**FISH OIL SUPPLEMENTATION ATTENUATES THE CHANGES
IN THE PLASMA LIPIDS CAUSED BY DEXAMETHASONE
TREATMENT IN RATS**

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FISH OIL SUPPLEMENTATION ATTENUATES THE CHANGES IN THE
PLASMA LIPIDS CAUSED BY DEXAMETHASONE TREATMENT IN RATS

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ABSTRACT

Dexamethasone is an anti-inflammatory that in excess, or after prolonged exposition, may alter the glucose and lipid homeostasis. Omega-3 fatty acids, present in fish oil (FO), can be used as potential modulators of intermediary glucose and lipid metabolism. Herein, we evaluate the effects of FO supplementation (1 g.kg B.W.^{-1}) on glucose and lipid metabolism in rats treated with dexamethasone ($0.5 \text{ mg.kg B.W.}^{-1}$) during 15 days. Adult male Wistar rats were distributed in four groups: CTL (saline $1 \text{ ml.kg B.W.}^{-1}$ and mineral oil 1 g.kg B.W.^{-1}); DEX (dexamethasone and mineral oil); FO (fish oil and saline); and DFO (fish oil and dexamethasone) for 15 days. Dexamethasone or saline were administered intraperitoneally, and fish oil or mineral oil by gavage. We evaluated functional and molecular parameters of lipid and glycemic profile at 8 days and at the end of treatment. The results showed that FO supplementation increased docosahexaenoic acid (DEX: 5.6 ± 0.7 ; DFO: $10.5 \pm 0.8\%$) and eicosapentaenoic acid (DEX: 0.3 ± 0.0 ; DFO: $1.3 \pm 0.1\%$) hepatic content and attenuated the increase of triacylglycerol, total cholesterol and non-HDL-C fraction plasmatic concentrations in the DFO rats compared with DEX. These effects seem not depend on hepatic expression of insulin receptor substrate-1, protein kinase B, peroxisome proliferator-activated receptor coactivator-1 and peroxisome proliferator activated receptors- γ proteins content. There was no supplementation effect on loss of body weight, fasting glycaemia and glucose tolerance in the rats treated with dexamethasone. In conclusion, we showed that the FO supplementation for 15 days attenuates the dyslipidemia induced by dexamethasone treatment.

Key-words: dexamethasone, fish oil, plasma lipids, triacylglycerol, metabolism, supplementation.

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INTRODUCTION

Dexamethasone (Dex) is an effective synthetic glucocorticoid (GC) to treat numerous immune-inflammatory related diseases like rheumatoid arthritis, asthma, allergy, autoimmune diseases, and organ transplant rejection. However, GC treatment can be associated with many metabolic disorders, such as hyperlipidemia, hepatosteatosis, hyperglycemia, insulin resistance and hypertension (Andrew et al. 2002; Czock et al. 2005; Rafacho et al. 2008).

Studies in rats treated with $1.0 \text{ mg.kg B.W.}^{-1}$ for 5 days have shown an increase in concentrations of plasma triacylglycerol (TAG) and increased visceral adiposity (Rafacho et al. 2008; Nunes et al. 2013; Motta et al. 2014). These alterations in lipid profile also can be observed in other studies in rats treated with higher doses of Dex (e.g. $5 \text{ mg.kg B.W.}^{-1}$ for 7 days) (Nicastro et al. 2012; Zanchi et al. 2012) and lower doses for prolonged time (e.g. $0.25 \text{ mg.kg B.W.}^{-1}$ for 28 days) (Chimin et al. 2014).

In human patients treated with glucocorticoids for asthma, cardiac and renal transplants and rheumatoid arthritis it has been shown elevations in total cholesterol (TC), triglycerides (TG) and low-density lipoprotein (LDL), and variable changes in high-density lipoprotein (HDL) (Sholter et al. 2000).

Dyslipidemia associated with GCs may result from direct and/or indirect action GC on lipolysis in adipose tissue, the production and turnover of free fatty acids (FFA), very low density lipoprotein synthesis (VLDL) and accumulation of fatty acids (FA) in the liver (Arnaldi et al. 2010). Additionally, GC use can disturb glucose homeostasis by inducing insulin resistance through modifications in the phosphorylation status of several proteins regarding insulin response (e.g. Akt/PKB and Insulin Receptor Substrate-1 (IRS-1)) (Motta et al. 2014).

The metabolic changes caused by treatment with Dex can be a limiting factor to its long-term use (Sholter et al. 2000; Arnaldi et al. 2010). Thus, strategies must be investigated in order to reduce the side effects of Dex. Some data suggest that omega-3 (n-3) polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA- 20:5n-3) and docosahexaenoic acid (DHA- 22:6n-3), present in fish oil (FO), are associated with alterations in the lipid homeostasis, resulting in the reduction of risk factors for various diseases such as dyslipidemia, cardiovascular disease, type 2 diabetes mellitus (DM2) and metabolic syndrome (Yang et al. 2011; Hirabara et al. 2012).

