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Dietetic effects of oral intervention with mare's milk on the Severity Scoring of Atopic Dermatitis, on faecal microbiota and on immunological parameters in patients with atopic dermatitis

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Abstract
In a double-blind, placebo-controlled crossover trial, 23 patients consumed 250 ml mare's milk or placebo for 16 weeks. The aim was to examine the effects of mare's milk on the characteristics of atopic dermatitis (AD), on faecal microbiota and on clinical and immunological parameters. The intensity of AD was examined using the Severity Scoring of Atopic Dermatitis (SCORAD) index. During the mare's milk period, the mean SCORAD value of patients ($n=23$; 17 females, 6 males) decreased from 30.1 to 25.3 after 12 weeks ($P<0.05$) and to 26.7 after 16 weeks ($P<0.1$). In a subgroup ($n=7$) the SCORAD index and especially the pruritus decreased by 30% through the mare's milk period ($P<0.01$). In this subgroup, the faecal bifidobacteria increased during the mare's milk period from 4.6% to 11.9% of eubacteria ($P<0.05$). The immunological parameters, except C-reactive protein, were unchanged.

Keywords: *Mare's milk, atopic dermatitis, endogenous eczema, Severity Scoring of Atopic Dermatitis, Bifidobacteria*

Introduction

The pathogenesis of atopic dermatitis (AD) is multi-factorial and, to date, not completely elucidated. The major immuno-pathogenic abnormality of AD is a predominance of T-helper type 2 cells over T-helper type 1 cells as well as a dysbalance of regulatory T cells. Additionally, cytokines and chemokines produced by keratinocytes in AD induce cutaneous immune responses that have a reduced ability to synthesize antimicrobial factors resulting in a specific cytokine profile (Kaminishi et al. 2002; Laouini et al. 2003).

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Mare's milk contains lysozyme, lactoferrin, and secretory IgA, which are responsible for the antibacterial, anti-inflammatory and immunomodulatory properties. In addition to its enzymatic effect on bacteria, lysozyme has antiviral (Lee-Huang et al. 1999), immunostimulating (Ibrahim and Aoki 2003; Murakami et al. 1997), fungicidal (Tobgi et al. 1988), anti-inflammatory (Ogundele 1998), and anti-carcinogenic properties (Sava et al. 1989). The iron-binding lactoferrin possesses antimicrobial (Orsi 2004) and anti-oxidative activity (Maneva et al. 2003; Sandomirsky et al. 2003). Furthermore, several positive effects on immuno-regulatory functions and inflammable processes due to lactoferrin have previously been demonstrated (reviewed by Orla 2001; Caccavo et al. 2002). In rat colon, lactoferrin reduced the degree of an induced colitis *via* a modulation of the immune system and *via* corrected cytokine imbalance (Togawa et al. 2002). In addition, lactoferrin showed antiviral (Grover et al. 1997; Marchetti et al. 1996) and fungicidal effects *in vitro* (Andersson et al. 2000).

The secretory IgA represents the main immunoglobulin in mature mare's milk. Human and equine secretory IgA homology was previously demonstrated with cross-reactions using human anti-IgA antiserum (Pahud and Mach 1972).

To date, only individual case reports and single clinical observations on the dietetic effects of mare's milk have been published. Controlled studies examining the therapy-supporting effect of mare's milk on AD have not been carried out. Therefore, the aim of this pilot study was to examine the effects of an oral intervention of mare's milk on the characteristics of AD, on selected faecal microbiota as well as on several clinical and immunological parameters.

Materials and methods

The Ethics Committee of the Medical Faculty of the Friedrich Schiller University, Jena, approved the study.

Subjects

Approximately 300 individuals were tested with regard to their suitability for participation in the study. Of these, 39 patients with proved mild to moderate expression of AD (Severity Scoring of Atopic Dermatitis [SCORAD] ≥ 10) were included in the study by means of strict inclusion and exclusion criteria:

Inclusion criteria

- A clear and definite diagnosis of AD.
- Willingness to use only the recommended drugs.
- Competence regarding the daily documentation of skin state and well-being.

Exclusion criteria

- Therapy with non-recommended drugs 1 month before start of the study.
- Active skin infection.
- Apparent asthma.
- Intolerance against milk.
- Long-term therapy with drugs.
- Symptomatic heart disease and/or internal disease.

