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Maternal anemia induces changes in immunological and nutritional components of breast milk

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Abstract

Objective: The effects of low maternal hemoglobin levels on the immunological and nutritional components of breast milk at different maturation stages were investigated.
Methods: Colostrum, transitional and mature milk were collected from 25 mothers with normal hemoglobin levels (control group) and 18 mothers with hemoglobin levels below 11 g/dL (anemia group). Total protein, antibodies, complement proteins, fat and calorie, lipase, iron, transferrin levels, total iron-binding capacity, latent iron-binding capacity (LIBC) and transferrin saturation index (TSI) were determined.
Results: In contrast to the control group, anemic mothers had higher total protein levels in milk, lower IgA and IgG levels in colostrum, lower C3 protein levels in milk, lower C4 protein levels in colostrum and transitional milk, higher fat in the colostrum and lower calorie content in mature milk. In both groups, lipase was lower in mature milk and iron concentration was similar. Transitional and mature milk from anemic mothers had higher LIBC and lower TSI values.
Conclusion: A decrease in maternal hemoglobin levels causes immunological and nutritional alterations in milk at different maturation stages. Special measures must therefore be taken for mothers at risk of developing anemia to ensure they can provide high-quality milk to their babies.

Keywords

Anemia, antibody, complement protein, enzymes, milk maturation

History

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Introduction

Breastfeeding provides optimum nutrition for infant growth and development. It is considered the first line of defense for newborns because breast milk is rich in soluble and cellular components that protect against gastrointestinal and respiratory infections. In addition, breastfeeding is economically valuable since it is one of the most efficient, cost-effective strategies for providing health benefits to both mothers and newborns [1,2].

The chemical composition of breast milk changes over time according to the nutritional needs of the infant, i.e. it adjusts to pregnancy stage (prepartum and postpartum) and time of day (night and daytime). Milk composition can also be modified by feeding habits and diseases contracted by the mother [3].

Breast milk can be classified as colostrum (1–7 d postpartum), which is rich in immunoglobulins, proteins, fat soluble vitamins, minerals and leukocytes [1]; transitional milk (7–15 d postpartum), rich in proteins, enzymes, vitamins and minerals but with low leukocyte levels; and mature milk (15 d or more postpartum), consisting of proteins, carbohydrates, lipids, minerals and vitamins and most importantly, enough water to supply child needs [2].

Breast milk is particularly rich in secretory IgA antibodies (sIgA – 1,2,3), which are able to block bacterial adherence to epithelial cells [4], neutralize toxins [5], prevent viral infections [4] and act as an opsonin to protect against various microorganisms [6,7]. It also contains immunoglobulins IgG and IgM, which play a complementary protective role in the respiratory mucosa and gastrointestinal tract of newborns [8], and complement system proteins, particularly C3 and C4, which act primarily as opsonins [9]. These proteins are also important for phagocytosis [10] and microbicidal activity [6,7].

The nutritional status of mothers should be evaluated during breastfeeding since it can affect the nutritional and immunological composition of milk [11]. For instance, deficiency in iron and other minerals might compromise the immune system [12].

Anemia is a grave and widespread public health problem in developing countries. It is especially serious for pregnant woman given the health risk for both mother and fetus [13]. However, the effects of anemia on maternal body changes must be further studied to assess their implications on pregnancy and breastfeeding. Iron levels in breast milk are

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known to be unaffected by maternal iron reserves or by ferritin and transferrin saturation [14]. The mechanisms involving iron transfer to breast milk are not fully understood, but this element is important because it provides bacteriostatic properties to milk. Although iron transfer is not compromised by maternal nutritional status [15], the effects of anemia on the nutritional and immune quality of breast milk at different maturation stages remain unknown. This issue is investigated in the present study, by evaluating the nutritional and immunological components of breast milk in anemic mothers at different maturation stages (colostrum, transitional and mature milk).

