The Effects of Refrigerated Storage Time on Sialic Acid and Nitric Oxide Levels and Oxidant Antioxidant System of Human Milk

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ABSTRACT

Objective: Human milk (HM) is a marvelous nutrition that serves all the needs of infants in the first six months with the vitamins, minerals, proteins, carbohydrates, and lipids it contains. For the first 4-6 months of a baby’s life, Nursing is accepted as the most beneficial and recommended feeding method. The greatest technique for providing nutrition in the absence of breastfeeding is through expressed HM. In this case, milk storage conditions become critical. The proper storage of HM is essential for preserving the nutritional and antioxidant properties of HM. This study aims to examine the effects of storing HM in the refrigerator.

Materials and Methods: The effects of storing HM in the refrigerator were examined for four days with regard to the protein profile oxidant-antioxidant balance and nitric oxide (NO) and sialic acid (SA) levels.

Results: Total protein (TP) levels decreased gradually over the four days. In the SDS-PAGE electrophoresis method, the heavy chain sIgA and κ-casein bands also disappeared in HM. While glutathione-S-transferase and superoxide dismutase activities decreased significantly during the first two days, their activities fell below the detection limit in the last two days. While the glutathione level and catalase activity also decreased gradually over the four days, the malondialdehyde, SA, and NO levels increased significantly.

Conclusion: HM can be safely stored in the refrigerator for two days due to the TP, SA, and NO levels, as well as the antioxidant enzyme activities, remaining unchanged from the first day of expressing HM.

Keywords: Human milk, storage, oxidant-antioxidant system, nitric oxide, sialic acid, SDS-PAGE electrophoresis

INTRODUCTION

Human milk (HM) is a valuable food that contains all the components required for the development and growth of an infant. HM is a liquid that varies depending on the mother’s age, medication use, diet, and health and is produced to satisfy the specific needs of each infant. Furthermore, HM serves important functions such as strengthening the immune systems of infants, protecting infection, supporting brain development, and improving digestive system functions.

HM contains many proteins, carbohydrates, lipids, minerals, vitamins, hormones, antioxidant agents, and immunoglobulins that play regulatory and structural roles, as well as antimicrobial peptides, growth factors, and small molecules, and is secreted by mammary epithelial cells. HM proteins consist of up to 70% whey proteins (e.g., α-lactalbumin, lactoferrin, secretory immunoglobulin A) and 30% caseins (α, β, and κ). HM proteins serve many biological functions such as nutrition, nutrient transport, enzymes, intestinal development, immune system regulation, prebiotics, and cognitive functions.

The main antioxidant enzymes of HM are superoxide dismutase (SOD), catalase (CAT), and glutathione-s-transferase (GST). Glutathione (GSH), a tripeptide that provides the infant with antioxidant protection, is also found in HM. The sialic acid (SA) bound to oligosaccharides accounts for around 75% of the total SA contained in HM. Human milk oligosaccharides (HMO) are prebiotic components that cannot be digested by the infant but instead stimulate the growth of beneficial bifidobacteria. HMO containing SA is acidic and contains SA in the terminal position of the chain. It constitutes 12-14% of the total HMO content. The trisaccharide sialyllactose, which consists of lactose at the reducing terminus and a Sia residue at the nonreducing terminus, is one of the main sialylligosaccharides in HMO sialyllactose and a key component of HMO. The SA levels in HM change with the geography, genetics, and diet of the breastfeeding mother. The high sialylligosaccharide content of HM appears to provide...
excellent conditions for the human newborn’s absorption and use of the milk Sia to satisfy developmental requirements. SA in HM plays an important role in brain and neural development. Its highest level occurs at the beginning of lactation (5.04 mM) and gradually decreases during the lactation period (1.04 mM). Also, another source of SA in HM is κ-casein, a glycoprotein with SA residues.

Nitric oxide (NO) is formed from arginine by the NO synthase enzyme in the endothelial cells and can be found in these cells in the form of a nitrate or nitrite. Recent studies have shown that xanthine oxidase in HM catalyzes the anaerobic reduction of inorganic nitrite to NO. This reaction is thought to be the source of NO in HM. In mammary gland epithelial cells, the xanthine oxidase enzyme bonds to the membrane of milk fat globules. NO is found in HM at around 100 μM. It regulates blood pressure, prevents infections, and regulates the immune system in infants.

