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n-3 Dietary supplementation and lipid metabolism: Differences between vegetable- and fish-derived oils

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ABSTRACT

The effect of flaxseed oil rich in linolenic acid (ALA), and a mixed oil (flaxseed oil and fish oil) rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on the lipid clearance and peroxisome proliferator activated receptors (PPARs) in liver and adipose tissue of rats fed for 30 days with the two oils was evaluated. The results showed that after treatment with the mixed oil the hematic triacylglycerol content was significantly decreased compared to control animals. Regarding the tissue distribution of the major omega-3 fatty acids, both oils were able to increase ALA, EPA, docosapentaenoic acid (DPA) in liver and adipose tissue; and DHA solely in the adipose tissue. Finally the treatment with either flaxseed or mixed oil increased hepatic PPAR-γ expression but only the mixed oil enhanced the hepatic expression of PPAR-α. No effect on adipose tissue PPAR-γ expression was observed with both oils' treatment.

1. Introduction

The use of nutraceutical and functional foods containing n-3 polyunsaturated fatty acids (n-3 PUFAs) has become a topic of great interest for the health of the world population, due to their reported and accepted beneficial effects. α-Linolenic acid (ALA) is the precursor of n-3 PUFAs from which eicosapentaenoic acid (C20:5n-3; EPA), docosapentaenoic acid (22:5n-3, DPA) and docosahexaenoic acid (C22:6n-3; DHA) are generated. A small amount of EPA and DHA (less than 5%) can be converted through enzymatic reactions by ALA in the human body (Brenna, 2002; Hoffman, DeMar, Heird, Birch, & Anderson, 2001). ALA and its metabolites are called essential fatty acids because they are fundamental for the human and animal growth, but they cannot be synthesized by the enzymatic pool, so they must be provided by diet (Burr & Burr, 1930; Lands, 1992). ALA is found in vegetable seeds like flax, rape, perilla, walnuts and chia and also in chloroplasts of leafy green vegetables (Adkins & Kelley, 2010). On the contrary, deep ocean fish are good sources of EPA and DHA, since algae, which are at the base of the marine food chain, have been shown to synthesize long chain PUFAs (Adkins & Kelley, 2010; Pereira, Leonard, Huang, Chuang, & Mukerji, 2004). The use of n-3 PUFAs plays an important role in the prevention and improvement of many cardiovascular diseases such as arrhythmia (Leaf, Kang, Xiao, & Billman, 2003), atherosclerosis (Thies et al., 2003), myocardial infarction (Harper & Jacobson, 2005; Rupp, 2006) and stroke (Iso et al., 2002; Skerrett & Hennekens, 2003). Randomized, double blind studies have found that fish oil supplementation has an anti-inflammatory effect against rheumatoid arthritis (Kremer et al., 1985; van der Tempel, Tulleken, Limburg, Muskiet, & van Rijswijk, 1990) and ulcerative colitis (Aslan & Triadafilopoulos, 1992).

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Several small studies demonstrated that depressed patients had significantly decreased serum concentration of total n-3 PUFAs and DHA, as well as an increased ratio of arachidonic acid (n-6 polyunsaturated fatty acid) versus n-3 PUFAs than controls (De Vriese, Christophe, & Maes, 2003; Frasure-Smith, Lespérance, & Julien, 2004; Maes et al., 1999). It is still controversial whether the supplementation of EPA and DHA in the diet could improve the depressive states. Some studies have denied this possibility (Jans, Giltay, & Van der Does, 2010; Rogers et al., 2008), but a recent work from Rondanelli et al. (2011) supports this hypothesis, since a supplementation of EPA and DHA for two months reduced the occurrence of depressive symptoms in elderly female patients, improved phospholipids fatty acids profile and health-related quality of life (Rondanelli et al., 2011). Finally, it has been demonstrated that the implemented diet with n-3 PUFA is positively associated with the prevention of neurodegenerative diseases (i.e. Alzheimer's disease) (Conquer, Tierney, Zecevic, Bettger, & Fisher, 2000; Jicha & Markesbery, 2010) and behavioural disorders (Brunner, Parhofer, Schwandt, & Bronisch, 2002; Perica & Delas, 2011).

