European Journal of Biology
Eur J Biol 2023; 82 (2): 243–250
DOI: 10.26650/EurJBiol.2023.1293969

RESEARCH ARTICLE

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

Begum Gurel Gokmen[1], Tugba Akbay[2]

[1]Marmara University, Institute of Health Science, Department of Biochemistry (Pharmacy), Istanbul, Turkiye
[2]Marmara University, Faculty of Dentistry, Department of Basic Medical Sciences, Istanbul, Turkiye

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.[1] Furthermore, HM serves important functions such as strengthening the immune systems of infants, protecting infection, supporting brain development, and improving digestive system functions.[2]

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.[1] HM proteins consist of up to 70% whey proteins (e.g., α-lactalbumin, lactoferrin, secretory immunoglobulin A) and 30% caseins (α, β, and κ).[3] HM proteins serve many biological functions such as nutrition, nutrient transport, enzymes, intestinal development, immune system regulation, prebiotics, and cognitive functions.[4] The main antioxidant enzymes of HM are superoxide dismutase (SOD), catalase (CAT), and glutathione-s-transferase (GST).[5,6] Glutathione (GSH), a tripeptide that provides the infant with antioxidant protection, is also found in HM.[7]

The sialic acid (SA) bound to oligosaccharides accounts for around 75% of the total SA contained in HM.[8] Human milk oligosaccharides (HMO) are prebiotic components that cannot be digested by the infant but instead stimulate the growth of beneficial bifidobacteria.[9] HMO containing SA is acidic and contains SA in the terminal position of the chain. It constitutes 12-14% of the total HMO content.[10] The trisaccharide sialyllactose, which consists of lactose at the reducing terminus and a Sia residue at the nonreducing terminus, is one of the main sialyloligosaccharides in HMO sialyllactose and a key component of HMO.[11] The SA levels in HM change with the geography, genetics, and diet of the breastfeeding mother. The high sialyloligosaccharide content of HM appears to provide

Corresponding Author: Begum Gurel Gokmen **E-mail:** bg.begumgurel@gmail.com
Submitted: 08.05.2023 • **Revision Requested:** 24.05.2023 • **Last Revision Received:** 25.05.2023 • **Accepted:** 27.05.2023 • **Published Online:** 31.08.2023

excellent conditions for the human newborn's absorption and use of the milk Sia to satisfy developmental requirements.[12] 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).[13,14] Also, another source of SA in HM is κ-casein, a glycoprotein with SA residues.[15]

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.[16] 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.[17] NO is found in HM at around 100 µM. It regulates blood pressure, prevents infections, and regulates the immune system in infants.[18]

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.[19] Many studies have shown storing HM in the refrigerator to affect the total protein content and antioxidant-oxidant balance over time.[5–7,20–23] 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.[24] 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.[25] 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 $M^{-1}cm^{-1}$ to represent the equivalent amount of MDA.

Determining the Antioxidant Parameters

This study investigates the changes over four days regarding GST[26], CAT[27] and SOD[28] activities and GSH level[29] 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^{-1} cm^{-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-dianisidine 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 H_2O_2 to H_2O 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.[23]

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%.[30]

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.[31] The results are presented as g/dL.

The SDS-PAGE method was carried out using the Laemmli system.[32] 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.[33]

