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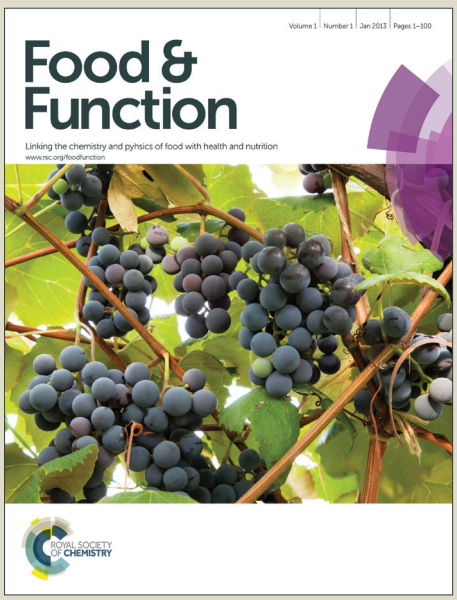

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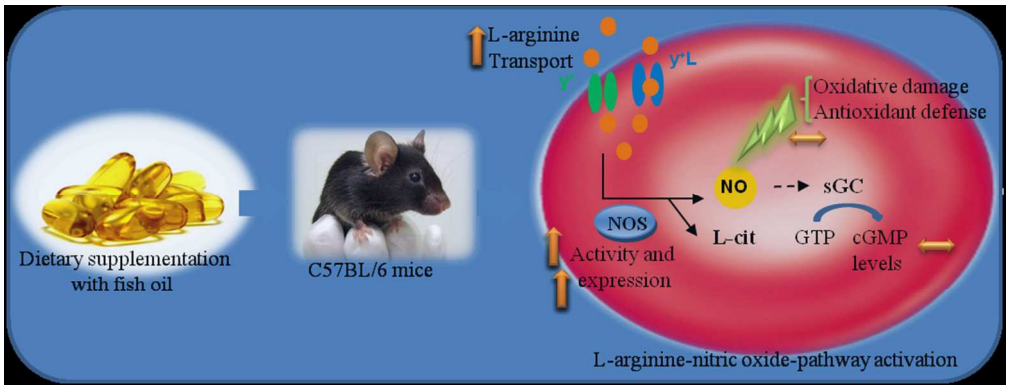

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DIETARY FISH OIL ON NITRIC OXIDE SYNTHASE ACTIVITY AND OXIDATIVE STATUS IN MICE RED BLOOD CELLS

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ABSTRACT

The consumption of *n*-3 polyunsaturated fatty acids (PUFAs) derived from fish oil concomitant with a reduction intake of saturated fats is associated with cardiovascular benefits, which may result from the participation of nitric oxide (NO). In contrast, PUFAs are vulnerable to peroxidation, which could affect oxidative stability of the cell and reduce NO bioavailability. Therefore, we investigated the effects of high fat diets with increasing amounts of fish oil (0-40% of energy) in place of lard on L-arginine-NO pathway, arginase pathway and oxidative status in mice red blood cells (RBC). We found that L-arginine transport, as well as NO synthase (NOS) expression and activity, was enhanced by highest doses of fish oil (30 and 40%). In contrast, diets rich in lard led to NOS expression and activity impairment. Arginase expression was not significantly affected by any of the dietary regimens. No significant difference in protein and lipid oxidative markers was observed among none of the fish-oil fed mice; only lard feeding induced protein damage in addition to a decreased superoxide dismutase activity. These data suggest that a substantial dose of fish oil, but not low doses, activate RBC L-arginine-NO pathway, without result in oxidative damage.

Keywords: fish oil; nitric oxide; L-arginine; oxidative stress; red blood cells.

1. Introduction

Improvements on dietary recommendations regarding the amount and sources of lipids have been considered a critical strategy for the reduction of cardiovascular risk. Along these lines, nutritional science has demonstrated that beneficial effects can be achieved with a reduction of the saturated fatty acids (FAs) intake associated with an increase of unsaturated FAs ingestion, essentially the consumption of fish oil [1, 2]. The *n*-3 polyunsaturated fatty acids (PUFAs) derived from fish oil (eicosapentaenoic and docosahexaenoic acids, EPA and DHA) have been reported in epidemiological and clinical trials to promote a variety of clinically relevant effects on cardiovascular health, reducing triglyceride levels, slowing down the expansion of atherosclerotic plaque and improving endothelial function [3, 4]

Accumulating evidence shows that one of the potential mechanisms responsible for the observed cardioprotective properties of *n*-3 PUFAs involves an up-regulation of the nitric oxide (NO) production. In rabbits, EPA reduces myocardial infarct size partially through NO-mediated mechanisms [5]. Likewise, DHA improves vascular function via endothelial NO synthase (eNOS) activation in forearm microcirculation [6]. Studies performed on endothelial cells have demonstrated that EPA and DHA increase NO synthesis and improve endothelium-dependent relaxation through multiple mechanisms, including inhibition of cyclooxygenase-2[7], alterations in the lipid composition of caveolae that induces translocation of eNOS [8, 9], an up-regulation of SIRT1 expression [10] and by activation of the AMP-Activated Protein Kinase via UCP-2 [11].

