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CLINICAL & BASIC RESEARCH

The Effects of Different Storage Conditions on Leukocytes in Human Breast Milk

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abstract: ***Objectives:*** Breast milk is the best baby food because it contains various nutrients and important factors for the baby's immune system, including leukocytes. This study aimed to determine the effects on morphology, number of cells and breast milk leukocytes count of various ways of storing breast milk based on different temperatures and storage durations. ***Methods:*** This study was conducted at the Biochemistry Laboratory, Integrated Laboratory and the Histology Laboratory, Universitas Indonesia, Jakarta, Indonesia from September 2022 to February 2023. Transitional breast milk samples from 7 breastfeeding mothers were utilised in the study. A total of 50 mL was 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 Centers for Disease Control and Prevention's (CDC) recommendations for breast milk storage. The breast milk cells were isolated to calculate the cell number and leukocyte population. Subsequently, the breast milk cells were stained with haematoxylin and eosin to analyse the number and morphology of leukocytes. ***Results:*** The findings showed a significant decrease in the breast milk's total number and population and changes in the morphology of breast milk leukocytes after storage. ***Conclusion:*** This study indicates that CDC storage recommendations do not affect the quantity of the CD45+ leukocyte population; however, there is a decrease in the total number of leukocytes and alterations in their microscopic morphology. Thus, additional research is recommended to determine whether these modifications influence the function of the breast milk cells.
Keywords: Human Milk; Leukocytes; Food Storage; Population; Breast Milk Expression.

Advances in Knowledge

- *The number of breast milk cells and the leukocyte subset population (lymphocytes, monocytes, eosinophils and basophils) decreased with different storage (cooling, freezing and thawing) conditions.*
- *Storage conditions also caused changes in the morphological structure and shape of leukocytes in breast milk.*

Applications to Patient Care

- *Information and knowledge on the storage management of expressed breast milk can be provided to breastfeeding mothers so that the contents of their stored breast milk remain optimal for the growth and health of their babies.*

Breastfeeding reduces infection risks and enhances immune system development in neonates.[1] Breast milk is a dynamic physiological fluid that provides, alongside other live cells, nutrients essential for healthy growth of the baby.[2] Breast milk comprises immunological and non-immunological cells. Non-immune cells, including epithelial cells such as ductal, alveolar, luminal-epithelial and myoepithelial, are essential for the lactating mammary glands' formation and function and contribute to 99% of the cellular components of human milk in healthy mothers and newborns.[3] Additionally, immune cells, such as leukocyte-group monocytes, T cells, NK cells, B cells and others, offer active immunity to infants by producing bioactive components, developing the newborn's immune system and altering the environment of the baby's gastrointestinal tract.[1]

The cells of breast milk include large leukocytes that depend on the lactation stage and the health status of the breastfeeding mother and baby.[4] Leukocytes in breast milk, such as granulocytes and mononuclear leukocytes (lymphocytes, monocytes and macrophages), are active, motile and interactive. Leukocytes provide active immunity in infants.[4] Additionally, breast milk leukocytes are also thought to protect the mammary glands from infection during breastfeeding.[4]

Trend *et al*. evaluated the leukocyte population in full-term and premature breast milk using multi-flow cytometry colour and found no differences in leukocyte concentration between preterm and term breast milk.[5] Ideally, breast milk should be stored at the right temperature and duration for optimal nutrient and bioactive component quality. The Centers for Disease Control and Prevention (CDC) states that freshly expressed breast milk can be stored in a cold container at room temperature (25°C) for ≤4 hours, in the refrigerator with a temperature of 4°C for ≤4 days and in the freezer with temperature ≤ –18°C for

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6–12 months.[6] Frozen breast milk can be defrosted at room temperature (≤ 25°C) for 1–2 hours or in the refrigerator at 4°C for 24 hours.[6] However, the effects of these storage condition's on leukocytes remains underexplored.

This study aimed to evaluate the changes in morphology and the number of leukocytes in breast milk based on the milk storage methods and conditions. 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.[7] It is hoped that the study's findings will provide information about the best storage conditions to maintain both the type and characteristics of leukocytes in human breast milk.

