

Ω 3-Polyunsaturated fatty acids prevent lipoperoxidation, modulate antioxidant enzymes, and reduce lipid content but do not alter glycogen metabolism in the livers of diabetic rats fed on a high fat thermolyzed diet

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Abstract Ω 3-Polyunsaturated fatty acids (Ω 3-PUFAs) are known to act as hypolipidaemics, but the literature is unclear about the effects that Ω 3-PUFAs have on oxidative stress in obese and diabetic patients. In this study, our aim was to investigate the effects of Ω 3-PUFAs on oxidative stress, including antioxidant enzyme activity and hepatic lipid and glycogen metabolism in the livers of diabetic and non-diabetic rats fed on a high fat thermolyzed diet. Rats were divided into six groups: (1) the control group (C), (2) the control diabetic group (D), (3) the high fat thermolyzed diet group (HFTD), which were fed a diet that was enriched in fat that was heated for 60 min at 180°C, (4) the high fat thermolyzed diet diabetic group (D + HFTD), (5) the high fat thermolyzed diet + Ω 3 polyunsaturated fatty acid group (HFTD + Ω 3), and (6) the high fat thermolyzed diet + Ω 3 polyunsaturated fatty acid diabetic group (D + HFTD + Ω 3). The most important finding of this

study was that Ω 3-PUFAs are able to reduce triglycerides, non-esterified fatty acid, lipoperoxidation levels, advanced glycation end products, SOD/CAT enzymatic ratio, and CAT immunocontent and increase SOD2 levels in the livers of diabetic rats fed with a HFTD. However, Ω 3-PUFAs did not alter the observed levels of protein damage, blood glucose, or glycogen metabolism in the liver. These findings suggest that Ω 3-PUFAs may represent an important auxiliary adjuvant in combating some diseases like diabetes mellitus, insulin resistance, and non-alcoholic fatty liver disease.

Keywords Advanced glycation end products · Glycogen metabolism · Diabetes · Oxidative stress · Ω 3-Polyunsaturated fatty acids · High fat diet

Introduction

Diabetes mellitus affects people worldwide and poses major public health and socioeconomic challenges. Hyperglycemic states are supposed to induce the overproduction of reactive oxygen species (ROS) through various mechanisms including the increased non-enzymatic formation of advanced glycation end products (AGEs), increased polyol pathway flux, and increased O₂-production through the activation of mitochondrial and protein kinase C (PKC)-dependent NAD(P)H oxidases in many tissues [1].

Recent studies suggest that oxidative stress increases with diabetes mellitus. This may be due to a decreased antioxidant potential, increased free-radical production, alterations in enzymatic pathways related to the redox state (such as the polyol and glyoxalase pathways), and changes in the compartmentalization of metal ions. Together, such

Marcos L. S. Perry—*In memoriam*.

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factors promote increases in oxidative stress levels in diabetic patients [2, 3].

Reactive derivatives from non-enzymatic glucose–protein condensation reactions as well as lipids and nucleic acids exposed to reducing sugars, such as glucose or glyceraldehyde, form a heterogeneous group of irreversible adducts called AGEs [4]. The formation and accumulation of AGEs in various tissues, which is known to progress during a normal aging process, is extremely accelerated in patients with diabetes mellitus [5, 6]. One important component of AGE accumulation with aging appears to be the sustained exposure to dietary AGEs (dAGEs), which contributes to the overloading of anti-AGE receptors and depletion of anti-oxidant reserves [7]. There is accumulating evidence that an interaction between AGEs and receptor for advanced glycation end products (RAGEs) elicits oxidative stress generation and subsequently modulates gene expression in various types of cells including hepatocytes and hepatic stellate cells [8, 9].

The liver is not only a target organ for circulating AGEs but also an important site for their clearance and catabolism. Indeed, the liver can sequester a number of circulating senescent macromolecules such as AGEs [10]. It has long been known that glucose is stored in the liver in the form of liver glycogen. Early studies on glycogen metabolism in type 1 diabetic patients using liver biopsies revealed conflicting results, reporting either increased or decreased liver glycogen concentrations [11].

Epidemiological studies also reported a lower incidence of type 2 diabetes mellitus in individuals who consumed Ω 3-polyunsaturated fatty acids (Ω 3-PUFAs), while intake of total saturated and/or monounsaturated fats was associated with an increased risk of type 2 diabetes in glucose-intolerant individuals [12]. The beneficial effects of Ω 3-PUFAs consumption on liver disease were mainly attributed to their effects in reducing triglyceride levels, increasing high density lipoprotein cholesterol, and improving endothelial function through anti-inflammatory mechanisms and reduced platelet aggregation [13]. Many studies show that it is very important to maintain a ratio at Ω 6 5:1 Ω 3 allowing a greater conversion of alpha-linolenic acid to docosahexaenoic acid [14]. However, this recommendation is more common for a normal situation (not pathological).

