Thawing rate and heating temperature effects on immunoglobulin A and lysozyme activity in human milk

Xuejing Li

Chiang Mai University Faculty of Medicine

krongporn ongprasert (✉ krongporn.o@cmu.ac.th)

Chiang Mai University <https://orcid.org/0000-0002-2766-7046>

Penprapa Siviroy

Chiang Mai University Faculty of Medicine

Jetsada Ruangsuriya

Chiang Mai University Faculty of Medicine

Nitthinan Yousaibua

Maharaj Nakorn Chiang Mai Hospital

Research Article

Keywords: human milk, immunoglobulin A, lysozyme activity, thawing rate, warming temperature

Posted Date: November 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1062409/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

The rate of infants receiving frozen HM is increasing, allowing critically ill preterm infants and infants with working mothers to benefit from the advantages of their mother's milk. The effects of thawing and warming on secretory immunoglobulin A (SIgA) and lysozyme activity in frozen human milk (HM) should be investigated to identify optimal methods for preserving immune factors in frozen HM.

Methods

Forty mothers that delivered full-term infants provided milk that was frozen and stored at -18°C two months before analyses. We compared the methods involving placing the container in a 4°C refrigerator overnight (slow thawing, ST) and placing it in a container of warm water (rapid thawing, RT). Additionally, we investigated the effect of warming temperature using room temperature (25°C) and physiological temperature (37°C). SIgA concentrations and lysozyme activities in the milk samples were determined by ELISA kits and fluorometric lysozyme activity assay kits, respectively. Data were analyzed by paired t-tests.

Results

SIgA concentrations and lysozyme activity were reduced by 16.5-52.1% and 16.8-39.3% in frozen HM compared to fresh HM, respectively. Significantly higher SIgA concentrations were maintained with slow thawing and warming at 37°C than with rapid thawing and warming at 25°C ($p < 0.001$). Greater lysozyme activity was retained at 25°C with slow thawing than with rapid thawing ($p < 0.001$) and more was preserved at 25°C than at 37°C with slow thawing ($p < 0.01$).

Conclusions

Thawing HM overnight in the refrigerator before warming has the potential to preserve SIgA levels and lysozyme activity better than thawing immediately after removal from the freezer. Broader temperatures ranges should be analyzed to determine the temperature that minimizes HM SIgA and lysozyme activity losses.

Trial registration

Not applicable

Background

The unique nutritional composition and nonnutritive bioactive factors in human milk (HM) promote adequate growth and healthy development of infants. Furthermore, numerous biologically active proteins and immune factors are essential for infants that are especially prone to a variety of infectious

pathogens, as their immune system is immature [1–3]. All the immunoglobulin isotypes (IgA, IgM, IgG, IgE, and IgD) are found in HM. The presence of secretory immunoglobulin A (SIgA) is considered the most important biological property of HM, and SIgA is the predominant immunoglobulin fraction (80 to 90%) of the antibodies in colostrum and mature milk [3, 4]. SIgA primarily binds invading microbes, preventing them from reaching the mucosa membranes, such as in the respiratory and gastrointestinal tracts, where they might cause infection [3]. Lysozyme is one of the major enzymes present in HM and is highly expressed in HM, which contains nearly 3000-fold more lysozyme activity than that of bovine milk [5, 6]. Lysozyme activity produces both anti-inflammatory action and bactericidal capabilities by degrading the outer wall of gram-positive bacteria and has the ability to kill gram-negative bacteria synergistically with lactoferrin and SIgA [1]. Furthermore, some reports have shown that lysozyme has antifungal and antiviral activities [3, 4, 6]. Lysozyme is highly heat stable under an acidic pH, but under a neutral pH, it becomes heat labile [7, 8].

