

## RESEARCH ARTICLE

# Effect of maternal smoking on colostrum and breast milk cytokines

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**ABSTRACT. Background:** Breast milk contains several immune modulator components. The transfer of numerous cytokines via mother's milk may add to an active stimulation of the infant's immune system. There are many factors in breast milk that could either facilitate or inhibit cytokine activities. Smoking negatively influences the immune system and changes the concentrations of important cytokines. **Objective:** The objective of this study was to assess the effect of smoking during pregnancy on the cytokines found in colostrum and mature human milk. **Methods:** The study population included 25 smoker and 27 non-smoker nursing mothers who gave birth to a term healthy infant via cesarean section. Breast milk was collected from the mothers on the 2<sup>nd</sup>-3<sup>rd</sup> and 21<sup>st</sup>-25<sup>th</sup> days postpartum during visits to examine the newborns. Samples were analyzed for IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF- $\alpha$  and TNF- $\beta$  cytokines by flow cytometric bead array. **Results:** We first saw that concentrations of IL-1  $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , and TNF- $\beta$  cytokines, but not IL-12, were measurable both in colostrum and in mature milk, being higher in colostrum. Next we observed that IL-1 $\beta$  and IL-8 levels were significantly lower in colostrum, and IL-6 was found to be significantly lower in the mature milk of smoking mothers. No significant effects of maternal smoking on breast milk concentrations of IL-2, IL-4, IL-5, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , and TNF- $\beta$  were observed. **Conclusions:** These findings indicate that maternal smoking alters the colostrum and mature milk levels of some cytokines. Therefore, it is thought that active smoking during pregnancy decreases the concentration of certain cytokines in breast milk, which might account for the newborn's increased susceptibility to infections.

**Key words:** maternal smoking, breastfeeding, infant, cytokine, immunology, human milk

Breast milk contains, not only the nutrients required for growth and development, but also immune modulator agents that help in the development of the immune system. It is known that breast milk protects infant against asthma, sudden infant death syndrome (SIDS), as well as infections of the gastrointestinal and respiratory systems and the middle ear [1]. Cytokines are small glycoproteins that take part in autocrine-paracrine mechanisms and that manage the development and functions of immune system, binding to specific cell receptors [2]. It has been suggested that breast milk contains cytokines that could influence the immune system of the newborn. Cytokine intake through human milk has the potential to influence maturation and development of immune cells in infants [2, 3]. B-cell growth, differentiation, and immunoglobulin production were among the first activities found in human milk and have been attributed to the presence of cytokines [4].

There are many factors present in breast milk that could either facilitate or inhibit the activities of cytokines. Smoking negatively influences the immune system; it impairs humoral and cellular immunity, and alters the concentrations of important cytokines [5]. Nicotine concentrations

in breast milk of smoking mothers have been reported to be 1.5 to 3 times higher than those found in maternal plasma [6]. Experimental studies have shown that nicotine suppresses primary and secondary immune response of the lungs, lymph nodes and spleen [7]. IL-1 $\alpha$ , IL-6 and TNF- $\alpha$  levels were found to be significantly lower in macrophages from the lungs of smokers [7, 8]. Ouyang *et al.* reported that cigarette smoke suppresses *in vitro* IL-1 $\beta$ , IL-2, IFN- $\gamma$  and TNF- $\alpha$  production [9]. Nonetheless, there are a limited number of studies demonstrating the influence of smoking on the protective effect of breast milk.

The present study investigated the effect of active maternal smoking during pregnancy on the cytokines present in colostrum and mature breast milk.

## DONORS AND METHODS

### Study population and design

The present study included women (n = 25) who smoked ( $\geq 5$  cigarettes per day) throughout pregnancy, and women (n = 27) who did not smoke at any stage of their pregnancy.

