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SHORT CONTRIBUTION

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Long chain polyunsaturate supplementation does not induce excess lipid peroxidation of piglet tissues

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Summary *Background* Addition of highly polyunsaturated fatty acids to infant formulas raises the possibility of increased lipid peroxidation. *Aim of the study* We determined the effects of increasing levels of dietary docosahexaenoic acid (DHA) and arachidonic acid (AA) on lipid peroxidation and peroxidative potential in piglet tissues. *Methods* Four groups of piglets (n = 6) were bottle-fed a formula containing one of four treatments: no long chain fatty acid (Diet 0) and three different levels of DHA/AA at 1-fold (0.3 %/0.6 % FA; Diet 1) 2-fold (0.6 %/1.2 % FA; Diet 2) and 5-fold (1.5 %/3 % FA; Diet 5) concentration used in some human infant formulas, and all with equal amount of vitamin E (5.7 IU/100 kcal formula) for four weeks. *Results* There were no significant differences between the groups in conjugated diene and glutathione (GSH) levels in the liver, and thiobarbituric acid-reactive substance (TBARS) in plasma. TBARS levels of the erythrocyte membranes increased in a dose-dependent manner when in vitro oxidation was induced with 10 mM hydrogen peroxide (H_2O_2) for 30 minutes. The TBARS levels of the liver homogenates of the Diet 5 and Diet 2 groups were significantly different than those of the membranes of the Diet 0 group when the in vitro oxidation was induced with H_2O_2. *Conclusion* The results show that dietary vitamin E effectively prevented lipid peroxidation at the LCP concentrations investigated and suggest that levels presently in infant formulas are sufficient.

Key words LCP supplementation – piglets – lipid peroxidation – vitamin E – red blood cells

Abbreviations

AA	arachidonic acid
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
GSH	glutathione
LCP	(C ≥ 20) long chain polyunsaturated fatty acids
PUFA	polyunsaturated fatty acid
RBC	red blood cells
SCO	single-cell oil
TBARS	thiobarbituric acid reactive substances

Introduction

Long chain polyunsaturated fatty acids (LCP) are recognized as playing a key role in the development of the human central nervous system in the perinatal period [1]. Starting in the mid-1990s, infant formulas with LCP have appeared in countries around the world, and recently in the USA and Canada. The goal of supplementation is to increase tissue content of LCP, and it is well known that supplementation is particularly effective at increasing tissue docosahexaenoic acid (DHA) content [2]. Tissues with greater unsaturation levels are at risk

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for increased oxidative susceptibility. In the absence of adequate endogenous antioxidant, the propagation of free radicals can lead to the reaction of secondary lipid autooxidation products with macromolecules such as membrane constituents, enzymes, and DNA. These concerns are relevant at all ages, but particularly for infants who are growing rapidly. An obvious choice for assisting in protection of LCP is vitamin E, which has long been known to function as an antioxidant for protection of polyunsaturated fatty acids in cell membranes through its peroxyl radical trapping activity [3].

In a recent study [4], we reported that increasing amounts of LCP fed to piglets in the first month of life did not alter serum chemistry or other measures of well-being. Here, we report the effects of increasing doses of dietary DHA and arachidonic acid (AA) and fixed amount of vitamin E supplementation on lipid peroxidation and in vitro susceptibility to oxidation in piglet tissues from that study.

Materials and methods

Animals and diets

All procedures involving animals were approved by the Cornell Institutional Animal Care and Use Committee (IACUC). Six sows from the Cornell University swine facility were delivered spontaneously at term and four healthy male piglets from each litter were randomly assigned to one of the treatment groups. Diet 0 served as an LCP-free control; Diet 1, Diet 2, and Diet 5 contained 34/17, 68/34 and 170/85 AA/DHA in units of *mg fatty acid per 100 kcal formula*. All diets contained 5.77 IU vitamin E per 100 kcal formula. Seventy-five percent of the vitamin E content was alpha-tocopherol with the reminder being of unspecified stereochemistry. The diet composition and preparation procedure is detailed elsewhere [4].

