Pediatrics and Neonatology (2018) xx, 1–6

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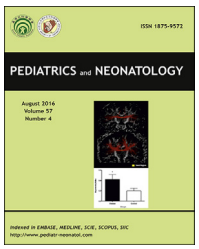

Original Article

Total antioxidant status in fresh and stored human milk from mothers of term and preterm neonates

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Received May 26, 2017; received in revised form Aug 2, 2017; accepted Feb 5, 2018
Available online ■ ■ ■

Key Words
early lactation;
human milk;
preterm;
term;
total antioxidant status

Background: Antioxidant defense of the body is assured by both endogenous and exogenous factors comprising several enzymes, vitamins, protein components and derivates and oligoelements. Breast milk has been proven to have important and essential antioxidant composition to prevent and protect against diseases in infancy. The objective of this study was to determine the total antioxidant status (TAS) of human milk and to evaluate the differences between premature milk and term milk at different moments of lactation (colostrum, transitional milk and mature milk). A second objective was to evaluate how TAS varies whether the human milk is refrigerated or frozen.

Methods: Pumped human milk samples of the third, seven and 30th day were collected from women who had term deliveries (30 cases) and preterm deliveries (60 cases). Samples were refrigerated (+4 °C) or frozen in domestic conditions (−20 °C) for various durations and TAS was determined using the ABTS® technique with Randox® reagents and compared for the two groups.

Results: Higher values were found in term versus preterm fresh milk at 30 days of lactation. A slight reduction in TAS was found after 72 h of refrigeration, while 1 week freezing produced significant decrease of total antioxidants. Freezing for 12 weeks reduced more than 50% of TAS in fresh milk.

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https://doi.org/10.1016/j.pedneo.2018.02.004

Conclusion: Breastfeeding provides the optimal antioxidant for neonates, regardless of gestational age. Fresh milk has the higher antioxidant power. When it is not available, refrigerated milk for 24 h is better than for 72 h and preferable than frozen milk. Freezing human milk for 3 months in household conditions markedly diminishes TAS.

1. Introduction

Free radicals are highly reactive molecules containing one or more unpaired electrons. They donate or gain electrons from other molecules in an attempt to pair their electrons and generate a more stable species.[1,2] Free radicals are normally produced in living organisms. Animal studies proved that, when produced in physiological concentrations, reactive oxygen species behave as important mediators of almost all cell functions. On the other hand, when excessively produced, they induce oxidative stress, which is responsible for cell and tissue injury.[3–5] Free radical reactions may cause alterations of macromolecules, such as polyunsaturated fatty acids and proteins.[6] Under physiological circumstances, free radicals are kept under control by an adequate antioxidant system whose activation depends upon the entity of the oxidative injury itself. Both in human and veterinary medicine, oxidative stress may be a cofactor in the development of many neonatal dysfunctions, leading to serious systemic effects and impairing vitality.[7–9]

Breast milk has been proven to have important and essential antioxidant composition to prevent and protect against diseases in infancy.

Many components of milk change with storage, including immune cells, which get inactivated by freezing.[10,11] Lipid peroxides formation in human milk stored at low temperatures was documented, which was probably caused by an increased presence of free fatty acids due to lipoprotein lipase activity during storage.[12] This could be ascribed to the higher susceptibility of human milk to degradation, a process that is not demonstrated in formula milk.[13]

Miranda et al. found an increase of malondialdehyde, a marker of oxidative stress, in refrigerated milk but not in frozen samples and a decrease of glutathione peroxidase activity in both refrigerated and frozen samples of human milk.[14]

In order to measure the overall antioxidant activity of plasma and body fluids, total antioxidant status (TAS) is used, as it includes the cumulative action of all the antioxidants, thus providing an integrated parameter rather than the simple sum of measurable antioxidants.[15]

2. Objective

The main study goal was to determine the total antioxidant status (TAS) of human milk and evaluate the differences between premature milk and term milk at different moments of lactation (colostrum, transitional milk and mature milk). A second objective was to evaluate how TAS varies whether the human milk is refrigerated (+4 °C) or frozen in domestic conditions (−20 °C), as is in our routine practice, in the absence of a milk bank.

3. Methods

The study involved two groups of lactating mothers admitted to a level III maternity hospital from eastern Romania, an area with the highest natality rate, but with low income and poor economic status: group 1 enrolled 60 mothers who gave birth prematurely (<37 weeks' gestation) and group 2 included 30 mothers who delivered term infants (≥37 weeks' gestation).

