

Effects of fish oil- and olive oil-rich diets on iron metabolism and oxidative stress in the rat

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The objective of the present study was to examine the effects of fish oil (FO)- and olive oil (OO)-rich diets on Fe metabolism and oxidative stress. Rats were fed for 16 weeks with diets containing 50 g lipids/kg; either OO, maize oil (MO) or FO. OO or MO diets contained a standard amount (100 mg/kg) of all-rac- α -tocopheryl acetate. FO diets were supplemented with 0, 100 or 200 mg all-rac- α -tocopheryl acetate/kg (FO-0, FO-1 or FO-2 diets, respectively). At the end of the feeding period, we measured non-haem Fe stores in liver and spleen, and erythrocyte and reticulocyte count. We also determined antioxidants and products derived from lipid peroxidation in plasma and erythrocytes. Our results showed reduced non-haem Fe stores in rats fed any of the FO diets. Reticulocyte percentage was higher in the rats fed FO-0 and FO-1. Plasma α -tocopherol was very low in rats fed the FO-0 diet. Rats fed the FO-1 and FO-2 diets showed higher α -tocopherol in plasma than the FO-0 group but lower than the MO or OO groups. We did not observe such differences in the α -tocopherol content in erythrocyte membranes. Superoxide dismutase and glutathione peroxidase activities were lower in the erythrocytes of rats fed the FO-0 diet. The products derived from lipid peroxidation were also higher in the FO groups. The administration of FO-rich diets increased lipid peroxidation and affected Fe metabolism. On the other hand, the OO-rich diet did not increase oxidative stress and did not alter Fe metabolism. Based on these results, we conclude that FO supplementation should be advised carefully.

Fatty acids: Iron metabolism: Oxidative stress: Vitamin E

The health benefits of fish oil (FO) have been extensively studied since the observation that the incidence of cardiovascular disease was lower among populations that consume large amounts of fish (Bang & Dyerberg, 1972). Since then, many clinical studies have indicated that diets rich in FO show not only cardiovascular protection (Singer *et al.* 1992; Engler, 1994), but also anti-inflammatory and anti-thrombotic properties (Ågren *et al.* 1997; Carbonell *et al.* 1997; Connor, 2000; Uauy & Valenzuela, 2000; Moreno *et al.* 2001). The component of FO responsible appears to be the high content of *n*-3 polyunsaturated fatty acids (PUFA), particularly eicosapentaenoic acid (20:5*n*-3) and docosahexaenoic acid (22:6*n*-3) (Bang & Dyerberg, 1987). Consequently, concentrates of *n*-3 PUFA have been used as medicinal and/or nutritional supplements (Drevon, 1992; Lands, 1992). Although the therapeutic effects of *n*-3 PUFA from FO have been well described, their impact on Fe metabolism remains unclear.

In a previous study (Rodríguez *et al.* 1996), we observed

that rats fed an FO-rich diet showed an increase in Fe absorption, when compared with rats fed a control diet. We suggested that this increase in Fe absorption was related to changes in oxidative stress. It has been demonstrated that the ingestion of *n*-3 PUFA derived from FO increases the susceptibility of erythrocyte membranes to lipid oxidation (Rodríguez *et al.* 1996) and their requirements for vitamin E (Ibrahim *et al.* 1997; McGuire *et al.* 1997). However, no studies have been carried out on the underlying mechanisms of FO involved in the alteration of Fe absorption.

Besides FO, olive oil (OO) is also related to cardiovascular health (Lairon, 1999). OO, the main source of fat in the Mediterranean diet, is rich in oleic acid, a monounsaturated fatty acid. Recent studies have shown that diets rich in monounsaturated fatty acids also have favourable effects on the coagulation process (Roche *et al.* 1998) and inflammation (Yaqoob *et al.* 1998; Moreno *et al.* 2001). Compared with FO, OO shows a lower rate of

Abbreviations: FO, fish oil; GPx, glutathione peroxidase; MDA, malondialdehyde; MO, maize oil; OO, olive oil; PUFA, polyunsaturated fatty acids; SOD, superoxide dismutase; TAS, total antioxidant status; TBARS, thiobarbituric acid-reactive substances.

