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MAIN TOPIC

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Polyunsaturated fatty acids influence neonatal monocyte survival

Abstract The n-3 and n-6 polyunsaturated fatty acids (PUFAs) are essential dietary constituents. They are potent modulators of the human immune response, and research has endeavoured to optimise the ratio of n-3 to n-6 fatty acids in the lipid emulsion component of total parenteral nutrition to harness their beneficial effects in the clinical setting. PUFAs modulate apoptosis of certain tumour cells and cell lines. Monocytes, which are major effector cells of the innate immune system, play a central role in the initiation, development, and outcome of the immune response. They are crucial in the defence against invading pathogens and are involved in the lysis of infected or malignant cells, wound healing, repair, and remodelling of tissues. In the present study we investigated whether PUFAs might evoke apoptosis in newborn monocytes. Purified cord-blood monocytes collected from uncomplicated full-term pregnancies were incubated for 24 h in complete medium in the presence or absence of one of the n-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic (EPA) or the n-6 PUFA arachidonic acid (AA). Following incubation, cells were triple-labelled with annexin V, CD14, and propidium iodide prior to flow-cytometric analysis to determine the degree of cell death. All experiments were performed in triplicate and data expressed as mean ±1 S.D. (%). In the absence of fatty acids, 30 ± 4% of control cord monocytes underwent apoptosis or necrosis after 24 h incubation. At a concentration of 50 μM, none of the PUFAs had a significant effect on monocyte cell death, but at a dose of 100 μM, DHA resulted in 60 ± 4% cell death ($P < 0.05$) while the other PUFAs had no significant effect. In contrast, at higher concentrations (200 μM), all the PUFAs significantly increased monocyte cell death (AA: 70 ± 5%, DHA: 86 ± 2%, EPA: 70 ± 4%). PUFAs thus exert a potent influence on cord monocyte cell survival in vitro. Their effect is dose-dependent and DHA appears to be the most potent of the fatty acids tested. The influence of PUFAs on neonatal monocyte-cell survival suggests a novel mechanism whereby PUFAs may modulate the immune response.

Keywords Parenteral nutrition · Neonatal monocytes · Polyunsaturated fatty acids · Apoptosis

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Introduction

The introduction of lipid emulsions for total parenteral nutrition (TPN) has been a major breakthrough in medicine. Lipids are a high-calorific source of energy, reducing the incidence of hyperglycaemia and fatty liver associated with the use of glucose as the only calorie source, and are the sole source of essential fatty acids in the TPN-dependent patient [1]. They are chiefly provided in the form of linoleic acid and α-linolenic acid. Humans lack the necessary enzymes to produce these fatty acids de novo, making them essential dietary constituents [2]. Linoleic acid and α-linolenic acid are precursors of long-chain polyunsaturated fatty acids (PUFAs) of the n-6 and n-3 series, respectively [3]. In utero, the fetus actively sequesters them transplacentally, where they play a key role in growth, neuroretinal cell-membrane development, and eicosinoid production [4, 5]. However, they have long been recognised to have potentially immunosupressive effects.

Fischer et al. [6] demonstrated that Intralipid, a commonly used lipid emulsion rich in n-6 PUFAs, impaired bacterial clearance and enhanced bacterial virulence in mice. Freeman et al. [7] determined that critically ill neonates fed intravenously with lipid solutions had an increased incidence of central-venous cathether-related sepsis. Further studies revealed that Intralipid impaired neutrophil function and depressed monocyte chemotaxis, phagocytosis, and complement and cytokine production [8]. The potency of the various n-3 and n-6 PUFAs tested is not uniform. One theory to account for this is the fact that eicosinoids derived from

n-3 PUFAs, compared to those derived from n-6 PUFAs, develop a similar quality but less intensity of action [9], which has been postulated to result in n-3 PUFAs producing less immunosupressive effects than their n-6 counterparts. Therefore, investigators have modified the fatty-acid content of conventional lipid emulsions by increasing the ratio of n-3 to n-6 PUFAs to try and reduce the unwanted immunosupressive side effects of TPN. Grimm et al. [10] administered lipid emulsions with variable n-3 to n-6 PUFA ratios to rats post-heart allotransplantation, and found that the more balanced the n-3 to n-6 ratios, the less immunosupressive the fat emulsion. It is hoped that the optimisation of this ratio in critically-ill, TPN-dependent patients can result in an optimal immune response and ultimately impact favourably upon prognosis.