The inclusion criteria were as follows: mothers of premature or term infants whose lactation started in the first 3 ± 1 days postpartum, aged 16—40 years, with normal recovery after delivery. Cases with any acute illness, treatment with antibiotics or any drugs that potentially pass in human milk, chronic treatment for epilepsy, cancer, thyroid conditions, arterial hypertension and breast infections were excluded.

Human milk from days 3 ± 1 (colostrum), 7 ± 1 (transitional milk), and 30 ± 1 (mature milk) was pumped under the same conditions, between 8:00 and 11:30 a.m., using the electric breast pump (Medela® Symphony Plus, McHenry, IL, USA) and divided in five aliquots/subject (2 ml sample in Eppendorf plastic tubes). Two samples were refrigerated at +4 °C, two were frozen at −20 °C and one, with fresh milk, was analyzed in the following 2 h.

TAS was measured from the following milk types: fresh (labeled FM), refrigerated for 24 h (labeled R24) and 72 h (R72), and frozen for 1 week (F1) and 12 weeks (F12), respectively. For each patient a total of 15 aliquots were analyzed.

The method used ABTS® spectrophotometric technique (Boeringer-Mannheim, Germany), Rx-IMOLA® analyzer (Randox Laboratories, Crumlin, County Antrim, Northern Ireland), RANDOX® reagents and calibrators.

Results were expressed as mmol/L. Samples where a concentration higher than 2.5 mmol/L was detected were diluted with a solution of double distilled water and remeasured. In the present study few samples were retested, as there were very few values of less than 2.5 mmol/L.

Informed consent was obtained from each subject and the study was approved by the "Grigore T. Popa" University's Ethics Committee.

Statistical analysis used SPSS V.20.1 (SPSS, Chicago, IL, USA) and MATLAB (MathWorks, Natick, MA, USA) software programs. The data were expressed as means ± standard deviation (SD). A paired-samples *t*-test was used for

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comparison of means at different times. Statistical comparisons between all groups were made by analysis of variance (ANOVA). The correlation between parameters was determined by Pearson correlation analysis. Statistical significance was defined as $p < 0.05$.

For the multivariate analysis, we used the "ENTER" method, where independent factors were included in a single step. The effect of independent variables on TAS values that was considered to be a dependent variable was described by unstandardized coefficients B, standardized coefficient Beta and p value. The association between independent variables and TAS values was expressed in regression model described by determination coefficient (R^2).

4. Results

The main characteristics of the two groups are briefly presented in Table 1.

Mother's age, marital and financial status were significantly different between the two groups ($p = 0.03253$, $p = 0.00911$, $p = 0.03110$, respectively). The groups were homogenous regarding delivery route, gender of the neonate, parity, BMI, residence, and educational level.

The mean values of TAS in the two groups increased from day 3 to day 30 (Table 2). This pattern was constant regarding fresh term milk (1.39 vs. 2.1 vs. 2.55 mmol/L), whereas in preterm milk TAS values were slightly higher in transitional FM compared to mature FM, although the difference was not significant (2.03 vs. 1.95 mmol/L) (Fig. 1). Term (group 2) FM had a higher TAS mean value than preterm (group 1) FM at each moment of lactation (1.39 vs. 1.27, 2.1 vs. 2.03, 2.55 vs. 1.95), with a statistically significant difference only for mature FM ($p = 0.038978$). A similar pattern was maintained if the milk was refrigerated for 24 h ($p = 0.033047$) or frozen for 1 week ($p = 005407$). For R72 the differences were maintained, but they were not statistically significant (Table 3).

The multivariate analysis showed that TAS was influenced by mother's age and parity: mothers >25 years had a significantly higher TAS than those <25 years ($\beta = 0.517$, $p = 0.013$, OR = 2.3) and multipara higher than primipara ($\beta = -0.226$, $p < 0.00001$, OR = 2.2) (Table 4). These findings needs to be confirmed by further studies in order to be better explained and understood. Neither social, nor financial status had any influence on TAS.

In preterm milk, the TAS increase stops after the first week at similar values with transitional term milk. In each phase of lactation refrigeration generated a slight decrease in TAS and there were no significant differences whether it was 24 or 72 h refrigeration time, excepting transitional term milk which showed a trend of preserving and even increasing the TAS content by refrigeration, although this finding was not significant (Fig. 1). These data suggest that 24 h refrigeration is better than 72 h, but 72 h of refrigeration for human milk remains a reasonable time in order to avoid severe decreases in TAS concentration.

