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ORIGINAL CONTRIBUTION

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Addition of vitamin E to long-chain polyunsaturated fatty acid-enriched diets protects neonatal tissue lipids against peroxidation in rats

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Summary *Background:* Tissue 20:4(n-6) and 22:6(n-3) status have been correlated with neonatal development and growth. Artificial formulas for neonates have been supplemented with long chain polyunsaturated fatty acids (LCP) from animal and marine sources which may enhance sensitivity of cellular membranes to oxidative damage. Diet-derived antioxidants like vitamin E play a key role in the protection of tissue lipids against oxidation.
Aim of the study: We seek to determine the influence of dietary vitamin E on tissue sensitivity to oxidative stress in rats fed for 4 weeks on diets enriched in (n-3) and (n-6) long-chain poly-unsaturated fatty acids.
Methods: Weanling rats received 10% fat diets that provided 18:1(n-9), 18:2(n-6) and 18:3(n-3) in a similar ratio to that of rat milk (group A), supplemented with fish oil (groups B and B+E) and supplemented with (n-6) and (n-3) LCP from an animal phospholipid concentrate (groups C and C+E). Vitamin E (500 mg vitamin E/kg fat) was added to diets B+E and C+E. Tissue fatty acid content and the activities of catalase, superoxide dismutase, glutathione transferase and glutathione peroxidase in liver and brain were measured. Glutathione status, vitamin E and the production of thiobarbituric acid reactive substances (TBARs) after incubation of erythrocyte, liver and brain lipids with inducers of enzymatic or non-enzymatic lipid peroxidation was measured.
Results: Group B registered significantly lower total superoxide dismutase acitvity than group B+E. Catalase activity was significantly higher in group C than in group C+E. Hepatic total and reduced glutathione levels were decreased in vitamin E supplemented groups compared to unsupplemented ones. TBARs production in erythrocyte lipids was significantly higher in groups B and C compared to vitamin E supplemented groups B+E and C+E.
Conclusions: This study shows that the addition of vitamin E protected erythrocyte and liver microsome lipids enriched in (n-3) and (n-6) LCP from lipid peroxidation during the postnatal development of rats. The protection was more effectively in group C+E than in group B+E.

Key words Long chain polyunsaturated fatty acids – peroxidation – vitamin E – weanling rats

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Introduction

Long chain polyunsaturated fatty acids (LCP) are the predominant structural fatty acids in the human brain and retina (1). These LCP accumulate rapidly in fetal and infant neural tissue during the periods of most rapid growth and development that is, during the last month of gestation and the first months of postnatal life. Breast milk contains LCP, but a number of formulas do not yet contain fatty acids longer than C18 under the assumption that infants could synthesize LCP from the essential C18 fatty acids, namely linoleic (18:2(n-6)) and alpha-linolenic (18:3(n-3)) acids, through elongase and desaturase systems. A decreased exogenous supply of 22:6(n-3) in preterm infants may decrease visual acuity and delay psychomotor development (2). In term infants, parameters of postnatal brain growth and cognitive behavior have been related to erythrocyte and plasma cholesteryl ester 22:6(n-3) contents and to dietary LCP supplementation, respectively (3, 4). As a result, the Nutrition Committe of the European Society for Gastroenterology and Nutrition (5), the Directive of the European Union for infant formulas (6) and the FAO (7) recommended that preterm formulas be supplemented with LCP, and various formulations are now commercially available.

Long chain polyunsaturated fatty acids are extremely susceptible to lipid peroxidation processes by oxygen free radicals (OFR) damage (8). Uncontrolled formation of lipid peroxides by OFR is considered to be a prevalent mechanism of cell damage and may lead to pathological conditions like inflammation and cardiovascular diseases (9). To avoid a dangerous overproduction of OFR, aerobic organisms have evolved several defence mechanisms that include an enzymatic defence system (superoxide dismutase, catalase, glutathione transferase, and glutathione peroxidase) and antioxidants (ascorbate, reduced glutathione, urate, vitamin E and ß-carotene) (10). Diet-derived antioxidants (vitamin E and ascorbate) play a key role in the antioxidant-defence system (9).

The incorporation of highly unsaturated fatty acids into tissue phospholipids modifies antioxidant defences in two ways. First, the incorporation of dietary LCP into membrane lipids provides target molecules to OFR attack and therefore increasing tissue susceptibility to lipid peroxidation (11, 12). Second, the administration of LCP changes the concentration of endogenous antioxidants and the activities of antioxidant and prooxidant enzymes in the body (13, 14). Although the protective role of dietary antioxidants, mainly vitamin E, has been demonstrated in a number of studies (13, 15), no report has yet assessed the effect of vitamin E intake on the susceptibility of tissue lipids enriched in (n-6) and (n-3) LCP from dietary origin to lipid peroxidation in an experimental model of postnatal development. For this reason, we investigated the effect of vitamin E on plasma, erythrocyte, liver and brain fatty-acid composition, on the susceptibility of erythrocyte membranes, liver microsome phospholipids, and brain homogenates to *in vitro* lipid peroxidation, and on the hepatic and cerebral activities of antioxidant enzymes in weanling rats that received diets supplemented with LCP of both the (n-3) and (n-6) series.

