Comparison of Fecal Calprotectin in Exclusively Breastfed and Formula or Mixed Fed Infants in the First Six Months of Life

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Abstract- We conducted this study to compare fecal calprotectin between exclusively breastfed and formula or mixed fed infants aged one month and six months. Sixty term infants were enrolled from the labor ward of Valiasr Hospital between Oct 2011 and July 2015 and their fecal calprotectin was checked by the ELISA method and Hycult biotech kits. The enrolled infants had a birth weight of 2500-4000 g and no perinatal insults or hospitalization. Stool sampling was done at 1±1 week and at 6n±1 months. The six-month infants had no recent disease, antibiotic use or vaccination. The mean fecal calprotectin was higher in exclusively breastfed infants at first and sixth months than formula and mixed fed infants (368.85±204.49 and 283.21±381.41 µg/g versus 152.59±139.13 and 113.62±92.75 µg/g respectively). (*P*=0.0001 and 0.018) Fecal calprotectin was higher in infants with GERD than healthy babies in the first and sixth months (*P*=0.0001 and 0.004). Based on the role of calprotectin in inflammation, its higher levels in exclusively breastfed infants is contrary to breast milk benefits and may be a sign of enhanced mucosal immune maturity in them.

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Keywords: Fecal calprotectin; Breastfed; Formula fed; Mixed fed

Introduction

Calprotectin is a 36.5 kDa calcium and zinc binding protein complex from the s100 protein family which constitutes about 60% of soluble cytosol proteins in human neutrophils (1,2).

It is also present in monocytes, macrophages and epithelial cells (1). It is suggested to regulate inflammatory processes and to have antimicrobial and antiproliferative properties (1,2). Calprotectin is mainly secreted from neutrophils and therefore can be potentially used as a non-invasive diagnostic screening test for intestinal inflammation. Its concentration in the stool is about six times that of plasma (3).

The mode of feeding is likely to interfere with the intestinal behavior during the first months of life, especially in terms of microflora, now recognized to have a major effect on the composition and functional differentiation of the immune cells (2). Conflicting results have been reported on the effect of the feeding method on the level of fecal calprotectin in infants. Some studies have shown higher stool calprotectin in exclusively breastfed infants, and some have shown no difference (4-6).

We designed this prospective cohort study to compare fecal calprotectin levels in relation to feeding strategy in infants. The aim of this study was to compare stool calprotectin between exclusively breastfed and formula or mixed-fed infants. We hypothesized that exclusively breastfed infants would have less intestinal inflammation than formula fed ones and their stool calprotectin should be lower.

Materials and Methods

This cohort study was conducted on infants born in Vali-e-Asr Hospital of Imam Khomeini Hospital Complex from October 2011 to July 2015. One hundred and ninety-three term newborns without prenatal or postnatal insults with a birth weight of 2500-4000 gr

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were enrolled in study. Informed consent was taken from their parents. Any infant with a history of the maternal disease or drug use, maternal eclampsia or preeclampsia, premature rupture of membranes, sepsis, respiratory distress, necrotizing enterocolitis, jaundice, congenital anomalies or admission in neonatal period was excluded from sampling.

Two stool samples were collected in 1±1 week and 6±1 months. Any enrolled infant without follow-up sampling at 6 months of age or a diagnosis of food allergy was excluded, too. One hundred and thirty-three infants were excluded, and 60 infants completed the study. The samples were stored at -20º C and calprotectin was measured by the ELISA method and Hycult biotech kits in µ/g of the stool. In 6-month infants, any history of recent infection as diarrhea, vaccination, or antibiotic use was checked, and a two-week interval was considered for sampling. The upper limit of fecal calprotectin is 50 µg/g in adults and 100 µg/g in children (2). Fecal calprotectin levels have been reported to be much higher during the first few weeks of life both in healthy full-term and preterm infants than in healthy adults and older children (3). Demographic characteristics including sex, day care attendance, feeding strategy (formula fed, breast fed, or mixed fed), gastroesophageal reflux disease (based on symptoms and signs including vomiting, weight loss or poor weight gain, unusual irritability, cough, choking, refusal to feed and Sandifer posture and the I-GER-R questionnaire), infantile colic (based on Wessel's criteria) and a family history of atopy were recorded in the questionnaires (7-9). Stool calprotectin was compared between two groups of infants: 1) exclusively breastfed babies and 2) formula or mixed (formula and breast milk) fed babies.