The 52 lactating mother had delivered a healthy, term infant via cesarean section and presented to the Newborn outpatient clinic of Bulent Ecevit University Medical Faculty, between June 2010 and June 2011. Only subjects who had undergone an elective caesarean delivery were considered for inclusion in order to minimize any confounding effects on the study of delivery method employed. Week of gestation, gender, birth weight and health status of infants, age, carrier and education status of mothers, number of births, chronic diseases (diabetes, hypertension, hypothyroidism, etc.), drug use, alcohol consumption and smoking status during pregnancy were recorded. Only infants without a history of prematurity (<37 weeks) or postmaturity (>42 weeks), with a birth weight of over 2,500 g and under 4,000 g, with no prenatal or postnatal problem (asphyxia, sepsis, congenital anomalous, intrauterine growth retardation, etc.), and mothers with no history of diabetes, hypertension or pre-eclampsia, the absence of any chronic, maternal diseases, and mothers that gave birth via cesarean section, were included in the study.

This study was approved by the regional ethics committee for medical research at the Bulent Ecevit University Faculty of Medicine. All mothers were received verbal and written information about the study, and their written and signed informed consents were obtained.

#### **Milk sample collection and processing**

Breast milk samples were collected from the mothers by manual expression on the 2<sup>nd</sup>-3<sup>rd</sup> and 21<sup>st</sup>-25<sup>th</sup> days postpartum, during their babies' check-up visits. Sample collection was standardized to reduce bias and potential diurnal variability in cytokine measurements. At least 5 ml of breast milk were obtained from each mother during control visits at the newborn polyclinic between 09:00 and 10:00 in the morning, within 1 hour of breastfeeding, and collected in sterile, polypropylene tubes.

The breast milk collected was centrifuged at 3,500 rpm for 10 minutes. The fatty supernatant portion was separated and the remaining liquid portion was placed into another tube and initially cooled to -20 °C. The samples continued to be cooled gradually, down to -70 °C, and stored until the day of examination.

#### **Measurement of breast milk cytokine concentrations**

IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IFN- $\gamma$ , TNF- $\alpha$ , and TNF- $\beta$  levels were measured by flow cytometry. Samples were analyzed using a Beckman Coulter Cytomix FC 500 (Miami-FL-USA) and a Flow Cytomix Human Th1/Th2 11plex Kit (eBioscience, Vienna-Austria). Data from flow cytometry were analyzed using FlowCytomix prosoftware (eBioscience, Vienna-Austria).

#### **Statistics**

Statistical analysis was performed using the SPSS 18.0 program. The Shapiro-Wilk test was used to assess whether the numerical variables were distributed normally. Numerical variables were represented as mean  $\pm$  standard deviation, whereas categorical variables were represented as number and percentage. The differences between the groups were analyzed using the Chi-square and Fisher's exact tests for categorical variables. For the comparison of two groups in

terms of numerical variables, a test for significance of the difference between two means was used if parametric test assumptions were provided, whereas the Mann-Whitney U test was used if parametric test assumptions were not provided. Repetitive measurements were evaluated by the test for significance of difference between paired samples if parametric test assumptions were provided; whereas the Wilcoxon, two-paired sample t-test was used if parametric test assumptions were not provided. The results were evaluated within a 95% confidence interval; a p value smaller than 0.05 was considered significant.

## **RESULTS**

There were no significant differences between the demographic characteristics of the smoking and nonsmoking mothers (*table 1*).

IL-1 $\beta$  and IL-8 concentrations in colostrum and IL-6 concentrations in mature milk were significantly lower in smoking mothers as compared to nonsmoking mothers (*table 2*).

Comparing colostrum and mature milk, cytokine concentrations found for smoking mothers, IL-6 ( $p = 0.001$ ), IL-8 ( $p < 0.001$ ) and TNF- $\alpha$  ( $p = 0.005$ ) were statistically significantly higher in colostrum. Although IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-10 and TNF- $\beta$  concentrations were higher in colostrum as compared to mature milk, the difference was not statistically significant. Comparing colostrum and mature milk cytokine concentrations for nonsmoking mothers, IL-1 $\beta$  ( $p < 0.001$ ), IL-4 ( $p = 0.049$ ), IL-6 ( $p = 0.009$ ), IL-8 ( $p < 0.001$ ), IL-10 ( $p = 0.032$ ), TNF- $\alpha$  ( $p = 0.008$ ) and TNF- $\beta$  ( $p = 0.019$ ) concentrations were higher in colostrum. Although IL-2 and IL-5 concentrations were higher in colostrum as compared to mature milk, the difference was not statistically significant.