The most ideal way to feed HM to a baby is by breastfeeding. However, breastfeeding may sometimes be discontinued due to problems arising from the mother or the infant. Storage conditions and duration are important for the nutritional content, antioxidant capacity, immunological content, and bacterial content of HM. Many studies have shown storing HM in the refrigerator to affect the total protein content and antioxidant balance over time. However, no study is found in the literature to have shown the effect of storing HM in the refrigerator with regard to SA and NO levels. Therefore, the results of this study provide a detailed and comprehensive understanding of the changes that occur during the storage of HM, as well as a new perspective to the studies in this field.

MATERIALS AND METHODS

Human Milk Collection

HM collection protocol was approved by the Marmara University School of Medicine Ethics Committee (Approval No. 09.2019.893). Eight breastfeeding mothers signed informed consent forms before donating fresh milk. HM samples were expressed with a pump and stored in milk bags (Lansinoh-Breastmilk Storage Bags). These bags were stored at +4°C during the four days of the experiment (Figure 1).

Determining the Oxidant Parameter

Malondialdehyde (MDA) levels were determined as an oxidant parameter for measuring the lipid peroxidation level in HM. This method reacts MDA and thiobarbituric acid to produce a pink-colored compound and measures the absorbance of this compound spectrophotometrically. The results are presented as μmol MDA/mL using an extinction coefficient of 1.56x105 M⁻¹cm⁻¹ to represent the equivalent amount of MDA.

Determining the Antioxidant Parameters

This study investigates the changes over four days regarding GST, CAT and SOD activities and GSH level as antioxidant parameters. In order to determine GSH levels, the study uses a method based on the spectrophotometric detection of the yellow product formed as a result of the reaction of the sulfhydryl groups of GSH with the Elmann reagent (5,5-dithio-bis-2-nitrobenzoic acid). The results are presented as mg% glutathione using an extinction coefficient of 13600 M⁻¹cm⁻¹.

The study investigates GST activity using spectrophotometric detection of the product formed as a result of the reaction of GSH and 1-chloro-2,4-dinitro-benzene at 340 nm. The results are presented as U/mL.

The study determined SOD activity, namely the oxidation of riboflavin sensitized o-dianside by the SOD enzyme, spectrophotometrically at 460 nm. The results are presented as U/mL.

The study determined CAT activity by monitoring the conversion reaction of H₂O₂ to H₂O with a decrease in absorbance at 240 nm. The results are presented as U/mL.

Determining the Nitric Oxide and Sialic Acid Levels

To determine the NO levels, vanadium (III) chloride causes the quantitative reduction of nitrate to nitrite. The complex diazonium compound is produced when N-(1-Naphthyl)ethylene diamine dihydrochloride reacts with nitrite in the presence of sulfanilamide. The absorbance of the resulting colored complex is measured spectrophotometrically at 540 nm. The results are presented as μM.

The study identified the SA levels spectrophotometrically at 549 nm through the product formed as a result of the reaction of β-formyl pyruvic acid (resulting from periodic acid oxidation) with thiobarbituric acid. The results are presented as g%.

The Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of HM

The total protein (TP) concentration of HM was determined from skim HM using the Lowry method over the 4 days. The results are presented as g/dL.

The SDS-PAGE method was carried out using the Laemmli system. The molecular weight of the protein bands was determined by comparing the migration rates of the protein standard (BioRad, Rome Italy). The changes in the protein band intensities were examined using ImageJ software. The bands’ intensities and peak areas were also calculated in ImageJ software.
Statistical Analysis

The results were evaluated using the GraphPad Prism 6.0 package program (GraphPad Software, San Diego, CA, USA). Data were obtained from at least 10 replicate experiments and presented as means ± standard deviations. Groups of data were analyzed using analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests, with a p<0.05 being regarded as significant.

RESULTS

The Oxidant Parameter of HM

The HM MDA level is a final product of lipid peroxidation and therefore a good marker of the degree of the oxidation process. Lipid peroxidation occurs as the result of the direct action of oxygen or other oxidative agents on unsaturated fatty acids and others in the cell membrane. This study saw the MDA level in the HM stored in the refrigerator to increase gradually and significantly over the four days (p<0.05; Table 1).

The Antioxidant Parameters of HM

This study presents the results of the changes in GSH levels and GST, CAT, and SOD enzyme activities as antioxidant parameters of HM over the four days in Table 1. The GSH level and CAT enzyme activity of HM stored in the refrigerator gradually and significantly decreased over the four days (p<0.05). On the other hand, the GST and SOD enzyme activities decreased significantly on the second day compared to the first day (p<0.05), with no further activity regarding the GST and SOD enzymes in HM being detected on Days 3 and 4.