Our group has previously demonstrated the deleterious effects of both endogenous and exogenous AGEs [15, 16]. de Assis et al. [17] showed that normal rats fed with a high thermolyzed fat diet for 12 months had significant increases in blood DNA damage and liver lipoperoxidation compared to those fed a normal high fat diet. Thus, the aim of this study was to investigate the effects of Ω 3-PUFAs on oxidative stress, antioxidant enzyme activity, and hepatic

lipid and glycogen metabolism in the livers of diabetic and non-diabetic rats fed with a high fat thermolyzed diet.

Materials and methods

Animals and chemicals

Sixty adult Wistar rats (male, 60 days old) from the Central Animal House of the Department of Biochemistry were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at room temperature ($22 \pm 2^\circ\text{C}$). All experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989, and all experiments were approved by our ethics committee for animal use at the Federal University of Rio Grande do Sul. D-[U- ^{14}C]-Glucose (297 mCi/mmol) was purchased from Amersham International (Little Chalfont, Bucks, UK). Alloxan monohydrate and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Treatments and diets

Diabetes mellitus was induced by an intraperitoneal (i.p.) injection of alloxan ($150 \text{ mg kg}^{-1} \text{ b.w.}$), and non-diabetic animals received a saline solution (NaCl 0.9%), also via an i.p. injection. After 10 days, blood samples were obtained from rats in fasting state to determine the plasma glucose concentration. Only animals that presented with 15–30 mmol/l glucose concentration were included in the study.

Rats were divided into six groups: (1) the control group (C, $n = 10$), which received standard laboratory rat chow; (2) the control diabetic group (D, $n = 10$), which received an injection of alloxan and the same diet as group 1; (3) the high fat thermolyzed diet group (HFTD, $n = 10$), which received a diet enriched with fats that had been heated for 60 min at 180°C to which a vitamin mixture was added after heating; (4) the high fat thermolyzed diet diabetic group (D + HFTD, $n = 10$), which received an injection of alloxan and the same diet as group 3; (5) the high fat thermolyzed diet + Ω 3 polyunsaturated fatty acid group (HFTD + Ω 3), which received a diet enriched with fats that had been heated for 60 min at 180°C to which a vitamin mixture and an Ω 3 fatty acid supplement was added after heating; and (6) the high thermolyzed fat diet + Ω 3-PUFA diabetic group (D + HFTD + Ω 3, $n = 10$), which received an injection of alloxan and the same diet as group 5. Diet thermolyzation was based on the study by Sandu et al. [18], which indicated a high AGE content (around 1 unit/ μg) in a high fat diet after thermolyzation. The salt and vitamin compositions used were the

same as those described by Horwitz [19]. During the treatment period of 4 weeks, the animals had free access to food and water.

Tissue preparation

Rats were killed by decapitation, and the blood was collected immediately in heparinized tubes. Retroperitoneal and epididymal fat tissues were dissected and weighed. The whole blood sample was then centrifuged at $2500\times g$ for 10 min to yield the plasma fraction, which was used for subsequent biochemical analyses. The liver was dissected and was then either weighed and cut into 0.3-mm slices using a McIlwain tissue chopper for glycogen synthesis or weighed and stored at -80°C for future analyses of oxidative stress, western blotting, glycogen, triglyceride, and cholesterol concentrations.

Blood biochemical parameters

Plasma glucose, triacylglycerol (TAG), total cholesterol, and HDL cholesterol levels were measured using commercial kits (Labtest, MG, Brazil). Reactions were performed using the Labmax apparatus (Labtest, MG, Brazil). The plasma non-esterified fatty acid (NEFA) activity was measured using a commercial kit (Roche Diagnostics, Germany). To analyze the amount of AGEs, we checked the concentration of Carboxymethyllysine in serum by ELISA anti-CML antibodies (R&D Systems, Minneapolis, MN, USA).

Oxidative stress and antioxidant enzyme activity

Measurement of protein carbonyl

The oxidative damage to proteins was measured by quantifying the number of carbonyl groups by reaction with 2,4-dinitrophenylhydrazine (DNPH). Proteins were precipitated by the addition of 20% trichloroacetic acid (TCA) and were resuspended in 10 mM DNPH, and the absorbance of the resulting solution at 370 nm was recorded [20]. Results are expressed as nmol carbonyl/mg protein.

Thiobarbituric acid reactive species (TBARS)

In order to assess the extent of lipoperoxidation, we detected TBARS formation through a heated and acidic reaction. This is widely adopted as a method for the measurement of lipid redox states, as previously described [21]. The samples were mixed with 0.6 ml of 10% TCA and 0.5 ml of 0.67% thiobarbituric acid and were then heated in a boiling water bath for 25 min. The level of TBARS was determined by measuring the absorbance of

each sample at 532 nm. The concentration of TBARS in the samples was then determined from a calibration curve using 1,1,3,3-tetramethoxypropane (which had been subjected to the same treatment as the supernatants) as a standard. Results are expressed as nanomoles of TBARS per milligram of protein.