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oxidation and production of free radicals (Ruiz-Gutiérrez *et al.* 1999). However, it is not known if OO modulates Fe metabolism.

The objective of the present study was to analyse the effect of FO- and OO-rich diets on Fe metabolism and oxidative stress. As FO is very susceptible to oxidation we prepared three FO diets with different levels of vitamin E (0, 100 or 200 mg all-rac- α -tocopheryl acetate/kg) and an OO-rich diet with 100 mg all-rac- α -tocopheryl acetate/kg. We measured Fe stores in liver and spleen, erythrocyte and reticulocyte count, and antioxidants and products from lipid oxidation in plasma and erythrocytes. The results were compared with those obtained from rats fed diets rich in MO and 100 mg all-rac- α -tocopheryl acetate/kg.

Methods

Animals and diets

Male Sprague–Dawley rats were purchased from Harlan Ibérica (Barcelona, Spain) and housed in the Department of Animal Care at the Faculty of Biology at the University of Barcelona. The experimental protocols were reviewed and approved by the Ethical Committee of the Faculty of Biology in accordance with the European Community guidelines.

After weaning, rats were fed isoenergetic semi-purified diets for 16 weeks (Table 1) containing 50 g lipids/kg, either as refined OO (rich in 19:1*n*-9), refined MO (rich in 18:2*n*-6), or FO (rich in 20:5*n*-3 and 22:6*n*-3) as refined menhaden oil. The fatty acid composition of the diets was determined according to Haan *et al.* (1979) (Table 2). The diets also contained 50 mg Fe/kg. Oils used in these experiments provided between 2.3 and 2.5 mg α -tocopherol/kg diet. The OO and MO diets contained 100 mg all-rac- α -tocopheryl acetate/kg (equivalent to 67 IU α -tocopherol/kg) and the FO diets contained 0 (FO-0), 100 (FO-1) or 200 (FO-2) mg all-rac- α -tocopheryl acetate/kg. No other

Table 1. Composition of semi-purified diets (g/kg)

Component	Amount
Casein	220
Maize starch	446
Sucrose	228
Cellulose	10
DL-Methionine	1
Mineral mix*	35
Vitamin mix†	10
Oil‡	50

* Mixture provided the following (g/kg diet): calcium carbonate, 12.5; mono-potassium phosphate, 8.75; sodium chloride, 2.59; potassium sulfate, 1.63; potassium citrate monohydrate, 0.98; magnesium oxide, 0.84; zinc carbonate, 0.058; manganese carbonate, 0.022; copper carbonate, 0.011; potassium iodate, 0.0035; sodium selenate anhydrous, 0.00036; ammonium molybdate-4H₂O, 0.00028; sodium meta silicate-9H₂O, 0.051; chromium potassium sulfate-12H₂O, 0.000097; boric acid, 0.0029; sodium fluoride, 0.0022; nickel carbonate, 0.0011; lithium chloride, 0.00061; ammonium vanadate, 0.00023.

† Mixture provided the following (g/kg diet): niacin, 30 mg; D-calcium pantothenate, 16 mg; pyridoxine-HCl, 7 mg; thiamine-HCl, 6 mg; riboflavin, 6 mg; folic acid, 2 mg; biotin, 0.2 mg; vitamin B₁₂ (0.1% trit), 25 mg; vitamin A palmitate 1.2 mg; vitamin D₃ (25 µg); menadione, 0.75 mg.

‡ Olive oil, maize oil or fish oil.

Table 2. Fatty acid composition (g/kg) of the diets

Fatty acids*	OO	MO	FO
14:0	1.02	0.80	3.90
16:0	5.25	6.55	9.38
16:1	0.32	nd	5.10
18:0	1.72	1.25	2.10
18:1 <i>n</i> -9	36.25	12.15	6.25
18:1 <i>n</i> -7	nd	nd	0.55
18:2 <i>n</i> -6	3.8	28.3	0.86
18:3 <i>n</i> -3	0.55	nd	0.92
18:4 <i>n</i> -3	nd	nd	2.45
20:1 <i>n</i> -9	nd	nd	0.81
20:4 <i>n</i> -6	nd	nd	0.70
20:5 <i>n</i> -3	nd	nd	8.73
22:5 <i>n</i> -6	nd	nd	0.20
22:6 <i>n</i> -3	nd	nd	4.27
24:1 <i>n</i> -9	nd	nd	0.34

OO, olive oil; MO, maize oil; FO, fish oil.

