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The Effect of Freeze-Drying on the Nutrient, Polyphenol, and Oxidant Levels of Breast Milk

Mariela Valentina Cortez[1,2] and Elio Andrés Soria[1]

Abstract

Objectives: Human milk banks need to extend the suitability of milk for breastfeeding, and for this technological advances are required. Our aim was to establish the capacity of freeze-drying to conserve milk properties without further oxidative deterioration.

Methods: One hundred sixteen healthy women participated from the city of Cordoba (Argentina). Proteins, glucose, triglycerides, polyphenols, and markers (nitrites, superoxide anion, hydroperoxides, lipoperoxides, and γ-glutamyl transpeptidase) were measured in their fresh milk. Samples were then separated for three treatments as follows: freezing and conservation for 6 months at −80°C (F: positive control); freeze-drying for 24 hours at ≤−70°C and ≤1.33 Pa and conservation for 6 months at 4°C (FD: treatment of interest); and freeze-drying for 24 hours at ≤−70°C and ≤1.33 Pa and conservation for 6 months at −80°C (FD+F). Next, analyses were repeated and compared by ANOVA and Tukey tests.

Results: Fresh milk showed these values per L as follows: proteins: 12.62 ± 2.51 g, glucose: 4.44 ± 0.25 g, triglycerides: 34.26 ± 0.59 g, polyphenols: 53.27 ± 8.67 mg, nitrites: 62.40 ± 19.09 mg, superoxide: 3,721.02 ± 198.80 OD, hydroperoxides: 7,343.76 ± 294.53 OD, lipoperoxides: 7,349.72 ± 398.72 OD, and γ-glutamyl transpeptidase: 4.66 ± 0.55 IU. Glucose was decreased after F treatment ($p < 0.05$), all variables were conserved by FD and were not improved by the FD + F combination.

Conclusions: Freeze-drying achieved suitable conservation and may improve bank functioning, by protecting nutritional properties, polyphenol-related functionality, and oxidative integrity of human milk through a 1-day treatment with easy maintenance.

Keywords: breastfeeding, macronutrient, milk banking, oxidative stress, phytochemical

Introduction

BREASTFEEDING PLAYS a central role in infant health worldwide, and human milk banks have been created to protect, promote, and support it.[1] These institutions require technological resources to extend the milk's suitability for feeding, which can be limited in developing countries. The technique commonly used by the banks is freezing, but different options are being studied to improve the preservation of important nutrients in milk and thus its health benefits.[2] Freeze-drying (lyophilization) has been proposed and this study assesses its effective application.

Human milk is important for its high content of macronutrients, such as proteins, carbohydrates, and lipids.[3] These, however, are susceptible to oxidative deterioration, which causes loss of nutritional value and increase of deleterious agents, such as nitrites, superoxide anion, hydroperoxides, and lipoperoxides.[4] Also, it is necessary to assay both amounts and bioactivity of proteins. The enzyme, γ-glutamyl transpeptidase (EC 2.3.2.2), can be used as a biomarker of catalytic activity and redox response.[5] Other molecules of interest are the polyphenols, given their functional properties as dietary antioxidants and chemopreventive agents,[6] and there is little information about their role in breastfeeding.

Thus, the aim of this work was to evaluate the effectiveness of freeze-drying for the long-term conservation of milk nutrients, polyphenols, and oxidative markers.

Materials and Methods

Design, treatments, and statistical analysis

This study was approved by the Institutional Ethics Committee of the Hospital Nacional de Clínicas, Universidad Nacional de Córdoba (Argentina), in accordance with current legal and ethical standards (Approval No. 145/2012).

[1]Instituto de Investigaciones en Ciencias de la Salud, Universidad Nacional de Córdoba, CONICET, FCM, Argentina.
[2]Comisión Nacional Salud Investiga, Ministerio de Salud de la Nación, Argentina.

One hundred sixteen women from Cordoba City, Argentina, participated during their first 6 months postpartum after 38.31 ± 0.19 weeks of gestation. This sample size collected from 2013 to 2014 was representative (formula $n = ND^2C^2/((N-1)E^2 + D^2C^2)$, where $n = 96$ (minimal participants), $N = 23{,}474$ total births, $D = 0.5$ distribution, $C = 1.96$ level (95% confidence), and $E = 0.1$ sample error limit). Inclusion criteria were as follows: healthy adult volunteers with normal obstetric controls, including negative serology for infectious diseases, who signed the corresponding informed consent to participate. Exclusion criteria were as follows: chronic use of drugs and nutraceuticals and current pregnancy. Participants were Caucasian, 28.08 ± 5.94 years old, with a current body mass index equal to 24.14 ± 0.45 kg/m^2, matching that expected according to national data.[7,8]

Milk (10 mL) was collected aseptically in a sterile vial and samples were maintained for up to 24 hours at 4°C, to be evaluated twice. These fresh samples were first analyzed chemically and nutritionally, with the results expressed as mean ± standard error. Pearson coefficients were calculated to establish correlations.