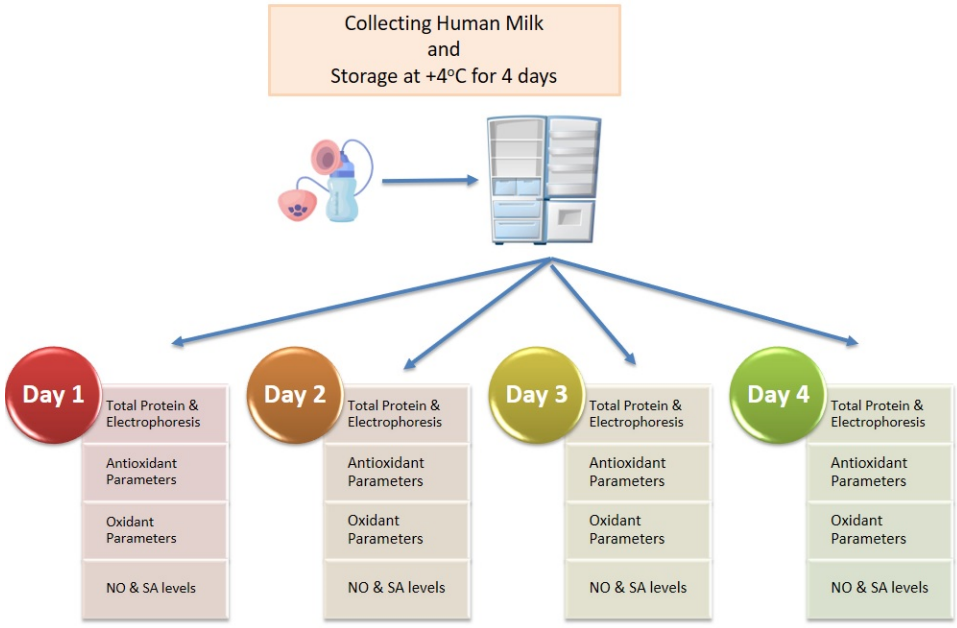

Figure 1. The experimental design.NO = nitric oxide, SA = sialic acid.

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.

Parameters	Day 1	Day 2	Day 3	Day 4
MDA (µmol/mL)	0.058 ± 0.009	0.924 ± 0.064 *	1.479 ± 0.087*ⁿ^A	2.239 ± 0.079*ⁿ
GSH (mg%)	0.469 ± 0.014	0.259 ± 0.014 *^A	0.189 ± 0.001*ⁿ^A	0.103 ± 0.005*ⁿ
GST (U/mL)	0.059 ± 0.003	0.041 ± 0.005 *	Under the Detection Limit	Under the Detection Limit
CAT (kU/mL)	2.035 ± 0.075	1.872 ± 0.027 *^A	1.764 ± 0.028*ⁿ^A	1.111 ± 0.132*ⁿ
SOD (U/mL)	4.308 ± 0.083	1.355 ± 0.178 *	Under the Detection Limit	Under the Detection Limit

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

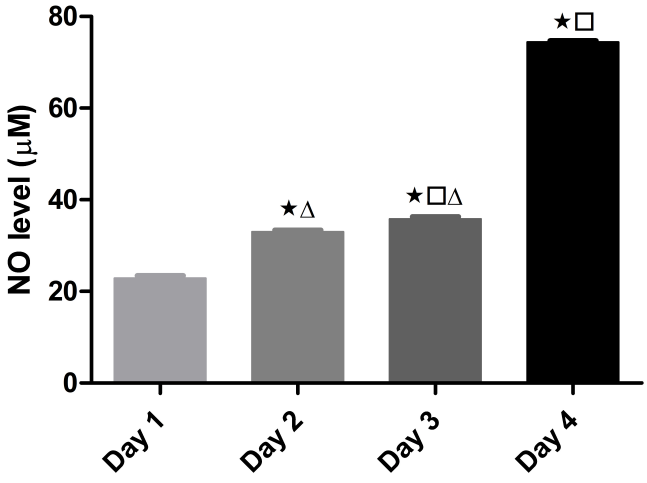

Figure 2. NO levels in human milk.NO = Nitric oxide.* signifies p<0.05 compared to Day 1; □ signifies p<0.05 compared to Day 2; Δ signifies p<0.05 compared to Day 4.