While several studies have shown that breastfeeding or feeding with fresh milk is the optimal way to preserve biological activity, the rate of infants receiving frozen HM is increasing, allowing critically ill preterm infants and infants with working mothers to benefit from the advantages of their mother's milk [9, 10]. International health authorities provided standard guidelines that recommended the optimum temperatures and storage durations for each type of HM (fresh, thawed, and left-over from a feeding) [10–12]. Although HM storage guidelines are clear, the evidence-based standards that recommend the optimal HM thawing method and feeding temperature for infants are limited. Several methods have been recommended for thawing frozen HM, including placing the container in the refrigerator overnight, running it under warm water, setting it in a container of warm water, or using a waterless warmer [10, 13]. Additionally, recommendations for feeding temperatures differ and include cold, room temperature and warm milk [10, 13], and prior surveys reported that feeding temperatures ranging between 22°C and 46°C were used in practice [14].

Previous studies found that the heating process elements, including the thawing rate and the warming temperature, has a variable effect on immunological proteins in frozen HM [15–18]. The different effects are probably due to the magnitude of protein denaturation caused by heat through proteolysis, refolding, or recrystallization [19–21]. Feeding temperatures are also associated with infant health, including feeding tolerance, body temperatures and gastric temperatures [22, 23]. Therefore, the optimum feeding temperature that is both safe for infant health and maintains most of the HM milk composition should be determined. However, there are only a few studies concerning the effect of the heating process on SIgA and lysozyme activity in HM and the reports have conflicting findings; moreover, most studies have been conducted at high temperatures (pasteurization) for donor milk in milk banks rather than in a real household setting [8, 15, 18, 24, 25].

The effect of the thawing method and warming temperature on SIgA and lysozyme activity in HM under normal daily conditions is an important consideration. To determine the optimal thawing method and warming temperature for preserving the SIgA concentration and lysozyme, we compared the methods involving placing the container in the 4°C refrigerator overnight (slow thawing, ST) and placing it in a

container of warm water (rapid thawing, RT). Additionally, we investigated the effect of warming temperature using room temperature (25°C) and physiological temperature (37°C). The findings of this study provide important information on the thawing and warming effects on SIgA and lysozyme activity, which should be considered when planning to improve HM storage guidelines.

Methods

Participants

Participants were recruited in July 2021 through study posters posted in the well-baby clinic and the lactation rooms of four hospitals in Chiang Mai City, Thailand. After interested mothers contacted the study staff via telephone, they were asked a set of questions corresponding to the inclusion and exclusion criteria. Lactating mothers who had given birth to a full-term infant aged 1 to 6 months were recruited for this study. The exclusion criteria were as follows: (a) any underlying disease in the mother or her offspring, (b) maternal age under 18 years or above 40 years, and (c) an inability of the mother to travel to our lactation room on her own. All eligible participants were then asked to make an appointment to collect milk samples (Figure 1). Before providing information and breast milk samples, all participants signed informed consent forms. All mothers gave written consent to participate in the research, and the study was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University (No. 078/2021).

Milk collection and acquisition of milk samples

Participants were required to provide milk samples in the lactation room of Mother and Child Hospital, Chiang Mai City, Thailand. To ensure sample uniformity, all breast milk samples were obtained between 8:00 AM and 11:00 AM on the same day using a Lactina electric selection pump (Medela, Switzerland). The pump was left on for approximately 15 min or until no further milk could be expressed for at least five minutes. Freshly expressed HM specimens were stored on ice from the time of collection and aliquoted within 4 hours after collection. Milk samples were divided into ten 10-mL aliquots corresponding to the different storage conditions (Figure 1). Two aliquots were stored at 4°C from the time of collection and analyzed within 24 hours to determine the baseline SIgA concentration and lysozyme activity (fresh). Eight 10-mL aliquots were stored at -18°C for 2 months.

Figure 1. Diagram of the study design

Thawing and warming process

Four 10-mL aliquots were thawed with the rapid thawing method and were placed in a water bath (Memmert GmbH+Co. KG., West Germany) with a constant temperature for 15 min. We divided the rapid thawing sample into two groups according to the water bath temperatures of 25°C (rapid thawing at 25°C, R25) and 37°C (rapid thawing at 37°C, R37). The final four 10-mL aliquots were thawed with the slow thawing method and were placing in a refrigerator (4°C) for 12 hours before warming. Two aliquots

were warmed with water-bath temperatures of 25°C (slow thawing at 25°C, S25). The remaining two aliquots were warmed at 37°C (slow thawing at 37°C, S37) (Figure 1).

