Alterations in the Host Defense Properties of Human Milk Following Prolonged Storage or Pasteurization

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ABSTRACT

Objectives: Preterm infants are often fed pasteurized donor milk or mother's milk that has been stored frozen for up to 4 weeks. Our objectives were to assess the impact of pasteurization or prolonged storage at −20°C on the immunologic components of human milk and the capability of the different forms of human milk to support bacterial proliferation.

Materials and Methods: The concentrations and activities of major host defense proteins in the whey fractions of mother's milk stored for 4 weeks at −20°C or pasteurized human donor milk were compared with freshly expressed human milk. Proliferation of bacteria incubated in the 3 forms of human milk was assessed.

Results: Relative to freshly expressed human milk, the concentrations of lysozyme, lactoferrin, lactoperoxidase, and secretory immunoglobulin A were reduced 50% to 82% in pasteurized donor milk and the activities of lysozyme and lactoperoxidase were 74% to 88% lower ($P < 0.01$). Proliferation of bacterial pathogens in pasteurized donor milk was enhanced 1.8- to 4.6-fold compared with fresh or frozen human milk ($P < 0.01$).

Conclusions: The immunomodulatory proteins in human milk are reduced by pasteurization and, to a lesser extent, by frozen storage, resulting in decreased antibacterial capability. Stringent procedure to minimize bacterial contamination is essential during handling of pasteurized milk.

Key Words: host defense proteins, human milk, lactoferrin, lactoperoxidase, lysozyme, muramidase, secretory immunoglobulin A

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Human milk is recommended by the American Academy of Pediatrics and the World Health Organization as the optimal form of nutrition for neonates, including low-birth-weight infants, and breast-feeding is pivotal to achieving the United Nations Millenium Development Goal 4 to reduce child mortality (1–3). Mother's milk has multiple short- and long-term benefits over cow's–milk–based formula (4). Its immunomodulatory components are particularly invaluable to the extremely low-birth-weight infants who are often hospitalized for an extended period in the neonatal intensive care unit (NICU) (4–7). Infants in the NICU are fed 1 of 3 forms of human milk (mother's own fresh milk, mother's milk that may have been stored at −20°C for up to 4 weeks, or pasteurized donor milk obtained from authorized milk banks) or cow's-milk–based infant formula. Donor milk is often advocated in preference to formula, in spite of its lower energy and protein contents, because it obviates exposure of the vulnerable preterm infants' gut to xenogeneic proteins and it is closer to mother's own fresh milk in its overall composition than formula.

Human donor milk is processed by Holder pasteurization to minimize the transmission of infectious agents to recipients. This process may alter the composition, concentration, and activity of humoral mediators of innate immunity in human milk. Although the benefits of mother's own fresh milk over formula, with respect to infection-related complications and necrotizing enterocolitis, have been well established (7,8), how many of these immune benefits are preserved following prolonged storage or pasteurization is unknown. In spite of conflicting data on clinical benefits, the use of pasteurized donor milk has gained widespread acceptance in the NICU setting. The goals of the present study are 2-fold: to compare the concentration and activities of major host defense proteins in the different forms of human milk available with preterm infants and to compare the capability of the different forms of human milk to support bacterial proliferation.

MATERIALS AND METHODS

Acquisition of Milk Samples

The 3 forms of human milk used in the present study are as follows: freshly expressed milk obtained through the Global Human Milk Research Collaborative, Cincinnati Children's Hospital; mother's milk that had been frozen at −20°C for 4 weeks, intended for feeding preterm infants in the NICU at the University Hospital, Cincinnati; and pasteurized donor milk from the Mother's Milk Bank of Ohio (Columbus). The study protocol was approved by the institutional review boards of Cincinnati Children's Hospital Medical Center and the University Hospital, Cincinnati. Milk specimens were handled as follows: freshly expressed human milk specimens were stored on ice from the time of collection and processed within 2 hours; frozen mother's milk or pasteurized donor milk specimens were thawed for 4 to 6 hours at 4°C before processing as described below.