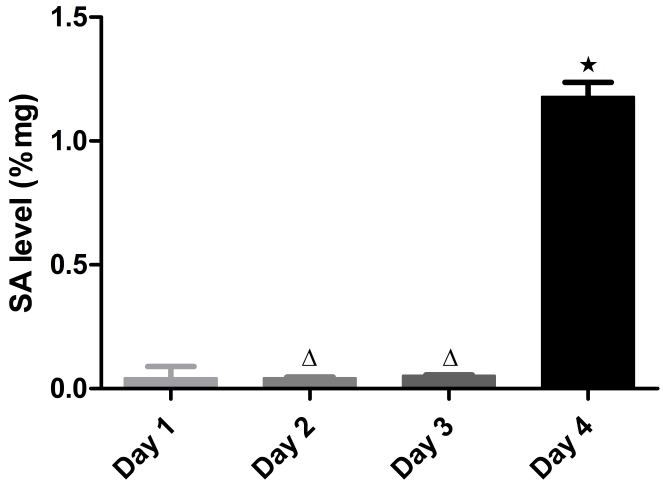

Figure 3. SA levels in human milk.SA = sialic acid .* signifies p<0.05 compared to Day 1; Δ signifies p<0.05 compared to Day 4.

creased 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

Table 2. Electrophoresis gel band image area analysis.

Area	Protein Bands	Protein	Protein Peaks Areas (cm^2)			
			Day 1	Day 2	Day 3	Day 4
1	a	Lactoferrin	100.2	100.6	69.0	68.9
	b	Serum albumin	40.2	40.4	23.7	21.2
	c	sIgA heavy chain	16.5	16.5	5.6	5.6
2	d	α casein	156.1	158.2	157.1	152.6
	e	β casein	92.7	92.7	92.4	92.3
	f	κ casein	6.59	6.54	0.04	0.04
	g	sIgA light chain	7.6	7.6	7.6	7.5
3	h	β lactoglobulin	19.6	19.7	19.7	19.6
	ı	α lactalbumin	185.3	185.6	185.3	185.2

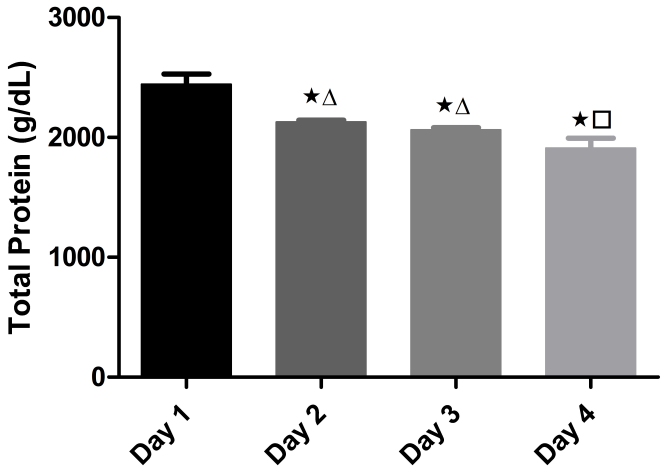

Figure 4. Total protein levels in human milk.* signifies p<0.05 compared to Day 1; □ signifies p<0.05 compared to Day 2; Δ signifies p<0.05 compared to Day 4.