## **DISCUSSION**

Human milk contains an array of cytokines such as IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, TNF- $\beta$ , TNF- $\alpha$  and IFN- $\gamma$  at physiologically important concentrations [10-21]. Concentrations of these cytokines fluctuate continuously through all phases of lactation [13, 14]. The present study investigated cytokine concentrations in colostrum and mature milk, and demonstrated that concentrations of IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , and TNF- $\beta$  cytokines, but not IL-12, are measurable both in colostrum and in mature milk, being

**Table 1**  
Demographic characteristics of the study groups.

	Smokers n = 25	Non-smokers n = 27	P
Maternal age (years)	29.2 $\pm$ 1.2	28.1 $\pm$ 0.8	0.450
Maternal education (years)	8.29 $\pm$ 2.6	8.30 $\pm$ 3.1	0.99
Parity (primiparous) (%)	20	37	0.65
Gestational age (weeks)	39.1 $\pm$ 0.3	38.4 $\pm$ 1.2	0.22
Birth weight (kg)	3.137 $\pm$ 0.64	3.219 $\pm$ 0.06	0.37
Sex (male/female) (%)	40/60	48.1/51.9	0.75

Table 2

Colostrum and mature milk concentrations of IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF- $\alpha$ , TNF- $\beta$  in smoker and non-smoker mothers.

	Colostrum			Mature milk		
	Smoker	Non-smoker	P value	Smoker	Non-smoker	P Value
IL-1 $\beta$ (pg/mL)	25.46 $\pm$ 5.01	45.51 $\pm$ 8.16	<b>0.037</b>	19.34 $\pm$ 4.39	18.00 $\pm$ 3.98	0.971
IL-2 (pg/mL)	84.44 $\pm$ 13.45	79.96 $\pm$ 13.05	0.707	62.06 $\pm$ 9.86	59.78 $\pm$ 10.41	0.700
IL-4 (pg/mL)	36.50 $\pm$ 3.86	36.86 $\pm$ 4.43	0.707	25.69 $\pm$ 3.43	26.36 $\pm$ 2.32	0.452
IL-5 (pg/mL)	14.05 $\pm$ 1.48	14.26 $\pm$ 1.53	0.862	10.80 $\pm$ 1.20	11.23 $\pm$ 1.07	0.790
IL-6 (pg/mL)	28.22 $\pm$ 7.04	30.17 $\pm$ 6.65	0.327	7.06 $\pm$ 1.37	12.40 $\pm$ 2.46	<b>0.022</b>
IL-8 (pg/mL)	1791.98 $\pm$ 249.96	2,662.83 $\pm$ 253.00	<b>0.015</b>	443.18 $\pm$ 136.02	524.73 $\pm$ 160.80	0.379
IL-10 (pg/mL)	14.31 $\pm$ 3.07	15.55 $\pm$ 2.67	0.735	7.24 $\pm$ 1.09	8.44 $\pm$ 0.98	0.707
TNF- $\alpha$ (pg/mL)	27.02 $\pm$ 4.83	28.32 $\pm$ 5.01	0.993	11.51 $\pm$ 1.68	11.20 $\pm$ 1.48	0.776
TNF- $\beta$ (pg/mL)	5.68 $\pm$ 0.84	9.05 $\pm$ 2.05	0.627	4.61 $\pm$ 0.83	4.49 $\pm$ 0.92	0.754

higher in colostrum. IL-6, IL-8 and TNF- $\alpha$  concentrations in particular, were found to be significantly higher in colostrum as compared to mature milk in both smoking and nonsmoking mothers.

