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# The Effect of Holder Pasteurization and Storage on Macronutrients in Donor Human Milk

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**Objective:** Milk delivered to human milk banks should be pasteurized and stored at -20℃ in order to inactivate any microbial agents that may be present. We aimed to quantify the changes in the macronutrient composition of donor human milk (DHM) that underwent Holder pasteurization (HoP) and subsequent storage.

**Methods:** A total of 54 breast milk samples from 26 healthy lactating mothers were collected at different time points after delivery, at intervals ranging from 1-6 months. We measured the carbohydrate, protein, fat, and energy content before and after HoP. After HoP, DHM was stored at -20℃, and the nutrients were measured at 1, 2, 4, 8, 12, 16, and 20 weeks after storage commenced.

**Results:** The difference in time between expression of milk and pasteurization did not affect the concentration of macronutrients. However, the protein, fat, and energy contents decreased significantly during HoP. The mean values of the protein, fat, and energy contents in DHM pre-HoP were 1.2 g/dL, 3.7 g/dL, and 72.1 kcal/dL, compared with post-HoP values of 1.0 g/dL, 3.1 g/dL, and 65.2 kcal/dL, respectively. Although HoP reduced the protein, fat, and energy contents of donor milk by 16.7%, 16.2%, and 9.6%, respectively, the carbohydrate content was not reduced. Moreover, there was a significant decrease in the content of all the analyzed macronutrients following storage for up to 20 weeks.

**Conclusion:** The post-HoP storage period affected nutrients, with several human milk components decreasing in content after HoP. As such, DHM after HoP may need fortification to ensure normal infant growth.

**Key Words:** Milk, human, Milk banks, Freezing, Nutrients

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## Introduction

Breast milk (BM) is considered the gold standard for infant nutrition, in terms of both nutrients and development.[1] Extensive evidence has shown that BM contains various physiologically active agents that affect brain development as well as the function of the gastrointestinal and immune systems.[2,3] In terms of clinical efficacy, donor human milk (DHM) is the first alternative, rather than artificial formula milk, when the mother's milk is either unavailable or insufficient, especially for premature infants.[4,5]

Unlike mother's milk, DHM should be pasteurized and stored at −20℃ to inactivate any microbial agents that may be present. The most frequently used pasteurization method is Holder pasteurization (HoP), which is a thermal process that consists of heating DHM at 62.5℃ for 30 minutes and subsequent fast cooling, according to the International Human Milk Bank Guidelines. After HoP, all milk samples are frozen and maintained in freezers at −20℃ for up to 6 months.[6-8] Although HoP and frozen storage are essential for eliminating the microbial content and maintaining the sterility of DHM, respectively, until it is consumed by infants, many studies have shown the detrimental effects of those processes on the milk components.[9-11]

Previous reports regarding the nutritional change of donated BM during the procedures performed in human milk banks (HMB) used various methods of pasteurization and a wide range of storage periods. In addition, the targeted components in those studies were diverse, and the data seem insufficient for estimating the change in nutrients due to pasteurization and frozen storage. As such, this study aimed to analyze the effect of HoP and storage on the macronutrient composition of DHM.

## Methods

The BM samples used in this study were retrieved from those donated to the HMB of Kyung Hee University Hospital at Gangdong. A total of 54 BM samples were collected from 26 mothers between April and August 2018. These mothers all had a full-term delivery, and the stage of lactation was less than 6 months after delivery. All donors signed a written consent form to donate their BM for research purposes. The informed consent requirements for this retrospective review were waived by the Institutional Review Board of the author's institution (approval number: KHNMC 2019-09-010).

For the donation process, BM was expressed manually or with an electric milk pump and stored in sterile polypropylene bottles provided by the HMB. All samples were collected within 24 hours at the donors' homes. After collection, the donor milk bottles were frozen and stored in a freezer at -20℃ at home and then transferred to the HMB using a post office home-delivery service. The number of days from collection to the first analysis was recorded to take into consideration the sample variation. The stage of lactation was also collected, which is the period between the infant's birth date and the day on which the milk was collected.

