Effects of Freezing on the Bactericidal Activity of Human Milk

Sahin Takci, Dolunay Gulmez, Sule Yigit, Ozlem Dogan, Kezban Dik, and Gulsen Hascelik

Objectives: Storage of human milk by freezing has been recommended for long-term storage. The present study analyzed the bactericidal activity of human milk on Escherichia coli and Pseudomonas aeruginosa and determined the changes in bactericidal activity following freezing at –20°C and –80°C for 1 month and 3 months.

Methods: Forty-eight milk samples were collected from 48 lactating mothers. Each sample was divided into 10 aliquots. Two of the samples were processed immediately and the others were stored at both –20°C and –80°C until analysis after 1 month and 3 months of freezing.

Results: All of the fresh milk samples showed bactericidal activity against E. coli and P. aeruginosa. Freezing at –20°C for 1 month did not cause statistically significant alteration in bactericidal activity (P > 0.017), whereas storage for 3 months lowered the degree of bactericidal activity significantly (P < 0.017) against E. coli. Bactericidal activity was protected when the samples were stored at –80°C. There was no statistically significant difference in the bactericidal activity of human milk against E. coli between freezing at –20°C and –80°C for 1 month (P > 0.017); however, when milk was stored for 3 months, –80°C was significantly more protective (P < 0.017). Freezing at –20°C and –80°C for 1 month and 3 months did not cause any significant change in bactericidal activity against P. aeruginosa (P > 0.05).

Conclusions: Storage by freezing at –80°C is more appropriate to keep bactericidal capacity of stored human milk >1 month if affordable and available, especially in intensive care settings.

Key Words: bactericidal activity, freezing of human milk, human milk, storage of human milk

(JPGN 2012;55: 146–149)

Human milk is the optimal source of nutrients for infants, providing them with a large number of antimicrobial substances (1) and exerting bacteriostatic and bactericidal action on various pathogenic microorganisms (2). This action of human milk is extremely important for premature and ill infants who are exposed to abundant pathogenic organisms during their stay in the neonatal intensive care unit (NICU). It has been clearly demonstrated that breast milk–fed infants in the NICU have less necrotizing enterocolitis and sepsis (3–5). Breast milk can be supplied for infants by their own mothers or by donor milk, which is available in breast milk banks for these infants. There is no doubt that mother’s own milk is the first choice for preterm and other high-risk infants; however, donor milk must be preferred for feeding when the mother’s own milk is not available in sufficient quantity.

In contrast to this, some small preterm infants cannot be fed or can receive only extremely small amounts of breast milk because of some problem, although the mother’s milk is available. In this situation the common practice is to store mother’s milk for the future. The storage of human milk under refrigeration for 2 to 8 days; however, for long storage periods, freezing is recommended (6–8). The common existing freezing temperature for storage is –20°C. Freezing of human milk at –70°C or –80°C is not a common storage practice, which is generally used in laboratory settings (9).

Numerous studies have focused on the effect of storage conditions on different milk components (nutritional and bioactive components) and the consequences of different protective systems (10–12). Reports about the effect of storage on bactericidal activity that is particularly important for critically ill infants are, however, limited.

The aim of the present study was to analyze the bactericidal activity of human milk on different pathogenic organisms and to determine the changes in bactericidal activity following freezing at –20°C and –80°C for 1 month and 3 months. The effects of freezing on the bactericidal capacity of colostrum and transitional milk were also assessed.

METHODS

The present study was carried out prospectively for >6 months (October 2010–April 2011) at Hacettepe University School of Medicine (Ankara, Turkey). Breast milk samples were collected from healthy donors between the 2nd and the 15th postpartum days. Before sampling, all of the donors gave informed consent. The study was approved by the ethics commission of Hacettepe University.

Sampling was carried out by careful manual expression, and samples were placed in sterile plastic tubes. A health care provider accompanied the donors while breasts were sucked by manual expression, to achieve hygienic control.

About 10 mL of milk from both breasts was collected and each sample was transported to the laboratory on ice and divided into 10 aliquots. Two of the samples were processed immediately after arrival at the laboratory. The others were stored at both –20°C and –80°C until analysis after 1 and 3 months of freezing. Frozen samples were thawed to room temperature before the procedure. All of the milk samples were analyzed in duplicate. A strain of Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 was cultured overnight on MacConkey agar (BBL; Becton Dickinson, Franklin Lakes, NJ), suspended in sterile 0.85 NaCl solution, and adjusted to a turbidity equal to McFarland no. 0.5 (approximately 1.5 × 108 cfu/mL). Fifty microliters of this solution was added to an Eppendorf tube with 200 µL of the milk sample.
The tubes were carefully mixed before and after incubation at 37°C for 2 hours. Control tubes containing Mueller-Hinton broth (BBL; Becton Dickinson) were included for each day of the study. Control samples were prepared mixing 50 μL of the bacterial solution with 200 μL of Mueller-Hinton broth. After incubation, dilutions from the tubes were subcultured on MacConkey agar and incubated at 36°±2°C for 24 hours, and bacterial counts were determined. Antibacterial effect was calculated as the difference between E. coli and P. aeruginosa counts in the control and milk samples, expressed as a percentage of the control sample counts.