The n-3 PUFAs like other fatty acids are well known ligands for peroxisome proliferator activated receptors (PPARs), nuclear receptors that act as transcription factors and modulate the expression of genes involved in lipid and glucose metabolism (Kersten, Desvergne, & Wahli, 2000; Kota, Huang, & Roufogalis, 2005). Three isotypes have been identified: (1) PPAR-α, highly expressed in brown adipose tissue and liver, that participates in lipids catabolism, (2) PPAR-β/δ and (3) PPAR-γ, which is manly expressed in adipose tissue where it is directly implicated in the lipid anabolic pathway (Kersten et al., 2000; Kota et al., 2005).

The purpose of the study was to compare the effect of flaxseed oil rich in ALA and a mixed oil derived from a mixture of flaxseed and fish oil on the lipid clearance and peroxisome proliferator activated receptors (PPARs) in liver and adipose tissue of rats fed for 30 days with the two oils.

2. Materials and methods

2.1. Rats and treatment

Thirty young (51–58 days) Sprague–Dawley male rats (Harlan Italy, Udine, Italy) weighing 210–238 g were used throughout the experiment in a controlled environment (air-conditioned room at 22 ± 1 °C and 60% humidity) maintained under a 12 h light/dark cycle. Rats had free access to water and food, a standard pellet containing carbohydrate 44.2%, crude protein 18.6%, neutral detergent fibre 14.7%, fat 6.2%, ash 5.3% and other nutrients 11.0% (Teklad Lab Animal Diets, Harlan, Italy). The animals were randomly assigned to one of the three groups (olive oil, flaxseed oil and mixed oil) and treated for 30 days, twice a day, by oral gavage. Treated animals received 0.47 ml of flaxseed oil (flaxseed oil group, FO; $N = 10$) and 0.96 ml of mixed oil (mixed oil group, MO; $N = 10$), corresponding to an ALA nominal content of 1.0 g/kg. The mixed oil was a combination of three different oils: flaxseed oil, rich in ALA; sunflower oil, rich in oleic acid and fish oil, rich in EPA and DHA. As control olive oil (olive oil group, OO; $N = 10$) was used at the single dose of 0.96 ml, since from our preliminary studies no difference between 0.47 and 0.96 ml was observed (data not published). The composition of the three oils is shown in Table 1. At the end of treatments, rats were anesthetized with CO_2 (g) and killed by decapitation. Rapidly liver and abdominal adipose tissue were removed and stored at −80 °C. Blood samples were also collected; plasma cholesterol and triacylglycerol levels were measured by a private medical laboratory.

All procedures were performed in accordance with the guidelines of the National (D.L. No. 116/1992) and European legislation (EEC No. 86/609) and of the National Institute of Health on the use and care of laboratory animals.

2.2. Fatty acids extraction, derivatization and RP-HPLC

The modified Dole extraction was used as previously described (Rustichelli, Avallone, Campioli, Braghiroli, & Baraldi, 2009). Tissues were homogenized with the extraction mixture (isopropanol/heptane/0.5 M H_2SO_4, 40:10:1, v/v/v). After 10 min heptane and water were added, then samples were sonicated in a cold ultrasonic bath for 1 min and centrifuged (800×*g*, 10 min at 4 °C). The extraction procedure was repeated twice and 2.5 ml of the combined organic layers were completely dried under a stream of nitrogen after addition of 300 μl internal standard (IS) eicosadienoic acid solution with acetonitrile. All the dried extracts were subjected to the derivatization procedure described below before RP-HPLC analysis. All reagents were purchased from Sigma–Aldrich Italy, Milano, Italy.