* Fatty acids not reaching 0.2 g/kg diet are considered not detected (nd).

antioxidants were present in the oils or diets. Oils and all-rac- α -tocopheryl acetate were provided by Sigma (St Louis, MO, USA). Food was provided daily and any food remaining was also removed daily. Diets were manufactured weekly and stored at -20°C under vacuum to prevent oxidation. No significant increase in the peroxidation index was observed during the 7 d storage of diets. The peroxide value of these non-oxidised FO diets was <10 meq O₂/kg when ready for consumption. Rats were kept at a constant temperature of $21-23^{\circ}\text{C}$ and humidity of 50–60% with a 12 h light–dark cycle.

At the end of the feeding period, rats were fasted overnight and exsanguinated by cardiac puncture. Plasma was stored at -80°C until used. The liver was perfused through the subhepatic vein with NaCl solution (9 g/l) to eliminate the blood in the organ. The liver and the spleen were then excised, washed with saline, weighed and stored at -80°C until analysed.

Iron stores: haematological and faecal analysis

Fe stores in liver and spleen were determined by the method of Torrance & Bothwell (1968). Packed cell volume and haemoglobin concentration (Drabkin & Austin, 1935) were measured in fresh blood samples. Erythrocytes were counted by flow cytometry. Reticulocytes were also counted by flow cytometry following Davis *et al.* (1995) and using Syto-13 (Molecular Probes, Eugene, OR) as a fluorescent probe. Plasma Fe concentration and total Fe-binding capacity were measured using the FerroZine kit (Boehringer Mannheim, Mannheim, Germany). The loss of blood by the gastrointestinal tract was measured by the porphyrin analysis of faeces periodically collected (Needham & Simpson, 1952).

Antioxidant analysis

α -Tocopherol was assessed by HPLC in plasma and erythrocyte membranes (Bieri *et al.* 1979). HPLC separation was performed on a 5 µm LiChrospher 100 RP-18 column (250 mm \times 4.6 mm internal diameter) (Merck,

Darmstadt, Germany) using α -tocopheryl acetate as internal standard. Samples were eluted with methanol at a flow rate of 1.5 ml/min and their ultraviolet absorption at 290 nm was recorded. Values of α -tocopherol in plasma are expressed in $\mu\text{mol/l}$ and in erythrocytes in $\text{nmol}/\mu\text{mol}$ phospholipids. Phospholipids were measured in the erythrocyte extracts using the Phospholipids commercial kit (Boehringer Mannheim).

Total sulfhydryl groups were measured in plasma according to Hu (1994) and are expressed in mmol/l . Plasma uric acid was determined using a kit (Boehringer Mannheim) and is expressed in $\mu\text{mol/l}$. Caeruloplasmin was assessed following the method described by Sunderman & Nomoto (1970) and the results are shown in mg/ml . Total antioxidant status (TAS) of plasma was measured using the TAS commercial kit (Randox Laboratories Ltd., Crumlin, UK) and the results are given in $\mu\text{mol/l}$.

The Cu, Zn-superoxide dismutase (SOD) (*EC* 1.15.1.1) activity in erythrocytes was measured according to the technique described by Marklund (1985) which is based on the inhibition of pyrogallol auto-oxidation in the presence of SOD. Results are expressed as U/g haemoglobin. Catalase (*EC* 1.11.1.6) activity in erythrocytes was determined at room temperature by following the decomposition of hydrogen peroxide at 240 nm (Aebi, 1984). The rate constant (k ; $k = 10^7 \text{ l}/(\text{mol} \times \text{s})$) for the first 30 s was calculated. Results are expressed as k/g haemoglobin. Glutathione peroxidase (GPx) (*EC* 1.11.1.9) was also assessed in erythrocytes by the Ransel commercial kit (Randox Laboratories Ltd.). Results are expressed in U/g haemoglobin.