Isolation of Whey Protein From Human Milk

Fifteen milliliters of each human milk specimen was centrifuged at 4000*g* for 30 minutes at 4°C to separate the lipid layer,

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which was subsequently discarded. Whey fractions were isolated from each milk sample by the sedimentation method as described previously (9). Briefly, defatted milk specimens were centrifuged at 105,000*g* for 1 hour at 4°C. The supernatants (whey fractions) were stored in 1-mL aliquots with or without protease inhibitors at −20°C until analyses.

Total Whey Proteins

The concentration of total protein in whey fractions was assessed by bicinchoninic acid assay method using bovine serum albumin as standard, as previously reported (10,11).

Host-defense Proteins

Secretory Component of Immunoglobulin A

The concentration of secretory component of immunoglobulin A (sIgA) in 500 μg of total protein from each sample of whey fraction was assessed by enzyme-linked immunosorbent assay, following the manufacturer's instructions (ALPACO Diagnostics, Salem, NH), as previously described (12).

Lysozyme, Lactoferrin, α-Lactalbumin, and Lactoperoxidase

Five hundred micrograms of whey protein was applied onto nitrocellulose membranes using a dot-blot filtration apparatus (Bio-Dot Microtiter System, Bio-Rad Laboratories, Hercules, CA) as described previously (13). Briefly, protein samples were adsorbed onto the membranes under vacuum suction. The membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS), washed twice for 5 minutes with PBS in 0.05% Tween 20 and once with PBS, and incubated for 4 hours with 1 of the following primary antibodies: antihuman lysozyme (Accurate Chemical and Scientific Corp, New York, NY); antihuman lactoferrin (from our laboratory); anti-α-lactalbumin (Abcam, Cambridge, MA); anti-lactoperoxidase antibody (gift from Dr Conner, University of Florida, Gainesville). Membranes were reacted with the appropriate horseradish peroxidase-conjugated secondary antibody (Calbiochem, La Jolla, CA) for 1 hour. Protein-antibody complexes were detected with an enhanced chemiluminescent system (Pierce, Rockford, IL) using Fotodyne Imaging System (Fotodyne Inc, Hartland, WI) and quantified using computer-assisted band densitometry with Gel-Pro Analyzer software (Media Cybernetic, Bethesda, MD). Each membrane included whey protein specimens, protein standards, and positive controls. Standard curves were generated with recombinant human lysozyme and iron-saturated lactoferrin (range 0–1000 ng, both from Ventria Bioscience, Sacramento, CA) or lactoperoxidase (0–500 ng, Worthington Biochemical Corp, Lakewood, NJ). To assess the specificity of each of the antibodies used for dot-blot assay, a subset of whey protein samples from the 3 forms of human milk were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the antibodies specified above, as previously described (9,14).

Protein Activity

Muramidase

Lysozyme enzyme activity was assessed by incubating 1 mL of killed *Micrococcus lysodeikticus* suspended in 0.4 mol/L potassium phosphate buffer (pH 6.7) at an optical density (OD) of 1 (450 nm wavelength) at 37°C with 50 μg of whey fraction protein as described previously (15). Change in absorbance was plotted against time for 30 minutes using a recording spectrophotometer. A standard curve was generated with recombinant human lysozyme (Ventria Bioscience). Muramidase activity was expressed as relative units (Δ OD 450 nm × 0.001/ng protein) (1 U of enzyme activity = 0.001 change in absorbance). Each sample of whey fraction was assessed in duplicate.

Peroxidase Activity

To assess peroxidase activity, 100 μg of whey protein was incubated with Amplex Red Peroxidase Assay reagent containing 2 μmol/L hydrogen peroxide at room temperature for 30 minutes as recommended by the manufacturer (Molecular Probes Inc, Eugene, OR), as described by Reszka et al (16). Lactoperoxidase activity was estimated from the rate of change in OD per second at a wavelength of 560 nm and correlated to a standard curve generated with horseradish peroxidase. Each sample of whey fraction was assayed in duplicate. To ascertain specificity of the peroxidase reaction, a parallel experiment was performed with each whey fraction sample in the presence of the inhibitor, methimazole (1 μmol/L), before adding Amplex Red reagent. To assess the distinct contribution of prolonged frozen storage from the effects of heat pulse at 62.5°C to simulate Holder pasteurization, a subset of freshly expressed human milk specimens (n = 10) was divided into 3 aliquots and processed as follows: 1 set of aliquots was processed within 4 hours of collection, and whey fractions were isolated following heat pulse for 30 minutes at 62.5°C or after storage at −20°C for 4 weeks.

