The Effect of Mare’s Milk Consumption on Functional Elements of Phagocytosis of Human Neutrophil Granulocytes From Healthy Volunteers

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(Original manuscript received 20 June 2001; revised manuscript accepted 19 January 2002)

We investigated functional parameters of phagocytosis in 18 healthy volunteers drinking 250 mL of mare’s milk, deep-frozen (FMM) or lyophilized (LMM), or cow’s milk (CM) daily for three weeks. Blood was taken before, weekly during, and one week after intervention. Chemotaxis of isolated polymorphonuclear leukocytes (PMN) was investigated by a micropore filter assay, migration was determined fluorimetrically. The activity of phagocytosis and respiratory burst of PMN in whole blood was analysed with Phago- and Bursttest® by flow cytometry. Contrary to phagocytosis activity, chemotactic index (CI) and burst activity diminished significantly in the group FMM. One week after intervention, CI rose tendentiously, burst activity significantly. In conclusion, immunostimulating effects ascribed to mare’s milk consumption could not be observed in healthy volunteers, at least concerning phagocytosis. Our results suggest that drinking FMM modulates inflammation processes by decreasing chemotaxis and respiratory burst, which might be favourable for giving relief to diseases with recurrent inflammation.

Keywords: mare’s milk, human neutrophils, chemotaxis, phagocytosis, respiratory burst

INTRODUCTION

Nearly 50 years ago, investigations by Kalliala et al. (1951) demonstrated that the chemical composition of mare’s milk is very different from that of cow’s milk and rather similar to that of breast milk. Mare’s milk is characterized by high lactose, low fat and low protein content in comparison with cow’s milk (Pagliarini et al., 1993; Solaroli et al., 1993).
The casein content of total protein is lower in mare’s than in cow’s milk in favour of higher content of whey proteins, among which are lysozyme and lactoferrin. Mare’s milk has a much higher content of medium chain and polyunsaturated fatty acids, particularly linolic and linoleic acids, which results in a higher ratio of unsaturated to saturated fatty acids of 1.32 (cow’s milk 0.45) (Pagliarini et al., 1993). As calculated from data by Glass et al. (1967), the ω-3 : ω-6 fatty acid ratio in equine milk is higher than in cow’s milk (0.85 vs 0.33).

These nutritional characteristics and the highly digestible protein and fat account for the growing dietary interest in mare’s milk in paediatrics and geriatrics, as well as for its use for debilitated or convalescent people in Central Europe (Solaroli et al., 1993). Consumption of mare’s milk improved the state of health of malnourished babies and children suffering from food allergies and atopic dermatitis (Bühlbäcker, 1996) and reduced transaminase activity in patients with chronic hepatitis (Baur, 1981). In Eastern Europe and Asia, mare’s milk has been used for treatment of tuberculosis and further gastrointestinal and respiratory diseases accompanied by cachexia and loss of strength (Müller-Dietz, 1959; Svoboda, 1969; Lozovich, 1995).

Mare’s milk has been traditionally used as a foodstuff in the Mongolian and Eurasian steppe regions for centuries. In Western countries, drinking mare’s milk is recommended in popular science articles as a tonic for healthy people. The invigorating effect is partly ascribed to immunostimulation.

Several studies have shown that whey proteins such as lactoferrin and lysozyme – characteristic components in mare’s milk – modulate phagocytosis of human neutrophils in vitro.

Bovine lactoferrin and its pepsin hydrolysate stimulated phagocytosis of neutrophils (Miyauchi et al., 1998). Iron-saturated human lactoferrin enhanced the generation of superoxide (Gahr et al., 1991) and hydroxyl radicals (Ambruso & Johnston, 1981) whereas chemotaxis and degranulation were not affected (Gahr et al., 1991).

