Turkish Journal of Immunology - Open Access
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Turk J Immunol 2019; 7(3):107–112

Association of the Reduced Level of Interleukin-13 in Breast Milk with Chronic Diarrhea in Infancy

Anne Sütü İnterlökin-13 Düzeyleri ile Kronik Bebek İshali Arasındaki İlişki

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Received: Jul 22, 2019
Accepted: Dec 15, 2019

https://doi.org/10.25002/tji.2019.1152

Abstract

Introduction: Chronic diarrhea (CD) is one of the major diseases frequently occur during infancy worldwide. The effects of various substances in breast milk on preventing inflammatory diseases such as chronic diarrhea are not fully elucidated. This study aimed to determine the concentration of inflammatory cytokines, including interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), and anti-inflammatory cytokines, including interleukin-4 (IL-4) and interleukin-13 (IL-13) through the mothers' breast milk feeding the infants with and without CD.

Material and Methods: Breast milk samples were obtained from 45 mothers feeding the infants with CD as the case group and 45 mothers feeding the healthy infants without CD (or any other inflammatory diseases) as the control group. The concentration of inflammatory and anti-inflammatory cytokines of breast milk was measured using ELISA technique.

Results: The mean of IL-13 concentration was significantly reduced in the case group compared to the control group ($p<0.001$). Whereas the mean of IL-4 concentration was significantly increased in the CD group in comparison to the control group ($p=0.001$).

Conclusion: The results indicated a lower IL-13 concentration and a higher IL-4 concentration in the mothers feeding the infants with CD. Therefore, low IL-13 as an anti-inflammatory cytokine in breast milk may be capable of predisposing the infants to CD. On the other hand, inflammatory cytokines may promote the immunity of infants with CD.

Keywords: breastfeeding, chronic diarrhea, cytokines, infant

Öz

Giriş: Kronik ishal (Kİ), tüm dünyada bebeklik çağında sık olarak görülen önemli bir hastalıktır. Anne sütünde olan ve kronik ishal gibi enflamatuar hastalıkları engelleyen maddelerin varlığı ve etkisi tam araştırılmamıştır. Çalışmada, interferon gamma (IFN-γ), tümör nekroz edici faktör alfa (TNF-α) gibi yengı artırıcı özelliği olan sitokinler ile intelökin-4(IL-4) ve interlökin-13 (IL-13) gibi yangı engelleyici sitokinlerin Kİ'i olan ve olmayan hastalardaki düzeylerine bakıldı.

Gereç ve Yöntemler: Anne sütü Kİ'li bebeği olan 45 anne ile, Kİ"i ya da başka bir yangısal hastalığı olmayan bebeği bulunan ve kontrol grubunu oluşturacak 45 anneden süt örnekleri alındı. Bu alınan süt örneklerinden yangısal ve yangı-karşıtı sitokin düzeylerine ELISA tekniği ile bakıldı.

Bulgular: IL-13 düzeyleri hasta grubunda kontrol grubundaki değerler ile karşılaştırıldığında istatistiksel olarak anlamlı düzeyde düşük bulundu ($p<0.001$).

Sonuç: Bulgulara göre Kİ'i bulunan bebeği olan annelerin sütlerinde kontrol grubuna göre daha düşük IL-13 ve daha yüksek IL-4 saptanmaktadır. Bu neden ile, anne sütünde saptanan ve yangı-karşıtı özellikleri olan IL-13 bebekleri, Kİ'a daha duyarlı hale getiriyor olabilir. Diğer yandan, sitokinler, Kİ olan bebeklerde bağışıklığı artırıyor olabilir.