Parameters of oxidative stress

Free malondialdehyde (MDA) in plasma was measured following the HPLC technique of Kawai *et al.* (1989) using a $5 \mu\text{m}$ Lichocart Lichrosphere RP-18 column (125 mm \times 4 mm internal diameter) (Merck) with a mobile phase consisting of sodium dihydrogenphosphate–acetonitrile–isopropanol (0.01 mol/l; 70:20:10, by vol.), and detection at 315 nm.

Thiobarbituric acid-reactive substances (TBARS) in plasma were determined by the method of Yagi (1984) with a minor modification: butylated hydroxytoluene and EDTA were added to the reaction mixture as antioxidants

at a final concentration of 0.01 % and $1.3 \mu\text{mol/l}$, respectively. TBARS values are expressed in terms of MDA equivalents as nmol/mg lipids. Lipids were measured using the Total Lipids commercial kit (Bio Mérieux, Lyon, France).

The determination of conjugated dienes in plasma is based on its maximum absorbance at 234 nm. Therefore, plasma samples were extracted according to Folch *et al.* (1957) and using dithiothreitol as antioxidant at a final concentration of 9 mmol/l . The results are expressed as absorbance/mg lipids. Total lipids were measured as described earlier.

The susceptibility of erythrocytes to lipid peroxidation was determined by measuring the TBARS (Stocks & Dormandy, 1971). Erythrocytes freshly suspended in PBS were exposed to hydrogen peroxide solution (10 mmol/l) in the presence of sodium azide (1 mmol/l). After 1 h incubation at 37°C , a portion was precipitated by half the volume of trichloroacetic acid–arsenite. Thiobarbituric acid was added to the supernatant fraction and samples were placed in boiling water for 15 min. Possible interferences were eliminated by measuring the difference in absorption between 540 and 620 nm. Results are expressed in $\text{nmol}/(\text{g}$ haemoglobin \times h).

Statistical analysis

The results are expressed as mean values with their standard errors, of seven to eight animals per group. Statistical analysis of the data was performed using GraphPad InStat statistical software v. 2.04a (1990). Data were analysed by one-way ANOVA with five groups per variable. The Student–Newman–Keuls multiple comparison test was used to detect significant differences between the groups ($P < 0.05$).

Results

After 16 weeks, rats fed the FO-0 diets had a lower body weight (467 (SE 9) g) than the rest of the groups (552 (SE 16) g in rats fed the MO diet) (Table 3). The absolute weights of the liver and the spleen were similar in the different groups of rats (Table 3). However, the relative weights in rats fed the FO-0 diet were significantly

Table 3. Weights and non-haem iron stores of liver and spleen in rats fed for 16 weeks on diets containing different oils* (Mean values with their standard errors for seven to eight rats per group)

Diet	Body weight (g)		Liver weight (g)		Liver iron stores (μmol Fe)		Spleen weight (g)		Spleen iron stores (μmol Fe)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
OO	537 ^b	14	14.5 ^a	0.8	29.4 ^{ab}	1.6	0.92 ^a	0.06	16.4 ^b	1.7
MO	552 ^b	16	15.6 ^a	1.0	32.7 ^b	2.3	0.94 ^a	0.07	18.2 ^b	1.4
FO-0	467 ^a	9	16.3 ^a	0.4	26.2 ^a	2.7	1.23 ^a	0.06	9.4 ^a	1.3
FO-1	557 ^b	22	15.7 ^a	0.8	23.3 ^a	2.5	1.02 ^a	0.08	8.7 ^a	1.3
FO-2	552 ^b	11	15.6 ^a	0.7	24.9 ^a	2.7	1.02 ^a	0.06	9.1 ^a	1.3

OO, olive oil; MO, maize oil; FO-0, fish oil with no all-rac- α -tocopheryl acetate; FO-1, fish oil with 100 mg all-rac- α -tocopheryl acetate/kg; FO-2, fish oil with 200 mg all-rac- α -tocopheryl acetate/kg.

^{a,b}Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

* For details of diets and procedures, see Tables 1 and 2, and p. 12.