NO plays a major role in cardiovascular homeostasis, with its effect mainly attributed to its action over the vascular wall. The biological effects of NO are primarily mediated through the activation of the enzyme soluble guanylyl cyclase (GC) with a subsequent increase in the intracellular levels of cyclic guanosine monophosphate (cGMP), which regulates relaxation of the vascular smooth muscle and inhibits platelet aggregation and adhesion [12, 13].

Besides endothelium, we and other have described nitric oxide synthases within RBC[14, 15] and it has been suggested to contribute to the intravascular NO pool. However the clinical implications of NO generated by RBC still remain controversial in the literature. An earlier theory has suggested that NO rapidly reacts with RBC-derived free hemoglobin limiting its bioactivity, while more recent reports designate RBC as a source of NO, essential for the oxidative (nitrite/nitrate) and nitrosative (iron nitrosylhemoglobin/ S-nitrosohemoglobin) transport forms of NO within blood [16].

In addition to nonenzymatic NO production, human and mice RBC possess both inducible (iNOS) and endothelial (eNOS) isoforms of NOS, and thus are capable of synthesizing their own NO [14, 15]. The NO produced by RBC contributes to its deformability, to the inhibition of platelet aggregation and to the modulation of the intravascular NO pool [17].

Similarly to the endothelial cells, RBC NO is synthesized through conversion of the semi-essential cationic amino acid L-arginine into L-citrulline and NO by the action of the enzyme NOS [16]. In different cell types, membrane transport of L-arginine seems to be a rate-limiting step for NO

synthesis [13]. Human and rodent RBC also express the enzyme arginase. Endothelial arginase competes with NOS for L-arginine and operates as an important regulator of NO production. Recent data suggest that increased arginase activity in RBC may also limit the NO synthesis [18].

In addition to the biosynthesis of NO, its bioavailability is another central factor that plays a key role in the cardiovascular homeostasis. Under conditions of oxidative stress, an imbalance between the pro-and antioxidant systems, the bioavailability of NO is known to be significantly reduced, leading to a loss of its cardioprotective actions. NO is also a free radical that can react with superoxide radicals to form peroxynitrite, which is one of the main mediators of cellular injury [19]. Physiological antioxidant defense mechanisms such as superoxide dismutase, glutathione peroxidase and catalase are involved in scavenging oxidant agents, protecting the cells from oxidative damage [20]. Lipids containing PUFAs are primarily susceptible to free radical oxidation [21], nevertheless the role of PUFAs as pro- or anti-oxidants remains an unanswered question.

Since it has been suggested as an important function by RBC in regulating vascular function via NO production, we hypothesized that the cardiovascular benefits of fish oil may be due, in part, to its capacity to stimulate L-arginine-NO pathway in RBC and consequently, NO production.

Therefore, the aim of the present study was to investigate the role of stepwise replacement of lard, rich in saturated fatty acid, by fish oil in nitric oxide production and oxidative status in RBC from C57BL/6 mice fed on a high fat diet.

2. Material and methods

2.1. Animals and diet

Three-month-old male C57BL/6 mice were divided into six groups based on the diet offered over a 12-week period: the control group (C group) was fed with a standard diet (10% of energy from fat) and the others groups were fed with isoenergetic high fat diets (50% of energy from fat) containing 0% (F0 group), 10% (F10 group), 20% (F20 group), 30% (F30 group), or 40% (F40 group) of fish oil (source of the n-3 PUFAs EPA and DHA; Sigma-Aldrich Chemical Co., St. Louis, USA). The high fat diets were composed of a mixture of lard and fish oil, with a gradual replacement of lard by fish oil. The diets were manufactured by PragSolucoes (PragSolucoes, Jau, SP, Brazil) in accordance with AIN-93M recommendations [22]; their detailed composition is described in table 1.

The animals were kept under standard conditions (12h light/dark cycles, 21 ± 2ºC and 15 min/h air exhaustion cycle) and had free access to food and water. The experimentation protocols were approved by the local Ethics Committee for the Care and Use of Laboratory Animals (CEUA/033/2011) and were performed in accordance with the guideline for ‘‘Care and Use of Laboratory Animals” (US National Institutes of Health 85-23, revised 1996).

2.2. Body mass and food intake

Body mass was measured every week and dietary intake monitored daily. Fresh food was freely allowed and its intake was checked daily by the difference between the amount of food supplied and the amount of food left in the cage. The energy intake was then calculated as the product of food consumption by the energy content of the diet (kcal/g of diet).

2.3. Preparation of RBC

At the end of the experimental period (12 weeks), the animals were anaesthetized with intraperitoneal sodium pentobarbital (0.42 mg/g). Blood samples obtained by cardiac puncture were collected into a tube containing heparin after a 6-h fast. The RBC were isolated by centrifugation, washed with saline, and then cells were suspended in saline to reach an haematocrit ranging between 5–10%. Haematocrit and haemoglobin were determined with a Coulter counter (Sysmex XT-2000i, Hyogo).

2.4. Measurement of L-[^{3}H]-arginine influx

L-[^{3}H]-arginine (100 μM) was added into RBC suspension, and the cells were incubated at 37ºC. After incubation, the cells were washed rapidly by centrifugation and resuspended in a cold isotonic medium (mM: $MgCl_2$ 107, MOPS 10, pH 7.4). The cells were then lysed using Triton X-100 and proteins precipitated with trichloroacetic acid. The radioactivity was measured

by a beta-scintillation counter (LS 6500 Liquid Scintillation Beckman Counter Inc., California, USA).

