

Mice fed fish oil diet and upregulation of brown adipose tissue thermogenic markers

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Received: 6 November 2014 / Accepted: 8 January 2015 / Published online: 23 January 2015
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Abstract

Purpose Fish oil (FO) elicits diverse beneficial effects. Reduction in or prevention of body mass (BM) gain in animal models may be associated with modulation of brown adipose tissue (BAT). We aimed to evaluate the effects of different high-fat diets with FO on BAT metabolism and thermogenic markers.

Methods C57BL/6 male mice (3-month-old) were fed different diets during 8 weeks: standard-chow diet (SC 10 % fat), high-fat lard diet (HF-L 50 % fat), high-fat lard plus FO diet (HF-L+FO 50 % fat), and high-fat FO diet (HF-FO 50 % fat). We evaluated BM and performed an oral glucose tolerance test. At euthanasia, plasma was collected for leptin, and triacylglycerol measurement and interscapular BAT was dissected and stored for molecular analyses.

Results HF-L group showed elevated BM; glucose intolerance associated with diminished TC10 and GLUT4 expressions; hypertriglyceridemia associated with increased CD36 and diminished CPT1 expression; elevated expression of pro-inflammatory cytokines; and reduced PPAR expression. Furthermore, these animals showed hyperleptinemia with increased expression of thermogenic markers (beta3-AR, PGC1alpha, and UCP1). Conversely,

HF-L+FO and HF-FO groups showed reduced BM gain with regularization of glucose tolerance and triglyceridemia, GLUT4, TC10, CD36, CPT1, and cytokines expressions. Both groups exhibited elevated PPAR and thermogenic markers expression in a dose-dependent way.

Conclusions FO improves metabolic profile and upregulates thermogenic markers, suggesting an elevated thermogenesis that leads to reduced BM gain.

Keywords Omega-3 fatty acids · Fish oil · Thermogenesis · Brown adipose tissue · Uncoupling protein 1

Introduction

Fish oil (FO) is rich in n-3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. First described to target hypertriglyceridemia, FO is now recognized with beneficial effects on cardiovascular and inflammatory diseases, among others. One of the mechanisms by which FO exerts its positive impact is through activation of the peroxisome proliferator-activated receptors (PPAR), transcription factors involved in gene expression regulation [1]. Several animal studies proved that the FO (or its components) can prevent or reduce weight gain [2–4]. Moreover, some studies have already confirmed that FO (i.e., n-3 PUFA) stimulates thermogenesis in brown adipose tissue (BAT), although the mechanisms are still to be elucidated [5, 6].

BAT can generate heat through uncoupling mitochondrial fuel oxidation from ATP production, characterizing non-shivering thermogenesis [7]. This ability of BAT is conferred by the brown fat-specific uncoupling protein 1 (UCP1) and is controlled by the *beta3* adrenergic receptor

Electronic supplementary material The online version of this article (doi:10.1007/s00394-015-0834-0) contains supplementary material, which is available to authorized users.

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(beta3-AR) pathway [8]. BAT activation can be achieved by cold or overfeeding (diet-induced thermogenesis). In relation to diet-induced thermogenesis, diet composition, more specifically fat composition, can chronically modulate BAT thermogenesis [9].

Recent evidences, which human adult BAT can be activated and can have a role in the metabolism rekindle studies in this area [10]. Therefore, we have the aim of studying the effects of different high-fat diets containing FO on BAT metabolism and thermogenesis.

Materials and methods

Animals and diet

All procedures were approved by the local ethics committee for animal experimentation (protocol number CEUA/018/2013) in accordance with the conventional guidelines for animal experimentation (NIH publication number 85-23, revised 1996). The animals were maintained under controlled conditions (20 ± 2 °C and 12 h/12 h dark/light cycle) with free access to food and water.

Forty C57Bl/6 male mice with 3 months of age were randomly assigned into four groups ($n = 10$ each group):

- standard-chow group (SC; 40 g soybean oil/Kg diet, 10 % of the total energy content from lipids);
- high-fat lard group (HF-L; 40 g soybean oil and 238 g lard/Kg diet, 50 % of the total energy content from lipids);
- high-fat lard plus FO group (HF-L+FO; 40 g soybean oil, 119 g lard and 119 g FO/Kg diet, 50 % of the total energy content from lipids); and
- high-fat FO group (HF-FO; 40 g soybean oil and 238 g FO/Kg diet, 50 % of total energy content from lipids).

The diets were elaborated with purified nutrients by Pragsolucos (Jau, SP, Brazil) based on American Institute of Nutrition's recommendations (AIN 93M) [11]. Table 1 shows the diet composition (FO from menhaden, approximately 25 % of EPA and DHA). Diets were offered over an 8-week period.

Body mass and food intake

The food intake was checked daily, and body mass (BM) weekly. Energy intake was the product of food intake by the energy content of the diet.

Indirect calorimetry and food quotient

The respiratory quotient (RQ) and the energy expenditure were measured (Apparatus Oxylet system, Panlab/

Table 1 Composition and energy content of the diets (AIN 93M-based diets)

Ingredients (g/kg)	SC	HF-L	HF-L+FO	HF-FO
Casein (≥ 85 % of protein)	140.0	175.0	175.0	175.0
Cornstarch	620.692	347.692	347.692	347.692
Sucrose	100.0	100.0	100.0	100.0
Soybean oil	40.0	40.0	40.0	40.0
Lard	–	238.0	119.0	–
Fish oil	–	–	119.0	238.0
Fiber	50.0	50.0	50.0	50.0
Vitamin mix ^a	10.0	10.0	10.0	10.0
Mineral mix ^a	35.0	35.0	35.0	35.0
L-Cystin	1.8	1.8	1.8	1.8
Choline	2.5	2.5	2.5	2.5
Antioxidant	0.008	0.060	0.060	0.060
Total mass	1,000.0	1,000.0	1,000.0	1,000.0
Proteins (% energy)	14	14	14	14
Carbohydrates (% energy)	76	36	36	36
Lipids (% energy)	10	50	50	50
Energy content (kcal/kg)	3,811	5,000	5,000	5,000

SC Standard-chow diet, HF-L high-fat lard diet, HF-L+FO high-fat lard plus fish oil diet, HF-FO high-fat fish oil diet

^a Mineral and vitamin mixtures are in accordance with AIN 93M

Harvard, Barcelona, Spain) at the end of the experiment. The monitoring system evaluated the respiratory metabolism by measuring the oxygen consumption (VO_2) and the carbon dioxide production (VCO_2). Data were collected during 48 h and averaged over the entire testing period.