The n-3 fatty acids, as EPA and DHA, alters metabolic pathways and modulates gene expression resulting in improved insulin sensitivity, attenuated dyslipidemia and hepatic TAG content. It has been suggest that these results provide a rational basis for the use of EPA and DHA as an alternative treatment for patients with metabolic syndrome (Castro et al. 2015). It is known that n-3 PUFA incorporation in phospholipid membranes is linked to modulation of tissues' insulin response (Calder 2012), a fact that can be related to plasma triglyceride content reduction (Yamazaki et al. 2011; Alexander-Aguilera et al. 2011). Furthermore, the n-3 PUFAs can modulate the activity of peroxisome proliferator activated receptors (PPARs), transcriptions factors that control the expression of genes involved in lipid and glucose metabolism (Ferreira et al. 2013).

Considering the biological effects of FO consumption on lipid and glucose metabolism in other models, it is possible to speculate a potential therapeutic effect of FO supplementation on the adverse metabolic effects caused by Dex. Therefore, in this study, we investigated the effects of supplementation with FO (1g.kg B.W.⁻¹) on changes in glucose and lipid metabolism caused by a 15-day treatment with Dex (0.5

mg. kg B.W.⁻¹). The hypothesis tested was that the FO intake attenuates the metabolic changes caused by Dex.

MATERIAL AND METHODS

Ethical approval

The experimental protocols and procedures were approved by Federal University of Santa Catarina Committee for Ethics in Animal Experimentation under protocol number PP00782.

Materials

Dexamethasone phosphate (Decadron[®]) was purchased from Aché (Campinas, SP, Brazil). Human recombinant insulin (Humulin[®]) was from Lilly (Indianapolis, IN, USA). Fish oil was purchased from Phytomare (Governor Celso Ramos, SC, Brazil). The reagents used in the glucose tolerance test, pyruvate tolerance test, lipolysis and hepatic glycogen protocols were from LabSynth (Diadema, SP, Brazil) and Sigma (St. Louis, MO, USA). For measurement of plasma lipids were used enzymatic colorimetric assays from Biotécnica (Varginha, MG, Brazil). For western blotting, the following anti-bodies were used: Akt goat polyclonal- A2611 (1: 500), p-Akt- (Ser 437) rabbit polyclonal C0211 (1: 200), IRS-1-A0311 polyclonal rabbit (1: 200), p-IRS (Tyr 989) rabbit polyclonal C3011 (1: 200), PPAR γ rabbit polyclonal- 1984 (1: 500) , PGC-1 rabbit polyclonal antibodies (1: 500) tubulin- α -mouse polyclonal (1: 300) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and β -actin- mouse polyclonal (1:3000) were from Abcam (Cambridge, MA, USA).

Animals

The experiments were performed using male Wistar rats (3-months old) from the Federal University of Santa Catarina Animal Breeding Center, kept in temperature-

controlled environment ($21 \pm 2^{\circ}\text{C}$) on a 12h light–dark cycle (lights on at 0600, lights off at 1800) with free access to food (commercial feed, Pure Trato[®]) and water.

Experimental Design

The rats received daily intraperitoneal injections of Dex ($0.5 \text{ mg.kg body weight (B.W.)}^{-1}$ using 0.5 mg.mL^{-1} Dex solution) or saline (0.9%) ($1 \text{ mL.kg B.W.}^{-1}$). Fish oil (1g.kg B.W.^{-1}) or mineral oil (1g.kg B.W.^{-1}) were administered orally by gavage, between 0800 and 0900, for 15 consecutive days. Saline was used as control to dexamethasone and the mineral oil used as a “placebo” to fish oil, since it is not absorbed by the animal. The dose of Dex was established based on the study by Rafacho et al. 2008, which observed changes in lipid metabolism after 5 days of Dex administration ($0.5 \text{ mg. kg B.W.}^{-1}$). The dose of fish oil was established based on previous studies (Yamazaki et al. 2011; Hirabara et al. 2012) using fish oil (1 g.kg B.W.^{-1}) showing reduction in plasma triglyceride and total cholesterol levels in animal models of metabolic disorders. The rats were distributed into four groups: a control group (CTL): treated with mineral oil and saline; Group dexamethasone (DEX): treated with mineral oil and dexamethasone; Group fish oil (FO): treated with fish oil and saline; and Group dexamethasone and fish oil (DFO): treated with fish oil and dexamethasone. On the eighth day were performed glucose and pyruvate tolerance tests in distinct animal groups. On the sixteenth day were performed glucose, pyruvate and insulin tolerance tests in different groups of animals and on the next day (17th day) the euthanasia was performed for collection of blood samples and tissue for further analysis (Figure 1).