These potential benefits may come at a price, however: n-3 PUFAs are susceptible to peroxidation, and there is evidence in the literature that the products of this process may result in cell death through apoptosis and necrosis [11]. They have been shown to induce cell death in a number of cell lines and tumour cells [12–14]. Coincubation of the monocytic cell line U9371 with eicosapentaenoic acid (EPA), an example of an n-3 PUFA, has been demonstrated to initiate apoptosis [15]. More recently, PUFAs have been shown to promote cell death of primary lymphocytes and colonic mucosa in rodent feeding studies [16, 17]. We hypothesized that PUFAs might also evoke apoptosis in neonatal monocytes.

Monocytes are the major effector cells of the innate immune system. They are crucial in the defence against invading pathogens and are involved in the lysis of infected or malignant cells, wound healing, and repair and remodelling of tissues [18]. They play a key role in the initiation and maintenance of the host immune response by differentiating into macrophages and dendritic cells and by producing cytokines [19]. Monocyte survival is exquisitely regulated in vivo by various pro- and anti-apoptotic signals, survival being promoted during the inflammatory response and diminished when they are surplus to requirements in the absence of inflammation [20]. In this study we investigated whether by modulating the tight control of monocyte survival PUFAs can influence the immune response.

Materials and methods

With ethical approval and informed maternal consent, peripheral-blood mononuclear cells were isolated from whole cord blood from uncomplicated term pregnancies by means of Ficoll Hypaque centrifugation. Platelet-free monocytes were positively selected using a CD14 immunomagnetic bead system (Miltenyi Biotec) with a modification of the manufacturer's protocol. A cation-free buffer containing 5mM ethylenediaminetetra-acetic acid was used to prevent cation-dependent binding of CD62P-positive platelets to the resultant purified monocytes. The viability of the freshly-isolated monocytes was over 90% as determined by ethidium bromide/acridine orange staining and over 90% of isolated CD45 cells were CD14-positive on flow-cytometric analysis. The cells were incubated in a 5% CO_2 incubator at 37 °C in Costar 24-well ultra-low attachment polystyrene flat-bottomed plates at a cell concentration of 10^6/ml in complete medium comprised of RPMI 1640 with 25 mM Hepes, L-glutamine (Biowhittaker), and 10% heat-inactivated fetal calf serum (FCS) with or without the various concentrations of the fatty acids to be studied.

Stock solutions of the n-3 PUFAs docosahexaenoic acid(DHA) and EPA and the n-6 PUFA arachidonic acid (AA) (Sigma-Aldrich) were made up in a nitrogen atmosphere using an Atmosbag (Sigma, Aldrich) in 100% ethanol and stored in the dark at −20 °C to prevent peroxidation. These stocks were diluted at least 1000-fold in complete medium to the test culture concentrations of 50, 100, and 200 μM. Apoptosis of monocytes was measured essentially according to the protocol of Schmidt et al. [21].

After 24 h incubation control monocytes, in complete medium only, or fatty-acid-treated monocytes were harvested with ice-cold PBS (Gibco), washed, and re-suspended in annexin V binding buffer (10 mM Hepes/NaOH [pH 7.4], 140 mM NaCl, and 2.5 mM $CaCl_2$) and triple-labelled with annexin V FITC (R&D Systems), CD 14 PE (Dako), and propidium iodide (PI) (Sigma); 10,000 events were acquired on 3 channels of a Becton-Dickinson FACscan flow cytometer and data were analysed with CellQUEST software. Cell death was quantified by measuring the number of apoptotic annexin V-positive and necrotic PI-positive events.

Data were analysed using ANOVA software (Instat) and expressed as mean ± 1 S.D. (%). A P value below 0.05 was deemed statistically significant. All experiments were performed at least in triplicate.