The food quotient (FQ) was calculated using the following equation: $(0.835 * \%protein) + (1.000 * \%carbohydrate) + (0.710 * \%fat)$ [12].

Blood analyses

The oral glucose tolerance test (OGTT) was achieved at the conclusion of the experiment using a 25 % glucose solution (1.0 g/kg) administered by orogastric gavage after a 6-h fasting period. Blood was obtained from the tail vein. Glycemia was measured before glucose administration (0 min) and 15, 30, 60, and 120 min after glucose administration (glucometer Accu-Check, Roche, SP, Brazil). The results are presented as the 'area under the curve' (AUC; arbitrary units, a.u.), calculated using the trapezoidal rule (GraphPad Prism version 6.05, La Jolla, CA, USA).

At killing, animals were deprived of food for 6 h and were intraperitoneally anesthetized with 150 mg/kg sodium pentobarbital. Blood samples were obtained, and the serum was separated by centrifugation (120 g, 15 min) and stored

individually at -80°C . Leptin was measured using the SinglePlex kit EZML-82K (Millipore, Billerica, MA, USA). The triacylglycerol was performed using an automated spectrophotometer (Bioclin System II, Quibasa, MG, Brazil).

Adiposity index

We dissected and weighed the following fat pads: the inguinal fat pad (subcutaneous fat located between the lower part of the rib cage and the mid-thigh), the retroperitoneal fat pad (connected to the posterior abdominal wall near the kidneys), and the epididymal fat pad (located in the lower part of the abdomen and connected to the epididymis). The adiposity index was determined as the ratio between the sum of the fat masses (epididymal, retroperitoneal, and inguinal) divided by the total BM, as a percentage [13].

Brown adipose tissue

The interscapular brown adipose tissue (iBAT) was dissected, and samples were rapidly frozen and stored at -80°C . Alternatively, the samples were kept in freshly prepared fixative solution (4 % formaldehyde w/v, 0.1 M phosphate buffer, pH 7.2).

RT-qPCR

Total RNA of iBAT was extracted using Trizol reagent (Invitrogen, CA, USA). Nanovue spectroscopy (GE Life Sciences) was used to determine RNA amount. Then, 1 μg RNA was treated with DNase I (Invitrogen, CA, USA). Afterward, oligo (dT) primers for mRNA and Superscript III reverse transcriptase (both from Invitrogen, CA, USA) were applied to the synthesis of first-strand cDNA. Quantitative real-time PCR used a Biorad CFX96 cycler and the SYBR Green mix (Invitrogen, CA, USA). The primers used are described in Table S1: beta3-AR; PPAR γ coactivator 1 α , PGC1 α ; UCP1; PPAR α ; PPAR β/δ ; PPAR γ ; cluster of differentiation 36, CD36; carnitine palmitoyltransferase-1, CPT-1; interleukin-6 (IL-6); and tumor necrosis factor α (TNF α). The endogenous beta-actin was used to normalize the expression of the selected genes. After the pre-denaturation and polymerase activation program (4 min at 95°C), 44 cycles of 95°C for 10 s and 60°C for 15 s were followed by a melting curve program (60°C to 95°C with a heating rate of $0.1^{\circ}\text{C}/\text{s}$). Negative controls consisted of wells in which the cDNA was substituted for deionized water. The relative expression ratio of the mRNA was calculated using the Eq. $2^{-\Delta\Delta\text{Ct}}$, in which $-\Delta\text{CT}$ represents the ratio between the quantity

of cycles (CT) of the target genes with the endogenous control.

Western blotting

Total proteins of iBAT were extracted in homogenizing buffer containing protease inhibitors. Equivalent quantities of total protein resuspended in SDS-containing sample buffer were heated for 5 min at 100°C and separated by SDS-PAGE. After electrophoresis, the proteins were electroblotted onto polyvinyl difluoride transfer membranes (Amersham Biosciences, Piscataway, N.J., USA). The blockade of the membrane was made with nonfat dry milk. Homogenates were incubated with the primary antibodies: beta3-AR (44 kDa; SC-1473; Santa Cruz Biotechnology); PGC1 α (91 kDa; SC-5816; Santa Cruz Biotechnology); UCP1 (33 kDa; SC-6529; Santa Cruz Biotechnology); PPAR α (52 kDa; SC-9000; Santa Cruz Biotechnology); PPAR γ (67 kDa; SC-7273; Santa Cruz Biotechnology); TC10 (24 kDa; T 8950; Sigma); and glucose transporter type 4 (GLUT-4, 55 kDa; 07-1404; Millipore). Beta-actin (SC81178; Santa Cruz Biotechnology) served as a loading control. We used ECL for protein expression detection system and the Bio-Rad Molecular Imaging ChemiDoc XRS Systems (Bio-Rad, Hercules, CA, USA). The chemiluminescence intensity of the bands was quantified using the ImageJ software, version 1.48 (NIH, imagej.nih.gov/ij, USA). The integral absorbance values were measured, and the results are shown as percentages of the SC group.

Immunofluorescence

For UCP-1 immunofluorescence, tissue sections (5 μm thick) were submitted to citrate buffer, pH 6.0, at 60°C for 20 min for antigen retrieval, glycine 2 %, and blocking buffer (PBS/5 % BSA). The iBAT sections were incubated overnight at 4°C with anti-UCP1 antibody (SC-6529; Santa Cruz Biotechnology), diluted 1:50 in PBS/1 % BSA, followed by incubation for 1 h at room temperature with fluorochrome-conjugated secondary antibody anti-goat IgG-Alexa 488 (Invitrogen, Molecular Probes, Carlsbad, CA, USA), diluted 1:50 in PBS/1 % BSA. After rinsing in PBS, the slides were mounted with SlowFade Antifade (Invitrogen, Molecular Probes, Carlsbad, CA, USA). Digital immunofluorescence images were captured using confocal microscopy (Nikon Confocal Laser Scanning Microscopy—Model C2; Nikon Instruments, Inc., New York, USA).

