Refrigerator Storage of Expressed Human Milk in the Neonatal Intensive Care Unit

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Objective To provide recommendations for refrigerator storage of human milk, the overall integrity (bacterial growth, cell counts, and component concentrations) of milk was examined during 96 hours of storage at 4°C.
Study design Fresh milk samples (n = 36) were divided and stored at 4°C for 0, 24, 48, 72, and 96 hours. At each time, pH, white cell count, and osmolality were measured and additional samples were stored at −80°C until analyzed for bacteria and concentrations of lactoferrin, secretory (s)IgA, fat, fatty acids, and protein.
Results There were no significant changes for osmolality, total and Gram-negative bacterial colony counts or concentrations of sIgA, lactoferrin, and fat. Gram-positive colony counts (2.9 to 1.6 × 10^5 colony-forming units per mL), pH (7.21 to 6.68), white blood cell counts (2.31 to 1.85 × 10^6 cells per mL), and total protein (17.5 to 16.7 g/L) declined, and free fatty acid concentrations increased (0.35 to 1.28 g/L) as storage duration increased, $P < .001$.
Conclusions Changes were minimal and the overall integrity of milk during refrigerator storage was preserved. Fresh mother's milk may be stored at refrigerator temperature for as long as 96 hours. *(J Pediatr 2010;156:26-8)*.

Human milk is the optimal nutrition for premature infants.[1] With the increasing use of human milk, guidelines for its quality control, to preserve the nutritional and immunologic constituents of the milk and to ensure optimal infection control, are an important part of hospital practices. After 3 days of refrigerator storage, previous studies have reported a reduction in the protective action of milk against bacteria and concluded that human milk should be stored at refrigerator temperature for no more than 48 hours.[2] Further studies, however, have shown that fortified human milk can be stored as long as 72 hours, based on declining bacterial colony counts, an indication of an active host defense in the milk.[3] These data are similar to those reporting a decline in bacterial colony counts during a 5-day refrigeration of unfortified human milk.[4] The Human Milk Banking Association of North America recommends up to 8 days for refrigerator storage of human milk.[5,6] Most recommendations for the optimal duration of milk storage, however, have focused solely on bacterial colony counts as measurements of milk contamination or lack of intrinsic host defenses in the milk. No study has addressed the effects of storage on milk components or byproducts of storage (such as pH or osmolality). For example, survival of white blood cells and viability of many of the immune proteins (IgA and lactoferrin) may be affected by storage temperature.[7] Thus, there is a wide variation in the determinants of how optimal storage conditions are assessed and in the recommendations for refrigerator storage of human milk in the neonatal intensive care unit (NICU). We hypothesized that refrigerator storage of human milk at 4°C for 96 hours would not affect its integrity, as defined by bacterial growth, white blood cell counts, pH, osmolality, and concentrations of selected immune factors and macronutrients.

Methods

Mothers of term and preterm infants in the NICU were asked to collect 80 mL of milk using an electric breast pump (Ameda SMB Electric Breast Pump, Piqua, Ohio) directly into breast milk storage containers (Medela, Inc., McHenry, Illinois) from either or both breasts. Mothers gave their fresh milk to the research team who aseptically separated the 80-mL sample into 5 milk storage containers that either were studied immediately (at time point 0) or stored in the NICU at refrigerator temperature (4°C) for 24, 48, 72, or 96 hours. The number of times each refrigerator was opened each day was tabulated. Refrigerator temperature verification was confirmed every 12 hours. At 0, 24, 48, 72, and 96 hours, milk was removed and analyzed immediately for pH, white cell count (hemocytometer), and osmolality (vapor pressure osmometer, Wescor Inc., Logan, Utah), and the remaining sample was stored at −80°C for subsequent analyses. For bacterial colony counts, milk was thawed rapidly and diluted serially with saline to enable detection of

NICU	Neonatal Intensive Care Unit
sIgA	Secretory IgA

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30 to 300 bacterial colonies per plate for total, Gram-positive and Gram-negative colony counts (Trypticase Soy agar, Columbia CNA, and MacConkey agar, Fisher Scientific, Pittsburgh, Pennsylvania). Milk samples were thawed and analyzed by ELISA for concentrations of lactoferrin (Calbiochem, LaJolla, California) and secretory IgA (ALPCO Diagnostics, Salem, New Hampshire). The protein concentration was measured by a modified Lowry Protein Assay (Pierce, Rockford, Illinois). Total lipid was determined by gas-liquid chromatography[8,9] and free fatty acid concentrations were determined by specific methylation of nonesterified fatty acids quantified by gas-liquid chromatography.[10]

Five samples of pasteurized donor human milk, heated to 62°C and subsequently frozen at −20°C, were thawed rapidly and subjected to the same storage conditions and analyzed for bacterial colony counts, pH, and concentrations of free fatty acids.

