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Full Length Research paper

Lactoferrin levels in human breast milk among lactating mothers with sick and healthy babies in Kaduna State, Nigeria

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Breastfeeding of babies has received worldwide recommendation and acceptance due to its high level of bioactive constituents. Lactoferrin, an iron binding glycoprotein is one of the major bioactive components of breast milk. Lactoferrin has many proposed biological functions which include antibacterial/anti-inflammatory activities, participation in local secretory immune systems in synergism with some immunoglobulins and other protective proteins among other functions. The levels of this protein (lactoferin) in human breast milk (colostrums, transitional and mature milk) were evaluated using mothers with healthy as well as sick babies. The aim was to ascertain if the level of lactoferrin in the human breast milk has any correlation with the health status of the baby with reference to the development of neonatal sepsis. From the result gotten, the mean lactoferrin levels in the breast milk of mothers with healthy babies were colostrum (9.55 ± 10.61 mg/ml), transitional milk (9.18 ± 10.02 mg/ml) and mature milk (9.19 ± 8.81 mg/ml). However, lower values were obtained that were statistically significant at P<0.05 for the lactoferrin levels in the breast milk of mothers with sick babies. The overall result showed that colostrum had the highest lactoferrin value as compared to transitional and mature milk even as the mean values in the mothers with sick babies were still significantly lower than those obtained from mothers with healthy babies. Age variations were also shown to play significant roles in the level of lactoferrin in breast milk. For the mothers with healthy babies at age 20 and below, the mean value for colostrum, transitional and mature milk were 9.00 ± 8.36, 14.00 ± 13.00 and 8.00 ± 9.00 mg/ml, respectively. The result for the mothers between 31–40 years showed 5.00 ± 1.00 mg/ml for colostrum, 12.00 ± 11.00 mg/ml for transitional milk and 8.00 ± 9.00 mg/ml for mature milk. Mothers with sick babies had lower values when compared to the corresponding ages of the mothers with healthy babies. The study thus showed that lower levels of lactoferrin in mother's breast milk could induce the development of neonatal sepsis and age variation was shown to be capable of affecting the level of lactoferrin in the breast milk.

Key words: Breast feeding, mothers, sick babies, healthy babies, lactoferin, colostrums, transitional, mature milk.

INTRODUCTION

There is a worldwide recommendation and acceptance of exclusive breastfeeding due to the fact that breast milk has been found to be rich in a lot of bioactive materials. Lactoferrin one of the major bioactive components was first isolated from cow's milk and subsequently from human milk (Losnedahl et al., 1998). It is an iron binding glycoprotein that consists of a single polypeptide chain of relative molecular mass (Mr) of 78kD with 703 amino acid residues (Zitka et al., 2007). It is the second most abundant protein in human milk (Masson and Heremans, 1971; Hennart et al., 1991). It is found in most exocrine secretions including tears, nasal secretions, saliva, intestinal mucus and genital secretions (Yu and Chen, 1993). This protein is also expressed and secreted by the

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secondary granules of polymorphonuclear neutrophils (Masson et al., 1969). In human milk and colostrum, the reported concentrations of lactoferrin are 2-4 and 6-8 g/l, respectively. In its natural state, lactoferrin is only partly saturated with iron (5-30%) (Losnedahl et al., 1998).

The polypeptide structure of lactoferrin comprises two homologous domains that appear to have arisen by intragenic duplication (Metz-Boutigue et al., 1984). The crystal structure of the protein has been resolved (Anderson et al., 1989; Anderson et al*.,* 1990) and each domain binds one ferric and one carbonate anion. In addition, each domain contains one glycosylated site to which N-linked glycan residues are attached (Spik et al*.,* 1988). Holo-lactoferrin is formed from one linear polypeptide chain forming two spherical domains (C- and N-terminal) with each domain containing one iron binding site (Zitka et al*.,* 2007). Lactoferrin is a member of the transferrin family of non-heme iron binding proteins (Aisen and Listowsky, 1980). The family includes transferrin, the major iron transporting protein in blood (MacGillivray et al*.,* 1983; Yang et al*.,* 1984), the egg-white protein, ovotransferrin (Jeltsch and Chambon, 1982), the melanocyte protein, melanotransferrin (Rose et al*.,* 1986) and a recently identified carbonic anhydrase inhibitor (Wuebbens et al., 1997). Members of the transferrin family are distinguished from other iron binding proteins by their unique anion requirement for binding of iron.

