ORIGINAL COMMUNICATION

Vitamin C in breast milk may reduce the risk of atopy in the infant

U Hoppu[1,2]*, M Rinne[1], P Salo-Väänänen[3], A-M Lampi[3], V Piironen[3] and E Isolauri[1]

[1]Department of Paediatrics, University of Turku, Turku, Finland; [2]Department of Biochemistry and Food Chemistry, University of Turku, Turku, Finland; and [3]Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland

Objective: To assess the effects of maternal dietary and supplement intake of vitamins C and E on breast milk antioxidant composition (vitamin C, α-tocopherol and β-carotene) and their protective potential against the development of atopy in the infant.

Design, subjects and methods: Mothers with atopic disease were recruited at the end of gestation and maternal sensitization was assessed by skin-prick testing. The 4-day food records of the mothers and breast milk samples were collected at the infants' age of 1 month. Infants' atopy was defined by the presence of atopic dermatitis during the first year of life and a positive skin-prick test reaction at 12 months of age ($n = 34$).

Results: Maternal intake of vitamin C in diet but not as supplement was shown to determine the concentration of vitamin C in breast milk. A higher concentration of vitamin C in breast milk was associated with a reduced risk of atopy in the infant (OR $= 0.30$; 95% CI 0.09–0.94; $P = 0.038$), whereas α-tocopherol had no consistent relationship with atopy. The group at risk of suboptimal vitamin C supply from breast milk was identified as infants whose mothers suffer from food hypersensitivity.

Conclusion: A maternal diet rich in natural sources of vitamin C during breastfeeding could reduce the risk of atopy in high-risk infants.

Introduction

In Western societies, the prevalence of atopic diseases has been increasing during the last decades. Notwithstanding extensive research, promotion of breastfeeding remains the single most effective means in the attempt to reverse this trend (Gore & Custovic, 2004). Conflicting findings on the protective effects of breastfeeding (Gdalevich *et al*, 2001; Bergmann *et al*, 2002; Sears *et al*, 2002) may be explained by the wide individual variation in breast milk composition.

The immunomodulatory and anti-inflammatory properties of breast milk are based on polyunsaturated fatty acids, nucleotides, cytokines, growth factors and antioxidants. The main diet-derived antioxidant compounds in breast milk are vitamin C, α-tocopherol and β-carotene (Garofalo & Goldman, 1999).

There is active research interest in the antioxidant regulation of atopic disease. Firstly, the inflammatory process results in the generation of oxygen free radicals, and antioxidant defence systems are important in controlling inflammation (Greene, 1999). Atopic diseases, in particular asthma and atopic dermatitis, are characterized by increased oxidative stress (Montuschi *et al*, 1999; Omata *et al*, 2001). Second, there is evidence that a low antioxidant intake may be associated with allergic symptoms; low vitamin C intake has been linked with wheezing (Bodner *et al*, 1999) and bronchial hyper-reactivity (Soutar *et al*, 1997) and an inverse association has been observed between dietary vitamin E intake and serum total IgE (Fogarty *et al*, 2000).

*Correspondence: U Hoppu, University of Turku, Itäinen Pitkäkatu 4 A, 5th floor, 20520 Turku, Finland.
E-mail: ulla.hoppu@utu.fi

Taking these observations together, the notion of enhancing the antioxidant defence potential of breast milk may become attractive. So far, the approach to reduce the risk of atopic disease in at-risk infants has centred on elimination of potentially allergenic foods from the maternal diet, with little success (Hattevig et al, 1999). Unbalanced elimination diets may contribute to low dietary intake of natural antioxidants that the use of synthetic supplements may not compensate (Stone et al, 2003).

The objective of the present study was to evaluate for the first time the antioxidant (vitamin C, α-tocopherol and β-carotene) composition of breast milk in atopic mothers, whose infants carry the greatest risk of atopic disease. Specifically, we demonstrate the succession of antioxidants from maternal diet to breast milk composition, and extend the conception of their impact on the development of atopic disease in the infant.

Subjects and methods

Mothers with atopic disease (allergic rhinitis, atopic dermatitis, asthma) were recruited at the end of gestation in antenatal clinics in the City of Turku ($n = 65$). Their infants were followed during their first year of life. The criteria for inclusion in the present study were that at the age of 1 month the infant was exclusively or predominantly breastfed and that a food record of the mother and a concomitant breast milk sample with metaphosphoric acid for the storage of vitamin C were available. Altogether 34 mother–infant pairs fulfilled the criteria and completed the follow-up. The research plan was approved by the Ethical Committee of the Hospital District of South West Finland. Written informed consent was given by all mothers.