Table 4. Erythrocytes and reticulocytes in rats fed for 16 weeks on diets containing different oils*

(Mean values with their standard errors for seven to eight rats per group)

Diet	Erythrocytes ($10^{12}/l$)		Reticulocytes (%)	
	Mean	SE	Mean	SE
OO	6.89 ^a	0.25	3.67 ^a	0.24
MO	7.10 ^a	0.23	3.58 ^a	0.28
FO-0	7.13 ^a	0.33	5.01 ^b	0.53
FO-1	7.22 ^a	0.30	5.13 ^b	0.41
FO-2	7.89 ^a	0.28	4.41 ^{ab}	0.20

OO, olive oil; MO, maize oil; FO-0, fish oil with no all-rac- α -tocopheryl acetate; FO-1, fish oil with 100 mg all-rac- α -tocopheryl acetate/kg; FO-2, fish oil with 200 mg all-rac- α -tocopheryl acetate/kg.^{a,b}Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

*For details of diets and procedures, see Tables 1 and 2, and p. 12.

increased when compared with the other groups. The relative weight of liver of rats fed the MO diet was 2.83 g/100 g body weight, while it was 3.51 g/100 g in rats fed FO-0, which represented a 24 % increase. In spleen such differences were even greater. In rats fed the MO diet, the relative weight of the spleen represented 0.17 g/100 g body weight while in FO-0 rats it was 0.27 g/100 g, a 59 % increase. The OO diet did not alter the relative weight of liver and spleen when compared with the MO group.

Total non-haem Fe stores in liver and spleen (Table 3) were significantly lower in rats fed the FO diets compared with rats fed the OO or MO diets (Table 3). However, the differences increased when values were expressed per g of organ. Packed cell volume, haemoglobin concentration, plasma Fe concentration and total Fe-binding capacity did not differ among groups (43.7 %, 129 g/l, 26.0 mmol/l and 72.4 mmol/l, respectively, for the MO group). The number of erythrocytes was the same for all groups of rats, but the percentage of reticulocytes was significantly greater in rats fed the FO-0 and FO-1 diets than in rats fed the OO or MO diets (Table 4). No blood loss was detected in the faeces during the present study.

Rats fed the FO-0 diets had significantly lower α -tocopherol in plasma than the rest of the groups

(Table 5). The MO and OO groups showed higher levels of α -tocopherol in plasma (15.52 μ mol/l for MO-fed rats) than those of the FO-1 (8.73 μ mol/l) and FO-2 (8.57 μ mol/l) groups. Total sulfhydryl groups, uric acid, caeruloplasmin and TAS showed no significant differences among the groups (Table 5).

The FO-0 rats also showed the lowest amount of α -tocopherol (0.24 nmol/ μ mol phospholipids) in erythrocyte membranes (Table 6). However, we observed no differences among the remaining groups. SOD and GPx activity were significantly lower in erythrocytes of rats fed the FO-0 diets than in the rest of the groups (Table 6). Rats fed the MO diet had 907 U SOD activity/g haemoglobin, while in rats fed the FO-0 diet such activity was only 609 U/g haemoglobin, which represents a 32 % reduction. GPx activity was 319 U/g haemoglobin in rats fed FO-0 diets, a 20 % reduction when compared with rats that received the MO diet (Table 6). The OO diet did not alter either SOD or GPx activity when compared with the MO group (Table 6). Catalase activity was similar in all groups (Table 6).

Free MDA was not detected in the plasma samples analysed. However, TBARS and conjugated dienes were significantly higher in rats fed the FO diets, irrespective of the amount of all-rac- α -tocopheryl acetate supplemented (Table 7).

Erythrocyte membranes of rats fed any of the FO diets showed a greater auto-oxidizing potential of added hydrogen peroxide measured as formation of TBARS (Table 7) than those rats fed the OO or MO diets.

