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Effect of cold storage on vitamins C and E and fatty acids in human milk

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Abstract

Vitamins C and E and fatty acid levels in human milk were determined when fresh, after refrigeration at 4 °C for 96 h, and after freezing at −20 °C or −80 °C for 12 months. Total vitamin C content at 4 °C (6 h), −20 °C (8 months) and −80 °C (12 months) was significantly decreased. Vitamin E levels did not change at either refrigeration temperature (under 24 h) or at freezing or ultrafreezing temperatures. Our analysis revealed that fatty acids are not affected by cold storage. In conclusion, we recommend a change in milk storage practices; specifically, it should be stored up to 3 h in a refrigerator, up to 5 months in a freezer or up to 8 months in an ultrafreezer (−80 °C). Alternatively, vitamin C supplementation may be considered. In addition, we propose vitamin C as a marker for human milk stability.

Keywords: Ascorbic acid; Fatty acid; Freezing; Refrigeration; Tocopherol; Vitamin

1. Introduction

Human breast milk is regarded as the most important nutrient for neonates, especially preterm infants (Lindemann, Foshaugen, & Lindemann, 2004). The early use of breast milk for preterm infants has resulted in a reduced incidence of necrotising enterocolitis, faster tolerance of enteral feeding and thus a reduced need for parenteral nutrition.

If the mother does not produce sufficient milk, frozen milk from a bank should be made available to all ill neonates until the mother's own production is established (Lindemann et al., 2004). Of equal value, however, is the practice of mothers collecting their own milk for later feeding (Ogundele, 2002). Possible influences on the stability of milk properties include temperature and storage. Current recommendations for storing human milk in neonatal units and at home vary: for refrigerator storage, from 24–48 h (Biancuzzo, 1999) to 3–5 days (Lawrence & Lawrence, 1999)–even up to 8 days (La Leche League International, 1998); for freezer storage at −18 °C, from 3 to 12 months (Biancuzzo, 1999; La Leche League International, 1998; Lawrence & Lawrence, 1999). In human milk banks, donor milk can be either stored at 4 °C in a refrigerator from 24 h (Baumer, 2004) up to 8 days (HMBANA, 2006), frozen at −20 °C from 3 months (Baumer, 2004) up to 12 months (HMBANA, 2006); or frozen at −70 °C for even longer periods (Baumer, 2004; HMBANA, 2006).

Vitamins E (specifically α-and γ-tocopherols) and C are crucial for anti-oxidant activity and immunomodulation (Jensen, 1995). Preterm infants have reduced antioxidant capacity and are often exposed to oxidative stress caused by infection, oxygen, mechanical ventilation, intravenous nutrition, and blood transfusions (Hanna et al., 2004). Preterm infants who ingest human milk rapidly increase their antioxidant concentrations (Sommerburg, Meissner, Nelle, Lenhartz, & Leichsenring, 2000; Van Zoeren-Grobben, Moison, Ester, & Berger, 1993).

Long-chain polyunsaturated fatty acids (LC-PUFAs), specifically arachidonic acid (C20:4*n*−6, AA) and

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docosahexaenoic acid (C22:6*n*−3, DHA), are of major importance during the perinatal period, during which the brain and retina are developing. They therefore have an influence upon visual acuity and learning abilities (Innis, 2004).

The usual cold storage conditions of human milk cited in the literature are as follows: refrigeration at temperatures between 4 and 6 °C for 48–72 h (Igumbor, Mukura, Makandirama, & Chihota, 2000; Lawrence, 2001; Ogundele, 2002) and under freezing conditions at temperatures between −20 °C and 70 °C for 15–90 days (Bitman, Wood, Mehta, Hamosh, & Hamosh, 1983; Buss, McGill, Darlow, & Winterbourn, 2001; Lawrence, 2001; Ogundele, 2002). These periods are used to minimise bacterial growth rather than preserve any nutritional properties. However, storage can result in the loss of nutrients sensitive to oxidation, such as vitamins C and E, since both are sensitive to light, oxygen and temperature (Miquel, Alegría, Barberá, Farré, & Clemente, 2004). Loss of LC-PUFAs can also occur as they contain a large number of double bonds.