Clinical evaluation and skin-prick testing of the mothers and infants

At the first study visit at 35–36 weeks of gestation, a detailed questionnaire on family history of atopic disease and other family characteristics was completed. Information on maternal dietary habits, including adverse reactions to foods, was collected by means of a questionnaire filled in in connection with a personal interview. At this visit, maternal sensitization was also assessed by means of skin-prick testing. Skin-prick tests (SPT) were carried out on the volar side of the forearm and reactions were read at 15 min. Half of the histamine (positive control) reaction size or more was recorded as positive on the condition that the mean diameter of the wheal was at least 3 mm and the negative control at the same time was 0 mm. Allergens tested included wheat, rice, milk, egg, cod, soy bean, peanut, hazelnut, latex, cat, dog, birch, alder, mugwort, a mixture of six local grasses, and house dust mite. Banana, potato and carrot were tested by prick–prick technique.

After delivery information on the infant's birth characteristics was recorded. The infants were clinically examined at 1, 3, 6 and 12 months of age, all signs and symptoms of atopic disease, specifically atopic dermatitis, being recorded. The diagnosis of atopic dermatitis was based on the criteria described by Hanifin (Hanifin, 1991). In the infants, skin-prick tests were performed at 12 months. The allergens tested were the same as in the mothers, excluding nuts, alder and mugwort. The diagnosis of infant's atopy, the outcome measure of the study, was based on the presence of atopic dermatitis during the first year of life and a positive SPT reaction at 12 months of age.

Dietary counselling and food records

Exclusive breastfeeding for 4–6 months and abstinence from smoking was encouraged. Oral and written information on the recommended diet for breastfeeding women including vitamin D supplementation (10 µg/day) during wintertime (Hasunen et al, 1997) was provided for all mothers by a nutritionist. Specifically, an abundant intake of fresh fruits, berries and vegetables during breastfeeding was recommended. Apart from the general recommendation of vitamin D during wintertime, mothers were neither advised nor forbidden to take vitamin or mineral supplements, but they were requested to carefully report such use in food records.

Mothers were given oral and written advice on how to complete 4-consecutive-day food records with household measures. At the 1-month visit food records were checked by a nutritionist and on this basis, mothers were given individual dietary counselling. Nutrient intakes were calculated with Nutrica® software (version 3.0, Research Centre of the Social Insurance Institution, Turku, Finland). As the Nutrica database does not include data on β-carotene composition of foods, this could not be calculated. Information on the vitamin contents of supplements was obtained from the manufacturers.

Collection and analysis of breast milk samples

Breast milk samples were collected at the infants' age of 1 month. All mothers were given standard written instructions as to how to collect the samples in the morning at home prior to the study visit. Mothers were asked to collect aside a few drops of breast milk by manual expression and then to collect samples of altogether 20 ml in plastic dark tubes. All samples were collected between 0700 and 1000 hours and were stored in a refrigerator at $+4°C$ for not more than 2 h before freezing. For the analysis of vitamin C, the breast milk samples were stored in 10% metaphosporic acid containing 1% oxalic acid (sample:acid ratio, 1:1). All samples were stored at $-70°C$ until analysed.

Prior to the sample preparation steps of all analyses, the samples were thermostated at 38–40°C for an hour and thoroughly mixed. All analyses were carried out in duplicate. Standard curves (four concentration levels) were obtained

daily by standard injections. Variations in detector response and retention times were obtained by standard injections, after every third or fourth sample injection.

Breast milk α-tocopherol and β-carotene concentrations were determined according to Salo-Väänänen *et al* (2000). Vitamins were extracted with *n*-hexane ethyl acetate (8:2) after saponification. The amount of sample was 0.5 ml and the amounts of all solutions used were halved. The analytical HPLC was based on a method previously described (Salo-Väänänen *et al*, 2000). Vitamins were separated by step-gradient elution: elution for the first 5 min with 0.3% diisopropyl ether in *n*-hexane and from 5.1 to 20 min elution with 8% diisopropyl ether in *n*-hexane. β-Carotene was detected with UV detector and α-tocopherol with fluorescence detector. Recoveries of added vitamins were good: 103% for α-tocopherol and 94% for β-carotene. Repeatability of the method was tested with in-house breast milk reference samples. The within-day variation was small, 2.4% for β-carotene and 1.6% for α-tocopherol (CV, $n = 6$), respectively. Variation between 8 days was also small, 4.5% (CV, $n = 16$) for β-carotene and 3.0% (CV, $n = 16$) for α-tocopherol.