Materials and methods

The protocol of this study was approved by the Committee of Animal Welfare of the University of Granada. Male Wistar rats at weaning were purchased from Interfauna Iberica S.A. (Barcelona, Spain). Animals were randomly divided into five groups of seven rats each and were housed seven per cage in a room with controlled temperature (21±1 ºC) and light (08.00-20.00 h).

Nutrition

Diets were prepared and packaged by the R&D department of PULEVA (Granada, Spain) and stored at 4 °C under nitrogen. All groups received a 10% fat semipurified diet, which differed in the source of dietary fat. The detailed composition of diets was previously described (16). Group A fat consisted of a mixture of olive oil (62.5%), soy oil (11.1%) and refined coconut oil (26.4%). Groups B and B+E received 7% of group A fat and 3% of deodorized sardine oil, kindly supplied by Dr. Valenzuela (INTA, University of Chile); groups C and C+E were fed with 7% of group A fat, 1.5% of the same fish oil concentrate and 1.5% of a purified animal tissue phospholipid concentrate, obtained from PULEVA (Granada, Spain). Diets B+E and C+E were also supplemented with 500 mg vitamin E/kg fat according to Valenzuela and Nieto (17). All animals were given free access to fresh diet and water daily.

Tissue preparation and methods

After 4 weeks of feeding, animals were deprived of food for 24 h, lightly anesthetized with diethyl ether and killed. Blood obtained by cardiac punction was collected into heparinized tubes. Plasma and membrane ghosts (18) were immediately prepared and stored at -80 °C until analysis. The liver and brain were removed, processed and stored at -80°C. Plasma lipids, erythrocyte membrane phospholipids, brain lipids and liver microsome phospholipids were extracted (19) and methylated (20). The fatty acid composition was analyzed by capillary gas chromatography on a Hewlett-Packard model no. 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame ionization detector and a 30 m capillary column filled with DB-2330-N stationary phase (J & W Scientific, Folsom, CA). The peroxidizability index (PI) was calculated as follows: PI = (%dienoic x 1) + (%trienoic x 2) + (%tetraenoic x 3) + (%pentaenoic x 4) + (%hexaenoic x 5) (21).

Total glutathione and reduced glutathione were determined in liver portions homogenized at 4 °C in five volumes of 5% 5-sulfosalicylic acid (previously flushed with N2) using a Teflon-glass homogenizer driven by a stirring motor at a constant speed. The homogenates were centrifuged in a microfuge at 12 000 x g for 10 minutes at 4 °C. Total glutathione was determined at 30 °C by the DTNB-GSSG reductase recycling assay as described by Anderson et al. (22) GSH was assayed at room temperature by the reaction with DTNB according to Akerboom and Sies (23).

The antioxidant enzyme activities were determined in liver and brain homogenized at 4 °C in 1 mM EDTA, 0.01% Digitonin, 0.1 M phosphate buffer pH 7.0 solution using a Teflon-glass homogenizer driven by a stirring motor at a constant speed. The homogenate was centrifuged at 13 000 x g for 15 min at 4 °C. The supernate was transferred to an eppendorf tube and stored at 4 °C until analysis. Total SOD activities in the supernate fraction were measured by the inhibition of cytochrome 3c reduction mediated via superoxide anions generated by the xanthine/xanthine oxidase system and monitored at 550 nm. MnSOD activities were determined with the same procedure in the presence of 10 μM potassium cyanide. Activities are expressed as units/mg protein where one unit of superoxide dismutase was defined as the amount required to cause half-maximal inhibition of cytochrome 3c reduction (24). CAT activity was determined according to Aebi (25) by following the decomposition of hydrogen peroxide at 240 nm. GPx activity was determined at 31 °C by NADPH oxidation in a coupled reaction system consisting of tert-butyl hydroperoxide and glutathione (26). GST activity was measured with 1-chloro-2,4-dinitrobenzene at 30 °C according to Warholm et al. (27).