Measurement of non-protein thiol content

An aliquot of the supernatant from homogenates precipitated with 20% TCA was diluted in 0.1% SDS and 0.01 M 5,5'-dithionitrobis-2-nitrobenzoic acid (DTNB) in ethanol was added. An intense yellow color developed, and the absorbance at 412 nm was recorded after 20 min [22]. Simultaneously, the absorbance of a blank sample, which was identical to the analyzed samples except for the absence of DTNB, was also recorded. The non-protein thiol content of each sample was calculated, after subtraction of the blank absorbance, using the following equation and the molar extinction coefficient of $13,600\text{ M}^{-1}\text{ cm}^{-1}$: $[(\text{final absorbance}/13,600)V]/1000/Q$, where "V" is the volume (in ml) of the sample used in the assay and "Q" is the amount of protein (in mg) in the sample used to perform the assay.

Antioxidant enzyme activity

Catalase (CAT) activity was measured as previously described [23]. The rate of decrease in absorbance at 240 nm was measured as a function of H_2O_2 degradation by CAT. Superoxide dismutase (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline autooxidation by recording the absorbance of the samples at 480 nm [24].

Glycogen metabolism

Hepatic glycogen synthesis

Levels of hepatic glycogen synthesis were measured as follows. The livers of rats were dissected and cut into 300 μm slices using a McIlwain tissue chopper (100–120 mg). The liver slices were incubated in a beaker with media containing Krebs–Ringer bicarbonate buffer (pH 7.4), 5 mM glucose, and 0.2 μCi $\text{D}[U\text{-}^{14}\text{C}]\text{glucose}$. Incubations were performed at room temperature in media that had previously been treated with a 95% O_2 :5% CO_2 mixture for 1 h. Liver slices (between 100 and 120 mg) were then incubated at 37°C for 1 h in a metabolic shaker (60 cycles/min) according to the method of Dolnikoff et al. [25]. The incubations were stopped by placing the bottles in ice, and subsequently, 1 ml of 60% KOH was added to each beaker. After 15 min in a boiling water bath, 3 ml of

96% ethanol was added to the tubes to precipitate the glycogen. After precipitation, glycogen was suspended in 0.2 ml of water, and scintillation liquid (Opti-Phase Hi-Safe3 from PerkinElmer-USA) was added. The samples were then assessed in a scintillation liquid counter.

Hepatic glycogen concentration

The hepatic glycogen concentration was measured using the Krisman method [26].

Hepatic lipids

In order to measure the hepatic lipid content, frozen liver samples were thawed on ice and homogenized in deionized water. Extraction and isolation of lipids to yield dried lipid extracts were performed using the technique described by Folch et al. [27]. The hepatic cholesterol and triglycerides content of the lipid extracts were then assayed enzymatically by colorimetry.

Western blotting analysis

Proteins (20 μ g) were separated by SDS-PAGE on 10% (w/v) acrylamide and 0.275% (w/v) bisacrylamide gels and were then electrotransferred onto nitrocellulose membranes. The membranes were then incubated in Tris-buffered saline Tween-20 [TBS-T; 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.05% (v/v) Tween 20] containing 1% (w/v) non-fat milk powder for 1 h at room temperature. Subsequently, the membranes were incubated for 12 h with the appropriate primary antibody (CAT, 1:6000; Superoxide dismutase, 1:2000 and β -Actin, 1:10000). After washing in TBS-T, blots were incubated with horseradish peroxidase-linked anti-immunoglobulin G (IgG) antibodies for 1.5 h at room temperature. Chemiluminescent bands were detected, and densitometric analysis was performed using Image-J[®] software.

Statistical analyses

Biochemical data are expressed as the mean \pm SD. All the analyses were performed using the Statistical Package for the Social Sciences (SPSS 16.0) software. Likewise, the differences between all groups were analyzed using ANOVA and Tukey's post-hoc test. *P* values were considered significant when *P* < 0.05.

Results

In Table 1, non-diabetic groups we had a significant (*P* < 0.05) increase in body weight and adipose tissue in

rats treated with high fat thermolyzed diets (HFTD and HFTD + Ω 3), this result was expected for us although treatment with the diet is only 1 month, for composition experimental diets details, see Table 2. Already in the diabetic groups, we had a reduction in body weight of all rats, though animals that received the high fat thermolyzed diets (HFTD and HFTD + Ω 3) showed a smaller reduction in body weight and a significant increase in adipose tissue in relation to the diabetic control group (D) Table 1.

Table 3 shows the biochemical parameters in our experimental groups. We observed a significant difference (*P* < 0.05) between the plasma glucose of diabetic and non-diabetic rats (an increase of about 400%), thus demonstrating that our model was effective in inducing diabetes mellitus. However, diabetic rats fed a HFTD or HFTD + Ω 3 diet showed a decrease in their glucose concentration. This effect was attributed to a lower amount of carbohydrate in these diets as compared to the control diet. We found no significant difference in the plasma cholesterol and HDL of the groups tested, although the groups that received only HFTD (both diabetic and non-diabetic) appeared to show higher levels of cholesterol. Diabetes mellitus and HFTD did not affect the level of plasma NEFAs but promoted an increase in plasma triglycerides. However, in adding polyunsaturated fatty acids in the diet, we noticed a significant reduction (*P* < 0.05) in plasma triglycerides and NEFAs in these groups. We also evaluated the concentration of AGEs in serum by measuring Carboxymethyllysine (CML) and we can observe that diet (HFTD and HFTD + Ω 3) increased significantly (*P* < 0.05) the amount of CML in non-diabetic rats. All diabetic rats had an increase of CML, however, supplementation with Ω 3-PUFAs having partially prevented this increase.