Vitamin C was expressed as the sum of ascorbic acid and dehydroascorbic acid. Ascorbic and dehydroascorbic acids were determined according to Kall and Andersen (1999) with slight modifications in sample treatment (Lykkesfeldt *et al*, 1995). To a 1.5 ml portion of the sample-acid mixture 1.5 ml of 0.5 M Trizma Base buffer (Sigma Aldrich Chemie GMBH, Steinham, Germany) was added and the sample solution was filtered through 5, 0.8 and 0.45 μm filters (Acrodisc 25 versapor, Schleicher & Schuell FP30 and GHP Acrodisc, respectively) prior to HPLC analysis. The analytical HPLC was based on the method of Kall and Andersen (1999). Ascorbic acid was detected directly after reverse-phase separation with UV detector and dehydroascorbic acid was detected indirectly with fluorescence detector after post-column reaction with *o*-phenyldiamide. In our method, the length of the reaction coil was 10 m, the temperature of the column oven 30°C and the injection volume 50 μl. Recoveries of added vitamin C compounds were good: 104% for ascorbic acid and 88% for dehydroascorbic acid ($n = 8$). Repeatability of the method was tested with in-house reference samples, stored in acid mixture at −70°C. The within-day variation was 0.9 % (CV, $n = 8$) for ascorbic acid and 1.1% for dehydroascorbic acid (CV, $n = 8$). The variation between days was slightly higher, 3.8% for ascorbic acid (CV, $n = 12$) and 8.3% for dehydroascorbic acid (CV, $n = 12$).

Since tocopherol and carotenoid concentrations are strongly correlated to fat and the fat concentrations of breast milk samples show significant variation, the α-tocopherol and β-carotene contents are expressed also in relation to fat (mg or μg/g fat). Breast milk fat content was expressed as triacylglycerols calculated from fatty acids analysed with a gas chromatographic method as previously described (Laiho *et al*, 2003).

Statistics
Results are expressed as mean with range or 95% confidence interval (CI) in parentheses. χ^2-test, Fisher's exact test and *t*-test for independent samples were used for comparisons between atopic and nonatopic groups. Correlation was analysed by Pearson's correlation analysis. Analysis of covariance (ANCOVA) was applied in assessing the effect of dietary (continuous variable) and supplement (dichotomised variable) intake of vitamins C and E on breast milk composition. Univariate and multivariate logistic regression analyses were used to study breast milk antioxidants as explanatory factors for infant's atopy. In logistic models, vitamin C, α-tocopherol and α-tocopherol/fat were included as continuous variables, maternal SPT reactivity and food hypersensitivity as dichotomous.

Results
Clinical characteristics
Altogether 23/34 (68%) mothers had positive SPT results. The most common positive test reactions were for cat 16/34 (47%), dog 15/34 (44%), birch 13/34 (38%), grass 13/34 (38%), alder 11/34 (32%) and mugwort 9/34 (26%). In addition, 15/34 (44%) of the mothers reported experiencing adverse reactions to foods (food hypersensitivity), mainly to nuts, some vegetables and fruits, for example, citrus fruits, kiwi and apples.

Altogether 13/34 (38%) infants were diagnosed with atopic dermatitis during their first year of life. Positive skin-prick test reactions occurred in 9/34 (26%) infants. Altogether 7/34 (20%) infants fulfilled the criteria of atopy; the occurrence of both atopic dermatitis during the first year and a positive SPT at the age of 12 months. Clinical characteristics of atopic and nonatopic infants are presented in Table 1.

Effect of maternal dietary intake on the concentration of antioxidants in breast milk
The mean energy intake of the mothers was 8.6 MJ/day (range 5.8–11.3) and the proportion of energy derived from fat was 33.0% (range 22.3–41.4). The mean intake of vitamin C from diet was 124 mg/day (range 40–279) and vitamin E (expressed as tocopherol equivalents) 8.3 mg/day (range 5.2–16.8). Altogether 17/34 (50%) of the mothers were using supplements containing vitamin C and 15/34 (44%) vitamin E, median intake from supplements 75 and 10 mg/day, respectively. None of the mothers was using supplements containing β-carotene. There was no significant difference in the dietary intake between mothers of atopic and nonatopic infants.

