Early docosahexaenoic and arachidonic acid supplementation in extremely-low-birth-weight infants

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BACKGROUND: Extremely-low-birth-weight (ELBW) infants accrue large deficits in docosahexaenoic acid (DHA) and arachidonic acid (ARA) and require improved supplementation strategies. We hypothesized that once daily DHA+ARA drops applied to buccal mucosa will increase blood levels.

METHODS: Thirty ELBW infants were randomized to receive DHA 20 mg/kg/d + ARA 40 or 60 mg/kg/d + ARA 120 mg/kg/d or *placebo* within 72 h of age for 8 wk duration. Red blood cell phospholipid levels of DHA (primary) and ARA (secondary) were measured at 2 and 8 wk of age.

RESULTS: Twenty-eight survivors with a median birth weight of 806 g completed dosing and sampling. Red blood cell levels were similar between the three groups at 2 wk (DHA: 4.62 wt% (interquartile range (IQR) 4.1–5.5) for all, $P = 0.29$ between groups; ARA: 21.1 wt% (IQR 18.78–22.6) for all, $P = 0.41$ between groups) and 8 wk (DHA: 6.0 wt% (IQR 5.1–7.1) for all, $P = 0.57$ between groups; ARA: 20.1 wt% (IQR 18.3–23.1) for all, $P = 0.63$ between groups). DHA in all infants showed a median increase of 31% from 2 to 8 wk ($P < 0.04$). ARA levels did not significantly change over time ($P > 0.6$).

CONCLUSION: Daily buccal DHA and ARA supplements did not affect fatty acid levels in ELBW infants.

Preterm infants receive inadequate amounts of the long-chain polyunsaturated fatty acids (LCPUFAs), docosahexaenoic acid (DHA, 22:6*n* − 3) and arachidonic acid (ARA, 20:4*n* − 6), during hospitalization in the neonatal intensive care unit (NICU) (1). Insufficient provisions and low red blood cell (RBC) DHA and ARA levels have been associated with poor growth and increased risk of chronic lung disease and sepsis (2–4). Deficiencies also contribute to select short-term visual and developmental morbidities in preterm infants (2,5,6).

Current formulations and delivery of parenteral and enteral nutrition to preterm infants in the NICU allow accumulation of significant deficits in DHA and ARA (7). The fully fed preterm infant absorbs <40% of expected fetal accretion rates (8), and intravenous lipid emulsions (IVLEs) used in the United States are devoid of these LCPUFA (9). Although lactating women may be taking supplemental DHA, breast milk levels still fail to meet the preterm infant's demands (8). Extremely-low-birth-weight (ELBW) infants are at greatest risk of deficit (8).

The best mechanism of providing LCPUFA to preterm infants remains to be determined. Supplements should be provided with relative ease of administration, good tolerability, and ideally remain independent of an infant's physiologic status and ability to tolerate enteral feedings. This would also account for immature pancreatic function and diminished intestinal absorption (10). Oropharyngeal application of small volumes of breast milk for nonnutritive purposes has been incorporated into some clinical practices, can occur independent of feeding status, and results in systemic absorption of bioactive factors (11,12). In addition, fatty acids have been safely applied and absorbed topically and to buccal and rectal mucosa as effective drug adjuvants (13–18). We therefore proposed the buccal administration of LCPUFA as a method of supplementation in a preterm population that depends on IVLE and may experience frequent interruptions in enteral feedings.

Correspondingly, we aimed to define the feasibility, efficacy, and tolerability of daily supplementation of DHA and ARA directly to the oral mucosa of ELBW infants. Our hypothesis is that this method would be a well-tolerated mechanism for raising systemic levels of LCPUFA after 8 wk of daily administration.

RESULTS

LCPUFA Levels and Infant Characteristics

LCPUFA supplementation with 20 mg/kg/d or 60 mg/kg/d DHA did not alter RBC phospholipid (PL) LCPUFA levels at 2 or 8 wk; levels were similar in all groups (**Table 1**). DHA increased over the sampling interval within all three groups ($P < 0.04$) and increases were of similar magnitude between groups (*Placebo*: 31%; Low: 38%; High: 17%; $P = 0.4$). Within assignment groups, ARA showed no significant increase between the sampling interval with no differences detected between groups (*Placebo*: 2.2%; Low: 2.4%; High: 3.2%; $P > 0.67$ within each group).