- Autoimmune diseases, immune defects, and malignoma.
- Alcohol and drug abuse.

In order to establish sensitization against mare's milk, skin prick tests were carried out and specific IgE for mare's milk was measured.

Of the 39 recruited patients, 23 patients (17 women, 6 men) completed the study. The patients were between 18 and 54 years old (30.2 ± 10.9 years) and had a base SCORAD value of 13–46 (29.1 ± 9.2). The high dropout rate was due to home and job changes or to an aversion to the test substances.

Study design

The study was conducted as a double-blind, placebo-controlled food challenge with a crossover design. The patients were randomized into two groups (Group 1 = 8 females and 3 males; Group 2 = 9 females and 3 males) using stratification by SCORAD baseline and gender (Group 1 started with mare's milk; Group 2 began with placebo). The participants received 250 ml mare's milk or placebo daily before breakfast during the 16-week intervention periods. Between the two intervention periods, a 4-week wash-out period without drinking any test substances was introduced.

Each patient started the intervention individually. Therefore the entire duration of the study lasted more than 1 year. Seasonal effects on the SCORAD index were controlled.

In this study, unpasteurized mare's milk was used. The placebo drink was based on a hypoallergenic infant formula (HA1 Hipp gmbH & Co. Pfaffenhofen, Germany) and enriched with lactose in order to obtain a taste similar to that of mare's milk. Both drink supplements were identically bottled and stored at –20°C. The similar colour and taste of the test products, the use of the identical bottles, the 'wash-out period' to obliterate the taste of the test products, and the explanation that patients would be testing 'summer and winter mare's milk' were factors used to sustain the double-blind character of the study.

All patients recorded a food frequency chart for a total of 5 days during each period. Additionally, the patients recorded daily the frequency of use of care cream as well as corticosteroid cream, which were allowed in the study, using a standardized patient diary.

Blood and faeces sampling

Blood samples were taken into a vacutainer (sodium–heparin, ethylenediamine tetraacetic acid, and serum, respectively) at the start of the study and subsequently after 8 and 16 weeks in order to examine the influence of mare's milk on immunological and inflammatory parameters. The serum was immediately aliquoted and stored at –80°C until analysis. Stool samples were taken at the beginning of the study and at the end of each intervention period.

Evaluation of atopic dermatitis

A detailed case history together with the AD symptoms was obtained for each patient before entry into the study by a trained and blinded dermatologist. The severity of AD was assessed by SCORAD index (Consensus Report of the European

Task Force on Atopic Dermatitis 1993) at the start of the study and subsequently at intervals of 4 weeks. The SCORAD evaluation included an assessment of the objective signs (extent and intensity) and the subjective symptoms (pruritus and sleep loss). Extent was calculated using Wallace's 'rule of nines' (Wallace 1951), which expressed the area of the affected skin surface. Intensity items were erythema, oedema/papulation, oozing/crusts, excoriations, lichenification, and dryness of uninvolved skin (0–3 points for each item). For the two subjective symptoms, an average value for the last 3 days/nights was calculated from scores between 0 and 10, given by the patients themselves. SCORAD is calculated using the equation $A/5+7B/2+C$ (A = extent, B = intensity and C = subjective criteria). The range of the total SCORAD index was between 0 and 103. Based on the SCORAD results, AD was classified into mild (<25), moderate (25–50) and severe (≥ 50) forms.

The patients assessed and recorded their daily condition in a patient journal during the entire study by means of four subjective criteria (inflamed dermis, pruritus, sleep loss, and feeling). Scores between 1 and 6 (very good to very bad) assessed the inflamed dermis, whereas each of the other three criteria was given a score between 1 and 3.

Clinical and immunological parameters

The concentration of C-reactive protein (CRP) was measured after 8 and 16 weeks of the intervention period by a turbidimetric assay using the Synchron LX®-System (detection limit = 3.0 mg/l; Beckman Coulter, gmbH, krefeld, Germany). The concentration of total IgE and of mare's-milk-specific IgE was determined using the UniCAP FEIA (Phadia AB, Uppsala, Sweden). Chemotaxis, phagocytosis, and oxidative burst of heparinizing blood were estimated at the end of each intervention period by flow cytometry (FACScan™; Cell Quest Software, Becton Dickinson) using the Migratest®, Phagotest®, and Bursttest® (Orpegen Pharma, Heidelberg, Germany), respectively.