Discussion

The ingestion of diets rich in FO and OO favourably affects atherosclerosis, CHD and inflammatory disease (Van der Tempel *et al.* 1990; Singer *et al.* 1992; Engler, 1994; Carbonell *et al.* 1997; Lairon, 1999; Connor, 2000; Perona & Ruiz-Gutierrez, 2000; Uauy & Valenzuela, 2000; Moreno *et al.* 2001). This has resulted in recommendations to increase the consumption of FO and OO (Lairon, 1997; Uauy & Valenzuela, 2000). Extensive literature exists concerning the optimal supply of monounsaturated fatty acids (Roche, 1999) and PUFA (Roche, 1999; Uauy

Table 5. Plasma α -tocopherol, total sulfhydryl (SH) groups, uric acid, caeruloplasmin and total antioxidant status (TAS) in rats fed for 16 weeks on diets containing different oils*

(Mean values with their standard errors for seven to eight rats per group)

Diet	α -Tocopherol (μ mol/l)		Total SH groups (mmol/l)		Uric acid (μ mol/l)		Caeruloplasmin (mg/ml)		TAS (mmol/l)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
OO	13.31 ^c	1.17	0.25 ^a	0.04	79.78 ^a	7.35	0.51 ^a	0.03	0.70 ^a	0.03
MO	15.52 ^c	2.32	0.27 ^a	0.07	94.77 ^a	18.26	0.52 ^a	0.03	0.65 ^a	0.02
FO-0	0.36 ^a	0.06	0.26 ^a	0.03	85.82 ^a	10.66	0.62 ^a	0.03	0.58 ^a	0.05
FO-1	8.73 ^b	0.67	0.25 ^a	0.03	61.55 ^a	5.38	0.59 ^a	0.03	0.67 ^a	0.05
FO-2	8.57 ^b	0.70	0.24 ^a	0.03	87.14 ^a	13.21	0.58 ^a	0.03	0.69 ^a	0.03

OO, olive oil; MO, maize oil; FO-0, fish oil with no all-rac- α -tocopheryl acetate; FO-1, fish oil with 100 mg all-rac- α -tocopheryl acetate/kg; FO-2, fish oil with 200 mg all-rac- α -tocopheryl acetate/kg.^{a,b,c}Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

*For details of diets and procedures, see Tables 1 and 2, and p. 12.

Table 6. α -Tocopherol and superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) activities in erythrocyte membranes in rats fed for 16 weeks on diets containing different oils*

(Mean values with their standard errors for seven to eight rats per group)

Diet	α -Tocopherol (nmol/ μ mol phospholipids)		SOD (U/g haemoglobin)		Catalase (k/g haemoglobin)†		GPx (U/g haemoglobin)	
	Mean	SE	SEM	SE	Mean	SE	Mean	SE
OO	5.24 ^b	0.53	1174 ^b	146	209 ^a	7	402 ^b	22
MO	4.17 ^b	0.28	907 ^b	66	187 ^a	4	398 ^b	12
FO-0	0.24 ^a	0.08	609 ^a	55	199 ^a	5	319 ^a	13
FO-1	4.21 ^b	0.71	821 ^b	48	200 ^a	9	357 ^b	18
FO-2	4.40 ^b	0.62	855 ^b	75	197 ^a	10	356 ^b	13

OO, olive oil; MO, maize oil; FO-0, fish oil with no all-rac- α -tocopheryl acetate; FO-1, fish oil with 100 mg all-rac- α -tocopheryl acetate/kg; FO-2, fish oil with 200 mg all-rac- α -tocopheryl acetate/kg.^{a,b}Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

* For details of diets and procedures, see Tables 1 and 2, and p. 12.

† k = 10⁷ l/(mol \times s).

et al. 1999) and there is a high degree of variation in the amount of *n*-3 PUFA that is recommended. However, the impact of FO- or OO-rich diets on Fe metabolism and oxidative stress has not been studied. It has been demonstrated that FO- or *n*-3 PUFA-rich diets reduce α -tocopherol content of plasma and membranes and increase oxidative stress (Garrido *et al.* 1993; Rodríguez *et al.* 1996; Kubo *et al.* 1997; McGuire *et al.* 1997), while OO-rich diets decrease the rate of peroxidation and production of free radicals (Ruiz-Gutiérrez *et al.* 1999). But, no clear relationship of these events to Fe metabolism has been established.