Lysozyme is of pharmacological interest due to its antiinflammatory effects which result from inhibition of neutrophils (Sava, 1996). Respiratory burst was decreased (Gordon et al., 1979) and phagocytosis was stimulated by human lysozyme (Klockars & Roberts, 1976). The results concerning the effect of human lysozyme on neutrophils’ chemotaxis are contradictory (Gordon et al., 1979; Nitzan et al., 1985). Lysozyme from equine milk is highly resistant to heat, acid (Jauregui-Adell, 1975) and protease digestion due to a calcium-binding loop (Kuroki et al., 1989). Provided that lysozyme from mare’s milk reaches the gut associated lymphatic tissue undigested, immunomodulating effects may be expected by lysozyme from mare’s milk consumption.

Moreover, phagocytosis of human neutrophils is modulated by ω-3 fatty acids which are characteristic for mare’s milk. Several intervention studies have shown a decline in chemotaxis, phagocytosis or respiratory burst in healthy volunteers by supplementation of ω-3 fatty acids (Lee et al., 1985; Fisher et al., 1986; Schmidt et al., 1989; Virella et al., 1989; Thompson et al., 1991; Sperling et al., 1993; Varming et al., 1995; Luostarinen et al., 1996).

Some components in mare’s milk might influence functional elements of phagocytosis of human neutrophils by consumption of mare’s milk. However, ex vivo and in vitro studies with lactoferrin, lysozyme and fatty acids from mare’s milk as well as clinical intervention studies proving immunomodulating effects by consumption of mare’s milk are still missing. There is no evidence for its clinical effectiveness and interaction with body defence (Doreau, 1991).

The objective of this pilot study was to determine if drinking different preparations of mare’s milk, either frozen mare’s milk (FMM) or lyophilized mare’s milk (LMM), influence functional parameters of phagocytosis of healthy volunteers. Examination of chemotaxis, phagocytosis and respiratory burst allows close-meshed investigation of main elements in the phagocytosis process.
EFFECT OF MARE’S MILK CONSUMPTION ON PHAGOCYTOSIS

MATERIALS AND METHODS

Subjects
Eighteen healthy volunteers (14 female, 4 male), aged 20–37 years (26 ± 5 years) participated in this study. Only subjects not suffering from allergies or food intolerance were included. Participants were instructed to neither change their dietary habits or to take vitamin or probiotic supplements. Moderate consumption of cow’s milk and its products (e.g., cheese, yoghurt) was tolerated. Informed consent had to be given. The study protocol was approved by the local ethical committee of the medical faculty, University of Bonn.

Experimental Design
Subjects were randomly assigned to drink either 250 mL of raw FMM, 25 g granule of raw LMM (both: Nativa®, Gestüt St Georg, Thomasburg, Germany) or 250 mL of pasteurized, frozen CM (Tuffi Campina Milchwerke, Köln/Wuppertal, Germany) daily over a period of three weeks. The dose of 25 g of granule is equivalent to 250 mL of liquid mare’s milk, recommended as daily dose by the marketer. The group FMM consisted of six female, the other groups of two male and four female subjects. The average age of the participants was 25.7 years in the FMM group, 26.2 years in the LMM group and 25.5 years in the CM group. Venous blood was taken before intervention, weekly during, and one week after intervention. The periodical checks allow a close-meshed investigation of the dynamics of immune response.

Chemicals and Reagents
Mono-Poly Resolving Medium® (M-PRM®) was purchased from ICN Pharmaceuticals, Eschwege, Germany, Dulbecco’s phosphate buffered saline (DPBS) from Biochrom, Berlin, Germany. Calcein acetoxyethyl ester (CAM) was obtained from Molecular Probes, Leiden, The Netherlands, Phago- and Bursttest® from Orpegen Pharma, Heidelberg, Germany. Calibrite® beads were purchased from Becton Dickinson, Heidelberg, Germany. All other chemicals came from Sigma, Deisenhofen, Germany.