Materials and methods

Subjects

After an informed consent form had been signed by the volunteers, about 15 mL of colostrum was collected manually from clinically healthy women, 18–35 years of age in the diurnal period [10]. During prenatal care, 18 pregnant volunteers were diagnosed with hemoglobin levels below 11 g/dL (anemia group), which defines the anemia of pregnancy and that women at nutritional risk (anemic group [16]) and 25 were confirmed to be normal hemoglobin levels (control group), at the Health System Program of Barra do Garças, Mato Grosso, Brazil. All the mothers had given birth to healthy term babies through vaginal delivery. The variables controlled in both groups during pregnancy were smoking status (yes/no), arterial hypertension (yes/no) and absence of infectious diseases. One breast milk sample was collected from each mother at 3 d postpartum (colostrum), 10 d postpartum (transitional milk) and 30 d postpartum (mature milk), a total of 129 samples. All procedures were submitted for ethical evaluation and obtained Institutional approval.

Creamatocrit analysis

The samples were heated to 40 °C for 15 min in a water bath and subjected to vortex mixing. Capillary tubes (2 µl) were approximately ¾ filled with sample, sealed with sealing wax and then centrifuged for 15 min. Centrifugation separated the samples into cream and serum. The cream column and the total column were measured and fat and Kcal content calculated using the following formulae:

(1) Fat content = % cream (mm) − 0.59/1.46,
where the % cream = cream column (mm) × 100/total column (mm);

(2) Kcal/L = (66.8% × cream) + 290.

Obtaining supernatant from colostrum and human milk

Colostrum and human milk supernatant was obtained by centrifugation (10 min, 160 g, 4 °C). The upper fat layer was discarded and the aqueous supernatant stored at −70 °C for later immunological and biochemical determination.

Total protein determination

Total protein was determined by the colorimetric method. Samples of human colostrum, transitional, mature milk and standard of 4 g/dL (Labtest, Minas Gerais, Brazil), were placed in 1.0 mL Biuret reagent (ions of copper in alkaline medium). The suspensions were mixed and incubated for 10 min at 37 °C. The reactions were read on a spectrophotometer at 540 nm.

IgA, IgM and IgG determination

Human colostrum, transitional and mature milk concentrations of IgA, IgG and IgM were determined by quantitative radial immunodiffusion according to Mancini et al. [17]. A tube containing 10 mL of 1% agarose was heated to fusion in a water-bath and then transferred to bath at 56 °C for temperature stabilization. Anti-human IgA, lamb serum (Biolab), anti-human IgM (Sigma, St Louis, MO, USA) and anti-human IgG (Sigma, St Louis, MO, USA) antibodies were added to the agarose and mixed by tube inversion. The mixture was placed between two glass plates separated by a spacer. After solidification, the plates were perforated and the samples applied. Antibody content in the milk samples was determined using the Kallestad standard curve.

C3 and C4 determination

The concentrations of C3 and C4 were determined by the turbidimetric method. Colostrum, transitional and mature milk samples were diluted at 1:12 (v/v) with a saline solution (9 g/L). C3 and C4 levels were determined in sample supernatants using C3 and C4 antisera (Bioclin, Minas Gerais, Brazil) diluted at 1:12 (v/v). A calibration curve obtained by the Multical (Bioclin, Minas Gerais, Brazil) calibrator was used to determine the standard curve. Samples of 10 µL of colostrum standard and positive or negative control sera (Bioclin, Minas Gerais, Brazil) were placed in a 500 µL buffer solution (sodium chloride 0:15 mol + l, Tris 50 mmol + L, 6.0000 PEG 50 g + L, sodium azide 15:38 nmol/L). The suspensions were mixed and incubated for 15 min at 37 °C. The reactions were read on a spectrophotometer at 340 nm.