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Table 1. Red blood cell phospholipid fatty acid levels at 2 and 8 wk (median IQR)[a]

Fatty acid	All, $n = 28$	*Placebo*, $n = 10$	Low dose, $n = 9$	High dose, $n = 9$	P[b]	Any DHA, $n = 18$	P[c]
2 wk							
LA	12.5 (11.0, 13.6)	12.2 (11.0, 13.2)	12.7 (10.4, 13.5)	12.7 (11.7, 13.9)	0.72	12.7 (11.0, 13.6)	0.70
ALA	0.2 (0.2, 0.2)	0.2 (0.1, 0.2)	0.2 (0.2, 0.2)	0.2 (0.2, 0.3)	0.32	0.2 (0.2, 0.3)	0.14
ARA	21.1 (18.8, 22.6)	21.6 (20.2, 23.0)	19.3 (17.6, 22.5)	20.3 (18.9, 21.3)	0.41	19.1 (18.8, 22.5)	0.20
DHA	4.6 (4.1, 5.5)	5.3 (4.8, 5.6)	4.0 (4.0, 4.8)	4.5 (4.4, 5.2)	0.29	4.4 (4.0, 5.2)	0.17
8 wk							
LA	12.0 (10.2, 13.6)	12.7 (11.7, 13.2)	13.7 (10.0, 15.0)	11.8 (9.9, 12.2)	0.30	11.8 (9.9, 14.7)	0.36
ALA	0.2 (0.1, 0.2)	0.2 (0.2, 0.2)	0.2 (0.1, 0.2)	0.1 (0.1, 0.2)	0.05	0.2 (0.1, 0.2)	0.10
ARA	20.1 (18.3, 23.1)	22.2 (19.1, 23.1)	19.9 (18.3, 21.1)	19.8 (17.8, 23.0)	0.63	19.9 (17.8, 23.0)	0.34
DHA	6.0 (5.1, 7.1)	6.8 (5.2, 7.4)	5.6 (5.5, 7.0)	5.5 (4.5, 7.1)	0.57	5.6 (5.0, 7.1)	0.4

ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; LA, linoleic acid.
[a]Levels reported as weight % (g/100 g). [b]Comparing all three intervention groups. [c]Comparing *placebo* and any supplement.

Thirty patients were enrolled, and two deaths occurred prior to the first blood sampling. One infant in the low-dose group developed necrotizing enterocolitis *totalis* and one in the high-dose group had severe respiratory failure and intraventricular hemorrhage (IVH). Surviving infants were born at a median of 26 wk gestation (interquartile range (IQR) 25–27) and 806 g (IQR 663–923). Maternal and infant perinatal characteristics at birth and measures of illness severity were similar in all three groups (**Table 2**) with infants in the high-dose group tending to be less mature and smaller at birth. Dosing administration (age at first dose: 2 d (IQR 2–3); age at last dose: 57 d (IQR 57–58)) and blood sampling (age at first sample: 16 d (IQR 15.5–17); age at second sample: 58 d (IQR 57–58)) for the entire cohort showed compliance with the protocol. No doses were interrupted due to apnea, bradycardia, or desaturation. One dose in one infant was not administered on the day of bowel perforation. Blood transfusions occurred in 75% of all infants; more of the infants receiving any DHA received RBC transfusions (*Placebo*: 50%; Any: 89%; $P = 0.06$).

Median growth velocity for all infants was 13 g/kg/d (IQR 11.6–14.1; $P = 0.68$ between groups). Infants receiving the high dose did not experience intestinal morbidity (**Table 2**). Rates of sepsis, bronchopulmonary dysplasia, and IVH were evenly distributed among the intervention groups; a trend toward higher rates of IVH appeared in the high-dose group. Three infants required intervention for retinopathy of prematurity.

