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Antioxidant and Radical Scavenging Activity of Human Colostrum, Transitional and Mature Milk

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Summary **Human milk from healthy women contains numerous nutrients such as antioxidants which are necessary for newborns. The aim of this study was to evaluate the changes of total antioxidant capacity (TAC) and free radical scavenging activity in human milk during the first six month period of lactation and also its relationship to maternal plasma. A total of 505 milk samples (colostrum, transitional and mature milks) collected from 115 healthy women with full term newborns. Blood plasma was obtained from 58 women at 3 months postpartum. The TAC of samples were measured by Ferric Reducing/Antioxidant Power assay and free radical scavenging activity were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. TAC was obviously higher in colostrums than transitional and mature milks. Similar results were observed for DPPH radical scavenging activity of the samples. There was a high significant correlation between the results of these two methods. The relationship between the antioxidant content of human milk and maternal plasma was also significant. These data suggest that using colostrum, with high antioxidant potential during the first days of life is vital; moreover, reduction in total TAC during the course of lactation may needs more attention about nutritional status.**

Key Words: **total antioxidant activity, radical scavenging activity, colostrum, transitional milk, mature milk**

Introduction

Human milk has been considered as a package of essential nutrients (vitamins, minerals, essential amino acids and fatty acids) and is commonly known as the best kind of nutrition for neonates and infants for the first six months of life. Studies documenting the protective effect of breast milk against various infectious diseases in infants are presented, including respiratory infections, diarrhea, otitis media, and infections in premature infants [*1*, *2*]. In addition to numerous clinically significance of breastfeeding, It seems human milk has bioactive components that protect newborns from a hyperoxic challenge due to transition of life to an environment far richer in oxygen than intrauterine environment [*3*, *4*].

Oxygen is potentially toxic, because of the production of Reactive Oxygen Species (ROS). Since these components

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have the ability to interact with and alter essential cell molecules, they are extremely cytotoxic. Antioxidant defense mechanisms of the body may prevent the production of ROS or neutralize them [*5*]. When the production of ROS exceeds the capacity of the body's antioxidant defenses to detoxify them, a condition known as oxidative stress occurs. It is well believed that oxidative stress is involved in the pathogenesis of numerous neonatal diseases such as bronchopulmonary displasia, retinopathy of prematurity and necrotizing enterocolitis [*6*, *7*].

Human milk has a number of enzymatic and non enzymatic antioxidant constituents, like superoxide dismutase, glutathione peroxidase, catalase, vitamin E, vitamin C, β-carotene, which may protect newborns against ROS at the early stage of life [*6*, *8*]. However, for the best benefits and functions, milk will always be recognized as a synergistic mixture of multiple interacting factors. In the other hand, the antioxidant status of breast milk seems to be affected by the maternal antioxidant status, which in turn, could influence the antioxidant status of the breast-fed infants [*9*]. Although data on the content of individual antioxidants in milk are available [*10–12*], there is a necessity in using methods for investigating the total antioxidant activity of milk. To measure all the antioxidant activity present in biological fluids, various methods have been devised [*13*, *14*]. These assays are useful in getting a global picture of related antioxidant activities in body fluids and how they change in different conditions. The aim of this study is to evaluate the total antioxidant and free radical scavenging activity levels in human milk during the first six month period of lactation and its correlation with maternal plasma total antioxidant capacity. This evaluation was done by using the ferric reducing/antioxidant power (FRAP) assay and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method as simple and reliable experiments adopted for this investigation.

Methods

This study was approved by the Ethics Committees of the Birjand University of Medical Sciences. Informed consent was obtained from every mother. A total of 115 healthy women (26.9 ± 5.3 year old) with normal pregnancy and delivery were included in this prospective consecutive study. The primary requisite for inclusion was intent to breastfeed exclusively. Exclusion criteria included gestation <37 weeks, birth weight <2.5 kg, multiple pregnancy, major illness requiring intensive care admission, and major congenital anomaly.