Analyses

Red blood cell (RBC) FA composition was determined by gas chromatography as previously described [4]. A representative coefficient of variation (CV) for components of minor abundance, e. g., LCP, is about 10 %. Determination of glutathione (GSH) in the liver was carried out by modification of the method of Ellman [5]. Briefly, liver homogenates (10 % w/v) were prepared and were combined with sodium azide (2 mM) and sulfosalicylic acid (4 % w/v). Following centrifugation, supernatants were analyzed for acid-soluble free sulfhydryl group content using 5,5'-dithiobis(2-nitrobenzoic acid), with representative CV of about 11 %. Conjugated dienes were assayed in an isooctane extract of liver homogenate by absorption at 234 nm and calibrated using a molar absorption coefficient of 29,500 $M^{-1}\,cm^{1}$ (CV~16 %). Plasma thiobarbituric acid reactive substances (TBARS) was measured by the method of Placer et al. [6]. Quantification of TBARS was performed by using 1,1,3,3 tetraethoxypropane standard (CV~8 %). Oxidative susceptibility was assessed by determination of TBARS concentration after exposure of RBCs to 10 mM hydrogen peroxide (H_2O_2) [7] (CV~10 %), and liver homogenates to a range of hydrogen peroxide concentrations (0, 1, 5, 10 mM) for 30 minutes (CV~14 %) [8].

Statistics

Kruskal-Wallis one-way ANOVA by ranks was applied to the data. Post hoc comparisons between pairs of means are made by using Wilcoxon rank sum test, with downward adjustment of the alpha level to compensate for multiple comparisons; significance was set at $P < 0.083$ (0.05/6 comparisons = 0.083) and performed by SPSS release 10.0 for Windows. For liver TBARS the effect of the diet was determined by the nonparametric Friedman two-way ANOVA procedure followed by a post-hoc test performed by SAS 8.1 (Cary, NC).

Results

The major results are presented in Table 1. Erythrocyte RBC membrane total lipid DHA and AA increased with dietary DHA/AA supplementation. These modifications resulted in a concomitant dose-dependent increase in the membrane unsaturation index. *In vivo* lipid peroxidation was assessed by measuring conjugated diene and glutathione levels in the liver, and by plasma TBARS. There were no significant differences between the groups in any of these parameters.

Oxidative susceptibility to 10 mM hydrogen peroxide was determined in RBC membranes. The TBARS level of Diet 1 was not different from the control Diet 0, or from Diet 2 and Diet 5. Diet 2 and Diet 5 had significantly greater TBARS than Diet 0, indicating greater susceptibility to this extreme oxidative insult, and consistent with great tissue unsaturation.

Fig. 1 shows graphically the liver homogenate oxidative susceptibility after incubation with increasing concentrations of hydrogen peroxide and measured by TBARS. With the Friedman procedure keeping the H_2O_2 effect constant, we found the effect of diet significant ($P < 0.05$). The significant differences were detected between Diet 0 vs. Diet 2 and Diet 0 vs. Diet 5 when the observations were pooled for all four H_2O_2 levels.

Table 1 Red blood cell fatty acid composition and antioxidant status of 4-wk-old piglets fed formulas with increasing amount of PUFA[1]

	Diet 0	Diet 1	Diet 2	Diet 5
Red blood cell DHA[2]	0.9±0.2[a]	2.8±0.5[b]	3.6±0.4[b]	4.0±0.5[b]
Red blood cell AA[2]	4.0±0.2[a]	5.4±0.9[b]	6.5±0.7[b, c]	8.2±0.4[c]
Red blood cell UI[3]	106	110	119	119
Liver glutathione (µmol/g wet tissue)	5.2±0.6	5.8±0.8	5.1±0.8	5.9±0.3
Liver conjugated dienes (nmol/g wet tissue)	35.5±19.4	27.3±9.6	30.0±13.1	36.2±7.8
Plasma TBARS (nmol/ml plasma)	10.0±1.9	9.4±0.8	10.0±2.1	9.2±1.4
RBC oxidative susceptibility[4] (nmol/g hemoglobin)	71.6±4.5[a]	78.8±8.5[a, b]	81.8±2.7[b]	92.6±10.8[b]

[1] Values are the means ± SD, n = 6. Different superscripts in a row indicate significantly different values ($P < 0.05$) by one-way ANOVA. The Diet 0 served as an LCP-free control, Diet 1 contains LCP in some infant formulas in the USA, Diet 2 and Diet 5 have approximately 2- and 5-fold greater concentration of LCP than Diet 1.
[2] Expressed as wt% of total fatty acids
[3] *UI* Unsaturation index = $\sum_i (F_x \times db_x)$, where F is percentage of fatty acid x, and db is number of double bonds in fatty acid x.
[4] TBARS measured after incubating RBC in 10 mM hydrogen peroxide for 30 minutes