Methods

This study was conducted at the Biochemistry Laboratory, Integrated Laboratory and the Histology Laboratory, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia from September 2022 to February 2023. Breast milk samples were received from mothers who met the following 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 gap of ≥2 years before the previous birth. Breastfeeding mothers who consumed alcohol, were smokers, were ill and who declined to provide their milk as a research sample were excluded. Demographic data were collected from the participants. For the breast milk samples, the participants were asked to clean their hands and nipples with antiseptic soap and rinse with clean water before expressing milk. Breast milk was expressed in the morning using an electronic milk pump with several parts sterilised. Breast milk samples were then collected in sterile 50 mL glass bottles and immediately taken to the laboratory (<4 hours). The milk samples were divided into 4 different treatments: (1) in an ice cooler box at 0–4°C for <4h (P1); (2) in the lower refrigerator/chiller at 0–4°C for 4 days (P2); in a freezer at –18°C for less than a month, thawed by placing it in the refrigerator at 0–4°C overnight (P3); and (4) thawed at room temperature of less than 25°C for less than 2 hours (P4).

The breast milk samples were centrifuged at 810 g for 20 minutes at 22°C. The resulting lipid layers and supernatants were carefully removed. Subsequently, the cell pellets were washed twice with phosphate-buffered saline (PBS). The resulting cell pellets were resuspended with PBS and the cells were counted using an automatic cell counter, LUNA-II™ (Logos Biosystem, Anyang-si, Republic of Korea), after adding trypan blue (1:1) and homogenising for segregation.

Approximately 1×10^5 breast milk cells were centrifuged at 2,500 rpm for 5 minutes to remove the supernatant then 300 µL of stain buffer was added. The cells were centrifuged at 2,500 rpm for 5 minutes, including 100 µL of the prepared antibody mixture (100 µL stain buffer and 2 µL Ab 1; CD45) before incubating for 30 minutes at room temperature in darkness. A 300 µL of stain buffer was then added to each tube and centrifuged at 2,500 rpm for 5 minutes. Then, 300 µL PBS was added to the remaining pellets. Subsequently, the samples were examined using a flow cytometer.

The pellets containing breast milk cells were cytocentrifuged onto glass slide using the Cytopro® Cytocentrifuge, Model 7622 (ELITechGroup Inc, Puteaux, France). Then, the cells were fixed using the method adopted by Tripathy and Singh and stained with haematoxylin and eosin (H&E) to study their cellular morphology.[7] The slides were fixed using a solution of ether and 96% alcohol (1:1) for 10 minutes before serial hydration in 100%, 95%, 80%, 70%, 50% and 30% alcohol for a minute each. They were then stained with haematoxylin for 3–4 minutes, followed by careful rinsing with running water. After that, the slides were immersed in distilled water for a minute and stained with eosin for 3–5 seconds. The next step was serial dehydration in 50%, 70%, 80%, 95% and 100% alcohol for a minute each. The final stage involved clarifying using xylol I and II for a minute each. Then, the slides were closed using Entellan® and a cover glass and observed under a light microscope at a specific magnification using OptiLab Viewer (PT MICONOS, Sleman, DI Yogyakarta, Indonesia), Zeiss Zen Microscopy (Zeiss Group, Oberkochen, Germany) and Image Raster software (PT MICONOS). The cell composition and morphology of specific cell types were then recorded. To reduce bias in the results, the findings were verified by a consultant histologist.

Statistical data analysis was performed using GraphPad Prism9 (GraphPad Software, San Diego, California, USA). The data were tested for normality and homogeneity using the Shapiro-Wilk test and processed using one-way analysis of variance. Subsequently, a post hoc Bonferroni and Tukey test was conducted. Since the data were not normally distributed and did not vary homogeneously, this was followed by the nonparametric Kruskal-Wallis test, wherein $P < 0.05$ indicated a significant difference, and a subsequent Dunn test.

This study conducted after approval from Ethical Committee of the Faculty of Medicine, Universitas Indonesia (KET-980/UN2.F1/ETIK/PPM.00.02/2022.).