Nevertheless, there is scant historical data addressing how vitamins C and E and fatty acids change during cold storage. Therefore, we investigated potential differences in the concentrations of antioxidant vitamins C and E, as well as in the percentage of fatty acids, between fresh and stored milk at three different cold temperatures: refrigeration (4 °C) and frozen at −20 °C or −80 °C. Thus, the objective of our study was to adopt recommendations on the cold storage of human milk, based on the stability of vitamins C and E and fatty acids in human milk stored at these temperatures.

2. Materials and methods

2.1. Sample collection

Identical volumes (50 ml) of mature human milk samples (term) were collected from both breasts by means of a Chicco manual breast pump (Chicco®, Italy), following the manufacturer's instructions, from ten healthy mothers (age 20–35 years) at the department's Extraction Unit. Human milk was collected into sterile, opaque bottles in the morning, at first expression. Milk samples from five mothers were directly pooled, obtaining two pools that were divided into 10 aliquots each. Fresh samples were immediately tested, and the rest of the aliquots were stored at 4 °C, −20 °C or −80 °C. These volumes were sufficiently large to provide twenty samples for every analysis (10 by each pool). Before processing, milk samples were thawed at room temperature.

2.2. Storage conditions

For this study, human milk aliquots were stored under three different conditions: in the refrigerator at 4 °C (for analyses at 3, 6, 9, 12, 24, 48, 72 and 96 h); in the freezer at −20 °C (for analyses at 3, 5, 8 and 12 months) and in the freezer at −80 °C (for analyses at 5, 8 and 12 months).

2.3. Total Vitamin C analysis

Total vitamin C content was measured following the direct method described by Romeu-Nadal, Morera-Pons, Castellote, and López-Sabater (2006a). In order to analyse the amount of vitamin C, dehydroascorbic acid was reduced to ascorbic acid with dithiothreitol. The latter was resolved by reversed-phase high-performance liquid chromatography (C-18) using a mobile phase of Milli-Q water with acetic acid (0.1% v/v) and methanol (in a relative proportion of 95:5 v/v) and was detected at 254 nm.

2.4. Vitamin E analysis

α- and γ-tocopherols were separated and quantified with reverse-phase high-performance liquid chromatography (C-18) using a mobile phase of acetonitrile:methanol:dichloromethane (60:38:2v/v) following the direct method described by Romeu-Nadal, Morera-Pons, Castellote, and López-Sabater (2006b). The human milk, with the addition of an internal standard (α-tocopherol acetate), was diluted in hexane. The dried sample was reconstituted in a dichloromethane:acetonitrile (3:1) solution. Detection was performed at 292 nm.

2.5. Fatty acids analysis

Fatty acid methyl esters (FAMEs) were prepared with sodium methylate and methanolic BF_3 and dissolved in hexane, following the method described by López-López, Castellote, and López-Sabater (2001). The fatty acids were separated and quantified with fast gas chromatography.

Fast gas chromatographic analyses were performed on a Shimadzu GC-2010 gas chromatograph (Kyoto, Japan) equipped with a flame ionisation detector and a Shimadzu AOC-20i Autoinjector. Separation of the FAME was carried out on a capillary column (10 m × 0.10 mm I.D) coated with a Varian VF-23 ms stationary phase (high cyanopropyl phase, 0.10 μm film thickness) from Varian (Palo Alto, USA). The operation conditions were as follows: the split-splitless injector was used in split mode with a ratio of 1:100. The injection volume of the sample was 1 μl. The injector and detector temperatures were kept at 250 °C and 270 °C, respectively. The temperature program was as follows: initial temperature: 120 °C, increased at 35 °C/min until reaching 175 °C (kept 0.5 min); increased at 20 °C/min until reaching 250 °C. Helium was used as the carrier gas, with a linear velocity of 59.4 cm/s (the average at 120 °C). Pressure: 482 kPa; detector gas flows: H2: 50 ml/min; air: 400 ml/min; make-up gas (N2): 50 ml/min; sampling frequency:

50 Hz. Data acquisition and processing were performed with Shimadzu-Chemstation software for gas chromatographic systems.

