The Influence of Prematurity and Long Chain Polyunsaturate Supplementation in 4-Week Adjusted Age Baboon Neonate Brain and Related Tissues

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ABSTRACT

Clinical studies show that docosahexaenoic acid (DHA) and arachidonic acid (ARA) supplemented formula improve visual function in preterm infants, however improved fatty acid status is known only for plasma and red blood cells (RBC) since target organs cannot be sampled from humans. Baboons were randomized to one of four groups: Term breast-fed (B); Term formula-fed (T-); Preterm formula-fed (P-); and Preterm DHA/ARA-supplemented formula-fed (P+). The P+ contained $0.61 \pm 0.03\%$ DHA and $1.21 \pm 0.09\%$ ARA, and breast milk had $0.68 \pm 0.22\%$ and $0.62 \pm 0.12\%$ as DHA and ARA, respectively. The B and P+ groups had significantly higher DHA concentration in all tissues than T- and P-. The P- group showed dramatically lower DHA content of 35%, 27%, 66%, and 75% in the brain, retina, liver, and plasma, respectively, compared with B. Supplementation prevented declines in DHA levels in the retina, and liver, and attenuated the decline in brain, plasma and RBC of preterm animals. In contrast, ARA was not significantly lower compared with B in any group in any tissue but was significantly elevated in liver and brain. RBC and plasma DHA were correlated with DHA in tissues; RBC/plasma ARA were uncorrelated with tissue ARA. We conclude that 1) DHA drops precipitously in term and preterm primates consuming formula without long chain polyunsaturates, while 22:5n-6 concentration rises; 2) tissue ARA levels are insensitive to dietary LCP supplementation

or prematurity, 3) plasma and RBC levels of ARA are uncorrelated with total ARA levels; 4) DHA levels are correlated with group effects and are uncorrelated within groups. (*Pediatr Res* 54: 244-252, 2003)

Abbreviations

ARA, arachidonic acid (20:4n-6)
B, breast-fed
CS, cesarean section
DHA, docosahexaenoic acid (22:6n-3)
EDTA, ethylenediaminetetraacetic acid
FA, fatty acid
FAME, fatty acid methyl ester
GC, gas chromatography
LA, linoleic acid (18:2n-6)
LCP, (≥ 20 carbons) long-chain polyunsaturated fatty acids
LNA, α -linolenic acid (18:3n-3)
PC, phosphatidylcholine
PE, phosphatidylethanolamine
PI, phosphatidylinositol
PL, phospholipids
PUFA, polyunsaturated fatty acid
RBC, red blood cells

Docosahexaenoic acid (DHA, 22:6n-3) is the major n-3 long chain polyunsaturated fatty acid (LCP) in the CNS of humans

and nonhuman primates. DHA deficiency induced by severe dietary restriction of n-3 fatty acids has long been known to produce characteristic functional deficits, including impaired electroretinograms and visual-evoked potential responses, and poorer visual acuity (1). Very premature human infants were the first pediatric group for which DHA was hypothesized to be conditionally essential, largely because the beginning of accelerated brain growth at about 28 wk gestation coincides with the conceptual birth age at which survival exceeds 50% (2). Early studies show that preterm infants consuming formulas containing DHA precursors but no DHA itself have poorer visual acuity than those who were supplemented with DHA (3-5).

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Concerns about overall growth raised by supplementation studies with DHA, as a component of high eicosapentaenoic acid (EPA) fish oil (6), led to the present consensus that arachidonic acid (ARA) should always be included with DHA supplementation (7). Formulas and baby foods supplemented with DHA/ARA are now available in the USA and in many countries worldwide.

Since the early 1980s, at least 12 randomized clinical trials of LCP supplementation in premature human infants have appeared, testing effects of LCP supplementation on biochemical and/or functional outcomes of preterm infants, as well as the alternative strategy to alter the n-6/n-3 precursor ratio to enhance DHA status (most reviewed recently by Gibson *et al.* (8)). Several showed improvements in visual development (9), consistent with findings from primate deficiency studies and the earliest human studies. Many studies have appeared for term infants as well, with rare exceptions showing either modest improvement or no differences. A major limitation to all these studies is that the target organs, specifically brain, retina, and liver, cannot be sampled in live human infants to assess how status changes with altered dietary intake. Red blood cells (RBC), and plasma phospholipid (PL) or total fatty acids (FA) are the usual proxies for organ concentrations, though buccal cell fatty acids obtained from cheek swab also respond to dietary fatty acid changes (10–12). From these studies there is abundant evidence that the absence of dietary DHA/ARA results in significant decreases in blood cell and plasma DHA and ARA (13–17). These data are consistent with autopsy studies of sudden-infant-death syndrome infants showing that brain lipids of unsupplemented formula-fed infants have as much as a 40% reduction in DHA compared with breast-fed infants, depending on brain region (18–21).

A convincing body of data shows that preterm infants suffer high rates of cognitive and behavioral deficits later in life (22–25). It is tempting to link these observations to the high CNS concentrations of DHA and ARA by the hypothesis that perinatal fatty acid nutrition limits ultimate neural development in preterms. Although preterms are capable of synthesizing DHA and ARA from their 18 carbon precursors (26, 27), the adequacy of the process to support the aggressive growth of the CNS is not known. There are no autopsy studies of tissue fatty acid composition to permit evaluation of DHA levels in premature infants with or without supplemented formula. Suitable

animal models in controlled studies are required to establish whether human brain DHA and ARA concentrations are likely to be compromised in infants consuming formula containing only precursors. Baboons are among the most common models for prematurity, and as omnivorous primates are among the best models for human fatty acid nutrition (28), however there are no primate studies yet reported testing diets that manipulate n-3 fatty acid composition in diets of preterm primates.