Results are presented as percentages of control sample counts. Data are presented as median and interquartile range (IQR). The Friedman test was used to determine the differences in bactericidal activity. Pairwise comparisons were evaluated by Bonferroni-corrected Wilcoxon test. Difference between the colostrums and transitional milk was calculated by the Mann-Whitney U test. Differences were considered to be statistically significant at P<0.05 when pairwise comparisons were applied; P value was considered to be 0.017 with Bonferroni correction.

RESULTS

Human milk was obtained between the 2nd and the 15th postpartum days, with mean 6.4±3.7 days from 48 mothers ages between 18 and 43 (mean 28.5±5.9) years who delivered at a mean gestational age of 38.4±1.9 weeks (range 32–41 weeks). Seventeen of 48 milk samples were collected before the fourth day of delivery, which is considered to be colostrum (10). The others were transitional milk. Eleven of 48 samples were premature milk (<37 weeks of gestation), and the mean gestational age for premature births was 33.5±1.2 weeks.

All of the fresh milk samples showed bactericidal activity against E. coli and P. aeruginosa. The median values of reduction in control sample counts were 84% (IQR 50.1%–98.2%) for E. coli and 95% (IQR 73.6%–98.5%) for P. aeruginosa.

Freezing at −20°C for 1 month did not cause statistically significant alteration in the bactericidal activity (P>0.017), whereas storage for 3 months lowered the degree of bactericidal activity significantly (P<0.017) for E. coli. The median value of reduction was 72.4% (IQR 19.8%–91.8%) for 1 month and 56.1% (IQR 5.5%–77.8%) for 3 months. Bactericidal activity was protected to some extent when the samples were stored at −80°C. Bactericidal activities following freezing at −80°C for 1 month and 3 months were 84% (IQR 57.1%–95.1%) and 65.6% (IQR 40.2%–85.5%), respectively, for E. coli; however, the alteration was not statistically significant (P>0.017). There was no statistically significant difference in the bactericidal activity of human milk against E. coli between freezing at −20°C and −80°C for 1 month (P>0.017); however, when milk was stored for 3 months, −80°C was significantly more protective (P<0.017) (Fig. 1).

Freezing at −20°C and −80°C for 1 and 3 months did not cause any significant change in bactericidal activity against P. aeruginosa compared with fresh samples (P>0.05). The median values of bactericidal activity under different freezing conditions were nearly the same.

The effect of nursing stage on bactericidal activity was also assessed. The median values of bactericidal activity for colostrum and transitional milk against E. coli were 86.7% (IQR 46.6%–98.6%) and 83.8% (IQR 50.6%–97.7%), respectively. The median values of bactericidal activity for colostrum and transitional milk against P. aeruginosa were 97.6% (IQR 82.4%–99.1%) and 91% (IQR 70.3%–98.4%), respectively (Fig. 2). Of the samples analyzed, both colostrum and transitional milk were influenced similarly from different storage conditions. The median values of reduction in control samples for colostrum and transitional milk for E. coli are seen in Table 1.

DISCUSSION

Human milk is an optimum source of nutrients acting in a unique innate medicinal role for infants. The bactericidal activity of human milk is the combined action of various biocomponents, some of which have already been identified and the others remain to be
We used 2 different species of bacteria. The strain of *E coli*, which is a common enteric pathogen in infants, was selected because it is commonly used (10,13). Also, we added a strain of *P aeruginosa*, which is a serious Gram-negative pathogen in infants, especially during the neonatal period. It is also noteworthy that *P aeruginosa* is an important nosocomial sepsis agent, especially for premature infants (14).

The *E coli* and *P aeruginosa* strains were inhibited in all of the fresh milk samples because growth of the bacteria was of a lower magnitude than in the control bacteria mixture. These findings are consistent with previous reports (10,13). Studies using *P aeruginosa* to analyze bactericidal activity of human milk are extremely rare in the literature (2,12). Dolan et al (2) showed inhibition of bacterial pathogens in human milk compared with infant formula in a broad range of bacteria, including *P aeruginosa*.

Numerous studies have been made to disclose the effect of storage condition on antimicrobial components of human milk showing different degrees of alteration in certain durations (11,15). These various antimicrobial components may be altered or irreversibly damaged by the processes such as refrigeration, freezing, thawing, and heating.

The influences of refrigeration on the bactericidal activity of human milk are well documented. It has been shown that the stability of bactericidal activity during refrigeration is protected for at least 48 hours (13,16). Although decreasing beyond 48 hours, bactericidal activity was detected up to 7 days with reduced levels, especially in colostrum milk (10). Therefore, the Human Milk Banking Association of North America recommends up to 8 days of refrigerator storage, considering the alterations of bioactive components, bacterial growth, and bactericidal effect of human milk (7,17). The advice of a certain storage time for human milk by refrigeration is controversial. Slutzah et al (18) recommended 4 days of storage by refrigeration at 4°C in NICU conditions.