The donor milk delivered from each home was stored at -20℃ until pasteurization. Before pasteurization was performed, the frozen samples were thawed in a refrigerator, and ten raw milk aliquots of 10 mL were collected from each mother's DHM. One of these aliquots was reserved to assess the microbiological safety after pasteurization, while another was used to quantify the carbohydrate, protein, fat, and energy levels prior to pasteurization. After HoP (heating at 62.5℃ for 30 minutes followed by fast cooling) was performed, the macronutrient content of the pasteurized BM was analyzed, followed by storage of the rest of the pasteurized milk samples (divided into seven bottles) at -20℃. At 1, 2, 4, 8, 12, 16, and 20 weeks of storage, an aliquot of the DHM was thawed, and the nutrient concentrations were assessed.

The milk bottles were heated up to 40℃ using a temperature-controlled bath to homogenize the samples appropriately. The analysis was performed on 10 mL samples and homogenized using a 1.5 s/mL ultrasonic homogenizer (Sonicator®; Biologics, Uppsala, Sweden), which ensured the best solubility of fat and destruction of casein micelles. The carbohydrate, protein, and fat concentrations were quantified using an infrared analyzer named the human milk analyzer ([HMA], Miris AB®; Miris, Uppsala, Sweden). This machine analyzes the fat, total nitrogen, carbohydrate, and energy contents using mid-infrared spectrometry. The device was calibrated after the analysis of every ten samples using the "Check" function, according to the manufacturer's instructions.

### 1. Statistical analysis

The concentrations of the macronutrients were expressed as means and standard deviations for continuous variables. The relationships between the pre-HoP storage period and the carbohydrate, protein, fat, and energy contents were expressed as scatter plots. A paired *t*-test was used to compare the pre- and post-HoP macronutrient concentrations of BM. The influence of the stage of lactation and pre-HoP storage period on the nutrient content changes before and after HoP was presented as the mean and standard deviation. A one-way analysis of variance was used to evaluate the alterations in nutrient concentrations during HoP at different stages of lactation and the pre-HoP storage period. Changes in the macronutrient content during the post-HoP frozen storage were analyzed via a trend test. All data were analyzed using the statistical program SAS 9.4 (SAS Institute Inc., Cary, NC, USA). A value of $P<0.05$ was considered statistically significant.

## Results

Over 4 months, 54 BM samples from 26 healthy donors were collected. The median postnatal age at the time of milk expres-

