

# Effect of storage on breast milk antioxidant activity

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*Arch Dis Child Fetal Neonatal Ed* 2004;89:F518–F520. doi: 10.1136/adc.2004.049247

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Accepted 7 June 2004

**Background:** Human milk, which contains compounds beneficial to infants, is often expressed and stored before use. Changes in its antioxidant activity with storage have not been studied.

**Objectives:** To measure antioxidant activity of fresh, refrigerated (4°C), and frozen human milk (–20°C), stored for two to seven days; to compare the antioxidant activity of milk from mothers delivering prematurely and at term; to compare the antioxidant activity of infant formulas and human milk.

**Methods:** Sixteen breast milk samples (term and preterm) were collected from mothers within 24 hours of delivery and divided into aliquots. Fresh samples were immediately tested for antioxidant activity, and the rest of the aliquots were stored at –20°C or 4°C to be analysed at 48 hours and seven days respectively. The assay used measures the ability of milk samples to inhibit the oxidation of 2,2'-azino-di-3-(ethylbenzthiazolinesulphonate) to its radical cation compared with Trolox.

**Results:** Antioxidant activity at both refrigeration and freezing temperatures was significantly decreased. Freezing resulted in a greater decrease than refrigeration, and storage for seven days resulted in lower antioxidant activity than storage for 48 hours. There was no difference in milk from mothers who delivered prematurely or at term. Significantly lower antioxidant activity was noted in formula milk than in fresh human milk.

**Conclusions:** To preserve the antioxidant activity of human milk, storage time should be limited to 48 hours. Refrigeration is better than freezing and thawing.

In 1997, the American Academy of Pediatrics issued a statement on breast feeding, summarising the benefits of breast feeding to the infant, the mother, and the nation, and set forth principles to guide the paediatrician.<sup>1</sup> Breast milk is considered an ideal nutrient for both term and preterm infants, benefiting host defences, digestion, and absorption of nutrients, gastrointestinal function, and neurodevelopment.<sup>2</sup>

Preterm infants have reduced antioxidant capacity<sup>3,4</sup> and are often exposed to oxidant stress caused by infection, oxygen, mechanical ventilation, intravenous nutrition, and blood transfusions. Many of the disorders of preterm infants, including chronic lung disease, necrotising enterocolitis, retinopathy of prematurity, and intraventricular-periventricular haemorrhage, are thought to be due to this imbalance between antioxidant capacity and oxidative stress.<sup>5</sup>

Human milk contains higher concentrations of scavengers of free radicals than cow's milk.<sup>6–8</sup> Part of the improved outcome of infants fed human milk especially with regard to necrotising enterocolitis may be related to its antioxidant activity. For feeding preterm infants, human milk is usually expressed and stored before use. Changes in antioxidant capacity of human milk with storage have not been well studied. The objective of our study was to measure antioxidant activity of fresh human milk in comparison with milk stored at refrigerator temperature (4°C) or freezer temperature (–20°C) for two to seven days. We also compared the antioxidant capacity of milk from mothers who delivered prematurely with that of mothers who delivered at term. In addition, we compared antioxidant capacity of infant formulas and human milk.

## METHODS

Breast milk samples were obtained within 24 hours of delivery from mothers who agreed to participate in the study, approved by the IRB of St Peter's University Hospital. Breast milk samples were collected in plain glass tubes, centrifuged, and divided into five aliquots. Fresh samples

were immediately tested, and the rest of the aliquots were stored in the freezer at –20°C or refrigerated at 4°C to be analysed at 48 hours and seven days.

Antioxidant capacity was measured using the method described by Miller *et al.*<sup>9</sup> Metmyoglobin and 2,2'-azino-di-(3-ethylbenzthiazolinesulphonate) (ABTS) in powder form were dissolved in phosphate buffered saline, 5 mmol/l, pH 7.4, for final reaction concentration of 6.1 µmol/l. Doubly deionised water or a known concentration of Trolox or an aliquot of breast milk were added to the metmyoglobin/ABTS solution in a cuvette, mixed by serial inversions, and absorbance at 600 nm was recorded. Then hydrogen peroxide (250 µmol/l) was added to the cuvette. Mixing was accomplished by serial inversion, and the cuvette incubated in a water bath at 37°C for three minutes. The second reading was taken exactly 3.5 minutes after the addition of the hydrogen peroxide. Total antioxidant status was calculated by comparing inhibition by breast milk with inhibition by Trolox. Results were expressed as Trolox equivalent antioxidant capacity.

Statistical analysis was performed using Statistica software (Statistica for Windows, 1984–1994; StaSoft, Inc, Tulsa, Oklahoma, USA). We compared data obtained from human milk and infant formula before and after storage at different temperatures. Continuous data are presented as mean (SD). Analysis of variance and post hoc comparisons of means by the Tukey honest significant difference test were used to analyse the serial measurements of milk and formula samples. All tests were two sided.  $p < 0.05$  was considered significant.