Lipase determination

Lipase was determined by the colorimetric method. Samples of 1.0 mL colostrum, transitional and mature milk were mixed with Tris buffer (100 mmol/L hydroxymetylamine methane, pH 8.5), phenylmethyl sulfonyl fluoride (8 mmol/L) and DTNB (3 mmol/L acid dithionitrobenzoic, 100 mmol/L sodium acetate, pH 6.0) and incubated for 2 min at 37 °C. After this period, 100 mL of tributyrate pyridine propanol (20 mmol/L and surfactant) was added to the solution and stirred for 30 min at 37 °C. The suspensions were subsequently added with 2.0 mL of acetone (p.a.), homogenized and kept still at room temperature for 3 min. The suspensions were then centrifuged at 400 × *g* for 5 min and read at 410 nm. A control assay was carried out without phenylmethysulfonyl fluoride (enzymatic inhibitor).

Determination of iron, latent and total iron-binding capacity, transferrin and transferrin saturation index

Latent iron-binding capacity (LIBC) was determined using the colorimetric method. Iron and ferric ions were dissociated from transferrin in acidic medium (pH = 4) and reduced to ferrous ions by the action of 144 mM hydroxylamine. The addition of 28 mm ferrozine formed a colored complex proportional to the

iron content in the sample and absorbance was measured in spectrophotometer at 560 nm. To determine LIBC, available sites in transferrin were saturated with 500 μg/dL standard iron (buffered at pH 8.3) and incubated at 37 °C for 10 min. Excess unbound iron was measured after incubation with 28 mM dye ferrozine at 37 °C for 10 min, producing a colored complex that was spectrophotometrically read at 560 nm. Iron levels and LIBC were used to calculate the values of total iron-binding capacity (TIBC), transferrin saturation index (TSI) and transferrin using the equations: TIBC (mg/dL) = Iron (mg/dL) + LIBC; TSI (%) = (Iron (mg/dL)/TIBC) × 100; Transferrin (mg/dL) TIBC × = 0.7 [18].

Statistical analysis

Two-way analysis of variance was used to evaluate the antibody concentration, complement protein, total protein, calories, fat, iron, LIBC and TIBC, transferrin and TSI, considering the post-partum stage as one factor and anemia as the other. Statistical significance was considered when $p < 0.05$.

Results

Subject characteristics

Body mass at early (control group = 57.4 ± 10.4; anemia group = 59.0 ± 13.5) and late pregnancy (control group = 67.4 ± 10.0; anemia group = 71.9 ± 13.1) was similar between the two groups studied. Mothers (control group = 9.9 ± 5.8; anemia group = 12.9 ± 3.6) from both groups had similar mass gain. Hemoglobin levels were lower in the anemia group (10.6 ± 0.2) than in control group (12.8 ± 0.4). The mean and standard deviation for gestational age were 39.0 ± 0.7 weeks in control group and 37.9 ± 0.7 weeks in anemia group and for newborns at birth were 2856.3 ± 341.8 control group and 2740.1 ± 392.1 in anemia group.

Total protein

Total protein levels were higher in the milk produced by anemic mothers, irrespective of maturation stage (Table 1).

Antibodies

IgA levels were low in colostrum, transitional milk and mature milk from anemic mothers. Colostral IgG levels were higher in the anemia group, but IgG levels in transitional and mature milk were similar between the groups. IgM levels were also similar between the groups (Table 1).

Complement proteins

C3 protein levels were lower in milk from anemic mothers, irrespective of milk maturation. The anemia group also had lower levels of C4 protein in colostrum and transitional milk (Table 1).

Fat, calories and lipase

In the control group, the highest fat and calorie content was found in transitional milk, whereas in the anemia group it was found in colostrum. Colostrum samples from the anemia group had higher fat and calorie content (Table 2). In the control group, calorie content was higher in transitional than in mature milk. In the anemia group, mature milk had the lowest calorie content. Lipase levels were lower in mature milk irrespective of anemic status (Table 2).