2.6. Statistical analysis

The values reported for each aliquot were compared using paired Student's *t*-tests. The level of statistical significance was set at 5%. The results were processed using the statistical package SPSS 10.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Effect of storage on total vitamin C concentration

Table 1 shows the concentration of total vitamin C in pooled human milk before and after storage at 4 °C for 96 h, as well as at −20 °C and −80 °C for 12 months.

When calculating total vitamin C levels it is important to measure both ascorbic and dehydroascorbic acid levels. Ascorbic acid is the principal biologically active form of vitamin C. However, dehydroascorbic acid also exhibits biological activity since the human body can easily convert it into ascorbic acid (Packer & Fuchs, 1997).

After 3 h refrigeration, the total vitamin C concentration was slightly lower than that found in fresh milk. As storage continued (6, 9, 12, 24, 48, 72 and 96 h) the total vitamin C levels in milk decreased significantly ($P < 0.05$). After 96 h, the decrease was equal to 63% with respect to initial concentration. These losses were lower when human milk was stored at −20 °C and −80 °C, decreasing 24% and 12% after 12 months, respectively. There were no differences in total vitamin C levels between fresh milk and stored milk (−20 °C) over 5 months, or of that stored in the ultrafreezer (−80 °C) for 8 months.

Table 1
Effects of cold storage on several compounds[a] (mg/L) in human milk

Milk samples	Total vitamin C[b]	α-Tocopherol	γ-Tocopherol
Fresh	34.7 ± 1.33	3.85 ± 0.16	0.37 ± 0.02
4 °C (3 h)	33.9 ± 0.79	3.88 ± 0.15	0.36 ± 0.02
4 °C (6 h)	32.6 ± 0.77*	3.92 ± 0.18	0.38 ± 0.01
4 °C (9 h)	32.9 ± 1.34*	3.93 ± 0.17	0.37 ± 0.01
4 °C (12 h)	32.1 ± 1.36*	3.93 ± 0.19	0.37 ± 0.02
4 °C (24 h)	28.4 ± 1.18*	3.74 ± 0.11	0.36 ± 0.01
4 °C (48 h)	24.4 ± 1.22*	3.25 ± 0.11*	0.32 ± 0.01*
4 °C (72 h)	16.9 ± 1.05*	3.08 ± 0.12*	0.29 ± 0.01*
4 °C (96 h)	12.8 ± 0.51*	2.88 ± 0.10*	0.26 ± 0.011*
−20 °C (3 months)	34.7 ± 1.33	3.81 ± 0.13	0.37 ± 0.02
−20 °C (5 months)	33.9 ± 1.35	3.80 ± 0.16	0.36 ± 0.01
−20 °C (8 months)	30.1 ± 1.09*	3.81 ± 0.17	0.35 ± 0.01
−20 °C (12 months)	26.5 ± 1.23*	3.77 ± 0.15	0.38 ± 0.02
−80 °C (5 months)	34.1 ± 1.35	3.76 ± 0.14	0.38 ± 0.01
−80 °C (8 months)	34.0 ± 0.90	3.73 ± 0.12	0.36 ± 0.01
−80 °C (12 months)	30.4 ± 0.65*	3.78 ± 0.14	0.37 ± 0.02

[a] Mean of twenty measurements (ten by each pool) ± standard deviation.
[b] Total vitamin C: ascorbic acid + dehydroascorbic acid.
* $p < 0.05$ vs. fresh human milk.

3.2. Effect of storage on α- and γ-tocopherol concentrations

Table 1 shows the concentration of α- and γ-tocopherols in pooled human milk before and after storage at 4 °C for 96 h or at −20 °C and −80 °C for 12 months.

The α- and γ-tocopherol concentrations did not change during refrigeration for 24 h or at freezing temperatures (−20 °C or −80 °C) over 12 months. However, refrigeration significantly decreased α- and γ-tocopherol concentrations after 96 h of storage, decreasing about 25% and 30% in α- and γ-tocopherols, respectively.