Furthermore, the immunological parameters eosinophilic cationic protein (ECP), soluble eosinophilic selectin (sE-selectin), macrophage-derived chemokine (MDC), and interleukin-16 (IL-16) in serum were also estimated at the end of each intervention period. ECP was measured using UniCAP FEIA (Phadia AB, Uppsala, Sweden). MDC, sE-selectin and IL-16 were estimated by quantitative sandwich enzyme-linked immunosorbent assay, according to the manufacturer's instruction (R&D Systems, Minneapolis, MN, USA) using a 96-well microplate precoated with murine monoclonal antibody against IL-16, human sE-selectin and MDC, respectively.

Microbiota in faecal samples

Entire fresh faecal samples were prepared for fluorescence *in situ* hybridization analysis within 2 h of defaecation. Aliquots were stored at –80°C until analysis. For determination of total bacteria (EUB 338), the fluorescence *in situ* hybridization method, as described by Klein et al. (2008), was used. The population of bifidobacteria was measured using the *Bifidobacterium* genus-specific probe Bif164 (Langendijk et al. 1995). The Lab 158 probe was used to quantify lactobacilli and

enterococci (Harmsen et al. 1999). Oligonucleotide probes were synthesized by Bioscience GmbH (Jena, Germany) and MWG-Biotech AG (Ebersberg, Germany).

Statistical analyses

The data were analysed using SPSS (version 11.5; SPSS, Inc., Chicago, IL, USA). The Wilcoxon test was applied for comparing changes between the SCORAD value at baseline and at 4, 8, 12, and 16 weeks of each intervention period. The differences between the concentrations of IgE, CRP, ECP, sE-selectin, MDC, and IL-16 in the two periods, mare's milk and placebo, were evaluated using Student's *t*-test or the Wilcoxon test depending on the data distribution. For CRP concentrations below the detection limit, a value of 3.0 mg/l was used. The relationship between the relevant parameters was tested by the Pearson correlation coefficient for normal data distribution and by the Spearman correlation coefficient for non-normal data distribution. Differences in the mean values between the two treatments were considered significantly at $P<0.05$.

Results

Severity of atopic dermatitis

The mean SCORAD value for all 23 patients decreased from 30.1 ± 9.7 to 25.4 ± 6.6 ($P<0.05$) after 12 weeks of mare's milk intake and to 26.7 ± 9.0 ($P<0.10$) after the 16-week period. During the placebo period, no significant changes of the SCORAD index were obtained (Table I). In particular, the pruritus as one part of the SCORAD value decreased during the 16-week mare's milk period from 5.4 ± 2.2 to 4.2 ± 2.4 scores ($P<0.01$), in contrast to the 16-week placebo period (from 4.9 ± 2.2 to 4.5 ± 2.7 scores; $P>0.05$). The SCORAD index did not show any seasonal variation in both intervention periods.

In a subgroup of patients ($n=7$) identified as 'responder', the symptoms of AD decreased continuously during the mare's milk period (Figure 1). The responder criterion was a decrease by at least 30% of the SCORAD value throughout the mare's milk period, but not throughout the placebo period. Among the responders, the SCORAD index was 37.5 ± 7.3 at the start of mare's milk intervention and 36.5 ± 12.7, 29.3 ± 8.6, 23.7 ± 5.8, and 22.6 ± 6.8 after 4, 8*, 12*, and 16* weeks, respectively ($^\star P<0.05$, compared with the start score).

In the responder group, the SCORAD items extent, erythema, oedema/population, oozing/crust and pruritus decreased due to the consumption of mare's milk (Table II, $P<0.05$).

Table I. Effect of mare's milk and placebo treatment on the SCORAD index ($n=23$ total patients).