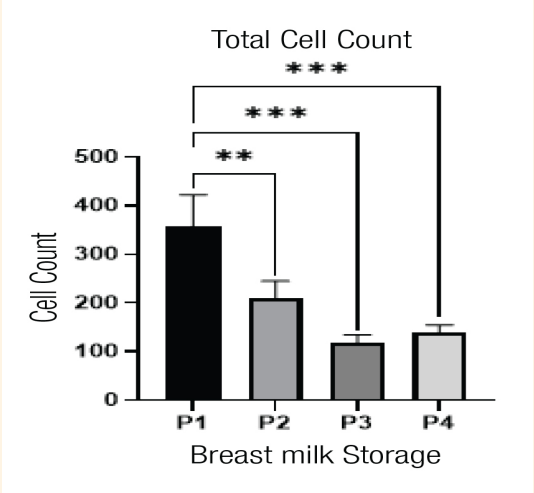

Figure 1: Total cells count of breast milk cells after various treatments.

*The total cell count of treatment P1 is significantly higher than those of treatments P2 (** P <0.01), P3 (***P <0.01) and P4 (***P <0.01).*

P1 = kept in an ice cooler box at 0–4°C < 4h; P2 = in the lower refrigerator/chiller at 0–4°C for 4 days; P3 = in a freezer at –18°C for less than a month and thawed by placing it in the refrigerator at 0–4°C overnight; P4 = thawed at room temperature of less than 25°C for less than 2 hours.

Results

A total of 7 mothers had given birth to full-term singleton infants and provided milk samples. The mean ± standard deviation for maternal age was 28.86 ± 3.132 years, the mean breastfeeding period was 11.00 ± 3.055 days, the mean number of births was 2.429 ± 1.512 and the mothers' mean body mass index was 22.71 ± 2.446 kg/m^2.

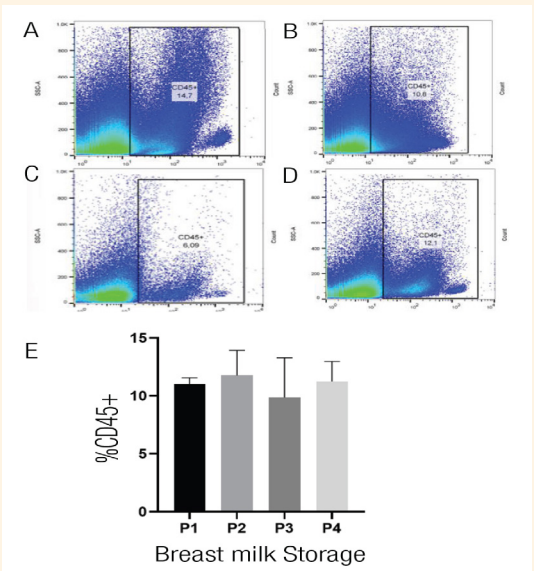

Figure 2: CD45+ percentage showing the leukocyte population of breast milk with regard to differences in breast milk storage. **A–D:** Graphical representation of CD45+ flow cytometry in breast milk. **E:** The breast milk leukocyte population had no significant percentage differences between treatment groups. Leukocyte population was highest in the P2, P4 and P1 treatments, with a decrease in the leukocyte population in P3.

P1 = kept in an ice cooler box at 0–4°C < 4h; P2 = in the lower refrigerator/chiller at 0–4°C for 4 days; P3 = in a freezer at –18°C for less than a month and thawed by placing it in the refrigerator at 0–4°C overnight; P4 = thawed at room temperature of less than 25°C for less than 2 hours. P1 = kept in an ice cooler box at 0–4°C < 4h; P2 = in the lower refrigerator/chiller at 0–4°C for 4 days; P3 = in a freezer at –18°C for less than a month and thawed by placing it in the refrigerator at 0–4°C overnight; P4 = thawed at room temperature of less than 25°C for less than 2 hours.