3.3. Effect of storage on fatty acid percentages

Table 2 shows the percentage of fatty acids in pooled fresh human milk. Saturated fatty acids (SFAs) constitute

Table 2
Fatty acids (%) found in fresh human milk

Fatty acid	Mean ± SD[a]
C8:0	0.16 ± 0.01
C10:0	1.04 ± 0.07
C12:0	4.74 ± 0.26
C14:0	5.55 ± 0.28
C14:1	0.11 ± 0.01
C15:0	0.16 ± 0.00
C15:1	0.05 ± 0.00
C16:0	19.8 ± 0.42
C16:1 $n-7+n-9$	1.69 ± 0.08
C17:0	0.25 ± 0.01
C17:1	0.18 ± 0.02
C18:0	7.34 ± 0.22
C18:1$n-9$	38.1 ± 1.84
C18:2$n-6$	17.3 ± 0.77
C18:3$n-6$	0.13 ± 0.01
C18:3$n-3$	0.66 ± 0.05
C20:0	0.25 ± 0.03
C20:1$n-9$	0.30 ± 0.02
C21:0	0.29 ± 0.02
C20:2$n-6$	0.24 ± 0.01
C20:3$n-6$	0.30 ± 0.01
C20:4$n-6$, AA[b]	0.44 ± 0.02
C22:0	0.08 ± 0.01
C22:1	0.11 ± 0.01
C22:2	0.05 ± 0.00
C20:5$n-3$	0.04 ± 0.00
C22:4$n-6$	0.13 ± 0.01
C22:5$n-6$	0.02 ± 0.00
C24:1	0.03 ± 0.00
C22:5$n-3$	0.10 ± 0.01
C22:6$n-3$, DHA[c]	0.25 ± 0.01
SFAs[d]	39.6 ± 3.23
MUFAs[e]	40.6 ± 3.41
PUFAs[f]$n-6$	18.2 ± 1.13
PUFAs[f]$n-3$	1.05 ± 0.09

$n = 20$ (ten by each pool).
[a] Standard deviation.
[b] AA: arachidonic acid.
[c] DHA: docosahexaenoic acid.
[d] SFAs: saturated fatty acids.
[e] MUFAs: monounsaturated fatty acids.
[f] PUFAs: polyunsaturated fatty acids.

Table 3
Effects of cold storage on selected fatty acids[a] (%) in human milk

Milk samples	AA[b] C20:4$n-6$	DHA[c] C22:6$n-3$	Linoleic acid C18:2$n-6$	Linolenic acid C18:3$n-3$	SFAs[d]	MUFAs[e]	PUFAs[f] $n-6$	PUFAs[f] $n-3$
4 °C (3 h)	0.42 ± 0.01	0.25 ± 0.01	17.9 ± 0.09	0.64 ± 0.02	38.9 ± 3.10	40.2 ± 3.43	18.8 ± 0.78	1.05 ± 0.09
4 °C (6 h)	0.43 ± 0.02	0.25 ± 0.01	17.9 ± 0.12	0.64 ± 0.01	38.9 ± 3.30	40.5 ± 3.22	18.7 ± 0.82	1.04 ± 0.09
4 °C (9 h)	0.44 ± 0.01	0.25 ± 0.01	17.8 ± 0.07	0.64 ± 0.01	39.0 ± 3.21	40.4 ± 3.71	18.7 ± 0.78	1.03 ± 0.09
4 °C (12 h)	0.43 ± 0.01	0.24 ± 0.01	17.8 ± 0.30	0.64 ± 0.01	38.6 ± 3.48	40.3 ± 3.78	18.6 ± 1.04	1.02 ± 0.05
4 °C (24 h)	0.43 ± 0.01	0.25 ± 0.01	18.0 ± 0.18	0.65 ± 0.01	39.4 ± 3.56	40.8 ± 3.11	18.8 ± 1.21	1.02 ± 0.08
4 °C (48 h)	0.43 ± 0.01	0.24 ± 0.01	17.9 ± 0.08	0.66 ± 0.02	39.1 ± 3.37	40.9 ± 3.56	18.7 ± 1.33	1.05 ± 0.08
4 °C (72 h)	0.43 ± 0.01	0.25 ± 0.01	17.9 ± 0.10	0.67 ± 0.02	38.7 ± 2.97	40.6 ± 3.26	18.8 ± 1.10	1.06 ± 0.08
4 °C (96 h)	0.44 ± 0.01	0.25 ± 0.01	17.9 ± 0.07	0.66 ± 0.01	38.7 ± 3.13	40.7 ± 3.41	18.8 ± 1.04	1.04 ± 0.06
−20 °C (3 months)	0.44 ± 0.02	0.25 ± 0.01	17.7 ± 0.70	0.65 ± 0.03	39.0 ± 3.18	40.2 ± 3.54	18.6 ± 1.19	1.04 ± 0.07
−20 °C (5 months)	0.44 ± 0.01	0.26 ± 0.01	17.8 ± 0.14	0.66 ± 0.01	39.7 ± 3.44	40.3 ± 3.51	18.7 ± 1.03	1.06 ± 0.08
−20 °C (8 months)	0.44 ± 0.01	0.25 ± 0.01	17.7 ± 0.03	0.66 ± 0.01	39.9 ± 2.93	40.1 ± 3.27	18.6 ± 1.12	1.07 ± 0.07
−20 °C (12 months)	0.44 ± 0.02	0.26 ± 0.01	17.6 ± 0.13	0.65 ± 0.01	40.9 ± 3.34	39.0 ± 3.01	18.4 ± 1.03	1.09 ± 0.07
−80 °C (5 months)	0.44 ± 0.01	0.25 ± 0.01	18.0 ± 0.06	0.65 ± 0.01	39.2 ± 3.51	40.3 ± 3.38	18.8 ± 1.26	1.04 ± 0.09
−80 °C (8 months)	0.45 ± 0.02	0.26 ± 0.01	17.9 ± 0.24	0.64 ± 0.01	40.0 ± 3.02	39.7 ± 3.19	18.8 ± 1.02	1.04 ± 0.08
−80 °C (12 months)	0.45 ± 0.01	0.25 ± 0.00	18.0 ± 0.18	0.64 ± 0.02	39.0 ± 2.94	40.6 ± 3.04	18.8 ± 1.41	1.04 ± 0.07