2.5. Measurement of RBC NOS activity

Basal NOS activity was determined by the conversion of L-[^{3}H]-arginine into L-[^{3}H]-citrulline. L-[3H]-arginine (500 μM) was added to RBC suspension, and the cells were incubated at 37ºC. All reactions were stopped by rapid centrifugation, followed by washes in a cold isotonic medium. The RBC was lysed with Triton X-100 and proteins precipitated with trichloroacetic acid. After centrifugation, the supernatant was applied to a Dowex cation exchange resin column. Recovered L-[^{3}H]-citrulline was eluted with water and radioactivity was measured by a beta-scintillation counter.

2.6. Western blot analysis for eNOS, iNOS and Arginase 1

RBC suspension was homogenized in a lysis buffer containing protease inhibitors (Sigma-Aldrich Chemical Co.) and the supernatant was assayed for total protein concentrations using the BCA Assay Kit (Thermo Scientific, Rockford, IL, USA). Amounts of 30 μg protein (50 μg for arginase 1) were electrophoretically fractionated in a 10% sodium dodecyl sulphate–polyacrylamide gel (Invitrogen, CA, USA), transferred to PVDF membrane and immunoblotted with mouse monoclonal antibodies against eNOS or iNOS (BD Transduction Laboratories, San Diego) followed by secondary goat anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

or rabbit polyclonal antibody against arginase 1 (Santa Cruz Biotechnology) followed by secondary goat anti-rabbit IgG-HRP antibody. Rabbit anti-β-tubulin (Santa Cruz Biotechnology) was used as a loading control. Immunoreactive bands were exposed to Enhanced Chemiluminescence System- ECL reagent (Amersham Biosciences, Piscataway, NJ, USA), the signal was visualized by autoradiography and measured using ImageJ 1.43u software (Wayne Rasband, National Institute of Mental Health, USA). Band density of proteins were normalized to the corresponding band density of β-tubulin (ratio NOS or arginase/β-tubulin).

2.7. Measurement of intraerythrocyte cGMP levels

Washed RBC were re-suspended at a haematocrit of 80% and incubated with 500 μM 3-isobutyl-1-methylxanthine (IBMX) to prevent degradation of cyclic nucleotides by phosphodiesterases. The incubation was terminated by the addition of perchloric acid (0.5 M). Cells were lysed by sonication followed by rapid freezing in liquid nitrogen and the tubes were centrifuged. Supernatant was removed, 8 M KOH were added and the tubes were underwent to another centrifugation. Thereafter, supernatant was removed and stored at -80°C until assay. The basal intraerythrocyte cGMP levels were detected using enzyme-linked immuno assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA).

2.8. Oxidative Status Parameters

Oxidative damage to proteins was assessed by determination of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH; Sigma, St. Louis, MO) using the method of Levine et al [23]. Carbonyl contents were determined from the absorbance at 370 nm and were expressed as carbonyl derivates (nM/mg protein). Total protein concentration was assayed using the BCA Assay Kit.

Lipid peroxidation was assessed by measuring the RBC expression of 4-Hydroxynonenal (HNE) protein adducts by western blot. 4-HNE is released during the oxidation of *n-6* PUFAs and it can efficiently react with sulfhydryl, histidine or lysine groups of proteins to form HNE-protein adducts. Western blot protocol were performed as earlier described; after electrophoresis, the RBC proteins (30 µg) were transferred to PVDF membranes which were incubated with goat polyclonal anti 4-HNE adduct antibody (Merck Millipore Corporation, Billerica, MA, USA) followed by donkey anti-goat IgG-HRP secondary antibody (Santa Cruz Biotechnology).

2.9. Antioxidant status

Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were determined in RBC lysate. CAT activity was measured in terms of the rate of decrease in hydrogen peroxide concentration at 240 nm [24]. SOD activity was assayed by measuring the inhibition of

adrenaline auto-oxidation as absorbance at 480 nm [25]. GPx activity was measured by monitoring the oxidation of NADPH at 340 nm in the presence of hydrogen peroxide [26]. All RBC enzyme activities were expressed per gram of haemoglobin (U/g Hb).

2.10. Statistical analysis

Data are shown as mean ± SEM. In the cases where homocedasticity of the variances were confirmed, comparisons among groups were checked by one-way analysis of variance (ANOVA) followed by a Holm-Sidak post-hoc test for all pair-wise comparisons. Otherwise differences were tested with a Kruskal–Wallis test and a Dunn's post-hoc test. In all cases, a value of $p \leq 0.05$ was accepted as statistically significant. Data were analyzed using the the latest version (6.0) of the Graph Pad Prism software (La Jolla, CA, USA).

3. Results

3.1. Food and energy intake

There was no significant difference in food intake (g) among the experimental groups, except for an increase in the amount of food consumed by F40 group compared to the F20 group (C: 3.9 ± 0.1; F0: 3.9 ± 0.1; F10: 4.0 ± 0.2; F20: 3.5 ± 0.1; F30: 4.0 ± 0.2; F40: 4.3 ± 0.2; $p \leq 0.05$). In relation to energy intake (kcal), the high fat diets resulted in greater energy intake than standard diet due to their high energy densities. Moreover, F40 group showed

higher energy intake than F20 group. (C: 14.8 ± 0.2; F0: 19.5 ± 0.6; F10: 20.2 ± 0.8; F20: 17.7 ± 0.3; F30: 20.3 ± 0.4; F40: 22.2 ± 0.9; $p \leq 0.05$).