Data analysis

Values are shown as the mean \pm standard deviation (SD). After confirmation of the homogeneity of variances, the

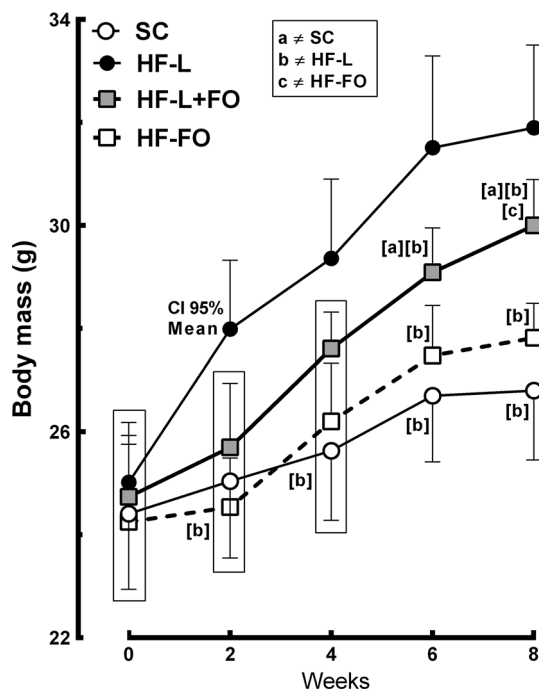


Fig. 1 Body mass evolution. Values are the mean \pm SD, $n = 5$ per group. Significant differences are indicated ($P < 0.05$), as determined by one-way ANOVA and Holm–Sidak post hoc test. Groups are standard-chow group (SC), high-fat lard group (HF-L), high-fat lard plus FO group (HF-L+FO), and high-fat FO group (HF-FO)

differences among the groups were tested using a one-way ANOVA followed by the Holm–Sidak post hoc test. In all cases, a P value ≤ 0.05 was considered statistically

Table 2 Food behavior, energy expenditure, and plasma analyses

Data	Groups			
	SC	HF-L	HF-L+FO	HF-FO
<i>Food behavior</i>				
Food intake (g)	2.97 \pm 0.11	2.87 \pm 0.20	2.97 \pm 0.31	3.02 \pm 0.38
Energy intake (kcal)	11.31 \pm 0.41	14.28 \pm 0.99 ^a	14.87 \pm 1.55 ^a	15.11 \pm 1.92 ^a
RQ	0.95 \pm 0.04	0.86 \pm 0.05 ^a	0.85 \pm 0.04 ^a	0.83 \pm 0.03 ^a
FQ	0.95	0.83	0.83	0.83
<i>Adiposity</i>				
Adiposity index (%)	1.97 \pm 0.07	3.34 \pm 0.73 ^a	2.69 \pm 0.60 ^{a,b}	1.43 \pm 0.22 ^{b,c}
<i>Energy expenditure</i>				
EE (kcal/day/kg ^{0.75})	195.70 \pm 1.48	176.20 \pm 8.95 ^a	197.30 \pm 4.20 ^b	207.50 \pm 2.58 ^{a,b,c}
<i>Plasma analyses</i>				
OGTT (AUC, a.u.)	17,832.00 \pm 387.50	23,191.00 \pm 25,780.00 ^a	17,599.00 \pm 1,996.00 ^b	18,073.00 \pm 1,379.00 ^b
Leptin (pg/mL)	1,063.00 \pm 355.80	18,357.00 \pm 2,281.00 ^a	2,352.00 \pm 2,663.00 ^b	2,474.00 \pm 1,608.00 ^b
Triacylglycerol (mg/dL)	71.50 \pm 3.60	79.21 \pm 3.69 ^a	72.22 \pm 3.96 ^b	68.22 \pm 3.27 ^b

Values are the mean \pm SD; $n = 5$ per group

SC standard-chow group, HF-L high-fat lard group, HF-L+FO high-fat lard plus fish oil group, HF-FO high-fat fish oil group, RQ respiratory quotient, FQ food quotient, EE energy expenditure, OGTT oral glucose tolerance test, AUC area under the curve, a.u. arbitrary units

In the signaled cases, $P \leq 0.05$ compared with the ^a SC group, ^b HF-L group, ^c HF-L+FO group (one-way ANOVA and Holm–Sidak post hoc test)

significant (GraphPad Prism version 6.05, La Jolla, CA, USA).

Results

BM and food intake

All animals started the experiment with similar BM. After 2 weeks of the diet, the HF-L group was heavier than the other groups (+12 %, $P = 0.006$, compared to the SC group). The difference remained until the end of the experiment (+19 %, $P < 0.0001$, compared to the SC group). The HF-L+FO group had higher BM than both the SC group (+12 %, $P = 0.0003$) and the HF-FO group (+8 %, $P = 0.008$), but lower BM than the HF-L group (–6 %, $P = 0.02$). The HF-FO group stayed similar to the SC group (Fig. 1).

The four groups showed similar food intake (Table 2). However, because the HF groups ingested a hyperenergetic diet, these animals had an elevated energy intake when compared with the SC group ($P < 0.05$). There were no differences between the HF groups.

Indirect calorimetry and food quotient (Table 2)

The HF groups showed reduced RQ, when compared with the SC group: –9 %, $P = 0.03$ for the HF-L group; –10 %, $P = 0.02$, for the HF-L+FO group; and –12 %, $P = 0.01$, for the HF-FO group. There were no differences between these three groups.

The calculated FQ was 0.95 for the SC diet and 0.83 for the HF diets.

In relation to the energy expenditure, animals from the HF-L group showed reduced values in comparison with the SC group (-10% , $P = 0.0005$). On the opposite way, the HF-L+FO group showed similar values to the SC group, and the HF-FO group demonstrated elevated values in comparison with the SC group ($+6\%$, $P = 0.02$). The HF-L+FO and the HF-FO groups showed elevated values when compared to the HF group ($+12\%$, $P = 0.0002$; $+18\%$, $P < 0.0001$; respectively). Lastly, the HF-FO group was elevated in comparison with the HF-L+FO group ($+5\%$, $P = 0.02$).

Blood analyses (Table 2)

The HF-L group was the only one to present an increase in the AUC from the OGTT test, being 30% higher than the SC group ($P < 0.0001$). Both HF-L+FO and HF-FO groups had similar AUC to the SC group and diminished AUC compared to the HF-L group (-24% , $P < 0.0001$; and -22% , $P < 0.0001$; respectively).

Plasma leptin was higher in the HF-L group than in the SC group ($P < 0.0001$), but standard in both FO groups (similar to the SC group). It is remarkable that the plasma leptin was diminished in FO groups in comparison with the HF-L group (-87% , $P < 0.0001$; -86% , $P < 0.0001$; respectively).

The triacylglycerol was found higher in the HF-L group ($+11\%$, $P = 0.0163$) and regularized in the HF-L+FO and the HF-FO groups, compared to the SC group. The HF-L+FO group was 9% lower ($P = 0.01$), and the HF-FO group was 14% lower ($P < 0.0001$) than the HF-L group.