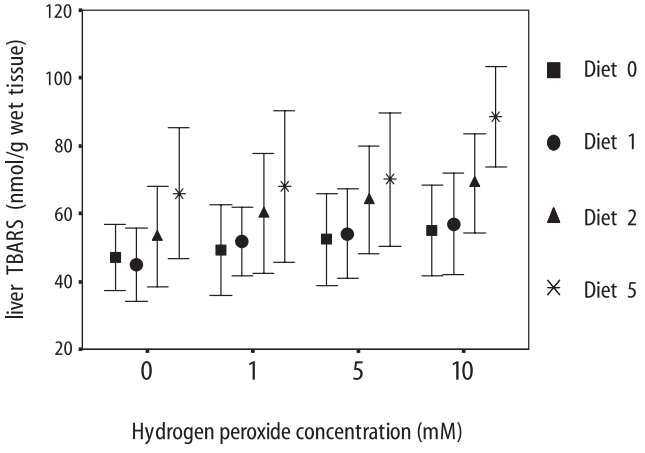

Fig. 1 Susceptibility of liver homogenate to hydrogen peroxide-induced lipid peroxidation. TBARS levels were measured in the liver homogenates after exposure of increasing concentration of hydrogen peroxide. Values are means ± SD (n = 6). The effect of the diet was significant ($P < 0.05$) keeping the H_2O_2 effect constant. The significant differences were detected between Diet 0 vs. Diet 2 and Diet 0 vs. Diet 5 when the observations were pooled for all four H_2O_2 levels

Discussion

Dietary long chain fatty acids are readily incorporated into cell membranes and the alteration in fatty acid composition is expected to make tissue and plasma more susceptible to free radical attack and lipid peroxidation. Increasing membrane unsaturation increases oxidative susceptibility and in principle increases the need for antioxidant protection. Therefore, the recommendation for vitamin E is higher when large amounts of LCP are consumed.

A human infant formula that has proven beneficial in a recent clinical trial provides 21.3 IU vitamin E per gram of LCP [9]. The piglet formulas Diet 1, Diet 2, and Diet 5 contain 113 IU, 57 IU, and 23 IU vitamin E, respectively, per gram of AA and DHA. Comparing the amount of vitamin E contained in the piglet formulas to the human infant formula, Diet 1 contained 5.3-fold, Diet 2 2.7-fold, and Diet 5 1.1-fold vitamin E per gram DHA and AA. Diet 5 therefore contained an equivalent amount of vitamin E/gram LCP as the human LCP-containing formula. Because greater relative amounts of vitamin E did not yield lower values for piglet tissue oxidation, e. g., in Diet 1 or Diet 2, we conclude that increased amounts of vitamin E would not improve oxidative status of tissues for human infants on that formula.

A dose response was observed for increasing oxidative susceptibility with increasing LCP. The hydrogen peroxide concentrations used in these experiments provided a much greater oxidative challenge than would be seen under physiological conditions, and were chosen to produce an effect. The diet effect was significant for liver TBARS and the RBC membrane responded to the 10 mM hydrogen peroxide concentration. For both the RBC and liver Diet 2 and Diet 5 produced significantly more TBARS than the controls. It is not clear if there are important physiological consequences to increasing oxidative susceptibility, and therefore these data alone do not support increasing vitamin E in formula.

The TBARS test is sensitive to many secondary products formed during lipid peroxidation, and it is accepted as an overall estimate of lipid peroxidation in tissues and plasma. This assay is thought to be preferable to other methods when the content of dietary fat is varied [10, 11]. The other indices of peroxidation that we measured, conjugated dienes and glutathione, are consistent with the TBARS data. Conjugated dienes are produced during the first phase of lipid peroxidation and glutathione is essential for maintenance of protein thiols and has other antioxidant functions.

There are many studies of human adults on fish oil supplementation, used as a source of DHA and eicosapentaenoic acid (EPA, 20:5n-3). Results conflict, showing either increased or decreased oxidative sensitivity [12, 13]. In preterm human studies, where formula was supplemented with 0.35 % DHA and 0.65 % EPA, RBC do not have elevated TBARS levels [14]. Supplementation of preterms with 0.4 % AA and 0.25 % DHA does not result in increased lipid peroxidation measured by the AA metabolite F2 isoprostanes [15].

In summary, our data collectively support the levels of vitamin E now used in US formulas. The data show that no additional protection against oxidation is necessary in supplemented piglet tissues at vitamin E to LCP levels five-fold greater than those used in commercial infant formulas in the USA.

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