Bacterial Proliferation Assay

Ten thousand colony-forming units of bioluminescent transformants of the common neonatal pathogens *Pseudomonas aeruginosa* (PA01), *Staphylococcus aureus* (both from Xenogen Corp, Alameda, CA), or *Escherichia coli* (gift from Dr Matti Karp, Kosice, Finland) were incubated at 37°C with each of the 3 forms of human milk (n = 8 specimens/group) in a 96-well plate. Luminescence was measured in a microtiter reader following 0, 2, 4, and 6 hours of incubation. For all bacteria, luminescence was correlated to colony-forming unit counts. Each sample of whey fraction was assessed in triplicate.

Statistical Analysis

Data are expressed as mean ± SD for protein concentrations, myeloperoxidase, and muramidase activities. Comparisons regarding protein concentrations and activity among the 3 forms of human milk were made with analysis of variance and the Tukey-Kramer adjustment for post hoc multiple testing. To investigate bacterial proliferation over time across milk types, analyses were made by repeated-measures mixed models. A multiple comparison adjustment using Tukey-Kramer for post hoc multiple comparisons was made for testing mean differences at each time point among the groups. All of the statistical analyses were performed with SAS version 9.2 software (SAS Institute, Cary, NC).

RESULTS

Demographic Characteristics of Milk Donors

A total of 20 frozen human milk (frozen), 15 pasteurized donor milk (pasteurized), and 18 freshly expressed human milk (fresh) specimens were analyzed. The demographic data on the donors are compared in Table 1. Fresh or frozen specimens were obtained from individual mothers, whereas each of the pasteurized specimens was pooled from 1 to 3 donors (personal communication,

TABLE 1. Demographic characteristics of donors of human milk and initial analyses of whey proteins

	Frozen milk (n = 20 donors)	Pasteurized milk* (n = 15 pooled specimens)	Fresh milk (n = 18 donors)	P
Gestational age, wk	29.6 (3.4)	38.8 (1.4)	38.9 (1.0)	<0.0001†
Maternal age, y	28.3 (5.7)	29.6 (5.3)	31.6 (4.5)	0.18
Parity‡	2.0 (1–5)	3.0 (1–5)	2.0 (2–4)	0.48
Total protein, mg/mL	55.5 (4.1)	46.9 (2.8)	58.5 (6.8)	<0.0001$
Day of lactation	14.3 (7.7)	NA	88.3 (53)	<0.0001

Values reported as mean with SD. Post hoc comparisons were conducted with Tukey multiple comparison adjustment.
* Specimens of pasteurized milk were pooled from 1 to 3 donors to the Mothers' Milk Bank of Ohio; total of 24 donors.
† Significant differences between frozen with both pasteurized and fresh ($P < 0.0001$). No significant difference between pasteurized and fresh ($P = 0.99$).
‡ Reported as median with range. Data were analyzed with Wilcoxon rank sum test.
$ Significant differences between frozen and pasteurized ($P = 0.0014$) and pasteurized and fresh (<0.0001). No significant differences between frozen and fresh ($P = 0.36$).

Georgia Morrow, Mother's Milk Bank of Ohio). There were no significant differences among the 3 groups with respect to maternal age and parity. The donors of fresh or pasteurized human milk were more likely than the donors of frozen specimens to have had previous breast-feeding experience. The median postpartum day at collection of milk (day of lactation) was significantly higher for fresh milk. For pasteurized human milk, each specimen had been subjected to Holder pasteurization at 62.5°C for 30 minutes and refreezing at −20°C until analyses. The 15 samples analyzed in the present study were donated by 24 mothers. The median number of donors per specimen of pasteurized milk was 2. Data on individual donors to the bank with respect to the day of lactation were not available. The concentration of total protein was significantly higher in fresh or frozen milk compared with pasteurized milk (Table 1).

