The Effect of Different Storage Condition on Leukocytes in Human Breast Milk

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Abstract

Objectives: Breast milk is the best baby food because it contains a complete nutritional composition and contains important factors for the baby's immune system, including leukocytes. Store breast milk to guarantee the baby receives it while the mother works. This study aims to determine the effect of morphology, number cells and the population of breast milk leukocytes on various ways of storing breast milk based on differences in temperature and storage duration.

Methods: We used transitional breast milk samples from 7 breastfeeding mothers. A total of 50 ml was then divided into 4 tubes of 12.5 ml each and treated based on temperature, storage time, and method of thawing frozen breast milk based on the recommendations of the CDC for breast milk storage. Then the breast milk cells were isolated to calculate the cell number and leukocyte population. After that, breast milk cells were stained with Hematoxylin and Eosin (HE) to analyze the number and morphology of leukocytes.

Results: The results showed a significant decrease in the total number, population in breast milk. Besides that, there were also changes in the morphology of breast milk leukocytes after storage. Conclusion: This study indicate that CDC storage recommendations have no effect on the quantity of the CD45+ leukocyte population; though, there is a decreased the total number of leukocytes as well as alterations in
their microscopic morphology. However, additional research is still required to determine whether these modifications also influence the function of these cells.

Keywords: Breast milk; leukocytes; storage; population; morphology.

Advances in Knowledge
- The number of breast milk cells and the leukocyte subsets population (lymphocyte, monocyte, eosinophils and basophils) of breast milk decreased with different storage (cooling, freezing, and thawing) condition.
- Changes in the morphologic structure and shape of leukocytes in breast milk are caused by storing breast milk.

Application to Patient Care
- Provide information and knowledge to breastfeeding mothers regarding the management storage of expressed breast milk so that the content can be optimal for the growth and health of the baby.

Introduction
Breastfeeding reduces the risk of infection in neonates and enhances the development of their immune systems. In addition to essential nutrients for the baby's healthy growth, breast milk is a dynamic physiological fluid containing a range of live cells. Breast milk contains immunological and non-immunological cells. Non-immune cells, including epithelial cells such as ductal and alveolar, luminal-epithelial, and myoepithelial, are essentials for the formation and function of lactating mammary glands and contribute up to 99% of the cellular components of human milk in healthy mothers and newborns. In addition, immune cells such as leukocytes groups monocytes, T cells, NK cells, B cells, and others have been demonstrated to offer active immunity to infants by producing bioactive components, aiding in the development of the newborn's immune system, and altering the environment of the baby's gastrointestinal tract. The total cells of breast milk have large leukocytes, which depend on the stage of lactation and the health status of the breastfeeding mother and baby. Leukocytes in breast milk, such as granulocytes and mononuclear leukocytes (lymphocytes, monocytes, and macrophages), are
active, motile, and interactive. Leukocytes can provide active immunity in infants. In addition to its function in nursing infants, breast milk leukocytes are also thought to protect the mammary glands from infection during breastfeeding.

Trend et al. evaluated the leukocyte population in full-term and premature breast milk using multi-flow cytometry color. There were no differences in leukocyte concentrations between preterm and term breast milk. Ideally, breast milk should be stored at the right temperature and duration for optimal nutrient and bioactive component quality. Centre for Disease Control (CDC) states that freshly expressed breast milk can be stored in a cold container at room temperature (25°C) for ≤ 4 hours, in the fridge chiller with a temperature of 4°C for ≤ 4 days, and in the freezer house with temperature ≤ -18°C for 6-12 months. The defrosting of frozen breast milk can be stored at room temperature ≤ 25°C for 1-2 hours or in the fridge chiller at 4°C for 24 hours. But the effect of this storage condition on leukocytes has not been studied.