In Table 4, we can observe the effect of different diets on hepatic lipids and hepatic glycogen metabolism. A high fat thermolyzed diet (HFTD) significantly increased (*P* < 0.05) hepatic triglycerides content in diabetic and non-diabetic rats. However, supplementation of their diet with Ω 3 (HFTD + Ω 3) reduced these values to normal levels (that of the control) in the non-diabetic group. A HFTD increased the levels of hepatic cholesterol, although this increase only proved to be significant (*P* < 0.05) in diabetic rats. In contrast to triglyceride content, supplementation with Ω 3 did not affect the levels of hepatic cholesterol. In addition, we noted increased levels of hepatic cholesterol in both diabetic groups compared to the non-diabetic groups, although the values were not statistically significant. We did not find a significant difference in the levels of hepatic glycogen metabolism in the experimental groups studied. However, diabetes mellitus has a tendency to decrease hepatic glycogen concentration.

We show the results of the analysis of oxidative stress and antioxidant enzyme activity in Table 5. A high fat

Table 1 Body parameters in diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD), and high fat thermolyzed diet + Ω 3 (HFTD + Ω 3)

Body parameters	C	D	HFTD + Ω 3	D + HFTD + Ω 3	HFTD	D + HFTD
Initial body weight (g)	282 ± 18.5	278 ± 14.3	285 ± 25.1	284 ± 22.5	275 ± 12.8	281 ± 17.4
Final body weight (g)	314 ± 23.2	226 ± 32.7*	331 ± 48.9	252 ± 39.3*	335 ± 22.4	242 ± 48.7*
Body weight gain (g)	32 ± 7.2	-52 ± 12.6**	46 ± 9.3*	-32 ± 5.9**	60 ± 11.8*	-39 ± 9.5**
Retroperitoneal adipose tissue (g)	2.1 ± 0.1	0.2 ± 0.0*	6.3 ± 1.9**	2.8 ± 0.6	8.6 ± 3.1**	3.2 ± 0.8
Epididymal adipose tissue (g)	1.2 ± 0.09	0.2 ± 0.0*	2.4 ± 0.4**	1.0 ± 0.2	3.5 ± 0.7**	1.2 ± 0.3*

Body weight and adipose tissue weight are expressed as grams (g), all parameters represented as the mean ± SD ($n = 10$ per group). *Different asterisks* on the same row indicate a significant difference ($P < 0.05$) and all groups were analyzed using ANOVA and Tukey's post-hoc test

Table 2 The composition of control (C), high fat thermolyzed (HFTD), and high fat thermolyzed + Ω 3 (HFTD + Ω 3) experimental diets that were fed to diabetic and non-diabetic rats

Composition	Control diet (%)	High fat thermolyzed diet (%)	High fat thermolyzed diet + Ω 3 (%)
Commercial bran	–	20.5	20.5
Soy protein isolate ^a	27.0	15.9	15.9
Corn starch	55.5	–	–
Sucrose	5.0	10.0	10.0
Vitamin mix ^b	1.0	1.0	1.0
Mineral salt mix ^c	4.0	2.0	2.0
DL-Methionin ^d	0.3	0.3	0.3
DL-Lysine ^e	0.3	0.3	0.3
Soy oil	5.0	1.0	1.0
Lard	–	49.0	46.0
Ω 3 fatty acid ^f	–	–	3.0

Salt and vitamin composition are according to Horwitz

^a Soy protein isolate, purity 97% (from Solae, Esteio, Brazil)

^b Vitamin mixture: mg/100 g of diet (from Roche, SãoPaulo, Brazil): vitamin A (retinyl acetate), 4; vitamin D (cholecalciferol), 0.5; vitamin E (DL- α -tocopheryl acetate), 10; menadione, 0.5; choline, 200; PABA, 10; inositol, 10; niacine (nicotinic acid), 4; pantothenic acid (calcium D-pantothenate), 4; riboflavin, 0.8; thiamin (thiamine hydrochloride), 0.5; pyridoxine (pyridoxine hydrochloride), 0.5; folic acid, 0.2; biotin [D-(+)-biotin], 0.04; vitamin B12, 0.003

^c Mineral salt mixture: mg/100 g of diet (from Roche, SãoPaulo, Brazil): NaCl, 557; KI, 3.2; KH₂PO₄, 1556; MgSO₄, 229; CaCO₃, 1526; FeSO₄·7H₂O, 108; MnSO₄·H₂O, 16; ZnSO₄·7H₂O, 2.2; CuSO₄·5H₂O, 1.9; CoCl₂·6H₂O, 0.09