Lactoferrin has many proposed biological functions, including antibacterial/anti-inflammatory activities, defense against gastro-intestinal infections, participation in local secretory immune systems in synergism with some immunoglobulins and other protective proteins, provision of an iron-binding antioxidant protein in tissues, and possibly promotion of growth of animal cells such as lymphocytes and intestinal cells (Losnedahl et al., 1998). The synergistic cationic effect of lactoferrin and lysozyme exhibit co-operative anti-staphylococcal properties. Binding of lactoferrin to lipoteichoic acid (LTA) is important in its synergy with lysozyme and interferes with the autolysins present on the LTA (Leitch and Willcox, 1999a). It appears that two different mechanisms involving two separate domains of the protein contribute to the antimicrobial functions of lactoferrin. The first mechanism is a bacteriostatic effect related to the high iron binding affinity of the protein that deprives iron-requiring bacteria of this essential growth nutrient (Arnold et al., 1977; Bullen et al., 1978; Reiter, 1978). Since the bacteriostatic properties of lactoferrin are due to its iron binding ability, the protein is capable of retarding the growth of a broad range of microorganisms including a variety of gram negative and gram positive bacteria and certain yeasts (Bullen et al., 1978; Reiter et al., 1975).

The objective of this study is to establish the lactoferrin levels in lactating mothers and compare the values between mothers whose babies developed sepsis with those mothers with healthy neonates. This will help determine if mothers with low levels of lactoferrin are predisposed to having babies born with the potential of developing sepsis.

MATERIALS AND METHODS

Breast milk samples

A total of 5-10 ml of breast milk was obtained from lactating mothers. The assistance of nurses on duty was employed to aseptically collect the samples. The mothers' consent was also obtained before collection. The milk samples collected based on time of delivery were colostrum, transitional and mature milk. These were stored into sterile sample bottles and transported on ice to the laboratory. A total of 500 women were involved in the study.

ELISA for lactoferrin

Kit content

The AssayMax human lactoferrin ELISA kit was obtained from ASSAYPRO (USA) for the test. All the reagents were allowed to warm up to room temperature (25°C) before use in accordance with the manufacturer's instruction. The kit contains plates pre-coated with polyclonal antibody against human lactoferrin, human lactoferrin standard, biotinylated lactoferrin antibody, streptavidin–peroxidase conjugate, sample diluent, wash buffer, chromogen substrate and the stop solution.

Assay procedure

The standard was diluted to obtain concentrations of 40, 10, 2.5, 0.625 and 0.156 ng/ml and 50 µl of the diluted standard solutions were added to wells 2, 3, 4, 5 and 6, respectively. Similarly, 50 µl of the test samples were added to the plates from wells 7 to the end of the other wells and the plate was incubated for 2 h at room temperature. The plates were aspirated after the incubation period and washed four times and then blotted to remove excess liquid from the plates. This was followed by addition of 50 µl of biotinylated lactoferrin to each well except the blank well and incubated for 1 h at room temperature. The plates were then washed again four times and blotted. Afterwards 50 µl of streptavidin–peroxidase conjugate was added to all the wells including the blank well. The plates were incubated again at room temperature for 30 min. This was followed by washing, blotting and the addition of the chromogen and a further incubation of 8 min for optimal colour development. The reaction was stopped by addition of 50 µl of the stop solution and the plates were read at a wavelength of 450 nm, using the microplate reader (SIGMA diagnostic ELISA reader).

RESULTS

The mean colostrum, transitional milk and mature milk lactoferrin levels in the breast milk of mothers with healthy babies were found to be 9.55 ± 10.61, 9.18 ± 10.02 and 9.19 ± 8.81 mg/ml, respectively. Similarly, the mean colostrum, transitional milk and mature milk levels in the mothers with sick babies were 6.85±7.03, 6.42 ± 7.85 and 3.62 ± 5.18 mg/ml, respectively. These results vary significantly at $P < 0.05$. This is presented in Tables 1 and 2.

Table 1. Mean values for mothers with healthy babies.

Milk	Mean (mg/ml)	N	Standard deviation
Colostrum	9.5487	78	10.60601
Transitional milk	9.1833	36	10.02493
Mature milk	9.1920	88	8.80739
Total	9.3282	202	9.70556

Table 2. Mean values for mothers with sick babies.