The Nitric Oxide and Sialic Acid Levels of HM

The NO values of HM increased significantly over the four days (p<0.05; Figure 2). The SA results of HM revealed no significant increase in the first three days, while observing a significant increase on Day 4 (Figure 3).

SDS-PAGE Results of HM

The TP level of HM decreased significantly over the four-day storage period. However, no significant difference occurred in the TP levels between Days 2 and 3 (Figure 4). Significant differences in the SDS-PAGE gel electrophoresis bands of HM were observed to be consistent with the change in TP. Three bands were seen on the electrophoresis gel images around 66 kDa, which belong to lactoferrin (Area 1, band a), serum albumin (Area 1, band b), and the sIgA heavy chain (Area 1, band c) from top to bottom, respectively. The bands around 24 and 36 kDa are the alpha-casein (Area 2, band d), beta-casein (Area 2, band e), kappa-casein (Area 2, band f), and sIgA light chain (Area 2, band g) from top to bottom, respectively. The band at 21 kDa is beta-lactoglobulin (Area 3, band h), and the band at 14 kDa is alpha-lactalbumin (Area 3, band i; Figure 5).

The protein intensity in the lactoferrin, serum albumin, sIgA heavy chain, and κ-casein bands in the gel significantly de-
Table 1. Oxidant-antioxidant parameters of human milk.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (gmol/mL)</td>
<td>0.058 ± 0.009</td>
<td>0.924 ± 0.064</td>
<td>1.479 ± 0.087*</td>
<td>2.239 ± 0.079*</td>
</tr>
<tr>
<td>GSH (mg%)</td>
<td>0.469 ± 0.014</td>
<td>0.239 ± 0.014*</td>
<td>0.189 ± 0.001*</td>
<td>0.103 ± 0.005**</td>
</tr>
<tr>
<td>GST (U/mL)</td>
<td>0.059 ± 0.003</td>
<td>0.041 ± 0.005</td>
<td>Under the Detection Limit</td>
<td>Under the Detection Limit</td>
</tr>
<tr>
<td>CAT (kU/mL)</td>
<td>2.035 ± 0.075</td>
<td>1.872 ± 0.027*</td>
<td>1.764 ± 0.028*</td>
<td>1.111 ± 0.132*</td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>4.308 ± 0.083</td>
<td>1.355 ± 0.178</td>
<td>Under the Detection Limit</td>
<td>Under the Detection Limit</td>
</tr>
</tbody>
</table>

SD = Standard deviation; * signifies p<0.05 compared to Day 1; ¤ signifies p<0.05 compared to Day 2; A signifies p<0.05 compared to Day 4.

increased on Days 3 and 4 compared to Days 1 and 2. The α-casein, β-casein, sIgA light chain, β-lactoglobulin, and α-lactalbumin bands in the gel did not change over the four days (Figure 6 and Table 2).

DISCUSSION

HM is a highly nutritious substance that is not only packed with essential nutrients for a growing infant but also contains several chemical substances and antioxidants that play a significant role in promoting infant health. The effect of HM storage conditions on its antioxidant properties mainly involves changes in the antioxidant activity and levels of antioxidant compounds present.34 HM should be stored at most for 3 hours at room temperature 25°C, 3 days in a refrigerator +4°C, or lastly up to 3 months in a freezer -20°C. 35 The nutritional content of HM has been determined to change when these periods are exceeded.36,37 Miranda et al. reported a significant increase in MDA values when HM had been stored in the refrigerator for 2 days compared to fresh milk.23 The current study found the MDA levels of HM to gradually increase over the four days, which is consistent with the literature.

Many studies have examined the change in the antioxidant capacity of HM based on storage conditions. Ankrah et al.’s study found a significant decrease in GSH levels when HM was kept at 4°C for 2 hours.7 Marinkovic et al.’s study observed a significant decrease in HM SOD activity when kept at -20°C for 7 days.5 These results are also consistent with the findings from the current study. No study is found in the literature to
Table 2. Electrophoresis gel band image area analysis.