^d DL-Methionin (from Merk, Rio de Janeiro, Brazil)

^e DL-Lysine (from Merk, Rio de Janeiro, Brazil)

^f Fish oil: docosahexaenoic acid (DHA) 5 : 1 eicosapentaenoic acid (EPA) (Naturalis SA, Brazil.)

thermolyzed diet increased SOD activity significantly ($P < 0.05$) in both diabetic and non-diabetic rats (HFTD and D + HFTD). When we added Ω 3 supplements, the SOD activity was reduced in non-diabetic rats (HFTD + Ω 3), and this reduction in activity was even more evident ($P < 0.05$) in diabetic rats (D + HFTD + Ω 3). CAT activity in non-diabetic groups increased only in the HFTD group ($P < 0.05$). Diabetes caused an increase in CAT activity in all diabetic groups (D, D + HFTD + Ω 3 and D + HFTD) relative to the non-diabetic rats. We did not find any difference in the SOD/CAT ratio in non-diabetic groups. However, in analyzing the SOD/CAT ratio in the diabetic groups, for this ratio, we verified an increase in the D and D + HFTD groups and a significant ($P < 0.05$) decrease in the

D + HFTD + Ω 3 group. The groups that received supplementation with polyunsaturated fatty acids (HFTD + Ω 3 and D + HFTD + Ω) showed significantly ($P < 0.05$) lower levels of lipoperoxidation compared to the other groups. All groups fed high fat thermolyzed diets (HFTD, D + HFTD/D, HFTD + Ω 3 and D + HFTD + Ω 3) showed a significant ($P < 0.05$) increase in protein carbonyl levels relative to the control groups (C and D). We did not observe any differences in the non-protein thiol content between groups.

A drastic reduction ($P < 0.05$) in the hepatic immuncontent of CAT was observed in groups for which diets were supplemented with polyunsaturated fatty acids (Ω 3-PUFAs) relative to groups that did not receive

Table 3 Biochemical parameters in diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD), and high fat thermolyzed diet + $\Omega 3$ (HFTD + $\Omega 3$)

	C	D	HFTD + $\Omega 3$	D + HFTD + $\Omega 3$	HFTD	D + HFTD
Glucose (mmol/l)	5.4 ± 0.5	27.5 ± 6.8*	5.8 ± 1.2	20.2 ± 4.0*	5.6 ± 0.4	19.9 ± 4.2*
Cholesterol (mg/dl)	56.9 ± 12.8	76.1 ± 16.4	59.0 ± 16.3	66.2 ± 14.3	77.1 ± 25.9	71.5 ± 10.4
Triglycerides (mg/dl)	73.4 ± 23.2	147.3 ± 60.4*	67.1 ± 18.6	104.7 ± 42.7*	140.9 ± 67.0*	262.7 ± 78.5**
HDL (mg/dl)	23.5 ± 5.3	26.0 ± 4.8	24.7 ± 5.6	29.5 ± 6.4	25.5 ± 5.7	25.6 ± 2.5
Non-esterified fatty acids (NEFA) (μM)	566.1 ± 60.5	583.0 ± 45.1	378.7 ± 60.7*	427.7 ± 143.5*	523.6 ± 126.5	616.4 ± 227.6
Carboxymethyllysine (% Control of CML)	100 ± 4.1	163.6 ± 13.9**	112.2 ± 6.3*	124.3 ± 16.4*	114.7 ± 5.5*	151.8 ± 11.3**

Cholesterol, high density lipoprotein (HDL), and triglyceride levels are expressed as mg/dl. Free fatty acid concentrations are expressed in $\mu\text{M}/\text{ml}$, the glucose concentration is expressed as mmol/l, and carboxymethyllysine are expressed by % of a control group. All the parameters represented as the mean ± SD ($n = 10$ per group). *Different asterisks* on the same row indicate a significant difference ($P < 0.05$) and all groups were analyzed using ANOVA and Tukey's post-hoc test

Table 4 Hepatic lipids and glycogen metabolism in diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD), and high fat thermolyzed diet + $\Omega 3$ (HFTD + $\Omega 3$)

	C	D	HFTD + $\Omega 3$	D + HFTD + $\Omega 3$	HFTD	D + HFTD
Hepatic triglycerides (mg/%)	2.29 ± 0.34	2.02 ± 0.51	2.90 ± 1.41	4.31 ± 0.94*	3.86 ± 1.03**	4.70 ± 1.17**
Hepatic cholesterol (mg/%)	1.03 ± 0.09	1.10 ± 0.15	1.26 ± 0.13	1.30 ± 0.09*	1.24 ± 0.16	1.31 ± 0.12*
Hepatic glycogen concentration (mg/%)	5.5 ± 0.8	4.8 ± 1.6	4.2 ± 0.4	3.1 ± 1.1	4.2 ± 0.4	3.6 ± 1.2
Hepatic glycogen synthesis (pmol/mg ⁻¹ tissue h ⁻¹)	75.0 ± 10.1	74.5 ± 13.0	82.1 ± 6.4	82.4 ± 13.8	80.0 ± 5.6	82.7 ± 8.9