Investigation of Phagocytosis Elements
Chemotaxis Peripheral blood polymorphonuclear leukocytes (PMN) were isolated by density centrifugation with M-PRM®. Three mL of the gradient was placed in a 15 mL centrifuge tube and 2.5 mL of EDTA-anticoagulated whole blood was layered onto the gradient. After centrifugation at 450 × g for 30 min at 20°C, the obtained PMN-fraction was washed with DPBS twice and PMN were resuspended in cell culture medium RPMI 1640 containing 10% fetal bovine serum. 1 × 10⁶ cells/mL were incubated with 2.5 μM CAM for 30 min at 37°C, 5% CO₂ in a humidified atmosphere. During incubation, cells were passively loaded with CAM, which was converted to the green fluorochrome Calcein by cell esterase activity. Due to loss of hydrophobicity, Calcein was retained as an intracellular marker. After labelling, cells were washed twice with DPBS and resuspended in RPMI 1640. Chemotaxis was investigated with a modified Boyden chamber technique (Boyden, 1962) using a 48 multiwell chamber (AP48, Nucleopore, Gaithersburg, MD, USA) and a PVP-coated polycarbonate membrane with a pore diameter of 3 μM (Corning Costar, Bodenheim, Germany). N-formyl-leucyl-phenylalanine (fMLP, 10⁻⁷ M) was used as a chemoattractant and RPMI 1640 served as control to assess random migration. Each sample was prepared in triplicates. The lower wells of the 48 multiwell chamber were filled with 24.5 μL medium or fMLP, the upper wells with 50 μL of the cell suspension. The chamber was incubated for 30 min at 37°C, 5% CO₂ in a humidified atmosphere. The filter was then removed and sedimented cells were carefully scraped off the upper side of the filter. Fluorescence on the lower side of the membrane was measured at 488 nm (FluorImager) and analysed with
Phagocytosis
The phagocytosis activity of PMN in heparinized whole blood was determined with Phagotest® (Hirt et al., 1994) using a modified protocol. Briefly, 100 μL heparinized whole blood was mixed with 20 μL precooled, fluorescein (FITC)-labeled opsonized Escherichia coli (E. coli) bacteria (5 μL suspension 1 + 3 v/v with washing solution) and incubated for 10 min at 37°C. Control samples remained on ice. Phagocytosis was stopped by placing all samples on ice simultaneously and adding 100 μL of quenching solution for suppression of signals from adherent, non-phagocytized bacteria. Samples were then washed twice with 3 mL washing solution. Erythrocytes were lysed by 2 mL diethylene glycol/formaldehyde solution during 20 min incubation at RT. After washing the samples with 3 mL of washing solution, cells were stained by addition of 200 μL DNA-staining solution containing propidium iodide (PI). Each sample was investigated in duplicates by flow cytometry, a standard technique for the examination of phagocytosis (Lehmann et al., 2000). Calibrite® beads were used to adjust instrument settings and to set fluorescence compensation.

Data were acquired with CellQuest® software on a FACScan flow cytometer (both: Becton Dickinson, Heidelberg, Germany). The event number was set to 15 000. WinMDI 2.7 software (J. Trotter, The Scripps Research Institute, La Jolla, CA, USA) was used for determination of phagocytosis activity. The PMN cluster was gated in the scatter diagram (lin FSC vs lin SSC), its green fluorescence and red fluorescence were plotted (log FITC vs log PI). A quadrant was set to differentiate between phagocytising (FITC⁺, PI⁺) and non-phagocytising (FITC⁻, PI⁺) PMN and to eliminate debris (FITC⁻, PIdim) and non-phagocytized bacterial aggregates (FITC⁺, PIdim). Phagocytosis activity was determined as a percentage of phagocytising PMN.

Respiratory Burst
The burst activity of PMN in heparinized whole blood was determined with Bursttest® (Hirt et al., 1994) using unlabelled opsonized E. coli bacteria. For stimulation, 20 μL precooled bacteria suspension were added to 100 μL heparinized whole blood. Instead, 20 μL of washing solution were added to the control. After 10 min of incubation at 37°C, 20 μL substrate solution were added and incubated for another 10 min at 37°C. The fluorogenic substrate dihydrorhodamine 123 was converted by reactive oxygen species (ROS) to green fluorochrome rhodamine 123 (R 123). The samples were taken out of the water bath simultaneously to stop this reaction. Erythrocytes were lysed by 20 min of incubation with lysing solution. After washing with 3 mL of washing solution, cells were stained with 200 μL DNA-staining solution containing PI. Analysis was done by flow cytometry as described above. The green fluorescence (R 123) of gated neutrophils was plotted against their red fluorescence (log R 123 vs. log PI). Elimination of debris and bacterial artifacts and determination of burst activity was performed according to the phagocytosis experiments.