Analyzing the frozen samples for 1 week (F1), the decrease of antioxidant capacity is constant, for every type of milk, in both groups, more dramatically in D3 and D7 term milk, and with similar values for both groups, term and preterm (0,93 and 0.97 mmol/L, vs. 1.06 and 0.99 mmol/L, respectively). These values are inferior to any value of TAS in fresh milk, even from D3. Compared with R24 and R72, F1 samples have significantly lower TAS content, suggesting that freezing, even for 1 week is worse than refrigeration for 3 days in terms of global antioxidant properties.

After 3 months of freezing, almost all antioxidant status is limited to around 0.8 mmol/L (with an interval of 0.79—0.81 mmol/L for group 1 vs. 0.68—0.9 mmol/L for group 2).

Table 1 Description of the 2 study groups.

Characteristics	Group 1 N = 60	Group 2 N = 30	p value
Mother's age			
<25 y	13 (21.7%)	13 (43.3%)	0.03253
≥25 y	47 (78.3%)	17 (56.7%)	
Gestational age			
Median (IQR)	32.5 (30.5—33.5)	38 (37—39)	
Delivery route			
Vaginal	27 (45%)	16 (53.3%)	0.45561
C — section	33 (55%)	14 (46.7%)	
Gender of newborn			
Male	33 (55%)	20 (66.7%)	0.28579
Female	27 (45%)	10 (33.3%)	
Parity			
Primipara	37 (61.7%)	19 (63.3%)	0.87782
Multipara	23 (38.3%)	11 (36.7%)	
Body Mass Index (BMI)			
<25 kg/m^2	26 (43.3%)	9 (30%)	0.22127
>25 kg/m^2	34 (56.7%)	21 (70%)	
Residence			
Urban	37 (61.7%)	17 (56.7%)	0.64808
Rural	23 (38.3%)	13 (43.3%)	
Educational level			
Primary	10 (16.7%)	4 (13.3%)	0.18334
High school	30 (50%)	10 (33.3%)	
Graduate level	20 (33.3%)	16 (53.3%)	
Marital status			
Single	0 (0%)	3 (10%)	0.00911
Couple/married	60 (100%)	27 (90%)	
Financial level[a]			
Low	12 (20%)	14 (46.7%)	0.03110
Medium	29 (48.3%)	10 (33.3%)	
Good	19 (31.7%)	6 (20%)	

IQR—interquartile range.
$p < 0.05$ was considered to be statistically significant.
[a] Low: <100 €/family member/month; medium: 100—200 €/family member/month; good: >200 €/family member/month.

5. Discussions

Oxidative stress is one of the major problems for ill neonates, especially because of the high rate of prematurity (over 14% in our population), so energy intake and antioxidant defense can be crucial for such neonates. They are frequently exposed to oxidative stress due to infection, oxygen therapy and mechanical ventilation (considering that in premature infants excess oxygen can secondarily

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Table 2 Mean values, SD and CI for TAS (Premature vs. term mothers).

TAS in human milk Mean (SD) 95%CI		FM	R24h	R72h	F1	F12
Term	Day 3	1.39 (0.3)	1.36 (0.3)	1.33 (0.4)	0.93 (0.2)	0.74 (0.2)
GROUP 2	95%CI	1.28–1.51	1.24–1.48	1.17–1.48	0.86–0.99	0.86–0.99
	Day 7	2.1 (1.7)	2.21 (1.3)	2.38 (2.1)	0.97 (0.6)	0.68 (0.3)
	95%CI	1.43–2.77	1.68–2.73	1.54–3.22	0.72–1.22	0.72–1.22
	Day 30	2.55 (1.7)	2.39 (1.6)	2.3 (2)	1.51 (0.7)	0.9 (0.4)
	95%CI	1.86–3.23	1.74–3.03	1.52–3.08	1.25–1.77	1.25–1.77
Preterm	Day 3	1.27 (0.3)	1.24 (0.3)	1.23 (0.3)	1.06 (0.4)	0.81 (0.3)
GROUP 1	95%CI	1.2–1.33	1.18–1.3	1.17–1.29	0.99–1.13	0.99–1.13
	Day 7	2.03 (1.8)	1.9 (1.3)	1.78 (1.4)	0.99 (0.4)	0.79 (0.3)
	95%CI	1.7–2.36	1.65–2.15	1.52–2.05	0.93–1.06	0.93–1.06
	Day 30	1.95 (1.2)	1.79 (1.2)	1.74 (1.2)	1.16 (0.6)	0.8 (0.4)
	95%CI	1.73–2.18	1.56–2.01	1.52–1.96	1.06–1.27	1.06–1.27

95% CI = 95% Confidence interval; SD = Standard deviation; Data given as mean (SD)/95% Confidence interval for means.
FM: fresh milk; R24, R72: milk refrigerated for 24, 72 h; F1,F12: milk frozen for 1, 12 weeks.