All the cells of the 4 treatments were examined from a CytoPro glass slide (ELITechGroup Inc.) under a light microscope with ×40 magnification Image Raster software (PT MICONOS) was used within a specified area measuring 290 µm in length and 215 µm in width, which was presumed to contain approximately

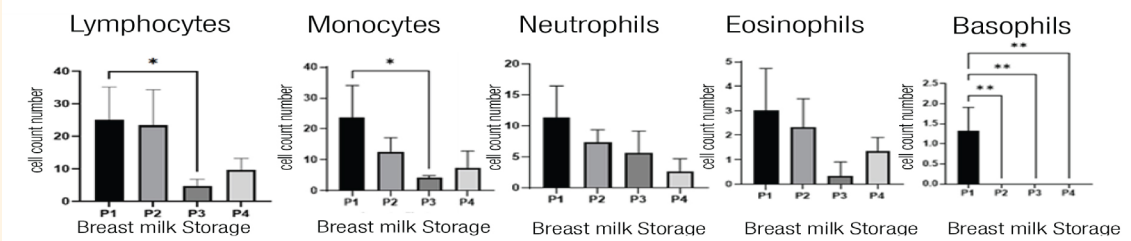

Figure 3: Calculation of the average type of leukocytes in breast milk, comparison of significance based on treatment groups. Lymphocytes showed significant differences in all P1–P4 treatment groups and a significant decrease in P1 to P3. Monocytes showed significant differences in all treatment groups P1–P4 and a significant decrease in P1 to P3. Neutrophils and eosinophils showed no significant difference. Basophils showed significant differences in all P1–P4 treatment groups and a significant decrease in P1 against P2, P3 and P4.

**P <0.05*

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P1 = kept in an ice cooler box at 0–4°C < 4h; P2 = in the lower refrigerator/chiller at 0–4°C for 4 days; P3 = in a freezer at –18°C for less than a month and thawed by placing it in the refrigerator at 0–4°C overnight; P4 = thawed at room temperature of less than 25°C for less than 2 hours.

500 cells. The total cell count exhibited a significant difference between the treatments. Further analysis with the post-hoc Bonferroni test showed that the number of cells in the P1 treatment sample was higher than those in the P2 ($P = 0.0089$), P3 ($P = 0.0004$) and P4 ($P = 0.0007$) treatment samples. When comparing the two procedures for thawing frozen breast milk, there was no significant difference between P3 and P4 in terms of total cell count. However, P4 (139.3 ± 16.01) exhibited a higher total cell count compared to P3 (117.7 ± 17.21) [Figure 1].

The percentage of CD45+ is one of the markers to determine the leukocyte population in a sample. The results of this study showed that the percentage of CD45+ was highest in the P2 treatment sample (11.78 ± 3.732), followed by the treatment samples P4 (11.24 ± 2.979), P1 (11.03 ± 0.9018) and, finally, P3 (9.863 ± 5.931), where it was the lowest. This experiment showed that the storage conditions did not significantly change the percentage of CD45+ in the breast milk. Moreover, when comparing the two thawing methods (P3 and P4), no significant change in the percentage of CD45+ was observed; although, the percentage of CD45+ in the P4 treatment (11.24 ± 2.979) was higher than in the P3 treatment sample (9.863 ± 5.931) [Figure 2].

Interestingly, when the primary subset leukocytes were counted, there were significant differences, especially in lymphocytes (25.00 ± 10.15; $P = 0.0299$), monocytes (23.67 ± 10.41; $P = 0.0246$) and basophils (1.333 ± 0.5774; $P = 0.0010$). Further analysis showed that compared to P1, there is a significant decrease in lymphocytes in P3 ($P = 0.0491$); monocytes are in P3 ($P = 0.0337$) and basophils are in P2 ($P = 0.0029$), P3 ($P = 0.0029$) and P4 ($P = 0.0029$) [Figure 3]. On the other hand, neutrophils and eosinophils showed different decreases after each treatment, although not significantly. Regarding the comparison between the thawing methods, the group thawed in a refrigerator showed lower counts of lymphocytes, monocytes and eosinophils than those thawed at room temperature, although the differences were not statistically significant.