The goal of this study was to determine the effect of prematurity and LCP supplementation on the fatty acid composition of brain, retina, liver, RBC, and plasma in neonatal baboons. Conceptual age matched randomized breast-fed and term groups served as controls. Correlations between ARA and DHA concentrations in plasma and erythrocytes *versus* those in developing tissues are examined to enable extrapolations in human studies.

MATERIALS AND METHODS

Animals and experimental design. All procedures involving animals were approved by the Cornell Institutional Animal Care and Use Committee (IACUC), and the animal facility is approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The characteristics of the animals are summarized in Table 1. Twenty-two pregnant baboons/neonates were randomly assigned to one of four categories: term breast-fed (B)($n = 5$); term formula-fed (T–; $n = 7$); preterm, formula-fed (P–; $n = 5$); preterm, formula + LCP-fed (P+; $n = 5$). Term and breast-fed animals were allowed to give birth spontaneously, around 182 d of gestational age. Formula-fed animals (T–) were transferred to nursery within 12 h of birth and fed a commercially available infant formula (Enfamil, Mead-Johnson, Evansville, IN, U.S.A.) without long-chain polyunsaturates (LCP) for 4 wk. There were two animals in the term group and one in the breast-fed group, which were kept for 6 wk. Their tissue fatty acid compositions were within the range defined by the 4-week animals and therefore all data were pooled. At about 151 d of gestation, females in the premature groups received two injections of antenatal betamethasone (175 mg/kg body weight/d) 48 h and 24 h before birth. Premature neonates were delivered by cesarean sections (CS) at 153 ± 4 d of gestational age as estimated by ultrasound measurement of head circumference

Table 1. Animal Characteristics

	B	T–	P–	P+
Gender	2F, 3M	5F, 2M	3F, 2M	3F, 2M
Gestational age at CS ^a (d)	Spontaneous labor	Spontaneous labor	156 $\pm$ 5	154 $\pm$ 2
Age at euthanasia (d) ^b	28	28	208 $\pm$ 2	208 $\pm$ 2
Birth weight (g) ^c	875 $\pm$ 65 ^a	821 $\pm$ 119 ^a	632 $\pm$ 61 ^b	608 $\pm$ 71 ^b
Body weight at euthanasia (g) ^c	1152 $\pm$ 177	1147 $\pm$ 113	1071 $\pm$ 187	1035 $\pm$ 220
Brain weight at euthanasia (g) ^c	103 $\pm$ 4	98 $\pm$ 6	93 $\pm$ 8	94 $\pm$ 9
Liver weight at euthanasia (g) ^c	29 $\pm$ 6	35 $\pm$ 4	31 $\pm$ 6	29 $\pm$ 6

Data are expressed as mean $\pm$ SD.

^a CS, caesarean section.

^b Reported as birth age for B and T groups and conceptual (gestational) age for the P– and P+ groups. The normal gestational period for baboons is 180–182 days. Conceptual age for B and T at euthanasia was within 2 days of that for P– and P+. Two animals in the T– group and one in the B group were 42 days old at euthanasia. Other than these, all animals were about 208 days post-conception at euthanasia.

^c Values that do not share common capital letter superscripts are significantly different ($p < 0.05$).

and head biparietal diameter of the fetus (29). In two cases, a lung surfactant (Survanta®, Ross Products Division, Abbott Laboratories, Columbus OH, U.S.A.) was administered to premature newborns because of mild respiratory distress. Five preterm neonates (P-) were fed a formula identical to the formula fed the T- group, and five received the LCP-supplemented formula (P+). All animals were fed six times a day, *ad libitum*, until they reached 4 wk adjusted age, corresponding to about 208 d since conception, when they were euthanized. The birth weight of premature neonates was significantly lower than that for term neonates, but at 4 wk adjusted age (208 d postconception) there were no significant differences among body, brain, and liver weights of animals in the four groups.

Diets. The P-/T- formula was a commercially available preterm infant formula (Enfacare, Mead-Johnson, Evansville, IN, U.S.A.). The P+ group was fed the same formula supplemented with microencapsulated ARA and DHA to provide $0.61 \pm 0.03\%$ wt (0.3%energy) DHA and $1.21 \pm 0.09\%$ wt (0.6%energy) ARA. Samples of breast milk were collected from lactating females on the day of neonate necropsy and analyzed for FA composition. Table 2 shows the FA composition of the different dietary regimes and the lactating female's chow. The breast milk and formula had similar linolenic acid (LNA, 18:3n-3) to linoleic acid (LA, 18:2n-6) ratios, the breast milk had about half the ARA, significantly more 22:5n-3 and similar DHA concentrations compared with the P+ formula. Eicosapentaenoic acid (EPA, 20:5n-3) was detected only in breast milk. Thin layer chromatography analysis verified that the ARA and DHA were in triglyceride form in the supplemented formula (data are not shown).

Sampling. Neonates were euthanized by exsanguination under halothane general anesthesia. Blood was drawn into EDTA-containing evacuated tubes, and RBC and plasma were separated by centrifugation. Liver and brain were removed quickly, weighed and placed individually in freezer bags. Both eyes were removed, and retinas were immediately dissected and stored in sterile saline. All samples were flash frozen and stored in liquid nitrogen until they were transferred to a -80°C freezer, where they were maintained until analysis.