Statistics
Results are expressed as median and the 25 and 75 percentile. SPSS 9.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Mann-Whitney U-Test was performed to compare the data of different groups at baseline. The differences within each group were examined by Wilcoxon test. Alpha was set to 0.05.
RESULTS
No significant differences in CI, phagocytosis and burst activity could be observed between the different groups at baseline.

Chemotaxis
The percental changes in CI in relation to baseline values are presented in Figure 1. CI diminished significantly in the FMM group during supplementation (Figure 1a). Decrease in CI started after the first week of supplementation. CI was 16% and 30% lower than baseline in week 2 and week 3, respectively. Decline in CI was significant after three weeks ($p = 0.046$). After one week washout, CI slightly increased towards baseline. CI in week 4 was not significantly different from baseline. In the LMM group, CI greatly varied throughout the study (Figure 1b). However, these changes were not significant. In the CM group (Figure 1c) CI declined until week 2 (11% below baseline), and increased in the following weeks. The only significant alteration was from week 2 to week 3 ($p = 0.043$).

![Chemotactic Index Graph](image-url)

FIG. 1. Relative alteration of chemotactic index in the group frozen mare’s milk (a), lyophilized mare’s milk (b) and cow’s milk (c). Week 0 = 0%. Results are expressed as median ± 25-, 75-percentile. $\alpha$ – $\gamma$: values differed significantly ($p < 0.05$) from value in week 0 ($\alpha$), week 1 ($\beta$) or week 2 ($\gamma$).
Phagocytosis activity

FIG. 2 Relative alteration of phagocytosis activity in the group frozen mare’s milk (a), lyophilized mare’s milk (b) and cow’s milk (c). Week 0 = 0%. Results are expressed as median ± 25-, 75-percentile. α, β: values differed significantly (p < 0.05) from value in week 0 (α), week 1 (β).

Phagocytosis
The percental changes in phagocytosis activity are presented in Figure 2. In the FMM group (Figure 2a) there was a variation in phagocytosis activity, but without significance. Concerning the LMM group (Figure 2b), phagocytosis activity increased during the first two weeks of intervention, decreased from week 2 to week 3, and rose in week 4. Apart from phagocytosis activity in week 2 which was significantly different from baseline value (p = 0.028), there were no significant alterations in phagocytosis activity.

There was a variation in phagocytosis activity in the group CM (Figure 2c). Only the difference in phagocytosis activity between baseline and week 1 (p = 0.028) as well as between week 1 and week 2 (p = 0.028) was statistically significant.

Respiratory Burst
The percental changes in burst activity relative to baseline are shown in Figure 3. Burst activity diminished significantly in the group FMM during supplementation (Figure 3a).
Decline in burst activity started after one week of intervention. Burst activity was significantly lower by 4% in week 2 ($p = 0.028$) and by 6% in week 3 ($p = 0.028$) compared with baseline and week 1 value. After the end of supplementation there was a significant increase in burst activity ($p = 0.046$). Burst activity was 2% lower in week 4 than baseline value, however this difference was not significant. In the group LMM, burst activity diminished by 6% after three weeks of supplementation and rose in the washout period up to baseline level, but without significance (Figure 3b). In the group CM, burst activity remained at baseline level throughout the study except for burst activity in week 2 which was significantly lower than in week 1 ($p = 0.046$) (Figure 3c).