They were then separated into three aliquots for the treatments as follows:

(1) Freezing for 6 months at −80°C in a Thermo Scientific 706 Rel#4 device (F: positive control);
(2) Freeze-drying for 24 hours at ≤−70°C and ≤1.33 Pa, using Thermovac equipment, and conservation for 6 months at 4°C to avoid temperature variations (FD: treatment of interest);
(3) Freeze-drying for 24 hours at ≤−70°C and ≤1.33 Pa and 6 months of conservation at −80°C (FD+F).

Subsequently, after the samples were reconstituted, the analyses were repeated to calculate the relative changes with respect to the amounts found in fresh milk (%). Treatment outcomes, expressed as mean of individual samples ± standard error, were compared by ANOVA followed by the Tukey test using the software InfoStat v.2012 (InfoStat Group, Argentina), with a significance at $p < 0.05$.

Chemical and nutritional analysis of milk

Nutrients (proteins, glucose, and triglycerides) were measured by spectrophotometry at 540 nm in a Promega GloMax-Multi Microplate Multimode Reader (USA) to calculate g/L units, using kits provided by Wiener Lab (Argentina) in accordance with the manufacturer's instructions.

Polyphenol concentration was measured spectrophotometrically at 720 nm using the Folin–Ciocalteu method[9] to calculate mg/L units with a standard curve of gallic acid (0–100 mg/L). In brief, samples were incubated for 30 minutes with 2 N Folin reactant, water, and a saturated sodium bicarbonate solution (1:1:6:2 v/v/v/v), in darkness at 37°C.

Nitrites, used as nitrosative stress markers, were assayed by the Griess reaction,[10] with reactants purchased from Wiener Lab. Samples were reacted with equal volumes of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 0.1 N HCl at room temperature for 15 minutes. A standard curve was used to calculate mg/L units at 550 nm.

Superoxide anion was measured at 600 nm by nitroblue tetrazolium staining.[11] Samples were mixed with it (9:1 v/v) for 30 minutes in darkness at 37°C. Then, they received dimethylsulfoxide and potassium hydroxide (2:1:1 v/v/v) before reading absolute optical density (OD/L).

Samples were mixed with a xylenol orange-based reactant (1:10 v/v) and incubated for 30 minutes to reveal hydroperoxides and lipoperoxides as OD/L at 540 nm.[12]

Activity of γ-glutamyl transpeptidase was measured using the kinetic Szasz method.[13] In brief, samples were reacted at 25°C with a substrate solution (pH 8.25, 100 mM Tris buffer, 2.9 mM L-γ-glutamyl-3-carboxy-4-nitroanilide, 100 mM glycylglycine). International units (IU/L) were calculated by multiplying the absorbance difference/minute by the constant 1,158. The reading was made at 405 nm, under conditions of initial velocity and linearity ranges.

Results

Three macronutrients were measured in fresh human milk as follows:

- Proteins: 12.62 ± 2.51 g/L
- Glucose: 4.44 ± 0.25 g/L
- Triglycerides: 34.26 ± 0.59 g/L

As a marker of protein enzymatic activity, γ-glutamyl transpeptidase was assayed (4.66 ± 0.55 IU/L of fresh milk).

Table 1. Milk Constituents Before (Fresh Milk) and After 6-Month Conservation by Different Methods

Nutrients and phytochemicals				
Methods	*Proteins (g/L)*	*Triglycerides (g/L)*	*Glucose (g/L)*	*Polyphenols (mg/L)*
None (fresh milk)	12.62 ± 2.51	34.26 ± 0.59	4.44 ± 0.25	53.27 ± 8.67
Freezing	12.65 ± 3.60	45.27 ± 10.65	2.08 ± 0.44[a]	76.50 ± 23.00
Freeze-drying	06.28 ± 1.80	25.41 ± 5.41	3.24 ± 0.44	51.19 ± 13.16
Freeze-drying+freezing	12.35 ± 3.37	36.22 ± 6.81	4.16 ± 0.58	69.41 ± 18.64
Oxidative markers				
Methods	*Lipoperoxides (OD/L)*	*Hydroperoxides (OD/L)*	*Superoxide anion (OD/L)*	*Nitrites (mg/L)*
None (fresh milk)	7,349.72 ± 398.72	7,343.76 ± 294.53	3,721.02 ± 198.80	62.40 ± 19.09
Freezing	11,577.3 ± 3,224.32	7,970.18 ± 1,764.70	5,059.10 ± 1,092.86	97.66 ± 36.44
Freeze-drying	8,637.39 ± 2,729.69	5,693.62 ± 1,623.70	6,203.68 ± 1,181.05	72.13 ± 23.71
Freeze-drying+freezing	9,323.85 ± 2,174.05	6,148.20 ± 1,346.85	5,580.04 ± 1,153.14	60.84 ± 14.29

[a]Significant difference with respect to fresh milk ($p < 0.05$).