In the present study we observed a decrease in the non-haem Fe stores in the liver and spleen of rats fed the FO diets when compared with those rats that received the OO or MO diets. Nevertheless, the supplementation of the FO diet even with 200 mg all-rac- α -tocopheryl acetate/kg (FO-2 group) did not increase Fe stores in both organs. These results are similar to those obtained by Rodríguez *et al.* (1996) who administered a sardine oil-rich diet with 100 mg all-rac- α -tocopherol/kg for 16 weeks. Moreover, in accordance with Rabbani *et al.* (1999), we observed an increase in the relative weight of liver and spleen in rats fed the FO-rich diets when compared with those that received the OO- or MO-rich

diets. In rats fed the FO diets, this increase counterbalanced the reduction of Fe stores to a greater degree in the liver than in the spleen. Although the non-haem Fe stores in liver and spleen were reduced, the rats used did not show symptoms of Fe deficiency. None of the haematological parameters studied related to Fe were affected by the diets, even when Fe stores were low, meaning that in rats fed FO diets, Fe levels in the blood are maintained through Fe stores.

Fe stores and Fe demand from the tissues are the main factors that regulate Fe absorption and metabolism. The mechanism responds even when there are little changes in the level of Fe in the storage organs (Hershko *et al.* 1987). One of the reasons why Fe stores could be reduced would be an increase in Fe demand, such as erythropoiesis. The formation of new erythrocytes can be analysed through the presence of reticulocytes. Reticulocytes move from the bone marrow to blood circulation where they continue their maturation for 1 or 2 d, when they become mature erythrocytes. The results obtained in the present study showed an increase in the percentage of reticulocytes in rats fed the FO-0 and FO-1 diets. An increase in the circulating reticulocytes together with a reduction of Fe stores indicates that there is an accelerated turnover of erythrocytes. Rats fed

Table 7. Thiobarbituric acid-reactive substances (TBARS) in plasma and erythrocytes and conjugated dienes (CD) in plasma in rats fed for 16 weeks on diets containing different oils*

(Mean values with their standard errors for seven to eight rats per group)

Diet	TBARS in plasma (nmol/mg lipids)		TBARS in erythrocytes (nmol/g haemoglobin)		CD (absorbance/mg lipids)	
	Mean	SE	Mean	SE	Mean	SE
OO	5.57 ^a	0.75	290 ^b	9	0.98 ^a	0.09
MO	5.26 ^a	0.84	295 ^b	15	0.85 ^a	0.15
FO-0	14.01 ^b	2.53	367 ^a	20	1.68 ^b	0.18
FO-1	13.16 ^b	1.26	362 ^a	17	1.69 ^b	0.18
FO-2	13.55 ^b	1.89	386 ^a	15	1.75 ^b	0.20

OO, olive oil; MO, maize oil; FO-0, fish oil with no all-rac- α -tocopheryl acetate; FO-1, fish oil with 100 mg all-rac- α -tocopheryl acetate/kg; FO-2, fish oil with 200 mg all-rac- α -tocopheryl acetate/kg.^{a,b}Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

* For details of diets and procedures, see Tables 1 and 2, and p. 12.

the OO diet showed similar percentage of circulating reticulocytes as rats fed the MO diet, which correlates with the maintenance of the Fe stores in the liver and spleen.

Changes in Fe metabolism and the increase in circulating reticulocytes were due to an increase on oxidative stress related to changes in the requirement of vitamin E. We showed that α -tocopherol levels in plasma and erythrocyte membranes of rats fed the FO-0 diet were very low compared with the rest of the groups. In the plasma of rats fed the FO-1 and FO-2 diets α -tocopherol levels were similar and higher, respectively, than those found in FO-0-fed rats, but never reached the values obtained in rats fed OO or MO diets. Farwer *et al.* (1994) also showed that when rats were fed an FO-rich diet with different amounts of α -tocopheryl acetate, the α -tocopherol levels of serum never reached those of the control groups. On the other hand, the levels of α -tocopherol in the erythrocyte membranes of rats fed the FO-1 and FO-2 diets were similar to those from rats fed the OO or MO diets. This would indicate that the level of α -tocopherol in the erythrocyte membranes was maintained through the level of α -tocopherol in the plasma, which was then reduced. However, due to the high presence of *n*-3 PUFA in the membranes from rats fed the FO diets (Rodríguez *et al.* 1996), α -tocopherol levels may not be enough to prevent the oxidative processes associated with these types of fatty acids.