Freezing is recommended if prolonged storage of human milk is required (approximately >1 week). Freezing has been carried out at −20°C, which is within the range of a home freezer, and at −70°C, which is within the range of a laboratory freezer (9,19,20). Breast milk can be stored at −20°C for up to 12 months (9,19). This recommendation is based on the knowledge that nutrient constituents remain stable at −20°C for up to 12 months; however, the information about bactericidal activity of human milk after long-term freezing is limited.

It has been shown that there are some effects of freezing on breast milk properties: it reduces the numbers and functions of bioactive cells (21) and increases lipolysis (11,22). In a study by Reynolds et al (23), 19% of cells in human milk remained viable, whereas the levels of IgA, IgM, lactoferrin, lysozyme, C3 and C4 complement components, and concentrations of amino acids and fatty acids were largely preserved or slightly changed after freezing at −20°C for 1 month. Akinbi et al (12) reported reduced concentrations of lysozyme, lactoferrin, lactoperoxidase, and secretory IgA at −20°C. Determination of bactericidal activity as a sum of these certain antimicrobial components in fresh milk and alterations by freezing at different temperatures and durations was the goal of the present study.

Bactericidal activity was stable for *E coli* at −20°C following freezing for 1 month; however, it declined after 3 months of storage in the present study. Ogundele (10) found decreasing bactericidal activity against *E coli* at −20°C at 28 days of storage. Hernandez et al (24) reported a significant loss of antimicrobial action of breast milk after 21 days of freezing, but the temperature was comparably high (−4°C). Silvestre et al (16) showed unaltered bactericidal activity of human milk frozen at −20°C for 7 days.

Milk can be stored safely for an unlimited time or >12 months at −70°C (6,9). Unchanged bactericidal activity of human milk at −70°C has been shown by Hernandez et al (24); however, human milk was freshly frozen at −70°C for 2 minutes. In the present study, bactericidal activity of human milk against *E coli* remained stable during storage at −80°C for 1 and 3 months. We suggest that the bioactivity of antimicrobial components is better protected by storage at −80°C. These data serve evidence for the consideration of storage safety at −80°C for a long period.

Freezing at −20°C and −80°C for 1 and 3 months did not cause any significant change in bactericidal activity against *P aeruginosa* compared with fresh samples in the present study. We do not have any explanation for why bactericidal activity for *E coli* was altered while bactericidal activity for *P aeruginosa* was not affected by freezing. We can say only that different antibacterial components probably act as bactericides for *E coli* and *P aeruginosa* and these components imply different sensitivities to freezing.

The effect of nursing stage on the bactericidal activity of milk was also examined and no significant differences were observed between fresh colostrum and transitional milk. The alterations in bactericidal activity of colostrum and transitional milk were similar during freezing. Ogundele (10) found that bactericidal activity of colostrum was more stable compared with transitional milk after refrigeration.

### Table 1. Median bactericidal activity and interquartile ranges of colostrum and transitional milk against *Escherichia coli* under different conditions

<table>
<thead>
<tr>
<th>Percentage reduction of control</th>
<th>Fresh</th>
<th>1 mo at −20°C</th>
<th>3 mo at −20°C</th>
<th>1 mo at −80°C</th>
<th>3 mo at −80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colostrum, n = 17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>86.7</td>
<td>71.1</td>
<td>59.1</td>
<td>81.4</td>
<td>60</td>
</tr>
<tr>
<td>25th percentile</td>
<td>46.6</td>
<td>13.1</td>
<td>0.5</td>
<td>41.3</td>
<td>25.3</td>
</tr>
<tr>
<td>75th percentile</td>
<td>98.6</td>
<td>89.5</td>
<td>82.3</td>
<td>93.6</td>
<td>73.2</td>
</tr>
<tr>
<td>Transitional milk, n = 31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>83.8</td>
<td>76.1</td>
<td>55.4</td>
<td>87.1</td>
<td>74.5</td>
</tr>
<tr>
<td>25th percentile</td>
<td>50.6</td>
<td>32.5</td>
<td>4.5</td>
<td>56.9</td>
<td>44.3</td>
</tr>
<tr>
<td>75th percentile</td>
<td>97.7</td>
<td>93.5</td>
<td>76.1</td>
<td>95.8</td>
<td>89.1</td>
</tr>
</tbody>
</table>
It is expected that the bactericidal properties of colostrum must be higher than those of transitional milk; however, in the present study we did not find any difference. It is probable that limited numbers of colostrum samples affected our results. Further studies are needed to clarify this issue.

In conclusion, human milk possesses a bactericidal function that remains stable during the first month of freezing storage; however, bactericidal activity is significantly reduced after 3 months of storage at −20 °C. Bactericidal activity does not change significantly by freezing at −80 °C for 3 months. Storage by freezing at −80 °C is more appropriate to keep the bactericidal capacity of stored human milk >1 month if affordable and available, especially in NICU settings.

REFERENCES