Adiposity index

The HF-L group had higher adiposity index compared to the SC group ($+73\%$, $P < 0.0001$). As observed in BM, the adiposity index in the HF-L+FO group continued elevated in comparison with the SC group ($+39\%$, $P = 0.0186$), but diminished in comparison with the HF-L group (-19% , $P = 0.0276$). The HF-FO group had similar adiposity index to the SC group, as shown in Table 2.

RT-qPCR

Regarding beta3-AR, PGC1alpha, and UCP1 mRNA expressions, the HF-L group ($+19\%$, $P = 0.007$; $+50\%$, $P = 0.04$; $+49\%$, $P = 0.002$; respectively), the HF-L+FO group ($+33\%$, $P < 0.0001$; $+111\%$, $P = 0.001$; $+129\%$, $P < 0.0001$; respectively), and the HF-FO group ($+73\%$, $P < 0.0001$; $+359\%$, $P < 0.0001$; $+239\%$, $P < 0.0001$; respectively) were elevated in comparison with the SC

group. Besides that, the HF-FO+L was elevated in comparison with the HF-L group ($+12\%$, $P = 0.02$; $+41\%$, $P = 0.04$; $+53\%$, $P < 0.0001$; respectively), and the HF-FO group was also elevated in comparison with the HF-L ($+45\%$, $P < 0.0001$; $+206\%$, $P < 0.0001$; $+127\%$, $P < 0.0001$) and the HF-L+FO group ($+30\%$, $P < 0.0001$; $+118\%$, $P < 0.0001$; $+48\%$, $P < 0.0001$) for all the three analyses (Fig. 2).

PPARbeta/delta gene expression was reduced in the HF-L group, in comparison with the SC group (-76% , $P = 0.04$) (Fig. 3). In the groups fed FO, the PPARbeta/delta gene expression was dose dependent: HF-L+FO group was 66% higher for PPARalpha ($P = 0.04$), 82% higher for PPARbeta/delta ($P = 0.04$), and 109% higher for PPARgamma ($P = 0.01$), and the HF-FO was 413% higher for PPARalpha ($P < 0.0001$), 227% higher for PPARbeta/delta ($P < 0.0001$), and 284% higher for PPARgamma ($P < 0.0001$), in contrast to the SC group.

Gene expression of CD36 was elevated in the HF groups compared to the SC group (more than 70% high; $P = 0.03$ for all analyses, but no difference was observed among the HF groups). In addition, the gene expression of CPT-1 was reduced in the HF-L group (-28% , $P = 0.002$) and elevated in both the HF-L+FO group ($+28\%$, $P = 0.002$) and the HF-FO group ($+22\%$, $P = 0.006$), compared to the SC group (Fig. 4).

IL-6 and TNFalpha gene expressions were increased in the HF-L group ($P < 0.0001$, for both genes) as observed in Fig. 5. The HF-L+FO and HF-FO groups had similar gene expressions to the SC group, but reduced gene expressions than the HF-L group (IL-6 -65% and TNFalpha -93% for the HF-L+FO group; and IL-6 -60% and TNFalpha -95% , for the HF-FO group; $P < 0.0001$, for all analyses).

Western blotting

Beta3-AR, PGC1alpha, and UCP-1 protein expressions were slightly elevated in the HF-L group ($+30\%$, $P = 0.04$; $+8\%$, $P = 0.03$; $+27\%$, $P = 0.008$; respectively), compared to the SC group. In contrast, they were elevated in the HF-L+FO group ($+76\%$, $P = 0.0003$; $+15\%$, $P = 0.0008$; $+59\%$, $P < 0.0001$; respectively) and highly elevated in the HF-FO group ($+118\%$, $P < 0.0001$; $+26\%$, $P < 0.0001$; $+83\%$, $P < 0.0001$; respectively), compared to the SC group. Thermogenic protein responses to FO were dose dependent (Fig. 2).

Both the PPARalpha and PPARgamma showed reduced protein expressions in the HF-L group (-21% , $P = 0.04$; -16% , $P = 0.04$; respectively). These protein expressions were elevated in the other HF groups (Fig. 3). Interestingly, the response of PPARalpha and PPARgamma protein expressions in FO groups was dose dependent. Consequently, the HF-L+FO group showed a 24% higher