Analyses. Total lipids were extracted from the tissue homogenates and the diets according to a modified Bligh and

Dyer protocol (30, 31) using a chloroform-methanol-water 2:1:0.8 mixture with 0.02% butylated hydroxy toluene as an antioxidant. Fatty acid methyl esters (FAME) were formed by saponification using sodium hydroxide followed by esterification with boron-trifluoride (BF_3) in methanol. Freshly prepared di-heptadecanoyl-phosphatidylcholine for tissue samples or tri-heptadecanoin for diets (Matreya, Inc Pleasant Gap, PA, U.S.A.) was added as internal standard before homogenization. Total FAME were analyzed by gas chromatography (GC) (HP 5890 GC with flame ionization detector; BPX-70 capillary column: 60 m $\times$ 0.32 mm ID $\times$ 0.25 μm film thickness (SGE), using H_2 carrier gas). FAME were identified by comparison with FAME standards, and the results were calibrated using response factors obtained from analysis of an equal weight FAME mixture of known composition using methyl heptadecanoate as an internal standard.

Statistics. Data are expressed as mean $\pm$ SD. A one-way ANOVA with unequal n Tukey honest significant difference (HSD) post-hoc test was used to test for significant differences at $p < 0.05$, performed by STATISTICA release 4.5 for Windows (StatSoft, Tulsa, OK, U.S.A.). Polynomial equations were fit to describe the change in % FA of different organs with increasing plasma or RBC FA concentrations.

To evaluate whether correlations between plasma or RBC and tissue DHA (or ARA) were explained fully by group effects, the SPSS general linear model was used (Chicago, IL, U.S.A.). The model was $y = ax + z$, where y is the organ DHA (or ARA), a is the slope of x , x is the either plasma or RBC DHA (or ARA) as covariate, and z is the group as a categorical variable. If the slope of blood borne DHA (a) is not significant after inclusion of the groups, then none of the remaining variation in the organ DHA (or ARA) is explained by the plasma or RBC DHA levels.

RESULTS

Visual cortex fatty acids. The visual cortex (brain) total FA composition is presented in Table 3, and the results for DHA and 22:5n-6/22:6n-3 (only) are shown graphically in Fig. 1. The B group had the highest brain DHA concentration while the T- and P- groups DHA concentrations were lower by 20% and 35%, respectively. Supplementation supported simi-

Table 2. Fatty acid composition of maternal diet, breast milk, LCP-free formula and LCP supplemented formula as wt % of total fatty acids (mean $\pm$ SD)

Fatty Acids	Breast milk	T-/P- Formula	P+ Formula	Female's Feed
Σ SFA	27.87 $\pm$ 8.49	30.22 $\pm$ 2.66	32.36 $\pm$ 0.23	30.37 $\pm$ 0.66
Σ MUFA	38.95 $\pm$ 8.37	44.72 $\pm$ 2.18	43.01 $\pm$ 0.35	35.27 $\pm$ 0.99
18:2n-6	27.23 $\pm$ 3.99	22.35 $\pm$ 0.42	20.11 $\pm$ 0.14	27.93 $\pm$ 1.36
20:4n-6	0.62 $\pm$ 0.12	ND	1.21 $\pm$ 0.09	0.24 $\pm$ 0.04
22:4n-6	0.22 $\pm$ 0.06	ND	ND	ND
18:3n-3	2.12 $\pm$ 0.53	2.33 $\pm$ 0.02	2.06 $\pm$ 0.05	2.58 $\pm$ 0.22
20:5n-3	0.34 $\pm$ 0.13	ND	0.12 $\pm$ 0.02	0.85 $\pm$ 0.11
22:5n-3	0.51 $\pm$ 0.15	ND	0.06 $\pm$ 0.09	0.20 $\pm$ 0.03
22:6n-3	0.68 $\pm$ 0.22	ND	0.61 $\pm$ 0.03	0.73 $\pm$ 0.03
18:2n-6/18:3n-3	12.84	9.59	9.76	10.83

* ND, non-detectable.

Table 3. Brain (visual cortex) fatty acid composition as wt% of total fatty acids (mean $\pm$ SD)

Fatty acid	B	T–	P–	P+
Σ SFA	44.26 $\pm$ 0.97 ^a	44.97 $\pm$ 0.31 ^{ab}	45.14 $\pm$ 0.61 ^{ab}	45.36 $\pm$ 0.41 ^b
18:2n-6	1.25 $\pm$ 0.13	1.24 $\pm$ 0.10	1.25 $\pm$ 0.15	1.05 $\pm$ 0.07
20:4n-6	12.90 $\pm$ 0.26 ^a	13.22 $\pm$ 0.67 ^a	14.26 $\pm$ 0.36 ^b	13.50 $\pm$ 0.63 ^{ab}
22:4n-6	5.58 $\pm$ 0.25 ^a	6.07 $\pm$ 0.46 ^{ab}	6.44 $\pm$ 0.31 ^b	5.96 $\pm$ 0.04 ^{ab}
22:5n-6	2.06 $\pm$ 0.27 ^{ab}	2.46 $\pm$ 0.45 ^{ab}	2.58 $\pm$ 0.16 ^a	1.96 $\pm$ 0.32 ^b
24:4n-6	0.16 $\pm$ 0.02	0.18 $\pm$ 0.02	0.19 $\pm$ 0.02	0.18 $\pm$ 0.01
Σ n-6	23.85 $\pm$ 0.76 ^a	25.32 $\pm$ 1.10 ^b	27.20 $\pm$ 0.26 ^c	24.37 $\pm$ 0.73 ^{ab}
22:5n-3	0.51 $\pm$ 0.07 ^a	0.46 $\pm$ 0.10 ^{ab}	0.58 $\pm$ 0.05 ^a	0.33 $\pm$ 0.02 ^b
22:6n-3	13.55 $\pm$ 0.37 ^a	10.74 $\pm$ 1.06 ^b	8.78 $\pm$ 0.26 ^c	12.18 $\pm$ 0.27 ^d
Σ n-3	14.06 $\pm$ 0.36 ^a	11.20 $\pm$ 1.00 ^b	9.37 $\pm$ 0.26 ^c	12.51 $\pm$ 0.25 ^d
22:5n-6/22:6n-3	0.15 $\pm$ 0.02 ^a	0.23 $\pm$ 0.07 ^{bc}	0.29 $\pm$ 0.02 ^b	0.16 $\pm$ 0.03 ^{ac}

Different superscripts in a row indicate significantly different values ($p < 0.05$).