[a] Mean of twenty measurements (ten by each pool) ± standard deviation.
[b] AA: arachidonic acid.
[c] DHA: docosahexaenoic acid.
[d] SFAs: saturated fatty acids.
[e] MUFAs: monounsaturated fatty acids.
[f] PUFAs: polyunsaturated fatty acids.

40% of the total acids in the lipids of mature breast milk. Palmitic acid (C16:0) accounts for 49.7% of the saturated fatty acids in mature milk saturates. Unsaturated fatty acids account for 60% of the total fatty acids in milk. These unsaturated fatty acids are mostly monounsaturated (MUFAs), constituting 41% of total fatty acids. The polyunsaturated content of both the linoleic (C18:2$n-6$) and linolenic (C18:3$n-3$) acid series constitute 18% and 1% of the total acids, respectively. AA and DHA account for 2.4% and 23.8% of the total polyunsaturated fatty acids $n-6$ and $n-3$, respectively.

Table 3 shows AA, DHA, essential fatty acids (linoleic and linolenic acids), SFAs, MUFAs, and the polyunsaturated fatty acids (PUFAs) $n-6$ and $n-3$ percentages in pooled human milk stored at 4 °C, −20 °C and −80 °C. Fatty acid percentages did not change at either refrigeration temperature over 96 h or at freezing and ultrafreezing temperatures for 12 months.

4. Discussion

The effect of storage on the various components of human milk has been studied extensively. However, most of these studies have focused on bacteriological and immunological effects (Igumbor et al., 2000; Lawrence, 2001; Ogundele, 2002), devoting scant attention to its effect on vitamins C and E and fatty acid levels.

The main findings of our study revealed a decrease in the total vitamin C concentration in human milk stored at both the refrigeration and freezing temperatures recommended by HMBANA (2006), La Leche League International (1998), and Lawrence and Lawrence (1999). Moreover, we observed a decrease in vitamin E concentration in human milk stored at refrigeration temperatures after 24 h, that is, storage at 4 °C for 3–8 days, even though this is a recommended temperature described by the aforementioned authors (HMBANA, 2006; La Leche League International, 1998; Lawrence & Lawrence, 1999). The maximum decrease in vitamins C and E was detected in human milk samples stored at refrigeration temperatures for 96 h. Refrigeration led to greater losses in vitamin C than did storage at freezing (−20 °C) or ultrafreezing temperatures (−80 °C).