Anahtar Kelimeler: Anne sütü ile besleme, kronik ishal, sitokinler, bebek

Introduction

Diarrhea is a general term used to describe loose/watery stools which occur three or more times within 24 hours which has a global mortality of less than 4%.[1,2] According to the World Health Organization (WHO), chronic diarrhea (CD) takes at least 14 days[3] and a series of factors are involved in causing the disease in a way that the most common cause of CD is probably irritable bowel syndrome. Other common causes include

intestinal parasitic infection, tuberculosis of intestines, malabsorption syndrome, and inflammatory bowel disease (IBD).[4] CD is one of the major diseases affecting children all over the world.[3] According to studies from Asia, Latin America, and Africa, the prevalence of the disease varies from 5 to 25%.[5] For various reasons breastfeeding is considered to be a preferred method of feeding the infants[6]; however, its role in preventing inflammatory diseases is under debate.[7] Many studies with contradictory results have been conducted to evaluate the relationship between breastfeeding and diarrhea. Some of these studies have shown a protective effect[8], while some of the other studies have indicated variable effects.[9]

Human milk contains a variety of cytokines.[10] These cytokines are secreted from the cells in the milk glands[11] and play a role in facilitating not only the development of the digestive system function but also in the development of the intestinal mucosal barrier.[12] In addition, the factors which are important modulators of immune responses and inflammatory reactions can play a key role in intestinal inflammation or suppress it.[13] Based on a report, CD has inflammatory nature and some inflammatory and anti-inflammatory cytokines are among inflammatory modulators, which are present at the site of inflammation (intestine).[14] According to the results of conducted studies, inflammatory cytokines such as IFN-γ and TNF-α and anti-inflammatory cytokines e.g. IL-13 and IL-4 are effective in either the development or prevention of inflammatory conditions such as CD, regardless of their origin.[15] Based on a report, large intestinal $CD4^+$ T cells were indicated to produce Th2-type cytokines (especially IL-4 and IL-13).[16] Moreover, Human milk-specific mucosal lymphocytes of the gastrointestinal tract show a TH2 cytokine profile i. e. IL-13.[17]

There is no report considering the relation of the desired cytokines in the breast milk and CD in infancy. Therefore, the present study was conducted for the first time to determine the IFN-γ, TNF-α, IL-13, and IL-4 levels in the mother's breast milk which feeds the infants with-and without CD.

Materials and Methods

Breast milk samples were obtained from 45 mothers feeding the infants with CD as the case group and 45 mothers feeding the healthy infants without CD (or any other inflammatory diseases) as the control group (age matched-between 6.54±3.25 months and gender-matched) from June to February of 2017 in AfzaliPour hospital of Kerman (a city located in the southeast of Iran). A pediatrician, hepatolgist and gastrointestinal specialist confirmed the disease, based on the clinical diagnostic criteria are including diarrhea >4 weeks, Clinical examination Stool examination x 3, evaluation of different types of disease such as fatty, watery and bloody diarrhea and finally exclude of infections, autoimmune enteropathy, etc.[4] A data collection form, including the age of breastfed infants and their mothers, the existence of any inflammatory disease, any kind of allergy, any drug consumption, the gender and assessment of other causes of diarrhea as idiopathic was completed by the participants. The infants who received any immunomodulating and immunosuppressive drugs including antihistamines, supplements or vitamins especially vitamin D and local and systemic steroid for two weeks were excluded from the study. The present study was evaluated and approved by the Ethics Committee of Kerman University of Medical Sciences, Kerman, Iran (Ethics code: CA/92/89).

The control group consisted of 45 mothers of age-matched between 7.3±4.2 months and gender-matched healthy infant subjects, without CD (or any other inflammatory diseases). The control group was selected among mothers referred to Health Centers of Kerman. All the mothers were healthy, with no acute or chronic disease (the inclusion criteria was being healthy of mothers, in particular being free from inflammatory intestinal disease, rheumatoid and collagen disorders, vascular and other autoimmune diseases). Indeed, the mothers with a history of recurrent infections, inflammatory diseases, any suspected immunological disorders, smoking, and any type of drug abuse were all excluded from the study. The mothers did not receive any immunomodulatory treatment such as vitamin D 6 months prior to milk collection.