Glycogen synthesis from D-[U-¹⁴C]-glucose is expressed in pmol of glucose incorporated into glycogen/100 mg tissue/h. Concentrations of hepatic glycogen, triglycerides, and cholesterol are expressed in mg/100 mg of tissue and are represented as the mean ± SD ($n = 10$ per group). *Different asterisks* on the same row indicate a significant difference ($P < 0.05$) and all groups were analyzed using ANOVA and Tukey's post-hoc test

Table 5 Oxidative stress and antioxidant enzymes in the livers of diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD), and high fat thermolyzed diet + $\Omega 3$ (HFTD + $\Omega 3$)

	C	D	HFTD + $\Omega 3$	D + HFTD + $\Omega 3$	HFTD	D + HFTD
Superoxide dismutase (U/mg protein)	73.1 ± 6.5	87.4 ± 18.1	92.6 ± 27.1	57.8 ± 7.9 *	124.4 ± 19.2**	113.1 ± 18.7**
Catalase (U/mg protein)	39.5 ± 5.1	50.1 ± 11.2	43.1 ± 4.4	61.3 ± 10.1*	71.1 ± 3.8*	64.2 ± 10.6*
SOD/CAT ratio (arbitrary units)	1.85 ± 0.1	2.20 ± 0.6	1.92 ± 0.5	0.95 ± 0.1*	1.75 ± 0.2	2.12 ± 0.4
Lipoperoxidation (nmol TBARS/mg protein)	0.055 ± 0.01	0.075 ± 0.03	0.033 ± 0.01*	0.049 ± 0.01*	0.056 ± 0.01	0.074 ± 0.01
Protein carbonyl (nmol/mg protein)	0.59 ± 0.29	0.53 ± 0.15	0.81 ± 0.11*	0.81 ± 0.16*	0.85 ± 0.13*	0.75 ± 0.14*
Non-protein sulfhydryl groups (nmol/mg protein)	0.030 ± 0.008	0.033 ± 0.006	0.029 ± 0.007	0.026 ± 0.005	0.041 ± 0.007	0.037 ± 0.008

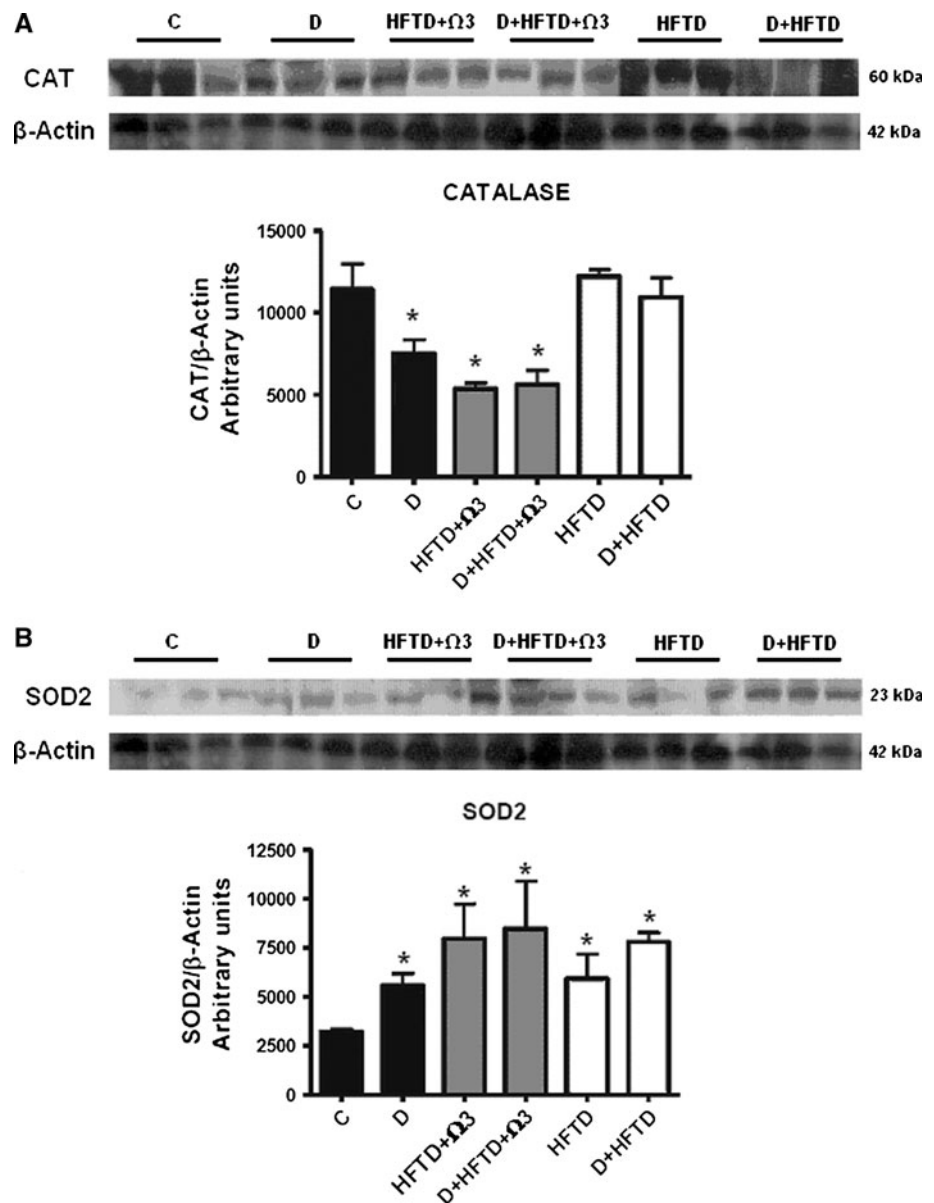
SOD and CAT activities are expressed in U/mg protein. The SOD/CAT ratio is expressed in arbitrary units. Lipoperoxidation, protein carbonyl and non-protein thiol contents are expressed in nmol/mg protein. All results are presented as the mean ± SD ($n = 10$ per group). *Different asterisks* on the same row indicate a significant difference ($P < 0.05$) and all groups were analyzed using ANOVA and Tukey's post-hoc test