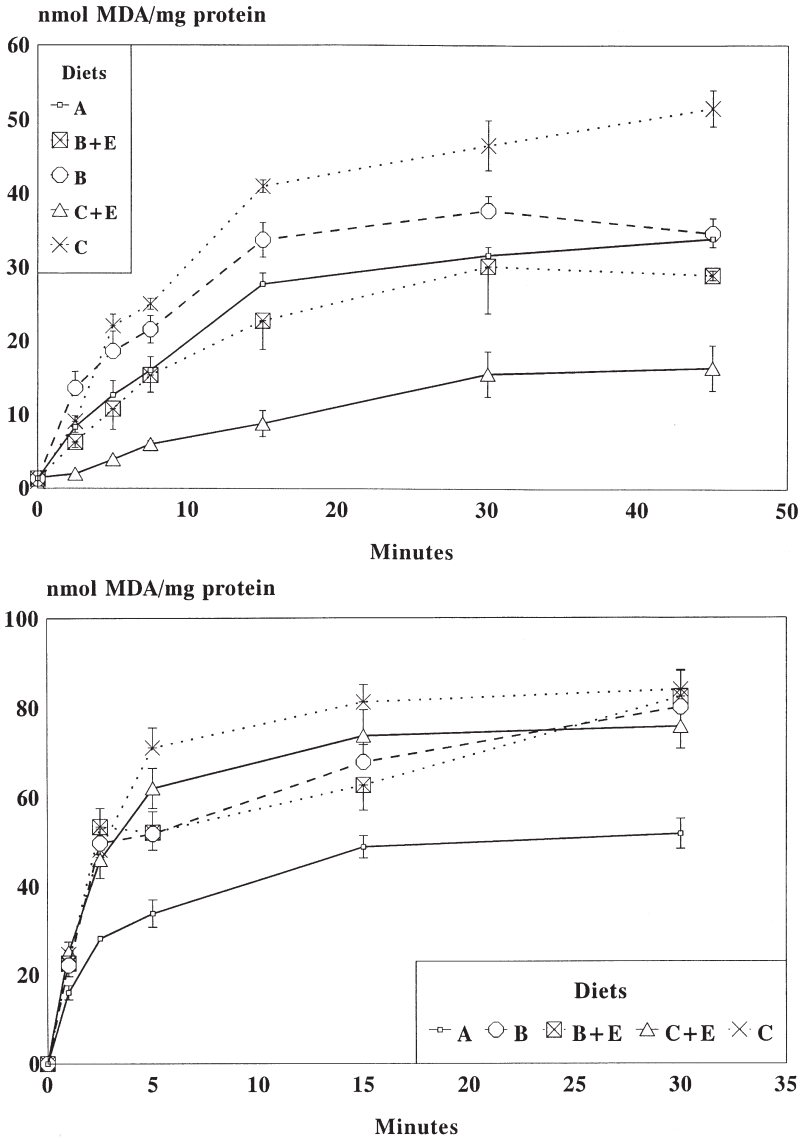

Fig. 1 Erythrocyte membrane (1A) and liver microsome (1B) peroxidation rates after induction with Fe^{2+}-ascorbate in rats at weaning. Diet symbols: A (*); B (open circle); B+E (crossed quadrangle); C (open triangle); C+E (cross). Results are expressed in nmol of malondialdehyde (MDA).mg $protein^{-1}$ as mean ± SEM; seven animals for each group.

Lipid peroxidation was induced *in vitro* in erythrocyte membranes with a Fe^{2+}/ascorbate solution (50mM/400μM) and in liver microsomes with either the same Fe^{2+}-ascorbate solution or with a NADPH/ADP/Fe^{3+} mixture (400μM/4μM/50μM) (28). After the induction of lipid peroxidation, thiobarbituric acid-reactive substances (TBARs) were assayed in the medium and expressed as nmol malondialdehyde (MDA) per mg of protein (29). Vitamin E content of liver microsomes was determined by HPLC according to Bieri et al. (30). For the evaluation of the effects of diets, a one-way anova was performed and adjusted means were compared *a posteriori* by the Bonferroni t-test. The level of statistical significance was determined at $p<0.05$.

Results

No significant differences were recorded in food intake, body weight or growth among rats fed the different diets. Percentage contributions of selected fatty acids to total plasma lipids, erythrocyte membrane lipids, liver microsome phospholipids and brain lipids as well as the sum of saturated fatty acids (SAT), monounsaturated fatty acids (MONO), (n-6) and (n-3) LCP and PI values are shown in Table 1. Activities of hepatic CAT, GPx, GST, total SOD and MnSOD activities as well as brain GPx, GST, total SOD and MnSOD activities are shown in Table 2. Total glutathione and GSH contents of rat livers and vitamin E content of liver microsomes are given in Table 3. Figure 1 shows the production of TBARs by erythrocyte membranes (1A) and liver microsomes after induction with Fe^{2+}-ascorbate (1B).

Our diets significantly altered the fatty acid pattern of rat tissues, particularly that of (n-3) and (n-6) LCP. In short, group A had the highest 20:4(n-6) and total (n-6) LCP values and the lowest levels of 22:6(n-3) and total (n-3) LCP in all tissues. The supplementation of diets with (n-3) LCP (groups B and B+E) significantly increased their individual and total presence compared with group A. These increases were counterbalanced by decreases in the content of 20:4(n-6) and total (n-6) LCP. The intake of both (n-3) and (n-6) LCP in groups C and C+E had a compensating effect on 22:6(n-3) and 20:4(n-6) levels in all tissues. 22:6(n-3) and total (n-3) LCP levels were significantly boosted in these groups compared with group A. Groups C and C+E had significantly higher 20:4(n-6) and total (n-6) LCP levels than did groups B and B+E. The addition of vitamin E to diets B+E and C+E did not bring about major differences in the percentage contributions of individual fatty acids compared with diets B and C. In plasma, 22:6(n-3) and total (n-3) LCP, values were significantly higher in group B+E than in group B. Unsaturation of erythrocyte membranes increased in group C+E compared with group C since the percentage contribution of saturated fatty acids was decreased.