Maternal cigarette smoking is a common yet avoidable factor associated with a significant increase in airway pathologies of childhood [22-24]. There is also emerging evidence that tobacco smoke can influence early immune function. This includes alterations in cytokine production by the fetoplacental unit, as was detected *ex vivo* in cord blood and in fetal mononuclear cell response patterns *in vitro* [25]. There is very limited information about the effects of cigarette smoking on breast milk, which is important for the development of the neonatal immune system. In our study, IL-1 $\beta$  and IL-8 concentrations were found to be significantly lower in colostrum, and IL-6 was found to be significantly lower in mature milk of smoking mothers. We observed no significant effect of maternal smoking on breast milk concentrations of IL-2, IL-4, IL-5, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , and TNF- $\beta$ . Nevertheless, it was observed that cytokine concentrations are lower in smoking mothers, particularly in colostrum. Comparing the colostrum and mature milk of nonsmoking mothers, cytokine concentrations seen in colostrum were found to be statistically significantly higher; cytokine concentrations were low in the colostrum of smoking mothers, similar to those found in mature milk.

Zanardo *et al.* found significantly lower IL-1 $\alpha$  concentrations in colostrum from mothers who smoked throughout pregnancy as compared to those for nonsmoking mothers, but they found no difference in terms of concentrations in transition milk. They propounded that SIDS and infections are more prevalent in infants of mothers who smoked during pregnancy, due to low IL-1 $\alpha$  concentrations in the colostrum [26]. Ermiş *et al.* demonstrated no significant difference between smoking and nonsmoking mothers in terms of IL-1 $\beta$  concentrations in transition milk on the 7<sup>th</sup> day postpartum [11]. In our study, we have shown that IL-1 $\beta$  concentrations are significantly lower in the colostrum of smoking mothers, but not in mature milk. Smoking-associated, decreases in IL-1 $\beta$  concentrations in colostrum might be a factor in the pathophysiology of the more frequent infections seen in the infants born to smoking mothers.

Prescott *et al.* reported that maternal smoking weakens several aspects of the toll-like receptor-associated, innate immune system of the newborn. They also found a negative correlation between cotinine levels and IL-6, IL-10 and TNF- $\alpha$  cytokine responses of smoking mothers [25, 27]. Likewise, Noakes *et al.* suggested that maternal smoking during pregnancy was associated with tendency to weaker neonatal IL-10 and IL-6 responses, although this did not achieve statistical significance either [28]. Ermiş *et al.* showed lower TNF- $\alpha$  concentrations in transition milk from smoking mothers as compared to nonsmoking mothers [11]. In the present study, significantly lower IL-6 concentrations in the mature milk of smoking mothers seem to corroborate these results. Nevertheless, there were no differences in IL-10 and TNF- $\alpha$  concentrations between smoking and nonsmoking mothers.

IL-8 is a potent, neutrophil chemotactic and activating factor. High IL-8 concentrations in breast milk facilitate the transition of maternal neutrophil, monocyte and lymphocytes into breast milk [14]. Moreover, IL-8 protects the intestinal cells against chemical injury. Maheshwari *et al.* reported that IL-8 in breast milk has a trophic function in intestinal system development [16]. Minekawa *et al.* suggested that IL-8 has an important role in the pathophysiology of necrotizing enterocolitis (NEC) and that breast milk is protective and curative in NEC disease in newborns [29]. Low IL-8 concentrations in the colostrum of smoking mothers indicate a weakened anti-inflammatory and protective effect of milk on tissue repair. Considering the trophic effect of IL-8 on the intestine, it could be said that development of NEC is more likely in the infants of smoking mothers.

In conclusion, active smoking during pregnancy influences IL-1 $\beta$  and IL-8 concentrations in colostrum, and IL-6 concentrations in mature milk. In addition to the effects on developing airways, maternal smoking in pregnancy also appears to influence early immune development via breast milk, and could be a factor for increased susceptibility to infections. Although further studies are required to examine this, there is no doubt that exposure to smoke has many other adverse effects; thus, better strategies aimed at reducing smoking should be of the highest priority.

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