<table>
<thead>
<tr>
<th>Area</th>
<th>Protein Bands</th>
<th>Protein</th>
<th>Protein Peaks Areas (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>1</td>
<td>a</td>
<td>Lactoferrin</td>
<td>100.2</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>Serum albumin</td>
<td>40.2</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>sIgA heavy chain</td>
<td>16.5</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>a casein</td>
<td>156.1</td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>β casein</td>
<td>92.7</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>κ casein</td>
<td>6.59</td>
</tr>
<tr>
<td></td>
<td>g</td>
<td>sIgA light chain</td>
<td>7.6</td>
</tr>
<tr>
<td>2</td>
<td>h</td>
<td>β lactoglobulin</td>
<td>19.6</td>
</tr>
<tr>
<td></td>
<td>i</td>
<td>α lactalbumin</td>
<td>185.3</td>
</tr>
</tbody>
</table>

Figure 4. Total protein levels in human milk.* signifies p<0.05 compared to Day 1; □ signifies p<0.05 compared to Day 2; Δ signifies p<0.05 compared to Day 4.

Figure 5. SDS-PAGE profiles for human milk. a = lactoferrin; b = serum albumin; c = sIgA heavy chain; d = α-casein; e = β-casein; f = κ-casein; g = sIgA light chain; h = β-lactoglobulin; i = α-lactalbumin.

have addressed the changes in GST and CAT activities of HM based on storage conditions. This study saw the GST, SOD, and CAT activities of the HM to gradually decrease during the four days. Even the GST and SOD activities fell under the detection limit after two days of storage. Padurarau et al. showed the total antioxidant capacity of HM to decrease when stored at 4°C for one to three days. Consistent with these studies, the current study detected significant decreases in GSH levels and antioxidant enzyme activities over the four-day storage period in the refrigerator.

A search of the literature search revealed no findings related to the changes in SA and NO levels in HM during storage in a refrigerator. This is why the current study focused on measuring SA and NO levels in HM. HM contains a significant amount of SA, primarily in the form of N-acetyl neuraminic acid. Wang et al. determined SA levels in colostrum, transition, and mature HM. This current study identified SA levels in mature milk to be at 0.01 g/dL, while Wang et al. found the total SA level in mature milk to be 0.045 g/dL. No study is
found in the literature regarding the change in HM SA levels based on storage conditions. Therefore, the SA findings from the current study are novel, which found the SA levels of HM to not change over the first three days of storage in a refrigerator but to increase very sharply on Day 4. In HM, 21%-28% of SA was bound to glycoproteins, with only 3% of SA being found in an unbounded form.39 The SA levels measured in the first three days are believed to have been the free SA found in HM. Because approximately 12-14% of the total SA in HM is bound to oligosaccharides and 21-28% to glycoproteins, this increase observed on the fourth day may have been due to the release of the SA bound to the oligosaccharide and glycoprotein through the activity of the sialidase enzyme. Because sialylation is important for cell functioning and affects the biological stability of glycoproteins, desialylation in milk is an undesirable reaction as it indicates the oligosaccharides or glycoproteins to which the SA had been attached have lost their function.

This study investigated the NO levels in HM as one of the novel parameters that change with storage in a refrigerator. NO is produced in breast tissue and may stimulate lactation in humans. Fernandes et al. found the nitrate value in HM to range from 42.6 µM to 96.6 µM.40 Yüksel et al. found the NO value in mature HM to be 93 µM.18 This study found the NO level of HM to be 22 µM on Day 1 and then to increase on Day 4. The literature search revealed no study to have addressed changes in the NO level of HM based on storage conditions. In this study, the increase in NO concentration of HM on Day 4 can be related to the NO produced by bacterial NO synthase. If storage exceeding 72 hours is required, cryopreservation is preferred to prevent bacterial growth.41

Iqbal et al. found HM protein content to decrease gradually over three days under refrigerated storage conditions.20 The current study similarly found the protein levels in HM to decrease. Meng et al.’s electrophoretic examination of HM showed the disappearance of sIgA bands after two days and of lactoferrin bands after five days for HM kept at 4°C.22 Another study on HM electrophoresis determined the β-casein bands to decrease at the end of seven days.21 The current study found the lactoferrin, serum albumin, sIgA, and κ-casein bands to decrease on Days 3 and 4 compared Days 1 and 2.

CONCLUSION

In conclusion, while storing HM at +4°C for three days did not significantly change the SA and NO levels, these parameters increased significantly on Day 4. Although the SA and NO levels didn’t increase until Day 4, storing HM for only two days is considered safer due to the decreased antioxidant capacity and increased lipid peroxidation on Day 2. This is in comparison to the three days of storage recommended in many guidelines.

Acknowledgement

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