Concentrations of Host Defense Proteins

Compared with fresh human milk, the mean concentrations of lysozyme were 32% or 60% lower in frozen human or pasteurized milk, respectively ($P < 0.001$; Fig. 1A). The concentration of lysozyme was significantly lower in pasteurized donor milk compared with frozen human milk ($P = 0.003$). Relative to fresh milk, concentrations of lactoferrin were 44% lower in pasteurized milk ($P = 0.002$), although there were no significant differences between fresh and frozen milk specimens ($P = 0.55$). The mean concentrations of sIgA were reduced by 51% or 60% in frozen or pasteurized specimens, respectively ($P < 0.0001$). No significant differences were detected in the concentrations of sIgA between frozen and pasteurized milk samples ($P = 0.25$). The mean concentration of lactoperoxidase was 66% to 82% lower in frozen or pasteurized milk than in fresh specimens, respectively ($P < 0.0001$). The SDS-PAGE/Western analysis of a subset of whey fractions from each of the human milk types confirmed the antibodies were monospecific and detected proteins of the predicted sizes (Fig. 1B).

Protein Activity

Muramidase activity in a subset of whey fractions from frozen or pasteurized milk specimens was 50% to 76% lower than that in fresh human milk ($P < 0.0001$; Fig. 2). Similarly, peroxidase activity was 69% to 88% lower in frozen or pasteurized milk, respectively ($P < 0.0001$). To assess the distinct contribution of freezing versus pulse heating to the diminution of protein activity, we compared peroxidase activities in a subset of whey fractions of fresh milk samples in which whey fractions were isolated on the day of milk collection, after storage at −20°C for 4 weeks or following pulse heating to 65°C for 30 minutes. Peroxidase activity was reduced 19% following freezing for 4 weeks and by 51% in whey fractions that were pulse heated to simulate pasteurization (Fig. 3). Peroxidase activity was inhibited ≥90.5% in specimens of whey fraction that were incubated with methimazole, indicating specificity of the peroxidase reaction (data not shown).

Proliferation of Bacteria Incubated in Human Milk

The capability of the 3 different forms of human milk to enhance bacterial proliferation was assessed by incubating milk with bacterial pathogens encountered commonly in neonates. Compared with the buffer control, the concentration of *S aureus* bacteria was significantly increased in fresh, frozen, or pasteurized milk specimens by 6 hours of incubation ($P < 0.01$; Fig. 4). No significant differences in bacteria concentration were detected between fresh milk and the buffer control at the 2- or 4-hour time points ($P > 0.1$). Relative to fresh human milk, bacterial counts were increased 1.8- to 2.3-fold at 2 hours, 2.8- to 3.8-fold at 4 hours, and 3.6- to 4.6-fold at 6 hours in the other forms of milk for all bacteria tested ($P < 0.05$; Fig. 4). Proliferation of the Gram-negative bacteria (*P aeruginosa* and *E coli*) was significantly lower in frozen milk than in pasteurized milk by 2 hours of incubation (*E coli*; $P < 0.003$) and at 4 and 6 hours (*E coli* and *P aeruginosa*; $P < 0.0001$).

DISCUSSION

The imperative by the United Nations in the Millennium Development Goal to reduce child mortality and the American Academy of Pediatrics statement (1–3) that mother's milk provides complete nourishment for newborn infants because of its positive effect on survival, growth, and development are supported by epidemiologic and case-control studies (8,17–20). Mother's milk may be especially important for extremely low-birth-weight infants because of deficiencies in innate immunity associated with preaturity. Although there are plausible reasons for alteration of the activities of host defense proteins, the benefits of fresh mother's own milk are often extrapolated to stored frozen human milk and pasteurized donor milk, the staple diet of infants in the NICU. The data from our study call the biological basis of this presumption into question because the concentrations and activities of major host defense proteins are reduced in frozen or pasteurized human milk compared with fresh human milk. Our data support the advantages of fresh human milk over other alternative diets available to an

