Effects of Extended Freezer Storage on the Integrity of Human Milk

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Objective To examine the integrity (pH, bacterial counts, host defense factors, nutrient contents, and osmolality) of freshly expressed and previously refrigerated human milk subjected to long-term freezer storage.

Study design Mothers donated 100 mL of freshly expressed milk. Samples were divided into baseline, storage at –20°C (fresh frozen) for 1, 3, 6, and 9 months, and prior storage at +4°C for 72 hours (refrigerated frozen) before storage at –20°C for 1 to 9 months. Samples were analyzed for pH, total bacterial colony count, gram-positive and gram-negative colony counts, and concentrations of total protein, fat, nonesterified fatty acids, lactoferrin, secretory IgA, and osmolality.

Results Milk pH, total bacterial colony count, and Gram-positive colony counts decreased significantly with freezer storage ($P < .001$); bacterial counts decreased most rapidly in the refrigerated frozen group. The gram-negative colony count decreased significantly over time ($P < .001$). Nonesterified fatty acid concentrations increased significantly with time in storage ($P < .001$). Freezing for up to 9 months did not affect total protein, fat, lactoferrin, secretory IgA, or osmolality in either group.

Conclusions Freezer storage of human milk for 9 months at –20°C is associated with decreasing pH and bacterial counts, but preservation of key macronutrients and immunoactive components, with or without prior refrigeration for 72 hours. These data support current guidelines for freezer storage of human milk for up to 9 months for both freshly expressed and refrigerated milk. *(J Pediatr 2016;177:140-3).*

Human milk is the optimal nutritional support for preterm infants and its use is encouraged in neonatal intensive care units (NICUs) and after discharge at home.[1,2] Freezer storage of expressed human milk for an extended time is a common practice for mothers of hospitalized infants in the NICU, those at home and/or returning to the workplace, and for donor human milk.[3] Evidence-based guidelines for the duration of freezer storage are necessary and needed to ensure the safety and integrity of the milk. Most reports of the stability of human milk during freezer storage focus on single attributes and do not present a composite evaluation of milk subjected to such storage conditions. Sample size, methods of milk collection and preparation, and duration of storage also vary among reports. For example, decreased antioxidant activity has been reported in milk after storage for 10-60 days at −18°C.[4] In contrast, after storage at −20°C for up to 1 month, no changes were reported in amino acid, fatty acid, and immune components, but there was 90% loss in cell viability.[5,6] Although decreases in bactericidal activity are observed, there is marked variability in the pattern of bacterial colony counts after storage.[7-10] Refrigeration of fresh human milk has been recommended for up to 96 hours with minimal changes,[11] but freezer storage after refrigeration has not been examined systematically.

The American Academy of Pediatrics currently recommends that unused human milk be stored frozen within 24 hours of collection and that it can be thawed and used, if stored appropriately, for up to 3 months, or 3-6 months if maintained undisturbed at −20°C. Although the Human Milk Banking Association of North America recommends storage at −20°C for up to 12 months, it suggests that <3 months is "optimal."[12] Because there are scant data justifying these strategies, this study was designed to examine the effects of extended freezer storage of both fresh and previously refrigerated human milk on its constituents and bacterial viability.

Methods

Random milk samples (100 mL) were collected from 40 mothers with infants in the NICU. Only mothers with a milk supply in excess of their infants' needs were approached for their voluntary informed participation. Milk was collected using

NICU	Neonatal intensive care unit
TBCC	Total bacterial colony counts
GPCC	Gram-positive colony counts
GNCC	Gram-negative colony counts
NEFA	Nonesterified fatty acids

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hospital-grade electric breast pumps following the standard double pumping procedure used in the NICU. Samples were obtained from 1-2 consecutive complete expressions (not >4 hours apart) during routine hospital visits.

Mothers participating in the study did not receive instruction or equipment beyond the routine for the NICU. Mothers with evidence of a breast infection or those receiving antibiotics within the past 7 days were excluded from participation.

Within 4 hours of collection, milk samples were divided into nine 10-mL aliquots. A baseline (time 0) aliquot was stored at −80°C until analyzed. Four 10-mL aliquots (fresh frozen) were stored in −20°C for 1, 3, 6, or 9 months, and then stored at −80°C until analyzed. The last set of 10 mL aliquots (refrigerated frozen) were placed in a refrigerator (+4°C) for 72 hours and then transferred to a freezer maintained at −20°C for 1, 3, 6, or 9 months, and finally stored at −80°C until analyzed. At the time of analyses, samples were thawed in a constant temperature water bath. A refrigerator-freezer unit similar to that used for clinical care in NICU was used. Storage temperatures were monitored and recorded daily.