A convenience sample of 36 mothers was chosen to detect a difference of 1 SD from the mean for each analysis. To determine changes over time, the data were analyzed by repeated-measures ANOVA. Significant differences were defined as $P < .01$ due to multiple comparisons. Unless indicated otherwise, data are presented as mean ± SD. Written informed consent from each mother was obtained, and the study protocol was approved by the Institutional Review Board of the North Shore-Long Island Jewish Health System.

Results

Milk was obtained at a median of 28 days postpartum (range, 7 to 150 days) from mothers who delivered at a median gestational age of 32 weeks (range, 25 to 41 weeks). The average time between milk collection and first analysis was approximately 2.4 ± 1.2 hours. The cumulative numbers of refrigerator "openings" at 24, 48, 72, and 96 hours were 21 ± 8, 38 ± 15, 56 ± 21, and 71 ± 27, respectively. Milk pH declined significantly, from 7.21 to 6.68 over 96 hours of storage (**Figure 1**). White blood cell count declined by 16% (95% CI, −27%, −6%), from 2.31 to 1.85 × 10^6 cells/mL ($P < .001$). Total protein concentration declined by 5%, from 17.5 to 16.7 g/L ($P < .001$). Gram-positive colony counts declined (median, 2 × 10^5; range, 2.9 to 1.6 × 10^5 colony forming units/mL) (**Figure 2**). There was a 3-fold increase in the concentration of free fatty acids, with duration of storage from 0.35 to 1.28 g/L (**Figure 3**). As a percentage of total fat, the concentration of free fatty acids increased with storage, from 1.3% to 4.8% ($P < .001$).

There were no significant changes in milk osmolality and concentrations of sIgA, lactoferrin, total fat, and total and Gram-negative colony counts during 96 hours of refrigerator storage. Percentage of free fatty acid concentrations at baseline, but not over time of storage, correlated with the postpartum age at the time of milk collection ($r = 0.48$, $P = .008$) but not with gestational age. There were no significant relationships between gestational age, postpartum age, and other milk component measures at baseline or with duration of storage.

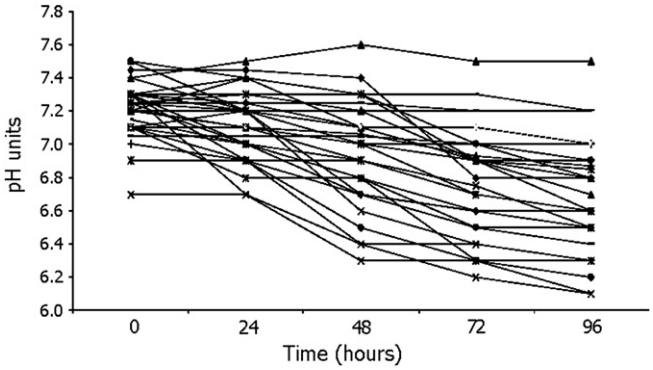

Figure 1. Milk pH declined over 96-hour refrigerator storage ($P < .001$). Each time point differs from the preceding value ($P < .05$).

There was a significant inverse relationship between milk pH and free fatty acid concentrations (**Figure 4**). Changes in pH and free fatty acid concentrations were not correlated with any of the other factors studied, including bacterial colony counts and the number of times the refrigerator was entered.

There were no bacteria isolated from the pasteurized donor milk. The pH and free fatty acid concentrations of 5 samples of pasteurized donor human milk did not change during the 96-hour refrigerator storage. Samples of pasteurized donor human milk had a mean pH of 6.3 ± 0.1 and percent free fatty acid concentration of 6.1% ± 2.2%.