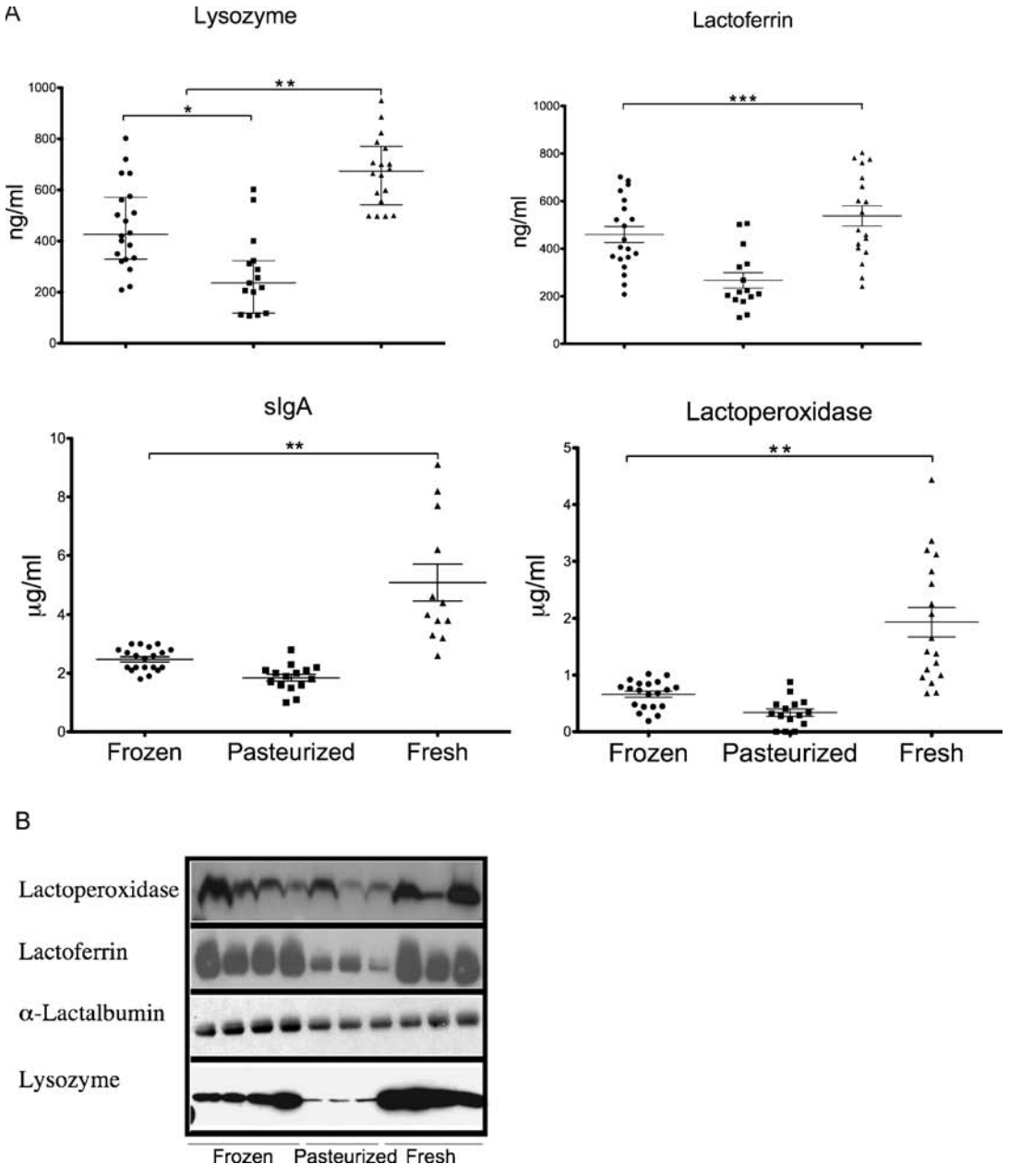

FIGURE 1. A, Concentrations of host defense proteins. The concentrations of lysozyme, lactoferrin, secretory immunoglobulin A, and lactoperoxidase, in whey fractions isolated from freshly expressed human milk (fresh), previously frozen human milk (frozen), or pasteurized donor human milk (pasteurized) were assessed as described in Materials and Methods. n = 20 frozen, 15 pasteurized, and 18 fresh specimens. $^{*}P = 0.003$ for frozen vs pasteurized milk; $^{**}P < 0.001$ for fresh vs frozen or pasteurized milk; $^{***}P < 0.05$ fresh or frozen vs pasteurized milk. B, Western analyses for host defense proteins. Ten to 50 μg of whey protein from a subset of fresh, frozen, or pasteurized were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and probed with respective antibodies and the appropriate conjugated secondary antibody. The protein-antibody complexes were detected with a chemiluminescent reagent.

infant in the NICU with respect to the abundance and activity of host defense proteins and the capability to stymie the proliferation of pathogens commonly encountered in infants in the NICU.