Metabolic measurements

Body weight, food intake and water were monitored daily from one day prior to the start of supplementation until the day of euthanasia. On the eighth day, after fasting for 12-14h, the rats had the blood collected from the tail to measure blood glucose levels with a glucometer (Accu-Check1 Performa; Roche Diagnostics GmbH, Mannheim, Germany) and it was also collected in a tube previously washed with saline, containing heparin as anticoagulant (Hemofol[®]). The plasma, obtained after blood centrifugation, was used to measure the TAG, total cholesterol (TC) and HDL-C by enzymatic colorimetric methods according to manufacturer's instructions (Biotécnica[®], Varginha, MG, Brazil) using a spectrophotometer (Abell et al. 1952; Burstein et al. 1970; Bucolo and David 1973). The fraction of non-HDL-C was obtained by the following calculation: TC - HDL-C.

On the sixteenth day, after 12-14h fasting, blood glucose levels were measured with a glucometer. In the next day, the fasted rats were euthanized (exposure to CO₂ followed by decapitation), the trunk blood was collected in tubes, previously washed with saline, containing heparin as anticoagulant (Hemofol[®]). The plasma obtained was used to measure the TAG, TC and HDL-C as previously described. Organs and tissues were carefully removed and weighed.

Intraperitoneal glucose tolerance test (ipGTT)

Conscious fasted (12-14h) rats had their tail tip cut for collection of blood. The first drop was discarded and the second was used for determination of glycemia (time 0) using a glucometer. Immediately after this, a 50% glucose solution (2 g.kg B.W.⁻¹, i.p.) was administered and blood samples were collected from the tail tip at 30, 60 and 120 min to measure the blood glucose levels. From these values, the area under curve (AUC) was calculated (Bergman et al. 1985).

Intraperitoneal pyruvate tolerance test (ipPTT)

Another set of fasted (12–14 h) rats was used for the ipPTT experiments. Conscious rats had their blood collected and glycemia measured (time 0) similar to the procedure for the ipGTT analysis. A pre-warmed (36 °C) 25% pyruvate solution was immediately administered ($1.5 \text{ g.kg B.W.}^{-1}$, i.p.), and blood samples were collected from the tail tip at 30, 60 and 120 min to measure the blood glucose levels. From these values we calculated the area under the curve (AUC) (Ferreira et al. 2012; Nagajyothi et al. 2013).

Intraperitoneal insulin tolerance test (ipITT)

Distinct groups of fed rats were used for ipITT experiments. Conscious rats had their blood collected and glycemia measured (time 0) similar to the procedure for the ipGTT analysis. Human recombinant insulin (Humulin[®], Indianapolis, IN, USA) (equivalent to $1 \text{ IU.kg B.W.}^{-1}$, i.p.) was immediately administered. Additional samples were collected at 5, 10, 15 and 20 min for blood glucose measurement. The constant rate for glucose disappearance (K_{ITT}) was calculated from the slope of the regression line obtained with log-transformed glucose values between 0 and 20 min after insulin administration (Bonora et al. 1989).

Hepatic triacylglycerol content

For determination of hepatic fat content, liver samples (100 mg) were transferred to test tubes containing 1 mol.L^{-1} NaCl and homogenized with T18 UltraTurrax (IKA; Staufen, Germany). Then, 2mL of methanol–chloroform solution (1:2 v/v) were added and the tubes were subsequently centrifuged for 5 min at $5000 \times g$. The methanolic phase was then transferred to another test tube and dried in boiling water bath. Then, the solution of methanol–Triton 100 (2:1 v/v) was added to resuspend the samples for determination of hepatic triacylglycerol content. The dosage was performed by enzymatic colorimetric triglyceride method according to manufacturer's instructions (Biotécnica[®]) (adapted from TRINH et al. 1998).

***Ex vivo* adipose tissue lipolysis**

The *ex vivo* adipose tissue lipolysis rate was assayed by incubating tissue samples and evaluating the glycerol release into the incubation medium. Epididymal and Retroperitoneal white adipose tissue fragments (100 mg) were incubated in aerated (5% CO₂: 95% O₂) Krebs buffer (pH 7.4) containing 1% bovine serum albumin for 60 min at 37°C. At the end of the incubation, the samples were collected and kept on ice. Glycerol levels were determined by an enzymatic colorimetric assay according to Bucolo and David (1973). Results are expressed as $\mu\text{g}\cdot\text{mg}^{-1}\cdot\text{h}^{-1}$.