Σ SFA, sum of the saturated fatty acids; Σ MUFA, sum of the monounsaturated fatty acids, were not significantly different among the groups and overall averaged $16.87 \pm 0.52\%$ wt.

Figure 1. (A) DHA concentrations in brain, retina, liver, red blood cell (RBC), and plasma. Different letters above bars indicate that groups have statistically different values ($p < 0.05$). In pools indicated by “*”, the treatments pair B/P+ and T–/P– are significantly different from one another ($p < 0.05$), but not different within a pair. (B) 22:5n-6/22:6n-3 ratio in the pools of (A). Letters and “*” have the same meaning as in (A).

lar concentrations in brain DHA as breast-feeding, though DHA in the P+ group was 10% lower than that of the B group ($p < 0.05$). Figure 1b illustrates the results for the 22:5n-6/22:6n-3 index, taken as an indicator of n-3 deficiency. Our results show that this index depended on the presence or absence of dietary DHA. The 22:5n-6/22:6n-3 ratios for the T– and P– groups were similar, as were the ratios for the B and P+ groups; however, the 22:5n-6/22:6n-3 ratios of the T– and P– groups were elevated by 2- to 5-fold compared with those of the B and P+ groups ($p < 0.05$).

In contrast to DHA results, a significantly greater ARA concentration was observed between the B group ($12.9 \pm 0.3\%$) and the P– group ($14.3 \pm 0.4\%$), while the T– and P+ group ARA levels were not significantly elevated relative to the B group ARA levels. Notable also is the effect of prematurity revealed by the significantly greater ARA concentration in P– compared with T– ($13.2 \pm 0.7\%$).

Retina fatty acids. Our results for retina FAME analysis are summarized in Table 4. Group B had the highest DHA levels at $22.6 \pm 1.6\%$, while both unsupplemented groups were significantly lower, at $18.6 \pm 1.4\%$ for the T– group and $16.5 \pm 1.8\%$ for the P– group. Unlike in the brain, supplementation maintained the P+ group retinal DHA concentrations ($22.1 \pm 1.2\%$) at a level similar to that of the B group. The 22:5n-5/22:6n-6 ratio was similar for the B and P+ groups, and was significantly elevated, by 2- to 3-fold, for the unsupplemented groups (P–, T–).

All four groups had similar levels of retinal ARA. However, the elongation products of ARA, 22:4n-6 and 22:5n-6, were elevated in the unsupplemented formula groups compared with the B group.

Liver fatty acids. As shown in Table 5 and Fig. 1a, liver DHA concentration of the unsupplemented groups was dramatically depleted compared with the levels in the B and P+ groups, which were not significantly different. The T– and P– groups had 37% and 34%, respectively, of the liver DHA concentration in the B group, while the 22:5n-6/22:6n-3 index was elevated 3- to 4-fold compared with the B and P+ groups. The EPA concentration was significantly elevated in the B group, possibly due to the nonzero EPA levels in breast milk. Although all the neonate diets contained similar amounts of LNA, the breast-fed group had about double the liver LNA of the formula groups.

The livers of the B group had reduced levels of ARA compared with the other three groups, which could be explained by the presence of EPA in breast-milk but not in the neonate formulas. The P+ group livers contained significantly less LA than the unsupplemented groups. Similarly, the unsupplemented groups showed elevated levels of 22:4n-6 and 22:5n-6.

RBC/plasma fatty acids. Quantitative FA profiles for RBC and plasma are presented in Tables 6 and 7, respectively.

Table 4. Retina fatty acid composition as wt% of total fatty acids (mean $\pm$ SD)

Fatty acid	B	T–	P–	P+
Σ MUFA	14.61 $\pm$ 0.95 ^a	16.51 $\pm$ 0.99 ^b	16.56 $\pm$ 0.36 ^b	15.55 $\pm$ 0.58 ^{ab}
18:2n-6	2.71 $\pm$ 0.20 ^{ab}	2.81 $\pm$ 0.30 ^{ab}	2.98 $\pm$ 0.34 ^a	2.36 $\pm$ 0.18 ^b
20:4n-6	10.39 $\pm$ 0.43	10.96 $\pm$ 0.41	11.11 $\pm$ 0.82	10.97 $\pm$ 0.39
22:4n-6	1.61 $\pm$ 0.28 ^a	2.34 $\pm$ 0.21 ^{bc}	2.68 $\pm$ 0.27 ^b	2.22 $\pm$ 0.19 ^c
22:5n-6	0.83 $\pm$ 0.18 ^a	1.71 $\pm$ 0.48 ^{bc}	2.28 $\pm$ 0.37 ^b	1.10 $\pm$ 0.24 ^{ac}
Σ n-6	17.90 $\pm$ 0.82 ^a	20.53 $\pm$ 1.18 ^b	21.70 $\pm$ 1.09 ^b	18.82 $\pm$ 0.30 ^a
22:5n-3	1.30 $\pm$ 0.29 ^a	0.86 $\pm$ 0.15 ^b	0.87 $\pm$ 0.03 ^b	0.78 $\pm$ 0.18 ^b
22:6n-3	22.56 $\pm$ 1.63 ^a	18.60 $\pm$ 1.36 ^b	16.46 $\pm$ 1.82 ^b	22.13 $\pm$ 1.21 ^a
Σ n-3	23.86 $\pm$ 1.88 ^a	19.46 $\pm$ 1.42 ^b	17.33 $\pm$ 1.83 ^b	22.9 $\pm$ 1.30 ^a
22:5n-6/22:6n-3	0.037 $\pm$ 0.011 ^a	0.094 $\pm$ 0.035 ^b	0.140 $\pm$ 0.024 ^c	0.050 $\pm$ 0.013 ^a

Different superscripts in a row indicate significantly different values ($p < 0.05$).