Results

Under standard culture conditions in complete media, in the absence of PUFAs 30 ± 4% of the cord monocytes died after 24 h as determined by flow-cytometric measurement of annexin V and or PI-positive events. Monocytes were analysed based on forward-side scatter characteristics and not on CD14-positive events, as has been performed by others [20], to avoid missing the significant CD14-negative population that arises during monocyte apoptosis [22]. At a concentration of 50 μM, none of the PUFAs had a significant effect on monocyte cell death compared to control cells, but at a dose of 100 μM DHA induced cell death in 60 ± 14% of monocytes ($P < 0.05$) while the other PUFAs had no significant effect. In contrast, at a higher concentration (200 μM), all the PUFAs significantly increased cell death (AA: 70 ± 5%, DHA: 86 ± 12%, EPA: 70 ± 4%). The very high purity of the freshly-isolated monocyte preparations precluded CD14-negative annexin V positivity being significantly attributable to contaminating non-monocytic cells.

Discussion

Monocytes, after circulating in the blood for 12–72 h, can migrate into the tissues and differentiate into macrophages [23]. The macrophage is a relatively long-lived cell, and the number of monocytes in the blood generally exceeds the number needed to replace dying macrophages in the periphery [19]. Therefore, large numbers of monocytes undergo apoptosis under physiological conditions to maintain cellular homeostasis. Mangan et al. [23] demonstrated that human adult monocytes cultured

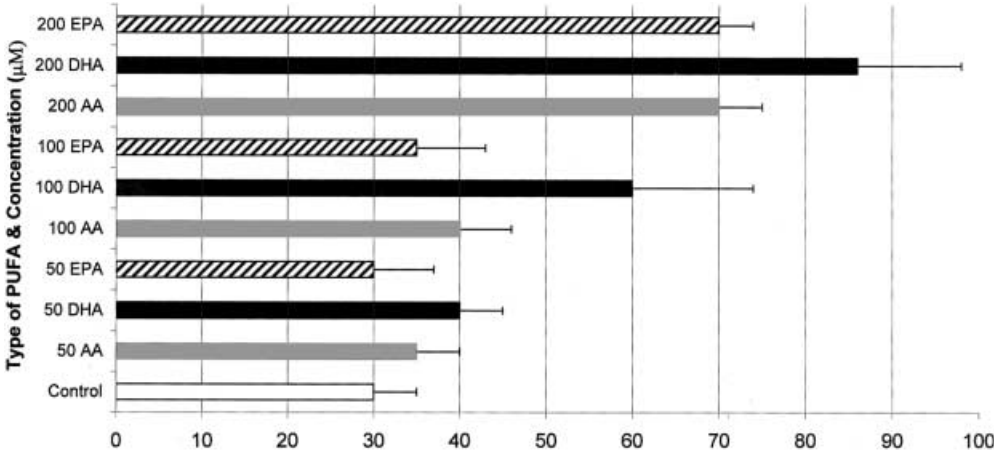
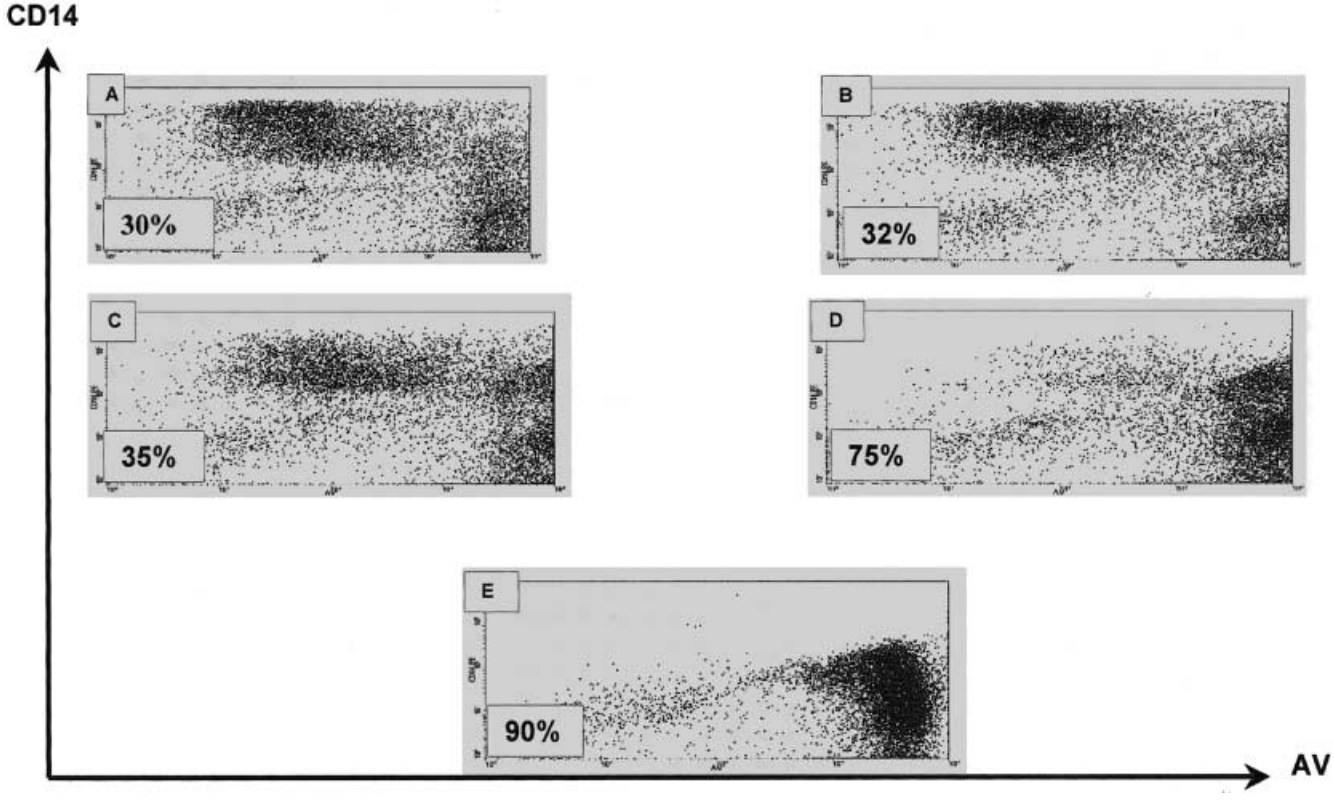