Moreover, the process of storing breast milk affected the morphology of leukocytes [Figure 4]. 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 in the P3 treatment, the

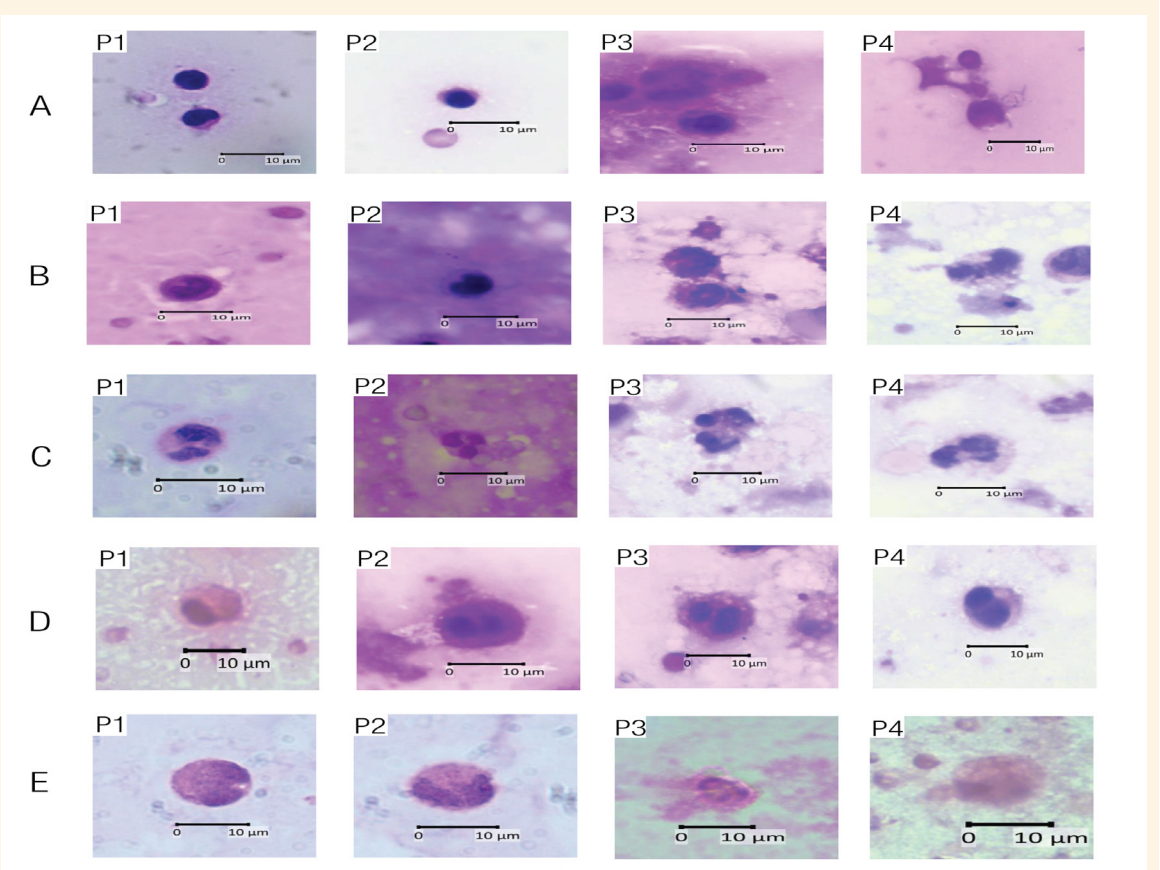

Figure 4: Haematoxylin and eosin stains at ×100 magnification with immersion oil of breast milk samples after various treatments showing morphological changes in **(A)** lymphocytes, **(B)** monocytes, **(C)** neutrophils, **(D)** eosinophils and **(E)** basophils.
P1 = kept in an ice cooler box at 0–4°C < 4h; P2 = in the lower refrigerator/chiller at 0–4°C for 4 days; P3 = in a freezer at –18°C for less than a month and thawed by placing it in the refrigerator at 0–4°C overnight; P4 = thawed at room temperature of less than 25°C for less than 2 hours.