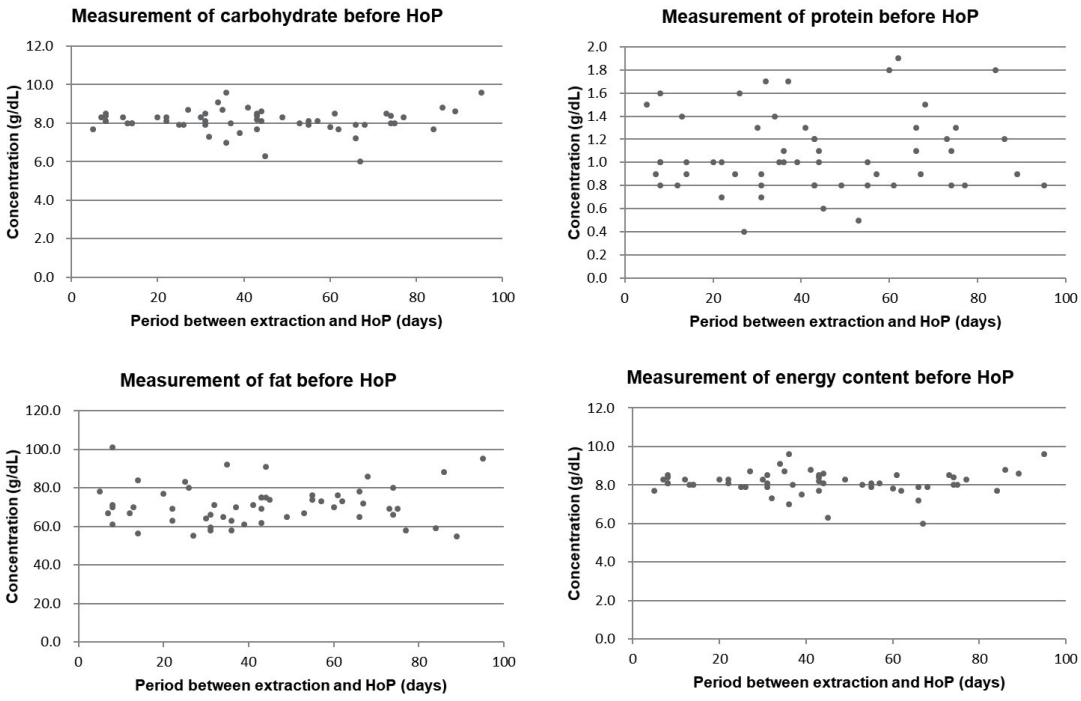


**Fig. 1.** Patterns of the macronutrient concentrations and energy content in the period between the expression of the mother's milk and Holder pasteurization (HoP).

**Table 1.** Changes in Nutrients upon Holder Pasteurization (HoP) (n=56)

| Variable | Pre-HoP | Post-HoP | *P*-value |
|---|---|---|---|
| Carbohydrates (g/dL) | 8.2±0.3 | 8.2±0.4 | 0.064 |
| Protein (g/dL) | 1.2±0.3 | 1.0±0.2 | <0.001 |
| Fat (g/dL) | 3.7±1.0 | 3.1±0.8 | <0.001 |
| Energy (kcal/dL) | 72.1±9.0 | 65.2±7.0 | <0.001 |

Values are presented as mean±standard deviation.

sion was 80 days (range 10–177 days) and the median of the number of days after milk expression until pasteurization was 40.5 days (range 6–96 days). As mentioned above, BM expressed at home was kept frozen until pasteurization at the HMB. As such, the correlation between the total pre-HoP storage time and the changes in the nutritional components were investigated. No significant differences were observed in the nutritional composition of DHM depending on the period between expression and HoP, as shown in Fig. 1.

Furthermore, the nutrient content was compared before and after HoP, as shown in Table 1. After HoP, the mean protein concentration decreased significantly by 16.7% (1.2–1.0 g/dL), as did the fat concentration by 16.2% (3.7–3.1 g/dL), and the energy content by 9.6% (72.1–65.2 kcal/dL). There was no change in the carbohydrate concentration, with a mean of 8.2 g/dL in both the pre- and post-HoP samples.

The alterations in macronutrient concentrations during the pasteurization process were analyzed in separated groups, based on the stage of lactation and pre-HoP storage period. When comparing groups with different stages of lactation, the total carbohydrate, protein, fat, and energy content before and after pasteurization showed no significant differences (Table 2). Similarly, the means were not significantly different when comparing groups with different pre-HoP storage periods (Table 3).

After pasteurization, changes in the levels of macronutrients might also occur in DHM samples stored at −20℃. Fig. 2 presents the changes in the carbohydrate, protein, fat, and energy contents during post-HoP frozen storage periods. The samples were grouped based on the stage of lactation, and it was noted that there was a decrease in the macronutrient content of each group as the duration of frozen storage increased. Even though the starting concentrations at day 0 were different for each group, the overall results show a consistent decline in the total macronutrient content for every group. Estimates and *P*-values were calculated without the separation of groups. Our results