For the derivatization, 200 μl acetonitrile, 100 μl *p*-bromophenacyl bromide solution (6.0 mg/ml in acetonitrile), 100 μl 18-Crown-6 solution (40 mg/ml in acetonitrile) and 10 mg K_2CO_3 were added to the dried extracts (all reagents were purchased from Sigma–Aldrich, Italy). The vials were placed in a heater block and heated at 80 °C for 45 min under magnetic stirring, then allowed to cool and, finally, centrifuged (24,000×*g*, 5 min). The obtained supernatants were directly subjected to RP-HPLC analysis.

The HPLC system was a JASCO high-performance liquid chromatograph (Jasco Europe S.R.L., Cremella, Lecco, Italy) equipped with two PU-2080 Plus pumps, an HG-980-30 solvent mixing module and a UV-2075 Plus UV–VIS detector. The chromatographic column LiChroCART Purospher RP-18e (125 × 4 mm; 5.0 μm) was preceded by a Purospher RP-18e (4.0 × 4.0 mm; 5.0 μm) guard column (both columns were pur-

Table 1 – Fatty acid composition of the three tested oils.

Fatty acids	Olive oil (g/100 g oil)	Flaxseed oil (g/100 g oil)	Mixed oil (g/100 g oil)
EPA (20:5n-3)	n.p.	n.p	2.9
DHA (22:6n-3)	n.p.	n.p.	1.6
ALA (18:3n-3)	0.9	58.0	25.9
Linoleic acid (18:2n-6)	8.9	15.0	14.1
Oleic acid (18:1n-9)	75.0	18.0	43.6
Palmitic acid (16:0)	12.2	6.0	7.9
Stearic acid (18:0)	3.0	3.0	4.0

ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; n.p., not present.

chased from Merck kGaA, Darmstadt, Germany). Analyses were performed at 35 °C by a JASCO CO-2067 Plus column oven (Jasco Europe S.R.L.) with a mobile phase consisting of acetonitrile and water (Rustichelli et al., 2009). The column eluates were monitored at 254 nm. The chromatograph was controlled and data were evaluated by Hercule Lite Chromatography Interface and Borwin Software, respectively (Jasco Europe S.R.L.). Individual fatty acid standards were purchased from Sigma–Aldrich, Italy.

2.3. Immunoblot

Tissues (liver and adipose tissue) were homogenized in an extraction buffer containing sodium chloride 140 mM, potassium chloride 2 mM, potassium phosphate monobasic 1.5 mM, sodium phosphate dibasic 15 mM, sodium deoxycholate 0.5%, sodium dodecyl sulphate 0.1%, Nonidet P40 1% (all purchased from Sigma–Aldrich, Italy). Fresh mixture protease inhibitor cocktail (Sigma–Aldrich, Italy) was added to homogenates. Protein extracts were obtained by centrifugation of the homogenate at 4 °C, and the protein concentration was measured with Bradford assay (Bradford, 1976).

Proteins were separated by SDS–PAGE, then transferred to nitrocellulose membrane (Invitrogen S.R.L., Milano, Italy) and blocked overnight at 4 °C in blocking buffer (20 nM Trizma Base, 100 mM NaCl, 1% Tween-20, 10% skimmed milk; Sigma–Aldrich, Italy). Membranes were first incubated for 3 h at room temperature with a rabbit IgG anti PPAR-α polyclonal antibody or IgG anti PPAR-γ polyclonal antibody (1:500 dilution; Cayman Chemical Co., Ann Arbor, MI, USA). Finally, membranes were incubated for 1 h at room temperature with a goat anti-rabbit IgG HRP (1:4000 dilution; Cayman Chemical Co.).