Analytical methods

SIgA levels

SIgA levels were determined with ELISA kits (Aviva System Biology, OKEH00516) following the manufacturer's protocol. Briefly, the milk samples from each condition were serially diluted up to 200,000× with deionized (DI) water and the assay diluent buffer. Then, both the diluted samples and the SIgA standard were loaded into each well at 100 µL per well of the ELISA plate. The plate was incubated at 37°C for 120 min, and the solution was discarded and replaced with biotinylated SIgA detector antibody. The plate was incubated at 37°C for 60 min, and the solution was discarded and washed. An avidin-HRP conjugate mixture was added and the solution was incubated at 37°C for another 60 min. TMB substrate was added after the solution was discarded and washed. Then, the plate was incubated in the dark at 37°C for 15 min. Finally, the stop solution was added, and the absorbance was read at 450 nm using a Synergy H4 Hybrid Reader (Bio-Tek, USA). SIgA concentrations in the milk samples from each condition were deduced from an SIgA standard curve (0–4000 pg/mL).

Lysozyme activity

Lysozyme activity in the milk samples was determined with a fluorometric lysozyme activity assay kit (MyBioSource, MBS846601). The milk samples were serially diluted up to 20,000× with DI water and the assay diluent prior to being loaded into each well of a 96-well plate. The synthetic substrate was then added, and the enzymatic reaction proceeded at 37°C for 180 min under light protection. The stop solution was added to each reaction well, and the fluorescent product was measured with a Synergy H4 Hybrid Reader (BioTek, USA) using an excitation wavelength of 360 nm and an emission wavelength of 445 nm (Ex/Em = 360/445 nm). The amount of fluorescent product was calculated with a standard curve of 4-methylumbelliferone (4-MU) ranging from 0–100 pmol/well. The lysozyme activity could be further calculated and expressed in nmol/min/mg protein in each milk sample.

Total protein

The total protein content in the HM samples was determined by Lowry's method using Folin-Ciocalteu solution (VWR Chemicals, 31360.264). The milk samples were diluted 100× with DI H₂O, and the diluted sample was mixed with an alkaline solution and the Folin-Ciocalteu solution. The mixture was incubated at room temperature for ten min, and the absorbance was read at 650 nm with a Synergy H4 Hybrid Reader (BioTek, USA). The concentrations of protein in each milk sample were calculated from a bovine serum albumin (BSA) (GE Healthcare, K41–001) standard curve with a concentration range of 0–100 mg/mL.

Statistical analyses

All statistical analyses were performed through SPSS for Windows version 22 (IBM Corp., Armonk, NY, USA). The participant characteristics were described. Continuous variables are presented as the mean $\pm$ standard deviation (SD), and categorical data are presented as frequencies and percentages. Outlier detection using boxplots of the concentration of SIgA and lysozyme values was used to detect and remove extreme values from the data. The normality of all parameters was evaluated with the Shapiro-Wilk test. The significance of differences in the content or activity resulting from the different frozen HM thawing methods and warming temperature were determined by paired samples t-test for parametric tests and Wilcoxon signed-rank test for nonparametric tests. Differences between means were considered statistically significant at $p < 0.05$.

Result

Demographic characteristics

Forty mothers who had delivered full-term infants provided milk samples. The mean ($\pm$ SD) values for maternal age and infant age were 28.55 ($\pm$ 4.77) years and 3.31 ($\pm$ 0.34) months, respectively. The characteristics of the pregnancies were primiparas (62.5%) and vaginal delivery (72.5%). The main characteristics of the mothers and infants are described in Table 1.

Table 1
The main characteristics of participants (n=40).