Nutritional Provisions and LCPUFA Levels by IVLE Duration

Initiation of IVLE occurred earlier in the *placebo* group compared with infants receiving any DHA, but otherwise parenteral nutrition exposures were similar in all infants including duration of IVLE (**Table 3**). To understand contributions of feedings to LCPUFA status, we evaluated feeding patterns including type of feedings. Although only 54% received human milk for all feedings throughout hospitalization, most formula exposure occurred after the 8-wk study period. During the first 4 wk, infants rarely received formula as the percentage of feedings as breast milk was highest with a median value of 100%. During the last 4 wk of intervention, 26 infants received ≥65% of feedings as human milk, almost exclusively from their own mothers, while 2 infants received <10% of feedings as human milk. Fifty-three percent of mothers consumed a DHA supplement during lactation and supplementation was similar between groups (**Table 3**). The use of supplement appears unrelated to RBC DHA ($P = 0.9$ at 2 and 8 wk). Four infants received donor milk as some portion of feedings. Feedings were started at a median age of 4 d (IQR 3–6) and infants in the *placebo* group achieved full enteral nutrition (FEN) somewhat but not significantly earlier (**Table 3**). Exposure to a DHA-containing breast milk fortifier was evenly distributed among the groups (data not shown).

Thirteen infants had IVLE discontinued prior to the median 16 d ("Short") and achieved FEN significantly sooner than those with at least 16 d ("Long") exposure to IVLE (Short: 18 d, IQR 17–22; Long: 41 d, IQR 37–56; $P < 0.001$). At 2 wk, infants with longer IVLE exposure showed higher RBC PL linoleic acid levels (Short: 11.8%, IQR 10.7–12.4; Long: 13.5%, IQR 11.7–14.4; $P < 0.001$) and α-linolenic acid levels (Short: 0.2%, IQR 0.1–0.2; Long: 0.2%, IQR 0.2–0.3; $P < 0.01$). ARA levels were not significantly different but DHA levels were lower with longer lipid exposure (Short: 5.2%, IQR 4.8–6.1; Long: 4.2%, IQR 4.0–4.5; $P = 0.008$). At 8 wk, linoleic acid remained higher with longer exposure (Short: 11.0%, IQR 9.9–12.3; Long: 13.2%, IQR 11.8–15.0; $P = 0.03$) and DHA remained lower (Short: 7.1%, IQR 6.2–7.5; Long: 5.5%, IQR 4.8–6.7; $P = 0.03$).

DISCUSSION

Buccal administration of a concentrated formulation of DHA and ARA once per day did not affect RBC LCPUFA levels in ELBW infants. Although we found no effect of supplementation, infants had a better status of DHA and ARA during hospitalization and avoided the declines anticipated from a previous publication (19). In contrast to the earlier study, infants in this study received human milk as their enteral nutrition while on IVLE. All human milk contains DHA and ARA, but many of the infants' mothers consumed a supplement of DHA, which is known to increase milk DHA (20). Increased attention to providing human milk feedings for preterm infants may have contributed to these findings.

Table 2. Infant and maternal characteristics (median IQR unless specified)

	All, *n* = 28	*Placebo*, *n* = 10	Low dose, *n* = 9	High dose, *n* = 9	*P*[a]	Any DHA, *n* = 18	*P*[b]
Gestational age, wk	26 (25, 27)	26.5 (25, 28)	26 (26, 27)	25 (25, 25)	0.14	25.5 (25, 27)	0.56
Birth weight, g	806 (663, 923)	852 (800, 910)	771 (700, 950)	730 (655, 820)	0.48	730 (655, 935)	0.31
Birth weight *Z*-score	−0.45 (−0.9, 0.6)	−0.3 (−0.9, 0.7)	−0.7 (−0.8, −0.2)	0.2 (−0.8, 0.5)	0.54	−0.5 (−0.8, 0.5)	0.52
Female gender, *n* (%)	11 (39)	4 (40)	5 (56)	2 (22)	0.40	7 (39)	0.63
Small for gestational age, *n* (%)	6 (21)	1 (10)	3 (33)	2 (22)	0.46	5 (28)	0.38
Maternal age, y	30 (28, 35)	30.5 (28, 33)	34 (29, 39)	29 (3, 28)	0.42	30 (28, 35)	0.75
Multiple gestation, *n* (%)	10 (36)	5 (50)	2 (22)	3 (33)	0.55	5 (28)	0.41
Preeclampsia, *n* (%)	6 (21)	2 (20)	3 (33)	1 (11)	0.64	4 (22)	0.64
Completed antenatal steroids, *n* (%)	23 (82)	8 (80)	7 (78)	8 (89)	0.30	15 (83)	0.10
Cesarean delivery, *n* (%)	18 (64)	5 (50)	9 (89)	5 (56)	0.22	13 (72)	0.41
Received surfactant, *n* (%)	24 (86)	8 (80)	8 (89)	8 (89)	1.0	16 (89)	0.60
SNAPPE-II	31.5 (24, 47.5)	31.5 (15, 50)	35 (26, 48)	31 (24, 47)	0.64	32.5 (24, 47)	0.46
Morbidities, *n* (%)							
BPD	14 (50)	4 (44)	5 (56)	5 (56)	0.80	10 (56)	0.70
SIP or NEC	3 (11)	1 (10)	2 (22)	0	0.51	3 (17)	1.0
IVH	7 (25)	2 (20)	1 (11)	4 (44)	0.31	5 (28)	1.0
ROP + Treatment	3 (11)	0	1 (1)	2 (22)	0.29	3 (17)	0.53
Sepsis	4 (13)	1 (10)	1 (11)	2 (22)	0.82	3 (17)	1.0
Cholestasis	2 (7)	0	0	2 (22)	0.19	2 (11)	0.52
Length of stay, d	107 (87, 135)	111 (80, 135)	115 (103, 127)	87 (87, 124)	0.58	107 (87, 127)	0.65
Discharge weight, g	3,105 (2,635, 3,775)	3,378 (2,580, 3,885)	3,457 (2,930, 3,530)	2,690 (2,315, 3,140)	0.28	43,003 (2,690, 3,530)	0.58
Discharge weight *Z*-score	−1.3 (−1.9, −0.8)	−1.1 (−1.6, −0.4)	−1.3 (−2.2, −0.9)	−1.8 (−2.2, −1.1)	0.4	−1.3 (−2.2, −1.1)	0.19