Fig. 1 Percent cell death of monocytes treated with different concentrations of polyunsaturated fatty acids (PUFAs) after 24 h (*EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *AA* arachidonic acid)

in medium lacking serum underwent apoptosis, but the addition of the pro-inflammatory cytokines tumor necrosis factor alpha and interleukin 1β and the gram-negative bacterial toxin LPS prevents apoptosis. Monocyte apoptosis has emerged as a central regulatory event in haemopoiesis and inflammation [24] and the elements of the complex systems regulating monocyte apoptosis are gradually emerging.

As this is the first time the effect of PUFAs on primary human monocytes has been assessed, preliminary experiments were conducted based on a protocol recently used by Diep et al. [25] to determine the influence of n-3 PUFAs on vascular smooth-muscle cells. They employed a serum-free model to test the effect of DHA on cell death at a dose range of 0–100 μM and found it to have potent effects. Therefore, we initially performed controlled, serum-free experiments on cord monocytes and found that within

Fig. 2A–E Annexin vs CD14 staining of control and fatty-acid-treated monocytes: **A** representative flow-cytometric data measuring positivity after 24 h in control cells. **B** Arachidonic acid 100 μM, **C** eicosapentaenoic acid 100 μM, **D** docosahexaenoic acid (DHA) 100 μM, **E** DHA 200 μM. Annexin V positivity shown in *bottom left corner* of each panel



6 h and at a 50 μM concentration AA, DHA, and EPA virtually completely destroyed all monocytes (data not shown). Next, in an effort to create a more physiological model, 10% FCS was used to produce a standard in-vitro culture system. The addition of serum protected against the pro-apoptotic effects of the PUFAs tested at lower concentrations (50 μM). At higher concentrations, DHA (100 μM and 200 μM), AA (200 μM), and EPA (200 μM) all had a statistically significant effect on cord monocyte viability (Figs. 1 and 2). At these levels, the PUFAs resulted in a significant increase in annexin positivity of the cell population on flow-cytometric analysis compared to controls. The majority of dying monocytes became CD14-negative in the process, as was first described by Heidenreich et al. [22], who noted that down-regulation of the CD14 receptor precedes apoptosis.