Characteristics	Mean $\pm$ SD or n (%)
Maternal	
Age (year)	28.55 $\pm$ 4.77
BMI (kg/m ²)	23.83 $\pm$ 3.40
Birth order (First/second)	25 (62.5)/15 (37.5)
Method of delivery (Vaginal delivery/cesarian section)	29 (72.5)/11 (27.5)
Infant	
Age (month)	3.31 $\pm$ 0.34
Gestation age (week)	38.72 $\pm$ 0.96
Birth weight (kg)	3.10 $\pm$ 0.34
BMI = Body mass index; Values are presented as the mean $\pm$ SD or number (%)	

Effects of thawing methods and warming temperatures on SIgA concentrations

A comparison of SIgA levels analyzed in the five different forms is shown in Figure 2. The SIgA concentration in frozen milk samples significantly decreased during the first two months of freezer storage at -18°C compared to that of fresh milk ($p < 0.001$). The mean SIgA concentrations of fresh HM and HM with rapid thawing at 25°C, rapid thawing at 37°C, slow thawing at 25°C, and slow thawing at 37°C were 27.33, 13.09, 18.72, 19.37, and 22.82 mg/dL, respectively (Table 2). After two different thawing temperatures (25°C and 37°C), slow thawing significantly maintained SIgA concentrations compared to that of rapid thawing ($p < 0.001$). With both thawing methods, warming at physiological temperature (37°C) maintained SIgA concentrations more effectively than warming at room temperature (24°C) ($p < 0.001$). When we compared milk attributes with thawing and warming processes, we discovered that slow thawing at 37°C preserved SIgA levels the best (16.5% decline), whereas rapid thawing at 25°C had the greatest reduction in SIgA levels (52.1%) (Table 2).

Figure 2. Mean and 95% CI of SIgA concentrations with heating for frozen HM

Figure 3. Mean and 95% CI of lysozyme activity levels with heating for frozen HM

Table 2
SIgA concentration and lysozyme activity levels comparison for fresh and frozen HM.

		Fresh sample	Thawing methods and temperature			
			Rapid		Slow	
			25°C	37°C	25°C	37°C
SIgA (mg/dL)	N	40	40	40	39	40
	Mean ± SD	27.33 ± 10.18	13.09 ± 4.27	18.72 ± 6.35	19.37 ± 6.65	22.82 ± 8.25
	Median	24.99	12.86	18.31	17.76	21.96
	Percentile (25th, 75th)	19.30, 34.90	10.36, 14.47	14.73, 22.39	15.43, 24.50	16.37, 29.60
	% Decrease	-	52.1	31.5	29.1	16.5
Lysozyme (nmol/mg protein)	N	39	38	39	40	40
	Mean ± SD	898.64 ± 194.55	545.74 ± 251.06	554.54 ± 133.47	747.78 ± 210.04	660.55 ± 343.86
	Median	928	471	535	718.50	659.50
	Percentile (25th, 75th)	737, 1037	331, 700.75	471, 607	587.25, 842.50	346.25, 926.50
	% Decrease	-	39.3	38.3	16.8	26.6
% Decrease = Percent decrease compared with fresh sample						

Effects of thawing methods and warming temperature on lysozyme activity

The lysozyme activity in four different forms of frozen HM was significantly decreased during the first two months of freezer storage at -18°C compared to that of fresh milk ($p < 0.001$, Figure 3). The mean lysozyme activity of HM samples warmed to 25°C using the rapid thawing method (545.74 nmol/mg protein) decreased significantly more than that of samples warmed to 25°C using the slow thawing method (747.78 nmol/mg protein) ($p < 0.001$). This study demonstrated that the thawing method had no effect on the lysozyme activity in a sample heated to 37°C. After the slow thawing method, warming at room temperature (25°C) preserved lysozyme activity more effectively than warming at physiological temperature (37°C), and the activity was 747.78 and 660.55 nmol/mg protein, respectively ($p < 0.01$, Figure 3). In comparison to fresh HM, slow thawing at 25°C results in less destruction (16.8% decline), whereas rapid thawing at 25°C results in the most destruction (39.3% decline) of lysozyme activity (Table 2).