**DISCUSSION**

The results of this study demonstrate that consumption of FMM over a period of three weeks could significantly decrease CI in healthy volunteers whereas significant alterations within a
trend did not occur after consumption of LMM and CM. Chemotaxis, the first step in phagocytosis, is an essential mechanism for efficient elimination of bacteria by phagocytes. Hence, inhibition of chemotaxis as observed in the group FMM can inhibit or at least delay steps following in the process of phagocytosis.

Regarding phagocytosis activity, strong variations could be observed in all groups, but without a marked trend in any group. Since *E. coli* bacteria in Phagotest® were opsonized, differences in the level of opsonization, which is well known to influence phagocytosis *in vivo*, can be excluded. Strong interindividual variations in phagocytosis activity, measured as mean fluorescence intensity using Phagotest®, were also found by Lun *et al.* (2000). Additional factors affecting ingestion of bacteria (e.g., adherence, fluidity of plasma membrane) may be responsible for these fluctuations.

Consumption of mare’s milk – either frozen or lyophilized – induced a continuous decline in burst activity which was significant only in the FMM group. Temporary stimulation of respiratory burst is a physiological response to microbial invasion. However, burst products – ROS – which attack the phagocytized bacteria, may induce structural or even functional alterations in the host’s cells. Therefore, neutrophil response must be controlled carefully to guarantee protection from pathological bacteria without harming the host himself. Permanent activation of chemotaxis and respiratory burst as observed in chronic inflammatory diseases results in tissue destruction. Provided that a significant decline in CI and burst activity also occurs in patients with inflammatory diseases, drinking FMM might support antiinflammatory therapy. It remains an open question whether the decline in CI and burst activity in healthy people favours susceptibility to infections or is advantageous by ROS burden.

The reasons for inhibition of chemotaxis and respiratory burst in the FMM group remain speculative. ω-3 fatty acids as well as lysozyme might be responsible for these effects. Decline in chemotaxis (Fisher *et al.*, 1986; Schmidt *et al.*, 1989; Thompson *et al.*, 1991; Sperling *et al.*, 1993), phagocytosis (Virella *et al.*, 1989) or respiratory burst (Lee *et al.*, 1985; Fisher *et al.*, 1986; Varming *et al.*, 1995; Luostarinen *et al.*, 1996) could be observed in healthy volunteers after supplementation of ω-3 fatty acids. However, the intake of ω-3 fatty acids by 250 mL of mare’s milk (444 mg, compared to 71 mg in cow’s milk, calculated from Glass *et al.* (1967) and Deutsche Forschungsgesellschaft für Lebensmittelchemie (1994)) was only a third of the minimal supplementation in those studies. The antiinflammatory effects of lysozyme reported by Sava (1996) as well as the inhibition of chemotaxis and respiratory burst *in vitro* (Gordon *et al.*, 1979) suggest that lysozyme might be associated with decline in CI and burst activity in the FMM group. The higher content of lysozyme in mare’s milk than in cow’s milk and the extraordinary stability of equine lysozyme (Jauregui-Adell, 1975; Kuroki *et al.*, 1989) may support this assumption. However, a significant decline in both CI and burst activity could not be observed in the LMM group. No information is available concerning the effect of lyophilization on equine lysozyme.

In conclusion, immunostimulating effects claimed by marketers could not be observed in healthy volunteers concerning phagocytosis. On the contrary, consumption of FMM, but not of LMM, significantly inhibited two elements of phagocytosis, chemotaxis and respiratory burst. The indications concerning anti-inflammatory effects should be scrutinized in further investigations considering a potential use for patients suffering from inflammatory diseases. To assess the effect of drinking mare’s milk on the immune system, further parameters including those of the specific cellular and humoral defence will have to be analysed.

ACKNOWLEDGEMENTS

We thank Kathrin Peter for technical assistance, Dr Jan Schmolling and Dr E. Radew, University Hospital and Medical Center for Gynaecology and Obstetrics, for blood sampling and Dr Christine Windemuth-Kieselbach, Institute of Medical Biometry, Informatics and Epidemiology, for statistical consultation. We are very grateful to the subjects who participated in this study. This study was funded by the Ministerium für Umwelt,
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