Liver fatty acids profile

The profile of fatty acids in liver was determined by High Performance Liquid Chromatography (HPLC), according to Nishiyama-Naruke et al. (1997). Fatty acids constituent of phospholipids, triacylglycerols, cholesterol esters, and free fatty acids were extracted using chloroform:methanol (2:1,v:v), adapting method described by Folch et al. (1957). Lipid extracts were suspended in methanol and the pH adjusted to ≥ 12 with 5 mol.L⁻¹ NaOH. The aqueous solution was acidified with hydrochloric acid (pH ≤ 3) and subjected to a new lipid extraction by hexane, followed by evaporation by N₂ gas at 37 °C. Fatty acids were derivatized with 4-bromomethyl-7-coumarin and acetonitrile according Abushufa, Reed and Weinkove (1994), and subsequently separated on reversed phase analytical column brand Sigma[®] - MV-C8 4.6 mm 25 cm id particles of 5 microns (Supelco[®]). The chromatographic analysis was performed with a Waters Alliance Separation Module e2695 (Waters, Milford, MA, USA). Were injected 1.6 μL of diluted derivatives, which were eluted isocratically by the binary gradient of water and acetonitrile (70-30%) at 80 minute run at a temperature between 18 to 21°C. The compounds were detected fluorometrically by fluorescence detection (fluorescence detector 2475 multi - waters), with excitation at 325 nm and emission at

398 nm and the data recorded and integrated by Empower Pro Version 2.0 software. We used standard curve containing fatty acids: myristic, lauric, palmitic, palmitoleic, oleic, stearic linoleic, alpha-linolenic, arachidonic, eicosapentaenoic and docosahexaenoic acid. Data were expressed as a percentage of grouped fatty acids.

Western blot analysis

Immediately after the death of animals, the liver tissue fragments were removed and frozen in liquid nitrogen for later analysis. These fragments were homogenized in lysis solution at 4°C (Cell Signaling, MA, USA) in ultraturrax (IKA®) for 3 sets of 15 seconds each. Subsequently were centrifuged at 13.000 x g for 50 min at 4°C (Eppendorf 5804R). The determination of the total protein concentration of the lysate was performed by the Bradford method (1976) according to the manufacturer's instructions (Bio-Rad, CA, USA). Aliquots of total protein (100 mg) were boiled at 100°C for 4 minutes in 25% of the volume of Laemmli buffer (1M sodium phosphate pH 7.0, 10% SDS, 10% β-mercaptoethanol, 50% glycerol, 0,1% bromophenol blue). The samples were subjected to electrophoresis (Mini Protean II - Bio-Rad) in polyacrylamide gel (6.5-15%). After electrophoresis, proteins were transferred to nitrocellulose membrane (Santa Cruz Biotechnology) in presence of 20% methanol and 1% SDS at 120V constant voltage for 2 hours (Shapiro et al. 1967). The lysate fragment for each animal was considered as n = 1. After 2 hours of blocking at room temperature the membranes containing the tissue lysate were washed in Tris buffer salt tween (TBST)/5% and incubated overnight with the following appropriate primary polyclonal antibodies in TBST/3% at 4°C. After washing in TBST, the membranes were incubated with the appropriate secondary antibody (Santa Cruz Biotechnology) in TBST/1%.

Antibody binding was detected by enhanced SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL, USA) according to the manufacturer's protocol. Chemiluminescence detection was performed with a Chemidoc MP system (BioRad, CA, USA) as described by the manufacturer. Band quantification was performed using the image processing and analysis software Chemidoc MP (BioRad, CA, USA).

Statistical analysis

All analyzes were performed using Graphpad prism v.5.01 (Graphpad Inc.; La Jolla, USA) software. The symmetry of the data was tested by Shapiro Wilk's test. Results are expressed as the mean $\pm$ standard error of the mean (SEM) when distribution was symmetric or as median and interquartile range when it was asymmetric. Analysis of variance (ANOVA) (one-way ANOVA) for unpaired groups followed by Tukey's *post-hoc* test were utilized for multiple comparisons of parametric data or Kruskal-wallis followed by Dunn's *post hoc* when variables presented asymmetric distribution. When the text refers to "their respective control groups" for DEX and DFO groups, it means DEX *vs.* CTL and DFO *vs.* FO groups, respectively. The significance level adopted was $p < 0.05$.

RESULTS

Treatment with dexamethasone decreases the body weight, food intake and changes the mass of tissues and organs.

The initial body weight of the rats was similar in all groups. After the second day of treatment, there was a progressive reduction in the weight of the animals treated

with Dex. There was a statistically significant reduction of food intake only between DEX and CTL groups, but in all groups the intake decreased during treatment (Table 1).

The relative mass of the epididymal adipose tissue and liver were significantly higher in rats treated with Dex as compared to their respective control. In addition, the mass of retroperitoneal adipose tissue and the adrenal were significantly reduced by use of Dex (Table 1). The supplementation with fish oil did not change any of these parameters.