Studies on the infection-related outcome in preterm infants fed the different forms of human milks tested in our study have yielded contradictory results (21–23). Immunomodulatory benefits against late-onset sepsis from feeding human milk to infants in the NICU can be inferred from its capacity to curb bacterial proliferation. This is arguably a better reflection of the composite of the activities of all of the immunomodulatory proteins present in mother's milk. In the present study, the proliferation of bacteria was significantly muted in fresh human milk and to a lesser extent in frozen milk compared with pasteurized human milk. Our data suggest that the effect of freezing and prolonged storage of

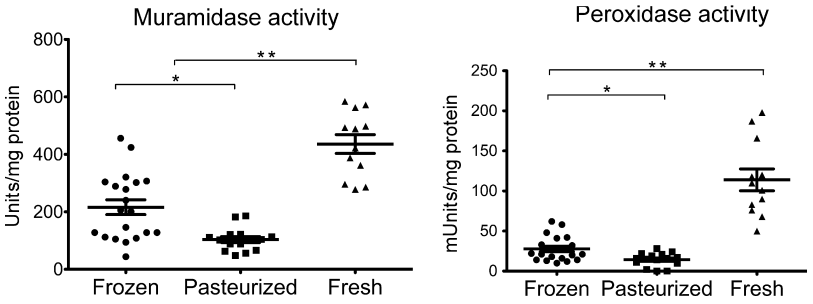

FIGURE 2. Protein activity. Lysozyme enzyme activity and lactoperoxidase activity were assessed in whey protein from 3 forms of human milk: freshly expressed human milk (fresh), previously frozen human milk (frozen), or pasteurized donor human milk (pasteurized) as described in Materials and Methods. n = 10 specimens per group; $^{*}P < 0.01$ fresh vs frozen and pasteurized; $^{**}P = 0.02$ frozen vs pasteurized.

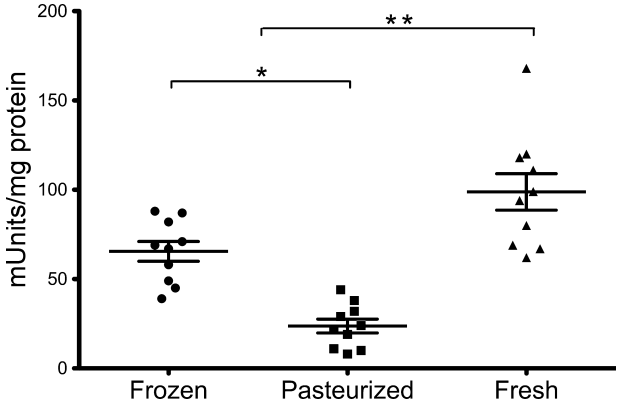

FIGURE 3. Distinct contribution of prolonged storage vs pulse heat on peroxidase activity in human milk. A subset of freshly collected human milk specimens (n = 10) were each divided into 3 aliquots, which were processed for whey proteins: within 2 hours of collection, following pulse heat at 65°C for 30 minutes or after 4 weeks of storage at −20°C. $^{*}P < 0.05$ fresh vs frozen or pulse-heated milk, $^{**}P < 0.01$ for frozen vs pulse-heated milk.

unprocessed human milk alone may be less important than the alteration of host defense proteins due to pasteurization.