	Week 0	Week 4	Week 8	Week 12	Week 16
Mare's milk	30.1 ± 9.7	28.0 ± 11.2	27.6 ± 8.2	$25.4\pm6.6^\star$	$26.7\pm9.0^\dagger$
Placebo	27.8 ± 8.5	25.7 ± 7.8	25.0 ± 8.7	24.9 ± 8.2	26.8 ± 9.0

$^\star P < 0.05$ and $^\dagger P<0.1$ (Wilcoxon test), compared with week 0.

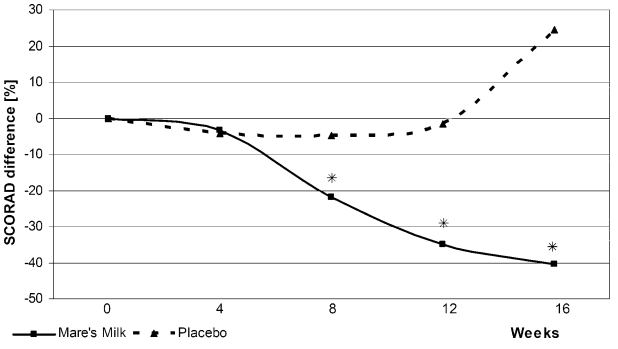

Figure 1. Percentage SCORAD development of responders ($n=7$). *Significant difference compared with the start score, $P<0.05$ (Wilcoxon test).

Conditions of patients and drug use

In general, the subjective conditions and drug use of the 23 patients did not significantly differ between the periods of mare's milk and placebo (Table III).

Immunological parameters

After 16 weeks of mare's milk intervention, the CRP increased compared with the placebo period ($P<0.05$). However, there was no difference in the total IgE between both intervention periods (Table IV). At the start of the study, the specific IgE against mare's milk in all patients was <0.35 kU/l and agreed with the normal value of <0.7 kU/l. Following the 16-week mare's milk period, the specific IgE in two patients increased to 4.01 kU/l and 1.03 kU/l, respectively. The immunological parameters ECP, sE-selectin, MDC and IL-16 as well as parameters for chemotaxis, phagocytosis and oxidative burst did not significantly differ between the mare's milk and placebo periods (Table IV and Table V).

Table II. Increase (+) and decrease (–) of the SCORAD items after the 16-week intervention.

	Responders ($n=7$)		Non-responders ($n=16$)	
	Mare's milk	Placebo	Mare's milk	Placebo
Extent	−3.6*	+1.1	+0.2	−2.7
Dryness	0	0	0	−0.1
Erythema	−1.0*	+0.4	+0.1	−0.1
Oedema/papulation	−1.0*	−0.1	+0.1	−0.1
Oozing/crust	−0.7*	+0.3	+0.1	0.2
Excoriation	−0.3	+0.3	+0.1	−0.1
Lichenification	−0.1	0	+0.2	−0.1
Sleep loss	−0.7	−0.6	+0.4	−0.6*
Pruritus	−2.4*	+0.6	−0.6	−0.8

*$P<0.05$ (Wilcoxon test), statistical differences between the start score and the end score of each intervention period.

Table III. Subjective conditions and drug use after the 16-week intervention ($n=23$ total patients).

	Mare's milk	Placebo	*P* value (Wilcoxon test)
Inflamed dermis	2.88±0.70	2.79±0.85	0.470
Pruritus	1.38±0.53	1.33±0.67	0.605
Sleep loss	0.42±0.43	0.36±0.44	0.197
Feeling	0.57±0.53	0.57±0.61	0.964
Dermatop®	0.35±0.35	0.31±0.37	0.621
Care cream	2.31±1.97	2.23±2.05	0.550

Faecal microbiota

For the evaluation of faecal microbiota, data of four patients were excluded due to use of antibiotics during the study. In general, these four patients were non-responders. The investigated faecal microbiota of the remaining 19 patients showed no significant differences between the placebo and mare's milk interventions. However, in the responder group ($n=7$), an increase in the population of bifidobacteria owing to mare's milk consumption was measured (mare's milk, 11.88±14.15% of eubacteria [EUB], placebo, 5.40±5.96% of EUB; one-sided paired *t*-test, $P<0.05$) (Figure 2). The rise from the study start to study end of the mare's milk intervention was up to 160% (study start, 4.56±4.47% of EUB; paired *t*-test, $P=0.088$). Thus, consumption of mare's milk resulted in a higher concentration of bifidobacteria in the responder group compared with the non-responder group ($P<0.05$; Figure 2).