BPD, bronchopulmonary dysplasia; DHA, docosahexaenoic acid; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis; ROP, retinopathy of prematurity; SIP, spontaneous intestinal perforation; SNAPPE, Score for Neonatal Acute Physiology-Perinatal Extension.
[a]Comparing all three intervention groups. [b]Comparing *placebo* and any supplement.

Table 3. Nutritional provisions for infants (median IQR unless specified)

	All, *n* = 28	*Placebo*, *n* = 10	Low dose, *n* = 9	High dose, *n* = 9	*P*[a]	Any DHA, *n* = 18	*P*[b]
Hour of life parenteral protein started	3.5 (2.8, 4)	3 (2, 4)	4 (3,4)	4 (3,5)	0.36	4 (3,5)	0.18
Hour of life IVLE started	25.3 (16.5, 32.5)	15 (4, 28)	29 (21, 33)	29 (22, 33)	0.04	29 (21, 32.5)	0.01
Maximum dose parenteral protein (g/kg/d)	4 (3.75, 4)	4 (4)	4 (3.5, 4)	4 (4)	0.25	4 (3.5, 4)	0.65
Maximum dose parenteral lipid (g/kg/d)	2.01 (2, 3)	2.9 (2, 3)	2 (1.2, 2.8)	2 (2, 2.9)	0.18	2 (2, 2.9)	0.07
DOL parenteral protein stopped	17 (14, 27)	14.5 (13, 16)	17 (15, 27)	21 (17, 27)	0.24	18 (15, 27)	0.11
DOL IVLE stopped	16 (12.5, 25.5)	13 (11, 16)	17 (13, 27)	17 (16, 24)	0.22	17 (14, 27)	0.11
DOL initiation of enteral nutrition	4 (3, 6)	4 (3, 5)	4 (3, 6)	5 (3, 6)	0.44	5 (3, 6)	0.22
DOL FEN achieved	20 (17.5, 33.5)	18 (17, 18)	22 (18, 36)	25 (20, 31)	0.13	24 (20, 36)	0.06
Breast milk for all feedings, *n* (%)	15 (54)	4 (40)	5 (56)	6 (67)	0.57	11 (61)	0.43
Mother taking DHA supplement, *n* (%)[c]	16 (53)	5 (50)	5 (50)	6 (60)	0.72	11 (55)	0.81
Any donor milk, *n* (%)	4 (13)	2 (20)	2 (22)	0	0.51	2 (11)	0.60

DHA, docosahexaenoic acid; DOL, day of life; FEN, full enteral nutrition; IVLE, intravenous lipid emulsion.
[a]Comparing all three intervention groups. [b]Comparing *placebo* and any supplement. [c]One unknown.