**Table 2.** Nutritional Changes before and after Holder Pasteurization (HoP) at Different Stages of Lactation

| | Stage of lactation | | | | | | |
|---|---|---|---|---|---|---|---|
| | <1 mo. (n=10) | 1-2 mo. (n=10) | 2-3 mo. (n=10) | 3-4 mo. (n=10) | 4-5 mo. (n=8) | 5-6 mo. (n=8) | *P*-value |
| Difference between post- and pre- HoP | | | | | | | |
| Carbohydrates (g/dL) | 0.3±0.4 | 0.1±0.7 | 0.3±0.9 | -0.3±0.5 | -0.1±0.9 | -0.2±0.3 | 0.351 |
| Protein (g/dL) | -0.2±0.4 | -0.3±0.3 | -0.4±0.2 | -0.1±0.4 | -0.2±0.4 | 0.1±0.2 | 0.071 |
| Fat (g/dL) | -0.6±0.8 | -0.4±0.9 | -0.4±0.9 | -0.4±1.0 | -1.2±1.0 | -0.2±0. 1 | 0.180 |
| Energy (kcal/dL) | -6.3±8.9 | -4.9±8.1 | -4.8±10.3 | -4.7±11.3 | -12.3±11.2 | -2.2±1.9 | 0.380 |

Values are presented as mean±standard deviation.
Abbreviation: mo., months.

**Table 3.** Difference of Nutrients before and after Holder Pasteurization (HoP), according to the Pre-Holder Pasteurization Storage Period

| | Pre-HoP storage period | | | |
|---|---|---|---|---|
| | <1 mo. (n=17) | 1-2 mo. (n=24) | 2-3 mo. (n=14) | *P*-value |
| Difference between post- and pre- HoP | | | | |
| Carbohydrates (g/dL) | 0.1±0.4 | 0.1±0.7 | 0.1±0.8 | 0.990 |
| Protein (g/dL) | -0.2±0.5 | -0.2±0.3 | -0.1±0.3 | 0.416 |
| Fat (g/dL) | -0.4±1.0 | -0.5±0.8 | -0.6±0.7 | 0.911 |
| Energy (kcal/dL) | -5.7±11.0 | -5.8±9.1 | -5.7±8.2 | 1.000 |

Values are presented as mean±standard deviation.
Abbreviation: mo., months.

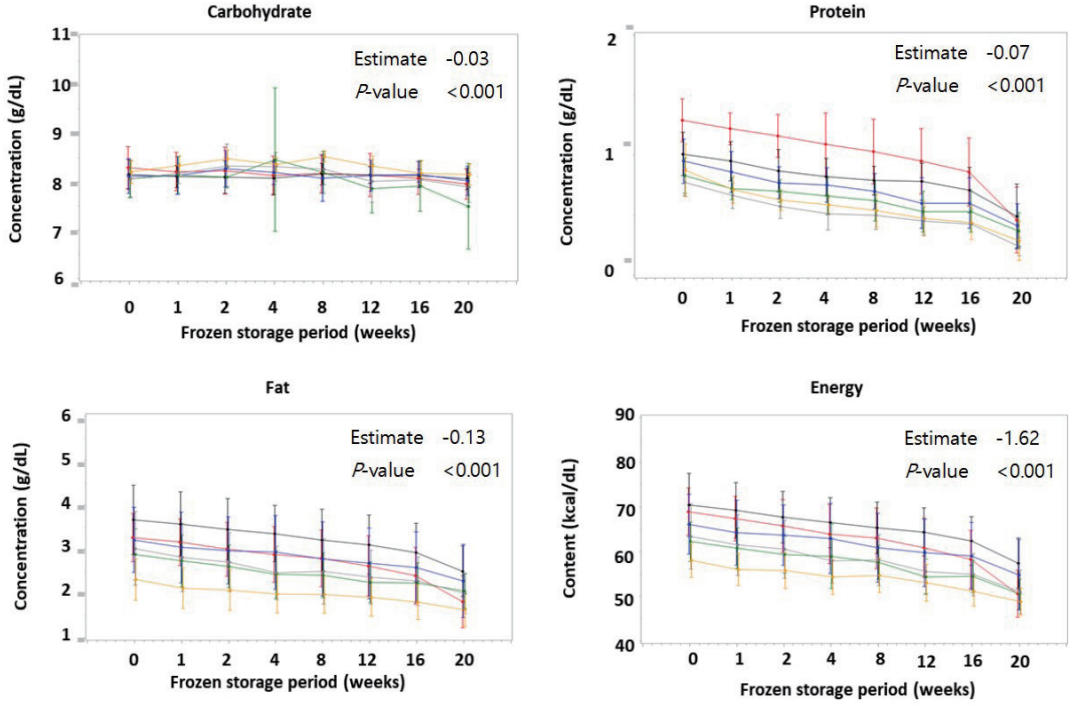


**Fig. 2.** Changes in the macronutrient concentrations and energy content according to the refrigeration period after Holder pasteurization.

show that longer storage periods lead to lower overall nutrient concentrations. The total energy content was shown to have the steepest decline, while carbohydrates were shown to decline in a relatively steady manner.