While clearly demonstrating potent effects of PUFAs (100–200 μM) on monocyte viability in our model, the question arises whether this effect reflects a pharmacological or physiological response. Rubin et al. [26] conducted a study on the influence of intravenously-administered fat emulsions on the plasma fatty-acid composition of premature infants. The baseline levels of the plasma PUFAs AA, DHA, and EPA were approximately 600, 185, and 25 μM, respectively. This would suggest that the concentrations tested in our model were within the physiological range, but there are two important caveats. Firstly, plasma fatty-acid levels may not reflect fatty-acid cell-membrane or tissue content. Secondly, 6 days of Intralipid emulsion infusion significantly increased plasma levels of linoleic acid and α-linolenic acid, but this was not accompanied by increased plasma levels of AA, DHA, or EPA.

Other longer-term feeding experiments have demonstrated that oral n-3 and n-6 PUFA supplementation can result in increased plasma levels of n-3 and n-6 PUFAs in both rodents and humans [27, 28]. Furthermore, these plasma changes have been reflected in changes in cell-membrane composition. Importantly, it has also been recognised that both premature and small-for-gestational-age infants could be particularly vulnerable to these potential lipid effects, as investigators have determined that this subgroup of patients have a diminished capacity to utilise and clear these lipids in the circulation [29].

How PUFAs mediate cell death has yet to be fully elucidated. It is recognised, however, that they are susceptible to lipid peroxidation and that the metabolites of this process are associated with increased oxidative stress [11]. Oxidative stress may be defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defences [30]. ROS resulting from oxidative stress have been implicated in the regulation of apoptosis. Fas-mediated monocyte apoptosis has been associated with increased intracellular levels of ROS and could be blocked completely with the antioxidant N-acetylcysteine [24]. Furthermore, the addition of hydrogen peroxide alone is sufficient to induce apoptosis [24]. Additionally, in a rodent model, Garrido et al. [31] have shown that dietary supplementation with PUFAs may produce a depletion of cellular antioxidants such as gluthathione, thus rendering cells more susceptible to oxidative stress.

In conclusion, this study demonstrates that in vitro, cord monocytes are susceptible to cell death in the presence of n-3 and n-6 PUFAs in a dose-dependent manner. DHA was the most potent of the PUFAs tested, and future studies will determine the role of lipid peroxidation in this process.

References

1. Garnacho Montero J, Shou J, Oritz Leyba C, Jiminez Jiminez FJ, Daly JM (1996) Lipids and immune function. Nutr Hosp 11: 230–237
2. Calder PC (1998) Dietary fatty acids and the immune system. Nutr Rev 56: S70–81
3. Miles EA, Calder PC (1998) Modulation of immune function by dietary fatty acids. Proc Nutr Soc 57: 277–292
4. Cambell FM, et al (1998) Placental membrane fatty acid-binding protein preferentially binds arachidonic acid and docosahexaenoic acids. Life Sci 63: 235–240
5. Makrides M, Neumann M, Simmer K, Pater J, Gibson R (1995) Are long-chain polyunsaturated fatty acids essential nutrients in infancy? Lancet 345: 1463–1468
6. Fischer GW, Hunter KW, Wilson SR, Mease AD (1980) Diminished bacterial defences with intralipid. Lancet 2(8199): 819–820
7. Freeman J, Goldmann DA, Smith NE, et al (1990) Association of intravenous lipid emulsion and coagulase-negative bacteremia in neonatal care units. N Engl J Med 323: 301–308
8. Calder PC (1997) N-3 polyunsaturated fatty acids and immune cell function. Adv Enzyme Regul 37: 197–237
9. Suchner U, Senftleben U (1994) Immune modulation by polyunsaturated fatty acids during nutritional therapy: interactions with synthesis and effects of eicosinoids. Infusionsther Transfusionmed 21: 167–182
10. Grimm H, Tibell A, Norrlind B, Blecher C, Wilker S, Schwemmle K (1994) Immunoregulation by parenteral lipids: impact of the n-3 to n-6 fatty acid ratio. J Parent Ent Nutr 18: 417–421
11. Reddy Avula CP, Fernandes G (1999) Modulation of antioxidant enzymes and apoptosis in mice by dietary lipids and treadmill exercise. J Clin Immunol 19: 35–44
12. Finstad HS, Dyrendal H, Wik Myhrstad MC, Heimli H, Drevon CA (2000) Uptake and activation of eicosapentaenoic acid are related to accumulation of triacylglycerol in Ramos cells dying from apoptosis. J Lipid Res 41: 554–563
13. Das UN (1999) Essential fatty acids, lipid peroxidation and apoptosis. Prostaglandins, Leukot Essent Fatty Acids 61: 157–163
14. Bougnoux P (1999) N-3 polyunsaturated fatty acids and cancer. Curr Opin Nutr Metab Care 2: 121–126
15. Finstad HS, Drevon CA, Kulseth MA, Stnstad AV, Knudsen E, Kolset SO (1998) Cell proliferation, apoptosis and accumulation of lipid droplets in U937-1 cells incubated with eicosapentaenoic acid. Biochem J 336: 451–459
16. Reddy Avula CP, Zaman AK, Lawrence R, Fernandes G (1999) Induction of apoptosis and apoptotic mediators in balb/c splenic lymphocytes by dietary n-3 and n-6 fatty acids. Lipids 34: 921–925
17. Calviello G, Palozza P, Maggiano N, Piccioni E, Franceschelli P, Frattucci A, Di Nicuolo F, Bartoli GM (1999) Cell proliferation, differentiation, and apoptosis are modified by n-3 polyunsaturated fatty acids in normal colonic mucosa. Lipids 34: 599–604