We tried to take sample milk at 5 different times but due to missing some mothers at some points, our final samples were consist of 115 samples of colostrum at the first 2 ± 1 days of postpartum, 97 samples of transitional milk at 7 ± 3 days postpartum and 293 samples of mature milks in three times (102 at 30 ± 3 days, 100 at 90 ± 7 days and 91 at 180 ± 10 days postpartum), among them we had just 68 cases with 5 complete samples. The samples were taken by manual expression of each breast into 10 ml sterile containers between 9.00–11.30 at morning and then aliquoted into 2 ml screw-top cryovials. Samples were stored at −70°C until analysis.

Blood samples were drawn by venipuncture (5 ml) into heparinized tubes but due to some limitations we just took blood samples at 90 ± 7 days postpartum for one time. The samples were immediately centrifuged to obtain plasma, which was then aliquoted into 2 ml cryovials and stored at −70°C until analysis.

Determination of total antioxidant activity

The FRAP assay, developed by Benzie and Strain [*15*] as a direct method for measuring the total antioxidant power of biological fluids, was adopted in this study. At low pH, reduction of a ferric 2,4,6-tripyridyl-s-triazine [Fe (III)-TPTZ] complex to the ferrous 2,4,6-tripyridyl-s-triazine [Fe (II)-TPTZ] complex, which has an intense blue color, can be monitored by measuring the change in absorption at 593 nm. The working FRAP reagent was prepared by mixing 10 volumes of 300 mmol/L acetate buffer, pH 3.6, with 1 volume of 10 mmol/L TPTZ in 40 mmol/L HCl and 1 volume of 20 mmol/L $FeCl_3$. A proper amount of sample (20 μl of breast milk or 50 μl of plasma) was mixed with 1.5 ml of freshly prepared FRAP reagent and incubated at 25°C for 10 min then reading was taken at 593 nm. To omit milk turbidity in experiments, 20 μl of breast milk and adequate volume of acetate buffer, was used as sample blank. Aqueous solutions of $FeSO_4 \cdot 7H_2O$ (100–1000 μM) were used for the calibration and the results were expressed as FRAP value (μM Fe (II)) of the samples.

DPPH radical scavenging activity

The free radical scavenging activity of milk samples were measured by the DPPH method proposed by Brand-Williams, Cuvelier, and Berset with a slight modification [*16*]. Briefly, 100 μl of each sample was added to 2 ml of DPPH in ethanol solution (100 mM) in a test tube. After incubation at 37°C for 30 min, 1 ml of chloroform was added and centrifuged at 3000 g for 5 min. the absorbance of clear solution was determined at 517 nm using spectrophotometer. An ethanolic solution of DPPH (100 mM) was used as control and the percentage of DPPH radical scavenging activity was calculated according to the following equation:

Scavenging activity (%) =
[(absorbance of the control – absorbance of the sample)
/absorbance of the control]* 100.

Table 1. Total antioxidant capacity, DPPH radical scavenging activity values of colostrum, transitional and mature milks

	Colostrum	Transitional milk		Mature milk	
	2 ± 1 days (n = 115)	7 ± 3 days (n = 97)	30 ± 3 days (n = 102)	90 ± 7 days (n = 100)	180 ± 10 days (n = 91)
Total antioxidant capacity (μmol/L)	1061.6 ± 500.6	915.3 ± 511.4*	816.3 ± 379.4*	862.7 ± 457.7*	724.7 ± 302.4*
DPPH radical scavenging activity (%) (μmol/L)	50.4 ± 19.7	40.8 ± 20.0*	41.9 ± 19.4*	44.6 ± 18.5*	38.2 ± 17.3*

Values are presented as Mean ± SD and * indicate significant difference in comparison with colostrums (p<0.05).

Statistical analysis

Statistical analysis was performed using the SPSS 11.5 package. The data were expressed as means ± standard deviation (SD). A paired-samples t test was used for comparison of means at different times with the colostrum samples and also statistical comparisons between all groups were made by analysis of variance (ANOVA) with repeated measures. The correlation between parameters was determined by Pearson correlation analysis. p values less than 0.05 were considered significant.