Σ SFA, sum of the saturated fatty acids, were not significantly different among the groups and overall averaged $43.0 \pm 1.2\%$ wt; Σ MUFA, sum of the monounsaturated fatty acids.

Table 5. Liver fatty acid composition as wt% of total fatty acids (mean $\pm$ SD)

Fatty acid	B	T–	P–	P+
Σ SFA	41.31 $\pm$ 1.67 ^a	38.03 $\pm$ 1.95 ^b	38.61 $\pm$ 1.77 ^{ab}	38.26 $\pm$ 0.57 ^b
Σ MUFA	10.83 $\pm$ 0.96 ^a	16.20 $\pm$ 2.20 ^b	16.11 $\pm$ 1.74 ^b	15.33 $\pm$ 2.11 ^b
18:2n-6	18.21 $\pm$ 0.54 ^a	16.67 $\pm$ 0.92 ^a	17.18 $\pm$ 2.20 ^a	13.34 $\pm$ 0.79 ^b
20:4n-6	11.85 $\pm$ 0.78 ^a	14.70 $\pm$ 0.91 ^b	15.36 $\pm$ 1.37 ^{bc}	17.16 $\pm$ 1.24 ^c
22:4n-6	0.40 $\pm$ 0.04 ^a	0.59 $\pm$ 0.06 ^{bc}	0.66 $\pm$ 0.08 ^b	0.54 $\pm$ 0.06 ^c
22:5n-6	0.58 $\pm$ 0.04 ^{ab}	0.86 $\pm$ 0.29 ^a	0.76 $\pm$ 0.13 ^a	0.38 $\pm$ 0.11 ^b
Σ n-6	33.90 $\pm$ 0.56 ^a	39.50 $\pm$ 2.07 ^b	39.33 $\pm$ 1.20 ^b	34.45 $\pm$ 0.89 ^a
18:3n-3	0.55 $\pm$ 0.11 ^a	0.34 $\pm$ 0.11 ^b	0.19 $\pm$ 0.10 ^b	0.29 $\pm$ 0.11 ^b
20:5n-3	0.36 $\pm$ 0.13 ^a	0.19 $\pm$ 0.04 ^b	0.17 $\pm$ 0.01 ^b	0.12 $\pm$ 0.02 ^b
22:5n-3	2.07 $\pm$ 0.40 ^a	1.33 $\pm$ 0.27 ^b	1.62 $\pm$ 0.17 ^{ab}	0.67 $\pm$ 0.08 ^c
22:6n-3	10.63 $\pm$ 0.79 ^a	3.98 $\pm$ 0.41 ^b	3.57 $\pm$ 0.32 ^b	10.62 $\pm$ 1.01 ^a
Σ n-3	13.25 $\pm$ 0.88 ^a	5.66 $\pm$ 0.33 ^b	5.38 $\pm$ 0.24 ^b	11.58 $\pm$ 1.02 ^c
22:5n-6/22:6n-3	0.06 $\pm$ 0.01 ^a	0.22 $\pm$ 0.07 ^b	0.22 $\pm$ 0.05 ^b	0.04 $\pm$ 0.01 ^a

Different superscripts in a row indicate significantly different values ($p < 0.05$).

Σ SFA, sum of the saturated fatty acids; Σ MUFA, sum of the monounsaturated fatty acids.

Table 6. Red blood cell (RBC) fatty acid composition as wt% of total fatty acids (mean $\pm$ SD)

Fatty acid	B	T–	P–	P+
Σ MUFA	12.57 $\pm$ 1.45 ^a	17.35 $\pm$ 1.83 ^b	19.04 $\pm$ 1.46 ^b	17.03 $\pm$ 0.90 ^b
18:2n-6	13.78 $\pm$ 1.72 ^a	13.77 $\pm$ 1.79 ^a	14.81 $\pm$ 1.78 ^a	9.97 $\pm$ 0.59 ^b
20:4n-6	18.19 $\pm$ 2.54 ^{ab}	17.25 $\pm$ 1.47 ^a	18.51 $\pm$ 1.85 ^{ab}	21.20 $\pm$ 0.54 ^b
22:4n-6	2.12 $\pm$ 0.33	2.60 $\pm$ 0.43	2.76 $\pm$ 0.53	2.36 $\pm$ 0.43
22:5n-6	1.00 $\pm$ 0.11 ^a	1.02 $\pm$ 0.22 ^a	0.83 $\pm$ 0.11 ^{ab}	0.65 $\pm$ 0.07 ^b
Σ n-6	37.62 $\pm$ 3.90 ^{ab}	38.30 $\pm$ 1.18 ^{ab}	40.13 $\pm$ 1.68 ^a	36.28 $\pm$ 0.75 ^b
18:3n-3	0.24 $\pm$ 0.02	0.32 $\pm$ 0.07	0.27 $\pm$ 0.06	0.20 $\pm$ 0.04
20:5n-3	0.87 $\pm$ 0.19 ^a	0.40 $\pm$ 0.10 ^b	0.32 $\pm$ 0.07 ^b	0.23 $\pm$ 0.05 ^b
22:5n-3	2.49 $\pm$ 0.16 ^a	1.54 $\pm$ 0.28 ^b	1.80 $\pm$ 0.16 ^b	0.89 $\pm$ 0.18 ^c
22:6n-3	8.92 $\pm$ 1.48 ^a	4.26 $\pm$ 0.43 ^b	3.17 $\pm$ 0.59 ^b	7.15 $\pm$ 0.72 ^c
Σ n-3	12.53 $\pm$ 1.31 ^a	6.53 $\pm$ 0.70 ^b	5.56 $\pm$ 0.64 ^b	8.48 $\pm$ 0.89 ^c
22:5n-6/22:6n-3	0.11 $\pm$ 0.02 ^a	0.24 $\pm$ 0.05 ^b	0.26 $\pm$ 0.02 ^b	0.09 $\pm$ 0.01 ^a