Milk	Mean (mg/ml)	N	Standard deviation
Colostrum	6.8529	120	7.02865
Transitional milk	6.4164	61	7.84558
Mature milk	3.6250	8	5.18066
Total	6.5754	189	7.23479

The mean obtained from the different age bracket of mothers is presented in Table 3. The overall result showed that colostrum had the highest lactoferrin value when compared to transitional and mature milk. The mean values in the mothers with sick babies were significantly lower than those obtained from mothers with healthy babies. In relation to age variation, the mean values recorded for mothers with healthy babies were significant at $P<0.05$. For the mothers with healthy babies at age 20 and below, the mean value for colostrum, transitional and mature milk were 9.00 ± 8.37, 14.00 ± 13.00 and 8.00 ± 9.00 mg/ml, respectively. The values for the mothers between 21–30 years varied from 10.00±13.00 mg/ml for colostrum, 6.00 ± 9.00 mg/ml for transitional milk and 10.00 ± 9.00 mg/ml for mature milk. The result for ages 31–40 years showed 5.00 ± 1.00 mg/ml for colostrum, 12.00 ± 11.00 mg/ml for transitional milk and 8.00 ± 9.00 mg/ml for mature milk. The figures for the 41–50 years category were few without statistical significance. Mothers with sick babies had lower values. The mothers at 20 years and below had 6.18± 5.08 mg/ml for colostrum and 2.55 ± 2.83 mg/ml for transitional milk. The mothers within the 21-30 years category had 7.01 ± 7.12 mg/ml for colostrum, 6.33 ± 6.94 mg/ml for transitional milk and 4.50 ± 5.82 mg/ml for mature milk. Similarly, the mothers within 31–40years had 9.81 ± 8.75 mg/ml for colostrum, 7.16 ± 6.96 mg/ml for transitional milk and 1.00 ± 0.00 mg/ml for mature milk. The figures for mothers within the 41 years and above were not statistically significant. The results are shown in Table 3.

DISCUSSION

The mean lactoferrin levels obtained from the mothers with healthy babies varied significantly ($P< 0.05$) from that of the mothers that had sick babies. This trend was observed for colostrum, transitional and mature milk samples. The mean values were higher in the mothers with healthy babies and was within the range described by Losnedahl et al. (1998). In human milk and colostrum, the reported concentrations of lactoferrin were 2-4 and 6-8 g/l, respectively (Losnedahl et al., 1998). This was also in agreement with their findings that the lactoferrin level in colostrum was higher than that for mature milk. The variation in the mean values was shown to be statistically significant. The low levels of lactoferrin obtained in the mothers with sick babies could account for the susceptibility of their babies to neonatal sepsis. This is because lactoferrin is one of the principal bioactive proteins present in milk (Losnedahl et al., 1998; Bayeye et al., 1999). Further research therefore should focus on the lethal levels of lactoferrin that is required for effective prevention of neonatal sepsis.

Neonatal sepsis may be categorized as either early or late onset (Ali et al., 2004). 85% of newborns with early-onset infection are present within 24 h, 5% within 24-48 h, and a smaller percentage of patients between 48 h- 6 days of life. Onset is most rapid in premature neonates. The microorganisms most commonly associated with the early-onset infection included group B *Streptococcus* (GBS), *Escherichia coli, Haemophilus influenzae,* and *Listeria monocytogenes.* The risk is greater in males than in females with a ratio of 2:1 (Mokuolu et al., 2002) and in newborns with congenital malformations, particularly of the gastrointestinal tract. The predominance of group B *Streptococcus* in neonatal sepsis has been documented by various studies (Lukacs et al., 2004; Ella et al., 2008). Ella et al. (2008) found that B *Streptococcus* was the prominent organism in Zaria metropolis, a principal city in Kaduna State. Other bacterial organisms implicated in neonatal sepsis in Kaduna state included *Enterobacter sp, Klebsiella sp, Escherichia coli* and *Citrobacter flexneri* (Ella et al., 2007; Ella et al., 2008b). It has been shown that 'natural' lactoferrin is bacteriostatic against a wide range of micro-organisms, including gram-negative bac-

Table 3. Lactoferrin levels in relation to age of mothers with healthy babies.

Health status	Age bracket (years)	Colostrum (mglml)	Transitional milk (mg/ml)	Mature milk (mg/ml)
Mothers with healthy babies	20 and below	9.00 ± 8.37	14.00 ± 13.00	8.00 ± 9.00
		N = 40	N = 10	N = 16
	21-30	10.00 ± 13.00	6.00 ± 9.00	10.00 ± 9.00
		N = 32	N = 18	N = 55
	31-40	5.00 ± 1.00	12.00 ± 11.00	8.00 ± 9.00
		N = 5	N = 8	N=15
	41-50	30.5 ± .00	-	5 ± 1.00
		N = 1		
Mothers with sick babies	20 and below	6.18 ± 5.08	2.55 ± 2.83	-
		N = 17	N = 12	
	21-30	7.01 ± 7.12	6.33 ± 6.94	4.5 ± 5.82
		N = 66	N = 29	N = 6
	31-40	9.81 ± 8.75	7.16 ± 6.958	1.00 ± 0.00
		N = 36	N = 19	N = 2
	41-50	10	4.10	