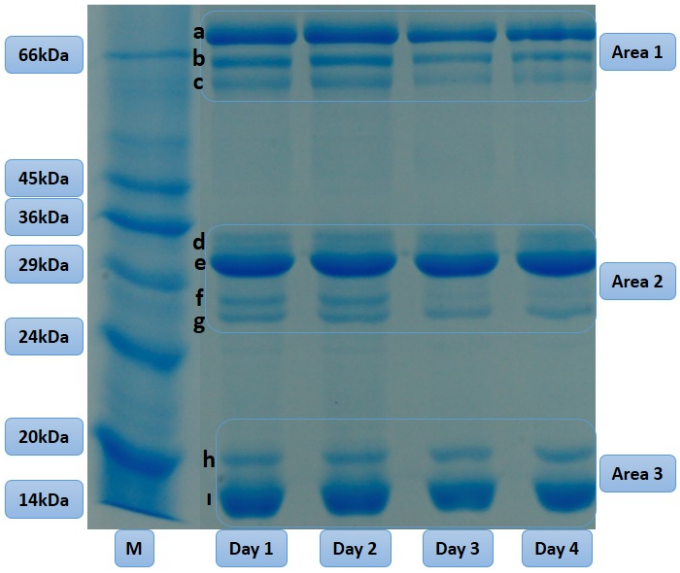

Figure 5. SDS-PAGE profiles for human milk. a = lactoferrin; b = serum albumin; c = sIgA heavy chain; d = α-casein; e = β-casein; f = κ-casein; g = sIgA heavy chain; h = β-lactoglobulin, i = α-lactalbumin.

for 7 days.[5] These results are also consistent with the findings from the current study. No study is found in the literature to 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. Paduraru et al. showed the total antioxidant capacity of HM to decrease when stored at 4°C for one to three days.[6] 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.[14] Wang et al. determined SA levels in colostrum, transi-

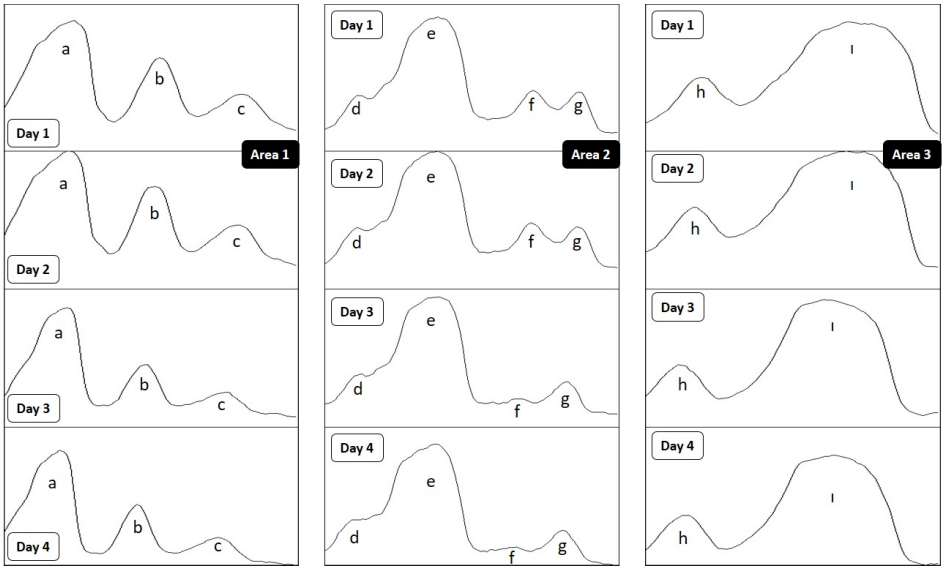

Figure 6. Image J protein band peaks. a = lactoferrin; b = serum albumin; c = sIgA heavy chain; d = α-casein; e = β-casein; f = κ-casein; g = sIgA heavy chain; h = β-lactoglobulin; i = α-lactalbumin.

tion, 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.[38] 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.

Ethics Committee Approval: HM collection protocol was approved by the Marmara University School of Medicine Ethics Committee (Approval No. 09.2019.893).

Informed Consent: Informed consent was taken.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- B.G.G., T.A.; Data Acquisition- B.G.G., T.A.; Data Analysis/Interpretation- B.G.G., T.A.; Drafting Manuscript- B.G.G., T.A.; Critical Revision of Manuscript- B.G.G., T.A.; Final Approval and Accountability- B.G.G., T.A.

Conflict of Interest: Authors declared no conflict of interest.