Data were analyzed using SPSS version 18. Descriptive statistics were evaluated as frequency, percentile, and mean±SD (standard deviation). Analytical statistics and comparison between groups were done using ANOVA and logistic regression tests. As all variables (especially the calprotectin level) were distributed normally (according to the Kolmogorov-Smirnoff Z test) the Mann-Whitney U test was not needed. Based on study of Dorosco *et al.*, in 2008, mean stool calprotectin level in exclusively breastfed infants was 60 mg/kg and in mixed feeds was 53 mg/kg so with a variance of 23, α=0.05 and β=0.20, we needed 40 cases in each group. We enrolled 193 newborns in the study, but unfortunately many of them did not complete the study. Eventually, we measured stool calprotectin in 60 infants aged 1 and 6 months.

This study was approved by Tehran University of Medical Sciences; registration code: 90-03-105-15017.

Results

Sixty term infants including 37 boys (61.7%) and 23 girls (38.3%) were enrolled in the study. All of them had a birth weight between 2500-4000 g. The infants in both groups were similar in terms of sex, GI problems and delivery route. Twenty-eight infants were exclusively breastfed, and 32 were formula or mixed fed. Complementary feeding was not begun in any of them. All of them were cared for at home, and none had day care attendance (Table 1).

Table 1. Demographic information between the two groups

	Feeding strategy	Exclusively breastfed (54)	Formula or mixed fed (48)	*P*.value
Sex	**Male (n-%)**	16(57%)	21(65%)	0.500
	Female (n-%)	12(43%)	11(34%)	
Delivery method	**C/S (n-%)**	21(76%)	23(73%)	0.728
	NVD (n-%)	7(24%)	9(27%)	
GI problems (n-%)	**GERD**	6(21%)	4(12.5%)	0.355
	Colic	7(25%)	9(28%)	0.785
	Both	0	0	

C/S. Caesarian Section, NVD: Normal Vaginal Delivery, GERD: Gastro Esophageal Reflux Disease

Twenty-nine infants had a negative history of GERD, or colic, 16 infants had a history of colic and 10 had GERD based on symptoms and signs and the I-GER-RQ (Infant Gastroesophageal Reflux Revised Questionnaire). Eight infants had a family history of atopy (one of them had colic, and two of them had

GERD too).

Calprotectin was higher in the first month versus the 6 months in all infants.

In exclusively breastfed infants, the mean stool calprotectin levels at first and sixth months were higher than exclusively formula fed and mixed fed babies. The difference was significant at both one and the six months. (*P*=0.0001 and 0.018 respectively) (Table 2,3).

Table 2. Mean stool calprotectin differences between groups based on feeding strategy

	Exclusively breastfed infants	Formula fed or mixed fed infants	*P*.value
Mean stool calprotectin	First month 368.85±204.49	First month 152.59±139.13	0.0001
	Sixth month 283.21±381.41	Sixth month 113.62±92.75	0.018

ANOVA test. $P \leq 0.05$ significant

Table 3. Logistic regression analysis of stool calprotectin between 2 groups

	β	*P*.value	Confidence interval
Calprotectin 1st mo	-0.584	0.0001	(-0.004)-(-0.001)
Calprotectin 6th mo	2.403	0.002	2.33-52.42

The median stool calprotectin in exclusively breastfed infants was 312 µg/g in the first month and 210.5 in the sixth month, while these quantities were 110 µg/g and 93.5 µg/g in formula or mixedfed infants (Figure 1 and 2).