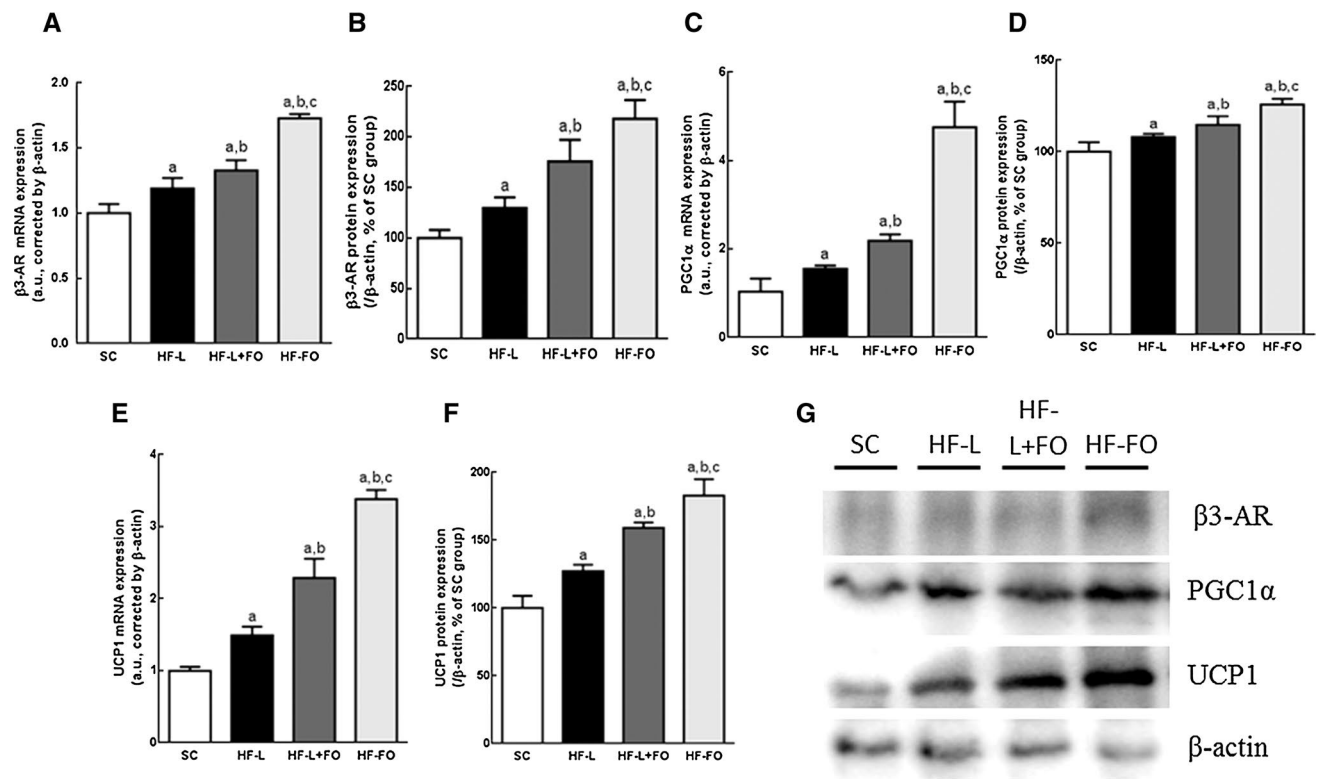


Fig. 2 Gene and protein expressions of beta3-AR, PGC1alpha, and UCP1 in iBAT. Values are the mean \pm SD, $n = 5$ per group. Significant differences are indicated ($P < 0.05$), one-way ANOVA and Holm–Sidak post hoc test: a \neq SC, b \neq HF-L, c \neq HF-L+FO.

PPARalpha expression ($P = 0.04$) and 29 % higher PPARgamma expression ($P = 0.002$) than the SC group. In addition, the HF-FO group showed a 47 % higher PPARalpha expression ($P = 0.0009$) and 100 % higher PPARgamma expression ($P < 0.0001$) than the SC group.

The HF-L group showed reduced TC10 and GLUT4 protein expressions than the SC group (-26 %, $P = 0.04$; -47 %, $P = 0.006$; respectively). Both the HF-L+FO and HF-FO groups showed higher protein expression of TC10 ($+38$ %, $P = 0.005$; $+44$ %, $P = 0.003$; respectively) than the HF-L group. The GLUT4 protein expression was similar comparing both the FO groups and the SC group (Fig. 4).

Immunofluorescence

The HF-L group showed a more intense UCP1 staining than the SC group. In addition, the FO groups showed more intense UCP1 staining than both the SC and HF-L groups (Fig. 6).

Discussion

We observed that the HF-FO diets could reduce/prevent weight gain and adiposity without affecting food intake

through stimulation of energy expenditure. The effects of FO on iBAT were associated with improvement in glucose intolerance and triacylglycerol levels, reduced gene expression of pro-inflammatory cytokines, and increased gene and protein expressions related to fatty acid/glucose uptake and oxidation in iBAT coupled with elevated protein and gene expressions of PPAR and thermogenic markers.

As expected, the HF-L group showed the greatest BM [13]. Interestingly, we observed that the ability of FO to prevent BM gain was dose dependent in agreement with previous studies. Rats fed a diet with 83 g/kg of n-3 PUFA had intermediate BM between high saturated fatty acids group and control group [4]. However, when 218 g/kg of FO was given to animals, there was a more reduced BM gain [2]. As illustrated by the adiposity index, this BM gain seen in the HF-L and the HF-L+FO groups was accounted for by increases in fat mass.

All the three HF groups showed similar food intake but elevated energy intake in comparison with the SC group due to high energy density of the HF diets [13]. N-3 PUFA does not alter the food intake as shown previously [14]. Therefore, the prevention of BM gain seen in the FO groups cannot be attributed to food intake changes rather than metabolic alterations.