Discussion

The composition of mare's milk is more similar to human milk than to milk of other species. Mare's milk is characterized by some nutritional properties; for example, a low content of casein (mare, 55%; human, 60%; cow, 80% of total protein) and a high content of bactericidal and immunological components. The bactericidal properties of mare's milk are based on lysozyme, lactoferrin, secretoric IgA and others (Hatzipanagiotou et al. 1998; Pahud and Mach 1972; Solaroli et al. 1993).

In the present study, the SCORAD value of all 23 patients decreased after 12 weeks of mare's milk intake, but not after placebo intake. After 16 weeks of mare's milk intake, a trend towards an improvement in the AD symptoms was seen. Although the absolute differences observed in the SCORAD index (Table I) were low, the dietetic mare's milk has individually clinically relevant effects. In the responder subgroup (seven patients), the SCORAD value decreased continuously by at least 30% (range,

Table IV. Clinical and immunological parameters after the 16-week intervention ($n=23$ total patients).

	Mare's milk	Placebo	*P* value (Wilcoxon test)
CRP (mg/l)	5.0±4.2	4.2±2.8	0.021
Total IgE (kU/l)	1,001±2,120	940±1,914	0.927
ECP (μg/l)	9.6±8.1	7.8±5.1	0.242
sE-selectin (ng/ml)[a]	25.2±11.5	25.0±12.7	0.858
MDC (pg/ml)	826±336	868±543	0.570
IL-16 (pg/ml)	207±46	224±104	0.358

[a]$n=21$ (values from two patients were not within the standard signal range).

Table V. Chemotaxis, phagocytosis and oxidative burst after the 16-week intervention ($n=23$ total patients).

	Mare's milk	Placebo	*P* value
Chemotaxis			
Chemotactic index[a]	11.3±9.4	10.6±6.6	0.628[b]
Phagocytosis			
Phagocytizing granulocytes (%)	99.2±1.0	99.0±0.9	0.149[c]
Mean fluorescence intensity	1914±885	1753±675	0.515[b]
Oxidative burst			
Oxidizing granulocytes (%)	98.9±1.6	96.6±8.1	0.109[c]
Mean fluorescence intensity	581±536	499±347	0.528[b]

[a]Fluorescence intensity of stimulated granulocytes/fluorescence intensity of unstimulated granulocytes. [b]*t*-test. [c]Wilcoxon test.

33–63%) during the 16 weeks of the mare's milk intervention. Moreover, these patients did not show any change of the SCORAD index during the 16-week placebo period. The observation that a subgroup of AD patients reacts to a particular food or to special food ingredients has also been observed in other studies: borage oil (Bahmer and Schaefer 1992; Henz et al. 1999), primrose oil (Steward et al. 1991), and probiotics (Rosenfeldt et al. 2003; Viljanen et al. 2005; Sistek et al. 2006). To date, comparable studies using mare's milk for the treatment of skin diseases are not available in the literature, although individual cases have been reported (Buehlbaecker 1996). Ellinger et al. (2002) found a decrease of the chemotactic index and the burst activity in healthy patients after a 3-week intervention with mare's milk.

In the present study, the IgE values correlated with SCORAD values ($P<0.01$; Table VI). This correlation was also observed in a study with 345 children (Laske and Niggemann 2004). However, an increased IgE value does not prove the existence of an atopy, and *vice versa*. During the mare's milk period, the concentration of mare's milk-specific IgE increased in two patients, signifying a sensitization against mare's milk proteins. A possible allergic reaction against mare's milk has already been described in the literature (Gall et al. 1996).