The vitamin C concentration in breast milk appeared to be dependent upon maternal dietary intake; there was a positive correlation between vitamin C intake from diet and the vitamin C concentration in breast milk ($r = 0.34$, $P = 0.047$). To assess the impact of dietary and supplement

Table 1 Clinical characteristics of atopic and nonatopic infants

	Atopic infants (n = 7)	Nonatopic infants (n = 27)
Family		
Father with atopic disease	2/7 (28%)	14/27 (52%)
Siblings	3/7 (42%)	15/27 (55%)
Siblings with atopic disease	3/3 (100%)	11/15 (73%)
Pets at home	1/7 (14%)	7/27 (26%)
Father smoking	2/7 (28%)	6/27 (22%)
Mothers		
Age (y)	30 (27–33)	30 (28–32)
Height (cm)	164 (161–167)	165 (163–167)
Weight at 1 month postpartum (kg)	67 (59–76)	69 (65–73)
Mother smoking before pregnancy[a]	2/7 (28%)	3/27 (11%)
University or vocational college education	2/7 (28%)	13/27 (48%)
Maternal positive SPT	7/7 (100%)	16/27 (59%)
Maternal food hypersensitivity	6/7 (85%)	9/27 (33%)
Infants		
Birth weight (kg)	3.7 (3.3–4.1)	3.5 (3.3–3.7)
Birth length (cm)	52 (50–54)	50.5 (50–51)
Head circumference at birth (cm)	35 (34–36)	35 (34.5–35.5)
Weight at 12 months (kg)	10.0 (8.9–11.2)	10.0 (9.6–10.4)
Length at 12 months (cm)	77 (74–80)	76 (75–77)
Duration of breastfeeding (months)		
Exclusive	3.2 (2.2–4.2)	3.5 (3.0–4.0)
Total	8.4 (5.5–11.3)	8.7 (7.5–10.0)

Data presented as mean (95% CI). Statistics: Fisher's exact test and *t*-test. No statistically significant differences between groups except maternal positive SPT ($P = 0.04$) and maternal food hypersensitivity ($P = 0.01$).
[a]None of the mothers were smoking during pregnancy or breastfeeding.

Table 2 Fat and antioxidants in breast milk consumed by atopic and nonatopic infants

	Atopic infants (n = 7)	Nonatopic infants (n = 27)	P
Fat (g/100 ml)	3.0 (2.4–3.6)	3.8 (3.4–4.3)	0.09
Vitamin C (mg/100 ml)	5.2 (4.6–5.7)	6.2 (5.8–6.6)	0.02
α-Tocopherol (mg/100 ml)	0.45 (0.27–0.62)	0.43 (0.37–0.48)	0.76
α-Tocopherol/fat (mg/g)	0.15 (0.11–0.19)	0.12 (0.10–0.13)	0.03
β-Carotene (µg/100 ml)	4.9 (3.6–6.2)	4.3 (3.6–5.0)	0.43
β-Carotene/fat (µg/g)	1.6 (1.2–1.9)	1.2 (1.0–1.4)	0.13

Data presented as mean (95% CI).

intake separately, an ANCOVA was applied, and it was found that only dietary intake of vitamin C increased the vitamin C concentration in breast milk ($P = 0.048$), while the intake from supplements had no effect ($P = 0.78$). Intake of vitamin E either from diet or from supplements was not associated with the α-tocopherol concentration in breast milk; $P = 0.34$ and 0.71 respectively.

Mothers with food hypersensitivity evinced lower concentrations of vitamin C in breast milk than those with no dietary restrictions, 5.5 mg/100 ml (95% CI 5.0–6.0) *vs* 6.4 mg/100 ml (95% CI 5.9–6.9); $P = 0.012$, although vitamin C intake was comparable between these groups. Vitamin C, α-tocopherol and β-carotene concentrations in breast milk did not differ between mothers with positive or negative skin-prick test results (data not shown).

Antioxidant concentrations in breast milk associated with atopy in the infant

Atopic infants had consumed breast milk with a lower concentration of vitamin C compared to nonatopic infants (Table 2). There was no difference in the absolute concentration of α-tocopherol between the groups, but because the fat concentration in breast milk samples was lower, the ratio of α-tocopherol to fat was higher in breast milk consumed by

atopic infants. Maternal positive SPT and maternal food hypersensitivity were found to be associated with atopy in the infant (Table 1). The relative risk (RR) of atopy in the infant was 3.4 (95% CI 0.5–24.0) in the case of mothers with positive *vs* negative SPT and 7.6 (95% CI 1.0–56.5) in food-hypersensitive mothers compared to mothers without food hypersensitivity. These variables were taken into account as confounding factors in a logistic regression model testing whether breast milk antioxidants have an impact on atopy in the infant.