3.2. Body mass

The body mass (g) of all animals was similar at the beginning of the study. After the experimental period of 12 weeks, the animals that received high fat diet containing the lowest percentages or absent of fish oil (0-20%) had higher body mass than animals fed with diet rich in fish oil (30% and 40%), which maintained body mass similar to that of the control group. As illustrated in Fig.1, the significant body mass gain of animals fed with high fat diet absent of fish oil (F0 group) started at week 2 and persisted until the end of the experimental period. The other differences were established from the sixth week.

3.3. L-arginine influx into RBC

As showed in Fig.2, diet containing the highest concentration of fish oil (40%) increased total L-arginine influx in RBC compared with the standard diet and diets containing 0-20% of fish oil.

3.4. Basal NOS activity in RBC

The highest dose of 40% of fish oil was able to increase total NOS activity in RBC compared to standard diet and 0-20% fish oil diets. Moreover,

an increase in NOS activity was also observed in the F30 group compared to the F0 group (Fig.3).

3.5. eNOS, iNOS and arginase 1 expression in RBC

In our previous study, an immunoblot analysis using positive control (endothelial cells lysate and macrophage lysate for eNOS and iNOS, respectively) demonstrated that both isoforms eNOS and iNOS were expressed in the RBC from C57BL/6 mice [15].

As showed in figure 4A, RBC eNOS expression from F0, F10 and F20 groups was similar and reduced compared to control, F30 and F40 groups.

When RBC iNOS expression was evaluated, it was observed that the F20 group had a lower expression than the control and F40 groups (Fig. 4B).

The expression of the arginase 1 did not differ among groups (Fig. 5).

3.6. cGMP levels in RBC

The differences in the intraerythrocyte cGMP levels (fmol/10^8cells) were not statistically significant among the groups (C: 36.7 ± 1.8; F0: 31.7 ± 6.6; F10: 36.1 ± 3.8; F20: 20.8 ± 2.7; F30: 21.9 ± 3.1; F40: 35.3 ± 7.2).

3.7. Oxidative Status

Intraerythrocyte carbonyl groups was found to be elevated in the F0 mice compared to control, F20, F30, and F40 mice (Table 2). Additionally,

there was a trend for F10 group to increase RBC protein oxidation (+164%) and lipid peroxidation (+126%) compared to control diet. No significant difference in protein and lipid oxidation (Fig. 6) was observed among none of the high fish-oil fed mice.

The antioxidant enzyme analysis (Table 2) showed that superoxide dismutase activity in RBC from F0 and F10 groups was decreased compared to control group. Greater amounts of fish oil did not affect this parameter. The activity of the other antioxidant enzymes, CAT and GPx, did not differ among groups, except for a reduction in GPx levels from the F40 group compared to the F20 group.

4. Discussion

The present study investigated the L-arginine-NO pathway in RBC from mice fed on a high fat diet with increasing amounts of fish oil in place of lard, a source of saturated FAs. The major finding is that the highest fish oil feeding induces NO biosynthesis by an activation of L-arginine transport associated with an increase in basal activity and expression of NOS enzyme in RBC without affecting body mass. On the other hand, we could not observe that activation of L-arginine-NO pathway affects RBC levels of cGMP.

In spite of the identical energy content of the high fat diets in lard and fish oil, diets containing highest concentrations of fish oil were able to prevent obesity. This is consistent with that seen in other animal studies showing that *n*-3 PUFAs derived from fish oil protect against the development of obesity in animals exposed to an obesogenic diet and reduce body fat when the animals

were already obese. Effects reported in body fat may, at least in part, result from changes of gene expression in liver and adipose tissues that suppress fat deposition and increase fat oxidation [27].

L-arginine uptake has a pivotal role for NO synthesis, and alterations of L-arginine transport into RBC were previously demonstrated by our group in essential hypertension, and chronic renal and heart failure [28-30]. In the current study, we showed that the highest concentrations of fish oil were able to induce an activation of L-arginine transport, compared to a standard chow and diets containing the lowest doses of fish oil. It might be possible that these effects were found in L-arginine transport as a consequence of alterations of the pattern of the FAs composition of the RBC lipid bilayer. Incorporation of EPA and DHA into erythrocyte membranes as a consequence of *n*-3 PUFAs supplementation was reported in several studies [31, 32]. The incorporation of PUFAs into membranes alters their physicochemical properties, thereby influencing the environment of transmembrane proteins, such as carriers, altering the manner in which they interact with their substrates [33].

We have also demonstrated an increased in total NOS activity in RBC from the groups that received diets rich in fish oil. In addition, an overexpression of eNOS in RBC from the animals that received high fish oil diets was observed. Another pathway which is able to compete with the L-arginine-NO pathway is the urea cycle. It is well known that NOS and arginase share the same substrate and these pathways are strongly interrelated In the present study, we did not demonstrate alterations in the expression of arginase I. It is possible that activation of L-arginine transport in RBC could explain the sustained elevated NOS activity, which was not affected by urea

cycle. The factors that modulate NOS expression and activity in RBC and in the precursor cells of RBC in mice need to be further investigated.