$\Omega 3$ -PUFA supplements (Fig. 1a). All diabetic groups and groups supplemented with a high fat diet, including groups that received $\Omega 3$ -PUFAs supplements, showed significant ($P < 0.05$) increases in the hepatic immunocent of SOD2 when compared to the non-diabetic control group (Fig. 1b).

Discussion

In this study, we evaluated the effect of a high fat thermolyzed diet on the liver of diabetic rats and the possible beneficial effect of $\Omega 3$ -PUFAs. The most important finding of this study was that $\Omega 3$ -PUFAs are able to reduce

Fig. 1 Representative and quantitative western blot analysis of the liver CAT (a) and SOD2 (b) immunocontent from diabetic and non-diabetic rats fed a control diet (C), high fat thermolyzed diet (HFTD), and high fat thermolyzed diet + Ω 3 (HFTD + Ω 3). The graphic represents the means \pm SD ($n = 3$ per group). Different asterisks indicate a significant difference ($P < 0.05$) and all groups were analyzed using ANOVA and Tukey's post-hoc test



triglycerides (in both plasma and the liver), NEFAs, lipoperoxidation and modulate antioxidant enzyme activity and immunocontent in the livers of diabetic rats supplemented with a high fat thermolyzed diet. However, these Ω 3-PUFAs did not affect the observed levels of protein damage, blood glucose, or glycogen metabolism in the liver.

The most common cause of obesity in man is a chronic intake of a hypercaloric diet rich in (saturated) fats. Obesity can lead to an increase in hepatic import and synthesis of triglycerides and a decrease in hepatic triglyceride export and oxidation. This results in the accumulation of triglycerides in hepatocyte, which promotes an increase in oxidative stress and cytokines proinflammation [28, 29]. It is known that fish oil diminishes the effect of lifestyle-related diseases like diabetes mellitus and hyperlipidemia [30].

The triglyceride-lowering effects of Ω 3-PUFAs have been thoroughly researched [31, 32], and studies have consistently demonstrated that supplementation with 2–4 g/d of Ω 3-PUFAs reduces circulating triglycerides up to 34% in hypertriglyceridemic patients [33]. In a recent study, Sato et al. [34] demonstrated that the anti-obesity effect of Ω 3-PUFAs in diet-induced obesity is associated with the suppression of hepatic lipogenesis and steatosis. Corroborating these authors, our results showed that both diabetes mellitus and a high fat thermolyzed diet for 1 month increases the levels of triglycerides in both plasma and the liver and that Ω 3-PUFAs have been effective in decreasing these values (Tables 3, 4). These results further emphasize the beneficial effect of Ω 3-PUFAs in both the protection from and the reversal of hyperlipidemia. Although levels of

NEFAs did not change in diabetic or high fat thermolyzed groups, we observed a beneficial effect of Ω 3-PUFAs (Table 3). These beneficial effects are largely mediated by peroxisome proliferator-activated receptors (PPARs). PPAR- α and PPAR- δ ($-\beta$) represent the main targets [35]; however, recent studies have shown the importance of the inhibition of SREBP-1c [36]. The hypolipidaemic and anti-obesity effects of Ω 3-PUFAs probably depends on the in situ suppression of lipogenesis and on an increase in fatty acid oxidation in several tissues [37, 38]. This metabolic switch might reduce accumulation of toxic fatty acid derivatives while protecting insulin signaling in the liver [39].