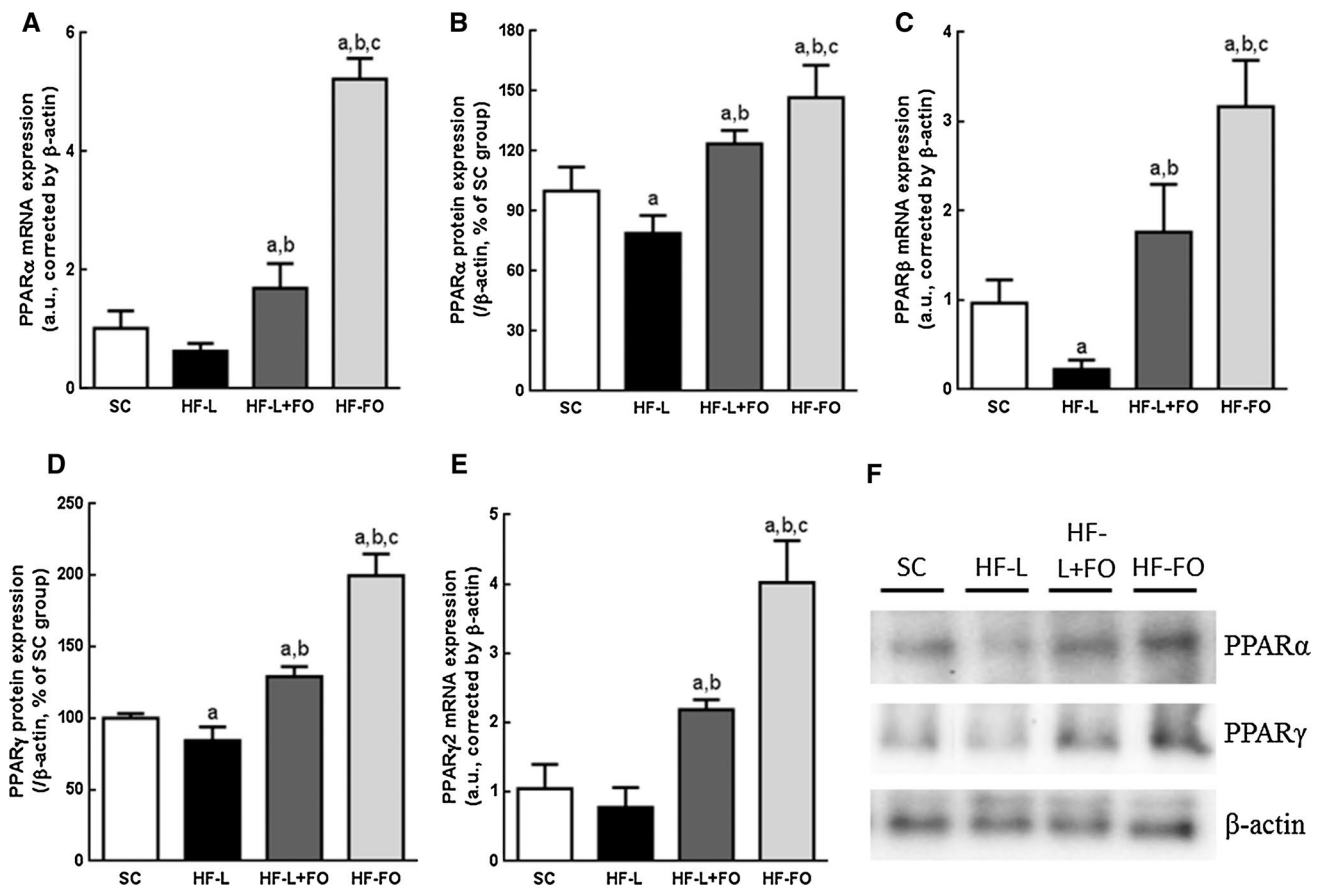


Fig. 3 Gene and protein expressions of PPAR in iBAT. Values are the mean \pm SD, $n = 5$ per group. Significant differences are indicated ($P < 0.05$), one-way ANOVA and Holm–Sidak post hoc test: $a \neq SC$,

$b \neq HF-L$, $c \neq HF-L+FO$. Groups are standard-chow group (SC), high-fat lard group (HF-L), high-fat lard plus FO group (HF-L+FO), and high-fat FO group (HF-FO)

RQ represents the fuel utilization and indicates unbalanced diets. In this way, the three HF diets were able to reduce it, representing increased fat oxidation. FQ, in comparison with RQ, is useful to predict energy homeostasis. A higher RQ than FQ means that the energy balance is positive. Therefore, energy intake is higher than energy expenditure leading to lipid accumulation and increased BM [15] as seen in the HF-L and the HF-L+FO. Mice fed a HF diet with lard showed slightly elevated RQ in comparison with FQ, indicating positive energy balance as seen in the present study [16].

When energy intake exceeds, energy expenditure (positive energy balance) obesity is established. In the present study, energy intake was similar among the three HF groups, but energy expenditure differed between the groups. HF-L group showed diminished energy expenditure compared to the SC group, justifying the positive energy balance and BM gain. On the other hand, the HF-FO group showed elevated energy expenditure compared with the SC group, which is indicative that the adaptive thermogenesis may be activated in these animals. Energy expenditure

comprises three main elements: obligatory energy expenditure (required for cells and organs normal functioning), physical activity, and adaptive thermogenesis. The thermogenesis is known to be induced by either cold or diet and, in rodents, is mostly developed by BAT [17].

Leptin is produced and secreted by adipocytes, being responsible for energy homeostasis. The more the adipose tissue increases; the more the leptin is secreted, leading to elevated plasma leptin levels in obese animals (as in the HF-L group) [13, 18]. Central actions of leptin include the activation of sympathetic nervous system (SNS), and although obese mice are leptin resistant in relation to appetite control, the same does not happen regarding thermogenic control. Obese mice with hyperleptinemia show increased stimulus to iBAT [18]. Elevated expression of thermogenic genes and proteins was also found in the present study (i.e., beta3-AR, PGC1alpha, and UCP1). Overfeeding is responsible for stimulating the SNS, which releases catecholamines that act via beta3-AR. This fact leads to a variety of intracellular changes resulting in the transcription of PGC1alpha, which is highly expressed in

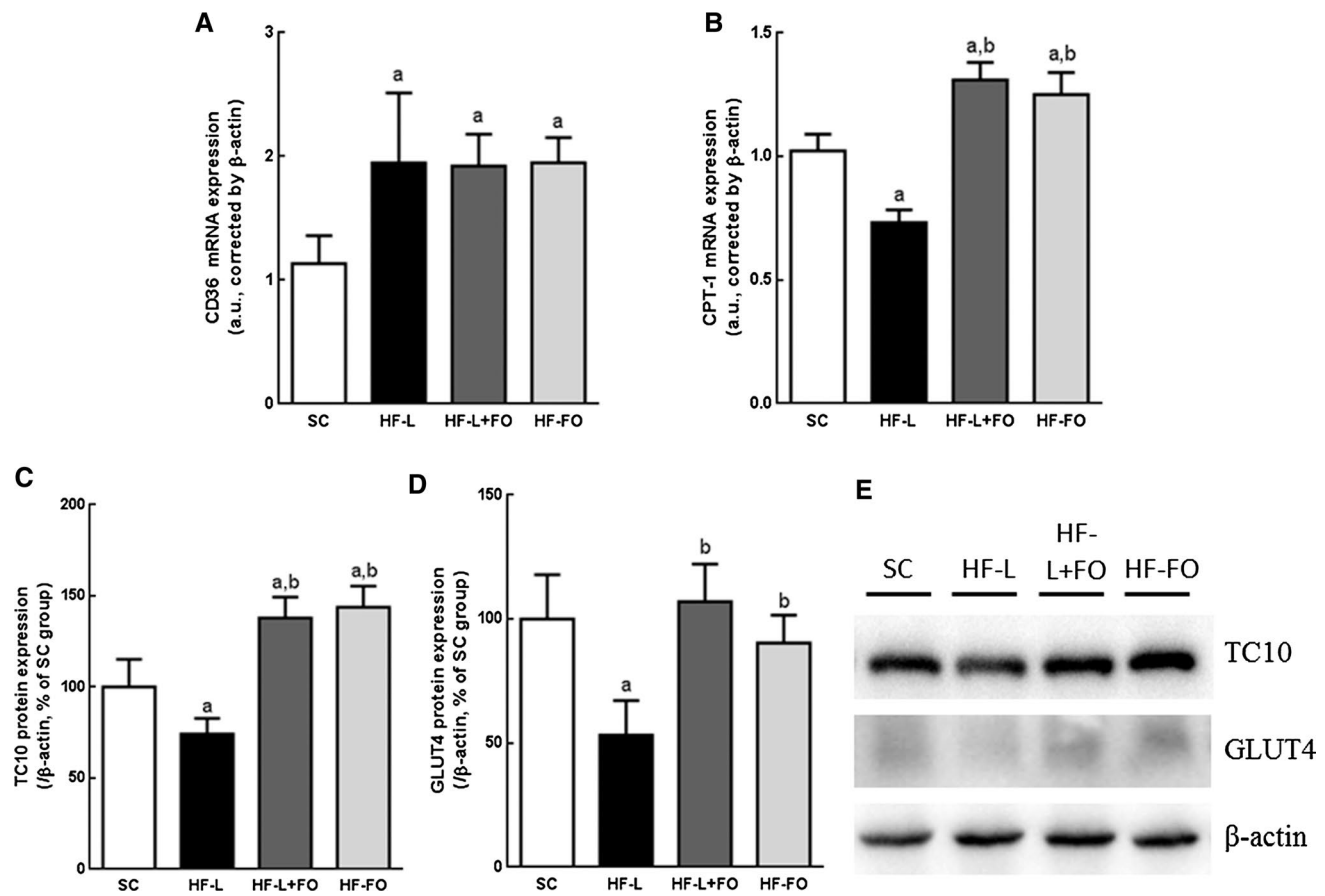
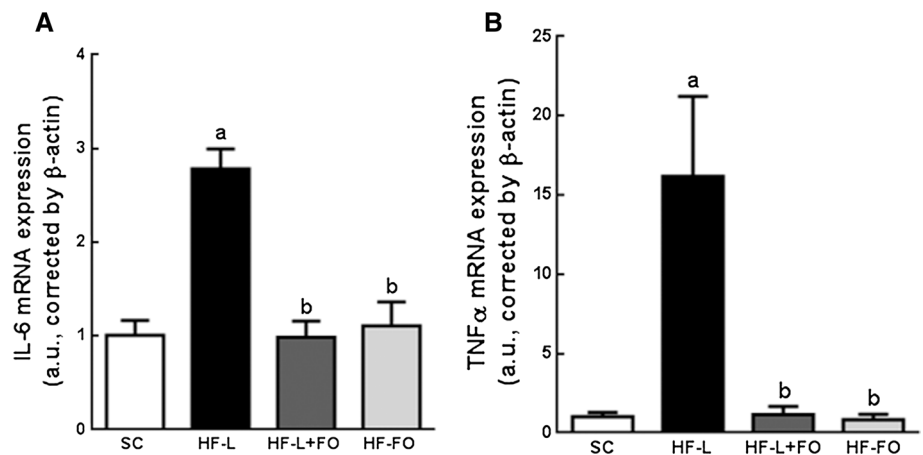