## RESULTS

Eight milk samples were from mothers who delivered prematurely (gestation 27.4 (2.7) weeks), and eight were from mothers who delivered at term (gestation 38.8 (1.5) weeks). Five formula samples were tested (Similac, Enfamil, and Isomil term formulas and PE20 and PE24 preterm formulas). Antioxidant capacity of the formulas was similar, so the results were combined. Antioxidant capacity of

**Table 1** Comparison of the antioxidant capacity of human milk and formula

	Fresh	4°C (48 hours)	4°C (7 days)	-20°C (48 hours)	-20°C (7 days)
Human milk (n = 16)	1.66 (0.06)	1.58 (0.06)	1.48 (0.05)	1.45 (0.05)	1.34 (0.04)
Formula (n = 5)	1.07 (0.02)*	1.08 (0.04)*	1.05 (0.02)*	1.05 (0.02)*	1.07 (0.04)*

Data are presented as Trolox equivalent antioxidant capacity.  
\*p < 0.05 compared with human milk.

fresh milk from mothers who delivered prematurely was 1.65 (0.03), similar to milk from mothers who delivered at term (1.67 (0.08)). Changes in the antioxidant capacity of human milk with different storage conditions and over time were also similar in samples obtained from mothers who delivered prematurely or at term. Consequently results from all human milk samples were combined for comparative purposes.

Table 1 presents the antioxidant capacity of human milk and formula under different storage conditions. The antioxidant capacity of human milk is significantly higher than that of formula, and this difference persists irrespective of the duration and temperature conditions of storage. Whereas the antioxidant capacity of the formula did not change with storage times and temperatures, the antioxidant capacity of human milk was altered significantly. Freezing significantly decreased it compared with refrigeration ( $p < 0.001$  when stored for either 48 hours or seven days). It also decreased with duration of storage (48 hours  $\nu$  7 days) both during refrigeration and freezing ( $p < 0.001$ ). Compared with fresh milk, the lowest antioxidant capacity was observed in human milk after freezing for seven days (1.34 (0.04);  $p < 0.001$ ). However, the antioxidant capacity of human milk after storage at 4°C for seven days is the same as after freezing at -20°C for 48 hours (1.48 (0.05)  $\nu$  1.45 (0.05);  $p > 0.05$ ).

## DISCUSSION

Fresh human milk has a higher antioxidant capacity than infant formula. This result is consistent with the study of Shoji *et al.*<sup>10</sup> who found increased urinary 8-hydroxy-2'-deoxyguanosine excretion in formula fed infants compared with breast fed infants. Schwarz *et al.*<sup>11</sup> found similar results by measuring breath ethane concentrations in breast fed and formula fed infants. Although most studies showed that human milk had a higher antioxidant capacity than formula, one study by Alberti-Fidanza *et al.*<sup>12</sup> using the oxygen radical absorbance capacity assay, suggested that formula may have higher antioxidant capacity than human milk. The difference in these findings may be due to the different methods used to determine the antioxidant capacity of human milk. An improved oxygen radical absorbance capacity assay has been developed and validated using fluorescein.<sup>13 14</sup> Friel *et al.*<sup>15</sup> showed that human milk has better antioxidant protection than formulas, perhaps because of the higher iron content of formulas. In this study, denaturing endogenous enzymes did not decrease the ability of human milk to resist oxidative stress. However, the antioxidant enzymes when added to formula were shown to provide increased protection against oxidative stress and lipid damage. Differences between human milk and formula are not only due to the source of milk but also the processing of milk to prepare the formulas. Infant formulas have higher preformed lipid oxidation products than human milk.<sup>16</sup> This may partially explain the greater lipid peroxidation in infants fed formula compared with breast milk.

The components of human milk responsible for its antioxidant activity are not entirely clear and include uric acid,  $\alpha$  and  $\gamma$  tocopherols, carotenoids, vitamin A and vitamin C, and enzymes such as catalase and glutathione peroxidase.<sup>6 7 17-19</sup> In addition, there is uncharacterised antioxidant activity in

the lipid fraction.<sup>20</sup> Preterm infants are born relatively deficient in antioxidant defences, and ingesting human milk rapidly increases antioxidant concentrations.<sup>6 7 16</sup> This may partly explain the protection afforded by human milk in the development of necrotising enterocolitis and retinopathy of prematurity.<sup>21</sup>

The effect of storage on various components of human milk has been studied. However, most of these studies have focused on the bacteriological, nutritional, and immunological effects of storage.<sup>22-31</sup> Vitamins A, D, and E have been found to be quite stable under various storage conditions for up to one week and after freezing at -20°C or -70°C for a longer time period.<sup>19 32</sup> Vitamin C concentration has been found to decrease with storage over time.<sup>28 29 33</sup> The study of the glutathione status of stored human breast milk by Ankrah *et al.*<sup>34</sup> showed a substantial loss of glutathione when breast milk was kept at -20°C, 4°C, or at room temperature for two hours compared with fresh unstored breast milk. The effects of different storage conditions on the activity of antioxidant enzymes have not been well studied, especially in premature human milk. The main finding of our study is a decrease in antioxidant activity with storage both with refrigeration as well as freezing at the currently recommended temperatures.

Many milk components change with storage, including immune cells, which are inactivated by freezing.<sup>26 27</sup> Storage also reduces lipase activity of the milk.<sup>35-38</sup> We studied the total antioxidant capacity of the milk and are unable to ascertain which components of milk are affected by storage. The antioxidant capacity of infant formula did not change with storage, which is consistent with previous studies suggesting stability of fat soluble vitamins with storage.<sup>19 32</sup> Thus it is likely that antioxidant enzyme activity of human milk degraded with storage.

In conclusion, we found that fresh human milk has the highest antioxidant capacity, which decreases with storage over time. To preserve antioxidant capacity, milk should only be stored for a short time at refrigerator temperature and not frozen.

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