Financial Disclosure: This research was financially supported by the Scientific and Technological Research Council of Türkiye (TUBITAKProject ID: 321S097) and the Marmara University Scientific Research Project Department (Project ID: TDK-2021-10306)

ORCID IDs of the authors

Begum Gurel Gokmen 0000-0002-3955-1948
Tugba Akbay 0000-0002-2091-9298

REFERENCES

1. Kim JH, Froh EB. What nurses need to know regarding nutritional and immunobiological properties of human milk. *J Obstet Gynecol Neonatal Nurs.* 2012;41(1):122-137.
2. Yi D, Kim S. Human breast milk composition and function in human health: From nutritional components to microbiome and MicroRNAs. *Nutrients*. 2021;13(9):3094. doi:10.3390/nu13093094
3. Hendricks G, Guo M. Bioactive components in human milk. *Human milk biochemistry and infant formula manufacturing technology.* Elsevier; 2014:33-54.
4. Donovan SM. Human milk proteins: Composition and physiological significance. *nestle Nutr Inst Workshop Ser*. 2019;90:93-101.
5. Marinković V, Ranković-Janevski M, Spasić S, et al. Antioxidative activity of colostrum and human milk: Effects of Pasteurization and storage. *J Pediatr Gastroenterol Nutr.* 2016;62(6):901-906.
6. Păduraru L, Dimitriu DC, Avasiloaiei AL, Moscalu M, Zonda GI, Stamatin M. Total antioxidant status in fresh and stored human milk from mothers of term and preterm neonates. *Pediatr Neonatol*. 2018;59(6):600-605.
7. Ankrah NA, Appiah-Opong R, Dzokoto C. Human breastmilk storage and the glutathione content. *J Trop Pediatr*. 2000;46(2):111-113.
8. Nakano T, Sugawara M, Kawakami H. Sialic acid in human milk: Composition and functions. *Acta Paediatr* Taiwan. 2001;42(1):11-17.
9. Ruhaak LR, Stroble C, Underwood MA, Lebrilla CB. Detection of milk oligosaccharides in plasma of infants. *Anal Bioanal Chem*. 2014;406(24):5775-5784.
10. Açar Y, Yassibaş E. Anne sütü oligosakkaritleri ve sağlık üzerine etkileri. *Gazi Sağlık Bilimleri Dergisi*. 2021;6(1):22-33.
11. Newburg DS. Oligosaccharides in human milk and bacterial colonization. *J Pediatr Gastroenterol Nutr*. 2000;30:S8-S17.
12. Liu F, Simpson AB, D'Costa E, Bunn FS, van Leeuwen SS. Sialic acid, the secret gift for the brain. *Crit Rev Food Sci Nutr.* 2022:1-20. doi:10.1080/10408398.2022.2072270
13. Jantscher-Krenn E, Bode L. Human milk oligosaccharides and their potential benefits for the breast-fed neonate. *Minerva Pediatr.* 2012;64(1):83-99.
14. Wang B. Sialic acid is an essential nutrient for brain development and cognition. *Annu Rev Nutr.* 2009;29:177-222.
15. Brignon G, Chtourou A, Ribadeau-Dumas B. Preparation and amino acid sequence of human κ-casein. *FEBS Letters*. 1985;188(1):48-54.
16. Martin HM, Hancock JT, Salisbury V, Harrison R. Role of xanthine oxidoreductase as an antimicrobial agent. *Infect Immun*. 2004;72(9):4933-4939.
17. Stevens CR, Millar TM, Clinch JG, Kanczler JM, Bodamyali T, Blake DR. Antibacterial properties of xanthine oxidase in human milk. *The Lancet*. 2000;356(9232):829-830.
18. Yuksel S, Yigit AA, Cinar M, Atmaca N, Onaran Y. Oxidant and antioxidant status of human breast milk during lactation period. *Dairy Sci & Technol*. 2015;95(3):295-302.
19. Ulukaya B. Storage of human milk: Effect of temperature and storage conditions on microbiota. Karabük University, Institute of Graduate Programs, Department of Midwifery, Master Thesis, 2022.
20. Iqbal M, Lestari LA, Kurdanti W, Mardiyati NL. Effect of temperature and storage duration on lactose, protein and fat content of breast milk. presented at: International Conference on Health and Well-Being; 2016.
21. Li X, Gu Y, He S, et al. Influence of pasteurization and storage on dynamic *in vitro* gastric digestion of milk proteins: Quantitative insights based on peptidomics. *Foods*. 2020;9(8):998. doi:10.3390/foods9080998
22. Meng F, Uniacke-Lowe T, Ryan AC, Kelly AL. The composition and physico-chemical properties of human milk: A review. *Trends Food Sci Technol*. 2021;112:608-621.
23. Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide*. 2001;5(1):62-71.
24. Miller EM, Aiello MO, Fujita M, Hinde K, Milligan L, Quinn EA. Field and laboratory methods in human milk research. *Am J Hum Biol*. 2013;25(1):1-11.
25. Ledwozyw A, Michalak J, Stepień A, Kadziołka A. The relationship between plasma triglycerides, cholesterol, total lipids and lipid peroxidation products during human atherosclerosis. *Clin Chim Acta.* 1986;155(3):275-283.
26. Habig WH, Jakoby WB. Assays for differentiation of glutathione S-transferases. *Methods Enzymol.* 1981;77:398-405.
27. Aebi H. Catalase. *Methods of enzymatic analysis*. Elsevier; 1974:673-684.
28. Mylroie AA, Collins H, Umbles C, Kyle J. Erythrocyte superoxide dismutase activity and other parameters of copper status in rats ingesting lead acetate. *Toxicol Appl Pharmacol.* 1986;82(3):512-520.
29. Beutler E. Reduced Glutathione. In: Bergmeyen HV, ed. *Glutathione in red blood cell metabolism: A manuel of biochemical methods.* Grune and Stratton; 1975:112-114.
30. Warren L. The thiobarbituric acid assay of sialic acids. *J Biol Chem.* 1959;234(8):1971-1975.