Treatment with fish oil attenuates the increase in plasma lipid concentrations in rats treated with Dex

The plasma concentrations of TAG, TC, HDL-C and non-HDL-C in the half and at the end of treatment with Dex and supplementation with FO are presented in Figure 2. The rats treated with Dex showed increased plasma concentrations of triacylglycerol on the eighth day of the treatment (CTL: 77.1 ± 4.8 ; FO: 96.6 ± 7.5 ; DEX: 197.0 ± 11.9 ; DFO: 182.6 ± 10.4 mg.dL⁻¹) and, as observed in Figure 2, the supplementation with fish oil had no effect in this parameter. The plasma concentrations of TC (CTL: 88.7 ± 7.4 ; FO: 98.9 ± 6.9 ; DEX: 101.3 ± 4.5 ; DFO: 105.0 ± 8.7 mg.dL⁻¹), HDL-C (CTL: 55.8 ± 4.0 ; FO: 56.4 ± 6.1 ; DEX: 58.6 ± 4.2 ; DFO: 58.7 ± 5.7 mg.dL⁻¹) and non-HDL-C (CTL: 34.9 ± 5.2 ; FO: 41.2 ± 3.6 ; DEX: 42.7 ± 2.5 ; DFO: 45.9 ± 4.8 mg.dL⁻¹) did not change after eight days of treatment with Dex and supplementation with FO.

After fifteen days of treatment, the rats treated with Dex showed increased plasma concentrations of triacylglycerol (CTL: 77.8 ± 5.5 ; FO: 85.2 ± 8.9 ; DEX: 162.5 ± 22.0 ; DFO: 108.2 ± 6.4 mg.dL⁻¹). However, on the DFO group, there was an attenuation of this increase, representing an effect of the supplementation with fish oil. Still regarding lipid profile, the rats treated with Dex showed increased TC (CTL: 130.7 ± 9.4 ; FO: 112.4 ± 6.4 ; DEX: 197.0 ± 18.8 ; DFO: 128.1 ± 9.5 mg.dL⁻¹) and non HDL-

C (CTL: 98.6 ± 5.4 ; FO: 80.0 ± 4.1 ; DEX: 134.0 ± 14.6 ; DFO: 77.3 ± 6.3 mg.dL⁻¹) concentrations when compared to their respective controls. The supplementation with fish oil was effective in decreasing these parameters, since there was a significant difference between DEX and DFO group.

Dex treatment causes changes in the content of hepatic triacylglycerol but does not change the liver total cholesterol content.

Supplementation with fish oil did not affect the elevation of the concentration of triacylglycerol in the liver caused by treatment with Dex (CTL: 3.8 ± 0.5 ; FO: 6.0 ± 0.7 ; DEX: 12.7 ± 2.3 ; DFO: 10.4 ± 1.6 mg.g⁻¹ of tissue) (Figure 3A). The total cholesterol content in liver was not different among groups (CTL: 1.3 ± 0.2 ; FO: 1.3 ± 0.2 ; DEX: 1.6 ± 0.3 ; DFO: 1.3 ± 0.2 mg.g⁻¹ of tissue) (Figure 3B).

Fish oil supplementation promotes incorporation of EPA and DHA in liver.

After 15 days of supplementation with fish oil, the incorporation of the fatty acids EPA and DHA in the liver was significantly increased, as shown in table 2. The proportion of saturated and other unsaturated fatty acids, however did not significantly change in the tissue.

Epididymal and Retroperitoneal fat lipolysis rate.

There was a significant increase in the basal glycerol release by the epididymal (CTL: 7.1 ± 0.6 ; FO: 8.3 ± 0.6 ; DEX: 11.1 ± 0.8 ; DFO: 10.8 ± 0.4 $\mu\text{g} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$) and retroperitoneal adipose tissues (CTL: 7.9 ± 0.7 ; FO: 8.7 ± 0.7 ; DEX: 11.3 ± 0.7 ; DFO: 12.4 ± 1.0 $\mu\text{g} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$) in Dex-treated groups compared to their respective controls, indicating increased basal lipolytic rate of these tissues (Figure 4A and 4B). Fish oil supplementation had no effect in basal lipolysis.

Supplementation did not alter the total and phosphorylated content of proteins involved in insulin signaling and total content of PGC-1 α and PPAR- γ in liver

Once we observed an incorporation of EPA and DHA in rats supplemented with FO for 15 days, we evaluated if this supplementation could alter the expression of the total and the phosphorylated form of the IRS-1. There was no significant change between the groups (IRS-1= CTL: 0.4 ± 0.1 ; FO: 0.4 ± 0.1 ; DEX: 0.5 ± 0.1 ; DFO: 0.4 ± 0.1 ; pIRS-1/IRS-1= CTL: 0.4 ± 0.01 ; FO: 0.5 ± 0.09 ; DEX: 0.5 ± 0.10 ; DFO: 0.5 ± 0.06) (Figure 5A and B). Similar result was obtained to Akt, since their total content (CTL: 1.0 ± 0.2 ; FO: 1.2 ± 0.2 ; DEX: 1.0 ± 0.1 ; DFO: 1.2 ± 0.2) and the ratio of p-Akt and Akt (CTL: 1.2 ± 0.2 ; FO: 1.2 ± 0.3 ; DEX: 1.1 ± 0.2 ; DFO: 1.7 ± 0.3) were not significantly different between groups (Figure 5C and D). To investigate other potential mechanisms related to changes in the lipid and glucose homeostasis, were evaluated the expressions of PPAR- γ and PGC-1 α in liver tissue. The total content of PGC-1 α (CTL: 1.4 ± 0.11 ; FO: 1.4 ± 0.05 ; DEX: 1.4 ± 0.13 ; DFO: 1.4 ± 0.03) and PPAR- γ (CTL: 0.9 ± 0.2 ; FO: 0.6 ± 0.1 ; DEX: 0.9 ± 0.2 ; DFO: 0.8 ± 0.1) was not significantly different between groups (Figure 5E and F).