Besides activation of L-arginine transport, other mechanisms can contribute to high activity of NOS in RBC. In endothelial cells [34] and possibly in RBC [17] eNOS is located in lipid rafts where it is associated with caveolin-1 (Cav-1), which maintains the enzyme in an inactive state [17]. Thus, as previously discussed, it is possible that the changes in the membrane lipid environment, as consequence of PUFAs incorporation, cause displacement of eNOS from the lipid rafts, which is critical for enzyme activation. Studies performed on endothelial cells have demonstrated that EPA and DHA modified the lipid composition of caveolae and reduced the association of eNOS and caveolin-1 [8, 9, 35]. Concurrently, these studies found elevated NOS activity. In agreement, another study demonstrated enhanced Cav-1 expression associated with decreased eNOS activity in the aortas of diet-induced obese rats [36]. In our previous study [15], we also demonstrated an impairment of NOS activity in the RBC of obese mice fed on a high fat diet containing 60% of lipids from an essentially saturated FA source. Despite the reduction in eNOS expression in RBC from obese mice fed with saturated FA, NOS activity was unaffected in the current study, where the amount of lipid in the diets was reduced by 10%. It is possible that an alteration in lipid quantity in the diet affects enzyme activity in RBC membrane.

The modulation of adipokine secretion by different types of dietary fat could also be a mechanism that regulation NOS activity. A potential relationship between insulin and NO production has been documented; insulin stimulates phosphatidylinositol 2-kinase/AKT pathway, which phosphorylates

and activates eNOS [37]. Moreover, an experimental study in hyperlipidemic rats demonstrated that adiponectin increased eNOS phosphorylation [38]. It is most likely that saturated FA rich diet would be expected to cause insulin resistance and reduce anti-inflammatory adipokine such as adiponectin, whereas the opposite effect might be expected in relation to unsaturated FA.

In general, NO formation activates cellular soluble GC to produce a second messenger molecule cGMP [39], however despite an upregulation of the L-arginine-nitric-oxide pathway observed in our assays, intraerythrocyte levels of cGMP were unaffected. It could be possible that NO derived from RBC was being scavenged by oxyhemoglobin (HbO_2) under normoxic conditions via conversion to methemoglobin and nitrate [40, 41] and thus inactivated. Such mechanism could account for the finding that NOS activation in RBC by shear stress is only observed in hypoxia, not in normoxia [42]. Therefore, the effect of NOS activation on cGMP levels in RBC might be limited to certain conditions, such as hypoxia and shear stress.

Another hypothesis for unchanged cGMP levels is the impairment of NO bioavailability due to oxidative stress. Paradoxically, despite the cardiovascular benefits provided by PUFAs, there has been concern that these fatty acids exhibit the highest sensitiveness to peroxidation [21], which could reduce NO bioavailability. Previous studies performed on RBC from animals and humans have shown that supplementation with these fatty acids can affect oxidative stability, depending on the dose [43-47]. Thus, oxidative damage rises as the amount of fish oil is increased, and when fish oil is administered at low doses, fish oil do not affect RBC susceptibility to lipid peroxidation.

In contrast, our study showed that the fish oil, even at high concentrations, was not able to induce RBC lipid peroxidation. This divergence might be due to the differences in research methods like specie, method of assessment employed to measure lipid peroxidation or duration and type of administration of fish oil.

Peroxidation of lipids can induce further harmful modifications in the assembly of the membrane, causing alterations in ion transport and enzymatic inactivation [21]. Venardos et al. [48] reported that peroxynitrite reduced endothelial L-[^{3}H]-arginine transport and intracellular concentration of L-arginine and L-citrulline. When we evaluate a protein oxidation marker, it was seen that the diets high in fish oil were not able to induce an increase of carbonyl content. Moreover, it was demonstrated that these diets led to an improvement in L-arginine uptake and enzymatic activity of NOS, which reinforces the suggestion that the concentrations of fish oil utilized in this study did not cause oxidative damage in RBC.

5. Conclusion

In conclusion, our study provides evidence that a substantial dose of fish oil, but not low doses, is associated to L-arginine transport activation and an increase in expression and activity of NOS enzymes in RBC. Furthermore, unlike the diet rich in saturated fatty acid, high fish oil diet neither induced oxidative damage in mice RBC nor increased body mass. Since RBCs present a functional NOS as demonstrated by our group and other authors [14,15], the

increase of the activity of this enzyme induced by fish oil may enhance intravascular NO pool.

Acknowledgements

We gratefully acknowledge the support of FAPERJ and PQ-CNPq.

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Table 1. Composition of the experimental diets.

Nutrient (U/kg diet)	Standard diet	High fat Diets				
	Control	F0	F10	F20	F30	F40
Casein (g)	140	175	175	175	175	175
Cornstarch (g)	620.7	347.6	347.6	347.6	347.6	347.6
Sucrose (g)	100	100	100	100	100	100
Soybean oil (g)	40	40	40	40	40	40
Fish oil (g)	**0**	**0**	**56**	**111**	**166**	**238**
Lard (g)	0	238	182	127	72	0
Fiber (g)	50	50	50	50	50	50
Mineral Mix (g)	35	35	35	35	35	35
Vitamin Mix (g)	10	10	10	10	10	10
L-cystine (g)	1.8	1.8	1.8	1.8	1.8	1.8
Choline (g)	2.5	2.5	2.5	2.5	2.5	2.5
Antioxidant (g)	0.008	0.06	0.06	0.06	0.06	0.06
Energy (kcal)	3800	5000	5000	5000	5000	5000
% as Carbohydrate	76	36	36	36	36	36
% as Protein	14	14	14	14	14	14
% as Fat	10	50	50	50	50	50
% as fish oil	**0**	**0**	**10**	**20**	**30**	**43**

HF- high fat; F0, HF diet with 0% of fish oil; F10, HF diet with 10% of fish oil; F20, HF diet with 20% of fish oil; F30, HF diet with 30% of fish oil; F40, HF diet with 40% of fish oil

Table 2. Oxidative stress parameters in RBC from mice fed with control diet or high fat diets with different amounts of fish oil.