PI values were altered by diet-induced changes. Incorporation of dietary LCP to tissue lipids resulted in higher tissue PI values in groups B, B+E, C and C+E than in group A. In erythrocyte membranes, group C+E had significantly lower PI values than did group B. Notably, groups C and C+E had significantly higher PI levels than did groups B and B+E. In terms of PI values in liver microsomes, group C+E had a significantly higher values than did group C.

No significant differences were found in brain enzymatic activities. Feeding rats with diets rich in LCP (groups B, B+E, C and C+E) decreased GPx activity compared with rats fed on unsupplemented diet A. The addition of vitamin E did not significantly change hepatic GST and Mn-SOD activities. Rats fed on diet B had significantly lower total liver SOD activity than did rats fed diets B+E, C and C+E. The addition of vitamin E in diet C+E significantly depressed CAT activity compared with diet C.

As for glutathione metabolism, rats fed diets C and C+E had the highest GSH and total liver-glutathione levels in all experimental groups. GSH content augmented significantly in group C compared with group A. Liver homogenates in rats fed diets C and C+E had significantly greater total glutathione contents than in rats fed diets A, B and B+E. Vitamin-E addition to diets B+E and C+E reduced the contents of total glutathione and GSH compared with unsupplemented diets B and C. No significant differences among groups were noted in vitamin-E contents in liver microsomes.

Vitamin-E addition to diets directly influenced the susceptibility of tissue fractions and homogenates to *in vitro* lipid peroxidation. Although erythrocyte lipids in group C+E were enriched in (n-3) and (n-6) LCP, the simultaneous intake of vitamin E significantly reduced its peroxidation rate even compared with unsupplemented group A. The reduction on the peroxidation of tissue lipids was highly significant in erythrocyte membranes of rats fed diet C+E compared with those fed diet C (Fig. 1). This effect but to a lesser extent was observed in erythrocyte membranes of rats fed diets B and B+E (Fig. 1). In liver microsomes, incorporation of LCP in groups B, B+E, C and C+E resulted in significantly higher Fe/Ascorbate-induced peroxidation rates compared with group A (Fig. 1). Vitamin E addition to diets B+E and C+E reduced the Fe/Ascorbate-induced peroxidation of liver microsomes compared to unsupplemented groups B and C. In this case, differences were significant between groups C and C+E. There were no differences between vitamin E supplemented and unsupplemented groups in NADPH/ADP/Fe induced lipid peroxidation of liver microsomes and in the peroxidation of brain homogenates (data not shown).

Table 1 Fatty acid levels and peroxidizability indexes (PI) in tissues of rats at weaning fed the experimental diets[1]