Other antioxidants in the plasma were not affected by the diets. Similar findings were observed by Cho & Choi (1994) with uric acid concentration when feeding rats for 8 weeks with FO diets supplemented with different levels of vitamin E. The TAS was also not affected. This technique analyses many antioxidants such as albumin, glutathione, uric acid, bilirubin, flavonoids and other substances (Prior & Cao, 1999). Total sulfhydryl groups, for example, were present in a concentration of about 0.25 mmol/l, while uric acid concentration was 90 μ mol/l. Changes in the concentration of plasma α -tocopherol would not necessarily affect the value of TAS.

The activity of some antioxidant enzymes in the erythrocytes was affected. Both SOD and GPx activity were reduced in FO-0-fed rats. The reduction in the expression of both enzymes in the presence of free radicals observed by Fujii & Taniguchi (1999) or the reduction in metal cofactors could explain our results. However, other enzymes such as catalase, or plasma caeruloplasmin were not affected. These results show that oxidative stress is increased, but each enzyme responds in a specific manner.

Weber *et al.* (1997) consider that the amount of vitamin E needed to protect against damage is at least 0.4–0.8 mg vitamin E/g PUFA and may be in excess of 1.5 mg vitamin E/g PUFA when diets contain higher-than-average levels of long-chain *n*-3 PUFA. It is evident that the administration of an FO diet, irrespective of the amount of all-rac- α -tocopheryl acetate provided, increased oxidative stress as shown by the reduction of antioxidant defences in plasma and erythrocytes. However, rats fed the OO or MO diet had similar antioxidant levels. This is also reflected by the results obtained when measuring products of lipid peroxidation. Cho & Choi (1994) found similar

results when feeding rats with FO diets for 8 weeks. Allard *et al.* (1997) also demonstrated that supplementing the human diet with FO resulted in an increase in lipid peroxidation, which was not suppressed by increasing vitamin E supplementation, even with 900 mg/d. Such an effect was not observed when OO was administered (Allard *et al.* 1997).

The results obtained with TBARS parallel those of conjugated dienes, being good indicators of oxidative stress. It is interesting to note that no free MDA was detected in the plasma. According to Largillière & Mélançon (1988), the amount of circulating free MDA is really low and, for example, in healthy human subjects it is not detected. The absence of MDA in plasma is not surprising considering the tendency of MDA to form complexes with proteins and amino acids and its fast enzymic degradation in the liver by aldehyde dehydrogenase (*EC* 1.2.1.5) (Largillière & Mélançon, 1988).

Lipid peroxidation did not only affect the fatty acids of the plasma, but also the fatty acids from membranes, such as erythrocyte membranes. Susceptibility to lipid peroxidation of erythrocyte membranes was increased in rats fed the FO diets, when compared with those that received the OO or MO diet. This increase would also enhance the requirements of α -tocopherol, and would indicate that the α -tocopherol present was not enough to prevent lipid peroxidation, especially in rats fed FO-0 but also in those fed FO-1 and FO-2.

Oxidative damage of erythrocytes has been implicated in erythrocyte ageing and removal (Pradhan *et al.* 1990; Signorini *et al.* 1995; Eda *et al.* 1997). It has also been shown that erythrocytes from vitamin E-deficient rats behave like old erythrocytes from normal rats (Kay *et al.* 1986). Old erythrocytes express in their membranes an antigen recognised by spleen mononuclear phagocytes, which then remove aged cells. It is probable that oxidatively modified erythrocytes had been eliminated from circulation, meaning that new erythrocytes were formed, which was confirmed by an increase in the percentage of reticulocytes. The formation of new reticulocytes increased the Fe demands and, consequently, Fe stores in the liver and the spleen were reduced. The administration of vitamin E should avert such effects. However, the supplementation of the FO diet with even 200 mg all-rac- α -tocopheryl acetate/kg was not enough to prevent the changes in Fe metabolism observed. On the other hand, OO diets did not alter oxidative stress or Fe metabolism parameters.

In conclusion, FO increases oxidative stress and alters Fe metabolism whilst OO shows none of these effects. Therefore, an FO-rich diet has to be adequately supplemented with antioxidants to avoid its adverse effects.

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