In some studies a positive correlation between the serum value of ECP and the intensity of AD was obtained (Czech et al. 1992; Kaegi et al. 1992; Pucci et al. 2000; Breuer et al. 2001). ECP can therefore function as a sensitive marker for judging

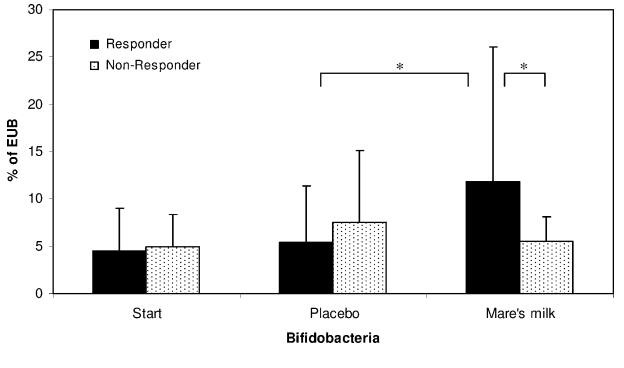

Figure 2. Percentage of faecal bifidobacteria related to eubacteria (EUB) in responders ($n = 7$) and non-responders ($n=12$). *$P<0.05$.

illness severity and can be used for the monitoring therapy success. In the present study, a correlation between the ECP and the SCORAD values was also obtained ($P=0.05$; Table VI). However, no differences were seen regarding the ECP concentration between the mare's milk and placebo periods.

In the present study, the SCORAD value correlated with IL-16 ($P<0.05$) and with MDC ($P<0.01$; Table VI) while the mean values of IL-16, MDC and sE-selectin did not differ between the mare's milk and the placebo periods. Compared with other studies, the measured serum concentrations of ECP, sE-selectin, MDC and IL-16 were similar to the healthy control individuals, but were slightly lower compared with AD patients (Czech et al. 1992; Furue et al. 1999; Tischendorf et al. 2000; Frezzolini et al. 2002; Kakinuma et al. 2002). This could be due to patients having a less severe form of AD. IL-16 was initially described in 1982 as having chemoattractant activity for human T cells. IL-16 is synthesized by a variety of immune and non-immune cells and has a pathobiological relevance for the onset and chronicity of AD (Cruikshank et al. 2000). Recently, Angelova-Fischer et al. (2006) showed the presence of a significantly higher concentration of IL-16 in patients with acute AD than in responders to treatment.

The increased number of bifidobacteria in the responders indicates that the effect of mare's milk is partly based on a modification of the microbiota in the gut. It is well known that the intestinal microbiota in humans has a strong influence on the immune system. For example, it has been shown that children with an allergy have a reduced intestinal colonization with bifidobacteria and lactobacilli (Kalliomaeki and Isolauri 2003). Likewise, a reduced number of bifidobacteria were measured in patients with AD compared with healthy control persons (Watanabe et al. 2003). In future studies, it may be interesting to research into species-specific effects of mare's milk on the bifidobacteria population. Apart from the quantitative differences seen in the bifidobacteria population of healthy and allergic infants, changes in the composition of the bifidobacteria flora were observed (Ouwehand et al. 2001). Finally, there are indications that the cytokine induction by bifidobacteria is a strain-dependent property that is related to a different immunologic status (He et al. 2002). The modulation of intestinal microbiota could be due to several factors. Lactoferrin has a growth-promoting influence on certain bifidobacteria and lactobacilli (Kim et al. 2004). Consequently, lactoferrin promotes the desired bacterial flora whilst suppressing pathogenic strains. With regard to the bactericidal effects of mare's milk, a synergy seems to be present between lactoferrin and lysozyme (Leitch and Willox

Table VI. Correlations between several blood parameters and the SCORAD index independent of the intervention.

Parameter	*R* value	*P* value
IgE ($n=92$)	0.471[a]	0.001
MDC ($n=46$)	0.452	0.002
IL-16 ($n=46$)	0.344	0.019
ECP ($n=46$)	0.291	0.050
CRP ($n=92$)	–0.067[a]	0.523
sE-selectin ($n=42$)	–0.064	0.688

$n=92$, parameter values after 8 and 16 weeks; $n=46$, parameter values after 16 weeks; $n=42$, parameter values after 16 weeks (values from two patients were not within the standard signal range).
[a]Spearman correlation coefficient.

1998). Moreover, the secretory IgA in mare's milk could possibly enhance defence against pathogens.

Although the number of patients was relatively small, the results indicated that the oral application of mare's milk achieved positive effects — for example, a reduced SCORAD index (in particular pruritus) and increased faecal bifidobacteria — in approximately one-third of the AD patients ('responders'). Future studies should aim to analyse the influence of dietary intervention with mare's milk on a larger cohort and to clarify its mode of action.

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