The breast milk samples (10 mL) were obtained from all participants and were centrifuged at 4000 g for 5 min. After the elimination of the fat layer, the water layer was separated and stored at -20°C until the time of the analysis. The concentration of inflammatory and anti-filamentary cytokines of breast milk was measured using commercial ELISA kits (U-Cy-Tech, The Netherlands) based on the manufacturer's instructions.

In brief, ELISA 96-well flat-bottomed plates (Micro-titer plates) were coated for 2 h at 37°C by 50 µl/well of concentrated antibody solution of considered cytokine in

Phosphate-buffered Saline (PBS) and filled up to 100 µl with PBS. Then the wells washed with Tween 20 containing 0.05% PBS (PBST) and subsequently blocked by 200 µl/well of Bovine serum albumin blocking buffer. After drain blocking buffer, 100 µl of standard and test samples were added to each well and the plate was incubated for 2 h at 37°C. After well washing, the plate was incubated for 1 h at 37°C by 100 µl/well of frozen aliquot containing biotinylated-detection antibody solution and horseradish peroxidase (HRP)-conjugated streptavidin diluted in 0.5 ml distilled water After re-washing of well, 100 µl/well of Substrate solution (a Tetramethylbenzidine, TMB tablet) was dissolved in 1 ml of Dimethyl Sulfoxide (DMSO). Then, 10 ml of substrate buffer was added to supply substrate and was completely mixed and added to each well and the plate incubated for 30 min at room temperature in the dark. Finally, the reaction was stopped by adding 50 µl of 2 M H_2SO_4 and the formed yellow solution was read using an ELISA reader device (ELX800, ELISA Reader Biotek US) at a wavelength of 450 nm (and the reference wavelength of 630 nm) with optical density (OD) comparison between test and standard. The concentration of each cytokine in breast milk samples was calculated in the concentration of pg/ml.

Data Analysis: The results were expressed as mean ± SD. The comparison between demographic variables of the two groups was made by Chi-square test. Differences in variables were analyzed using unpaired t-test and Mann-Whitney test as appropriate and *P*-values of less than 0.05 were considered significant. The unadjusted and adjusted Odds ratios (ORs), as well as 95% confidence interval (CIs) of the risk factors of CD were assayed by binary logistic regression model. The data were analyzed by SPSS statistical software (version 18, Chicago, IL, USA).

Results

The demographic data of mothers and their infants are summarized in Table 1. In the present study, the age range of infants was 1 to 16 months {6.94±3.74 (Mean ± SD)} (n=90). The concentrations of IL-13 and IL-4 anti-inflammatory cytokines are shown in Figure 1 A and B. The median (IQR) the concentration of IL-4 was significantly increased in mothers feeding the infants with CD [4.40 (2.85–5.4)] in comparison to mothers feeding the healthy infants [2.9 (2.50–3.25), (p=0.001). In contrast, the concentration of IL-13 was significantly decreased in the case group [1.4 (1.2–1.5)] compared to the control group [4.5 (1.4–5.9)], (p<0.001). IFN-γ and TNF-α were comparable as shown in Figure 1 C and D.

Table 1. Demographic characteristics of mothers and their breastfed infants in the two groups

Variable	Patients' mothers (n=45)	Controls (n=45)	*P*-value
Age of mothers (years: mean ± SD)	27.9±4.4	28.2±5.0	0.72
Age of infants (months: mean ± SD)	6.5±3.3	7.3±4.2	0.30
Sex			
Male	25 (54.3)	21 (45.7)	0.40
Female	20 (45.5)	24 (54.5)	

A relative increasing was apparently observed in IFN-γ concentration in the case group [43 (38–48)] compared to the control group[42 (32.6–45)] but the difference was not statistically significant (p=0.47). However, TNF-α concentration did no differ (not statistically significant) in the milk of mothers feeding the infants with CD [2.30 (1.80–2.70)] compared to mothers feeding the infants without CD [2.30 (1.40–2.70), (p=0.78)].