Fig. 4 Gene and protein expressions of CD36, CPT1, TC10, and GLUT-4 in iBAT. Values are the mean \pm SD, $n = 5$ per group. Significant differences are indicated ($P < 0.05$), one-way ANOVA and Holm–Sidak post hoc test: a \neq SC, b \neq HF-L, c \neq HF-L+FO.

Groups are standard-chow group (SC), high-fat lard group (HF-L), high-fat lard plus FO group (HF-L+FO), and high-fat FO group (HF-FO)

Fig. 5 Gene expression of IL-6 and TNF α in iBAT. Values are the mean \pm SD, $n = 5$ per group. Significant differences are indicated ($P < 0.05$), one-way ANOVA and Holm–Sidak post hoc test: a \neq SC, b \neq HF-L, c \neq HF-L+FO. Groups are standard-chow group (SC), high-fat lard group (HF-L), high-fat lard plus FO group (HF-L+FO), and high-fat FO group (HF-FO)



BAT and functions as a coactivator of a range of nuclear receptors that upregulate UCP1 gene expression [7, 19]. As reported in our study, HF-obese mice and rats fed a cafeteria diet had elevated mRNA expression of beta3-AR and UCP1, compared to lean mice [20, 21].

On the contrary, although high-PUFA diets also yield augmentation of BAT adaptive thermogenesis, they are thought to modulate this tissue in a leptin-independent way [22]. Mice fed with a high-PUFA diet had elevated capacity for non-shivering thermogenesis and upregulated UCP1

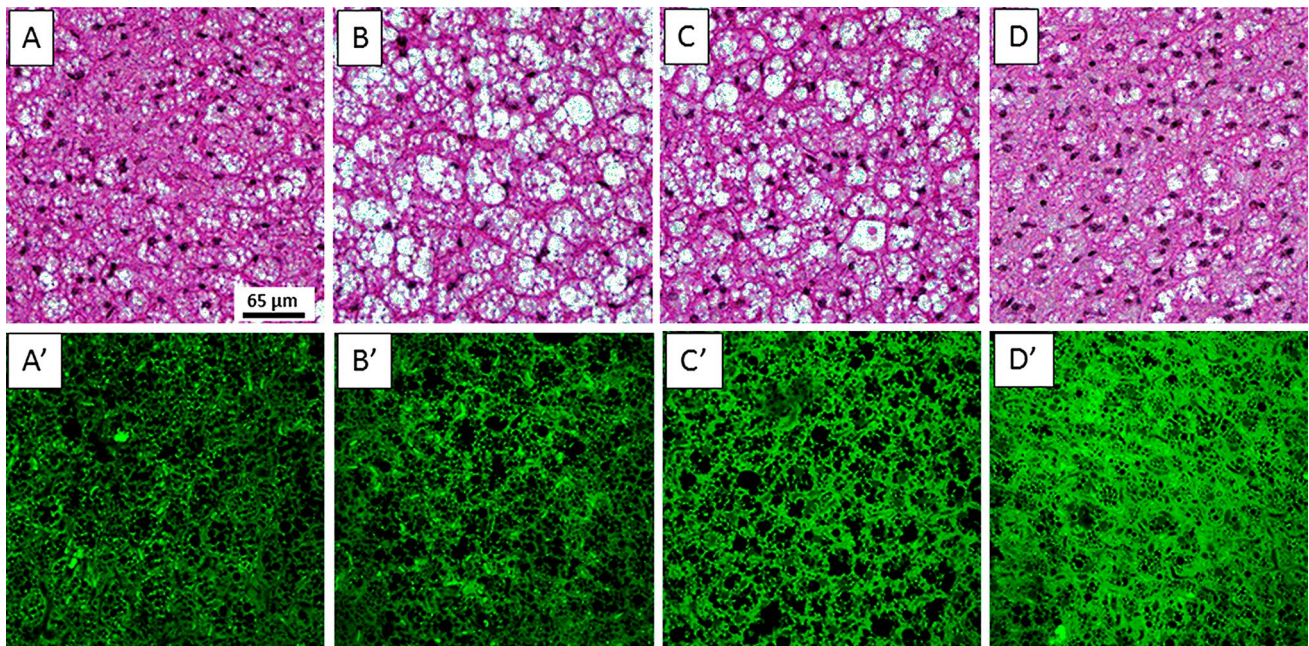


Fig. 6 iBAT hematoxylin and eosin staining and immunofluorescence. SC group showed typical multilocular brown adipocytes (a), whereas HF-L (b) and HF-L+FO (c) groups showed larger lipid droplets within iBAT. FO restored brown adipocyte cytoarchitecture in HF-FO (d). Likewise, tissues were labeled for UCP1 and showed

different intensity of positive immunoreactions. Less intensity was found in SC group (a'), followed by HF-L group (b'). The FO in the diet complied with higher intensity of UCP1 labeling in a dose-dependent manner: in HF-L+FO group (c'), intermediate intense labeling, and HF-FO group (d'), the most intense labeling

