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Original Article

The total antioxidant capacity of infant feeds at various handling temperatures - a comparative study

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Abstract

Among the several mechanisms that the infant body possesses to counteract damage by free radicals and other reactive oxygen species (ROS), a diet rich in antioxidants enhances this defense capacity to ward off diseases. This research aimed to compare the total antioxidant capacity (TAC) of various available infant feeds that were subjected to varying temperatures and storage durations. Ten samples each of cow's fresh milk, pasteurized milk and four infant formulas were collected. Cow's milk and pasteurized milk were assessed at room temperature immediately, 48 h post refrigeration (4°C) and after heating with low and high flame followed by 2-h flasking. Infant formulas were assessed for their TAC at room temperature, following refrigeration and freezing for 48 h at 4°C and –8°C respectively by phospho-molybdenum method. The results showed pasteurized cow's milk to have significantly lesser antioxidants than fresh cow's milk. The 2-h flasking of heated milk samples produced a less significant increase in TAC levels. A high statistical difference was observed in the TAC comparing freshly prepared infant formulas to those when refrigerated and frozen. We concluded that fresh cow's milk possessed the highest TAC followed by pasteurized milk and infant formulas at all storage temperature and durations.

INTRODUCTION

Milk is considered to be the most nutritionally complete food containing nearly all the elements of nutritional importance to humans. It has long been recognized as a source of macro- and micronutrients, immunological and biologically active substances, which not only allow growth, but also promote health in mammalian newborns. Many milk lipids, lipid-soluble substances, and their digested products are bioactive, including vitamins and vitamin-like substances [1].

The American Academy of Pediatrics recommends that preterm infants be fed sufficient amounts of all nutrients to achieve postnatal rates of growth and nutrient accretion that approximate those of a normal fetus during the same period [2]. According to the findings of the National Family Health Survey (NFHS)-1, it was concluded that breastfeeding with supplements is more beneficial than exclusive breastfeeding even for children at very young ages (less than 4 months) [3].

Besides breast milk, the medical community considers infant formulas to be nutritionally acceptable for infants under the age of one year. Although cow's milk is the basis of almost all infant formula, plain cow's milk is totally unsuited for infants because of its high protein and electrolyte (salt) content, which may put a strain on infant's immature kidneys. Also, the infant intestine is not properly equipped to digest non-human milk and this may often result in diarrhea, intestinal bleeding and malnutrition [4]. Yet, many communities from the lower socio-economic strata feed their infants with fresh cow's milk in the early stages following birth.

The general notion among public is that pasteurized milk would serve the basic requirements of infant feed. However, studies have shown that pasteurization destroys enzymes, diminish the vitamin content, denature fragile milk proteins, destroys Vit B_{12} and Vit B_6, kills beneficial bacteria, promotes pathogens and is also found to be associated with allergies, increased tooth decay, colic in infants, growth problems in children etc [5, 6].

Although the human body has an inherently antioxidative system (such as superoxide dismutase, glutathione peroxidase and uric acid) to protect itself from damage caused by peroxidants, these systems are not sufficiently effective to totally prevent such damage [7]. Hence, there is an increasing interest in finding natural antioxidants from food, because it is believed that they can protect the human body from attack of free radicals and retard the progress of many chronic diseases, as well as retarding the lipid oxidative rancidity in foods [8]. A diet rich in antioxidants provides the infant with biological immunity to fight against free radicals, and to maintain systemic or oral health [9].

Modernization and challenging life styles have led to an increase in double income parents with time constraints for the maternal feeding practices. Thus, feeding of infants with stored forms of milk is on a rise [10]. A previous research by Xavier *et al* focusing on the variations in levels of antioxidants of expressed breast milk (EBM), showed declining TAC levels upon long term refrigeration and freezing respectively, when compared to the baseline value recorded at room temperature immediately following expression [11]. However, the lack of sufficient research-based data that compared the total antioxidant concentrations (TAC) of various available infant feeds, subjected to varying temperatures and storage durations has necessitated carrying out this study. As an effective way to begin an infant's life long program of preventive care, allied medical and dental professionals must be involved as partners in providing anticipatory guidance to mothers regarding the effect of milk handling on its TAC and the subsequent implications on the infant's well-being.

MATERIALS AND METHODS

The study was carried out over a period of 18 months through 2009-2011 in the Department of Pediatric Dentistry of A.B. Shetty Memorial Institute of Dental Sciences, Mangalore. Ethical clearance for the study was provided by the institutional committee for ethics and research, affiliated to the Rajiv Gandhi University of Health Sciences, Bangalore, Karnataka, India.