Inadequate endogenous production from the parent essential fatty acids, linoleic acid and α-linolenic acid, warrants direct provision of ARA and DHA in preterm infants (21). Past methodologies to increase DHA and ARA delivery relied on maternal supplementation, sonication of FA with milk prior to feeding as well as direct administration via feeding tubes (6,22,23). Adherence to the feeding tube and impaired intestinal absorption likely decreased the total dose delivered with those methods. Buccal absorption occurs for some nutrients and bioactive factors but it does not appear efficacious in regards to acyl fatty acids used in this study's formulation. Alternatively, the dosing in this

study may have been insufficient to impart changes in blood levels.

Dosing assignments in this study approximated amounts provided in commonly used feeding regimens (low dose) and estimates of fetal accretion rates (high dose) (8). Multiple factors may have prevented efficacy. Despite the presence and reported function of lingual lipase proximally (24,25), cleavage of the acyl fatty acid may have been insufficient to affect levels. We expected some of the concentrated liquid to be swallowed with continued opportunity for gut metabolism. However, low pancreatic lipase function, a lack of coordination with feedings (which increases stomach acid production and facilitates lipase function), as well as the theoretical chance that the lipid adhered to the external surface of the enteric tube in the esophagus, all remain potential interfering factors. Infants tolerated the procedure well and the route of administration and small volumes appeared safe and might be considered for other nutrient interventions.

Despite no observed effect from the intervention, RBC PL DHA levels increased between the 2nd and 8th wk and no declines in ARA occurred. We speculate that key characteristics related to feeding of the participants promoted increases in DHA levels and stable ARA levels when, otherwise, this population accrues large deficits throughout hospitalization. Compared with our prior investigation, this population had higher exclusive breast milk use during the study period (1). Although observed rates of exclusive breast milk feedings throughout hospitalization might not be categorized as high, formula feedings were initiated most frequently after the intervention period. In that context, approximately half of mothers of infants participating in this study took DHA supplements during lactation, although we do not detect an apparent effect from this. DHA concentrations in breast milk reflect maternal intake (26). Population-based sampling shows marked variability in DHA yet many samples reveal concentrations much higher than that found in preterm infant formulas (27). Our previously analyzed samples from women in an urban Midwestern region of the United States showed DHA concentrations as high as 1%, amounts almost five times greater than in preterm infant formulas (1). Although we did not collect milk during this study, we expected milk fed to infants in this study to have LCPUFA concentrations that match our prior measurements. One participating center used a breast milk fortifier that contained DHA; however, infants enrolled at the site not using a DHA-containing fortifier still showed increased levels over time. The observed increase in DHA and stable ARA status over time were unexpected findings; we can only speculate that these patterns are attributable to high rates of human milk feedings. With ongoing efforts by clinicians to increase human milk use in NICU's and the possibility of continued increases in mother's taking DHA supplements, we support that future investigations involving LCPUFA supplementation in the context of human milk–fed infants must involve sampling of human milk.

Infants assigned to *placebo* reached FEN at a median 6 d sooner than infants receiving DHA supplements and had slightly higher 8-wk DHA levels. Overall, these are consistent findings which support improved DHA status resulting from breast milk feedings. In consideration of these increases, 8 wk values still did not approximate a theoretical target level of 9%, a plateau level documented in breast milk–fed term infants whose mothers consumed DHA supplements during lactation (28). We note that randomization placed fewer small-for-gestational-age (SGA) infants in the *placebo* group which may have contributed to the earlier achievement of FEN. Enteral nutrition advancement in SGA infants often occurs more slowly than infants born at an appropriate weight for age (29).

The characteristics of this ELBW cohort included multiple-gestation pregnancies, pregnancy complications including pre-eclampsia and rates of SGA status higher than the expected 10%. Although *in-utero* DHA and ARA transfer in these pregnancies may have been reduced (30), this study focused on postnatal provisions and absorption. To be sparing in blood collections in a vulnerable population, and based on the fact that levels at 2 and 8 wk should reveal effects of supplements, we excluded blood sampling at birth in this feasibility trial. We previously measured LCPUFA levels at birth and throughout hospitalization in ELBW infants, observing that postnatal levels from 2 wk until 8 wk correlated with nutritional provisions (1). We believe these pregnancy-related circumstances would not be expected to impact postnatal absorption and future, larger studies will be powered to account for relevant factors including SGA status.