[5]. Regardless some reports that every PUFA is capable of inducing thermogenesis to the same extent [22, 23], other studies affirm that different types of fatty acids modulate different responses in UCP1. For instance, mice fed a beef tallow diet (saturated and monounsaturated fatty acids) had lower stimulus to thermogenesis than corn oil-fed mice (PUFA) [24]. A high-FO diet given to rats elevated UCP1 mRNA expression to a bigger extent than a high-safflower diet (rich in n-6 PUFA) [6]. The gene and protein expression pattern observed in the present HF groups is in agreement with the previous studies.

One of the potential regulators of UCP1 and adaptive thermogenesis in this alternative HF diets (i.e., PUFA diets) is the PPAR [21]. PPARalpha and PPARgamma are well-known targets for FO [1]. PPARalpha is highly expressed in BAT and regulates gene expression of PGC1alpha and UCP1 [25]. Besides that, PPARalpha is also responsible for the transcription of genes involved in fatty acid oxidation, a process that is essential for thermogenesis [26]. PPARgamma presents the same roles as PPARalpha in BAT: gene expression regulation of PGC1alpha and UCP1 [27]. The activation of PPAR can lead to activated thermogenesis, which could explain our findings (HF-L+FO and HF-FO groups). The PPARbeta/delta was proven to increase fatty acid oxidation and thermogenesis (UCP1 expression) [28]. However, the PPARbeta/delta upregulation may not be associated with n-3 PUFA intake but rather with

endogenous fatty acid ligands generated in BAT thermogenesis [29].

Although elevated UCP1 mRNA expression could represent recruitment of BAT, when only verifying this gene expression we cannot conclude that BAT function, i.e., thermogenic capacity, is activated. Recent publication considered that to estimate heat production in BAT, it is necessary to measure total UCP1 protein level per mouse [30]. In the present study, we did not evaluate the total UCP1 protein level per mouse. However, not only the gene expression was measured, but the protein expression of UCP1, which reinforces the results. Thus, it is prudent to conclude that thermogenic markers were upregulated in iBAT, without any inferences in the thermogenic capacity of the tissue.

Adaptive thermogenesis requires the fuel utilization from fatty acids or glucose. More importantly, this use may account for a whole-body metabolic improvement [22, 31]. Mice lack CD36 when exposed to cold for 12 h had a drastic reduction in body temperature. When exposed to cold for 24 h, the mice died, demonstrating that CD36 performs a crucial role in fatty acid uptake for thermogenesis in BAT [32]. In the same way, mice submitted to cold displayed an augmentation of CPT1 gene expression, representing an enhancement of fatty acid degradation [31]. The increased fatty acid substrate availability to thermogenesis was seen in the HF-L+FO and the HF-FO groups, with improvement of plasma triacylglycerol. Of interest, FO was already

proven to elevate gene expression of CD36 and CPT1 in the liver [33, 34]. Our findings in the HF-L group, despite the growth in fatty acid uptake (represented by CD36), indicate that the mitochondria entrance for thermogenesis was compromised (diminished CPT1). Therefore, thermogenesis was probably affected.

An augmentation of glucose uptake for oxidation can be explained by elevated GLUT-4 [32] as we saw in the HF-L-FO and the HF-FO groups, leading to an improvement in glucose tolerance. Cold exposure in rats promoted augmentation of GLUT-4 expression and glucose uptake in BAT [35]. In this regard, TC10 is crucial for GLUT4 translocation in the cell membrane and glucose transportation within the cell [36]. FO fed mice had increased GLUT-4 gene expression in skeletal muscle associated with reduced plasma glucose [37]. On the contrary, a reduction in GLUT4 expression, as observed in the HF-L group, can represent diminished glucose transport within the cell and decreased substrate for thermogenesis. Female rats fed a high-fat diet showed reduced GLUT-4 protein expression, leading to insulin resistance [38].

Diabetic obese mice have elevated inflammation in BAT with increased immunostaining for IL-6 and TNF α [39]. In agreement with this report, the HF-L group showed iBAT inflammation, as indicated by elevated gene expressions of IL-6 and TNF α . Unlike the HF-L group, the groups receiving FO showed a pro-inflammatory pattern similar to the SC group, in which the dose was not decisive for the result. The n-3 PUFA are well-known anti-inflammatory compounds, acting, at least partially, via PPAR activation [1], and diminishing inflammation in the liver and white adipose tissue in mice models of obesity [3, 40]. Interestingly, BAT inflammation is associated with insulin resistance [41]. Therefore, it is possible to hypothesize that the reduced inflammation in the FO groups contributed to the improvement of their glucose tolerance and nutrient utilization.

In addition, the present study used a high fish oil dose in order to compare the effects of equivalent HF diets (same amount of energy and lipids) that only differ in fatty acid composition. The authors know that such dose (that is a non-physiological dose) cannot be translated to humans, and this is a limitation of the present study. However, the study may give an actual indication of the beneficial effects of fish oil.

In conclusion, HF diets rich in FO can prevent/reduce BM gain and adiposity and improve metabolic profile (glucose tolerance and triacylglycerol levels). The changes are related to elevated expression of proteins and genes associated with glucose (TC10 and GLUT4) and fatty acid uptake and oxidation (CD36 and CPT1), diminished gene expression of cytokines (IL-6 and TNF α), upregulation of PPAR (the three isoforms), and elevated expression of

thermogenic markers (beta3-AR, PGC1 α and UCP1). The results indicate that the FO has beneficial effects on mice iBAT.

Acknowledgments The authors would like to thank Mrs. Thatiany Marinho, Mrs. Aline Penna, Mrs. Gezileia Lau, and Mrs. Michele Soares for their technical assistance. This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

Conflict of interest The authors declare that they have no conflict of interest.

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