This study aimed to evaluate the changes in morphology and the number of leukocytes in breast milk based on the method of storing breast milk with different storage conditions. Related to this study, preparing cytological smears and applying other staining procedures can provide a more comprehensive view of the underlying tissue architecture and the complete cellular morphology of the involved tissue or the physiological fluids. It is hoped that the findings of this study will provide information about the best storage condition to maintain both the type and characteristics of leukocytes in human breast milk.

**Methods**

The Ethical Committee of the Faculty of Medicine, Universitas Indonesia, approved this study's ethical approval. We conducted this research at the Biochemistry Laboratory, Integrated Laboratory, and the Histology Laboratory, Faculty of Medicine, University of Indonesia. We received breast milk samples from mothers who met the inclusion criteria: breastfeeding mothers who willingly provided their milk as a research sample were in good health, gave birth to full-term babies, had babies aged around 1-4 weeks, and had a spacing of previous births ≥ 2 years. The exclusion criteria were as follows: the researcher was not present at the sampling site, breastfeeding mothers who consumed alcohol and were smokers, mothers who were sick, and
mothers who declined to provide their milk for research samples. We asked participants to clean their hands and nipples with antiseptic soap and rinse with clean water before expressing milk. Breast milk is expressed in the morning using an electronic milk pump with several parts sterilized. Breast milk samples were collected in sterile 50 ml glass bottles and immediately taken to the laboratory (< 4 hours). We divided the milk sample into four different treatments:
keeping it in the ice cooler box at a temperature of 0-4°C (P1), placing it in the lower refrigerator/chiller at 0-4°C for four days (P2), storing it in the freezer at -18°C for less than a month and thawing it by placing it in the refrigerator at 0-4°C overnight (P3), thawing it at room temperature of less than 25°C for less than 2 hours (P4).

Breast milk samples were centrifuged at 810 g for 20 minutes at 22°C. We carefully removed the resulting lipid layer and supernatant. Following that, we washed the cell pellet twice with PBS. The resulting cell pellet was resuspended with PBS (Phosphate buffered saline). We carefully removed the resulting lipid layer and supernatant. After that, the cell pellet was washed twice with PBS. We resuspended the resulting cell pellet with PBS (Phosphate-buffered saline) and counted cells using an automatic cell counter, LUNA-II™ (Logos Biosystem), after adding trypan blue (1:1) and homogenizing for segregation.

About 1x10^5 breast milk cells were centrifuged at 2500 rpm for 5 minutes to remove the supernatant and then added 300 µL of stain buffer. We centrifuged the cells at 2500 rpm for 5 minutes and 100 µL of the prepared antibody mixture (stain buffer 100 µL and Ab 1; CD45 2µL) before incubating for 30 minutes at room temperature and dark conditions. After that, 300 µL of stain buffer was added to each tube and centrifuged at 2500 rpm for 5 minutes. To the remaining pellets, 300 µL PBS was added. Subsequently, we examine the sample using a flow cytometer.

We cytocentrifuged pellets containing breast milk cells using the Cytopro®Cytocentrifuge model 7622 (ELITechGroup Inc) onto object glass. Then, we fixed them using the method adopted by Tripathy et al. and stained them with Hematoxylin Eosin (HE) to study cellular morphology. We fixed the slides using a solution of ether and 96% alcohol (1:1) for 10 minutes and then slides were serially hydrated in 100%, 95%, 80%, 70%, 50%, 30% alcohol for 1 minute each. Then stained with hematoxylin for 3 to 4 minutes, then rinsed with running water carefully. After that,
We immersed the slides in distilled water for 1 minute and stained with Eosin for 3-5 seconds. The next step is dehydration in a series of 50%, 70%, 80%, 95%, 100% alcohol, each 1 minute. The final stage is clarified using xylol I and II for 1 minute each. Then, the slide is closed using an entellan and a cover glass and observed under a light microscope at a specific magnification and using Optilab, Zeiss and Image Raster software. We recorded the cell composition and morphology of specific cell types, and then, to reduce bias in the results, the findings were corrected again by a consultant histologist.