Iron, LIBC and TIBC

Iron concentration in breast milk at the different maturation stages did not vary between the groups (Table 3). Transitional and mature milk from anemic mothers had higher LIBC compared to the control group. The lowest LIBC was observed in transitional and mature milk from the control group (Table 3). TIBC was similar between the groups and types of milk (Table 3).

Transferrin concentration and TSI

Overall transferrin levels were similar between the groups and within each group among the types of milk (Table 3). However, transferrin saturation was lower in transitional and mature milk from the anemia group, while transitional and mature milk from the control group had the highest transferrin saturation (Table 3).

Table 1. Total protein, immunoglobulins and complement protein concentrations in colostrum, transitional milk and mature milk from anemic mothers.

Parameter	Groups	Colostrum	Transitional milk	Mature milk	Statistical
Total protein (mg/dL)	Control	4.5 ± 0.8	3.7 ± 0.5	2.6 ± 0.8*	$F(2, 30) = 5.6772$; $p = 0.0082$ (comparing the kind of milk)
	Anemia	5.7 ± 0.7†	5.5 ± 0.6†	4.4 ± 0.4†	$F(1, 30) = 14.1690$; $p = 0.0010$ (comparing the groups)
IgA (mg/dL)	Control	331.9 ± 49.8	300.9 ± 45.7	293.1 ± 47.5	$F(2, 48) = 0.1153$; $p = 0.89$ (comparing the kind of milk)
	Anemia	214.4 ± 50.1†	268.2 ± 49.1	252.4 ± 55.4	$F(1, 48) = 4.7911$; $p = 0.0315$ (comparing the groups)
IgG (mg/dL)	Control	150.7 ± 32.1	103.6 ± 12.1*	110.1 ± 32.6	$F(2, 36) = 2.5964$; $p = 0.0467$ (comparing the kind of milk)
	Anemia	102.9 ± 19.7†	83.2 ± 5.2†	87.8 ± 10.1†	$F(1, 36) = 12.2433$; $p = 0.0016$ (comparing the groups)
IgM (mg/dL)	Control	135.8 ± 29.6	116.3 ± 30.6	121.4 ± 31.9	$F(2, 42) = 0.3389$; $p = 0.7193$ (comparing the kind of milk)
	Anemia	93.0 ± 23.4	134.3 ± 21.8	113.4 ± 11.6	$F(1, 42) = 0.8320$; $p = 0.6300$ (comparing the groups)
C3 (mg/dL)	Control	155.6 ± 55.6	188.1 ± 51.7	130.9 ± 15.6	$F(2, 42) = 1.6635$; $p = 0.2001$ (comparing the kind of milk)
	Anemia	87.0 ± 33.5†	77.3 ± 22.2†	73.7 ± 29.6†	$F(1, 42) = 32.8928$; $p = 0.00001$ (comparing the groups)
C4 (mg/dL)	Control	47.5 ± 9.7	52.8 ± 31.7	39.4 ± 15.7	$F(2, 42) = 0.2153$; $p = 0.8093$ (kind of milk)
	Anemia	22.5 ± 6.3†	27.0 ± 14.5†	35.0 ± 3.1	$F(1, 42) = 7.6673$; $p = 0.0082$ (comparing the groups)

Results are expressed as the mean and SD.
*Statistical differences between milk collection period for a same group.
†Statistically significant differences between the control and anemic mothers, considering the same kind of milk.

Table 2. Calorie, fat and lipase concentrations in colostrum, transitional milk and mature milk from anemic mothers.