We validated our earlier findings that the duration of IVLE exposure affects DHA levels (1). Differences appeared at 2 wk of age, emphasizing early detrimental effects of current nutritional provisions. Perhaps more notable, IVLEs in infants with longer exposure were discontinued at a median age of 31 d and yet the DHA levels at 8 wk of age remained significantly lower. This late effect highlights the compounding and lasting effects of prolonged exposure to a lipid source lacking DHA followed by suboptimal provisions in feedings.

Inadequate ARA and DHA contribute to impaired growth (2,31), but investigators must continue to assess for negative effects of increased doses on growth. The high-dose group was discharged at a smaller weight than the other groups. In review of their gestational age at birth, birth weight, and length of stay, we believe that this reflects their clinical circumstances and not an effect from supplements, particularly in the face of no detectable uptake of the supplements.

ELBW infants represent a population at high risk of suboptimal DHA and ARA provisions. Early feedings appear safe in these infants yet some feeding protocols involve the withholding of initial feedings for 7–14 d in ELBW infants (32). Slow feeding advances delay enteral provisions even if a mother's milk DHA concentration happens to be high. Ongoing investigations of methods to promptly and adequately provide these critical LCPUFA are necessary. These methodologies should ideally operate independently of feeding status. Access to IVLE

that contains these LCPUFA will aid in early provision that is independent of feeding status. It is noteworthy, though, that IVLE formulations containing longer chain fatty acids do not mimic amounts transferred *in utero* and as compared with breast milk contain higher concentrations of unknown significance (9).

In conclusion, DHA and ARA administration to the buccal mucosa of ELBW infants, as formulated into high oleic sunflower oil, did not affect RBC PL LCPUFA levels in this study. Nonetheless, we observed an unexpected increase in DHA and stable ARA levels in ELBW infants over their first 8 wk. This was presumably attributable to factors related to breast milk feedings and not to buccal administration of a supplement, although even in breast milk–fed infants, the levels remained below achievable values in term infants. Eliminating deficits in these important LCPUFA in preterm infants should be expected to improve growth and reduce morbidity. The best methodology of supplementation remains to be determined. Continued attention to optimizing prompt feeding of breast milk with sufficient DHA in the maternal diet and additional infant supplementation appears to be an important foundation for both clinical and research interventions aimed at improving LCPUFA delivery to preterm infants.

METHODS

We prospectively screened and then randomized infants born <1,000 g and at gestational ages <34 wk with informed, signed consent by parent or legal guardian before 72 h of age. Exclusion criteria included metabolic disorders, gastrointestinal anomalies, or being deemed inappropriate for study enrollment by the attending neonatologist. Study enrollment commenced in October 2013, and the final participant completed the study supplement in March 2015. Institutional Review Boards of NorthShore University HealthSystem, Northwestern University, and Lurie Children's Hospital approved the study.

Randomization and Supplement Dosing

Randomization was computer generated, and participants were assigned to receive 0.5 ml/kg daily of one of three supplements involving the use of two supplement oils to formulate doses (100% high oleic sunflower oil and ARA 240 mg/ml + DHA 120 mg/ml; DSM Nutritional Products): (i) *Placebo* without DHA, 100% high oleic sunflower oil; (ii) Low-dose DHA, 20 mg/kg/d with ARA 40 mg/kg/d; and (iii) High-dose DHA, 60 mg/kg/d with ARA 120 mg/kg/d. Group assignment and dose preparation were performed by research pharmacy staff to maintain blinding of investigators, clinical staff, and participating families to the assignment group. Multiple births were assigned to the same group. The daily dose was dispensed in two syringes, half of the dose in each syringe. Trained neonatal nurses placed the tip of the syringe alongside the buccal mucosa and administered the syringe volume over ≤2 min. Administration was paused until after recovery if apnea, bradycardia, or desaturation occurred. The procedure was repeated on the opposite side with the second syringe. Routine oropharyngeal suctioning by clinical staff was withheld for at least 1 h after administration if clinically appropriate. The intervention continued through 8 wk of age or discharge, whichever occurred first.

Outcomes and Clinical Measures

The primary outcome was RBC PL DHA levels at 2 wk after randomization. Secondary outcomes included all other LCPUFA levels at 2 and 8 wk, or at time of discharge if sooner. Demographic data collected were standard maternal and infant characteristics including fetal and neonatal growth status at birth, maternal DHA supplementation in pregnancy and lactation, and measures of severity of illness (Score for Neonatal Acute Physiology—Perinatal Extension II (33)).

