic (C18:2n-6)	19.77	66.50	53.87	52.78
α-Linolenic (C18:3n-3)	2.19	4.03	25.90	25.87
n-6/n-3 ratio	9.03	16.50	2.08	2.04

Control, AIN-93M group; HFF, high-fat and high-fructose group; Chia seed groups, HFF with 13.3% (w/w) chia seed; Chia oil groups, HFF with 4% (w/w) chia oil.

[a] Amount was calculated based on protein content equal to 86% to provide 12 g of protein/100 g of diet.

[b] Fatty acids expressed in g/kg diet and determined by gas chromatography [39,40] using a capillary GC Agilent 6850 Series GC System, equipped with DB-23 capillary column Agilent (50% cyanopropyl-methylpolysiloxane, 60 m x 0.25 mm×0.25 μm), with flame ionization detector (FID).

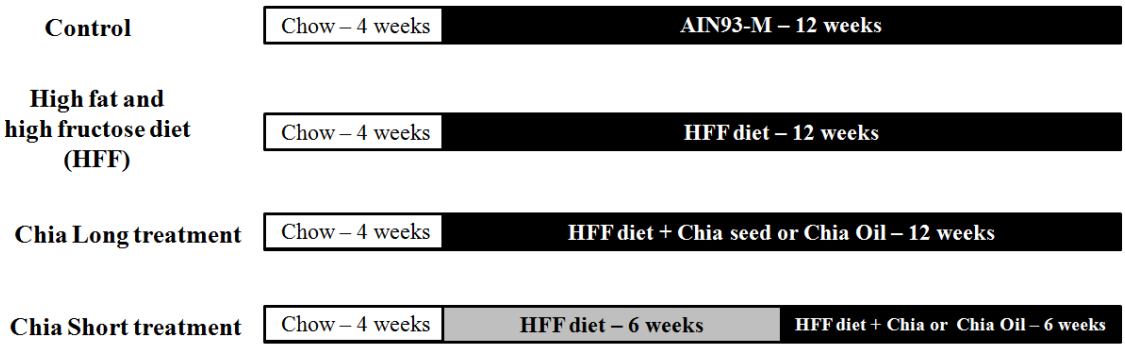

Figure 1. Experimental design. The animals were maintained in chow diet during 4 weeks for growth. After 4 weeks they were divided in six groups (n=8/group): Control (lean control) received a standard diet; High-fat and high-fructose group (HFF containing 4% (w/w) soybean oil, 31% (w/w) lard and 20% fructose (w/w); chia seed long and short treatments received HFF diet with chia seed 13.3% (w/w); chia oil long and short treatments received HFF with chia oil 4% (w/w). Animals from long treatment were fed with HFF containing chia seed or chia oil for 12 weeks. Short groups were initially fed only with HFF diet for 6 weeks, followed by 6 more weeks with HFF containing chia seed or chia oil. All diets were based on the AIN-93M diet.