Parameter	Groups	Colostrum	Transitional milk	Mature milk	Statistical
Fat (%/mL)	Control	2.31 ± 0.8	3.90 ± 0.7*	2.8 ± 0.7	$F(2, 30) = 3.80$; $p = 0.033$ (comparing the kind of milk)
	Anemia	4.3 ± 0.3*†	3.40 ± 0.8	2.30 ± 1.0	$F(1, 30) = 4.23$; $p = 0.04$ (comparing the groups)
Calorie (Kcal/mL)	Control	525.3 ± 72.2	697.0 ± 70.5*	584.7 ± 91.9	$F(2, 30) = 4.4341$; $p = 0.021$ (comparing the kind of milk)
	Anemia	710.3 ± 61.5*†	684.3 ± 75.6*	553.7 ± 98.5	$F(1, 30) = 2.2941$; $p = 0.0451$ (comparing the groups)
Lipase (UI/mL)	Control	27.5 ± 1.4	25.3 ± 3.6	22.8 ± 1.2*	$F(2, 30) = 4.7$; $p = 0.016$ (comparing the kind of milk)
	Anemia	30.3 ± 2.2	24.4 ± 7	20.8 ± 3.2*	$F(1, 30) = 0.0911$; $p = 0.7623$ (comparing the groups)

Results are expressed as the mean and SD.
*Statistical differences between milk collection period for a same group.
†Statistically significant differences between the control and anemic mothers, considering the same kind of milk.

Table 3. Iron concentration, LIBC, TIBC, TSI in colostrum, transitional milk and mature milk from anemic mothers.

Parameter	Groups	Colostrum	Transitional milk	Mature milk	Statistical
Iron (μg/dl)	Control	86.2 ± 37.3	166.2 ± 53.3	157.1 ± 51.2	$F(2, 30) = 0.5457$; $p = 0.5902$ (comparing the kind of milk)
	Anemia	134.7 ± 58.2	112.1 ± 47.3	116.9 ± 58.1	$F(1, 30) = 0.3869$; $p = 0.5454$ (comparing the groups)
LIBC (μg/dL)	Control	376.8 ± 47.6	266.8 ± 42.0*	275.1 ± 42.4*	$F(2, 30) = 4.2989$; $p = 0.0223$ (comparing the kind of milk)
	Anemia	393.2 ± 34.7	371.6 ± 30.2†	371.2 ± 7.6†	$F(1, 30) = 18.8420$; $p = 0.0003$ (comparing the groups)
TIBC (μg/dL)	Control	428.6 ± 82.7	447.9 ± 75.7	454.5 ± 43.3	$F(2, 30) = 1.2265$; $p = 0.3076$ (comparing the kind of milk)
	Anemia	535.7 ± 85.5	467.1 ± 74.0	457.4 ± 39.2	$F(1, 30) = 1.2524$; $p = 0.2714$ (comparing the groups)
Transferrin (mg/dl)	Control	324.2 ± 70.1	313.5 ± 53.0	318.2 ± 30.3	$F(2, 30) = 0.6143$; $p = 0.5524$ (comparing the kind of milk)
	Anemia	369.6 ± 54.4	327.0 ± 51.8	320.2 ± 27.5	$F(1, 30) = 0.5873$; $p = 0.5444$ (comparing the groups)
TSI (%)	Control	18.0 ± 8.9	45.5 ± 8.7*	45.3 ± 12.9*	$F(2, 30) = 5.9216$; $p = 0.0070$ (comparing the kind of milk)
	Anemia	26.5 ± 7.0	23.1 ± 7.5†	18.1 ± 7.1†	$F(1, 30) = 8.7347$; $p = 0.0061$ (comparing the groups)

Results are expressed as the mean and SD.
*Statistical differences between milk collection periods for a same group.
†Statistically significant differences between the control and anemic mothers, considering the same kind of milk.

Discussion

Anemia is defined as a deficiency in the amount of oxygen-carrying hemoglobin in the red blood cells. Individual differences that influence circulating hemoglobin levels are not necessarily caused by nutritional disabilities [19]. Low hemoglobin levels are likely to affect both mother and fetus [20]. Women in the anemia group had hemoglobin values below the normal range. However, anemic status did not affect body mass or weight gain of mothers and newborns.