After conjugation with the secondary antibody, the complex was visualized using an Amersham chemiluminescence kit (GE Healthcare Europe GmbH – Filiale Italiana, Milano, Italy). To verify the uniformity of protein loading, each sample was also incubated with anti β-actin (1:1000 dilution; Cell Signaling, Euroclone S.p.A., Pero, Milan, Italy) or β-tubulin (1:1000 dilution; Upstate, Millipore Corporation, Billerica, USA).

Band intensity was quantified by densitometry measurements of autoradiographs analysed by a Bio-Rad GS-690 Scanner (Bio-Rad, Segrate, Milano, Italy). Optical densities of samples were normalized to that of β-actin or β-tubulin.

2.4. Statistical analysis

Data, expressed as mean ± SEM and N, were analysed with one-way ANOVA followed by Dunnett's post hoc test between control group and treated groups by using the GraphPad Prism program (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were taken as the level of significance.

3. Results

3.1. Rats weight

After 30 days of treatment with oils, both treated rats fed with either flaxseed or mixed oil showed no significant change in body weights ($P > 0.05$) compared with the control group fed with olive oil (Fig. 1).

3.2. Changes in plasma lipids profile

The levels of the total cholesterol, HDL, LDL and triacylglycerols in rats' plasma were determined. After 30 days of treatment with the mixed oil, a decrease ($P < 0.05$) in the triacylglycerol content (67.60 ± 7.72; mean ± SEM, $N = 10$) was observed compared to the control group (113.40 ± 15.80; mean ± SEM, $N = 10$) (Table 2). On the contrary the total cholesterol, HDL and LDL were not significantly modified after the chronic treatment. The use of flaxseed oil for 30 days, likewise, did not change the aforementioned lipids profile.

3.3. n-3 Fatty acids RP-HPLC

Since rats' diet was supplemented with n-3 PUFAs, RP-HPLC was used to monitor the tissue levels of four well known n-3 PUFA: ALA, EPA, DPA and DHA.

After 30 days of treatment with flaxseed oil, ALA in the liver was significantly increased (6124 ± 546 ng/g tissue, $P < 0.001$) compared to the olive oil (1513 ± 204 ng/g tissue) (Table 3). Both EPA and DPA levels were significantly increased: EPA 14,151 ± 682 ng/g tissue ($P < 0.001$) compared to the olive oil (9379 ± 369 ng/g tissue) and DPA 2693 ± 200 ng/g tissue ($P < 0.001$) compared to the olive oil (1567 ± 123 ng/g tissue) (Table 3). After chronic treatment with the mixed oil, ALA in the liver was significantly increased (5561 ± 246 ng/g tissue, $P < 0.001$) compared to the olive oil (1513 ± 204 ng/g tissue) (Table 3). EPA hepatic levels were significantly increased

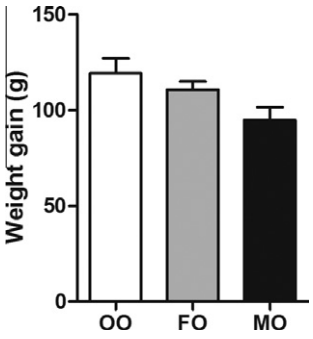

Fig. 1 – Flaxseed oil (FO) and mixed oil (MO) did not increase rats' weight after 30 days of treatment compared with the olive oil (OO) control. All values are expressed as mean ± SEM, N = 10.

Table 2 – Effect of dietetic oils on total cholesterol, HDL, LDL and triacylglycerol plasma levels.

Lipid (mg/dl blood)	Olive oil	Flaxseed oil	Mixed oil
Total cholesterol	81.80 ± 1.32	81.20 ± 2.85	78.80 ± 2.06
HDL	33.79 ± 0.86	35.40 ± 1.12	34.14 ± 0.88
LDL	10.60 ± 0.51	10.20 ± 0.58	11.60 ± 0.40
Triacylglycerols	113.40 ± 15.80	103.00 ± 11.63	67.60 ± 7.72*

Values are means ± SEM ($N = 10$).
* $P < 0.05$, one-way ANOVA followed by Dunnett's post hoc analysis.