Results

The total antioxidant capacity and radical scavenging activity of breast milk in different times of lactation was presented in Table 1. According to the paired-samples t test, there was a significant higher level of total antioxidant capacity measured by FRAP assay in colostrum in comparison to transitional and mature milks. The total antioxidant levels showed a trend to decrease from 1061.6 ± 500.6 μmol/L in colostrum to 724.7 ± 302.4 μmol/L after six months of study. In the DPPH test for radical scavenging activity, the colostrums were more potent (50 ± 20%) to reduce the stable radical DPPH in comparison with transitional and mature milks. Also statistical comparison were made by analysis of variance with repeated measures in cases with complete sampling (n = 68). These balanced data showed significant differences between the means of values at different times (p = 0.015).

There was a significant correlation between the results of FRAP and DPPH methods for total antioxidant capacity and radical scavenging activity of the samples of breast milk (r = 0.562, p<0.001) (Fig. 1).

The mean of maternal plasma antioxidant capacity was 842.0 ± 123.5 μmol/L ranged from 565.2 to 1193.4 μmol/L. This level was closely near to the mean of breast milk antioxidant capacity at 90 ± 7 days that was 862.7 ± 457.7 μmol/L and ranged from 121.5 to 3816 μmol/L and there was a significant correlation between the maternal plasma and breast milk antioxidant capacity (r = 0.267, p<0.05) (Fig. 2).

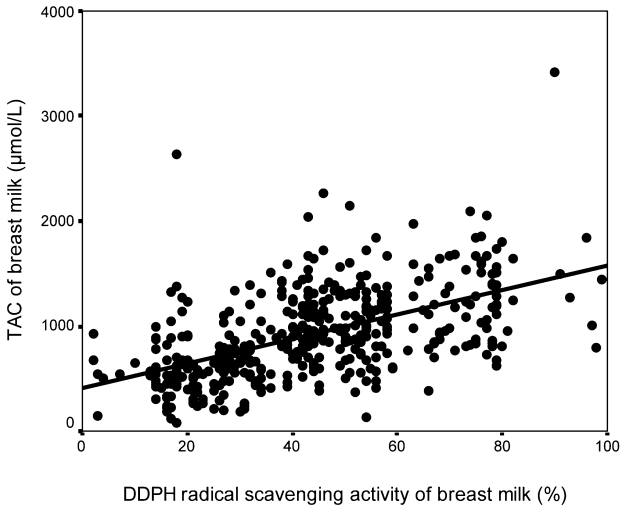

Fig. 1. The correlation between the results of TAC by FRAP method and radical scavenging activity using DPPH radicals in human colostrum, transitional and mature milk samples (r = 0.562, p<0.001).

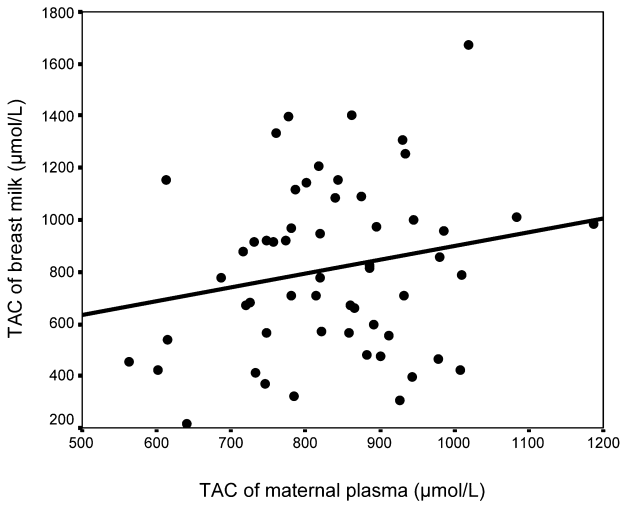

Fig. 2. The correlation between TAC of breast milk and maternal plasma (r = 0.267, p<0.05).