The effects of breastfeeding on the growth, development and protection of the child as well as the epidemiological and emotional aspects involved have been extensively studied in recent years [1,2,6,7,21,22]. The first clinical use of breast milk was as a vehicle for passive immunity transfer, but its immune compounds, which contain high immunoreactivity, change over time [2].

The relationship between the immunological components of human milk and physiological characteristics of mothers is a matter of discussion. Some studies suggest that maternal characteristics cause variations in immune components of colostrum and milk [1,23], whereas others report changes driven by nutritional status [24]. Our study shows the influence of maternal nutrition on milk composition. Colostrum, transitional and mature milk from anemic mothers had higher levels of total proteins, but this was not reflected in the secretion of immunoreactive proteins (antibodies and complement proteins), which was similar between the groups. The anemia group had lower IgA, IgG and C3 levels in breast milk at all maturation stages and lower levels of C4 protein in transitional milk.

Human milk IgA plays a protective role against a number of microorganisms, acting as opsonins [8,25], blocking bacterial adherence to epithelial cells [4], neutralizing toxins and preventing viral infections [4]. These functions are complemented by the antibodies IgM and IgG [8] and particularly by complement proteins C3 and C4 [10]. Secretory IgA in breast milk serves as optimal antigen-targeted passive immunization of the breastfed infant's gut. Breastfeeding is therefore the best defense against mucosal infection in developing countries. The protection offered by breastfeeding depends not only on immunoglobulin levels or other immunoreactive proteins [8,10], but also on the amount, time and type of milk consumed [2,8].

The complexity of human milk makes this secretion the ideal food source for babies for at least the first 6 months of their life. This early nutrition is an important environmental input that can exert lifelong effects on child metabolism and development.

Breastfeeding can support normal child development when fat and calorie content is balanced. The nutritional composition of breast milk, particularly of the fat fraction, can be modulated by mother's diet, age, parity, nutritional status and socioeconomic conditions as well as prematurity and retarded intrauterine fetal growth [26].

Lipase levels were higher in colostrum and decreased with milk maturation in both anemia and control groups. In the anemia group, fat and calorie content in colostrum was higher.

Our results support the hypothesis that changes in maternal nutrition affects lipid metabolism in the mammary gland. This increase in fats and calories in colostrum is likely associated to the higher energy supply received by children of anemic mothers, which is suitable for adequate growth and development.

Anemia is a multi-causal, complex etiology that involves cultural, socioeconomic, geographic and biological factors. Effective strategies for anemia prevention must be based on nutritional education to promote proper dietary supplementation during pregnancy and lactation [26].

Iron levels in milk are correlated with maternal hemoglobin levels, but not with ferritin levels [27]. In the present study, reduction of maternal hemoglobin was not associated to a reduction in LIBC or TSI in transitional and mature milk. The mechanisms involved in iron transfer from blood to milk are only partially understood. Factors affecting maternal iron metabolism, such as infections, are not found in breast milk [15].

Breast milk may contain low iron content to allow lactoferrin to remain mostly unsaturated, thereby maintaining its bacteriostatic properties. Other studies on iron distribution in breast milk fractions observed low transferrin saturation and lactoferrin content [28]. These enzymes, natural milk proteins that transport and fixate iron, have bacteriostatical action [29,30]. Maternal nutritional factors, such as iron levels, may play an important role not only in milk iron levels [27] but also in the immune defenses of both mother and newborn [30].

In conclusion, our findings indicate that a decrease in maternal hemoglobin levels can influence the quality of breast milk at different stages of maturation, in terms of immunological and nutritional properties. Owing to the importance of iron for anemia prevention as well as the role of breastfeeding in child development, new public health policies should be developed to promote nutrition education for mothers at risk for anemia and ensure high-quality milk for their children.

Declaration of interest

The authors declare no conflict of interest and non-financial competing interests.

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