Storage at -80°C Preserves the Antioxidant Capacity of Preterm Human Milk

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Background and Objective: It is essential to establish optimum parameters for maintaining the quality of stored milk until the moment of consumption with minimal deterioration of its properties. The aim of the study was to evaluate total antioxidant capacity (TAC) and total oxidation status (TOS) of fresh and freeze-stored samples (at -80°C) of preterm human milk (HM). **Methods:** Samples of colostrum were collected from 98 healthy women within the first 4 days after delivery. The total milk volume collected (6 ml) was divided in two aliquot parts: 3 ml for the fresh analysis which was done immediately after the extraction and 3 ml for storage under freezing conditions at -80°C for three months. The antioxidant status and oxidative stress of the

fresh and stored breast milk were assessed via determination of TAC and TOS levels. **Results:** The mean gestational age and the birth weight of the infants were 31.26 ± 2.93 weeks and 1620 ± 581.91 g; respectively. There were no significant correlations between maternal age, route of delivery and milk oxidative stress. There was no significant difference between the levels of TAC, TOS and the oxidative stress index in fresh and freeze-stored samples of colostrum in preterm HM ($p > 0.05$). **Conclusion:** Freeze storage of preterm HM at -80°C for three months preserves the antioxidant capacity without changing oxidative status of HM, which could be noteworthy for the preterm infant nutrition. J. Clin. Lab. Anal. 28:415–418, 2014. © 2014 Wiley Periodicals, Inc.

Key words: total antioxidant capacity; preterm infant; human milk; freeze stored

INTRODUCTION

Neonates are exposed to strong oxidative stress as they transition from a hypoxic intrauterine to a normoxic extrauterine environment at birth (1, 2). This increased oxidative stress must be controlled by an antioxidant defense system that matures during the course of gestation (3–8). Preterm infants may be at increased risk of free radical damage because of a poorly developed antioxidant system that may be overwhelmed by excessive reactive oxygen species (ROS). Excessive production of ROS in preterm infants has been implicated in the pathogenesis of the major complications of prematurity, including necrotizing enterocolitis, bronchopulmonary dysplasia, retinopathy of prematurity, and intraventricular hemorrhage (9). Furthermore, there is increasing evidence that alterations in the epigenetic regulation of gene expression triggered by ROS in the fetal or neonatal period of life are associated

with conditions that appear in adulthood, such as type 2 diabetes, hypertension, and obesity (10–12).

Because human milk (HM) contains many antioxidant constituents such as superoxide dismutase, glutathione peroxidase (GPx), catalase, vitamins A, C, and E, and β -carotene that may protect against damage by ROS, breastfeeding is important to buffer oxidative stress during the neonatal period and could attenuate deleterious consequences later in life (13–17). However, many preterm infants need to stay in the hospital for a period of time,

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and continuing maternal lactation requires extraction and storage of the mother's milk until it is supplied to the infant. It is essential to establish optimum parameters for maintaining the quality of stored milk until the moment of consumption with minimal deterioration of its properties. A few studies have demonstrated alterations in the oxidative state of milk during storage at different temperatures and over different storage times (18–20). However, little information is available on optimal storage conditions of preterm HM to preserve its antioxidant properties.

Thus, the present study was performed to evaluate the antioxidant capacity and oxidative status of fresh and freeze-stored samples (at -80°C) of preterm HM for 3 months.

MATERIAL AND METHODS

This prospective study was performed in the Zekai Tahir Burak Maternity and Teaching Hospital Neonatal Intensive Care Unit between September 2009 and July 2010. A total of 98 healthy, nonsmoking women with a single preterm infant were included in the study.

Samples of colostrum secreted within the first 4 days after delivery were obtained with a vacuum pump (Medela Lactina Select) from one breast of each mother before infant feeding. A 6.0 ml sample of HM was collected from each participant between 11:00 AM and 1:00 PM into sterile plastic tubes. Samples were packed in dry ice and transferred to the hospital laboratory. At the laboratory, milk samples from each mother were distributed in two aliquots: (a) 3 ml for the first analysis, which was performed immediately after extraction without refrigerating the samples; and (b) 3 ml for storage at -80°C for 3 months. The stored samples were analyzed as soon as possible after thawing.