	Control	F0	F10	F20	F30	F40
Carbonyl derivates (nmol/mg protein)	21.8 ± 6.6	77.3 ± 9.9[a]	57.5 ± 19.9	18.7 ± 4.2[b]	19.8 ± 7.2[b]	28.4 ± 8.8[b]
Superoxide dismutase (U/g Hb)	731 ± 80	174 ± 110[a]	112 ± 73[a]	567 ± 56	548 ± 42	577 ± 61
Catalase (U/g Hb)	0.058 ± 0.009	0.060 ± 0.007	0.050 ± 0.006	0.075 ± 0.007	0.069 ± 0.009	0.097 ± 0.017
Glutathione peroxidase (U/g Hb)	3.6 ± 0.6	3.5 ± 0.5	4.8 ± 0.7	6.1 ± 0.8	3.6 ± 0.8	2.9 ± 0.4[d]

Data denote mean ± S.E.M. [a] $p \leq 0.05$ vs control; [b] $p \leq 0.05$ vs F0; [d] $p \leq 0.05$ vs F20; (n=7-9).

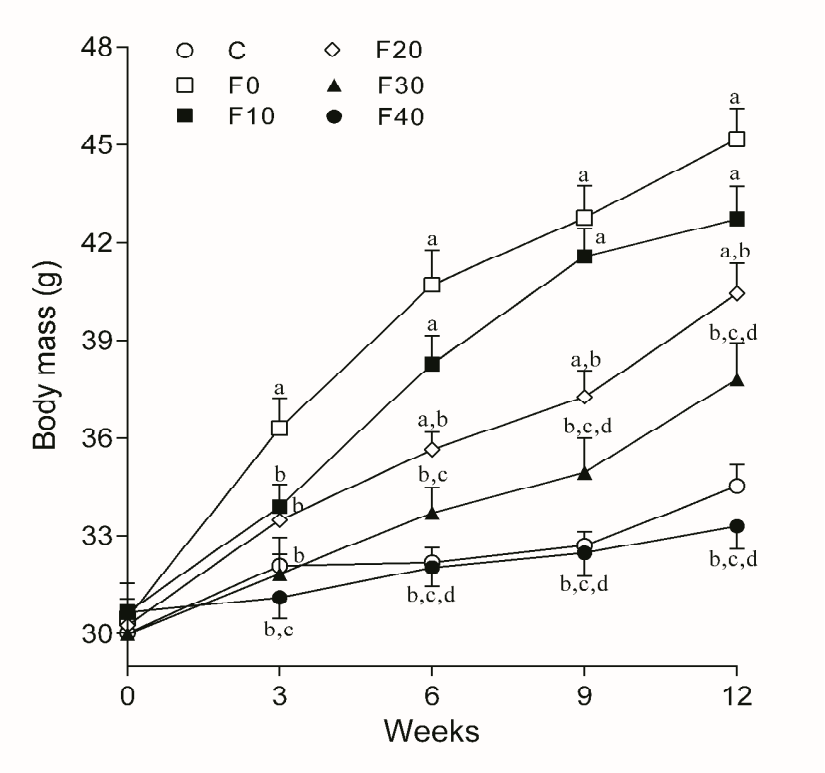

Figure 1. Effects of different concentrations of fish oil in body mass evolution (n=10-13). Mice were fed with control diet (C) or a high-fat diet containing 0%, 10%, 20%, 30% or 40% of fish oil (F0-F40 groups). Data denote mean ± S.E.M. [a] $p \leq 0.05$ vs control; [b] $p \leq 0.05$ vs F0; [c] $p \leq 0.05$ vs F10; [d] $p \leq 0.05$ vs F20.

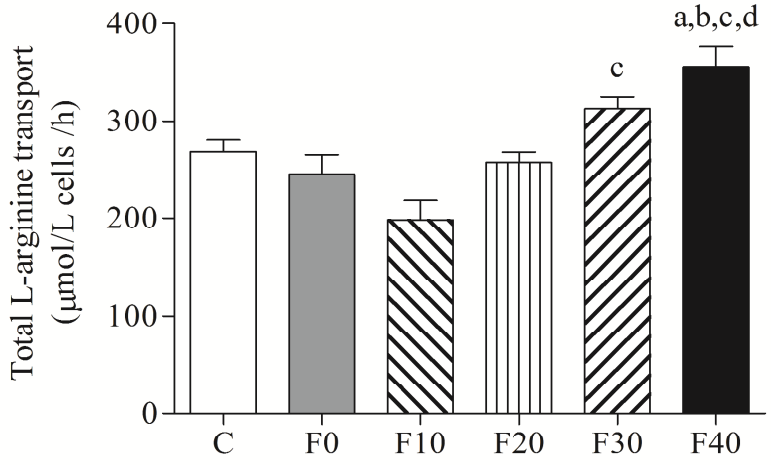

Figure 2. Effects of different concentrations of fish oil in L-arginine transport in mice red blood cells (n=6). Mice were fed with control diet (C) or a high-fat diet containing 0%, 10%, 20%, 30% or 40% of fish oil (F0-F40 groups). Data denote mean ± S.E.M. [a] $p \leq 0.05$ vs control; [b] $p \leq 0.05$ vs F0; [c] $p \leq 0.05$ vs F10; [d] $p \leq 0.05$ vs F20.