Discussion

These studies were conducted to evaluate the effect of thawing rate and warming temperature on the stability of SIgA and lysozyme in frozen HM. Compared with fresh HM, the SIgA level and lysozyme activity in frozen HM at -18°C for 2 months were significantly lower after the heating processes, which was in accordance with previous studies that demonstrated a significant loss in SIgA concentration [8, 24, 25] and lysozyme activity [8, 25] following storage and heating processes. Our results highlighted that slow thawing at physiological temperature (37°C) results in minimal changes in SIgA levels, whereas slow thawing at room temperature (25°C) has a high potential for preserving lysozyme activity.

We discovered that the slow thawing method significantly maintained SIgA levels at either warming temperature (25°C or 37°C) and significantly maintained lysozyme activity at 25°C in comparison to that of the rapid thawing method. The reason that slow thawing preserves more recovery of SIgA activity and lysozyme than that of rapid thawing is probably due a slower thawing rate, which minimizes more damage caused by the recrystallization process. Recrystallization exerts additional interfacial tension or shear on the entrapped proteins and causes further damage after small ice crystals are formed by the freezing process [20]. It is difficult to compare our results to those of other studies, such as the results of a small study that examined the effect of thawing methods in a residential setting, because the effect of thawing rate on SIgA levels and lysozyme activity was minimal. A previous study examined the effect of various thawing methods on 40 frozen HM samples and discovered no significant difference in SIgA levels between the samples thawed at 4°C for 24 hours before the warming process and those that continued warming immediately after the thawing process [26]. The effect of the thawing rate on the higher temperature (pasteurization process) has been described, but the results were inconclusive. Our findings contradicted previous research that found that short-term high-temperature pasteurization (72°C

x 5-15 sec) preserved SIgA and lysozyme more effectively than longer-term low-temperature pasteurization (62.5°C x 30 min) [15].

The effect of the warming temperature was then investigated for SIgA and lysozyme activity with rapid and slow thawing. This study demonstrated that thawing at a physiological temperature (37°C) preserved more SIgA concentration than thawing at room temperature (25°C) using both rapid and slow thawing methods. On the other hand, warming at room temperature (25°C) better preserved lysozyme activity than a physiological temperature (37°C) using slow thawing method. Previous research examined changes followed by warming at 40°C and 60°C. Higher temperatures (60°C) resulted in more SIgA reduction than that of lower temperatures (40°C), and higher temperatures (60°C) preserve more lysozyme activity than that of lower temperatures (40°C), but the difference was not statistically significant [24]. An earlier study also demonstrated a progressive loss of SIgA concentration and lysozyme activity at heating temperatures of 60°C, 62.5°C, 65°C, 67.5°C, and 70°C following freezing at -20°C [18] which was similar to Ogundele et al. [16] who concluded that the heating process results in progressive loss of IgA. There has been evidence that as the temperature rises, more milk protein degradation occurs [27, 28]. Although the majority of evidence indicates that heat causes more milk protein degradation, the inverse result was observed in our study in terms of SIgA levels. This variation could be explained by the temperature used in the study, as our study focused on lower temperatures than that of other studies. Additionally, Akazawa-Ogawa et al. [19] reported that milk proteins have varying degrees of heat stability depending on their structure; for example, each immunoglobulin domain unfolds at a different temperature. As a result, antibodies exhibit a mixture of folded and unfolded structures at different temperatures. Their findings may help to explain why the effect temperature on SIgA has been inconsistent and the reports are highly variable.

Previous investigators have examined the effect of thawing rate and warming temperature on the immunologic component of HM. However, determining an optimal rate of thawing and warming temperature is challenging due to the differences in previous study protocols, such as storage duration, the number of samples exposed to the freeze-thaw cycle, freezing rate and milk donors between sample groups. Additionally, drawing conclusions and applying them to a household setting should be done cautiously, as most previous studies used storage temperatures between the range of a home freezer (-18°C) and a laboratory freezer (-80°C). Therefore, we investigated the changes in SIgA and lysozyme that can occur in household conditions. To minimize the effect of storage time and individual variation, we collected all milk samples within three hours, which is a strength of our study. Some limitations should be noted. First, each sample contained 10 ml of milk, which is significantly less than the volume of milk that is generally stored and may affect the difference result. Milk samples with a greater volume should be utilized in future studies to reduce the effects of these variables. Second, we investigated two different warming temperatures and were unable to determine the best temperature to preserve both SIgA and lysozyme. Additional research is needed to determine a broader range of warming temperatures that are clinically acceptable to make the most suitable recommendation for warming temperatures in both home-based and hospital settings.