No significant difference was seen in the mean stool calprotectin between male and female infants (*P*=0.94 and 0.40 in the first and sixth month).

The mean stool calprotectin level was significantly higher in GERD patients versus non-GERD patients at one and six months (*P*=0.0001 and 0.004).

In infants with a history of colic, the mean stool calprotectin level in the first and six-month was lower than infants without colic but the difference was not significant (*P*=0.172 and 0.584).

Infants with a family history of atopy had higher mean stool calprotectin levels than those without a history of atopy (358.37±263 and 248.50±160 µg/g versus 237.38±189 and 184.19±254 in first and sixth month, respectively), but the difference was not significant (*P*=0.117 and 0.550) (Table 4).

Table 4. Mean stool calprotectin in GERD, colic and atopy history compare to healthy infants and negative history

	GERD *vs* healthy	Colic *vs* no Colic Hx	Atopy Hx *vs* no Hx
Mean Stool Calprotectin 1st month	540.90±278.43 *vs* 196.04±122.79	193.93±90.34 *vs* 275.18±227.61	358.37±263.52 *vs* 237.38±189.94
***P*.value**	0.0001	0.172	0.117
	GERD Vs healthy	Colic vs. no colic Hx	Atopy Hx vs. no Hx
Mean Stool Calprotectin 6th month	420.70±630.71 *vs* 147.18±92.62	159.56±78.93 *vs* 204.84±323.73	248.50±160.11 *vs* 184.19±294.25
***P*.value**	0.004	0.584	0.550

ANOVA test. $P \leq 0.05$ significant
GERD: Gastro Esophageal Reflux Disease; Hx: history