We performed statistical data analysis using GraphPad Prism9. Data were tested for normality and homogeneity using the Shapiro-Wilk test. Statistical data will be processed using one-way ANOVA. Next, we carried out a Post Hoc Bonferroni and Tukey test, while the data were not normally distributed and did not vary homogeneously, followed by the nonparametric Kruskall-Wallis test (p <0.05 indicating a significant difference) and a follow-up test with the Dunn test.

**Result**

We presented the key characteristics of the participants in Table 1, which included demographic information for the seven mothers who had given birth to full-term singleton infants and provided milk samples. The mean (± SD) values for maternal age were 28.86 (±3.132) years. The characteristics of the breastfeeding period 11.00 (±3.055) days, number of births 2.429 (±1.512) and, for the mother’s BMI 22.71 (±2.446).

We examined the total cells of the four treatments from a cytopro object glass under the light microscope with 40x magnification. We used Image Raster software within a specified area measuring 290 µm in length and 215 µm in width, which was presumed to contain approximately 500 cells. The total cell count showed that there was a significant difference between treatments. Further analysis with Post Hoc Bonferroni test showed that the cells in the treatment P1 group had a higher number of cells when compared to treatments P2 (p = 0.0089), P3 (p = 0.0004), and P4 (p = 0.0007) (Figure 1). When comparing the two procedures for thawing frozen breast milk, there was no significant difference between P3 and P4 in terms of total cells. However, P4 (139.3 ± 16.01) exhibited a higher total cell count compared to P3 (117.7 ± 17.21).
The percentage of CD45+ is one of the markers to determine the level of leukocyte population in a sample. The results of this study showed that the CD45+ presentation had the highest levels in treatment P2 (11.78 ± 3.732), followed by P4 (11.24 ± 2.979), P1 (11.03 ± 0.9018) and the lowest percentage of CD45+ was by P3 (9.863 ± 5.931) (Figure 2). This experiment showed that the storage condition did not significantly change the CD45+ percentage in human breast milk. In addition, when comparing the two thawing methods (P3 and P4), there is no significant change in the percentage of CD45+, although the percentage of CD45+ at P4 (11.24 ± 2.979) was higher than at P3 (9.863 ± 5.931).

Interestingly, when we counted the primary subset leukocytes, there were significant differences, especially in lymphocytes (25.00 ± 10.15), monocytes (23.67 ± 10.41), and basophils (1.333 ± 0.5774). Further analysis shows that compared to P1, there is a significant decrease in lymphocytes; monocytes are in P3, and basophils are in all P2, P3, and P4 (Figure 3). On the other hand, neutrophils and eosinophils showed different decreases after each treatment, although not significantly. In the comparison of thawing methods, the group thawed in a refrigerator showed lower counts of lymphocytes, monocytes, and eosinophils than the room temperature group, although the differences were not statistically significant.

Moreover, the process of storing breast milk affects the morphology of leukocytes. As seen in Figure 4, we observed that lymphocytes, neutrophils, eosinophils, and basophils experienced a change in pyknosis in P2. The changes in morphology were extreme when the breast milk was frozen and thawed. When it thawed in the refrigerator at 0 – 4°C overnight (P3), the changes observed were almost similar in all subtypes of leukocyte, as in cytoplasmic and nucleus vacuolation, swelling of the cell nucleus, incomplete cytoplasm, and cell membranes in all leukocytes subtype. Nevertheless, when it thawed at room temperature for less than 2 hours (P4), the changes were different between each cell type (Table.2). The lymphocytes and monocytes showed changes in cytoplasmic and cell nucleus vacuolation, nuclear fragmentation, cell disintegration, a non-intact cell membranes and cytoplasm; neutrophils and basophils showed all above except cell disintegration and eosinophils still have intact cell membrane and cytoplasm.
**Discussion**