	diets				
	A	B	B+E	C	C+E
Plasma					
20:4(n-6)	23.9 ± 1.7a	8.7 ± 0.6c	9.6 ± 0.9c	15.1 ± 0.8b	17.4 ± 1.2b
22:6(n-3)	3.7 ± 0.2c	9.9 ± 0.7b	11.7 ± 0.4a	9.1 ± 0.3b	9.47 ± 0.3b
SAT[1]	33.4 ± 1.1ab	31.5 ± 1.0bc	31.9 ± 0.8bc	30.8 ± 0.6c	31.7 ± 0.89c
MONO[1]	24.0 ± 1.2	24.9 ± 1.2	25.0 ± 1.0	25.4 ± 0.7	24.1 ± 0.9
(n-6) LCP[1]	25.4 ± 1.7a	9.3 ± 0.3c	10.3 ± 1.1c	16.1 ± 0.8b	18.0 ± 1.4b
(n-3) LCP[1]	4.3 ± 0.3c	15.6 ± 0.4b	19.0 ± 0.9a	14.4 ± 0.8b	14.6 ± 0.7b
Erythrocyte					
20:4(n-6)	27.9 ± 0.7a	18.4 ± 0.3c	18.1 ± 0.4c	21.7 ± 0.4b	22.7 ± 0.8b
22:6(n-3)	4.2 ± 0.2c	8.7 ± 0.2a	8.2 ± 0.2a	7.3 ± 0.3b	7.3 ± 0.2b
SAT[1]	42.2 ± 0.8ab	42.5 ± 0.4ab	42.9 ± 0.5ab	43.7 ± 0.3a	42.0 ± 0.2b
MONO[1]	16.6 ± 0.5a	14.9 ± 0.4b	14.5 ± 0.3b	14.5 ± 0.3b	15.5 ± 0.6ab
(n-6) LCP[1]	30.6 ± 0.5a	19.5 ± 0.4c	19.0 ± 0.4c	22.8 ± 0.4b	23.3 ± 0.7b
(n-3) LCP[1]	6.1 ± 0.3c	17.6 ± 0.2a	16.8 ± 0.4a	13.0 ± 0.4b	13.2 ± 0.7b
PI[1]	125 ± 29c	135 ± 2a	132 ± 3ab	133 ± 3ab	130 ± 3bc
Liver microsomes					
20:4(n-6)	18.0 ± 1.5a	11.3 ± 0.5b	10.7 ± 1.0b	15.5 ± 0.9a	16.3 ± 1.2a
22:6(n-3)	7.6 ± 1.d	10.4 ± 1.2cd	12.9 ± 1.0bc	14.3 ± 1.1ab	17.7 ± 1.4a
SAT[1]	47.8 ± 2.3	49.3 ± 2.4	47.4 ± 2.9	45.8 ± 1.5	44.0 ± 2.7
MONO[1]	14.8 ± 1.7a	14.1 ± 0.6a	13.0 ± 1.0a	11.0 ± 0.6b	10.5 ± 0.7b
(n-6) LCP[1]	19.4 ± 1.7a	12.1 ± 0.4b	11.5 ± 1.0b	16.6 ± 0.9a	17.7 ± 1.2a
(n-3) LCP[1]	8.6 ± 1.2c	14.6 ± 1.7b	16.6 ± 1.5ab	17.6 ± 1.4ab	20.6 ± 1.6a
PI[1]	106 ± 9c	114 ± 9c	113 ± 8c	142 ± 8b	162 ± 7a
Brain					
20:4(n-6)	10.9 ± 0.3a	8.6 ± 0.4c	8.2 ± 0.3 c	9.7 ± 0.3b	10.1 ± 0.4b
22:6(n-3)	17.3 ± 0.7b	21.8 ± 0.8a	20.9 ± 1.3a	19.8 ± 0.7a	19.7 ± 0.7a
SAT[1]	38.1 ± 1.2	38.3 ± 1.5	38.0 ± 1.1	37.6 ± 1.3	37.9 ± 0.8
MONO[1]	24.5 ± 0.8	23.4 ± 1.2	24.3 ± 2.0	24.3 ± 1.6	25.1 ± 1.5
(n-6) LCP[1]	16.9 ± 0.4a	13.3 ± 0.4c	12.6 ± 0.4c	15.3 ± 0.6b	15.2 ± 0.4b
(n-3) LCP[1]	17.4 ± 0.7b	22.4 ± 0.6a	21.6 ± 1.1a	20.3 ± 0.7a	20.0 ± 0.6a
PI[1]	140 ± 3	151 ± 5	141 ± 6	148 ± 3	145 ± 3

[1]Fatty acids are expressed as mol/100mol ± SEM of total fatty acid methyl esters for seven animals in each group. PI index and diet composition refer to Materials and Methods. Numbers with different superscript (a-c) are significantly different, $p<0.05$. SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids; LCP, total (n-3) fatty acids longer than 18 carbon atoms.

Discussion

LCP, which have been recently added to commercial preterm and term infant formulas, are prone to lipid peroxidation. The sensitivity increases as a function of the number of double-bonds of each individual fatty acid. Modifications of the fatty acid composition of enteral and parenteral diets to improve growth and development may modify the sensitivity of the newborn baby to oxidative stress. In preterm infants, supplementation of fatty acids gave conflicting results: polyunsaturated fatty acids in parenteral formula increased lipid peroxidation products (31) whereas TBARS production was not modified or was decreased by enteral LCP supplementation (12, 32). In adults, supplementation of diets with LCP increased lipid peroxidation products and oxidative sensitivity (33).

Protection against oxidative damage is conferred by dietary antioxidants, such as vitamin E and vitamin C (9). No study has assessed the protective effect of dietary vitamin E against tissue oxidative sensitivity in an experimental model of postnatal development when animals are simultaneously fed with LCP supplemented diets. In the present study, we report the effect of a low dosage of vitamin E on liver and brain antioxidant defence systems and on tissue susceptibility to lipid peroxidation in wean-

Table 2 Effect of vitamin E and LCPs intake on liver and brain antioxidant defence[1]

	diets				
	A	B	B+E	C	C+E
Liver					
GPx	134 ± 29a	94 ± 21ab	59 ± 15ab	66 ± 7b	73 ± 14ab
GST	69 ± 2	73 ± 2	72 ± 3	71 ± 2	69 ± 2
total SOD	279 ± 7ab	262 ± 7b	291 ± 8a	284 ± 3a	281 ± 4a
MnSOD	43 ± 01	40 ± 1	44 ± 1	40 ± 1	39 ± 1
CAT	257 ± 32ab	260 ± 25ab	261 ± 32ab	255 ± 16a	215 ± 15b
Brain					
GPX	5.5 ± 0.5	4.9 ± 0.3	5.1 ± 0.3	5.1 ± 0.4	5.3 ± 0.3
GST	25.0 ± 0.1	25.2 ± 0.1	25.0 ± 0.1	25.6 ± 0.1	26.5 ± 0.2
total SOD	188.5 ± 7.5	197.0 ± 9.5	200.2 ± 9.8	201.3 ± 13.5	202.7 ± 9.3
MnSOD	32.6 ± 1.6	33.8 ± 1.7	33.8 ± 1.7	31.5 ± 1.7	32.7 ± 1.7

[1] Results are expressed as mean ± SEM; seven animals for each group. Diet composition refer to Materials and Methods. Activities of antioxidant enzymes are expressed as: catalase (CAT), μmol H_2O_2 decomposed.min-[1]mg protein-[1]. Total superoxide dismutase (total SOD), manganese superoxide dismutase (MnSOD), glutathione transferase (GST) and glutathione peroxidase (GPx), units.min-[1]mg protein-[1]. Numbers with different superscript (a,b) are significantly different, $p<0.05$.