Table 3 – Effect of flaxseed and mixed oils on hepatic ALA, EPA, DPA and DHA levels (ng/g tissue).

Fatty acid	Olive oil	Flaxseed oil	Mixed oil
ALA (18:3n-3)	1513 ± 204	6124 ± 546***	5561 ± 246***
EPA (20:5n-3)	9379 ± 369	14151 ± 682***	18774 ± 1216***
DPA (22:5n-3)	1567 ± 123	2693 ± 200***	2919 ± 91***
DHA (22:6n-3)	13171 ± 696	12191 ± 637	14808 ± 839

ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Values are means ± SEM (N = 10).

*** $P < 0.001$ versus olive oil, one-way ANOVA followed by Dunnett's post hoc analysis.

(18,774 ± 1216 ng/g tissue; $P < 0.001$) compared to the olive oil (9379 ± 369 ng/g tissue) as well as DPA hepatic levels (2919 ± 91 ng/g tissue; $P < 0.001$) compared to the olive oil (1567 ± 123 ng/g tissue) (Table 3). Regarding DHA, no increase in DHA hepatic level was observed after treatment with either flaxseed or mixed oil.

After 30 days of treatment with flaxseed oil, ALA in the adipose tissue was significantly increased (90.02 ± 13.32 mg/g tissue, $P < 0.001$) compared to the olive oil (11.70 ± 1.51 mg/g tissue) (Table 4). Also EPA, DPA and DHA levels were significantly increased: EPA 16.17 ± 1.77 mg/g tissue ($P < 0.001$) compared to the olive oil (4.74 ± 0.33 mg/g tissue), DPA 2.75 ± 0.26 mg/g tissue ($P < 0.05$) compared to the olive oil (1.46 ± 0.27 mg/g tissue) and DHA 37.24 ± 5.97 mg/g tissue ($P < 0.05$) compared to the olive oil (20.26 ± 0.79 mg/g tissue) (Table 4). After chronic treatment with the mixed oil, ALA in the adipose tissue was significantly increased (70.52 ± 7.98 mg/g tissue, $P < 0.001$) compared to the olive oil group (11.70 ± 1.51 mg/g tissue) (Table 4). EPA levels were significantly increased (15.70 ± 1.51 mg/g tissue) compared to the olive oil (4.74 ± 0.33 mg/g tissue) as well as DPA levels (2.64 ± 0.27 mg/g tissue; $P < 0.05$) compared to the olive oil (1.46 ± 0.27 mg/g tissue) and DHA levels (44.01 ± 8.71 mg/g tissue; $P < 0.05$) compared to the olive oil (20.26 ± 0.79 mg/g tissue) (Table 4).

Table 4 – Effect of flaxseed and mixed oils on adipose tissue ALA, EPA, DPA and DHA levels (mg/g tissue).

Fatty acid	Olive oil	Flaxseed oil	Mixed oil
ALA (18:3n-3)	11.70 ± 1.51	90.02 ± 13.32***	70.52 ± 7.98***
EPA (20:5n-3)	4.74 ± 0.33	16.17 ± 1.77***	15.70 ± 1.51***
DPA (22:5n-3)	1.46 ± 0.27	2.75 ± 0.26*	2.64 ± 0.27*
DHA (22:6n-3)	20.26 ± 0.79	37.24 ± 5.97*	44.01 ± 8.71*

ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Values are means ± SEM (N = 10).

* $P < 0.05$ and *** $P < 0.001$ versus olive oil, one-way ANOVA followed by Dunnett's post hoc analysis.