Different superscripts in a row indicate significantly different values ($p < 0.05$).

Σ SFA, sum of the saturated fatty acids, were not significantly different among the groups and overall averaged $36.2 \pm 2.8\%$ wt.; Σ MUFA, sum of the monounsaturated fatty acids.

Comparisons between the liver and plasma FA compositions show several similarities consistent with the role of the liver as an important site of LCP synthesis. As expected, the P+ group had higher levels of DHA in the RBC and plasma than did the P– group. Similar to the brain results, DHA levels in the plasma and RBC of the P+ group were significantly lower than that of the B group. Plasma DHA in the T– and P– groups was further depressed, at levels 70% and 75% below the B group values, respectively. Plasma 22:5n-6/22:6n-3 indexes

were similar to that of the liver, but the T– and P– groups were elevated by 4- to 5-fold compared with the B and P+ groups.

Similar to the results for other tissues, plasma and RBC LA concentrations were significantly lower in the P+ group whereas LNA levels did not show any significant differences. Significantly higher ARA levels found in the P+ group plasma probably reflect the high dietary intake compared with the B group. However, it is notable that B, T–, and P– groups were

Table 7. Plasma fatty acid composition as wt% of total fatty acids (mean $\pm$ SD)

Fatty acid	B	T–	P–	P+
Σ MUFA	12.68 $\pm$ 1.67 ^a	23.75 $\pm$ 2.65 ^b	23.53 $\pm$ 1.66 ^b	20.61 $\pm$ 2.51 ^b
18:2n-6	28.00 $\pm$ 1.03 ^a	24.37 $\pm$ 2.00 ^b	24.32 $\pm$ 1.11 ^b	19.23 $\pm$ 1.18 ^c
20:4n-6	8.71 $\pm$ 0.96 ^a	8.08 $\pm$ 0.93 ^a	8.29 $\pm$ 0.75 ^a	13.75 $\pm$ 1.11 ^b
22:4n-6	0.51 $\pm$ 0.10	0.53 $\pm$ 0.05	0.54 $\pm$ 0.02	0.54 $\pm$ 0.07
22:5n-6	0.46 $\pm$ 0.07 ^{ab}	0.52 $\pm$ 0.19 ^a	0.39 $\pm$ 0.06 ^{ab}	0.27 $\pm$ 0.06 ^b
Σ n-6	40.14 $\pm$ 1.39 ^a	38.53 $\pm$ 1.49 ^{ab}	37.41 $\pm$ 1.82 ^b	36.45 $\pm$ 0.66 ^b
18:3n-3	0.55 $\pm$ 0.08	0.53 $\pm$ 0.11	0.62 $\pm$ 0.11	0.46 $\pm$ 0.04
20:5n-3	0.75 $\pm$ 0.17 ^a	0.18 $\pm$ 0.03 ^b	0.19 $\pm$ 0.04 ^b	0.18 $\pm$ 0.05 ^b
22:5n-3	1.98 $\pm$ 0.21 ^a	0.91 $\pm$ 0.18 ^b	1.06 $\pm$ 0.05 ^b	0.53 $\pm$ 0.07 ^c
22:6n-3	6.39 $\pm$ 0.69 ^a	1.89 $\pm$ 0.28 ^b	1.60 $\pm$ 0.06 ^b	5.21 $\pm$ 0.63 ^c
Σ n-3	9.67 $\pm$ 0.91 ^a	3.52 $\pm$ 0.24 ^b	3.48 $\pm$ 0.81 ^b	6.28 $\pm$ 0.84 ^c
22:5n-6/22:6n-3	0.07 $\pm$ 0.01 ^a	0.28 $\pm$ 0.09 ^b	0.25 $\pm$ 0.01 ^b	0.05 $\pm$ 0.01 ^a

Different superscripts in a row indicate significantly different values ($p < 0.05$).

Σ SFA, sum of the saturated fatty acids, were not significantly different among the groups and overall averaged $35.2 \pm 1.7\%$ wt.; Σ MUFA, sum of the monounsaturated fatty acids.

similar in plasma ARA concentrations despite the presence of ARA in breast milk but not in the unsupplemented formula groups. Unlike in the other tissues, 22:4n-6 in plasma and RBC did not change, and there was a modest trend in 22:5n-6 levels favoring the term groups (B, T–).

Regression analysis. Neither the plasma nor the RBC ARA levels were correlated with the ARA levels of the other tissues. Figure 2C shows the relationships between liver DHA *versus* RBC or plasma DHA, and the corresponding best-fit equations. The unsupplemented groups are clearly separated from B and P+, consistent with the role of the liver as a storage site for LCP. Figs. 2A and B present the linear and quadratic relationships between DHA levels in brain and retina *versus* plasma or RBC. After group effects were included in the regression model, further analysis showed that no remaining variability in tissue DHA could be explained by RBC or plasma levels.