Treatment with fish oil did not alter the glucose homeostasis in rats treated with Dex

The fasting glucose levels were significantly increased in rats treated with Dex compared to their respective control in the half and at the end of the treatment, no effect of supplementation with fish oil was observed. On the eighth day of treatment, glucose tolerance and pyruvate tolerance tests showed a significant increase in the AUC of rats treated with Dex. The supplementation with FO had no effect in these parameters. However, at the end of treatment the glucose tolerance test, pyruvate tolerance test and insulin tolerance test showed no significant difference between all groups (Table 3). Revealing that the Dex effects in glucose metabolism are time dependent.

DISCUSSION

The main finding of the current study was the attenuation of dyslipidemia (e.g. TAG, TC and non-HDL-C) caused by Dex treatment after 15 days of concomitant fish oil ingestion. Another important aspect, showed by this study, was that the effect of plasma TAG was not evident on the first measure (on the 8th day of supplementation), but only after the second measure (on the 17th day). Noteworthy, in this model, the Dex-induced dyslipidemia (characterized by increased TAG levels) is present on the 8th or 15th day of treatment. Such information might be useful for future planning of studies regarding fish oil effects on serum lipids.

It has been suggested that changes in lipid profile (e.g. TAG, TC levels and in non HDL-C levels) promoted by the use of corticosteroid are a result of several effects. Some examples of such effects are: enhanced activity of acetyl-coenzyme A carboxylase and free fatty acid synthase in liver, increased hepatic synthesis of VLDL, down-regulation of LDL receptor activity, increased activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Kobashigawa and Kasiske 1997), augmented hormone-sensitive lipase activity (Slavin et al. 1994), and inhibition of lipoprotein lipase (Franco-Colin et al. 2000). Independent of the involved mechanisms, the dyslipidemia caused by the Dex use can be one among several limiting factors for long-term use of such important pharmacologic strategy (Arnaldi et al. 2010). Studies have suggested that diets containing n-3 fatty acids decrease plasma triglyceride concentrations in mice by reducing endogenous triglyceride synthesis, increasing LPL activities and enhancing the blood clearance of triglyceride-rich particles (Qi et al. 2008). Such aspects were not investigated in the present study and could be the objectives of futures studies.

It is known that the liver is responsible for the clearance of plasma free fatty acids leading to the synthesis of new TAG molecules (Nguyen et al. 2008). In the liver, n-3 fatty acids seem to stimulate β -oxidation and decrease lipogenesis (Takahashi et al. 2002). A review performed with studies in humans showed that the n-3 PUFAs, EPA and DHA, have shown to decrease fasting TG concentrations. This conclusion was based on studies that used EPA+DHA in the form of FO capsules at doses $>2 \text{ g}\cdot\text{day}^{-1}$ and showed 25–35% reductions in TG concentrations (Musa-Veloso et al. 2010).

Other studies with n-3 PUFA supplementation in obesity animal models showed reduction in plasma cholesterol (Hassanali et al. 2010; Yamazaki et al. 2011; Hirabara et al. 2012). Apparently, fish oil ingestion can decrease HMG-CoA reductase (Livar et al. 1996) and sterol regulatory element-binding protein 1 (SREBP-1c) mRNA resulting in reduction of cholesterol synthesis. In addition, the supplementation can influence the expression of the CCAAT-enhancer binding protein (C/EBP), promoting changes in gene expression of enzymes (e.g. fatty acid synthase), causing reduction in TG synthesis and enhanced fatty acid β -oxidation (Kim et al. 1999). Such mechanisms might be involved on the attenuation of the TAG and TC levels observed in the present study and can be investigated in future studies.