## Discussion

Studies on the effect of pasteurization on macronutrients have shown different results over the years. Among them, studies analyzing protein content modifications seem to agree that protein levels decrease after pasteurization.[12,13] Studies on carbohydrates have investigated the saccharide and lactose levels to try to quantify the total carbohydrate content. When using saccharides as an index, many studies have found no change in the carbohydrate content following pasteurization.[13,14] However, when using lactose, studies have found conflicting results. Some studies identified a decrease in carbohydrate levels when analyzing lactose concentrations, while others found no change after pasteurization.[15] The HMA used in this study showed the total carbohydrate content as the sum of the lactose and saccharides. The majority of studies regarding fat concentrations observed reduction in fat levels following pasteurization,[12,15-17] which is consistent with the results of the present study. However, several previous studies found no change in fat concentrations after pasteurization.[13,17]

To our knowledge, this is the first study focusing on the effects of the stage of lactation and pre-HoP storage period on the change in macronutrient content during pasteurization. This study aimed to investigate whether stored BM could lose nutritional value under the influence of external conditions, such as the lactation stage or pre-HoP storage days. The results of this study showed no meaningful differences between the groups. As such, it is concluded that these two factors do not cause BM to be more easily degraded during pasteurization.

Several studies have revealed a decline in the fat content of thawed BM after frozen storage.[15,17] Previous studies have attributed this loss to fat adhering to the container wall. When analyzing the protein and carbohydrate contents, studies have shown conflicting results, mainly due to variations in the quantification methods and storage duration. As such, these conclusions may be valid for specific experimental situations. In contrast to the lack of effect that the storage period had on the nutritional content before HoP, once the milk has been pasteurized, the reduction in nutrient levels seem to have a positive correlation with the storage period. No previous studies appear to have focused on this idea. Therefore, it can be concluded that structural changes during pasteurization may negatively affect nutritional components, leading to the destruction of macronutrients during freezing.

Preterm infants require more vigorous nutritional support for optimal growth during the neonatal period than at any other time of their life.[18] However, it is widely recognized that the protein content of all BM, both the mothers' and the donors', is generally insufficient to meet the requirements of growing preterm infants without fortification.[19,20] In this study, it has been demonstrated that the HoP and storage of DHM lead to the reduction of macronutrients in BM after a certain time point. Therefore, the appropriate fortification and consumption of BM soon after pasteurization is necessary to ensure optimal quality.

A limitation of this study is that the fat content was assessed as total fat and not in its different biological forms, such as triglycerides, free fatty acids, phospholipids, or cholesterols. Although HoP is currently the gold standard method of pasteurization, there are other ways to pasteurize BM, such as high-temperature short-time pasteurization (HTST), high-pressure processing (HPP), ultraviolet-C (UV-C) irradiation, and ultrasonication. HTST has shown promising results with respect to its efficacy in eliminating inoculated lipid-enveloped viruses and bacteria.[21] In HPP, the high variability of the results with respect to the reduction in microbiological activity warrants further studies.[22] Studies regarding UV-C irradiation and ultrasonication have also revealed their effects on eliminating bacterial and viral activity. However, data regarding these techniques are insufficient and do not warrant their routine use in HMBs. As such, optimal environmental and technical points need to be determined to achieve targeted biological safety and retain nutritional components.

The results of this study reveal the changes in the macronutrient content of BM following the DHM handling process in HMBs. This study demonstrates that HoP modifies the protein and fat concentrations of the DHM, and the subsequent frozen storage at −20℃ reduces the carbohydrate concentration as well as the protein and fat concentrations in BM.

## Conflict of interest

No potential conflict of interest relevant to this article was reported.

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