Finally, the effect of IL-13 and IL-4 concentrations were simultaneously analyzed using logistic regression to compare the effect of each of the variables in the presence of each other and the results were represented in the form of odds ratio (OR) with 95% confidence interval (CI) as it has been shown in Table 2. According to the unadjusted model, there was a significant difference between IL-13 and IL-4 concentrations of the two groups (p=0.001 for both of the cytokines). Thus, both cytokines play considerable roles in the prognosis of CD. However, in adjusted test, there was a significant difference only in the concentrations of IL-13 between the two groups (p=0.001). In addition, the evaluation of OR and CI showed that the increase of OR in IL-13 resulted in the higher power of this cytokine as a predictive factor. Finally, for evaluating the accuracy of our results, the relationship between the mean production level of IL-4 and IL-13 was measured. There was no statistically significant correlation between the mean concentration of IL-13 (1.4) and the mean concentration of IL-4 (4.3) (p=0.22). However, there was a significant direct correlation (p<0.001) between the levels of IL-4 and TNF-α (r=0.63) (Figure 2).

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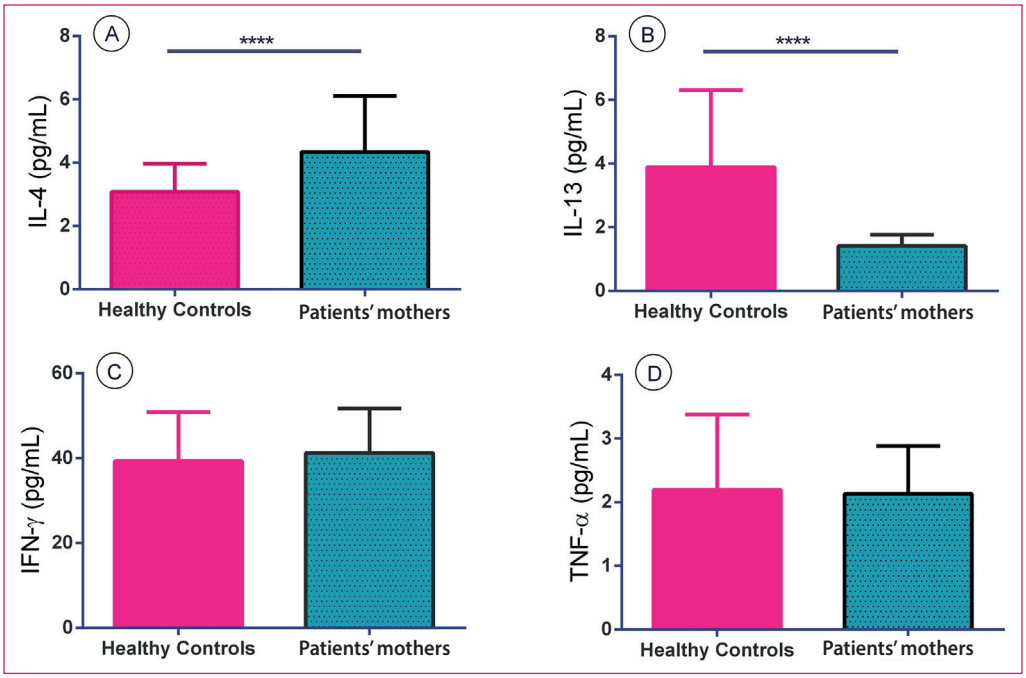