Table 1: Morphological changes of leukocytes in a unit area of breast milk CytoPro glass slides of the experimental subject.

Cell type	Cell change	Treatment storage
Lymphocytes	No changes	P1
	Pyknosis	P2
	- Cytoplasmic and nuclear vacuolation - Swelling of the nucleus -The form of cytoplasm and membrane cells were not intact	P3
	- Cytoplasmic and nuclear vacuolation - Nucleus fragmentation - The form of cytoplasm and membrane cells were not intact - Cell disintegration	P4
Monocytes	No changes	P1
	Pyknosis	P2
	- Cytoplasmic and nuclear vacuolation - Swelling of the nucleus - The form of cytoplasm and membrane cells were not intact	P3
	- Cytoplasmic and nuclear vacuolation - Nucleus fragmentation - The form of cytoplasm and membrane cells were not intact - Cell disintegration	P4
Neutrophils	No changes	P1
	Pyknosis	P2
	- Cytoplasmic and nuclear vacuolation - Swelling of the nucleus - The form of cytoplasm and membrane cells were not intact	P3
	- Cytoplasmic and nuclear vacuolation - Nucleus fragmentation - The form of cytoplasm and membrane cells were not intact	P4
Eosinophils	No changes	P1
	Pyknosis	P2
	- Cytoplasmic and nuclear vacuolation - Swelling of the nucleus - The form of cytoplasm and membrane cells were not intact.	P3
	- Cytoplasmic and nuclear vacuolation - Nucleus fragmentation	P4
Basophils	No changes	P1
	Pyknosis	P2
	- Cytoplasmic and nuclear vacuolation - Swelling of the nucleus - The form of cytoplasm and membrane cells were not intact	P3
	- Cytoplasmic and nuclear vacuolation - The form of cytoplasm and membrane cells were not intact	P4

P1 = kept in an ice cooler box at 0–4°C < 4h; P2 = in the lower refrigerator/chiller at 0–4°C for 4 days; P3 = in a freezer at −18°C for less than a month and thawed by placing it in the refrigerator at 0–4°C overnight; P4 = thawed at room temperature of less than 25°C for less than 2 hours.

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 1]. 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 the above except cell disintegration, and eosinophils still had 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, than stored human milk. Pittard and Bill found that milk cellular components, such as macrophage and neutrophil concentration, were significantly reduced after 48 hours of refrigeration.[8] The current study supports this finding by demonstrating the further decreased total number of breast milk cells after prolonged storage. This phenomenon is likely caused by reduced antioxidant activity in stored breast milk; Hanna *et al.* highlighted the decrease in the antioxidant capacity of breast milk stored at 4°C and –20°C when compared to fresh breast milk.[9] Decreased antioxidant activity can produce oxidative stress and an imbalance between reactive oxygen species and intra- and extracellular antioxidant systems that cause damage to cells.[10]

Additionally, the current study found 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 recrystallisation, is more effective.[11] Theoretically, recrystallisation generates additional interfacial tension or shear on entrapped proteins, leading to further damage by forming tiny ice crystals during the freezing process.[11] Further research using a large sample is still needed to explore the effect of different thawing processes on breast milk cell population.

Further analysis on the effect of different storing and thawing methods on leukocyte population found that the percentage of leukocytes CD45+ in human breast milk did not significantly change when flow cytometry was used [Figure 2]. The thawing process also did not significantly change the CD45+ population, although room temperature thawing showed a little higher CD45+ count than when thawed in a refrigerator. The results showed that overall CD45+ population in breast milk exhibited resilience during storage and thawing processes. This finding aligns with CDC guidelines; mothers who cannot directly supply breast milk to their infants can therefore confidently use breast milk stored in accordance with these guidelines.