31. Lowry O, Rosebrough N, Farr A, Randall R. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193(1):265-275.
32. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227(5259):680-685.
33. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods.* 2012;9(7):671-675.
34. Chang YC, Chen CH, Lin MC. The macronutrients in human milk change after storage in various containers. *Pediatr Neonatol.* 2012;53(3):205-209.
35. Abramovich M. *Human Milk Storage Conditions in regard to safety and optimal preservation of nutritional properties.* University of Manitoba (Canada); 2011.
36. Place M. *Donor Breast Milk Banks: The Operation of Donor Milk Bank Services*. London: National Institute for Health and Clinical Excellence (NICE); February 2010.
37. Aschberger K, Castello P, Hoekstra E, et al. Bisphenol A and baby bottles: Challenges and perspectives. *Luxembourg: Publications Office of the European Union.* 2010;10:5-50.
38. Wang B. Sialic acid is an essential nutrient for brain development and cognition. *Annu Review Nutr.* 2009;29:177-222.
39. Hauser J, Pisa E, Arias Vásquez A, et al. Sialylated human milk oligosaccharides program cognitive development through a non-genomic transmission mode. *Mol Psychiatry.* 2021;26(7):2854-2871.
40. Fernandes JO, Tella SOC, Ferraz IS, Ciampo LAD, Tanus-Santos JE. Assessment of nitric oxide metabolites concentrations in plasma, saliva, and breast milk and their relationship in lactating women. *Mol Cell Biochem.* 2021;476(2):1293-1302.
41. Silvestre D, Löpez M, March L, Plaza A, Martinez-Costa C. Bactericidal activity of human milk: Stability during storage. *Br J Biomed Sci.* 2006;63(2):59-62.

How cite this article

Gurel Gokmen B, Akbay T. The Effects of Refrigerated Storage Time on Sialic Acid and Nitric Oxide Levels and Oxidant Antioxidant System of Human Milk. Eur J Biol 2023; 82(2): 243–250. DOI: 10.26650/EurJBiol.2023.1293969