The samples were centrifuged for 10 min in plastic tubes at a speed of 3,500 rpm. The supernatant was evaluated for total antioxidant capacity (TAC) and total oxidation status (TOS). TAC levels were measured according to the method described by Erel (21), which is based on bleaching the characteristic color of a more stable 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation by antioxidants. The results were expressed in millimoles Trolox equivalent per liter. TOS was measured with Erel's TOS method (22), which is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidative species in acidic medium and measurement of the ferric ion by xylenol orange. The results were expressed in micromoles $\text{H}_2\text{O}_2/\text{l}$. Erel's TAC and TOS methods are colorimetric and automated, and the precision is excellent at $<3\%$ (21, 22). The TOS-to-TAC ratio was defined as the oxidative stress index (OSI), an indicator of the degree of oxidative stress (23). To perform the calculation,

the unit for TAC (millimoles of Trolox equivalent per liter; mmol/l) was changed to micromoles of Trolox equivalent per liter ($\mu\text{mol/l}$), and the OSI value was calculated as $\text{OSI} = (\text{TOS}, \mu\text{mol/l}) / (\text{TAC}, \mu\text{mol Trolox equivalent/l} / 100)$.

This study was approved by the Zekai Tahir Burak Maternity and Teaching Hospital ethics committee. Written informed consent was obtained from each mother. Assistance for milk sampling was provided to all participating women during the study period.

STATISTICAL ANALYSES

Descriptive analysis was performed for demographic and clinical characteristics of the patients. Data were expressed as the arithmetic mean $\pm$ standard deviation (SD) or median (interquartile range), as appropriate. Wilcoxon test was used to analyze the repeated data because distribution was normal. Spearman's rho test was used to analyze the correlation between maternal age, route of delivery, and milk's oxidative stress status. Statistical analysis was performed with SPSS software version 13.0 and statistical significance was set at $P < 0.05$.

RESULTS

A total of 98 samples of fresh HM were obtained from the mothers of preterm infants born with a mean $\pm$ SD gestational age of 31.26 ± 2.93 weeks (range, 24–36 weeks) within the first 4 days of lactation.

The characteristics of the mothers and preterm infants included in the study are shown in Table 1. The TAC and TOS levels and the OSI of fresh and stored samples of preterm HM are shown in Table 2. There was no significant difference between the levels of TAC and TOS and the OSI value in fresh and freeze-stored samples. There were no significant correlations between maternal age, route of delivery, and milk oxidative stress (Table 3).

TABLE 1. General Characteristics of the Mothers and Preterm Infants

Mothers ($n = 98$)	
Maternal age, years (mean $\pm$ SD)	27.63 ± 5.54
Body mass index (prepregnancy), kg/m^2 (mean $\pm$ SD)	25.09 ± 4.45
Weight gain, kg (mean $\pm$ SD)	12.5 ± 5.4
Route of delivery (vaginal/cesarean delivery), n	31/67
Preterm infants ($n = 98$)	
Male gender, n (%)	50 (51.5)
Gestational age, week (mean $\pm$ SD)	31.26 ± 2.93
Birth weight, g (mean $\pm$ SD)	1.620 ± 581.91

TABLE 2. Levels of TAC, TOS, and OSI in Fresh and Stored Preterm Human Milk Samples

	Fresh milk	Stored milk	<i>P</i>
TAC (mmol Trolox equivalent per liter)	3.2 (1.9–4.7)	3.8 (1.9–5.4)	0.301
TOS ($\mu\text{mol H}_2\text{O}_2$ per liter)	76.1 (39.2–98.2)	60.7 (42.9–98.3)	0.681
OSI (arbitrary unit)	0.2 (0.1–0.3)	0.1 (0.1–0.2)	0.341

All data are median (interquartile range).