teria with high iron requirements (coliforms, which are major mastitis pathogens), and also against some gram-positive organisms such as *Staphylcoccus aureus* (also a major mastitis pathogen), bacillus species, and *Listeria monocytogenes* (Losnedahl et al., 1998). Lactic acid bacteria in the stomach and intestine have low iron requirements and are generally not affected (Losnedahl et al., 1998). The efficacy of bacteriostasis is dependent on the iron status of lactoferrin and is overcome by saturation of lactoferrin with iron (Griffiths and Humphreys, 1977).

The second antibacterial property of lactoferrin is due to a direct bactericidal function within the protein. Secreted lactoferrin exerts antimicrobial action by chelation of iron or destabilization of bacterial membranes (Bayeye et al., 1999). Studies have suggested that, on binding to the anionic LTA of *Staphylococcus epidermidis*, the cationic protein lactoferrin decreases the negative charge, allowing greater accessibility of lysozyme to the underlying peptidoglycan (Leitch and Willcox, 1999b). There is also evidence that on certain streptococcal mutants and *Vibrio cholerae*, lactoferrin can exert a direct, bactericidal effect that is independent of iron-deprivation (Losnedahl et al., 1998).

Lactoferrin has a direct bactericidal effect against some gram negative and gram positive bacteria that cannot be attributed to simple iron deprivation. Apolactoferrin can cause a rapid loss of bacterial viability that cannot be reversed by the addition of exogenous iron to the growth medium (Arnold et al., 1977; Arnold et al., 1981). At physiological concentrations, apolactoferrin directly damage the outer membrane of gram negative bacteria by causing the release of lipopolysaccharides (LPS) (Ellison et al., 1988). Synthetic peptides containing this cationic domain (amino acid residues 18–40) of human lactoferrin have a more potent bactericidal effect (Bellamy et al., 1992) and lead to a greater release of LPS (Yamauchi et al., 1993) than intact lactoferrin (Bellamy et al.,1992).

A significant body of evidence has accumulated in recent years to support a role for lactoferrin in the regulation of host immunity (Crouch et al., 1992; Machnicki et al., 1993). Lactoferrin is expressed in neutrophil secondary granules (Masson et al., 1969) and has been reported to have both positive (Sawatzki and Rich, 1989) and negative (Broxmeyer et al., 1987; Hangoc et al., 1991) regulatory effects on myelopoiesis. Systemic infection with bacteria is accompanied by a rapid rise in serum levels of lactoferrin secreted from granulocytes (Gutteberg et al., 1989) and a concomitant decrease in serum iron levels (hypoferremia). Both *in vitro* and *in vivo* studies suggest that this prophylactic effect of lactoferrin involves an inhibition of production of several cytokines including tumor necrosis factor (TNF-) and interleukin-1ß (IL-1ß) that are key mediators of the inflammatory response leading to death from toxic shock (Crouch et al., 1992; Machnicki et al., 1993). It has been proposed that this inhibition of TNF- release by lactoferrin is due to its ability to act as an anti-endotoxin by binding to the lipid A moiety of LPS released from lysed bacteria thereby inhibiting subsequent binding of LPS to CD14 receptors on macrophages where it initiates a proinflammatory response (Appelmelk et al., 1994). However, the identification of receptors for lactoferrin on the surface of myeloblasts (Birgens et al., 1984), monocytes (Birgens et al., 1983), macrophages (Van Snick and Masson, 1976) and lymphocytes (Mazurier et al., 1989), in addition to epithelial cells are involved in the local production of TNF. Lyer and Lonnerdal (1993) suggested that lactoferrin may have a direct effect on regulation of cytokine production by these cells via receptor

mediated signaling pathways.

Conclusion

Colostrum obtained from the mothers with healthy babies was significantly higher than that from mothers with apparently sick babies. Similarly, the mean lactoferrin levels obtained from the mothers with healthy babies varied significantly ($P < 0.05$) from that of the mothers that had sick babies in all the categories. The mean values were higher in the mothers with healthy babies and was within the range described by many authors. In human milk and colostrum, the reported concentrations of lactoferrin were 2 - 4 and 6 - 8 g/l, respectively. The low levels of lactoferrin obtained in the mothers with sick babies could account for the susceptibility of their babies to neonatal sepsis.

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