Fresh human milk contains a higher total number of breast milk cells, including lymphocytes, monocytes, neutrophils, eosinophils, and basophils, in comparison to stored human milk. Previous study by Pittard et al., found that milk cellular components, such as macrophage and neutrophil concentration, were significantly reduced after 48 hours of refrigeration.\(^8\) Our result sharpen this finding by showing that prolong storage can further decreased the total number of breastmilk cells. This phenomenon is likely caused by reduced antioxidant activity in stored breast milk, as Hanna et al., (2004) stated that the decrease in the antioxidant capacity of breast milk stored at 4°C and -20°C compared to fresh breast milk.\(^9\) Decreased antioxidant activity can produce oxidative stress and an imbalance between ROS and intra- and extracellular antioxidant systems that cause damage to cells.\(^10\)

Additionally, our observations suggest that thawing human milk in a refrigerator (4°C for 24 hours) tends to preserve the total cell count to a lesser extent than immediate rapid thawing at room temperature (25°C for 2 hours). Although the result is not significant, it contradicts the theory that a slower thawing rate, intended to reduce damage from recrystallization, is more effective.\(^14\) Theoretically, recrystallization generates additional interfacial tension or shear on entrapped proteins, leading to further damage by forming tiny ice crystals during the freezing process.\(^14\) Further research using large sample is still needed to explore the effect of different thawing process on breast milk population.

We further analysis the storing and thawing effect on leukocyte population and found that the percentage of leukocytes CD45+ in human breast milk did not significantly change after different storing and thawing procedure using flow cytometry (Figure 2). The thawing process also not significantly change the CD45+ population although room temperature thawing showed a little higher in CD45+ than those thawing process at chiller. Our results showed that overall CD45+ population in breast milk exhibit resilience during storage and thawing process, and thus align with CDC guidelines. Therefore, mothers who cannot directly supply breast milk to their infants can confidently use breast milk stored in accordance with the guidelines.
Previous study by Trend S et al. indicated that leukocytes expressing CD45+ present in breast milk decrease as lactation progresses. While there are minor differences in leukocyte subset frequencies between preterm and term breast milk, there are no significant differences in leukocyte concentration. As each leukocytes are contribute to the immune defense of breast milk and may play a role in protecting infants from infection. We tried to see the storing and thawing effect on different leukocyte subsets. Our result showed that although it does not change the overall CD45+ population, the storage and thawing processes can reduce the levels of leukocyte subtypes, especially in lymphocytes, monocytes and basophils (Figure 3).

Several previous studies have stained breast milk preparations to observe the morphology of the cells in colostrum and mature breast milk using histological stains such as Hematoxylin Eosin and Giemsa. In our study also observed the morphological changes in leukocytes of human breast milk in different storing and thawing process. The cell nuclei experienced pyknosis, especially in lymphocytes, neutrophils, and eosinophils (Figure 4) by refrigerating for several days. This morphologic characteristics changes by different thawing process. Thawing of frozen breast milk at room temperature (P4) caused damage to the shape and structure of cells, but not to the point of causing cell death (Table 2), but thawing frozen breast milk by placing it in a chiller for 24 hours (P3) led to damage to the structure and shape of the cells, which pointed to more cell death than P4.

These results are in line with previous study that stated the changes in the shape and structure of the cells due to the cooling, freezing and thawing processes according to their normal state. Pyknosis is a degenerative cell nucleus condition characterized by cell shrinkage and increased nuclear compactness or density that can lead to karyorrhexis. This destroyed nucleus cell leaves chromatin fragments scattered in the cell. Later, the dead cell nucleus loses its ability to be stained and disappears, called karyolysis. The cause of these morphological changes is probably caused by cell damage that occurs at low temperatures. Low temperatures disturbed the permeability of the cell membrane, reduced the ability to control intracellular components of the cell plasma membrane, and consequently caused cell damage. A previous study by Kadam et al. observed this morphological changes in the cell nucleus of white blood cell smears when cooled at 4°C in karyolysis, lobulation, vacuolation, and degeneration. We are the first to
observe the cooling of the human breastmilk may as well cause morphological changes in breastmilk leukocytes.