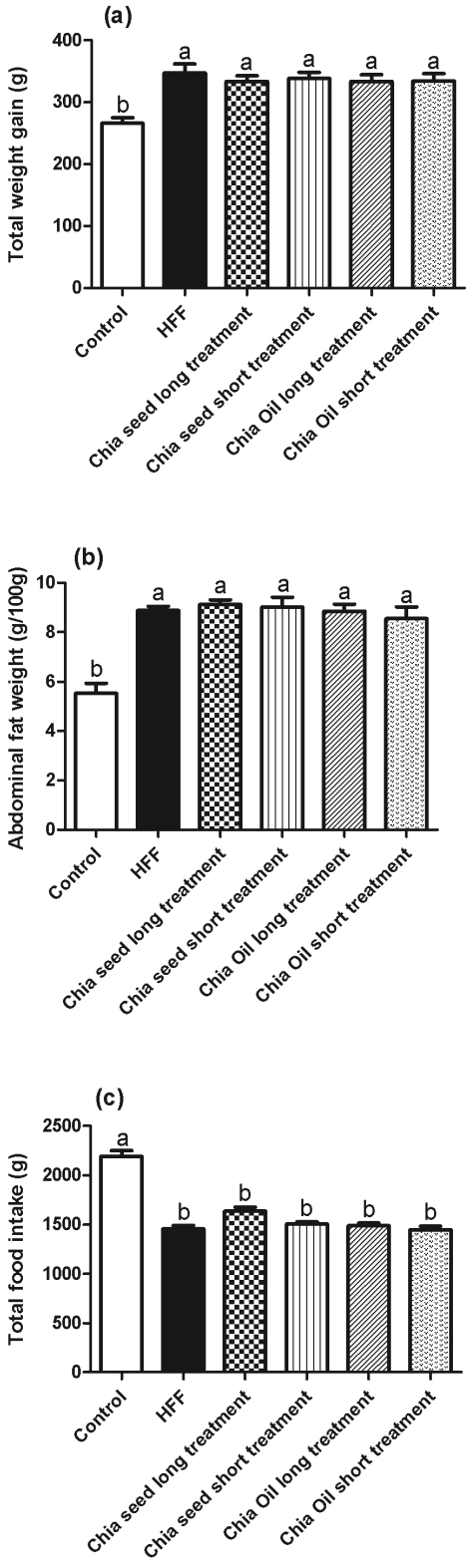

Figure 2. Mean and standard error for: (a) total weight gain, (b) abdominal fat weight, (c) total food intake. Treatments: long (12 weeks) and short (6 weeks) with chia seed or chia oil. Different letters represent significant differences ($p < 0.05$).

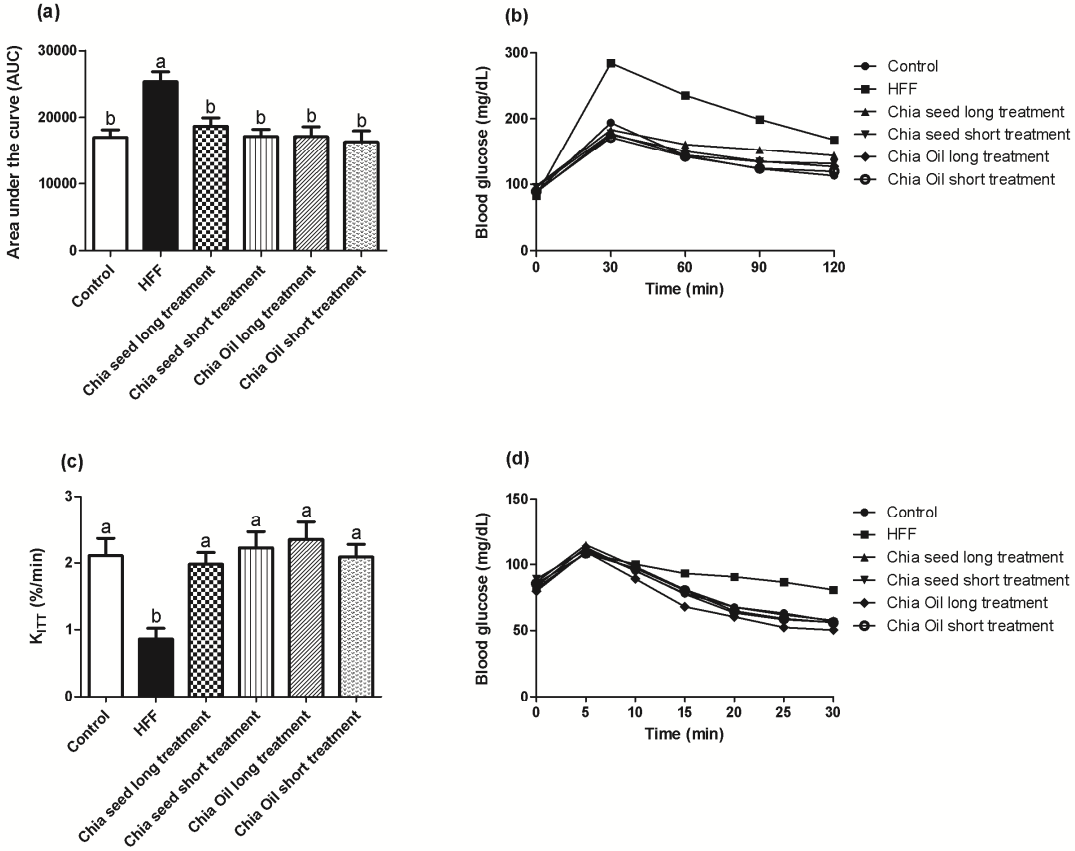

Figure 3. Mean and standard error for Glucose Tolerance Test (iGTT) and Insulin Tolerance Test (ITT) (n=8 per group): (a) Glucose AUC during intraperitoneal glucose tolerance test ($iGTT_{AUC}$), (b) Mean blood glucose levels after intraperitoneal infusion of glucose solution, (c) K_{ITT} during insulin tolerance test, (d) Mean blood glucose levels after intraperitoneal infusion of insulin. Treatments: long (12 weeks) and short (6 weeks) with chia seed or chia oil. Different letters represent significant differences ($p < 0.05$).