Discussion

In our research, the most significant result is that colostrums in comparison to transitional and mature milks has more total antioxidant activity decreasing during the course of lactation. There are only few reports that measured TAC and free radical scavenging activity of breast milk. Recently, the same pattern for total antioxidant capacity was reported by Quiles *et al.* [*17*]. They showed that the total antioxidant capacity of milk registered significantly higher values for colostrums compared with mature and transitional milk. These authors used 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS+) assay for total antioxidant capacity of breast milk. Ezaki *et al.* reported that TAC tends to decrease with the passage of time [*18*]. They used biological antioxidant potential (BAP) test for measuring of TAC which is principally very similar to FRAP assay. Also, Fidanza *et al.* reported a high, but not significant antioxidant capacity in colostrums [*19*]. The reason for this difference is probably due to the type of the methods used for total antioxidant capacity of milk and the number of samples. They used ORAC (oxygen radical absorbent capacity) assay with 30 samples whereas we used FRAP assay with 115 samples.

In the FRAP method the ability of the sample to reduce the ferric ion is used as a criterion on antioxidant capacity. Ascorbic acid, α-tochopherol, uric acid, Bilirubin and phenolic compounds were found to have ferric-reducing activity, but this method was suggested to be unsuitable for proteins, glutathione and lipoic acid [*15*, *20*]. DPPH is a stable commercial available free radical using as a popular marker for screening of free radical scavenging activity of compounds or biological samples. This method measures hydrogen atom or electron-donating activity and the scavenging activity related to the structure of the active substances [*16*]. Since the activities of antioxidant may vary in different biological systems, it is necessary to employ several methods to measure total antioxidant capacity based on different principles [*14*]. In our results, it is shown that there is a positive correlation between the results of the FRAP and DPPH assays. So these simple, speedy and inexpensive methods may be considered as practical indicators of total antioxidant activity of biological samples and useful in all the studies concerning the evaluation of oxidative stress.

High antioxidant capacity in colostrums can be effective in preventing newborns from exposure to an environment rich in oxygen after birth, 4 to 5 times as much as interauterine environment [*4*]. There is some evidence that susceptible newborns, especially preterm ones, are potentially vulnerable to oxidative stress due to the inefficiency of their antioxidant defense system or increase in free radical production [*21*]. In this situation damage to main molecules, lipids, proteins, carbohydrates and nucleic acids may increase [*22*]. In this way, oxygen therapy in newborns with respiratory problems which can induce and intensify oxidative stress should be considered more cautiously. Also it has been recently demonstrated that phototherapy for jaundiced neonates is related to increased oxidative stress and should be used with care [*23*].

Hence newborn feeding with the breast milk especially colostrums can be useful to neutralize free radicals and improve antioxidant system. It has been shown that 8-OH deoxyguanosine levels as a sensitive marker for oxidative stress is lower in infants fed with breast milk compared with those fed with formula [*24*]. In another report an increase was also found in the frequencies of sister chromatid exchange of infants not breast-fed compared to those who were breast-fed [*25*]. In addition according to epidemiologic studies, a reverse correlation between breast feeding and some diseases such as diabetes mellitus, cancer and cardiovascular diseases was found [*1*].

In this study total antioxidant capacity of the breast milk showed a significant decrease during the course of lactation which can be a natural result of decline in antioxidant storage of the mothers. In addition, a large variation was observed between total antioxidant values. Mothers with low values need more attention with regard to their nutrient intake, especially natural antioxidants during lactation.

Moreover, a significant correlation was observed between maternal plasma antioxidant capacity and their breast mature milk at 3rd month, which this situation may be observed in colostrum and transitional milk. This is in accordance with the result of Vanderjagt *et al.* and can be used as a predictor of breast milk antioxidant capacity [*26*]. Despite closeness between the mean values of maternal plasma and breast milk antioxidant capacity, the variation among breast milk values is remarkable. On the other hand, since the uric acid concentration is lower in breast milk in comparison with plasma [*27*], equal and higher levels of breast milk antioxidant capacity indicate that milk is concentrated for some antioxidants.

Conclusion

These data suggest that using colostrum, with high antioxidant capacity and radical scavenging activity during the first days of life is vital; moreover, reduction in total antioxidant capacity during the course of lactation and its relation to maternal antioxidant status needs more attention about nutritional status of mothers. More investigations prefer to evaluate the *in vivo* efficacy of breast milks with different levels of total antioxidant capacity.

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