DISCUSSION

To isolate the effects of prematurity on neonates maintained on an unsupplemented diet, we directly compare the P– and T– groups since they were on identical diets and differed only in gestational age. Brain DHA was 18% lower in the P– group compared with the T– group ($p < 0.05$), and a similar, nonsignificant trend was consistently found in other pools. The 22:5n-6/22:6n-3 ratio was significantly elevated in retina but not in brain or in the other tissues. These results likely reflect the longer period that the P– group was without the benefit of maternally derived DHA *via* placental transfer. In contrast, brain ARA concentrations were significantly greater in the P– group compared with the T– group, and again, a similar, nonsignificant trend is evident for the other tissues. The importance of this seemingly paradoxical increase in ARA in prematurity is not apparent, nor is the reason for an increase in ARA products.

The effects of supplementation on FA composition can be evaluated by comparisons between the P+ and P– groups, and secondarily the B *versus* T– groups. All pools showed greater DHA concentrations when DHA was present in the diet. In contrast, supplementation did not significantly influence ARA levels in most tissues, though plasma ARA in the P– group

was less than that in the P+ group, and in the T– livers ARA levels were greater than in the B livers. Comparing the brain data of the premature groups (P– *versus* P+), the ARA product 22:5n-6 decreased with supplementation. The B and T– groups did not show significant changes in this FA, but the sum of n-6 fatty acids was significantly different as result of the trend in favor of the T– group. Overall, we conclude that DHA supplementation is effective at improving tissue DHA concentrations in premature baboons, but that ARA concentrations are more tightly controlled.

In the absence of dietary LCP, liver DHA levels were lower by 63% and 66% (T– and P–, respectively), plasma DHA was lower by 70% and 75%, RBC DHA was lower by 52% and 64%, but brain DHA was lower by only 21% and 35% compared with the B group. Assuming there are no significant differences in the concentrations of DHA among the groups at birth, these values represent decreases in DHA concentration in unsupplemented groups compared with the breast-fed group. These declines occurred even though the T–/P– formula contained LNA levels found in conventional formulas. The dramatic decreases in liver DHA concentrations and in blood pools (plasma and RBC) suggest that the liver capacity for LCP synthesis is limited. The smaller decrease observed in the visual cortex of the T– and P– groups is consistent with rapid uptake of LCP by the developing brain, which is spared the decrease in DHA seen in other tissues. Brain requirements appear to deplete plasma DHA and, in turn, decrease DHA levels in liver and other tissues. This conclusion is supported by evidence that brain LCP accretion requires mobilization of LCP from other organs (32).

The 22:5n-6/22:6n-3 ratio in tissues or blood is commonly used as an indicator of n-3 fatty acid deficiency, based in part on empirical observations showing that this ratio rises in n-3 fatty acid deficiency. This phenomenon has been observed in preterm infants on formulas with varying amounts of n-3 fatty acids (13). It is thought that 22:5n-6 is a structural analogue to DHA and can serve similar biochemical functions, though functional deficits suggest that 22:5n-6 is not a fully effective replacement for DHA (33). In the present study, all tissues from the unsupplemented groups showed significantly elevated

Figure 2. Legends for all panels are the same as shown in the first plot. (A) Brain DHA correlations to RBC and plasma DHA expressed as weight%, with each point representing one animal. The least squares equations are: $\text{Brain}_{\text{DHA}} = -0.070 \text{ RBC}_{\text{DHA}}^2 + 1.6 \text{ RBC}_{\text{DHA}} + 4.9$ ($r^2 = 0.86$) and $\text{Brain}_{\text{DHA}} = 0.75 \text{ Plasma}_{\text{DHA}} + 8.5$ ($r^2 = 0.76$). (B) Retina DHA correlations to RBC and plasma DHA expressed as weight%, with each point representing one animal. The least squares equations are: $\text{Retina}_{\text{DHA}} = -0.19 \text{ RBC}_{\text{DHA}}^2 + 3.3 \text{ RBC}_{\text{DHA}} + 8.3$ ($r^2 = 0.67$) and $\text{Retina}_{\text{DHA}} = -0.26 \text{ Plasma}_{\text{DHA}}^2 + 3.2 \text{ Plasma}_{\text{DHA}} + 12.9$ ($r^2 = 0.70$). (C) Liver DHA correlations to RBC and plasma DHA expressed as weight%, with each point representing one animal. The least squares equations are: $\text{Liver}_{\text{DHA}} = -0.13 \text{ RBC}_{\text{DHA}}^2 + 3.0 \text{ RBC}_{\text{DHA}} - 5.1$ ($r^2 = 0.85$) and $\text{Liver}_{\text{DHA}} = -0.24 \text{ Plasma}_{\text{DHA}}^2 + 3.5 \text{ Plasma}_{\text{DHA}} - 1.5$ ($r^2 = 0.95$).

22:5n-6/22:6n-3, with plasma and RBC being particularly high. This indicates that plasma and RBC should be sensitive probes of tissue 22:5n-6/22:6n-3 ratios, and our data are direct evidence that the plasma and RBC are markers for increases in this ratio in liver, brain and retina.

The large decreases in plasma and RBC DHA concentrations observed in our data are even greater than those reported in the most similar of the existing human preterm studies. Plasma DHA levels were lower by about 61% in preterms (2- and 6-wk-old) fed a formula with no DHA compared with a formula supplemented with 0.76%wt DHA, and by about 50% compared with a breast-fed reference group (14). Plasma DHA levels dropped 51% in 6 week old preterm infants fed an unsupplemented soy-oil-based diet compared with a supplemented diet (13), and by about 25 wk of age the decrease was 59% (34). In those 6-week-old preterms, total RBC DHA was lower by 24% in a soy diet compared with a DHA supplemented diet (13). In another study, RBC DHA was lower by

41% at 7 wk in DHA supplemented preterms compared with controls (35). Variability in total body fat at birth is one plausible factor that may contribute to the relatively wide range of plasma and RBC DHA decreases reported in human studies, as well as the generally milder decreases in humans compared with baboons. The human infant accumulates uniquely large body fat stores before birth (36, 37), and these stores contain significant amounts of DHA (38). Although preterm infants have much less body fat than term infants, the preterms in the cited studies averaged more than 30 wk gestational age, when the period of body fat accumulation is underway.