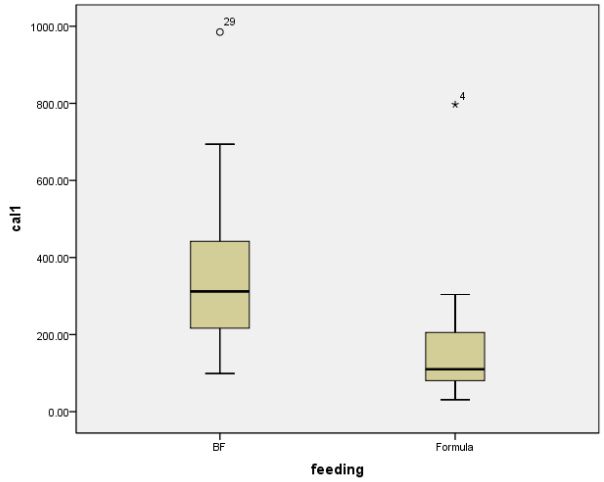

Figure 1. The first month Fecal Calprotectin Median

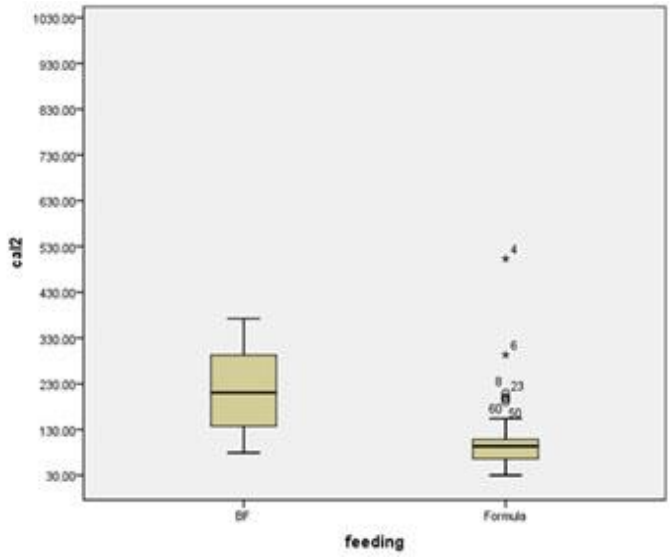

Figure 2. The sixth month Fecal Calprotectin Median

Discussion

Calprotectin, a complex of two calcium and zinc-binding proteins that belong to the S100 protein family, is abundant in the cytosolic fraction of neutrophils and has important extracellular activities. Although its exact biological function is not known, it has been shown to have bactericidal and fungicidal properties, and various data suggest that it may also be involved in the regulation of the inflammatory process (1,2). Its fecal concentration has been associated with the degree of disease activity in inflammatory bowel disease in adults and children (3,10-12). Recently, it has been shown by 'point-of-care testing' (POCT) that stool calprotectin indicates inflammation in the bowel (10). Fecal calprotectin is a simple and non-invasive method for assessment of the excretion of macrophages into the gut lumen (2). Fecal calprotectin levels have been reported to be higher during the first few weeks of life both in healthy full-term and preterm infants than healthy adults and children suggesting that the intestinal mucosa in young infants is potentially at risk for inflammatory processes (2,3). We found that the fecal calprotectin level was higher in the first month versus the sixth

month of age which may be due to the immaturity of the gut barrier and exaggerated inflammatory response.

Compeotto *et al*., also showed that compared with healthy adults, newborns have high calprotectin concentrations in the first days of life (2). We found significantly higher stool calprotectin levels in one-month and six-month infants who were exclusively breastfed as compared those who were formula or mixed fed. This finding may be due to higher inflammation or better immunity response in breast fed infants but requires more studies to clarify what happens in the intestine at a mucosal level.

Our finding is in line with Li *et al.,* Dorosco *et al*., and Savino *et al.,* findings and contra wise to Campeotto *et al*., and Rosti *et al*., findings (2), (2,4,6,13,14). Since fecal calprotectin is an objective and non-invasive test reflecting various pathological processes occurring in the gut mucosa of pediatric patients, we hypothesized that it must be lower in exclusively breastfed infants (3,11). On the other hand, fecal calprotectin is a marker of macrophage excretion into the gut lumen (11). Immune enhancing properties of the breast milk such as cytokines, other immunostimulating factors and growth factors, may have a role in this paradox (15,16). Nonetheless Oswari *et al*., showed no detrimental effects of formula feeding on the biomarkers of mucosal health and microbiota in infants and no significant difference in fecal calprotectin (5). Rosti *et al*., showed stool calprotectin was not different between breastfed and formula fed infants. They found higher fecal calprotectin levels in infants with colic or a family history of allergy (14). In our study, we did not find a significant difference between infants with colic or a family history of atopy, but fecal calprotectin was higher in GERD patients. Liu *et al*., showed higher fecal calprotectin levels in rural versus urban infants which are accounted for lower length-for-age Z-score of them (17). This finding can represent the negative effect of inflammatory mediators such as calprotectin on growth, and higher fecal calprotectin in breastfed infants is in contradiction with breast milk advantages (15). Assuming that gut microbiota plays an important role in the development of immunologic and gastrointestinal functions of the infants, Urwin, *et al*., showed differences in gut microbiota and fecal calprotectin between breast and formula fed infants (18). Urban *et al*., showed the antifungal activity of neutrophils calprotectin and Li *et al*., showed that breastfeeding may protect the infant against some infections (19,20). These studies emphasize the protective role of breast feeding and its role in the infants' health that may be applied by immune mediators such as calprotectin. Rouge *et al*., showed higher fecal calprotectin levels in preterm infants and its direct correlation with the enteral feeding volume and linked it to gut microbiota establishment. (21) Canani *et al*., showed higher fecal calprotectin levels in inflammatory disorders of the stomach and Rhoads *et al*., showed higher stool calprotectin in colic, but we did not find any significant differences in fecal calprotectin in infants with a history of colic (22,23).

Further immunologic investigations are needed to better clarify the mechanism underlying the relationship between the feeding pattern and fecal calprotectin levels in infants. One of our study limitations was that many infants were lost to follow-up.

Based on the role of calprotectin in inflammation, its higher levels in exclusively breastfed infants is contrary to breast milk benefits and may be a sign of enhanced mucosal immune maturity in them.

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