generate retinopathy of prematurity or chronic lung disease) — so oxygen is a required treatment but also a potential dangerous "drug". Parenteral nutrition, blood transfusions, necrotizing enterocolitis, intraventricular/periventricular hemorrhage, and retinopathy of prematurity are all thought to be consequences of this imbalance between antioxidant capacity and oxidative stress,[16] added to an already demonstrated reduced antioxidant capacity. Also, for severely asphyxiated infants, either preterm or term, total antioxidant status is reduced, together with different fractions of radical scavenging activity in plasma. Therefore, to counteract this high risk, enteral nutrition, when possible, should provide maximum protection. Zarban et al.'s extensive study on milk samples (colostrum, transitional and mature milk) collected from 115 healthy women only with full-term neonates demonstrated that TAS was obviously higher in colostrum than transitional and mature milk.[17] These data suggest that using colostrum, during the first days of life is vital, due to its high antioxidant potential. Quiles studied coenzyme Q10 as a marker of total antioxidant capacity and found higher concentrations for colostrum and transition milk in the full-term vs. the preterm group, but values decreased through lactation in mothers delivering full-term infants.[18] The major downside of this study is enrolling only 30 cases.

Ezaki et al.'s study, on 56 cases of premature delivering Japanese mothers found that TAS tended to decrease in time when studying milk's composition up to 150 days, with TAS values homogenously distributed during the first month of lactation.[19] Ezaki's TAS values are remarkably higher compared to other studies, which is probably due to the method used or ethnic or geographical particularities. Ezaki used the biological antioxidant potential (BAP) test for measuring of TAS, which is very similar as principle to the FRAP assay used by Zarban et al.[17] Fidanza reported a high, but not significant antioxidant capacity in colostrum, using

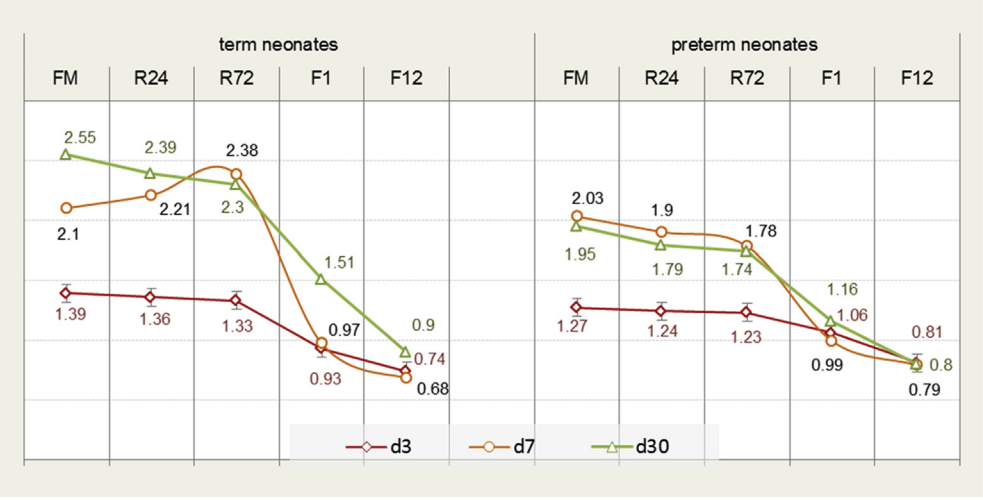

Figure 1 TAS dynamics depending on storage method of different types of human milk (mmol/L). FM—fresh milk; R24, R72—refrigerated milk for 24, and 72 h, respectively; F1, F12—frozen milk, for 1 and 12 weeks, respectively; d3, d7, d30 — days 3, 7, 30 of lactation.

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Table 3 Level of significance *(p)* in comparison of TAS values in human milk from term and preterm mothers.

Day of lactation	p[a] (fresh milk)	p[a] (R 24 h)	p[a] (R 72 h)	p[a] (F1)	p[a] (F12)
	TAS: preterm vs. term				
Day 3	0.079330	0.081682	0.180936	0.055115	0.201615
Day 7	0.860016	0.285988	0.078413	0.771669	0.095554
Day 30	0.038978[a]	0.033047[a]	0.056289	0.005407[a]	0.235300

TAS—total antioxidant status.
[a] p-value <0.05 was considered to be statistically significant.