Study samples and collection

Five milliliters of fresh milk from ten domestically reared cows in and around the region of our study and ten samples of commercially packaged pasteurized milk (Nandini; Mangalore, Karnataka, India) were collected for assessment. Also, ten samples each of four commercially available and commonly consumed infant formulas namely, Lactogen 1 and Lactogen 2, Nan 1 and Nan 2 (all from Nestle India Ltd; New Delhi) were included for our analysis.

Study design and sample preparation

Cow's milk and pasteurized milk were assessed for the TAC at room temperature (mean temperature 30°C) immediately after collection, 48 h post refrigeration and after heating with a low as well as high flame till the boiling temperature of milk was achieved. A part of the milk samples heated in a low and high flame were also flasked (Vacuum Flask, Dragon-Sky International Co, Guangzhou, PR China) for a period of 2 h was also a part of our experimentation. The infant formulas were assessed at room temperature immediately following preparation, 48 h post refrigeration and freezing for the total antioxidant levels using the phospho-molybdenum method [12]. Samples of milk that were refrigerated and frozen were stored in a 180 l refrigerator cum freezer, where refrigerating and freezing temperature was set as 4°C (39.2°F) and –8°C (17.6°F), respectively.

The Asian population at large uses gas operated stoves for cooking and heating food/consumables at homes. These gas stoves have burners of varying sizes operated by simmers that can be adjusted by either high or low flames based on the type of food items being prepared. A circular burner of 8 cm diameter was used for boiling milk with both the lowest and highest flames. It was noticed that 100 ml of cow's fresh and pasteurized milk achieved boiling temperature at 1.25 min with the highest flame, while 3.45 min with the lowest flame.

Estimation of total antioxidant concentration (TAC)

The phospho-molybdenum method was used for assessing the TAC of the samples. An aliquot of 0.1 ml of the sample solution containing a reducing species (ethanol) was combined in an eppendorf tube. To this, 1 ml of reagent solution, containing 28 mM of sodium monophosphate, 0.6 mM of sulphuric acid and 4 mM of ammonium heptamolybdate, was added. The eppendorf tubes were capped and incubated in a thermal block (Rotek Co, Ernakulam, Kerala, India) at 95°C for 90 min. The samples were then allowed to cool at room temperature. The absorbance of the aqueous solution for each sample was measured at 695 nm against a blank solution using a spectrophotometer (Spectrophotometer 106, Systronics Co, Ahmedabad, Gujarat, India). The typical blank solution contained 1 ml of reagent alone without the test solution and was incubated under the same conditions as the remaining samples.

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Statistical analysis

Data was analyzed using Student's t-test, one way analysis of variance (ANOVA), with Bonferroni multiple comparison test. The statistical Package for the social sciences (SPSS Inc, Chicago, IL, USA), version 15.0, was used for all analyses. The one way ANOVA analysis statistically assessed the TAC variations in fresh cow's milk, pasteurized milk and infant formulas subjected to temperature alterations. The unpaired t-test compared the variations in TAC between fresh cow's milk and pasteurized milk following a particular temperature treatment. The TAC variations were also compared between the three infant feeds of the current paper to the concentrations of human breast milk samples performed in our initial research, using the one-way ANOVA analysis. $P < 0.05$ was considered significant.

RESULTS

The TAC of cow's milk was statistically compared using ANOVA analysis for the values obtained following various temperature treatments ($P < 0.05$). Pair-wise comparisons showed a high statistical difference ($P < 0.005$) between each experimented group, when compared to the baseline room temperature (Table 1). A high statistical significance was observed between TAC of samples heated in a low and high flame ($P < 0.005$). The 2 h flasking of both the heated milk samples produced a statistical less significant increase in TAC, when compared to their corresponding initial flame treatment ($P < 0.001$).

The pasteurized milk samples also showed a similar pattern of TAC variation, as did cow's fresh milk (Table 1), when subjected to the various temperature treatments ($P < 0.005$). Bonferroni multiple comparison test showed a very high statistical difference between the inter-groups, except for the refrigerated samples at 48 h and those heated with a high flame, indicating both the temperature treatments to have similar effects on TAC alterations in milk. The unpaired t-test inter-compared all the variables of cow's fresh milk to pasteurized milk and showed a very high statistical significance ($P < 0.005$), implicating a wide variation of TAC between both the samples.

The one-way ANOVA compared the TAC of infant formulas subjected to varying storage temperatures, showing a high statistical difference between the groups ($P < 0.005$). The Bonferroni post hoc pair-wise tests also showed a very high statistical difference (Table 2) when the 48 h post refrigerated and frozen samples were compared to the baseline TAC assessed at room temperature ($P < 0.005$) with all the infant formulas, except Lactogen 1 following the 48 h refrigeration ($P < 0.438$). No statistical difference was noticed in TAC between the 48 h post refrigerated and frozen samples of all infant formulas. The TAC comparison between the 4 infant formulas at room temperature also showed no statistical difference ($P < 0.08$).