Rats treated with Dex only had a TAG increment in the liver, but the same did not happen with the DFO group, suggesting an attenuator effect of FO. Some data suggest that PPAR- γ expression can be related to the increment of fat accumulation in liver in high fat diet experimental models (Fraulob et al. 2012; Al Sharif et al. 2014). Since Dex treatment also induced hepatic lipid accumulation, we tested the expression of such transcription factor and one of its regulatory proteins (PGC-1 α - Peroxisome proliferator-activated receptor coactivator-1 α). However, besides the increment in liver TAG caused by Dex in our model, neither dexamethasone nor the supplementation with

fish oil for 15 days altered the total PPAR- γ or PGC-1 α content. On the other hand, as we did not evaluate the activation of PPAR- γ , we cannot claim that there is no relationship between the activation of PPAR- γ and changes in lipid profile.

Furthermore, the dexamethasone treatment is commonly used to study changes in glucose homeostasis (Saad et al. 1993; Burén et al. 2008). The supplementation with fish oil for 15 days did not attenuate the changes in body weight and glycemic parameters caused by treatment with Dex. In rats supplemented with 4.9% fish oil in the diet for 4 weeks and treated with Dex (1 mg.kg B.W.⁻¹) for 5 days there was incorporation of PUFA n-3 in liver, but also in skeletal muscle and adipose tissue. However, the fish oil did not affect the increase in blood glucose, insulin, and the plasma concentrations of non-esterified fatty acids (NEFA), caused by Dex treatment (Corporeau et al. 2006; Le Foll et al. 2007). Besides the production of similar data, compared to the present study, the parameters related to Dex treatment and fish oil supplementation are far distinct from the ones applied on the present study for a proper comparison.

The increase on blood glucose concentrations during the fasting state is also one of the effects caused by treatment with Dex. This may be associated with increased gluconeogenesis and/or hepatic glycogenolysis, as suggested by studies showing decreased hepatic glycogen content and higher glycaemia curves during the pyruvate tolerance test (Beaudry et al. 2013; Cummings et al. 2013; Rafacho et al. 2014). Additionally, increased basal glucagon concentrations (Rafacho et al. 2014) in rats treated with Dex may contribute for such higher fasting glycaemia. In rats, it has been shown that glucocorticoids induce expression of key gluconeogenic enzymes and also phosphoenolpyruvate carboxylase (Jin et al. 2004). Such increment may contribute to enhance endogenous glucose production, potentially increasing the fasting glucose.

Previous studies have shown that the treatment with Dex promotes reduction of glucose tolerance (Burén et al. 2008; Rafacho et al. 2008; Chimin et al. 2014). However, in our model, glucose tolerance was decreased only after 8 days of Dex administration at $0.5\text{mg.kg B.W.}^{-1}$, returning to control levels after additional 7 days of treatment. Our study was not designed to investigate such unusual result and we are not aware of other studies regarding this subject. Future might investigate this interesting topic, as the reproduction of the same response was homogenous in three different sets of rats (data not show).

In conclusion, fish oil (1 g.kg B.W.^{-1}) ingestion caused a significant attenuation on increased plasma triacylglycerol and total cholesterol levels caused by Dex treatment, during 15 days ($0.5\text{ g.kg B.W.}^{-1}$). However, no effect of the supplementation was evidenced, on the development of the same changes, on the initial 8 days of treatment and supplementation. Such time dependent effect need to be considered in future studies. Furthermore, fish oil ingestion does not seem to modify Dex-induced changes on glucose homeostasis (e.g. in fasting glycaemia). Therefore, fish oil can be a potential nutritional strategy to attenuate the dyslipidemia caused by Dex administration.

Conflicts of interest

The authors state that there are no personal conflicts of interest in the present study.

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Table 1. Changes in body weight (g) and food intake (g.kg BW⁻¹) and tissues weight (mg.g BW⁻¹) in CTL, FO, DEX and DFO animals in half and the end of treatment with Dex and/or FO supplementation.

		CTL	FO	DEX	DFO
Body weight^a (T1-T0)	8th day	8.00[3.50;11.50]	5.00[4.00;7.50]	-42.00[-52.5;-37.50] [#]	-40.00[-57.00;-30.00] [#]
	15th day	17.00[13.00;21.50]	15.00[9.00;17.50]	-67.50[-86.25;-54.00] [#]	-69.00[-87.25;-62.25] [#]
Food Intake^a (T1-T0)	8th day	-2.68[-2.95;-2.52]	-0.14[-2.59;0.03]	-6.59[-7.97;-5.20]	-6.64[-8.24;-5.88]
	15th day	-1.90[-2.90;-0.87]	-1.83[-1.96;-1.48]	-4.95[-5.69;-3.76] [#]	-2.55[-4.64;-1.16]
Liver	17th day	32.74±0.68	33.63±1.01	38.99±1.75 [#]	40.40±2.38 [#]
Adrenal	17th day	0.16±0.01	0.16±0.00	0.11±0.01 [#]	0.11±0.01 [#]
Epididymal Fat	17th day	10.76±0.54	10.30±0.90	15.01±0.91 [#]	14.56±1.36 [#]
Retroperitoneal Fat	17th day	13.51±1.22	14.10±1.34	7.41±0.92 [#]	8.55±1.28 [#]

Values are means ± SEM; n = 10. BW: body weight, T0: before treatment and supplementation, T1: after treatment and supplementation. [#]indicates significantly different compared with their respective control groups (DEX vs. CTL and DFO vs. FO) using ANOVA with Tukey's post-hoc test, ^aKruskal Wallis test (p < 0.05).