Trend *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.[5] Each leukocyte contributes to the immune defence of breast milk and may play a role in protecting infants from infection.[5] The current study's findings on the effect of different storing and thawing methods on different leukocyte subsets showed that although they do 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 H&E and Giemsa.[2,7] This study's observation on the morphological changes in human breast milk leukocytes in different storing and thawing processes showed the cell nuclei experiencing pyknosis, especially in lymphocytes, neutrophils and eosinophils, after refrigeration for several days [Figure 4]. This morphological characteristic changes with different thawing processes. Thawing of frozen breast milk at room temperature (P4) damaged the shape and structure of cells, but not to the point of cell death; yet, thawing frozen breast milk by placing it in a refrigerator for 24 hours (P3) damaged the structure and shape of the cells and led to more cell deaths than in P4 [Table 2].

These results are in line with a previous study highlighting the changes in the shape and structure of cells due to the cooling, freezing and thawing processes according to their normal state.[12] Pyknosis is a degenerative cell nucleus condition characterised by cell shrinkage and increased nuclear compactness that can lead to karyorrhexis. This destroyed nucleus cell leaves chromatin fragments scattered in the cell. The dead cell nucleus loses its ability to be stained and disappears, which is called karyolysis.[13] The cause of these morphological changes can probably be traced to cell damage that occurs at low temperatures. Kadam *et al.* observed these morphological changes in the cell nucleus of white blood cell smears when cooled at 4°C in karyolysis, lobulation, vacuolation and degeneration.[12] Low temperatures disturb the permeability of the cell membrane, reduce its ability to control intracellular components of the cell plasma membrane and consequently cause cell damage.[14] The current study is the first to observe the morphological changes in human breast milk leukocytes caused by cooling.

As storage can cause the morphological degeneration of leukocytes in human breast milk, there is a requirement for a quantitative approach

to characterising these leukocytes. Therefore, further studies 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. Previous study by Zhang *et al*. showed that different thawing manners (placing in the air at 4°C for 10 h, placing in the air at 25°C for 1 h and shaking in tepid water at 45°C for 1 min) did not change the content of bioactive proteins, such as the levels of IgG and IgA in human milk.[15] Lymphocytes generate both IgA and IgG.[16] Li *et al*. found that thawing frozen human milk overnight in a refrigerator before reheating to 25°C or 37°C for 30 minutes can preserve the SIgA concentrations and lysozyme activity more effectively than immediately thawing at room temperature after removal from the freezer.[1] Cells experience gradual damage if frozen at temperatures below 0°C. Meanwhile, when thawing from a frozen state, the cell's extracellular solution is hypotonic, which causes the cell membrane to rupture.[14] 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.[17]

The primary strength of this study lies in its simulation of 4 pertinent storage conditions, in line with recommendations from the CDC. These conditions are commonly encountered in scientific research and home storage of human milk. Such storage conditions are relevant not only for the milk's bacterial contaminants, nutritional quality and bioactive peptide content but also for its immunological cellular components, such as leukocytes. The findings of this study contribute to the field of research concerning the staining of breast milk using H&E 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.

Potential limitations of this study must be noted. First, the sample processed was 12.5 mL each, which is a small volume and could affect the research results. The breast milk cell population used was transitional breast milk, which is why the samples were so small, especially when obtained through the expressed method. Future studies are recommended to use a larger sample volume. Second, further research should be done regarding changes in the characteristics of breast milk leukocytes using several leukocyte subset markers and quantitative methods to complement the microscopic morphological observation data. Third, future research could 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, the current study only analysed the number and viability of breast milk cells and leukocyte populations using 2 different methods of thawing, and the results were not significant, indicating that the thawing method was the best. Further studies are needed to examine more widely the cellular components and other bioactive factors, and the various thawing methods that can be applied well and safely at home and in hospitals.

Conclusion

The process of storing, freezing and thawing breast milk does not affect the human breast milk cell viability and its CD45+ count. 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. This is the first study to demonstrate that frozen breast milk thawed at room temperature (25°C) for 2 hours has a higher leukocyte concentration than when thawed in a chiller (0–4°C) for 24 hours. Thus, even though there was a decrease in the cellular components of breast milk, especially leukocytes, in the previously recommended breast milk storage process, the current study notes that when thawing frozen breast milk, it is more advisable to leave it at room temperature for 2 hours than thawing it gradually over a period of 24 hours.

AUTHORS' CONTRIBUTION

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

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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