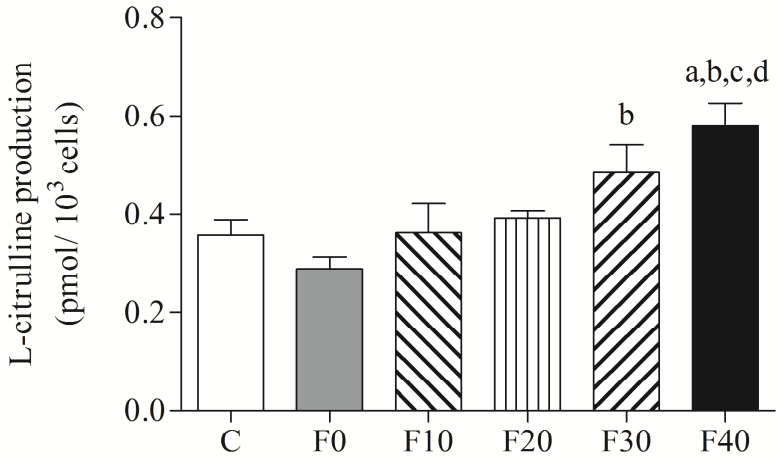

Figure 3. Effects of different concentrations of fish oil in basal nitric oxide synthase (NOS) activity in red blood cells (n=6). NOS activity was measured by conversion of L-[3H]- arginine to L-[3H]-citrulline. Mice were fed with control diet (C) or a high-fat diet containing 0%, 10%, 20%, 30% or 40% of fish oil (F0-F40 groups). Data denote mean ± S.E.M. a $p \leq 0.001$ vs control; b $p \leq 0.05$ vs F0; c $p \leq 0.05$ vs F10; d $p \leq 0.05$ vs F20.