Because the storage can cause the morphological degeneration of leukocytes in human breast milk, there is a requirement for a quantitative approach to characterize these leukocytes. Therefore, further study must employ various subset markers to complement the microscopic morphological observations. It is also necessary to examine the functionality of these leukocytes to determine potential effects. Research conducted by Zhang et al. showed differences in the process of freezing breast milk at −18 °C and −60 °C and thawing in three manners (placing in the air at 4 °C for 10-h stationary, placing in the air at 25 °C for 1-h stationary and shaking in the tepid water at 45 °C for 1 min, respectively). Different thawing manners did not change the content of bioactive proteins, such as levels of IgG and IgA in human milk. Lymphocytes generate both IgA and IgG. Li et al. found that sIgA levels in frozen human milk remained stable after thawing overnight in a refrigerator and reheating to 37°C. Thawing overnight in the refrigerator and then warmed to 25°C or 37°C for 30 minutes can preserve sIgA concentrations and lysozyme activity more effectively than immediately thawing at room temperature after removal from the freezer. Cells will experience gradual damage if it has frozen at temperatures below 0°C. Meanwhile, when thawing from a frozen state, the cell's extracellular solution is hypotonic, which will cause the cell membrane to rupture. The cellular function of various types of cells can be affected by the shape of the cell, such as its structure, the shape of the nucleus, and the cytoplasm content.

**Conclusion**

This research shows that the process of storing, freezing, and thawing breast milk does not affect the human breast milk cell viability, CD45+ population. However, these processes do affect the counts of lymphocytes, monocytes, and basophils and lead to morphological changes that damage the shape and structure of breast milk leukocytes.

The primary strength of this study lies in its simulation of four pertinent storage conditions, in line with recommendations from the Centers for Disease Control and Prevention. These conditions are commonly encountered in scientific research and home storage of human milk.
Such storage conditions are relevant not only for bacterial contaminants, nutritional quality, and bioactive peptide content but also for immunological cellular components, such as leukocytes in human milk; the findings of this study can contribute new information to the field of research regarding the staining of breast milk using HE and Giemsa histology staining methods, which can provide a clear image of breast milk cells, especially leukocytes so that researchers can identify leukocytes and their morphological changes under a microscope with a specific magnification. This study is the first study that describes thawing frozen breast milk at room temperature 25°C for 2 hours has higher leukocyte concentration than thawing in a chiller at 0-4°C for 24 hours. Thus, even though there was a decrease in the cell components of breast milk, especially leukocytes, in the previously recommended breast milk storage process, it should be noted that in the process of thawing frozen breast milk, it is more advisable to leave it at room temperature for 2 hours.

Potential limitations need to be considered. First, the sample processed was 12.5 mL each, which is a small volume and could affect the research results because the breast milk population used was transitional breast milk, which is why the breast milk samples were so small, especially when taken using the expressed method. In further studies, it is recommended to use a larger sample volume and, second, required confirmation of data from further research regarding changes in the characteristics of breast milk leukocytes using several leukocyte subset markers using quantitative methods to complement the microscopic morphological observation data. Third, it is necessary to study in more depth the mechanism of damage to breast milk cells due to the storage process and the function and benefits of these cells when consumed by babies. Fourth, we analyzed the number and viability of breast milk cells and leukocyte populations with two different methods of thawing breast milk, and the results were not significant, indicating that the thawing method was the best. Further studies are needed to examine more widely in terms of cell components and other bioactive factors and various thawing methods that can be applied well and safely at home and in hospitals.

Authors’ Contribution
RS conceptualized and designed the study. DKP obtained the research grant and performed the experiments. AAJ and DKP analysed the data. All authors drafted the manuscript and approved the final version of the manuscript.