Table 3 Influence of vitamin E and LCPs intake on hepatic total glutathione, reduced glutathione and vitamin E[1]

	diets				
	A	B	B+E	C	C+E
GSH	3.3 ± 0.3b	3.8 ± 0.4ab	3.6 ± 0.4ab	4.6 ± 0.3a	4.0 ± 0.2ab
total GSH	1.9 ± 0.1b	2.1 ± 0.2b	2.0 ± 0.1b	3.3 ± 0.3a	2.9 ± 0.1a
vitamin E	0.16 ± 0.06	0.21 ± 0.07	0.22 ± 0.06	0.21 ± 0.01	0.26 ± 0.05

[1] Results are expressed as mean ± SEM; seven animals for each group. Diet composition refer to Materials and Methods. Reduced glutathione (GSH) and total glutathione are expressed as μmol.g-1 of liver, and vitamin E as μg.mg protein-[1]. Numbers with different superscript (a,b) are significantly different, $p<0.05$.

ling rats which received LCP from (n-3) and (n-6) series. The supply of vitamin E (50 mg/kg diet) added to diets B+E and C+E is very close to the minimun requirement for long-term maintenance of laboratory animals (34). Thus, our results would show the effect of the minimum nutritionally safe dosage of vitamin E on tissue lipid peroxidation while receiving dietary LCP during postnatal development. Like human milk, rat milk supports saturated, monounsaturated and essential fatty acids as well as 20:4(n-6) and 22:6(n-3). Our diets were designed to provide amounts of saturated fatty acids, 18:1(n-9) and essential fatty acids (18:2(n-6) and 18:3(n-3)) similar to those provided by the mother's milk. LCP were included in our experimental diets B, B+E, C and C+E to support preformed 20:4(n-6) and 22:6(n-3) as rat milk does.

The most notable finding was that susceptibility of erythrocyte membranes to lipid peroxidation was highly sensitive to dietary vitamin E during LCP supplementation. Groups B+E and C+E had lower rates of induced lipid peroxidation in erythrocyte membranes and liver microsomes than did unsupplemented groups B and C. This antioxidant effect was significantly higher in rats fed (n-3) and (n-6) LCP (group C versus group C+E). Red cells are continously exposed to intracellular oxidative stress due to the generation of superoxide anion by the self-oxidation of hemoglobin (35). A role for OFR as mediators of tissue injury has been proposed in hyperoxia (36). Under certain physiopathological circumstances, hyperoxic ventilatory therapy is provided to premature infants to compensate for decreased oxygen intake due to lung immaturity. Since free oxygen radical-induced lipid

peroxidation was correlated with outcome in very low birth weight infants (37), this result may have implications in infant-formula design. In liver microsomes, protection against oxidation was only noticable in group C versus group C+E when lipid peroxidation was nonenzymatically induced. Given that the vitamin E contents in liver microsomes were similar in all groups, our data suggest that other dietary factors related to the composition of the animal phospholipid concentrate may influence the antioxidant protection of liver lipids. Dietary vitamin E did not alter the susceptibility of brain lipids to *in vitro* lipid peroxidation, suggesting that antioxidant protection may require higher vitamin E intake. A higher degree of protection against oxidation in rat liver and heart was previously observed when diets included vitamin E concentrations higher than 50 mg/kg diet (15, 38).

As we previously described, LCP supplemented diets significantly increased LCP content in tissues compared with unsupplemented diet A (16). Our present data show that vitamin E intake does not modify the percentage distribution of 20:4(n-6) and 22:6(n-3) in rat tissues, except for plasma 22:6(n-3) levels, which were significantly higher in group B+E compared with group B. Given that 22:6(n-3) is the most susceptible fatty acid to lipid peroxidation, this result may be related to the protection against oxidation provided by vitamin E and oleic acid in plasma lipoproteins (39).