TAC, total antioxidant capacity; TOS, total oxidation status; OSI, oxidative stress index.

TABLE 3. The Correlation Between Maternal Age, Route of Delivery and Milk Oxidative Stress

	Maternal age		Route of delivery	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Fresh milk				
TAC	0.251	0.221	0.059	0.583
TOS	0.285	0.289	0.116	0.279
OSI	0.175	0.111	0.111	0.299
Stored milk				
TAC	−0.081	0.452	−0.078	0.456
TOS	0.111	0.304	−0.008	0.938
OSI	0.258	0.215	0.077	0.466

TAC, total antioxidant capacity; TOS, total oxidation status; OSI, oxidative stress index; *r*, correlation coefficient.

DISCUSSION

We have provided evidence for the TAC and TOS levels and OSI values of fresh and freeze-stored samples of preterm HM. Our study showed that the TAC and oxidation status of preterm HM did not change by storing at -80°C for 3 months. To our knowledge, this study is the first to evaluate the TAC and oxidation status of fresh and freeze-stored (-80°C) samples of preterm HM in a large series.

Among the different types of milk, colostrum may be the most susceptible to lipid peroxidation. Thus, colostrum could be the most vulnerable milk during manipulation and storage in the hospital milk bank. Because oxidative damage can be much more severe during the first days after birth, especially up to 72 hr after birth, we used colostrum to evaluate the TAS and TOS levels and OSI in this study.

Frozen storage is often used by milk banks to save expressed HM for later use, especially in preterm infants. Most studies on the effect of the freezing of breast milk have preferred -20°C as the storage temperature, which is the normal practice in milk banks. Miranda et al. (24) reported that storage of HM at -20°C for 10 days led to

a decrease in GPx activity, resulting in lower antioxidant activity and a nonsignificant increase in the malondialdehyde (MDA) concentration. However, Turoli et al. (19) showed no difference in the TAC levels of colostrum between fresh samples and those stored at -20°C in their study. Hanna et al. (18) reported that the antioxidant activity of preterm and term HM at both refrigeration and freezing (-20°C) temperatures was significantly decreased. In the same study, freezing resulted in a greater decrease than did refrigeration, and storage for 7 days resulted in lower antioxidant activity than storage for 48 hr.

HM is more susceptible to degradation at -20°C because lipoprotein lipase activity, which is responsible for increased lipid oxidation products, remains active during freezing at -20°C (19, 25). Based on these alterations in the lipid fraction of milk, researchers have recommended that milk banks use the usual freezing temperature of -20°C to lower temperatures for the storage of breast milk to decrease lipase activity (25). Additionally, freezing results in loss of the antioxidant features of breast milk, and this loss increases with the period of storage and change in intensity according to the temperature. A recent study evaluated the glutathione GPx activity and MDA concentration of term HM when stored frozen, comparing the effects of two temperatures (-20°C and -80°C) and different storage times (26). The study showed that freezing induced loss of antioxidant properties of term HM associated with the duration of storage. Silvestre recommended HM storage at -80°C for a period of less than 30 days, rather than for shorter time periods, at the usual temperature of -20°C to preserve the antioxidant properties of term HM. However, no significant change was observed in the TAC levels of the preterm HM in stored samples at -80°C for 3 months in our study. Variations in the heat stability of antioxidative agents in preterm and term HM may explain this effect. Our study results also suggest that a potential decrease in the activity of lipoprotein lipase at -80°C may play role in the nonsignificant change over time in the TOS levels in stored samples of HM.

In conclusion, we found that the TAC of preterm HM did not change by storing at -80°C for 3 months. This finding is thought to be noteworthy in terms of the preservation of preterm HM in hospital milk banks. However, we are unable to confirm the advantages of using a freezing temperature lower than the usual temperature of -20°C with this single study. Therefore, the results of our study should be seen as preliminary, and further research is needed to evaluate the preservation of antioxidant capacity with minimum deterioration by storage at different temperatures and storage times in preterm HM.

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