Effects of storage practices on long-chain polyunsaturated fatty acids and lipid peroxidation of preterm formula milk

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IN, MK and SD contributed to conception and design. IN contributed to data acquisition and analysis. IN and SD contributed to interpretation of data and manuscript drafting. IN, SD, and MK reviewed it critically and provided final approval of submitted version.

Abstract

Background Preterm formula milk (FM) is often prepared in advance, potentially affecting nutritional quality. Long-chain polyunsaturated fatty acids (LCPUFAs), important for brain and immune system function, are prone to lipid peroxidation, which correlates with comorbidities of prematurity. Effects of clinical storage practices on LCPUFA content and lipid peroxidation of preterm FM were investigated.

Methods U.K. liquid and powder preterm FM (2017) from two manufacturers, were subjected to routine storage conditions (liquid: refrigeration ≤10 hours; powder: weekly preparation according to manufacturer's instructions and refrigeration ≤24 hours for 4 weeks). LCPUFA content, thiobarbituric acid reactive substances, and 4-hydroxy-2-nonenal (HNE) content were analysed.

Results Storage did not significantly decrease LCPUFA content. ESPGHAN recommended LCPUFA intake, but not *in-utero* accretion rates could be achieved with both FM brands (liquid and powder). Lipid peroxidation was evident on opening, with 6x higher levels in powder. No effect of ≤10 hours refrigeration on peroxidation was seen in liquid FM. In powder FM it increased over refrigeration (HNE opening: 6.5-9.7 μg/mL *vs.* day 28, 24 hours: 16.6-36.5 μg/mL) with a significant interaction between storage time and refrigeration ($p = 0.015$), with higher HNE at 4 hours on day 0, 7, 14, and 21 (all $p < 0.05$).

Conclusions Results suggest preterm FM and storage conditions do not support *in-utero* accretion rates for LCPUFAs. Although results suggest different susceptibility of liquid and powder FM to peroxidation upon refrigeration, they are too preliminary to make specific recommendations. We suggest minimising storage time of fresh and prepared powder FM, wherever possible.

[1]

[1] Abbreviations:
ARA: arachidonic acid
DHA: docosahexaenoic acid
ESPGHAN: European Society for Paediatric Gastroenterology, Hepatology and Nutrition
FM: formula milk
HNE: 4-hydroxy-2-nonenal
LCPUFA: long-chain polyunsaturated fatty acid
MDA: malondialdehyde
TBARS: thiobarbituric acid reactive substances

1. INTRODUCTION

When preterm infants have access to insufficient or no maternal milk, formula milk (FM) may be used. Preterm infants receive specialised FMs, with greater energy density, protein and carbohydrate than term FM. Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are omega-3 and -6 long-chain polyunsaturated fatty acids (LCPUFAs), respectively, essential for optimal brain development, and immune system function [(1)]. Preterm infants have high LCPUFA requirements, due to premature interruption of maternal transfer, low adipose tissue stores, limited conversion from precursors, and limited intestinal absorption [(2)]. LCPUFAs are prone to peroxidation [(3)] and FMs containing LCPUFAs have higher peroxidation levels than those without [(4)]. Preterm infants are particularly susceptible to oxygen radical associated diseases, such as bronchopulmonary dysplasia, necrotising enterocolitis, and retinopathy of prematurity, which may be exacerbated by increased intake of peroxidation products [(5)].

The effects of different storage conditions on LCPUFA content and lipid peroxidation of FMs have been explored under a range of conditions, but with inconsistent results. Increased malondialdehyde (MDA), a non-specific peroxidation product [(3)], has been found in FMs stored for 12 months [(6)]. Furthermore, opening powder FM significantly increases MDA after 21 days storage at room temperature [(7)]. Similarly, daily opening and stirring powder FM significantly increases 4-hydroxy-2-nonenal (HNE) and 4-hydroxy-2-hexanal, omega-6 and -3 LCPUFA peroxidation products, respectively, after 10 days [(8)]. However, these studies do not accurately reflect clinical practice as although manufacturers and the WHO recommend using prepared powder FM immediately, the WHO acknowledges the need to prepare FM and recommend a maximum storage of 24 hours at 4°C [(9)]. The present study investigated for the first time the effects of clinically relevant storage practices on LCPUFA content and lipid peroxidation of preterm FM.