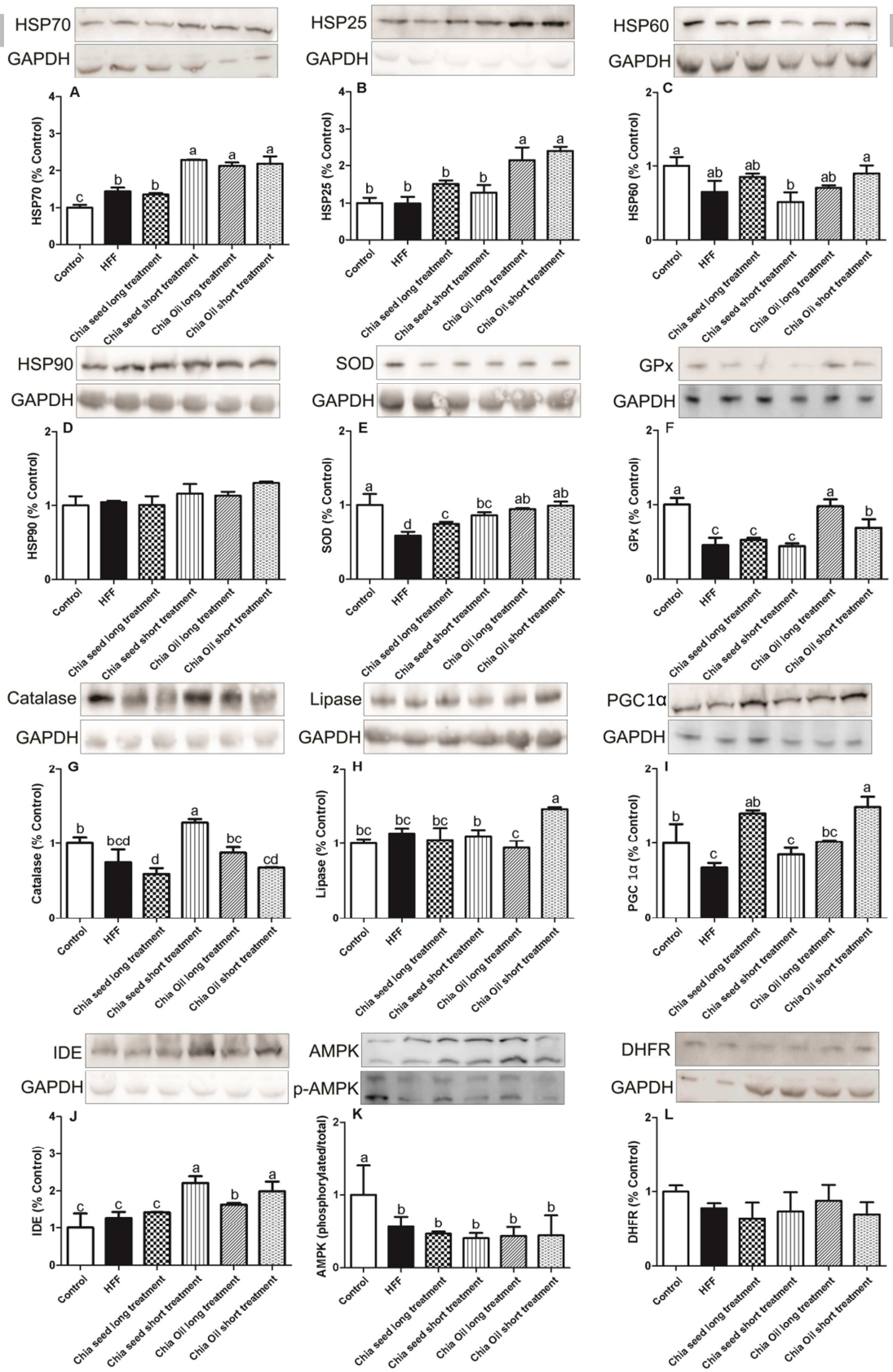

Figure 4. The data were obtained from soleus skeletal muscle (n=8 per group). Mean and standard error for the Western blot analysis of: (a) HSP70, (b) HSP25, (c) HSP60, (d) HSP90, (e) SOD, (f) GPx, (g) Catalase, (h) Lipase, (i) PGC-1α, (j) IDE, (k) AMPK, (l) DHFR. All values were reported as % Control. Treatments: long (12 weeks) and short (6 weeks) with chia seed or chia oil. Different letters represent significant differences ($p < 0.05$).