Discussion

This multifactorial approach determined that the integrity of fresh mother's milk was not affected by 5 days of storage at

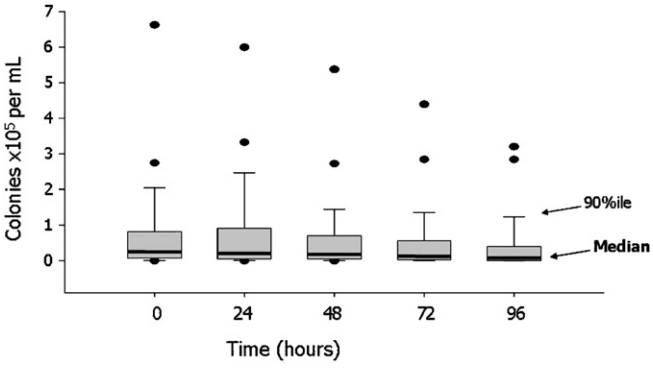

Figure 2. Gram positive bacterial colony counts declined over 96-hour storage ($P < .001$).

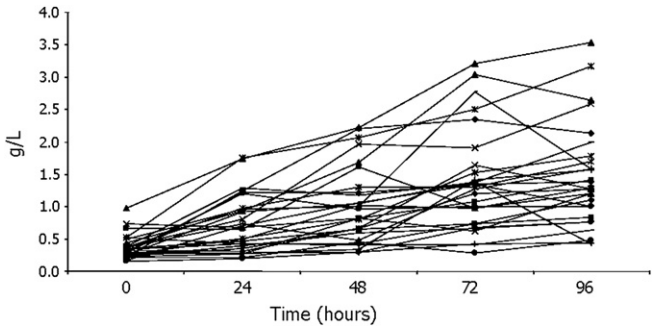

Figure 3. Free fatty acid concentrations increased 3-fold over 96-hour storage ($P < .001$).

refrigerator temperature. We observed how often the refrigerator was opened each day, yet the temperature was maintained and verified by serial monitoring. The lack of significant increases in bacterial colony counts is important. Indeed, the decline in Gram-positive colony counts is indicative of an active host defense system in the milk. Although the changes in bacterial counts are reassuring to the integrity of milk, the lack of major changes in macronutrients and immune factors, such as sIgA, lactoferrin, total fat, and total protein, further supports the safety of refrigerator storage for 96 hours.

The study did find some changes in milk during refrigerator storage, exemplified by declines in pH and white cell counts, and a rise in free fatty acid concentrations. The changes in pH were minimal and compared with the ranges others have investigated. For example, storage at refrigerator temperature of 15°C for 24 hours was associated with a decline in pH by approximately 7%, a level that did not affect the activity of milk enzymes or the recommendation for the use of that milk.[11] That change in milk pH was of similar magnitude as we observed in our study.

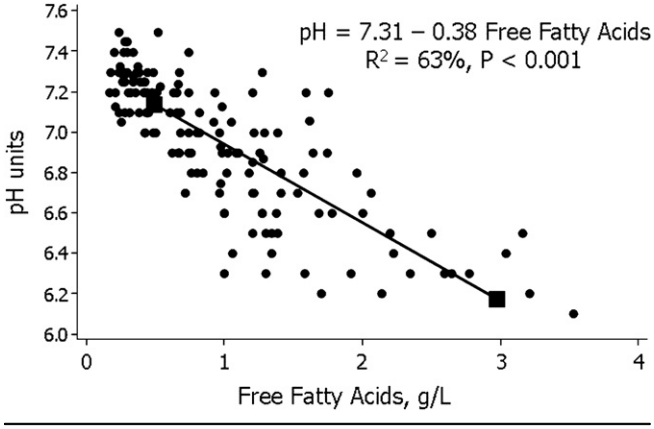

Figure 4. Inverse relationship between pH and free fatty acid concentrations ($r = 0.79$, $P < .001$).

The rise in free fatty acid concentrations observed in our study is indicative of active lipolysis. Lipolytic products may be beneficial because they are associated with antimicrobial activity against bacteria, viruses, and protozoa.[12] The changes in free fatty acids were similar to those reported for storage at higher temperatures for 24 hours and were deemed compatible for feeding. Moreover, the changes in free fatty acid concentrations with storage were no greater than that found in usual pasteurized human milk. Despite the decline in white blood cell counts, more cells remain after storage to 96 hours than after freezing or pasteurization. It is therefore doubtful whether these changes would be detrimental to the NICU infant.

Mother's milk may be stored in the NICU at refrigerator temperature of 4°C for 96 hours without compromising its overall integrity, as assessed by bacterial colony counts, white blood cell counts, osmolality, pH and concentrations of sIgA, lactoferrin, protein, total fat, and free fatty acids. ■

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