Antioxidant enzymes constitute a major defensive barrier against oxygen radical damage and participate in the control of membrane damage and lipid peroxidation in cells. As the antioxidant enzymes GPx and CAT detoxify hydroperoxides, and SOD detoxifies superoxide radicals, they all play along with antioxidant nutrients, a key role in the antioxidant defence system. Alterations in the activities of antioxidant enzymes may predispose tissues to free radical injury. Nutritional intervention with different fat sources has been shown to modify the cellular antioxidant defence mechanism. In monkeys, the addition of vitamin E (270 mg/kg diet) to a 10% fish oil diet reduced liver CAT, SOD and GPx activities compared with unsupplemented diet (40). Higher hepatic CAT, SOD, GST and GPx activities have been reported in rats fed a 10% fish oil diet supplemented with 200 mg/kg diet vitamin E than in rats fed the same diet devoid of vitamin E (13). Differences in study design may explain the discrepancy in results reported. In our study, the addition of vitamin E increased liver total SOD activity in group B+E compared with group B whereas decreased hepatic CAT activity in group C in relation to group C+E. The absence of marked changes in liver and brain antioxidant defence may be due in part to the low amount of both LCP (3%) and vitamin E (50 mg/kg diet) in relation to other studies (LCP, 10-20 wt%; vitamin E, 200-300 mg/kg diet).

The glutathione ratio is a sensitive marker of oxidative stress. The addition of vitamin E to diets B+E and C+E reduced the amounts of total glutathione and GSH compared with unsupplemented diets B and C. In 1994, Cho and Choi (15) reported that hepatic glutathione levels in rats fed an (n-3) LCP diet progressively declined as dietary vitamin E amounts ranging from 3 IU/kg to 209 IU/kg were increased. Detoxification of lipid hydroperoxides by GPx and inactivation of aldehyde products of lipid peroxidation by GST require the presence of GSH. Reduced synthesis of glutathione in livers of rats fed vitamin E may be related to a sparing effect induced by vitamin E. At the first line of defence, vitamin E would inhibit the initiation of lipid peroxidation by the reduction of lipid hydroperoxide formation, consequently reducing the requirement for glutathione in the cell.

In conclusion, this study shows that a low amount of vitamin E protected erythrocyte lipids enriched in (n-3) and (n-6) LCP from *in vitro* lipid peroxidation during the postnatal development of rats. Dietary vitamin E intake does not interfere with (n-3) and (n-6) LCP accretion to tissue lipids in weanling rats. Vitamin E more effectively decreased *in vitro* TBARs production in tissues from rats fed diet C+E high in (n-3) and (n-6) LCP than in tissues from rats fed diet B+E high in (n-3) LCP. The recommendation of the Nutrition Committe of the European Society for Gastroenterology and Nutrition (5), the Directive of the European Union for infant formulas (6) and the FAO (7) states that preterm infants who do not receive human milk should be fed formulas supplemented with (n-3) and (n-6) LCP. The analysis of our data suggest that safe infant formulas for the perinatal period should include an amount of vitamin E higher than 500 mg/kg fat to guarantee incorporation and protection of dietary 20:4(n-6) and 22:6(n-3) into organ lipids in neonates. More studies are needed to determine what an adequate ratio of dietary vitamin E to total LCP should be for the design of infant formulas.

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References

1. Crawford MA (1993) The role of essential fatty acids in neural development: implications for perinatal nutrition. Am J Clin Nutr 57(suppl):703S–710S
2. Hoffman DR, Birch EE, Birch DG, Uauy RD (1993) Effect of supplementation with w-3 long-chain polyunsaturated fatty acids on retinal and cortical development in premature infants. Am J Clin Nutr 57(suppl):807S–812S
3. Woltil HA, Van Beusekom CM, Schaafsma A, Muskiet FA, Okken A (1998) Long-chain polyunsaturated fatty acid status and early growth of low birth weight infants. Eur J Pediatr 157:146–152
4. Willats P, Forsyth JS, DiModugno MK, Varma S, Colvin M (1998) Effect of long-chain polyunsaturated fatty acids in infant formula on problem solving