Figure 1. a–d. The comparison of cytokine levels between the healthy mothers of the patients and controls by ELISA method. There was a significant increment in IL-4 concentration in patients [4.40 (2.9–5.4)] compared with healthy controls [2.9 (2.5–3.3)], (p= 0.001) **(a)**. **B.** there was a significant decrement in IL-13 concentration in patients [1.40 (1.20-1.50)] compared with healthy controls [4.5 (1.4-5.9) (p<0.001) **(b)**. There were no significant differences in IFN- γ (p=0.47) and TNF-α (p=0.78) levels between healthy controls and patients **(c, d)**. ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Table 2. Binary logistic regression assay was performed for analysis risk (Unadjusted and adjusted ORs with 95% CIs) for CD in infancy

Variable	Univariate analysis	*P*-value	Multivariate analysis	*P*-value
	Unadjusted OR (95% CI)		Adjusted OR (95% CI)	
IL-13 level in milk	0.30 (0.150–0.603)	0.001	0.34 (0.181–0.640)	0.001
IL-4 level in milk	2.06 (1.364–3.103)	0.001	1.54 (0.983–2.416)	0.06

Binary logistic regression assay was performed for analysis. Variables that had a p<0.25 or were significant in the univariate analysis, were entered in the logistic statistical model. First, each variable was individually and then all variables were examined together.
IL, interleukin; OR, odds ratio; CI, confidence interval.

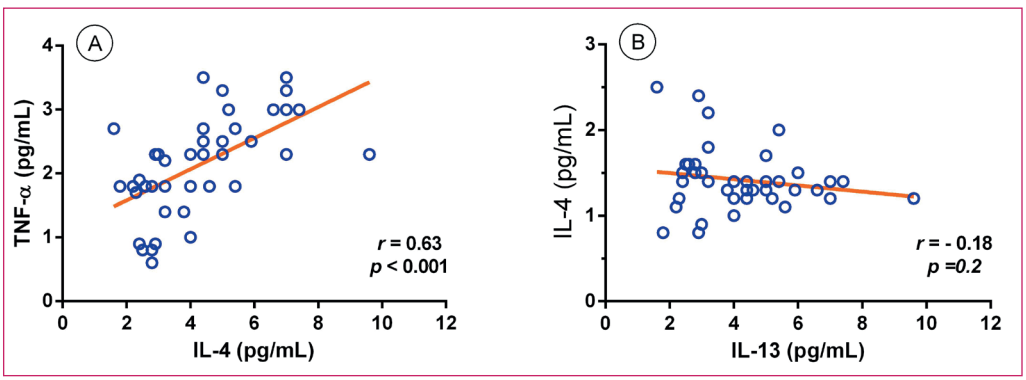

Figure 2. The linear regression (spearman correlation) between cytokine levels in the healthy mothers of the patient group. There was a significant correlation between TNF-α and IL-4 levels (r= 0.63) **(a)**. IL, interleukin; TNF, tumor necrosis factor.

Discussion

CD is one of the major diseases of children in the world.[18] Due to gastrointestinal immaturity in the infants, proteins and cytokines are easily absorbed even without epithelial damage.[19] According to a study by Djuardi et al. in 2009, it was found that maternal cytokine responses are important as indicators of neonatal cytokines during the first year of life.[20] Moreover, in a study by Moradkhani et al., the presence of IFN-γ, TNF-α, IL-13, and IL-4 in breast milk was proven.[21] In our study, a relative increasing (with no significant difference) was observed in IFN-γ level in infants with CD compared to the healthy infants. There was also no significant difference between

the infants of two groups in TNF-α concentration. These results might be due to the fact that the investigation of changes in IFN-γ and TNF-α is almost challenging, because pro-inflammatory cytokines are generally found at lower levels in breast milk, and decreased over lactation. According to the mentioned findings, there may not be a significant relationship between IFN-γ and TNF-α inflammatory cytokines present in breast milk and CD in infants. Moreover, it is reported that TNFα present in breast milk is neutralized by soluble TNF receptors, thus most TNF-α may not be freely active to influence chronic diarrhea. Although the role of inflammatory cytokines in breast milk is still under investigation, it is reported that they might be involved in several processes such as enhancing intestinal development and immune system. [22–24]