Conclusions

Thawing HM overnight in the refrigerator before warming has the potential to preserve SIgA levels and lysozyme activity better than thawing immediately after removal from the freezer. Further research is necessary to analyze a broader range of temperatures to determine the warming temperature that minimizes SIgA and lysozyme activity losses in HM.

Abbreviations

BMI
Body mass index
Lys
lysozyme
RT
rapid thawing
R25
rapid thawing at 25°
R37
rapid thawing at 37°C
SIgA
secretory immunoglobulin A (SIgA)
ST
slow thawing
S25
slow thawing at 25°C
S37
slow thawing at 37°C

Declarations

Ethics approval and consent to participate

The protocol for this study was approved by Research Ethics Committee 4, Faculty of Medicine, Chiang Mai University No. 078/2021. This study complied with the principles established by the Declaration of Helsinki (1964) and all of its subsequent amendments. Written informed consent was obtained from all participants.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interest

The authors declare that they have no competing interests.

Funding

The study was financially supported by the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

Author's contributions

X.L. and K.O. conceptualized the experiment, created the data collection instruments, performed the analyses, and drafted the manuscript. X.L., P.S. and J.R. reviewed the manuscript. K.O. and P.S. analyzed the data. J.R. supervised the sample analysis. K.O. and N.Y. supervised the sample collection. K.O. critically reviewed the manuscript and revised the manuscript.

Acknowledgements

We would like to thank the mothers who provided milk samples for the project. Chotiros Phanpong, Darunee Limtrakul, and Pikul Suppansan were also crucial in assisting the facility with the recruitment process and collecting samples from study participants.

Author's information

Instructor at Community Medicine Department, Faculty of Medicine, Chiang Mai University

References

1. Riordan J. The biological specificity of breastmilk. In: Breastfeeding and human lactation. Burlington, MA: Jones & Bartlett Learning; 2016. p. 121-69.
2. Wu X, Jackson RT, Khan SA, Ahuja J, Pehrsson PR. Human milk nutrient composition in the United States: current knowledge, challenges, and research needs. *Curr Dev Nutr*. 2018;2:nzy025.
3. Hanson L. The role of breastfeeding in the defense of the infant. In: Hale T, Hartmann P, editors. Hale and Hartmann's textbook of human lactation. New York, NY: Springer Publishing Company; 2017. p. 159-92.
4. Palmeira P, Carneiro-Sampaio M. Immunology of breast milk. *Rev Assoc Med Bras* (1992). 2016;62:584-93.
5. Shahani KM, Kwan AJ, Friend BA. Role and significance of enzymes in human milk. *Am J Clin Nutr*. 1980;33:1861-8.