Infants in the NICU are often fed frozen mother's milk or pasteurized donor milk in preference to formula when fresh mother's milk is unavailable or is inadequate. Pasteurized donor milk undergoes extensive handling from the time of collection, including at least 2 cycles of freezing and thawing interspaced by heating to 62.5°C in Holder pasteurization, before shipment to the NICU. The stability and activity of heat-labile humoral mediators of innate immunity in human milk could be adversely affected during pasteurization. From our data, it appears that the stability of the different protein components of human milk is extremely variable. In contrast to lysozyme, lactoferrin, lactoperoxidase, and sIgA, the concentrations of α-lactalbumin and serum albumin (data not shown) are not significantly altered by either storage at −20°C or pasteurization. In correlation to reduced protein concentrations, the activities of host defense proteins are decreased by pasteurization and, to a smaller extent, by prolonged storage. Our data suggest that the pulse heating involved in Holder pasteurization may contribute more to reduced activity of host defense proteins in human milk than storage at −20°C. The correlation of the degree of diminution of protein concentration and activity in the different forms of human milk to clinical outcome is unclear, although our data from the bacterial proliferation study suggest this may be of clinical relevance. Diminished concentration and activity of host

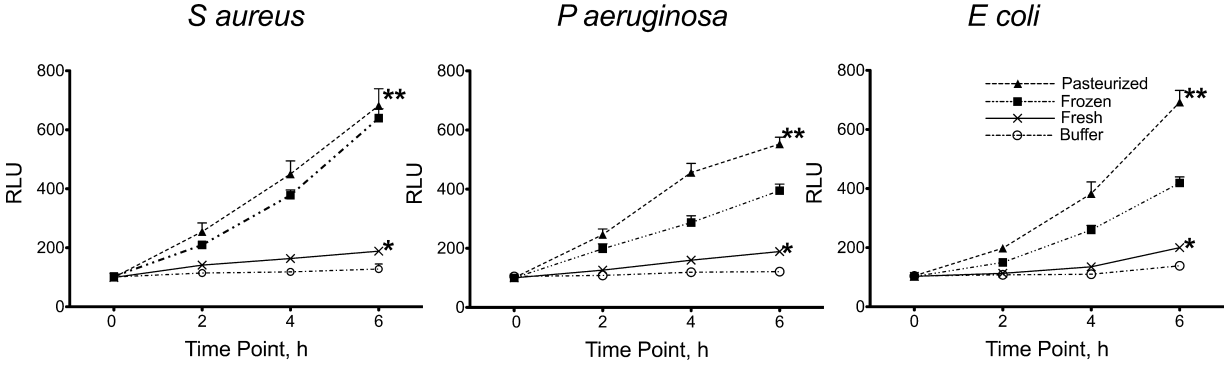

FIGURE 4. Bacterial proliferation assay. The propensity of the different forms of milk to support bacterial growth was assessed by incubating 10,000 colony forming units of bioluminescent *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or *Escherichia coli* in phosphate-buffered saline, in freshly expressed human milk (fresh), previously frozen human milk (frozen), or pasteurized donor human milk (pasteurized) at 37°C. Luminescence was measured at 2- , 4- , or 6-hour incubation in RLU. n = 5 individual specimens per group. $^{*}P < 0.001$, fresh vs other types of milk; $^{**}P < 0.02$ frozen vs pasteurized. RLU = relative light units.

defense proteins in pasteurized donor milk is consistent with the report that the risk of infection-related complications is mitigated by feeding fresh mothers' own milk but not pasteurized donor milk (21,24). Other forms of sterilizing human milk, such as high-temperature short-term, pasteurization of 72°C for 15 seconds, used in the dairy industry, may be compared with Holder pasteurization to determine whether the functions of host defense proteins are better preserved (25).

The differences reported between fresh and frozen milk specimens should be interpreted with caution because of the differences in the demographic characteristics between the 2 sets of donors (26–28). Some of the alterations detected between fresh and pasteurized milk may be due to that a higher proportion of donor milk may have come from hindmilk, which is enriched in fat but has a lower protein content than foremilk. Our study analyzed a finite number of host defense proteins. Other immunomodulatory proteins not assessed in our study may retain activity after Holder pasteurization and confer some protection to infants fed pasteurized banked milk in preference to cow's–milk–based formula.

In conclusion, the concentration and activity of host defense proteins are adversely affected by pasteurization and, to a lesser extent, by prolonged freezing. It is uncertain whether pasteurized donor milk offers the same immunologic benefits as associated with feeding mothers' own fresh milk. Mothers should be encouraged to provide fresh milk to derive maximum protection for very-low-birth weight infants. Based on the findings in the present study, caution is warranted in the use and handling of any other forms of milk fed to low-birth-weight infants.

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