Milk pH was measured using a standard electronic pH meter. Total bacterial colony counts (TBCC), gram-positive colony counts (GPCC), and gram-negative colony counts (GNCC) were measured by serial dilutions with saline to enable detection of 30-300 bacterial colonies per plate (Trypticase Soy agar, Columbia CNA, and MacConkey agar, Fisher Scientific, Pittsburgh, Pennsylvania) after incubation at 37°C. Total protein concentration was measured by Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, California), and concentrations of lactoferrin (Calbiochem, LaJolla, California) and secretory IgA (ALPCO Diagnostics, Salem, New Hampshire) were assessed by enzyme-linked immunosorbent assay according to manufacturer's directions. Total lipids were extracted using chloroform:methanol (2:1), and percent lipid determined gravimetrically.[13] The lipid was then resuspended in methanol:chloroform (7:3) and assayed in triplicate for nonesterified free fatty acid concentrations using the HR Series NEFA-HR(2) assay kit (Wako Chemicals USA, Inc, Richmond, Virginia) with oleic acid as a standard.[13] Milk osmolality was measured using the Model 3320 Osmometer (Advanced Instruments, Norwood, Massachusetts).

A sample size of 36 was established to enable detection of 1 standard deviation from the mean for each analysis. Data were analyzed using repeated measures ANOVA to detect both changes over time and differences between methods (fresh frozen vs refrigerated frozen). Significant differences were defined as $P < .01$ owing to multiple comparisons. Data are expressed as mean values ± SEM. The study was approved by the Institutional Review Board of the North Shore-Long Island Jewish Health System and informed consent was obtained from mothers.

Results

Forty mothers donated freshly expressed milk, with mean maternal age of 32 years (range, 19-46), mean gestational age of infants at birth 30 weeks (range, 23-41), and mean postpartum age at the time of milk collection of 61 days (range, 5-270). The average time between expression and initiation of refrigerator or freezer storage was 2.2 ± 0.2 hours.

Milk pH decreased significantly during the first month of freezer storage ($P < .001$), and continued to decrease to 9 months (**Figure 1**). TBCC and GPCC also declined similarly, approaching zero after 3 months of storage. This decrease in viable bacteria occurred more rapidly in refrigerated frozen milk, with lower colony counts observed in refrigerated frozen milk after 1 month of freezer storage. GNCC were present in 10 samples (25%) and decreased during the first 3 months of storage, but did not differ between refrigerated frozen and fresh frozen milk. Changes in pH correlated positively ($P < .001$) with changes in TBCC ($r = 0.44$) and GPCC ($r = 0.47$), independent of time. Freezer storage for up to 9 months did not affect total protein, fat, lactoferrin, secretory IgA, or osmolality (291 ± 0.9 mOsm/kg, mean ± SD).

Nonesterified fatty acid (NEFA) concentrations increased with time in freezer storage up to 9 months (**Figure 2**).

An inverse relationship ($r = −0.82$; $P < .005$) between NEFA concentrations and pH was observed. There were no relationships between maternal age, gestational age at birth, postnatal age at the time of milk expression, and the time elapsed from milk expression to initiation of freezer storage and the baseline values of pH, TBCC, GPCC, GNCC, or NEFA.

Discussion

We examined the effects of freezer storage of human milk for up to 9 months, using a design that simulated common patterns of use and storage in the NICU. We found that freezer storage for up to 9 months was associated with a decline in milk pH that was not affected by prior refrigeration up to 72 hours before freezing at −20°C. The decline in bacterial colony counts during the first 3 months indicates that there is ongoing biological activity in milk with long-term storage at −20°C. The more rapid decrease in GPCC when compared with GNCC may be related to the milk lysozyme, which has greater activity against gram-positive than gram-negative bacteria.[14] In addition, prior refrigeration also results in decreases in bacterial counts, which contributed to the more rapid decrease in the refrigerated frozen groups.[11] Decreased GNCC after prolonged freezer storage suggests that such storage is safe. However, the marked decrease toward zero in bacterial counts might be associated with diminished health benefits attributed to prolonged frozen storage of human milk. In this regard, prior refrigeration before freezer storage may accelerate these potentially deleterious effects on milk microflora. Intestinal bacteria and its diversity are important in neonatal health and disease, so future studies on the effects of human milk in preterm infants should account for the prior milk storage conditions.[15] The data suggest an evaluation of feeding fresh versus stored milk is warranted.

The observed decreases in milk pH with proportional increases in fatty acid concentrations are likely a result of continued lipolytic activity with storage. We have previously reported[16] a similar observation with refrigerator storage of human milk. As free fatty acid concentrations increased with