2. MATERIAL AND METHODS

2.1 FM samples and study design

At the time of the study, two manufacturers (A and B) provided preterm FM in the U.K., including two ready-to-feed liquid (L) FMs for preterm/low birthweight infants (<1800 g; A1L and B1L),two ready-to-feed liquid (L) post-discharge FMs (A2L, B2L) and two powder (P) post-discharge FMs (A2P and B2P; **Supplementary Table 1**). Preterm FMs are only available on prescription in the U.K., and samples were provided by manufacturers, who had no involvement in the study. Opened liquid FM and prepared powder FM may be stored for up to 12 and 24 hours, respectively, at 4°C in

hospitals. Therefore, liquid FMs (A1L: n=5; B1L: n=3; A2L: n=5; B2L n=3) were analysed after opening, and after 1, 4, and 10 hours at 4°C. Powder FM (A2P: n=6) was tested on day 0, 7, 14, 21, and 28 at 0, 4, and 24 hours storage at 4°C after preparation, respectively (**Figure 1**). All FMs were analysed before their use by dates. To simulate normal use, powder FM boxes were opened 3x per day, stirred, and one scoop removed, as described previously [10, 11]. Powder FM was prepared according to manufacturers' instructions. Samples for fatty acid analysis were processed directly, samples for lipid peroxidation analysis were stored under N_2 at -70°C to prevent further lipid peroxidation, and analysed within three months [4, 12].

2.2 Fatty acids

Fatty acids were analysed as described previously [13], with tricosanoic acid as internal standard, using gas chromatography (7820A, Agilent Technologies, U.S.A) and Omegawax™ 100 column (15 m x 0.1 mm x 0.5 µm, Sigma-Aldrich, U.K).

2.3 Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS), an indirect MDA measurement, were analysed using a commercial assay (Cayman Chemical, U.S.A.) following manufacturer's instructions. Absolute levels were adjusted according to determined recovery rates (A1L 39%, A2L 61%, A2P 49%), since the FM matrix can affect recovery [14]. Absolute values were corrected for published fat content. Intra-assay coefficient of variation was 7.8%, inter-assay coefficient of variation was 6.1%. TBARS could not be measured in B1L and B2L as they contain partially hydrolysed whey protein that could not be precipitated and interfered with measurements.

2.4 HNE

HNE adducts were quantified using an OXISelect HNE adduct competitive ELISA assay (Cell Biolabs, U.S.A) following manufacturer's instructions. Values were corrected for published fat content.

2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism, and non-parametric tests were used due to the sample size. Comparing between FMs, Mann Whitney test or Kruskal-Wallis test with Dunn's multiple comparisons test were used. Wilcoxon matched pairs test or Friedman test with Dunn's multiple comparisons test were used for comparison over refrigeration time. Influence of opened storage (days) and refrigeration (hours) was tested using repeated two-way ANOVA with Dunnet's post-hoc test. Wilcoxon signed rank test was used to compare LCPUFA content to manufacturers' values. Enteral LCPUFA supply, based on analysed LCPUFA content, was calculated for a hypothetical 28 week gestational age infant, weighing 1000 g at birth and receiving full enteral feeding (150 mL/kg/day) with infant FM and later on (2000 g bodyweight) with post-discharge FM. Values were compared to European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommendations [15], using Wilcoxon signed rank test. LCPUFAs available for accretion were also calculated for this hypothetical infant and compared with estimated *in-utero* accretion, as described [16]. For correlations, Spearman correlation was used. Data presented as means ± SD and 95% CI and considered statistically significant at $p < 0.05$.

3. RESULTS

3.1 LCPUFA content

LCPUFAs were measured at opening and compared to manufacturers' information. A1L and A2L ARA (15.5 ± 5.5 and 23.8 ± 2.2 vs. 20 mg/100 mL, respectively) and DHA (12.0 ± 4.5 and 13.4 ± 2.4 vs. 10 mg/100 mL, respectively), were consistent with published values (**Figure 2**). A2P had significantly lower ARA (11.5 ± 2.1 vs. 20 mg/100 mL, $p = 0.031$), but not DHA (8.2 ± 1.9 vs. 10 mg/100 mL). B1L and B2L had comparable ARA (11.5 ± 0.8 vs. 15 mg/100 mL, 10.4 ± 2.0 vs. 14.4 mg/100 mL, respectively) and DHA (10.5 ± 1.0 vs. 15 mg/100 mL, 10.4 ± 1.9 vs. 14.4 mg/100 mL, respectively) to published levels. A2L provided significantly more ARA than B2L and A2P (both $p < 0.05$), and DHA was significantly higher in A2L than in A2P ($p < 0.01$; **Figure 2**). These values are 1:1 convertible to mg/100 kcal.

3.2 Effects of storage on LCPUFA content

There was no effect of 4 hours refrigeration on ARA or DHA content of A1L, A2L, or B2L. There was a significant effect of storage days ($F(1,6) = 5.64$, $p = 0.047$) and refrigeration ($F(1,6) = 7.66$, $p = 0.027$) on ARA content of A2P (**Table 1**); which was further explored by post-hoc analysis, which revealed ARA content on day 21 at 24 hours was significantly different, although this was

within 95% CI of ARA content at opening. Only storage time (days) had an effect on DHA concentration in A2P ($F(2,11) = 9.11$, $p = 0.005$; **Table 1**). However, post-hoc test revealed no significant differences.