3.4. *Immunoblotting*

The protein expression of PPAR-α and γ in rats' livers and PPAR-γ in the adipose tissue was monitored by Immunoblotting. Immunoblots were analysed by densitometry and normalized to olive oil (OO). After chronic treatment with mixed oil (MO), a significant increase of both hepatic PPAR-α (1.78 ± 0.19, mean ± SEM; $N = 10$; $P < 0.05$) and PPAR-γ (1.60 ± 0.12, mean ± SEM; $N = 10$; $P < 0.05$) was observed compared to OO (Fig. 2A and B). On the contrary, after 30 days of treatment with flaxseed oil (FO), a significant increase was observed in hepatic PPAR-γ (1.73 ± 0.20, mean ± SEM; $N = 10$; $P < 0.01$) compared to OO (Fig. 2B). Regarding PPAR-γ in the adipose tissue, no change in its expression was observed either with FO or MO (Fig. 2C).

4. Discussion

Studies on the use of fish oils rich in n-3 PUFAs started in the late 60s, when it was shown that after chronic administration

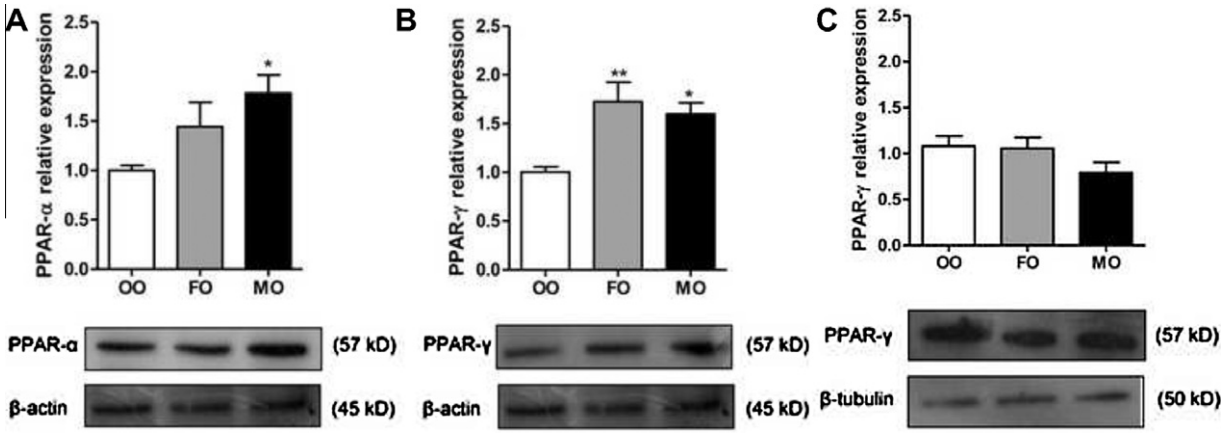

Fig. 2 – Chronic treatment for 30 days with flaxseed oil (FO) and mixed oil (MO) increases PPAR-γ hepatic protein expression, but only MO is able to increase PPAR-α hepatic protein expression. (A) Immunoblot of PPAR-α in rats' liver with densitometric analysis. (B) Immunoblot of PPAR-γ in rats' liver with densitometric analysis. (C) Immunoblot of PPAR-γ in rats' adipose tissue with densitometric analysis. Results are expressed as mean ± SEM, $N = 10$; * $P < 0.05$, ** $P < 0.01$, one-way ANOVA followed by Dunnett's post hoc test versus olive oil (OO).

of fish oil a general reduction in plasma triacylglycerol was observed (Engelberg, 1966). Only at the beginning of the 80s, different research groups demonstrated that the beneficial effect of fish oil in the reduction of plasma lipids was due to long chain n-3 PUFAs, EPA and DHA (Kobatake, Kuroda, Jinnouchi, Nishide, & Innami, 1984; Singer et al., 1983, 1984). In agreement with these studies and the current literature, after chronic treatment with the mixed oil enriched in EPA and DHA we observed a decrease in plasma triacylglycerols. This finding is very interesting because it confirms that the use of fish oil or long chain n-3 PUFAs-enriched oil is preferable to the one containing only ALA.