DISCUSSION

Numerous disorders in early infancy have been linked to oxidative damage, where reactive oxygen species (ROS) have been known to play a significant role. Free radicals are produced in the body as by-products of normal metabolism and as a result of exposure to radiation and some environmental pollutants [13]. Nutritional support of infants has been found to compensate for their metabolic and gastrointestinal

Table 1. Total antioxidant concentration of cow's milk and pasteurized milk from the baseline room temperature

Milk sample	Mean TAC (± SD) in µg/dl					
	Room Temp	Refrigeration 48hrs (4°C)	Heating		Heating + Flasking (2hrs)	
			Low flame	High flame.	Low flame.	High flame.
Cow's milk	75.92 ± 7.85	69.01 ± 8.96**	72.74 ± 6.96**	69.33 ± 7.52**	73.25 ± 6.35**	71.25 ± 7.26**
Pasteurized Milk	55.87 ± 5.12	47.83 ± 5.72**	52.01 ± 4.91**	46.94 ± 4.93**	54.81 ± 5.14*	50.81 ± 4.98**

*Not significant, **$P < 0.005$

Table 2. Total antioxidant concentrations of four infant formulas from the room temperature as baseline

Infant formula	Mean TAC (± SD) in µg/dl		
	Room Temperature	Refrigeration 48 h (4°C)	Freezing 48 h (–8°C)
Lactogen 1	34.35 ± 2.28	33.86 ± 2.48*	33.04 ± 1.9**
Lactogen 2	36.56 ± 1.79	32.52 ± 1.83***	33 ± 1.79***
Nan1	34.33 ± 1.31	29.73 ± 1.12***	25.22 ± 1.49***
Nan 2	37.96 ± 2.28	34.82 ± 2.34***	32.29 ± 1.94***

*Not significant, **$P < 0.05$, ***$P < 0.005$

immaturity, immunologic insufficiencies, and the demands of such co-morbid conditions. As a preventive measure against these diseases, antioxidant rich infant feeds or antioxidant fortified supplements are expected to be of great benefit [13]. A pediatric specialist should thus, be successful in providing effective counseling to mothers and members of her support system regarding the importance of antioxidants in the infant's normal growth and development, health maintenance and disease, through effective medical/dental home strategies.

In a number of infant medical conditions like tachypnea, poor suckling reflex with prematurity, cleft palate, hypoglycemia and maternal illness, such as agalactia, diabetes, cancers *etc*, breastfeeding expression becomes difficult and the mothers will have to resort to other infant feeds [14]. When breast milk is no longer enough to meet the nutritional needs of the infant, complementary foods should be added to the diet of the child. The transition from exclusive breastfeeding, to other food, typically covers the period after 6 months of age, and is a very vulnerable period. It is the time when malnutrition starts in many infants, contributing significantly to the high prevalence of malnutrition in children less than 5 years of age worldwide [2]. Every infant should be managed individually according to their needs. For the healthy normal birthweight full-term infant born in an industrialized country, current research and the World Health Organisation (WHO) supports the benefit of exclusive breast milk feeding until 4-6 months followed by weaning with complementary food [15].

The upswing in breastfeeding has been accompanied by a deferment in the average age of introduction of other foods (such as cow's milk), resulting in increased use of both breast milk and infant formula between the ages of 3-12 months [16]. Infant formula is necessarily an imperfect approximation of breast milk, as the exact chemical properties of breast milk are still unknown [17] and it is been found that a mother's breast milk changes over time in response to the feeding habits of her baby, thus adjusting to the infant's growth and development [18].

Our study assessed the total antioxidant capacity of 4 infant formulas commonly used by mothers in the location of our study. The antioxidant levels were found to show a high statistical difference upon refrigeration and freezing for 48 h when compared to at room temperature, except in Lactogen 1. Our results are in variance to a study which reported that the antioxidant capacity of formula wasn't affected by time or temperature: Hanna *et al* [19] reported that fresh human milk has a higher concentration of lipid peroxidation products when compared to formula milk, probably caused by an increased presence of free fatty acids due to lipoprotein lipase activity during storage at reduced temperatures. Interestingly, the high content of lipid peroxides did not correspond to a low total antioxidant capacity in either breast or formula milk, signifying these levels might be present naturally in human milk, but are subjected to strong peroxidation either at room temperature or at –20°C.

Considerable proportion of the Indian population roots from rural areas tied down by social, economic and educational backwardness, responsible for the ignorance regarding the importance of breastfeeding in infant health. This leads to cultivation of practices like rearing the child on fresh cow's milk much earlier than recommendations.