Table 4 *Multivariate analysis concerning factors that influence TAS values.*

TAS Model predictors	Sig. p-value[a]	Adjusted Odd Ratio	95%CI for adjusted OR	
			Lower	Upper
Mother's age (≥25 y)	.017	2.311	1.878	2.936
Marital status (Couple/married)	.967	1.023	.358	2.923
Financial level (Medium, good)	.462	.861	.577	1.284
Parity (Multipara)	.023	2.263	1.928	3.718

95% confidence intervals, OR- Adjusted odds ratio.
[a] p-value <0.05 was considered to be statistically significant.

ORAC (oxygen radical absorbent capacity) assay on only 30 samples.[20] Abuhandan et al. found that the oxidants and antioxidants in the milk of mothers of premature infants were significantly higher than those of full-term birth mothers.[21] Although the study groups enrolled similar number of subjects, the prematurity focused on 30—32 weeks' gestation, and the method used differed in terms of technique (Erel method) and units, so absolute values cannot be compared with ours. Moreover, the samples were collected on day 5 and preserved for an unspecified interval of time at −80 °C, so comparison with our data would be inappropriate.

In a recent study on 15 subjects in different stages of lactation, Mehta showed the identified enhancement of the antioxidant capacity of human milk by bio-active proteins that are lacking in commercial formula, and this study supports consideration of breast milk as the ideal nutrition for preterm-born neonates.[22]

Many studies were conducted in this field, but only one measured the antioxidant activity of fresh, refrigerated and frozen milk at the same temperature as in our study.[23] Hanna et al. only studied 16 subjects and used different techniques. We chose to use the ABTS® technique because according to the latest studies and meta-analysis it is the most reliable method in vivo and in vitro.[24,25]

Endogenous antioxidants in breast milk such as catalase, glutathione peroxydase, and superoxide dismutase were thought to increase with the passage of days after birth.[26,27] This explains our results, as TAS in fresh breast milk increased gradually during the first postnatal month.

Our data show that preterm milk has lower TAS levels than term milk at every moment of lactation we studied, but differences are not significant until 30 days. The discrepancy with other studies may be due to ethnic particularities, nutritional habits and tradition.[28] As the antioxidants generally accumulate better during the last trimester of pregnancy as demonstrated by blood determinations, it is probable that a woman who has given birth prematurely would not synthetize a higher TAS-concentrated milk as she would when delivering at term. We also consider that plausible reasons for these results are stress, prolonged hospitalization, concerns about infant's health and frustrations generated by separation from the child and limited access to his care during the initial intensive care time, which contribute to a lower level of antioxidants at this category of mothers. In preterm milk TAS does not increase after 7 days, suggesting that premature infants benefit most from the antioxidant capacity of colostrum and transitional milk.

In terms of the storage method, our data were consistent with other studies in demonstrating that refrigeration for 3 days was better than freezing human milk at −20 °C.

In a study by Xavier et al. performed on 20 term and preterm delivering mothers, the highest TAS was found in colostrum and decreased over time, more through freezing (−8 °C) than refrigeration, with no difference between term and preterm milk, which emphasizes the need of awareness and curtailment of the practice of storing and later use of human milk in medical practice and home care.[29]

In term of mother's age, Matos et al. found no statistically significant differences in TAS contents when comparing < 30 years with >30 years.[30] By decreasing the limit to 25 years of age, we found significant differences.

According to the Spanish Pediatric Association,[31] the study of different ways to preserve the antioxidant capacity of breastmilk throughout lactation and of factors to improve mother and infant antioxidant status are important fields of research. However, many questions are still to be answered. More research on methods for extraction and storage of expressed breast milk is needed to best preserve antioxidant properties and constituents of breast milk.

This is the first such study performed in our country exploring antioxidant activity in human milk. A larger, multicenter prospective study on Romanian population, including endogenous antioxidants components such as catalase, glutathione peroxidase, and superoxide

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dismutase, along with specific oligo elements (Cu, Zn, Mg and Se) would be of real benefit, to better confirm and explain the present results.

6. Conclusions

Breastfeeding remains an essential tool to help in the protection against free radicals, oxygen reactive species and oxidative stress. Fresh human milk has the highest antioxidant capacity. When fresh milk is not available, preserving milk in the refrigerator for a short time up to 72 h is a better option than freezing. Regardless of the type of preservation, human breastmilk remains highly beneficial in terms of providing antioxidant protection.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgement

All authors had an equal contribution to this study.
This study was supported by the 'Grigore T. Popa' University of Medicine and Pharmacy, Iasi, Romania, as part of the internal research grant 30881/30.12.2014.
The first author expresses her gratitude to all mothers that participated in this study.

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Please cite this article in press as: Păduraru L, et al., Total antioxidant status in fresh and stored human milk from mothers of term and preterm neonates, Pediatrics and Neonatology (2018), https://doi.org/10.1016/j.pedneo.2018.02.004