Conflict of Interest
The authors declare no conflict of interest.

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References
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Table 1: Main characteristics of the participants (n = 7)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (± SD)</th>
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<tr>
<td>Mother’s Age (years)</td>
<td>28.86 (± 3.132)</td>
</tr>
<tr>
<td>Breastfeeding periods (days)</td>
<td>11.00 (± 3.055)</td>
</tr>
<tr>
<td>Number of births</td>
<td>2.429 (±1.512)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.71 (±2.446)</td>
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*BMI body mass index, SD standard deviation
*Values are presented as the means ± SDs

**Fig 1**: Total cell count of breast milk cells after treatments. The total cell count of treatment P1 show a significantly higher from treatment P2 (**p < 0.01), P3 (***p < 0.01), and P4 (***p < 0.01).

**Fig 2**: CD45+ percentage which shows the leukocyte population of breast milk to differences in how
breast milk is stored. (A-D) Graphical representation of CD45+ flow cytometry in breast milk.

A. treatment P1; B. treatment P2; C. treatment P3; D. treatment P4. E. The breast milk leukocyte population had no significant percentage differences between treatment groups. Descriptively the highest was in the P2, P4 and P1 treatments, while the decrease in the percentage of leukocytes was in P3.

**Fig 3**: Calculation of the average type of leukocytes in breast milk, comparison of significance based on treatment groups P1-P4 (A-E). A. Lymphocytes showed significant differences in all P1-P4 treatment groups \( (p < 0.05) \) and a significant decrease in P1 to P3 \( (*p < 0.05) \). B. Monocytes showed significant differences in all treatment groups P1-P4 \( (p < 0.05) \) and a significant decrease in P1 to P3 \( (*p < 0.05) \). C, D. Neutrophils and eosinophils showed no significant difference. E. Basophils showed significant differences in all P1-P4 treatment groups \( (p < 0.05) \) and a significant decrease in P1 against P2, P3 and P4 \( (**p < 0.05) \).

**Table 2**: Morphological changes of leukocytes in a unit area of breast milk cytopro object glass of the experimental subject.

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Cell changes</th>
<th>Treatment storages</th>
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<tbody>
<tr>
<td>Lymphocytes</td>
<td>No changes</td>
<td>P1</td>
</tr>
<tr>
<td></td>
<td>Pyknosis</td>
<td>P2</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic and nuclear vacuolation. Swelling of the nucleus. The form of cytoplasm and membrane cells are not intact.</td>
<td>P3</td>
</tr>
<tr>
<td>Cell Type</td>
<td>Changes</td>
<td>Description</td>
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<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>Monocytes</td>
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</tr>
<tr>
<td></td>
<td>Pyknosis</td>
<td>P2</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic and nuclear vacuolation.</td>
<td>P3</td>
</tr>
<tr>
<td></td>
<td>Nucleus fragmentation.</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>No changes</td>
<td>P1</td>
</tr>
<tr>
<td></td>
<td>Pyknosis</td>
<td>P2</td>
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<td></td>
<td>Cytoplasmic and nuclear vacuolation.</td>
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<td></td>
<td>Swelling of the nucleus.</td>
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<tr>
<td>Eosinophils</td>
<td>No changes</td>
<td>P1</td>
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<td>Basophils</td>
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<td>P1</td>
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<td></td>
<td>The form of cytoplasm and membrane cells are not intact.</td>
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</tr>
<tr>
<td></td>
<td>Cytoplasmic and nuclear vacuolation.</td>
<td>P4</td>
</tr>
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Fig 4: Leukocytes subset degenerative morphological changes after treatments (green arrow: nucleus). A. Lymphocytes (HE, 100x with immersion oil). B. Monocytes (HE, 100x with immersion oil). C. Neutrophils (HE, 100x with immersion oil). D. Eosinophils (HE, 100x with immersion oil). E. Basophils (HE, 100x with immersion oil).