The total mean vitamin C concentration detected in our fresh samples (35.1 ± 1.42 mg/L) was in agreement with that reported by Buss et al. (2001) (23.3–80.4 mg/L). The 18% loss of total vitamin C in refrigerated human milk after 24 h was less than the 35% decrease found by Buss et al. (2001). The loss of vitamin C in milk caused by storage may stem from the increased conversion of ascorbic to dehydroascorbic acid and then, in turn, to diketogulonic acid (Naidu, 2003). Buss et al. (2001) observed that vitamin C losses were partly due to lactoperoxidase activity since adding the peroxidase inhibitor potassium cyanide (KCN) to human milk samples provided some protection against these losses.

The mean α- and γ-tocopherol concentrations in our fresh samples were 3.85 ± 0.16 mg/L and 0.37 ± 0.02 mg/L, respectively. The α-tocopherol levels detected in this study were similar to those reported by Hoppu et al. (2005) in human milk (3.7–4.8 mg/L). Coinciding with our own findings, other authors have reported that vitamin E content in human milk is quite stable under various storage conditions, for up to one week, as well as freezing at −20 °C or −70 °C for a longer time period (Van Zoeren-Grobben et al., 1993).

As the stability of vitamin C proved lower than that of vitamin E, it may constitute an effective indicator of human milk stability. This vitamin has already been proposed as a marker of oxidative stress in biological samples (Lykkesfeldt, Loft, & Poulsen, 1995).

Our observation that vitamins C and E, especially the former, are lost during certain cold storage periods indicates that infants receiving stored human milk receive fewer anti-oxidant compounds than those given fresh milk (Miranda et al., 2004), since these vitamins are the components responsible for anti-oxidant activity (Sommerburg et al., 2000). The maximum decrease in vitamins C and E was detected at the refrigeration temperature over 96 h.

In neonatal units, infants may be fed stored milk for extended periods of time, the oldest milk being given first. While many of these infants are supplemented with vitamin C, term infants usually do not receive such supplementation. They may still be given stored milk, though usually not exclusively. Our main point of concern is the loss of vitamin C that occurs in stored samples. Storage time should therefore be kept as brief as possible. If long-term stored milk constitutes a major and regular proportion of consumption, vitamin C supplementation may be necessary.

Our results indicate that recommended storage conditions for human milk used in neonatal units, in the home and in milk banks (Baumer, 2004; Biancuzzo, 1999; HMBANA, 2006; La Leche League International, 1998; Lawrence & Lawrence, 1999) exert no significant effect upon fatty acid percentages. Previous published studies found that LC-PUFAs were unaffected by heat treatment, such as pasteurisation at 62.5 °C for 30 min (Fidler, Sauerwald, Demmelmair, & Koletzko, 2001; Henderson, Fay, & Hamosh, 1998; Lepri, Bubba, Maggini, Donzelli, & Galvan, 1997). Our observation that LC-PUFAs are unaffected by cold storage indicates that infants receiving this stored milk are not deprived of these crucial components. The stability of these LC-PUFAs during cold storage probably stems from the high anti-oxidant activity of human milk (Buescher & McIlehan, 1992; Henderson et al., 1998). In particular, arachidonic acid and docosahexaenoic acid, which we detected in fresh milk at levels of 0.44% and 0.25% respectively, were similar to those reported by Koletzko, Mrotzek, Eng, and Bremer (1988) (0.36 and 0.22%), Lepri et al. (1997) (0.42 and 0.21%) and Henderson et al. (1998) (0.52 and 0.21%) in pooled human milk.

5. Conclusions

In conclusion, fatty acid percentages did not decrease at the recommended refrigeration, freezing or ultrafreezing temperatures. The same was observed for vitamin E content in human milk stored at both freezing and ultrafreezing temperatures. On the contrary, the extent of vitamin C loss during storage was considerable. Thus, we recommend a change in some human milk storage practices, specifically, it should be stored up to 3 h in a refrigerator, up to 5 months in a freezer or up to 8 months in an ultrafreezer. Alternatively, vitamin C supplementation may be considered. In addition, we propose vitamin C as a marker for human milk stability.

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