Regarding phytochemicals, the total polyphenol concentration in milk was 53.27 ± 8.67 mg/L.

The oxidative markers measured in fresh human milk were as follows:

- Nitrites: 62.40 ± 19.09 mg/L
- Superoxide: 3,721.02 ± 198.80 OD/L
- Hydroperoxides: 7,343.76 ± 294.53 OD/L
- Lipoperoxides: 7,349.72 ± 398.72 OD/L

Additional assessments showed that the polyphenol concentration was inversely correlated with the concentrations of lipoperoxides and hydroperoxides (coefficient = –0.34 and –0.29, respectively, $p < 0.05$). On the contrary, glucose concentration was directly correlated with the levels of these oxidative markers (coefficient = 0.36 and 0.43, respectively, $p < 0.05$).

After 6 months of conservation, a decrease in glucose was found after the F treatment ($p < 0.05$), but all the study variables were conserved by FD, and were not improved by the FD + F combination (Table 1). Also, the activity of γ-glutamyl transpeptidase with respect to that found in fresh milk was as follows: F: 043.60% ± 43.0%, FD: 096.10% ± 25.0%, and FD+F: 130.30% ± 35.0%.

Discussion

The amounts of polyphenols found by this study in the breast milk of Argentinean women were higher than those found in other populations.[14,15] Moreover, although diet supplementation has been proposed as antioxidant,[16] the mothers in the study had significant milk levels of antioxidant polyphenols without supplementation, due to the exclusion criteria. These diet photochemicals bound to plasma albumin reach mammary parenchyma by cellular affinity,[14,16] which can be promoted by several factors. These include the total time dedicated to breastfeeding previous and current children, which is positively associated with milk polyphenolic concentration ($p < 0.0001$, unpublished data), which supports the chemopreventive benefits of breastfeeding through polyphenols.[17–19]

Regarding milk nutrients, the protein content was in accordance with scientific literature, with the sample mean being higher than the general mean, but nonsignificantly.[3] Protein activity, that is, γ-glutamyl transpeptidase,[10] was also conserved.[20,21] Triglycerides represent about 98% of total milk lipids, and the content found also matched previous reports.[3] On the contrary, free glucose levels were higher than expected,[22] because glucose can be released from lactose of fresh milk maintained at 4°C.[23] In addition, there is a deleterious interplay between metabolic and oxidative pathways,[24] as confirmed by the positive relationship of milk peroxides and glucose in the study samples. Moreover, obesogenic diathesis impairs milk lipogenesis,[25] which can reduce triglyceride secretion and increase milk peroxides ($p < 0.05$, unpublished data).

Freeze-drying prevents microbial proliferation.[26] We found in this study that it also preserved nutrients and oxidative markers and was better than freezing, which is one of the commonest methods used for human milk banking,[27] but which did not protect glucose during long-term storage. This loss depends on nonoxidative glucose instability in aqueous biological samples.[27,28]

Proteins were better preserved by freezing, since freeze-drying stores the samples at a higher temperature, and the remaining moisture enables protein reduction.[29] Nonetheless, no significant differences were found in protein quantity and enzymatic activity, using γ-glutamyl transpeptidase as a marker of protein functionality.[10] Protein functionality has been found to be protected by freeze-drying and lost by heating treatments.[30] After freezing, a concomitant reduction of this enzyme and of glucose confirmed the role of this sugar protecting protein functional integrity, which is more efficient in FD (micromolar ranges) than in F (molar ranges).[26]

On the contrary, although both FD and F prevented oxidative stress as expected because of cold,[31] freeze-drying was not improved by additional freezing under the combined treatment (FD+F). This is important because milk oxidation initiated by superoxide produces peroxides from nutrients, leading to nutritional loss and exposing infants to free radicals.[32]

Conclusion

Freeze-drying is seen to achieve suitable conservation and may improve bank functioning, because it protects the nutritional properties, polyphenol-related functionality, and the oxidative integrity of human milk, through a 1-day treatment followed by simple maintenance.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Elio Andrés Soria, MD, PhD
Instituto de Investigaciones en Ciencias de la Salud
Universidad Nacional de Córdoba CONICET
FCM Córdoba 5014
Argentina

E-mail: easoria@fcm.unc.edu.ar

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