Clinical outcomes were collected and included: respiratory distress syndrome requiring surfactant, IVH, periventricular leukomalacia, spontaneous intestinal perforation or necrotizing enterocolitis, bronchopulmonary dysplasia (oxygen requirement at 36 wk postmenstrual age), retinopathy of prematurity requiring intervention, sepsis (positive blood culture thought not to be a contaminant by primary team), cholestasis, and mortality. RBC transfusions and hospital stay were recorded.

Nutritional data collected included details of parenteral nutrition provisions, feeding initiation, and timing of FEN (enteral feedings provide ≥110 kcal/kg/d). Nutritional management was at the discretion of the attending neonatologist, as were all other aspects of clinical management. Centers initiated parenteral dextrose and protein solutions promptly after birth. IVLE (Intralipid, 20% solution; Fresenius Kabi) began within 48 h of age. Feedings started when deemed appropriate (≤20 ml/kg/d) and advanced at 20 ml/kg/d as tolerated. Maternal breast milk was provided when available, and donor breast milk used for supplementation until infants weighed 1,500 g.

Blood Sampling

Whole blood was drawn at 2 and 8 wk of dosing and collected into EDTA-containing phlebotomy tubes for RBC LCPUFA analysis. RBCs were separated and stored under nitrogen at −80°C or on dry ice in transfer until fatty acid analysis. Samples were analyzed within 20 mo of collection. The staff completing assays was masked to the assignment groups.

Preparation of RBC PLs

Total lipids in RBC samples were extracted according to a modified procedure of Folch *et al.* (34), and the PLs were isolated by thin layer chromatography (Silica gel G plates, 250 μm thickness; Analtech, Newark, DE) with an 80:20:1 hexane–diethyl ether–acetic acid solvent (35). PL bands were transmethylated with 1 ml boron trifluoride (Sigma-Aldrich, St. Louis, MO), and the resulting fatty acid methyl esters were extracted with pentane (36); pentane was vaporized under nitrogen and the sample dissolved in dichloromethane for storage at −80°C until analysis.

Analysis of RBC PL LCPUFA

Fatty acid methyl esters were separated by gas chromatography in a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) on an SP-2560 capillary column (L × I.D. 100 m × 0.25 mm, d_f 0.20 μm; Supelco). Carrier gas was helium at 1.2 ml/min, and the split ratio was 25:1. Column temperature was held constant at 140°C for 5 min then advanced at 4°C/min for 25 min and held constant at 240°C for 12 min. Injector and detector (flame ionization) were programmed at 260°C. Individual fatty acids were identified by comparing retention times with fatty acid mixtures (Supelco 37 and PUFA 2, Sigma-Aldrich) and weight percent determined by comparison of the area under the curve (OpenLab ChemStation C.01.06, Agilent Technologies) to weighed standards (Supelco 37).

Statistical Analyses

Using levels from our initial investigation in a similar population (1) for sample size calculations, we determined that a sample size of 10 in each intervention group would allow detection of an increase in RBC PL DHA levels from 3.6 weight (wt) %, SD 1 at 2 wk of age to 5 wt%, SD 1 with power = 0.8 and $\alpha = 0.05$. Nonparametric tests were used for all statistical analyses. Due to past evidence that duration of IVLE impacts LCPUFA levels and to measure effects of IVLE duration, the median IVLE exposure in the entire cohort was calculated to be 16 d in order to stratify infants based on shorter or longer IVLE exposures. Two-group comparisons of LCPUFA levels were performed based on IVLE duration. Gender specific standard deviation (*Z*) scores for birth and discharge weight were calculated using the Fenton curve (37). Analyses were performed using Stata v12.1 (StataCorp), and statistical significance was defined using $\alpha = 0.05$. All fatty acid levels are reported as wt % (g/100 g).

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and B.F. There has been no study sponsorship involvement in any of the following areas: (i) study design; (ii) the collection, analysis, and interpretation of data; (iii) the writing of the report; and (iv) the decision to submit the paper for publication. The first draft was written by D.T.R and B.F., and no authors received an honorarium, grant, or any form of payment for the production of this manuscript. This trial was registered prior to first enrollment at ClinicalTrials.gov, registration number NCT01955044.

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