at 10 months of age. Lancet 352:688–691
5. Aggett PJ, Haschke F, Heine W, Hernell O, Koletzko B, Launiala K, Rey J, Rubino A, Schoch G, Senterre J, Tormo R (1991) ESPGAN Committee on Nutrition. Acta Paediatr 80:887
6. Directive 96/4/CE of the European Union for Infant Formulas, February 16th, 1996
7. FAO (1993) Lipids in early development. In: FAO (eds) Fats and oils in human nutrition. FAO Foods and Nutrition Papers, Roma, pp 49–56
8. Halliwell B, Murcia MA, Chirico S, Aruoma O (1995) Free radicals and antioxidants in food and in vivo: What they do and how they work? Crit Rev Food Sci Nutr 35:7–20
9. Halliwell B (1996) Antioxidants in human health and disease. Ann Rev Nutr 16:33–50
10. Cotgreave IA, Moldeus P, Orrenius S (1988) Host biochemical defense mechanisms against prooxidants. Ann Rev Pharmacol Toxicol 28:189–212
11. Suarez A, Faus MJ, Gil A (1996) Dietary supplementation with long-chain polyunsaturated fatty acids increases susceptibility of weanling rat tissue lipids to in vitro lipid peroxidation. J Nutr Biochem 7:252–260
12. Jacobs NJ, Van-Zoeren-Grobben D, Drejer GF, Bindels JG, Berger HM (1996) Influence of long chain unsaturated fatty acids in formula feeds on lipid peroxidation and antioxidants in preterm infants. Pediatr Res 40:680–686
13. Pulla-Reddy AC, Lokesh BR (1994) Alterations of lipid peroxides in rat liver by dietary n-3 fatty acids: modulation of antioxidant enzymes by curcumin, eugenol and vitamin E. J Nutr Biochem 5:181–188
14. Bouglé D, Nouvelot A, Billeaud C, Sarda P, Entressangles B, Descomps B, Mendy F (1996) Relationships between red blood cell vitamin E and polyunsaturated fatty acid in the premature infant. Ann Nutr Metab 40:325–330
15. Cho S-H, Choi Y-S (1994) Lipid peroxidation and antioxidant status is affected by different vitamin E levels when feeding fish oil. Lipids 29:47–52
16. Suárez A, Ramírez MC, Faus MJ, Gil A (1996) Dietary long-chain polyunsaturated fatty acids influence tissue fatty acid composition in rats at weaning. J Nutr 126:887–897
17. Valenzuela A, Nieto, S (1996) Synthetic and natural antioxidants: food quality protectors. Grasas y Aceites 47: 186–196
18. Burton GW, Ingold KU, Thompson KE (1981) An improved procedure for the isolation of ghost membranes from human red blood cells. Lipids 16:946–950
19. Kolarovic L, Fournier N (1986) A comparison of extraction methods for the isolation of phospholipids from biological sources. Anal Biochem 156:244–250
20. Morrison MR, Smith LM (1964) Preparation of fatty acids methyl esters and dimethylacetals from lipids with boron fluoride methanol. J Lipid Res 5:600–607
21. Skúladóttir GV, Shi-Hua D, Brodie AE, Reed DJ, Wander RC (1994) Effect of dietary oils and methyl ethyl ketone peroxide on in vivo lipid peroxidation and antioxidants in rat heart and liver. Lipids 29:351–357
22. Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. Meth Enzymol 113:548–555
23. Akerboom TPM, Sies H (1989) Transport of glutathione, glutathione disulfide, and glutathione conjugates across the hepatocyte plasma membrane. Meth Enzymol 173, 373–382
24. Flohé L, Otting F (1984) Superoxide dismutase assays. Meth Enzymol 105:93–104
25. Aebi H (1984) Catalase in vitro. Meth Enzymol 105:121–126
26. Flohé L, Gunzler WA (1984) Assays of glutathione peroxidase. Meth Enzymol 105:114–12127
27. Warholm M, Guthenberg C, Von Bahr C, Mannervik B. (1985) Glutathione transferases from human liver. Meth Enzymol 113:499–503
28. 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 24:833–835
29. Esterbauer H, Cheeseman KH (1990) Determination of aldehydic lipid peroxidation products: Malondialdehyde and 4-hydroxynonenal. Meth Enzymol 186:408–421
30. Bieri JG, Tolliver TJ, Catignani GL (1979) Simultaneous determination of alpha-tocopherol and retinol in plasma and red cells by high pressure liquid chromatography. Am J Clin Nutr 32:2143–2149
31. Wispe JR, Bell EF, Roberts RJ (1985) Assessment of lipid peroxidation in newborn infants and rabbits by measurements of expired ethane and penthane: influence of parenteral lipid infusion. Pediatr Res 19:374–379
32. Hoffman DR, Uauy R (1992) Essentiality of dietary w-3 fatty acids for premature infants: plasma and red blood cell fatty acid composition. Lipids 27:886–895
33. Van Gossum A, Shariff R, Lemoyne M, Kurian R, Jeejeebhoy K (1988) Increased lipid peroxidation after lipid infusion as measured by breath pentane output. Am J Clin Nutr 48:1394–1399
34. Reeves PG, Nielsen FH, Fahey GC (1993) AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc Writing Committee on the reformation of the AIN-76A rodent diet. J Nutr 123:1939–1951
35. Misra HP, Fridovich I (1972) The generation of superoxide radical during the autoxidation of hemoglobin. J Biol Chem 247: 6960–6962
36. Wispe JR, Roberts RJ (1987) Molecular basis of pulmonary oxygen toxicity. Clin Perinatol 14:651–656
37. Pitkanen OM, Hallman M, Andersson SM (1990) Correlation of free oxygen radical-induced lipid peroxidation with outcome in very low birth weight infants. J Pediatr 116:760–764
38. Leibovitz BE, Hu M-L, Tappel AL (1990) Lipid peroxidation in rat tissue slices: effect of dietary vitamin E, corn oil-lard and menhaden oil. Lipids 25:125–129
39. Parthasarathy S, Khoo JC, Miller E, Barnett J, Witztum JL, Steinberg D (1990) Low density lipoprotein rich in oleic acid is protected against oxidative modification: implications for dietary prevention of atherosclerosis. Proc Natl Acad Sci U S A 87:3894–3898
40. Kaasgaard SG, Holmer G, Hoy C-E, Behrens WA, Beare-Rogers JL (1992) Effects of dietary linseed oil and marine oil on lipid peroxidation in monkey liver in vivo and in vitro. Lipids 27:740–745