Necessity regarding the boiling or pasteurization of milk has been a matter of controversy as it is found to seriously impair its nutritional value or affect the host-defense mechanism [5, 6]. Although there is little evidence that bacterial contamination of milk poses a hazard to the infant, especially human milk, standard processing in most institutions includes heat treatment. The number and type of microorganisms as well as the exact temperature and time of heating seem to influence the efficiency of decontamination. The holder process recommended by several authors seems to be a compromise between "acceptable" decontamination and preservation of the bacteriostatic properties in milk [20, 21].

Since most proteins will denaturate when exposed to heat, heat treatment should have an adverse effect on the milk. Available data are confined mainly to the effects on milk components with antimicrobial properties [22]. In this study, the authors compared the TAC of cow's milk using ANOVA statistical analysis following various temperature treatments. As the samples showed statistical significance, pair-wise comparison was carried out between each experimental group when compared to the baseline temperature and showed a high statistical difference (Table 1). A high statistical significance was also observed between TAC of samples heated in a low and high flame. The viable cells in milk are very sensitive to heat and even mild heat treatment will lead to an almost complete destruction. The oxidative deterioration of milk has been attributed to reactive sulfhydryl groups that formed as a result of heat treatment [23]. Taylor and Richardson [21] reported that the antioxidant activity and total sulfhydryl content of raw skim milk did not change during storage at 5°C, which was contradicting to the findings of our study, as a high statistical difference was observed upon storage at 4°C [21].

Preserving the temperature of the heated milk is generally practiced by keeping such milk in thermos flasks for usage at a later period of time. However, the effects on its nutritional value have not been cited in

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previous literature. Our study observed a slight rise in the TAC following 2 h flasking of the heated milk samples ($p<.001$). The basis of this finding though fully unclear, may be related to the fact that the temperature of the heated milk samples reduced over time and in the event reacted with the atmospheric oxygen, neutralizing the overly excited free radicals.

Pasteurized milk samples were also assessed for the TAC following subjection to various temperature treatments, as are the commonly practiced milk handling methods at homes in the Indian community. The pasteurized milk samples also showed similar pattern of TAC variations as did cow's fresh milk (Table 1). But while comparing the TAC levels, cow's fresh milk was found superior to pasteurized milk. A scientific thought has to be given to the variable feeding patterns of the herbivores, which is the main source of TAC of the fresh and pasteurized cow's milk studied. Refrigeration was found to deplete the TAC by 5-10 μg/dl compared to room temperature.

Heating milk with a higher flame had a negative bearing on the TAC compared to while heating with a low flame, and was of high statistical significance. Heated skim milk (30 min at 110°C) has been found to rapidly loose its sulfhydryl groups after heat treatment unlike raw skim milk, at a slower rate [24]. Generally, increased temperature denatures proteins more rapidly, while freezing (that will not cause decontamination) is found to be less harmful [25].

Studies justify that the ordinary sterilization or preheating temperatures used in the preparation of evaporated and powdered skim milks decrease the protein efficiency of milk in proportion to the degree of heat used. While the damage is not marked at the temperatures commonly used, it may be increased by careless overheating [6]. It cannot be assumed that only one amino acid was affected, nor even that only one group was affected, since varying temperatures and times of exposure to heat can exert varying degrees of damage upon the nutritive value of proteins, particularly of milk proteins. It seems possible that one amino acid or group of amino acids might be first affected and that different proteins might appear to have different sensitivities to heat, in proportion to the amount or the molecular exposure of such heat-sensitive amino acids. Secondary damage resulting from further heat treatment might be due to attack upon another amino acid or group of amino acids, larger in amount [26].

Human milk has excellent antioxidant properties, known to protect against the potentially-harmful effects of oxidative stress. It contains vitamin C and E and enzymes, including superoxide dismutase, catalase, and glutathione peroxidase [13]. Apart from its role in maintaining the viability and texture of human tissue cells, it also modulates immune-mediated mechanisms in the body for a healthy survival. In a previous research, the TAC of human breast milk samples was time-tested by Xavier *et al* through the various phases of lactation [11]. Though a decline in the TAC was observed from colostrum through the various phases of lactation [11], their levels in milligrams/deciliter are found far higher than the 3 infant feeds researched in this work. With the growing concerns in today's society over maintaining a healthy lifestyle, one of the keys to protection against oxygen radical disease could be rethinking what the infant should be fed each day.

It should be the supreme responsibility of all health professionals, especially pediatric specialists, to provide anticipatory guidance to mothers regarding the health benefits of infant feeds, especially breast milk and their handling precautions. Medical/dental homes should compulsorily provide prenatal and postnatal counseling to mothers regarding the importance of providing infants with the richest antioxidant feed.

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COMPETING INTERESTS

All co-authors contributed substantially and approved the final version of his manuscript. The authors declare no conflict of interest pertaining to this research.

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