The conversion efficiency of short chain n-3 to long chain n-3 is below 5% in men and it is related to the concentration of n-6 fatty acids and long chain polyunsaturated fatty acids in the diet (Brenna, 2002; Gerster, 1998; Hoffman et al., 2001). Some studies suggest that dietary intake of ALA increased ALA itself, EPA and DPA, but it failed to change DHA in various animal tissues (muscle, liver and kidney) and plasma (Barceló-Coblijn, Collison, Jolly, & Murphy, 2005; Blank, Neumann, Makrides, & Gibson, 2002; Matthews, Homer, Thies, & Calder, 2000). Barceló-Coblijn, Collison, Jolly, and Murphy (2005) showed that rats fed on a diet containing EPA (13.63%) and DHA (11.85%) but no ALA dramatically increased hepatic and cardiac DHA content (Barceló-Coblijn et al., 2005). The Δ6 desaturase is an enzyme involved in the creation of the carbon/carbon double bond for the formation of highly unsaturated fatty acids. Δ6 desaturase is the rate limiting step for the conversion of ALA into stearidonic acid (18:4n-3, SDA) and tetracosapentaenoic acid (24:5n-3) into tetracosahexaenoic acid (24:6n-3). Finally after a β-oxidation process tetracosahexaenoic acid is finally converted into DHA. Portolesi, Powell, and Gibson (2007) demonstrated that the competition between tetracosapentaenoic acid and ALA for Δ6 desaturase may limit the accumulation of DHA in HepG2 cell membranes (Portolesi et al. 2007), because of its higher affinity for ALA than tetracosapentaenoic acid (D'andrea, Guillou, & Jan, 2002). In the *in vivo* model no change was observed in DHA hepatic content after chronic treatment with both oils, conversely hepatic ALA, EPA and DPA levels were significantly increased without affecting the total body weight. The explanation for the failure to increase DHA may be due to the limited content of EPA and DHA, but especially the high tone of ALA, that as described above has a very high affinity for the Δ6 desaturase.

The treatment with mixed oil increased the hepatic protein concentration of PPAR-α, on the other hand after dietary supplementation with only ALA (flaxseed oil) no change was observed. It was previously reported that a 4 week chronic treatment with a fish oil enriched diet increased hepatic PPAR-α mRNA in C57B1/6 mice (Larter et al., 2008). Tanaka et al. (2010) showed that chronic treatment for 12 weeks with EPA, instead, did not change hepatic PPAR-α mRNA in the mouse. These results suggest that the effect of n-3 fatty acids on hepatic PPAR-α may be due to DHA, but further studies are needed to evaluate this idea. After chronic treatment with either flaxseed or mixed oil, we observed an increase in hepatic PPAR-γ protein expression. Kim, Lee, Park, Jeon, and Choi (2008) found that ob/ob mice treated for 12 weeks with a high EPA and DHA diet increased the hepatic content of PPAR-γ mRNA. The role of hepatic PPAR-γ had not yet been well clarified because of its low abundance in non-steatotic liver. Gavrilova et al. (2003) demonstrated that PPAR-γ contributes to triacylglycerol homeostasis, regulating triacylglycerol clearance and the lipogenic program in both lipoatrophic AZIP mice and mice with diet-induced obesity. Finally, from our results the expression of PPAR-γ in the adipose tissue after chronic treatment with flaxseed and mixed oil did not change. Conversely, the adipose tissue content of n-3 PUFA was increased for all the four tested fatty acids. This result is very interesting because it shows how the use of n-3 PUFA may interfere with the lipid catabolism without affecting the fat accumulation and weight gain.

In conclusion we demonstrated that the chronic administration of marine-derived n-3 PUFAs improves the lipid clearance compared with a diet containing only vegetable-derived n-3 PUFAs. Our results would better explain the different role and potency of each n-3 PUFA in the lipid metabolism control, in particular DHA that appeared to be the most interesting among the n-3 fatty acid pool.

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