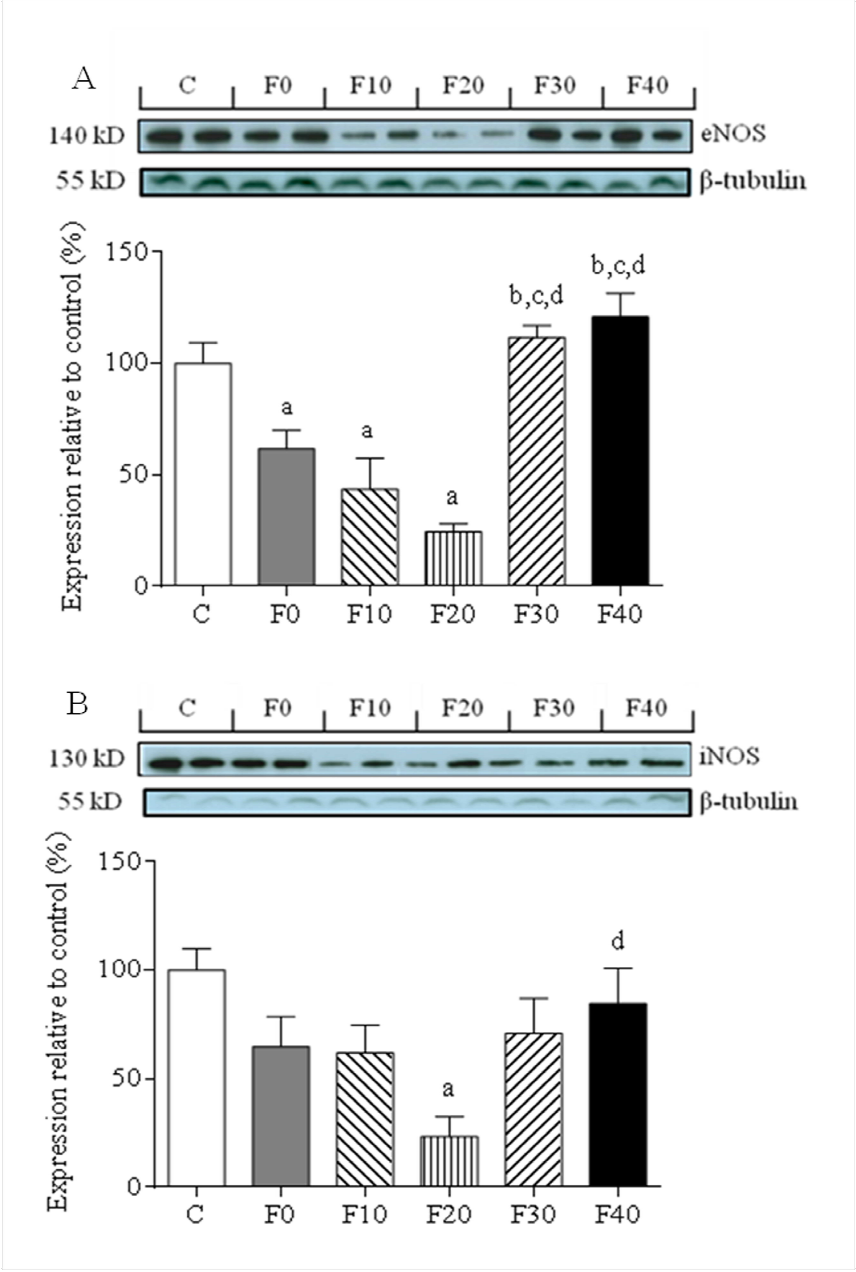

Figure 4. Effects of different concentrations of fish oil on expression of nitric oxide synthase (NOS) in mice red blood cells (n=5-6). (A) Expression of endothelial NOS (eNOS), (B) Expression of inducible NOS (iNOS). Representative western blots of eNOS or iNOS and β-tubulin are shown (insets). Bars represent mean ± S.E.M. ratio of NOS/β-tubulin from

densitometric analysis. Mice were fed with control diet (C) or a high-fat diet containing 0%, 10%, 20%, 30% or 40% of fish oil (F0-F40 groups). [a] $p \leq 0.05$ vs control; [b] $p \leq 0.05$ vs F0; [c] $p \leq 0.05$ vs F10; [d] $p \leq 0.05$ vs F20.

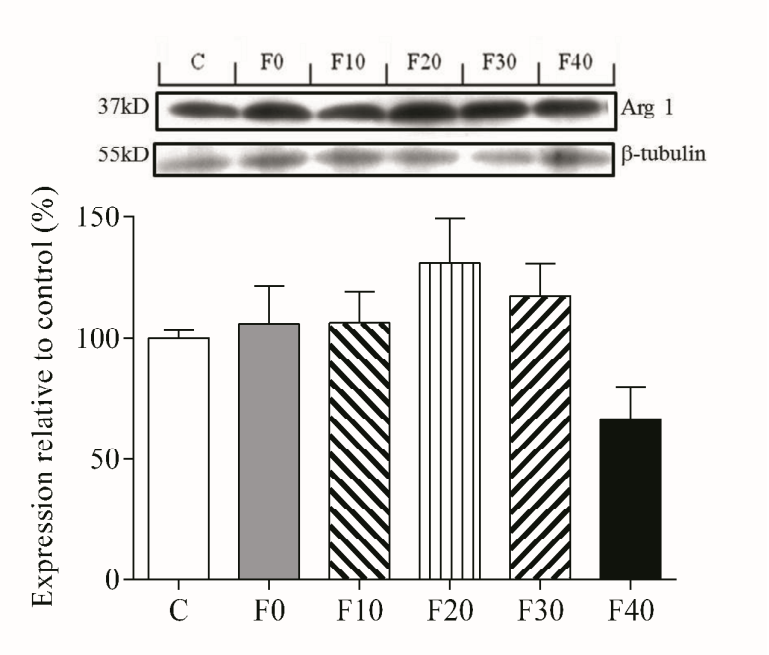

Figure 5. Effects of different concentrations of fish oil on expression of Arginase 1 (Arg 1) in mice red blood cells (n=5). Representative western blots of Arg 1 and β-tubulin are shown (insets). Bars represent mean ± S.E.M. ratio of NOS/β-tubulin from densitometric analysis.

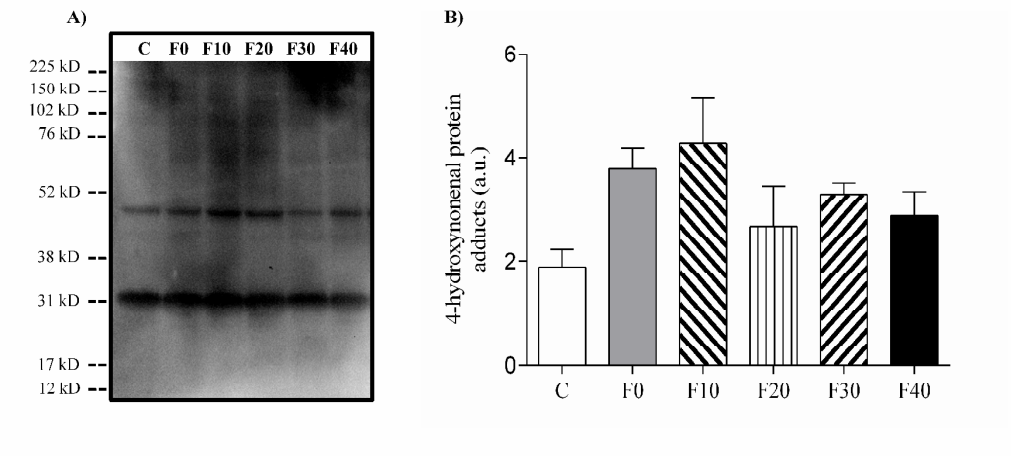

Figure 6. Expression of 4-hydroxynonenal protein adducts (4-HNE PAs) in mice red blood cells (n= 5). A) Representative western blots of 4-HNE PAs are shown. B) Bars represent mean ± S.E.M. of 4-HNE PAs bands quantification from densitometric analysis; a.u.: arbitrary units.