3.3 Estimated LCPUFA intake

LCPUFA intake from A1L and B1L was calculated for a hypothetical preterm infant (1000 g bodyweight) based on analysed fatty acid content, assuming full enteral feeding (150 mL/d). A1L and B1L provided 23.2 ± 7.2 and 17.3 ± 1.0 mg/d ARA, respectively, for a 1000 g infant, and although not significantly below ESPGHAN minimum recommendations (18-42 mg/kg/d) [15], mean values for B1L were 0.7 mg/kg/d lower than recommended. DHA similarly achieved recommendations (18.05 ± 5.8 and 15.8 ± 1.2 vs. 12 mg/d for a 1000 g infant; range 12-30 mg/kg/d).

LCPUFA intake from A2L, B2L, and A2P was calculated for a hypothetical preterm infant (2000 g bodyweight), assuming full enteral feeding (300 mL/d). ARA levels provided by A2L (71.3 ± 6.0 mg/d for a 2000 g infant) were within ESPGHAN recommendations (18-42 mg/kg/d, i.e. 36-84 mg/2kg/d), while levels provided by B2L and A2P (31.3 ± 5.1 and 34.4 ± 5.7 mg/d for a 2000 g infant, respectively) were lower but not statistically significant different. ARA levels differed significantly between FMs ($p = 0.011$; **Figure 3A**). Daily DHA intake provided by A2L, B2L and A2P was within ESPGHAN recommendation (12-30 mg/kg/d, i.e. 24-60 mg/d for a 2000 g infant), at 40.3 ± 6.5, 31.1 ± 4.6 and 24.7 ± 5.1 mg/d; however, it was significantly different between FMs ($p = 0.009$; **Figure 3B**).

LCPUFAs available for accretion were also estimated for the hypothetical infant. Full enteral feeding with A1L and B1L provided 45.5 ± 5.8 and 40.7 ± 0.8 mg/d of ARA, respectively, representing 22% and 19% of *in-utero* accretion. DHA from A1L and B1L provided 62% and 58% of the *in-utero* accretion (26.8 ± 4.5 and 25.0 ± 0.9 vs. 43 mg/d, respectively). There was no significant difference in ARA and DHA available for accretion between FMs.

A2L, B2L and A2P provided ARA below estimated *in-utero* accretion rates at 111.2 ± 4.9 (26%), 78.8 ± 4.1 (19%), and 81.3 ± 4.7 (19%) vs. 424 mg/d, respectively. ARA available for accretion was significantly higher in A2L than B2L and A2P (both $p < 0.05$). A2L, B2L and A2P provided 56.8 ± 5.1, 49.6 ± 3.6, and 44.6 ± 4.0 mg/d DHA for accretion. These levels were below estimated *in-utero* accretion rates (86 mg/d), representing 66%, 58%, and 52%. DHA availability was significantly higher from A2L than A2P ($p < 0.01$).

3.4 TBARS

There were no significant differences in TBARS on opening (A1L: 4.8 ± 0.4 µM, A2L: 4.0 ± 0.4 µM, A2P: 4.5 ± 0.5 µM), and no significant differences following storage A1L or A2L, or A2P (**Table 2**).

3.5 HNE

There were significant differences in HNE between FMs on opening ($p = 0.003$; **Figure 4**), with A2P levels 6x higher than A2L. Refrigerated storage did not significantly alter HNE levels of liquid FMs. There was a significant effect of opened storage (days) ($F(2,3) = 24.89$, $p = 0.009$) and refrigeration (hours) ($F(1,2) = 26.74$, $p = 0.034$), as well as a significant interaction between storage time and refrigeration ($F(2,4) = 17.26$, $p = 0.015$) in A2P (**Table 3**). Post-hoc analysis, confirmed significantly higher HNE at 4 hours on day 0, 7, 14, and 21.Although mean levels were further increased at 24 hours on days 21 and 28, these increases were not statistically significant due to a wider variability in measurements.

4. DISCUSSION AND CONCLUSION

This study investigated for the first-time effects of clinically relevant storage conditions on LCPUFAs and lipid peroxidation of liquid and powder first and post-discharge preterm FM. ARA and DHA content were not significantly altered by storage. However, lipid peroxidation was evident on opening, with higher HNE in powder than liquid FMs. Refrigeration of liquid FM for up to 10 hours did not alter TBARS or HNE levels, and opened storage of powdered FM for 28 days similarly did not increase peroxidation. Refrigerated storage of prepared powder FM led to significantly increased HNE at later time-points, and increased variability in the levels with time, therefore suggesting increased susceptibility to lipid peroxidation with storage time.