6. Yang B, Wang J, Tang B, Liu Y, Guo C, Yang P, et al. Characterization of bioactive recombinant human lysozyme expressed in milk of cloned transgenic cattle. *PLoS One*. 2011;6:e17593.
7. Farkye NY, Bansal N. Enzymes indigenous to milk. In: Fuquay J, Fox P, McSweeney P, editors. *Encyclopedia of dairy science*. Amsterdam, Netherlands: Academic Press; 2011. p. 327-4.
8. Sousa SG, Delgadillo I, Saraiva JA. Effect of thermal pasteurisation and high-pressure processing on immunoglobulin content and lysozyme and lactoperoxidase activity in human colostrum. *Food Chem*. 2014;151:79-85.
9. Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. *Pediatr Clin North Am*. 2013;60:49-74.
10. Eglash A, Simon L. ABM clinical protocol #8: human milk storage information for home use for full-term infants, revised 2017. *Breastfeed Med*. 2017;12:390-5.
11. World Health Organization. Global strategy for infant and young child feeding. 2003. <https://www.who.int/publications/i/item/9241562218>
12. American Academy of Pediatrics. American academy of pediatrics (AAP) policy on breast feeding. 2020. <https://www.aap.org/en-us/advocacy-and-policy/aap-health-initiatives/Breastfeeding/Pages/default.aspx>. Accessed 13 January 2021.
13. Center for Disease Control and Prevention. Proper storage and preparation of breast milk. 2021. https://www.cdc.gov/breastfeeding/recommendations/handling_breastmilk.htm. Accessed 13 January, 2021.
14. Lawlor-Klean P, Lefaiver CA, Wiesbrock J. Nurses' perception of milk temperature at delivery compared to actual practice in the neonatal intensive care unit. *Adv Neonatal Care*. 2013;13:E1-10.
15. Hamprecht K, Maschmann J, Müller D, Dietz K, Besenthal I, Goelz R, et al. Cytomegalovirus (CMV) inactivation in breast milk: reassessment of pasteurization and freeze-thawing. *Pediatr Res*. 2004;56:529-35.
16. Ogundele MO. Techniques for the storage of human breast milk: implications for anti-microbial functions and safety of stored milk. *Eur J Pediatr*. 2000;159:793-7.
17. Goldblum RM, Dill CW, Albrecht TB, Alford ES, Garza C, Goldman AS. Rapid high-temperature treatment of human milk. *J Pediatr*. 1984;104:380-5.
18. Evans TJ, Ryley HC, Neale LM, Dodge JA, Lewarne VM. Effect of storage and heat on antimicrobial proteins in human milk. *Arch Dis Child*. 1978;53:239-41.

19. Akazawa-Ogawa Y, Nagai H, Hagihara Y. Heat denaturation of the antibody, a multi-domain protein. *Biophys Rev*. 2018;10:255-8.
20. Cao E, Chen Y, Cui Z, Foster PR. Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. *Biotechnol Bioeng*. 2003;82:684-90.
21. Lawrence RA. Storage of human milk and the influence of procedures on immunological components of human milk. *Acta Paediatr Suppl*. 1999;88:14-8.
22. Dumm M, Hamms M, Sutton J, Ryan-Wenger N. NICU breast milk warming practices and the physiological effects of breast milk feeding temperatures on preterm infants. *Adv Neonatal Care*. 2013;13:279-87.
23. Eckburg JJ, Bell EF, Rios GR, Wilmoth PK. Effects of formula temperature on postprandial thermogenesis and body temperature of premature infants. *J Pediatr*. 1987;111:588-92.
24. Chang JC, Chen CH, Fang LJ, Tsai CR, Chang YC, Wang TM. Influence of prolonged storage process, pasteurization, and heat treatment on biologically-active human milk proteins. *Pediatr Neonatol*. 2013;54:360-6.
25. Akinbi H, Meinzen-Derr J, Auer C, Ma Y, Pullum D, Kusano R, et al. Alterations in the host defense properties of human milk following prolonged storage or pasteurization. *J Pediatr Gastroenterol Nutr*. 2010;51:347-52.
26. Handa D, Ahrabi AF, Codipilly CN, Shah S, Ruff S, Potak D, et al. Do thawing and warming affect the integrity of human milk? *J Perinatol*. 2014;34:863-6.
27. Qian F, Sun J, Cao D, Tuo Y, Jiang S, Mu G. Experimental and modelling study of the denaturation of milk protein by heat treatment. *Korean J Food Sci Anim Resour*. 2017;37:44-51.
28. Zimmerman S, Jeon IJ, Shirley JE, McVay L, Ferdinand E, Sukup D, et al. Bacterial degradation of milk components is affected by storage temperature and time. 2001. <https://core.ac.uk/download/pdf/5170638.pdf>. Accessed 01 January, 2001.

Figures

Figure 1

Diagram of the study design

Figure 2

Mean and 95% CI of SIgA concentrations with heating for frozen HM

Figure 3

Mean and 95% CI of lysozyme activity levels with heating for frozen HM