According to the univariate logistic regression analysis, vitamin C in breast milk was related to a lower risk of atopy in the infant (OR = 0.30; 95% CI 0.09–0.94; $P = 0.038$). The absolute amount of α-tocopherol in breast milk had no effect, but high levels of α-tocopherol/fat tended to be associated with an increased risk (OR = 12.46; 95% CI 0.95–163.9; $P = 0.055$). These factors were shown to retain their effect even when the maternal SPT reactivity and food hypersensitivity were taken into account in multivariate analyses.

Discussion

This study provides new insight into the protective role of breastfeeding against atopic diseases. It was shown for the first time that vitamin C in breast milk may be associated with a reduced risk of atopy in high-risk infants. Our data may be taken into account in research aiming at identification of the factors responsible for the increased prevalence of atopic diseases, currently constituting the most common chronic diseases of childhood in industrialized countries.

In this study, we identified a distinct dietary risk group for atopic disease; infants whose mothers have food hypersensitivity. These mothers may have a greater need for antioxidants due to the oxidative stress associated with atopic disease but a lower intake of antioxidants due to food restrictions. The low antioxidant status of the mother may be transferred via breast milk to the infant at a vulnerable age.

It is obvious that mothers eliminate foods, mainly fruits and vegetables causing adverse reactions for themselves also

during breastfeeding. In addition, elimination of food antigens associated with food allergy in the infant, such as milk, wheat and soy, has been practiced in approach to reduce the risk of infant's atopic disease but with little success (Zeiger & Heller, 1995). Consequently, supplementation of the maternal diet with certain immunomodulatory compounds such as probiotics, antioxidants or fatty acids has been promoted recently (Duggan et al, 2002). Successful intervention strategies are nonetheless not possible before the basic effects and interactions of diet and supplements have been evaluated. In fact, our results here emphasise the importance of a varied and balanced diet.

The important finding in this study was that maternal intake of vitamin C from the diet but not from supplements influences the concentration of vitamin C in breast milk. There may be an intrinsic regulatory mechanism for the transfer of vitamin C from diet to breast milk to prevent excessive loads. At low intakes, there may be a dose–response effect, but above a certain saturation level, large supplement intakes are not reflected in breast milk vitamin C (Thomas et al, 1980; Byerley & Kirksey, 1985). Low vitamin C concentrations in breast milk were associated with a higher risk of atopy in the infant. We may thus extend previous demonstrations linking low vitamin C intake with symptoms of atopic disease (Fogarty & Britton, 2000) to the development of the condition.

As in some previous studies (Chappell et al, 1985; Ali et al, 1986), dietary intake of vitamin E was not reflected in breast milk α-tocopherol, most likely because fat-soluble vitamins can be mobilised from maternal stores. Although a low dietary vitamin E intake has also been associated with current symptoms of atopic disease (Bodner et al, 1999; Hijazi et al, 2000), we found no consistent relationship between vitamin E (α-tocopherol and its ratio to fat) in breast milk and the development of atopic disease in the infant. The most plausible explanation for the discrepancy could be methodological differences. Previous, mainly cross-sectional studies have reported dietary intake collected by food-frequency methods and have not systematically distinguished between intakes from diet and supplements. Such a distinction is of the utmost importance, as shown by Troisi et al (1995), who reported that vitamin E intake from diet but not from supplements was inversely related to asthma. This could be explained by different biological activity of natural and synthetic vitamin E (Stone et al, 2003). It should also be noted that the concentration of antioxidants in serum may differ from those in target organs (Kelly et al, 1999). Consequently, further validation of methods for measuring antioxidant intake and status in atopic and at-risk individuals is thus called for in future studies.

Finally, our results underline the importance of evaluating the complex inter-relationships between antioxidants. A diet rich in vegetables, berries and fruits contains a large variety of bioactive compounds that may also synergistically contribute to antioxidative and immunomodulatory effects (Knekt et al, 2002). In like manner, the diet is always a

combination of many foods and nutrients and their inter-actions may be more crucial than is currently understood. There appears to be no short cut in reducing the risk of atopic and other chronic diseases; a healthy balanced diet may outweigh the use of single dietary supplements.

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