The unsupplemented group brain DHA concentrations were 34% to 37% lower than the B group, which is within the low end of the range reported in human autopsy studies. Those data showed decreases of 10% to 40% in brain DHA levels in unsupplemented formula-fed human infants, compared with breast-fed infants (18–21). Statistically valid comparisons between breast-fed and formula-fed infants are not possible in

human studies because randomization is not possible. However, our results showing larger changes in plasma and RBC in baboons, leading to changes in brain DHA that are within the low end of human autopsy results, are consistent with the human data. If, indeed, preterm baboons have lower stores of DHA at birth than preterm humans, and if the primate brain is protected against low DHA at the expense of other organs, then plasma, RBC, and liver DHA depletion should lead to brain depletion, as we observe.

There are no animal data available, in any species, with which to compare our data on the effects of supplementation on prematurity. There are many studies of LCP supplementation in sub-primates, and our data are in good qualitative agreement with them. They generally show that most tissues increase DHA concentration in response to supplementation, while for ARA only plasma and RBC respond to supplementation. After 12 wk of feeding formula supplemented with ARA/DHA (0.9%wt/0.6%wt) to guinea pigs, Abedin and coworkers found that the DHA tissue proportions improved by 1.4-fold in brain phosphatidylethanolamine (PE), 1.8-fold in retinal PL, and by 14-fold in the liver. We found 1.4-fold, 1.3-fold, and 3-fold increases in brain, retina, and liver DHA respectively with similar levels of supplementation. There were no changes in brain or retina ARA proportions even at the highest level of supplementation, however liver ARA was significantly elevated compared with controls (39). These data are in accord with our observations that ARA and DHA supplementation in the P+ group did not increase retina and brain ARA levels compared with the T- and P- groups, while liver ARA increased significantly compared with the T- group only.

Piglets fed formula containing 0.5%wt ARA and 0.4%wt DHA showed greater tissue DHA levels compared with unsupplemented controls, with the most pronounced changes observed in liver and plasma (>2-fold greater), while the retina and brain PE showed a modest 1.1-fold increase. ARA levels in plasma and RBC PE were greater, but were not different in brain and retina PE in comparison to no-LCP controls (40, 41). In our study, ARA and DHA provided at 1.21%wt and 0.61%wt, respectively, to the P+ group resulted in elevated ARA only in plasma and not in the other tissues when compared with the P- group. In another piglet study, inclusion of ARA and DHA in the diet caused a moderate but significant improvement (<1.2-fold) in frontal cortex PE and phosphatidylinositol (PI) but no changes in other PL classes. Addition of ARA/DHA to the formula did not lead to higher levels of ARA in the frontal cortex or hippocampus; on the contrary, in the latter the ARA level was lower in the supplemented group (42). Craig-Schmidt *et al.* reported increases in retinal phospholipid DHA for piglets supplemented with 1%wt ARA and 0.8%wt DHA. They found 1.3-fold higher ARA level in the retina phosphatidylcholine (PC) ARA but not in PE ARA after supplementation (43).

Artificially reared rats fed diets with >2%wt ARA and >2%wt DHA showed that the DHA content of forebrain phospholipids were significantly elevated after 3 wk and 9 wk of supplementation. The RBC DHA levels showed an average 2.3-fold increase after 9 wk in the different PL classes (44, 45). We also found a 2.3-fold greater level for this tissue in

baboons. In rhesus macaque infants, the breast-fed animals had the highest level of plasma DHA even though the breast milk contained less than half the DHA concentration of the supplemented formula, which had 1%wt ARA and 1%wt DHA. At 4 wk of age, the plasma DHA of the supplemented animals increased by 3.4-fold and ARA by 1.6-fold. These figures are very close to our 3.3-fold and 1.7-fold increases in the plasma levels for DHA and ARA, respectively, in premature baboons (46).

We examined the correlation between plasma and RBC ARA *versus* organ ARA levels. Other than one significant but weak correlation, our analysis shows that RBC or plasma ARA is not predictive of organ ARA concentrations. On the other hand, plasma and RBC DHA were predictive of group tissue DHA in all cases examined, as shown in Figs. 2A–C. Additionally, analysis accounting for group effects as categorical data show that all variability is explained by group effects, and that within a group, levels were uncorrelated. We conclude that that RBC and plasma DHA concentrations are useful proxies for brain, retina, and liver DHA levels, and that caution is warranted when comparing these levels over a small range.

CONCLUSION

In conclusion, our results indicate that unsupplemented formula caused a precipitous drop in DHA of brain and related tissues, and that formula supplemented with 0.3 energy% DHA and 0.6 energy% ARA prevented this drop in retina and liver. Retina and brain DHA concentrations are more resistant to dietary manipulation than liver or blood DHA, and tissue ARA levels are less sensitive than DHA to LCP supplementation and prematurity, and may decrease with supplementation. RBC and plasma total ARA is unresponsive to changes in tissue ARA and thus cannot be used as a proxy for tissue ARA. Finally, brain, retina, and liver DHA concentrations are significantly correlated to plasma and RBC levels, and these correlations were fully explained by group effects.

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