18. Mangan DF, Wahl SM (1991) Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. J Immunol 147: 3408–3412
19. Ralph P (1989) Colony stimulating factors in human monocytes. In: Zembala M, Asherson GL (eds) Human monocytes. Academic Press, New York, p 227
20. Fahy RJ, Doseff AI, Wewers MD (1999) Spontaneous human monocyte apoptosis utilizes a caspase-3-dependent pathway that is blocked by endotoxin and is independent of caspase-1. J Immunol 163: 1755–1762
21. Schmidt M, Pauels HG, Lugering N, Lugering A, Domschke W, Kucharzik T (1999) Glucocorticoids induce apoptosis in human monocytes: potential role for IL-1β (1999) J Immunol 163: 3484–3490
22. Heidenreich S, Schmidt M, August C, Cullen P, Rademaekers A, Pauels H-G (1997) Regulation of human monocyte apoptosis by the CD14 molecule. J Immunol 159: 3178–3188
23. Mangan DF, Welch GR, Wahl SM (1990) Lipopolysaccharide, tumor necrosis factor-α, and IL-1β prevent programmed cell death (apoptosis) in human peripheral blood monocytes. J Immunol 146: 1541–1546
24. Um HD, Orenstein JM, Wahl SM (1996) Fas mediates apoptosis in human monocytes by a reactive oxygen intermediate dependent pathway. J Immunol 156: 3469–3477
25. Diep QN, Intengan HD, Schiffrin EL (2000) Endothelin-1 attenuates ω3 fatty acid-induced apoptosis by inhibition of caspase 3. Hypertension 35: 287–291
26. Rubin M, Moser A, Naor N, Merlob P, Pakula R, Sirota L (1994) Effect of three intravenously administered fat emulsions containing different concentrations of fatty acids on the plasma fatty acid composition of premature infants. J Pediatr 125: 596–602
27. Vilaseca J, Salas A, Guarner F, Rodriguez R, Martinez M, Malagelada JR (1990) Dietary fish oil reduces progression of chronic inflammatory lesions in a rat model of granulomatous colitis. Gut 31: 539–544
28. Endres S, Ghorbani R, Kelley V, et al (1989) The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 an tumor necrosis factor by mononuclear cells. N Engl J Med 320: 265–271
29. Andrew G, Chan G, Schiff D (1976) Lipid metabolism in the neonate. J Pediatr 88: 273
30. Betteridge DJ (2000) What is oxidative stress? Metabolism 49 [Suppl 1]: 3–8
31. Garrido A, Garrido F, Guerra R, Valenzuela A (1989) Ingestion of high doses of fish oil increases the susceptibility of cellular membranes to the induction of oxidative stress. Lipids 24: 833