Table 2. Fatty acids (%) in the liver of rats CTL and/or treated with Dex and/or supplemented with fish oil for 15 days.

	CTL	FO	DEX	DFO
EPA+DHA	5.5±0.3	8.8±0.7*	5.9±0.7	11.9±0.7*
Other unsaturated fatty acids	57.7±2.1	54.1±2.5	55.3±1.5	49.5±2.2
Saturated fatty acids	36.8±2.2	37.1±3.1	38.8±1.5	38.6±2.6

Values are means ± SEM; n = 7. EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid. Asterisk (*) indicates significantly different compared with non-supplemented group using ANOVA with Tukey's post-hoc test ($p < 0.05$).

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Table 3. Fasting blood glucose, intraperitoneal glucose (ipGTT) and pyruvate (ipPTT) tolerance in two distinct moments and insulin tolerance (ipITT) at the end of treatment with Dex and supplementation with fish oil.

		CTL	FO	DEX	DFO
Blood glucose (mg.dL ⁻¹)	8th day	98.1±2.6	95.2±2.1	115.1±3.7 [#]	113.8±5.5 [#]
	16th day	96.2±2.7	100.1±1.8	132.3±8.2 [#]	123.4 ±3.5 [#]
AUC for ipGTT	8th day	15926±1042	11024±1401	25456±2804 [#]	21186±2664 [#]
	16th day	16783±1452	15208±950	17423±1728	16474±2288
AUC for ipPTT^a	8th day	4434[3062;7834]	6049[4392;8971]	27080[18633;29693] [#]	22728[20572;26160] [#]
	16th day	10243[3550;13435]	5466[4350;6487]	26668[13085;33000] [#]	14476[6935;25626] [#]
K_{ITT} for (%.min ⁻¹)	16th day	2.77 [1.90;3.78]	3.85 [2.66;4.23]	2.42 [2.11;2.93]	2.70 [2.30;5.54]
ipITT^a					

Values are means ± SEM or median [interquartile interval], n = 10. [#]indicates significantly different (p < 0.05) compared with their respective control groups (DEX vs. CTL and DFO vs. FO) using ANOVA with Tukey's post-hoc test or ^aKruskal Wallis test with Dunn's post-hoc test. AUC: Area under the curve (mg.dL⁻¹.120min⁻¹).

Legends:

Figure 1. Study design

Figure 2. Plasma lipid profile after treatment with Dex and supplementation with fish oil. Plasma concentration of triacylglycerol (A), total cholesterol (B), HDL-C (C) and non-HDL-C (D) in the eighth and seventeenth day. Values are means $\pm$ SEM, $n = 10$. HDL-C: high-density lipoprotein cholesterol. #indicates significantly different compared with their respective control groups (DEX vs. CTL and DFO vs. FO) and asterisk (*) indicates significantly different compared with DEX using ANOVA with Tukey's post-hoc test ($p < 0.05$).

Figure 3. Hepatic triacylglycerol and total cholesterol content. (A) Hepatic triacylglycerol content was significantly increased in DEX-group compared with CTL-group. (B) The content of hepatic total cholesterol content was not altered by DEX or FO treatment. Data are median [interquartile interval]. #indicates significantly different compared with their respective control groups (DEX vs. CTL and DFO vs. FO) using Kruskal-Wallis test with Dunn's post-hoc test ($p < 0.05$, $n = 10$).

Figure 4. Glycerol release by Epididymal fat (A) and Retroperitoneal (B) fat fragments from CTL, DEX, FO and DFO rats. Values are means $\pm$ SEM, $n = 10$. #indicates significantly different compared with their respective control groups (DEX vs. CTL and DFO vs. FO) using ANOVA with Tukey's *post-hoc* test ($p < 0.05$).

Figure 5. Total content and phosphorylated IRS-1 and Akt in liver rats treated with DEX are not decreased. Total IRS-1 content (A). Ratio of phosphorylated IRS-1 to their total content (B). Total Akt content (C). Ratio of phosphorylated Akt to their total content (D). Total PGC1- α content (E). Total PPAR γ content (F). Data are presented as mean $\pm$ SEM. We used the one-way ANOVA followed by post hoc Tukey, $p < 0.05$, $n = 6$. Representative bands were selected for each group. IRS-1: Insulin receptor substrate-1; Akt: Protein kinase B; PPAR- γ : Peroxisome Proliferator-Activated Receptor- γ ; PGC-1 α : Peroxisome proliferator-activated receptor coactivator-1. It was necessary to select bands to make them representative to each group.