On the other hand, the mean concentration of IL-13 was decreased significantly in the case group compared to the control group. Also, the determination of IL-13 concentration in breast milk may be useful as a prediction model for the diagnosis of CD. Whereas the mean concentration of IL-4 was increased insignificantly in mothers feeding the infants with CD compared to mothers feeding the infants without CD. Since there was no significant difference in IL-4 concentrations between the two groups in adjusted test, IL-4 cannot be probably considered an independent factor in predicting the susceptibility to CD and it is affected by other factors. As it was mentioned before, there was a statistically significant correlation between the mean concentrations of IL-4 and TNF-α.

IL-13 is effective at limiting ischemia consistent with similar effects on inflammatory cytokine activity in the gut and periphery.[25,26]

IL-13 independent of IL-4 plays an important role in stimulating helper T-type 2 (Th2) production.[19] IL-4 also is considered to be the inducer cytokine of Th2 cells responses.[27] In the present study, elevated levels of IL4 have been demonstrated in patients' mothers' milk. A reason for this controversy may be the difference in sample type. IFN-γ induces helper type 1 T (Th1) cells and inhibits Th2 cells-related cytokine and Immunoglobulin E (IgE) production.[28] It has been reported that TNF-α is a mediator of both Th1 and Th2 responses.[24] To our knowledge, few studies have ever been conducted on the relation of the CD and our studied cytokines in the mothers' milk, until now. Further studies are needed.

According to the results of this study and also other studies, it can be declared that IL-13 and IL-4 play important roles in the prevention and susceptibility of CD, receptively. More research is necessary on stimulatory or inhibitory effects of inflammation for other cytokines in addition to four cytokines used in the present study.

There are few reports regarding the association between inflammatory and anti-inflammatory cytokines of breast milk in the mothers of infants with CD. It would be possible to reduce the symptoms of this disorder by avoiding the infant from feeding with breast milk containing inflammatory cytokines or in the other infants, preventing cessation of breastfeeding to avoiding them being deprived of this important and immunological food source. According to the relationship between these cytokines and CD, a statistical model was provided by which it would be possible to predict the probability of CD risk.

In conclusion, the results indicated a lower IL-13 concentration and a higher IL-4 concentration in the mothers feeding the infants with CD. Therefore, a lower concentration of IL-13 as anti-inflammatory cytokine in breast milk may be capable of predisposing the infants to CD. On the other hand, inflammatory cytokines may promote the immunity of infants with CD; however, there was no evidence regarding this issue in mothers' breast milk feeding the infants with CD. We found that the low level of IL-13 in breast milk might be a valuable biomarker for predicting CD in infancy. More research is necessary to validate the prediction models for CD in infancy using levels of milk cytokines. It would be useful to resolve the limitations of current research (lack of tracking infants and measurement of cytokines concentration in terminal phase of disease) along with previous studies (small sample size, lack of random selection, short-term period of breastfeeding and deficiency of planning a unilateral blind study) and conducting further interventional studies to identify the efficacy or lack of efficacy of breastfeeding on CD.

Acknowledgments: We are thankful to the Department of Immunology, Kerman University of Medical Sciences, Kerman, Iran to carry out this study.

Ethics Committee Approval: The present study was evaluated and approved by the Ethics Committee of Kerman University of Medical Sciences, Kerman, Iran (Ethics code: CA/92/89).

Peer-review: Externally peer-reviewed.

Authorship Contributions: *Concept:* SM; *Design:* SM, SAR; *Data Collection or Processing:* MRB, FS; *Analysis or Interpretation:* MK, MRB; *Literature Search:* SM, FM, MV; *Writing:* SM, FM, MV; *Critical Review:* SM, MMM.

Conflict of Interest: This study was financially supported by the Cardiovascular & Physiology Research Center, Institute of basic and clinical Physiology Sciences, Kerman University of Medical Sciences, Kerman, Iran. The authors declare no conflict of interest.

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