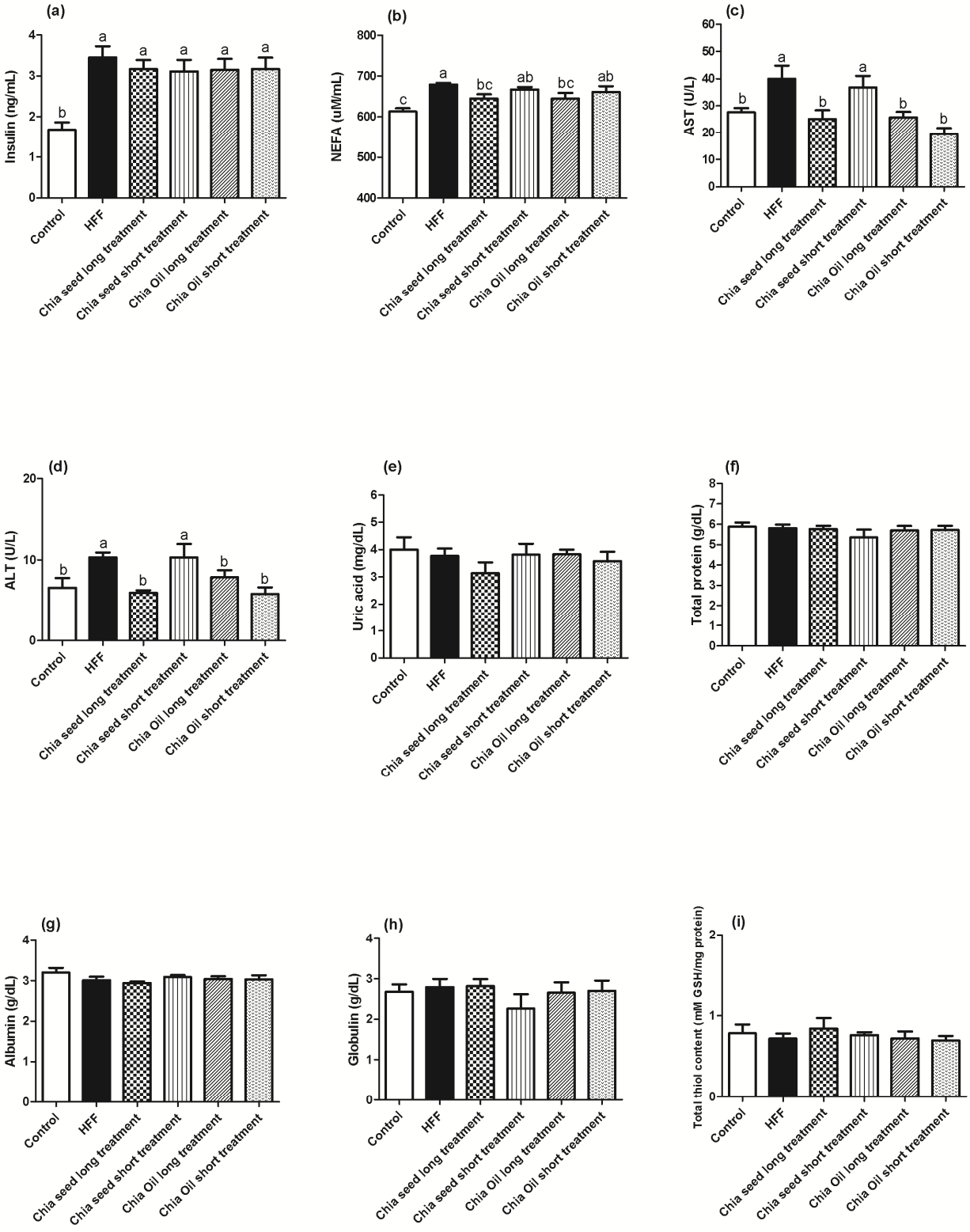

Figure 5. Mean and standard error for blood analysis (n=8 per group): (a) Insulin (fasting), (b) NEFA, (c) AST, (d) ALT, (e) Uric acid, (f) Total protein, (g) Albumin, (h) Globulin, (i) Total thiol content. Treatments: long (12 weeks) and short (6 weeks) with chia seed or chia oil. Different letters represent significant differences ($p < 0.05$).

Highlights

Chia seed and chia oil improve glucose and insulin tolerance in obese *Wistar* rats.

Chia oil induces HSP70 and HSP25 expression in skeletal muscle.

Chia oil restores antioxidant system expression changed by HFF diet.

Chia seed and chia oil restore PGC-1α expression reduced by HFF diet.

Chia seed and chia oil increase expression of tissue protection proteins.