Although we did not observe significant differences in the metabolism of glycogen, our results suggest a decrease in the hepatic glycogen content of diabetic rats. This reduction is more drastic in the diabetic groups that received a high fat thermolyzed diet (Table 4). An in vitro study showed that AGEs decreased tyrosine phosphorylation of IRS-1 and subsequently reduced glycogen synthesis in insulin-exposed Hep3B cells and the association of the p85 subunit of phosphatidylinositol 3-kinase with IRS-1 [40]. These results may justify our finding that a high fat diet enriched in AGEs (HFTD) reduced observed glycogen concentrations. Insulin promotes storage of glucose in the form of glycogen, and as expected in this study, alloxan-induced diabetes mellitus led to decreased glycogen content in the liver. Human studies have also reported lower glycogen content in the livers of type 2 diabetics [41, 42]. This phenomenon is already well described in the literature [43], but little is known about the impact of hepatic glycogen content on the regulation of glucose metabolism in the liver from in vivo models. In a recent study, the author suggests that the beneficial effects of Ω 3-PUFAs on glucose metabolism are most strongly related to their interaction with PPARs. On the other hand, SREBP-1c has been shown to play a more important role in lipid metabolism in obese mice [44]. Our results demonstrate that lipid metabolism is more susceptible to the beneficial effects of supplementation with Ω 3-PUFAs over a 4-week period than glucose metabolism. However, studies with longer periods of supplementation appear to show that Ω 3-PUFAs have more beneficial effects on glucose metabolism [45].

Obesity and diabetes mellitus represent chronic inflammatory states that lead to increases in ROS, AGEs, and several immunomodulatory factors that can contribute to non-alcoholic fatty liver disease (NAFLD) and liver injury [46]. Many earlier studies have used Ω 3-PUFAs to prevent oxidative stress that results from NAFLD, a high fat diet or diabetes mellitus. However, results in the literature are contradictory. Some studies suggested that the intake of a diet high in fish oil results in high Ω 3-PUFA content in membrane lipids and enhances lipid peroxidation in the

organs, blood, and urine of experimental animals and humans [47, 48]. Some others, however, suggest that low doses of fish oils do not significantly affect lipid peroxidation in vivo [49, 50]. In our study, we have shown that Ω 3-PUFAs were effective in reducing and preventing increased lipid peroxidation that is promoted by diet and diabetes mellitus (Table 5). We believe that this beneficial result is observed as a consequence of the intermediate size of the dose used in this study (3 g Ω 3-PUFAs/100 g diet) as compared to data from the literature and the fact that we used this dose over a short period of time (4 weeks). We can also correlate the decrease in NEFAs (Table 3) with the reduction of lipid peroxidation in rats supplemented with Ω 3-PUFAs (Table 5) because it is already known that NEFAs induce a stress-signaling pathway in the liver. In our study, we identified an increase in protein carbonyl levels in rats fed with a high fat thermolyzed diet (Table 5), including rats whose diets were supplemented with Ω 3-PUFAs. These findings corroborate the study of Matsuzawa-Nagata et al. [51] who reported that protein carbonyl levels were increased by 35% in the livers of mice fed a high fat diet, compared to control mice, after 6 weeks. In our study, we showed that diabetes mellitus, a high fat thermolyzed diet and Ω 3-PUFAs could modulate enzyme activity. We observed an increase in SOD activity in HFTD and D + HFTD, suggesting that O_2^- production is increased during treatment with this diets, however, SOD activity of HFTD + Ω 3 did not change and D + HFTD + Ω 3 decreased suggesting an attenuation in the increase of SOD activity caused by HFTD. The immunoblot of SOD2 increased in this groups making a regulation in the substrate level and decreasing the enzymatic activity. These results suggesting that O_2^- production is decreased which corroborating the result of lesser lipoperoxidation by HFTD + Ω 3 (Table 5). Bauer et al. [52] demonstrated that an increase in NEFA and impaired adiponectin bioactivity both contribute to reduced levels of SOD2 protein in monocytes of type 2 diabetes patients. Indeed the study of Bauer et al. was conducted with subjects with DM2 and our model (alloxan) is DM1, however, once installed the pathology of diabetes mellitus, the pro-inflammatory and pro-oxidant response are very similar in both types (DM1 and DM2). [53, 54]. The study of Flachs et al. [55] observed that feeding mice with a high fat diet enriched with EPA/DHA concentrate (6% EPA, 51% DHA) for 5 weeks leads to elevated systemic concentrations of adiponectin and suggested that this increase could explain, to some extent, the anti-diabetic properties of these Ω 3-PUFAs.

In this study, supplementation with Ω 3-PUFAs decreased levels of lipoperoxidation and hepatic lipids and increased the SOD2 immunocontent. A combination of prior evidence from the literature and the results presented

here lead us to propose that Ω 3-PUFAs reduce the amount of NEFAs and increase the concentration of adiponectin. This, in turn, leads to increases in the levels of SOD2, which subsequently prevents HFTD-mediated increases in lipoperoxidation and oxidative stress. Our findings confirm the beneficial effects of Ω 3-PUFAs on lipid metabolism and oxidative stress in the liver, which may represent an important adjuvant to therapies currently used in the treatment of some diseases like diabetes mellitus, insulin resistance, and non-alcoholic fatty liver disease.

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