The lack of effects on ARA and DHA confirm previous observations that LCPUFA levels are stable during short-term storage after opening [10, 11]. The calculated daily ARA and DHA intake levels from FMs all met ESPGHAN recommendations [15] for the hypothetical infants, but were below estimated *in-utero* accretion rates, and may therefore not provide optimal levels of intake. ESPGHAN recommendations do not cover extremely preterm infants (< 28 weeks gestation, < 1000 g), the most vulnerable group for LCPUFA deficits. Furthermore, early LCPUFA deficits are not considered, and these recommended levels are based on safety evidence and breast milk levels [15].

Suboptimal LCPUFA intake can have long lasting effects in preterm infants, as they are essential for brain development [2], and insufficient intake is associated with comorbidities of prematurity, such as respiratory distress syndrome, sepsis, and intraventricular haemorrhage [17], whereas higher intakes might reduce delays in cognitive development [18].

Our study also considered the effects of storage on lipid peroxidation, as significant increases in lipid peroxidation can occur without changes in LCPUFAs [19]. Powdered FM had significantly higher HNE than liquid FM on opening, indicating increased lipid peroxidation, as has been found by others [20]. This discrepancy may be due to differences in processing, as spray drying of FM powder uses high temperatures (180-220°C), which can increase lipid peroxidation [6], whereas, the lower temperature of bottle sterilisation of liquid FM does not [21].

Lipid peroxidation of liquid FM was not found to increase following refrigeration, or opened storage of powder FM, whereas, refrigeration of prepared powder FM increased HNE. Other studies show increased lipid peroxidation of FM after opening and storage [6-8, 10]. This dissonance may be due to differences in antioxidant composition of FM and the protection provided. For example, higher vitamin A and E content of FM is associated with lower peroxidation [22, 23]. Further work should seek to explore the optimal antioxidant profile for FM, since different antioxidants provide different protection and synergistic effects may occur.

There are currently no guidelines for safe intake ranges of lipid peroxidation products in preterm infants, and the levels of TBARS and HNE found in FM are lower than the levels previously reported in U.K. preterm breast milk and donor human milk by us [13]. Others have similarly found higher lipid peroxidation in fresh Italian term breast milk than term FM [22], although this observation is not universal, with lower levels in fresh Spanish and American term breast milk than in term and preterm FM, respectively [7, 24]. This suggests levels identified in the present study may not severely increase the oxidative load of preterm infants. However, preterm infants have an underdeveloped antioxidant system [25] and an increased intake of lipid peroxidation products is associated with development of preterm morbidities such as, retinopathy of prematurity, bronchopulmonary dysplasia, intraventricular haemorrhage, necrotising enterocolitis, and sepsis [26, 27]. Protecting parenteral lipid emulsions from light to decrease lipid peroxidation, is associated with 30% reduction in bronchopulmonary dysplasia [5], highlighting the importance of decreasing exposure to lipid peroxidation products. Therefore, to minimise dietary sources of lipid peroxidation products we recommend FM is used on “first in, first out” basis and refrigerated storage time minimised whenever possible.

A strength of the study is that effects of current clinical storage conditions on LCPUFA and lipid peroxidation of preterm FM were simulated for the first time. Furthermore, dietary intake levels of measured LCPUFA content were compared to EPSGHAN guidelines and *in-utero* accretions rates, to provide clinically relevant perspectives on intake. Limitations are that lipid peroxidation was not measured immediately for logistical reasons; however, specific storage conditions were employed to inhibit further lipid peroxidation, and since preterm FM is only available on prescription in the U.K., analysis was limited to samples provided by manufacturers, so not all available FMs were analysed. However, although sample numbers were limited, the analysis compares favourably with work by others, where sample sizes of one have been reported [7, 8, 12, 19, 28]. Nevertheless, these results need corroboration with larger studies in order to provide specific details of best practice for refrigerated storage times.

In conclusion, current storage conditions did not significantly decrease LCPUFA content of preterm FMs tested here, and although ESPGHAN recommended intake levels [15] could be achieved, none provided sufficient intake for estimated *in-utero* accretion rates. Furthermore, the differences in lipid peroxidation on opening, and increased susceptibility of powdered FM to lipid peroxidation, suggest powder FM has a greater potential to contribute to the oxidative load of preterm infants than liquid FM. Storage of both fresh and prepared powder FM should be minimised wherever possible.

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TRANSPARENCY DECLARATION

The lead author affirms this manuscript is an honest, accurate, and transparent account of the study being reported. The lead author affirms that no important aspects of the study have been omitted.

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